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CHAPTER 1

Anaerobic Biodegradation of Methyl *tert*-Butyl Ether (MTBE) and Related Fuel Oxygenates

Max M. Häggblom,* Laura K. G. Youngster,* Piyapawn Somsamak,† and Hans H. Richnow‡

Contents

I. INTRODUCTION

Methyl tertiary (*tert*)-butyl ether (MTBE), an octane enhancer and a fuel oxygenate in reformulated gasoline, has received increasing public attention. As a consequence of over a decade of extensive use, MTBE has become one of the most frequently detected groundwater contaminants. MTBE is a synthetic chemical which is added to gasoline as a fuel oxygenate. Other fuel oxygenates in use include ethyl *tert*-butyl ether (ETBE), methyl *tert*-amyl ether (TAME), *tert*-butyl alcohol (TBA), and isopropyl

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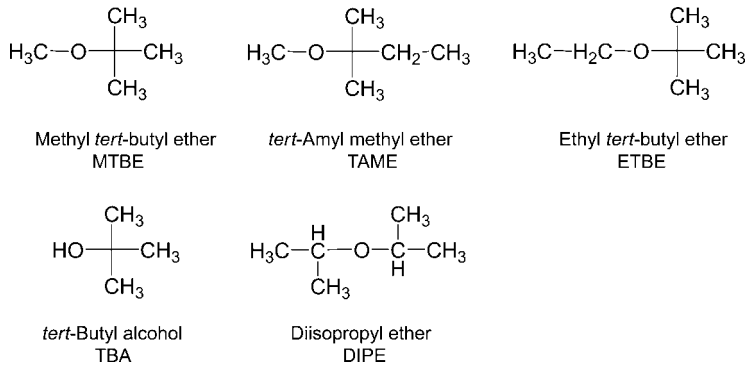

FIGURE 1.1 Structure of MTBE and related fuel oxygenates.

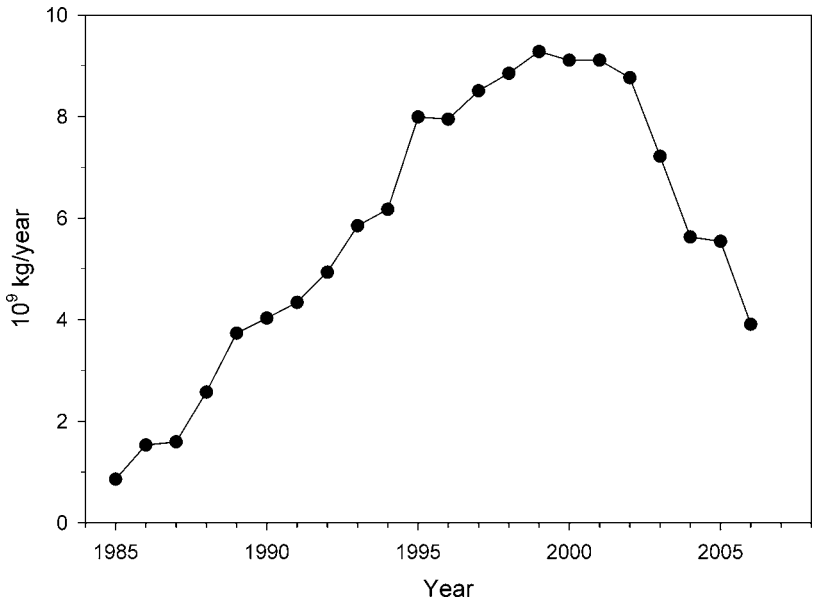

FIGURE 1.2 Annual production of MTBE in the Unites States from 1983 to 2006 (sources: Energy Information Administration, US Department of Energy; Chemical Engineering News, 1994, 1996, American Chemical Society).

ether (Fig. 1.1), although only relatively minor amounts of these are used in gasoline. MTBE was first used in gasoline as an octane enhancer, and later to reduce emissions of carbon monoxide and formation of ozone as mandated by the Clean Air Act Amendments of 1990 (Franklin *et al.*, 2000). Because of its low cost, ease of production, and favorable transfer and blending characteristics, MTBE became the most commonly used

fuel oxygenate (Shelly and Fouhy, 1994). By 1995, oxygenates were added to 30% of US gasoline, 87% of which was MTBE (US EPA, 1999, 2000). The volume of MTBE produced in 1995 was the second highest of all synthetic organic chemicals in the United States, and US production peaked at ~200,000 barrels per day (Fig. 1.2). At present the use of MTBE is being banned or restricted in many states and in most cases replaced with ethanol. In Europe, MTBE is progressively being replaced by ETBE and TAME.

II. FUEL OXYGENATES AS CONTAMINANTS OF WATER SOURCES

As MTBE use increased in the mid-1990s, the frequency and extent of contamination were quickly visible across the United States. The widespread use of MTBE to reduce air pollution has instead resulted in major contamination of water resources (Achten *et al*., 2002a,b; Ayotte *et al*., 2005; Dernbach, 2000; Heald *et al*., 2005; Johnson *et al*., 2000; Pankow *et al*., 1997; Reuter *et al*., 1998; Squillace *et al*., 1996, 1999; Toran *et al*., 2003). Point sources of contamination include leaking gas tanks, pipelines, landfill sites, dumps, spills, and refueling facilities. There have been reports of MTBE contamination in lakes and coastal environments as a result of motorized watercrafts. Other possible sources include precipitation, discharge from contaminated groundwater, stormwater runoff, effluents from refineries, and treated municipal wastewater (Brown *et al*., 2000, 2001; Reuter *et al*., 1998; Squillace *et al*., 1996). The US Geological Survey reported that MTBE was one of the most frequently detected volatile organic compounds in groundwater based on sampling of close to 3000 wells between 1985 and 1995 (Squillace *et al*., 1999). Generally, high-level contamination is associated with direct release of gasoline, such as leaking underground storage tanks, with concentrations up to 200 mg/liter measured in groundwater near sites of gasoline leakage (Zogorski *et al*., 1997). The very high aqueous solubility of MTBE (~48 g/liter) makes it very mobile in groundwater systems resulting in widespread plumes.

MTBE has been found in source water or finished drinking water from more than 1850 public water systems in 29 US states, serving over 15 million people (Environmental Working Group, 2005). Over 700 communities have been identified with source or finished water with >2 ppb MTBE. Several municipal water supplies have been closed due to MTBE contamination (Franklin *et al*., 2000; Weaver *et al*., 1996). MTBE has a very strong turpentine-like taste and smell and can only be tolerated in drinking water at very low levels. While there is no federal regulation regarding MTBE allowance in water, the US Environmental Protection Agency has issued a recommended limit of 20–35 ppb in drinking water

(US EPA, 1997). In the interest of preserving drinking water quality, many states have adopted lower thresholds of 13–14 ppb (Ayotte *et al*., 2005). In addition to the unpleasant odor and taste, MTBE is a skin and respiratory irritant and can be carcinogenic in rats and mice, although at very high concentrations (Bird *et al*., 1997; Werner *et al*., 2001). Studies of the potential health hazards to humans have been inconclusive and the US EPA lists MTBE as a possible human carcinogen. MTBE has not been shown to be toxic to aquatic life at environmental concentrations, but there is some concern that likely degradation products, such as TBA and formaldehyde, may pose health problems (Mormile and Suflita, 1996; Werner *et al*., 2001). A report shows that environmentally significant concentrations of MTBE (as low as 0.11 mg/liter) had estrogenic effects on male zebra fish and caused reduced sperm motility (Moreels *et al*., 2006).

III. ENVIRONMENTAL FATE

Once fuel oxygenates are released to the environment, their behavior is largely determined by how they partition in gasoline, air, water, and onto subsurface solids (Squillace *et al*., 1997). The physical and chemical properties of MTBE make environmental contamination a challenging problem. Relative to other gasoline components, such as benzene, toluene, and other aromatic hydrocarbons, MTBE has a higher vapor pressure, and will volatilize easily from the nonaqueous phase (US EPA, 2004). This may cause greater atmospheric concentrations and distribution by precipitation. MTBE is more soluble in water than aromatic hydrocarbon compounds. The solubility of pure liquid MTBE in water (about 48 g/liter) can result in high concentrations of MTBE in surface water and groundwater near gasoline-release site (Squillace *et al*., 1997). Furthermore, the very high aqueous solubility of MTBE makes it very mobile in groundwater systems resulting in widespread plumes. Although pure phase MTBE is very volatile (vapor pressure of 250-mm Hg at 25°C), it is less volatile once dissolved into water and is difficult to remove from water by air stripping. MTBE also has a lower soil adsorption coefficient (K_{oc}) than the BTEX components, making it less susceptible to removal by frequently used carbon-based adsorption methods. Together these properties mean that when MTBE is spilled, it is likely to dissolve in water and migrate quickly throughout the water system without being hindered by volatilization or adherence to soil (Stocking *et al*., 2000). MTBE is also difficult and expensive to remove by methods used for the treatment of other gasoline components (US EPA, 2004). There is thus considerable interest in developing effective technologies to remediate MTBE-contaminated sites and groundwater plumes.

IV. MTBE BIODEGRADATION

The ultimate fate of MTBE and related fuel oxygenates in the environment is dependent on its susceptibility to microbial transformation and degradation. Ether compounds are often recalcitrant to biodegradation because ether bonds have a high dissociation energy of ~360 kJ/mol (Kim and Engesser, 2004). Specifically, the tertiary carbon structure and ether linkage are two features of MTBE that render it relatively recalcitrant to microbial degradation. MTBE was initially thought to be completely resistant to biodegradation. However, Salanitro *et al*. (1994) first demonstrated aerobic microbial biodegradation of MTBE. Since then, numerous laboratory studies have clearly demonstrated that a variety of microorganisms, including both bacteria and fungi, are capable of utilizing MTBE aerobically as a primary source of carbon and energy or are able to cometabolize MTBE following growth on another substrate (see reviews by Deeb *et al*., 2000; Fayolle *et al*., 2001; Fiorenza and Rifai, 2003; Stocking *et al*., 2000). Initial attack on MTBE is mediated by a variety of oxygenases, including methane monooxygenase (Liu *et al*., 2001), toluene monooxygenases (Vainberg *et al*., 2006), cytochrome *P*450 monooxygenases (Steffan *et al*., 1997), propane monooxygenase (Smith *et al*., 2003; Steffan *et al*., 1997), as well as toluene dioxygenase, ammonium monooxygenase, and propylene monooxygenase (Hyman and O'Reilly, 1999). These enzymes initiate the oxidation of MTBE to *tert*-butoxymethanol, which is unstable and dismutes to TBA and formaldehyde (Rohwerder *et al*., 2006), or is degraded to *tert*-butylformate, which is further hydrolyzed to TBA and formate. TBA is thus a key intermediate of aerobic MTBE degradation.

On the basis of these studies, a number of technologies for remediation of contaminated groundwater were developed (Fortin and Deshusses, 1999; Liu *et al*., 2006; Stocking *et al*., 2000; Zaitsev *et al*., 2007; Zien *et al*., 2004, 2006). However, MTBE-is spilled commonly in a matrix of gasoline and biodegradation leading to rapid consumption of oxygen and generally anoxic conditions. Thus, most MTBE-contaminated sites are subsurface with insignificant amounts of oxygen. This limits the utility of enhanced *in situ* aerobic bioremediation, and the fate of MTBE in the environment is thus mainly dependent on anaerobic processes.

Information on the microbial degradation of MTBE under anoxic conditions is still quite limited. There have been just around a dozen reports of anaerobic MTBE biodegradation since 1993. However, there is little information on these anaerobic microbial communities and their role in the biodegradation of MTBE in the absence of oxygen. MTBE has been shown to be biodegradable under a variety of anaerobic conditions (Table 1.1), including methanogenic (Bradley *et al*., 2001a; Mormile *et al*., 1994; Somsamak *et al*., 2005, 2006; Suflita and Mormile, 1993; Wilson *et al*., 2000), nitrate-reducing (Bradley *et al*., 2001b; Fischer *et al*., 2005),

TABLE 1.1 Examples of studies demonstrating anaerobic biodegradation of MTBE

Inoculum source	Anaerobic condition	MTBE concentration, incubation time, and extent of degradation	Number of microcosms showing degradation vs not showing degradation	References
Oligotrophic soil, Virginia	Methanogenic	100 mg/liter, 270 days, 80–100%	Three sites tested under three conditions. Degradation only at one site under one condition	Yeh and Novak, 1994
River sediment, Ohio	Methanogenic	48 mg/liter, 152 days, 46%	Degradation only seen in one of triplicate identically prepared microcosms	Mormile *et al.*, 1994
Aquifer material, South Carolina	Fe(III) reducing	U-^{14}C-MTBE, 73,000–666,000 dpm, 7 months, 3% production of radiolabeled CO_2.	Several conditions tested, MTBE degradation only seen under one	Landmeyer e*t al.*, 1998
Aquifer material, North Carolina	Methanogenic	3.1–5.7 mg/liter, 490–590 days, 99%	MTBE degradation in both alkylbenzene-supplemented and unsupplemented culture conditions	Wilson *et al.*, 2000

Surface water sediment, South Carolina, Florida, New Jersey	Methanogenic, sulfate reducing, nitrate reducing, Fe(III) reducing, Mn(IV) reducing	U-^{14}C-MTBE, 1.3–1.6 mg/liter, 166 days, 10–80%	80% MTBE mineralization in Florida sediments under sulfate-reducing conditions. Only 10–20% mineralization at other sites and other anaerobic conditions	Bradley *et al.*, 2001a
Streambed sediment, South Carolina	Nitrate reducing	U-^{14}C-MTBE, 1.5–1.8 mg/liter, 77 days, 25%	Significant MTBE mineralization seen under nitrate-reducing conditions, but not under methanogenic or sulfate-reducing	Bradley *et al.*, 2001b
MTBE-contaminated aquifer sediment, South Carolina	Fe(III) reducing	50 mg/liter, 275 days, 100%	MTBE degradation seen in 1 out of 5 conditions tested. Only one of 3 Fe(III)-reducing replicates showed degradation	Finneran and Lovley, 2001
Estuarine sediment, New Jersey, New York	Sulfate reducing	100 mg/liter, 1160 days, 100%	MTBE degradation only under sulfate-reducing conditions (out of 4 conditions tested) and only in some replicates. No MTBE loss observed in methanogenic, nitrate-reducing, or Fe(III)-reducing cultures	Somsamak *et al.*, 2001

(*continued*)

TABLE 1.1 (*continued*)

Inoculum source	Anaerobic condition	MTBE concentration, incubation time, and extent of degradation	Number of microcosms showing degradation vs not showing degradation	References
Aquifer material, New Jersey	Unidentified	~9 mg/liter, 199 days, 10–99%	MTBE degradation seen in 5 out of 12 replicates	Kolhatkar *et al.*, 2002
Estuarine sediment, New Jersey, New York, California	Sulfate reducing, methanogenic	100 mg/liter, 246–1160 days, 100%	Out of 3, 9 sites tested showed degradation in 1 out of 3 replicates of each	Somsamak, 2005; Somsamak *et al.*, 2005, 2006
Bioreactor sludge, Texas	Fe(III) reducing	5 mg/liter, 380 days, 100%	Similar results for all 72 active microcosms	Pruden *et al.*, 2005
Groundwater samples from contaminated wells, Leuna, Germany	Sulfate reducing, nitrate reducing	~50 mg/liter, 180 days, 60%	Out of 20 microcosms, only 1 sulfate-reducing and 3 nitrate-reducing cultures showed MTBE degradation	Fischer *et al.*, 2005

manganese(IV)-reducing (Bradley *et al*., 2002), iron (III)-reducing (Bradley *et al*., 2001b; Finneran and Lovley, 2001; Pruden *et al*., 2005), and sulfate-reducing conditions (Bradley *et al*., 2001a; Fischer *et al*., 2005; Somsamak *et al*., 2001, 2006). These studies have been conducted with microcosms inoculated with sediments, groundwater, or bioreactor sludge from eight different US states and one location in Germany. Initial MTBE concentrations have ranged from 1.3 to 100 mg/liter and the reported extent degradation ranged from 3 to 100%. Degradation rates were generally slow, as the minimum time reported for 100% initial loss of MTBE was over 240 days. Complete degradation has been observed under all but Mn (IV)-reducing conditions. Most studies examining several different degradation conditions or inocula observed degradation in only a few samples under a few conditions, indicating that the microorganisms mediating this process are rare. Furthermore, degradation was frequently not observed in every replicate culture established from the same inocula and under the same conditions. Additionally, many studies testing for anaerobic MTBE degradation have yielded negative results (Borden *et al*., 1997; Chen *et al*., 2005; Somsamak, 2005; Suflita and Mormile, 1993). TBA has often been detected as an intermediate of anaerobic MTBE biodegradation, suggesting that cleavage of the ether bond is the initial step in the degradation pathway, similar to that reported for aerobic degradation. Under both aerobic and anaerobic conditions, the slow degradation of TBA indicated by an enrichment of these components suggests that the degradation of this metabolite is a crucial step in MTBE mineralization.

We have evaluated the anaerobic biodegradability of MTBE and the related fuel oxygenates under different anaerobic conditions. Using different sediment inocula under conditions promoting either denitrification, sulfate reduction, Fe(III) reduction, or methanogenesis, we investigated the potential for anaerobic degradation of MTBE, and two other fuel oxygenates, ETBE, and TAME (Somsamak *et al*., 2001, 2005). In our initial study, we demonstrated the biodegradation of MTBE and TAME under sulfate-reducing conditions (Somsamak *et al*., 2001). Complete loss of MTBE and TAME was observed under sulfate-reducing conditions, concomitant with the reduction of sulfate. Stoichiometric amounts of TBA and *tert*-amyl alcohol (TAA) accumulated, indicating that cleavage of the methyl group was the initial step in MTBE and TAME biodegradation under sulfate-reducing conditions. No transformation of MTBE or TAME was observed under the other electron-accepting conditions over 3 years (further demonstrating the recalcitrance of these compounds). Under all conditions tested, there was no biotic loss of ETBE observed. MTBE and TAME were depleted on refeeding the sulfidogenic cultures. The accumulation of TBA and TAA, products of *O*-demethylation of MTBE and TAME, respectively, indicated that biodegradation of these intermediates might be the rate-limiting step for complete degradation of the parent compounds.

Additional microcosms were established using inocula from various sites, including several sediments from New Jersey and sites in San Diego Bay area (Somsamak, 2005; Somsamak *et al.*, 2005, 2006). Anaerobic sediment microcosms degraded MTBE and TAME with the accumulation of TBA and TAA, respectively, under both methanogenic and suldfidogenic conditions (Fig. 1.3). The microcosms were successfully transferred into fresh medium with enrichment of MTBE-degrading methanogenic and sulfidogenic populations, respectively. MTBE-enriched cultures also exhibited biotransformation of TAME to TAA. The cultures, however, did not biodegrade the ethyl ether, ETBE (Fig. 1.4). This observation is

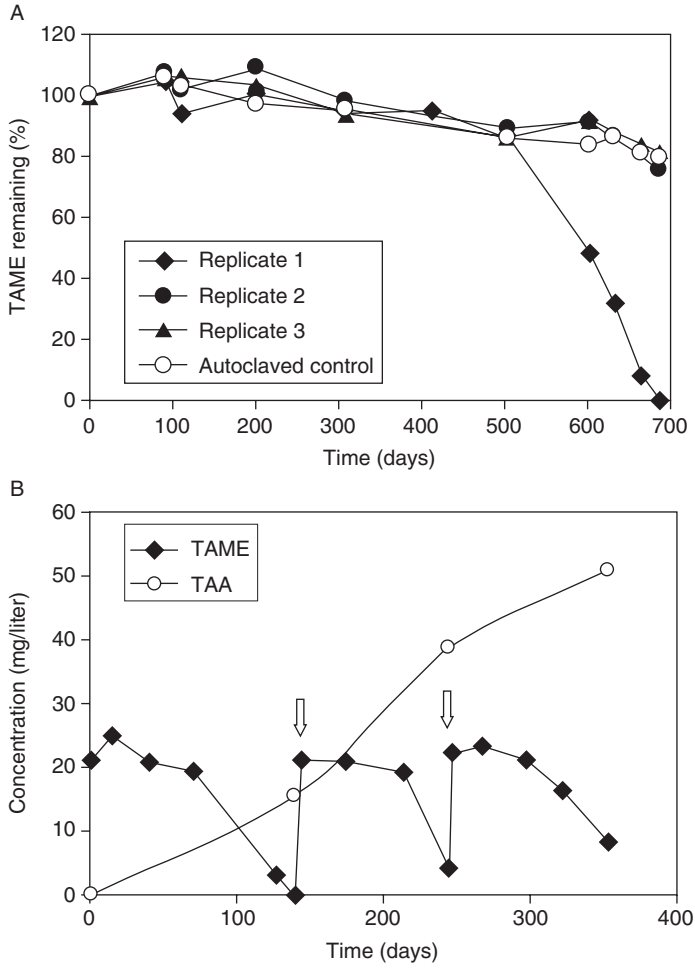

FIGURE 1.3 Biotransformation of TAME (initial concentration 100 mg/liter) to TAA by anaerobic sediment cultures of New York Harbor sediment (A) and in enrichment cultures on respiking (B). Adapted from Somsamak (2005).

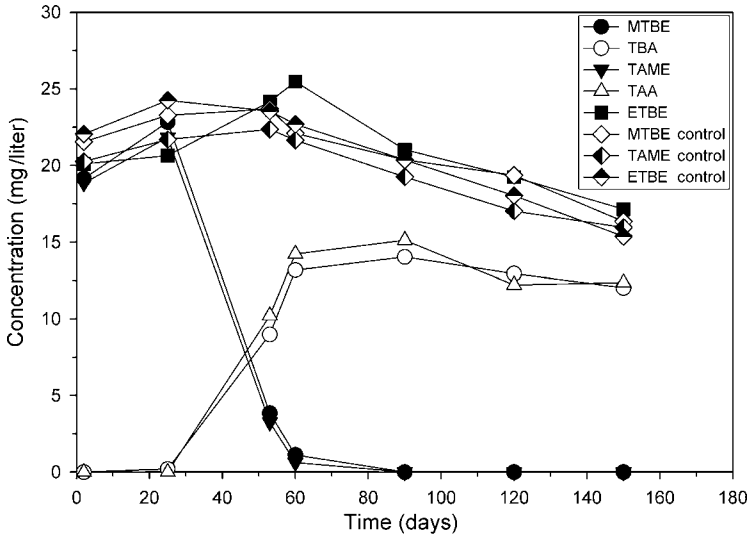

FIGURE 1.4 Utilization of MTBE, TAME, and ETBE by a methanogenic MTBE-degrading culture enriched from anaerobic Arthur Kill sediment (adapted from Somsamak, 2005).

consistent with the characteristics of sulfidogenic MTBE-degrading cultures reported (Somsamak *et al.*, 2001).

The role of methanogens was investigated by adding bromoethanesulfonic acid (BES), the methanogenesis inhibitor. With BES, methane production was greatly reduced, but there was no inhibition on MTBE degradation (Somsamak *et al.*, 2005). Similarly, MTBE degradation in the sulfidogenic cultures was not inhibited by sodium molybdate, an inhibitor of sulfate reduction (Somsamak, 2005; Somsamak *et al.*, 2006). This suggests that microorganisms other than sulfate reducers or methanogens were responsible for cleaving the ether bond of MTBE, although they might ultimately utilize biodegradation product(s) coupled to methanogenesis or sulfate reduction, respectively. Although BES addition did not inhibit MTBE degradation, a prolonged lag period was seen, which demonstrates the importance of the entire microbial community for MTBE/TAME degradation, and the effect of BES on the microbial community interactions may provide information for optimizing and maximizing biodegradation rates in the environments.

V. MONITORING NATURAL ATTENUATION

Monitored natural attenuation is being increasingly implemented for a number of environmental pollutants (Hunkeler *et al.*, 2005; Kao *et al.*, 2006; Reisinger *et al.*, 2005). Natural processes, including dispersion, sorption,

dilution, volatilization, and biodegradation, control plume migration and reduce MTBE concentration. Among these processes, biodegradation is the most effective to reduce the mass of contaminant in the environment in a sustainable way. One challenge, however, is to accurately assess the efficiency of the remediation techniques *in situ*. Generally, the contaminant concentration needs to be recorded to demonstrate the losses over time. Other lines of evidence to demonstrate *in situ* biodegradation may be the detection of degradation intermediates, as well as the depletion of electron acceptor. In the case of MTBE, the information collected is not always conclusive. TBA, the intermediate of MTBE degradation, is also a by-product of MTBE production. Moreover, the biodegradation of relatively more biodegradable gasoline components, such as BTEX, might lead to the depletion of electron acceptors. Therefore, novel techniques are needed as a tool to assess on-going *in situ* MTBE biodegradation and to document *in situ* MTBE biodegradation in natural attenuation approaches.

Compound-specific stable isotope analysis has received increased attention as a tool for assessing *in situ* biodegradation of environmental pollutants (Hunkeler *et al.*, 2001; Mancini *et al.*, 2003; Meckenstock *et al.*, 2004; Richnow *et al.*, 2003). This method is based on the fractionation of stable isotopes that occurs during microbial degradation of the compound leading to an enrichment of heavier isotopes in the residual fraction. Thus, the resulting shift in the ratio for carbon, hydrogen, or another element can be used as an indicator for *in situ* biodegradation. With the appropriate compound-specific isotopic enrichment factor (ε) or isotope fractionation factor (α) determined from controlled laboratory experiments, the extent of biodegradation (C_t/C_o) can be estimated by using the following equation:

$$\delta^{13}\mathrm{R}_t = \delta^{13}\mathrm{R}_0 + \varepsilon \ln\left(\frac{C_t}{C_0}\right)$$

where δ^{13}R represents the carbon isotope ratios or hydrogen of MTBE, C is the MTBE concentration and the index (0, t) describes the beginning (0) and the reaction time (t).

To quantify *in situ* biodegradation, isotope fractionation factors representative for the biogeochemical conditions governing *in situ* biodegradation are essential. For a proper assessment of biodegradation by the compound-specific stable isotope analysis, it is important to choose the right laboratory-derived α (or ε) for the initial step of biodegradation, which is dominant in the investigated environment. Since the geochemical conditions are not always well known and several electron-accepting processes can occur concomitantly, this is not such a simple task.

The combined use of hydrogen and carbon isotope analysis was proposed as a tool to characterize the biodegradation pathway of MTBE in the field (Zwank *et al*., 2005). This hypothesis presumes that degradation pathways can be clearly distinct by the isotope fractionation pattern of the residual fraction. Studies on the variability of carbon and hydrogen isotope fractionation have shown that the simultaneous analysis of carbon and hydrogen isotope composition to analyze pathways of MTBE in the field may have some potential; however, the carbon and hydrogen fractionation pattern of aerobic biodegradation can also lead to similar pattern as expected from anaerobic degradation (Rosell *et al*., 2007). Nevertheless, the extent of anaerobic carbon and hydrogen fractionation seems to be higher than aerobic biodegradation. Therefore, two-dimensional isotope approaches open opportunities to analyze the predominant degradation process in the field, but further studies on the variability of hydrogen and carbon fractionation during anaerobic biodegradation are needed for a final evaluation.

To date, there have been only four studies reporting carbon isotope fractionation studies during anaerobic MTBE degradation. As previously discussed, we have established a variety of anaerobic MTBE-degrading microcosms using inocula from various contaminated sediments (Somsamak *et al*., 2001, 2005, 2006). Over the past several years, these cultures have been successfully transferred into fresh medium with enrichment of MTBE-degrading populations. These stable enrichment cultures were then used to determine the carbon isotopic fractionation during anaerobic biodegradation of MTBE and TAME under different anaerobic conditions (Somsamak *et al*., 2005, 2006).

The studies have conclusively demonstrated that compound-specific stable isotope analysis can be used as a tool to demonstrate active *in situ* MTBE biodegradation (Somsamak *et al*., 2005, 2006). Moreover, with the appropriate isotopic enrichment factor (ε), the extent of biodegradation can be estimated. A number of factors, including the microorganism, degradation mechanisms, growth conditions, and terminal electron-accepting processes, impact isotope fractionation. Thus, in order to accurately assess anaerobic *in situ* MTBE degradation through carbon isotope analysis, the isotope enrichment factor needs to be determined for different microbial communities and electron-accepting conditions. We demonstrated that anaerobic MTBE transformation to TBA under methanogenic and sulfidogenic conditions is accompanied with significant enrichment of ^{13}C in the residual MTBE. The isotopic enrichment factors (ε) estimated for each enrichment were almost identical ($-13.4‰$ to $-14.6‰$, $r^2 = 0.89$–0.99). An ε value of $-14.4 \pm 0.7‰$ was obtained from regression analysis ($r^2 = 0.97$, $n = 55$, 95% confidence intervals), when all data from our MTBE-transforming anaerobic cultures were combined (Fig. 1.5). Similar fractionation was observed also when methanogenesis

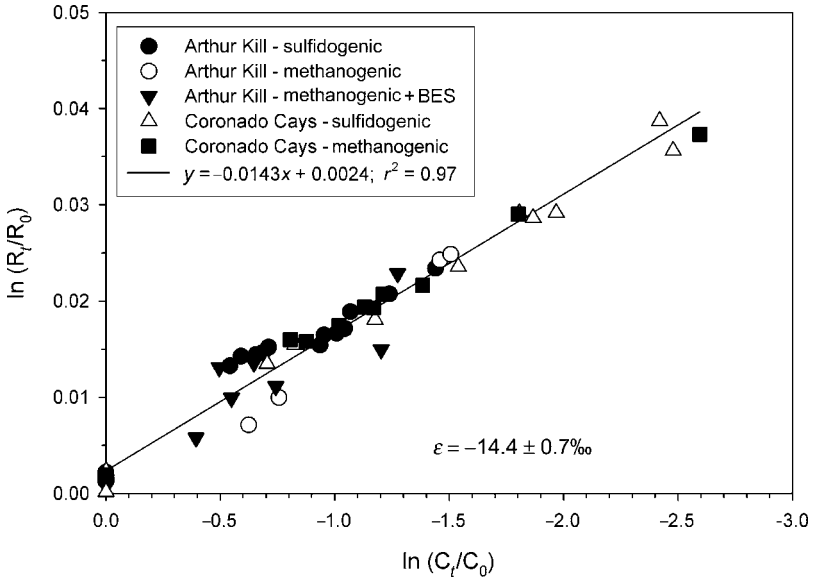

FIGURE 1.5 Double logarithmic plots according to Rayleigh equation of the isotopic composition versus the residual substrate concentration. Data obtained from sulfidogenic, methanogenic, and bromoethane sulfonic acid (BES)-inhibited methanogenic enrichments of Arthur Kill and Coronado Cays sediments. The isotopic enrichment factor ε was determined from the slope of the curve (b) with $b = 1/\alpha - 1$ and $\varepsilon = 1000 \times b$. (Data from Somsamak *et al*., 2005, 2006.)

or sulfate reduction was inhibited. This is a crucial step because the extent of *in situ* MTBE biodegradation can be estimated using the isotopic enrichment factor determined in our studies. This approach has the potential to be an effective and reliable tool to assess the natural attenuation and properly manage MTBE-contaminated environments. These values are similar to those estimated in other studies. Kolhatkar *et al*. (2002) demonstrated carbon isotope fractionation during anaerobic MTBE degradation at a field site and in laboratory microcosms although the electron-accepting processes were not identified. Kuder *et al*. (2005) monitored carbon isotope fractionation during anaerobic MTBE by enrichments compared to fractionation in groundwater at nine gasoline spill-sites. These values are in contrast to the much lower carbon isotope fractionation factors observed for aerobic biodegradation of MTBE (Gray *et al*., 2002; Hunkeler *et al*., 2001; Rosell *et al*., 2007; Zwank *et al*., 2005).

The similar magnitude of carbon isotope fractionation observed in all the anaerobic enrichments regardless of culture or electron-accepting condition (Fig. 1.5) suggests that the terminal electron-accepting process may not significantly affect carbon isotope fractionation during anaerobic

MTBE degradation. The current information available suggests that the initial step of degradation is similar among studied MTBE-degrading cultures. For instance, all enrichments produce TBA as an intermediate or end-product of degradation, suggesting that cleavage of ether linkage is the initial step of MTBE degradation. Both the methanogenic and sulfidogenic enrichments continued to utilize MTBE even when the electron-accepting process of the community was inhibited, even though retardation of overall utilization rate occurred (Somsamak *et al*., 2005, 2006). The finding suggests that MTBE-degradation is not directly coupled to sulfate reduction or methanogenesis. It is thus possible that the same microorganisms are responsible for MTBE degradation in both methanogenic and sulfate-reducing communities. The possible hypothesis is that MTBE-degrading microorganisms cleave the ether linkage and produce a C-1 compound or acetate through acetogenic pathways, which consequently serve as a carbon source for the overall methanogenic or sulfate-reducing communities. Therefore, these MTBE-utilizing microorganisms could function in various types of environments and electron-accepting conditions. However, two acetogenic bacteria tested, *Acetobacterium woodii* and *Eubacterium limosum*, were unable to degrade MTBE (Mormile *et al*., 1994). To date, the anaerobic MTBE-degrading bacteria enriched from different sediments have not been identified and the mechanisms of anaerobic MTBE degradation have yet to be elucidated.

VI. SUMMARY

MTBE, used as an octane enhancer and fuel oxygenate in reformulated gasoline, has become a major contaminant of water resources. MTBE is resistant to common methods used for efficient removal of other fuel components from groundwater and is relatively resistant to biodegradation. There has been increasing interest in the development of effective technologies to remediate MTBE-contaminated sites, for instance pump-and-treat techniques, biostimulation, and bioaugmentation. The full-scale implementation of these techniques is, however, considerably costly and is currently economically applicable for a few sites with highly sensitive receptors. Therefore, the fate of MTBE in the environment is mainly dependent on natural remediation processes. Although anaerobic MTBE degradation has been demonstrated in the laboratory, it is not known how efficiently anaerobic degradation occurs in contaminated environments. Furthermore, no organisms have yet been identified from the anaerobic MTBE-degrading consortia, nor is the anaerobic biodegradation mechanism known. Our information about the role of anaerobic microbial communities involved in the biodegradation of MTBE is thus limited. Methods for *in situ* monitoring of natural attenuation are being developed

through studies of the stable isotope fractionation values for anaerobic MTBE degradation. Compound-specific stable isotope analysis has emerged as a useful tool to demonstrate active *in situ* MTBE biodegradation and will contribute to the development of appropriate remediation measures.

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CHAPTER 2

Controlled Biomineralization by and Applications of Magnetotactic Bacteria

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I. INTRODUCTION

The magnetotactic bacteria represent a morphologically, physiologically, and phylogenetically diverse assemblage of motile, mostly aquatic prokaryotes that passively align along geomagnetic field lines as they swim (Bazylinski and Frankel, 2004). The serendipitous discovery of these intriguing microorganisms by R. P. Blakemore in 1974 (Blakemore, 1975, 1982) proved to have a serious impact not just in microbiology, but also in a number of diverse research fields including geology, mineralogy and biomineralization, crystallography, chemistry, biochemistry, physics, limnology and oceanography, and even astrobiology. In addition, the ever increasing amount of information on these bacteria has put these organisms in the midst of several important scientific controversies, some still even hotly debated today (Thomas-Keprta *et al.*, 2000, 2001, 2002).

Magnetotactic bacteria are fastidious with regard to their growth requirements and are difficult to isolate in pure culture and cultivate in the laboratory. Because of this, research in this area has been painfully slow at times. Despite over 30 years of study, the genetic and molecular basis of magnetotaxis has only started to become realized in the last decade with the development of tractable genetic systems in these organisms. In this chapter, we describe general features of the magnetotactic bacteria and their magnetic mineral inclusions, but focus mainly on the molecular and genetic basis for controlled biomineralization of these organisms as well as on applications of the magnetotactic bacteria and their intracellular magnetic mineral crystals.

II. FEATURES OF THE MAGNETOTACTIC BACTERIA

A. General features

It is important to understand that the term ''magnetotactic bacteria'' has no taxonomic meaning and members of this group should be looked on as a diverse assemblage of prokaryotes that share a common trait: the ability to biomineralize magnetosomes and express magnetotaxis (Bazylinski and Frankel, 2004). Magnetosomes are defined as intracellular crystals of a magnetic mineral surrounded by a lipid bilayer membrane (Balkwill

et al., 1980; Gorby *et al.*, 1988; Komeili *et al.*, 2004). Despite their differences, the magnetotactic bacteria share several features. All known species or strains (1) are Gram-negative prokaryotes phylogenetically associated with the domain bacteria, (2) are motile by means of flagella, (3) grow only microaerophilically with oxygen or anaerobically or both, (4) with one exception possess a solely respiratory form of metabolism, (5) display nitrogenase activity and thus are able to fix atmospheric dinitrogen, (6) are mesophilic with respect to growth temperatures, and (7) all possess magnetosomes (Bazylinski and Frankel, 2004).

Most of all cultivated strains of magnetotactic bacteria are facultatively anaerobic microaerophiles. However, physiology and metabolism of the strains are very different and the physiological state of the cells (e.g., how they are grown), in some cases, can have a profound effect on magnetosome formation and cellular magnetism (Bazylinski and Williams, 2007).

B. Distribution and ecology

Magnetotactic bacteria are distributed throughout the world and have been found on every continent. However, on a local basis, these bacteria are confined to a very specific habitat, a relatively thin, microaerobic zone called the oxic–anoxic interface (OAI) within water columns or sediments with vertical chemical stratification (Fig. 2.1). They can reach relatively high cell numbers (e.g., 10^4 cells/ml) at the OAI and the anoxic regions of the habitat or both (Bazylinski and Moskowitz, 1997; Bazylinski *et al.*, 1995; Simmons *et al.*, 2004).

In almost all freshwater systems, sulfate concentrations are zero or very low, and the OAI is generally located at the water–sediment interface or several millimeters below it. Although the situation in many deep sea sites is similar to this, it can be strikingly different in mostly undisturbed, marine, coastal habitats. Seawater contains ~28-mM sulfate and anaerobic sulfate-reducing bacteria reduce sulfate to hydrogen sulfide through a respiratory process. The hydrogen sulfide diffuses upward from sediments into the water column causing the OAI to occur in the water column. What results is an inverse oxygen; hydrogen sulfide concentration double gradient where oxygen diffuses downward from air at the surface and hydrogen sulfide (H_2S) diffuses upward from the anoxic zone of the water column or sediments (Fig. 2.1). Magnetotactic bacteria are known to produce two types of minerals: irons oxides and iron sulfides. Those that produce iron oxides only biomineralize magnetite (Fe_3O_4) (Frankel *et al.*, 1979) and those that produce iron sulfides biomineralize greigite (Fe_3S_4) (Mann *et al.*, 1990) in addition to some mineral precursors to greigite (Pósfai *et al.*, 1998a,b). Only iron oxide-producing types have been found in freshwater systems while both iron oxide and iron sulfide producers are present in marine environments. In marine systems, the iron oxide producers are generally found

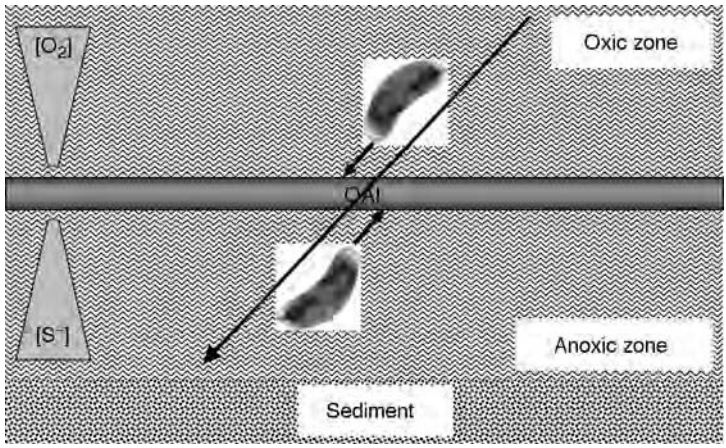

FIGURE 2.1 Schematic of the oxic–anoxic interface (OAI) occurring in the water column due to an inverse concentration gradient of O_2 from air at the surface and H_2S from the reduction of sulfate by sulfate-reducing bacteria present in the sediment and anoxic region of the water column. Cells of magnetotactic bacteria align and swim along the Earth's geomagnetic field lines (shown as arrows) and use chemotaxis to locate and maintain position at an optimal O_2 concentration. The geomagnetic field lines are inclined because of the vertical component of the Earth's geomagnetic field. At the equator, the direction of the field is horizontal and field lines become increasingly more inclined going to either magnetic pole where the field lines are vertical.

at the OAI and the iron sulfide producers reside slightly below the OAI where O_2 is absent and S^{2-} is present. Iron sulfide-producing magnetotactic bacteria have only been found in marine habitats.

C. Phylogeny and taxonomy

As previously mentioned, all known cultured magnetotactic bacteria are phylogenetically affiliated with the domain Bacteria. In addition, all retrieved 16S rDNA sequences for uncultured species from environmental samples are consistent with this generalization. However, there appears to be no good reason why some members of the Archaea cannot be magnetotactic especially when taking into consideration the evidence that the genes for magnetosome synthesis have been distributed to many types of bacteria through lateral transfer. Perhaps, many types of habitats where Archaea are the dominant prokaryotes (e.g., hyperthermic environments) have not been sampled widely enough for magnetotactic bacteria.

Cultured and uncultured iron oxide-producing magnetotactic bacteria are associated with the α- and δ-subgroups of the Proteobacteria, a very large collection of Gram-negative bacteria, and the *Nitrospira phylum*. Those in the α-subgroup include species of the freshwater genus *Magnetospirillum*; all the marine and freshwater magnetotactic cocci studied thus far; a marine

spirillum known as strain MMS-1 (Bazylinski and Williams, 2007); a group of marine vibrios (e.g., strain MV-1) (Bazylinski *et al.*, 1988); and some uncultured magnetotactic bacteria collected from different aquatic environments (Spring *et al.*, 1992, 1993, 1995) (Fig. 2.2). Only one iron oxide-producing magnetotactic bacterium, the freshwater sulfate-reducing species *Desulfavibrio magneticus* strain RS-1, is phylogenetically associated with the δ-subgroup (Fig. 2.2).

The genus *Magnetospirillum* phylogenetically lies within the Rhodospirillaceae (α-1 group) and is nested within a cluster of α-Proteobacteria which includes the phototrophic genera *Phaeospirillum* and *Rhodospirillum*, and the nonphototrophic genus *Azospirillum* (Burgess *et al.*, 1993). Strain MC-1, the only magnetotactic coccus isolated in pure culture thus far, and

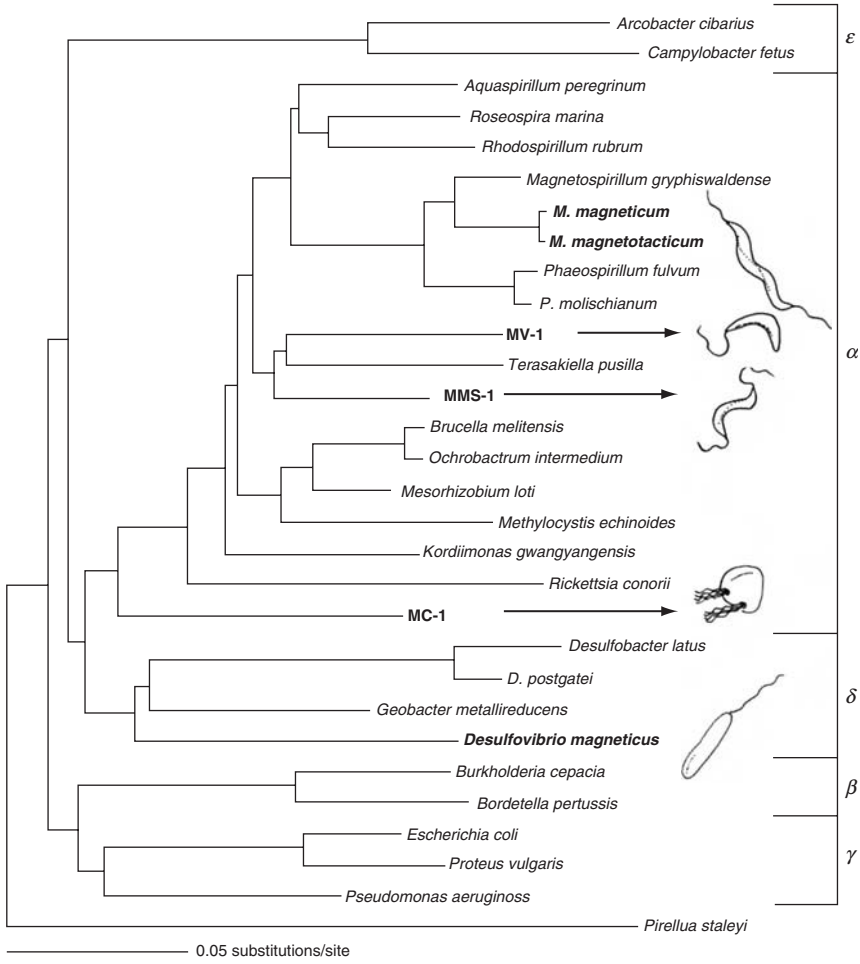

FIGURE 2.2 Phylogenetic relatedness of some magnetotactic bacteria in the different subgroups of the Proteobacteria. Magnetotactic bacteria are in bold.

other uncultured marine and freshwater magnetotactic cocci in general show no close affinity to any other α-Proteobacteria and appear to constitute a unique lineage that diverged early from the main branch of the α-Proteobacteria (DeLong *et al.*, 1993; Fernandez de Henestrosa *et al.*, 2003) and are only distantly related to other magnetotactic α-Proteobacteria. Phylogenetically, marine vibrioid strains like MV-1 and the marine spirillum strain MMS-1 represent new genera within the α-Proteobacteria and have no known close phylogenetic relatives (Bazylinski and Williams, 2007; DeLong *et al.*, 1993). *D. magneticus* strain RS-1 clearly fits into the genus *Desulfovibrio* in the family Desulfovibrionaceae and order Desulfovibrionales within the δ-Proteobacteria (Kawaguchi *et al.*, 1995), its closest relative being *D. burkinensis* (similarity 98.7%) (Sakaguchi *et al.*, 2002). The cellular fatty acid profile of *D. magneticus* is also consistent with its inclusion in the genus *Desulfovibrio* (Sakaguchi *et al.*, 2002). A very large rod-shaped bacterium that contains multiple bundles of chains of magnetosomes, *Candidatus* Magnetobacterium bavaricum (Spring *et al.*, 1993), is phylogenetically affiliated with the Nitrospira phylum group and not with the Proteobacteria (Spring and Bazylinski, 2000; Spring *et al.*, 1993).

Little is known regarding the phylogeny of the iron sulfide-producing magnetotactic bacteria. Two organisms, the many-celled magnetotactic prokaryote (MMP) and a large rod, have been partially characterized morphologically and phylogenetically. The MMP is an unusual, large, multicellular bacterium that consists of about 10–30 cells arranged in a roughly spherical manner, and is motile as a unit but not as separate cells (Rodgers *et al.*, 1990). Each cell is multiflagellated on one side. Phylogenetically, the MMP is affiliated with the sulfate-reducing bacteria in the δ-Proteobacteria suggesting that it is also a sulfate-reducing bacterium (DeLong *et al.*, 1993) (Fig. 2.2). The MMP has been found in brackish-to-marine habitats all over the world and, based on its unique morphology, it was generally assumed that it represented a single species. However, Simmons and Edwards (2007) reported 16S rDNA gene sequences from a natural population of MMPs and found the sequences representing five lineages separated by at least a 5% sequence divergence. Because of this great unexpected phylogenetic diversity, the MMP should be considered a separate genus that contains several species in the *δ*-Proteobacteria rather than a single species as previously thought (Simmons and Edwards, 2007). A marine rod-shaped iron sulfide producer was found to be affiliated with γ-Proteobacteria suggesting a metabolism based on iron rather than sulfur (Simmons *et al.*, 2004).

D. Physiology

1. *Magnetospirillum* species

The species of this genus are the most studied of the magnetotactic bacteria. Known species include *M. magnetotacticum* strain MS-1 (Maratea and Blakemore, 1981), *M. gryphiswaldense* strain MSR-1 (Schleifer *et al.*, 1991),

and *M. magneticum* strain AMB-1 (Matsunaga *et al.*, 1991). All are obligate microaerophiles when grown with oxygen as the sole terminal electron acceptor but are capable of denitrification converting nitrate to dinitrogen gas (Bazylinski and Blakemore, 1983; D. Schüler, personal communication). However, unlike most denitrifiers that perform this process anaerobically, *M. magnetotacticum* requires very small amounts of oxygen even when grown with nitrate (Bazylinski and Blakemore, 1983). All three species biomineralize cubo-octahedral crystals of magnetite within their magnetosomes (Fig. 2.3A–C) and only under microaerobic and/or anaerobic conditions depending on the species.

All three *Magnetospirillum* species are known as chemoorganoheterotrophs as they have been routinely grown in this fashion but they have the potential for autotrophic growth because all three species have a complete ribulose-1,5-bisphosphate carboxylase/oxygenase (RubisCO) gene within their genome indicating the potential to synthesize at least one Calvin-Benson-Bassham cycle enzyme (Bazylinski *et al.*, 2004; Matsunaga *et al.*, 2005; D. Schüler personal communication). However, no electron donor has yet been found to support growth of these organisms. Grown under chemoorganoheterotrophic conditions, *Magnetospirillum* species utilize only certain organic acids as electron and carbon sources. All three species show nitrogenase activity and thus are capable of fixing atmospheric dinitrogen (Bazylinski *et al.*, 2000).

2. *D. magneticus* strain RS-1

D. magneticus strain RS-1 is a strictly anaerobic chemoorganoheterotrophic vibrio that utilizes lactate, pyruvate, malate, oxaloacetate, and glycerol as electron donors and carbon sources (Sakaguchi *et al.*, 2002). Cells respire with fumarate, sulfate, and thiosulfate, converting the latter two compounds to sulfide, but not with sulfite, nitrate, elemental sulfur, Fe(III), or O_2 as terminal electron acceptors. This species is the only magnetotactic bacterium known to ferment as cells ferment pyruvate to acetate, CO_2, and H_2 in the absence of a terminal electron acceptor (Sakaguchi *et al.*, 2002). This strain is the only magnetotactic bacterium in pure culture that biomineralizes tooth-shaped crystals of magnetite in their magnetosomes (Fig. 2.3D–E).

3. Strain MC-1

Strain MC-1 is the only magnetotactic coccus in pure culture. Cells are roughly spherical, are 1–2 μm in diameter, and are bilophotrichously flagellated (having two bundles of flagella on one side of the cell) (Frankel *et al.*, 1997). The magnetotactic behavior of this species, like all other known magnetotactic cocci, defines polar magneto-aerotaxis (Frankel *et al.*, 1997).

This strain does not exhibit much in the way of metabolic versatility. It is an obligate microaerophile that grows chemolithoautotrophically on

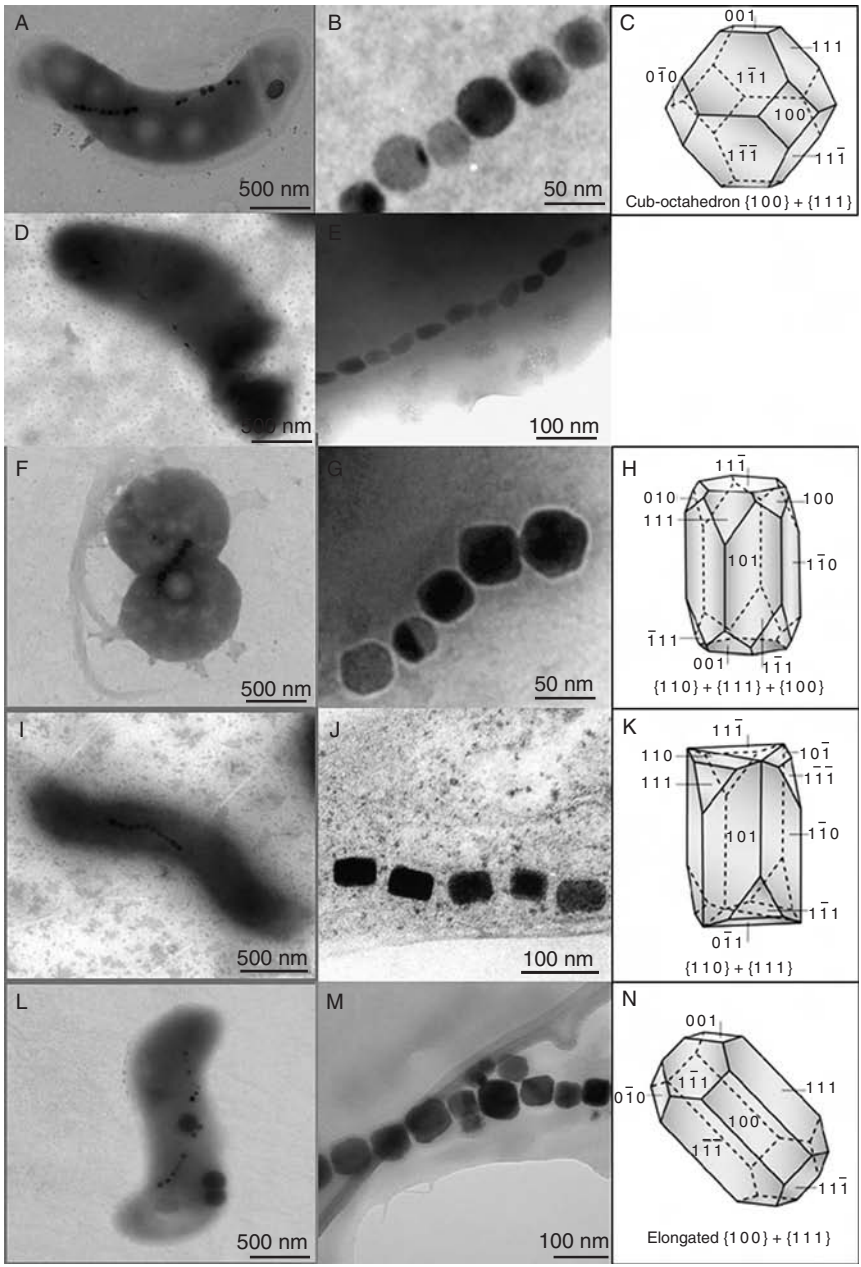

FIGURE 2.3 Transmission electron micrographs of cells of different species of cultured magnetite-producing magnetotactic bacteria (first column) with accordant magnetosomes (second column) and idealized crystal habits as determined by high-resolution TEM (third column). Freshwater *Magnetospirillum* species (A–C) all biomineralize cubo-octahedral

sulfide and thiosulfate as electron donors and chemoorganoheterotrophically with acetate (Bazylinski and Williams, 2007; Williams *et al.*, 2006). Unlike other autotrophic magnetotactic bacteria, this strain utilizes the reverse tricarboxylic acid cycle for carbon dioxide fixation and autotrophy (Williams *et al.*, 2006). Cells of strain MC-1 biomineralize a single chain of magnetosomes that contain elongated prismatic Fe_3O_4 crystals (Meldrum *et al.*, 1993a) (Fig. 2.3F–H). Strain MC-1, like *Magnetospirillum* species, also appears to be capable of dinitrogen fixation (Bazylinski and Williams, 2007).

4. Strain MV-1 and other related magnetotactic vibrios

Strain MV-1 is a small marine vibrio that is a facultatively anaerobic microaerophile. It is comma-shaped or helical in morphology and has a single polar flagellum (Bazylinski and Williams, 2007). Cells of strain MV-1 grow chemoorganoheterotrophically as well as chemolithoautotrophically and chemoorganoautotrophically (Bazylinski *et al.*, 2004). Chemoorganoheterotrophic growth is supported by O_2 microaerobically or by N_2O anaerobically as terminal electron acceptors, and certain organic acids and amino acids are used as electron donors for chemoorganoheterotrophic growth (Bazylinski *et al.*, 1988). Strain MV-1 utilizes sulfide, thiosulfate, and possibly soluble Fe(II) but not tetrathionate or Fe(II) as FeS or $FeCO_3$ (siderite) microaerobically as electron donors to support autotrophy (Bazylinski *et al.*, 2004). Chemoorganoautotrophic growth occurs through the oxidation of formate as an electron source (Bazylinski *et al.*, 2004). Autotrophy is through the Calvin-Benson-Bassham cycle (Bazylinski *et al.*, 2004).

Some recent evidence suggests that this strain may be a complete denitrifier, converting nitrate to dinitrogen gas. Cells are capable of fixing dinitrogen (Bazylinski and Williams, 2007). Cells of strain MV-1 and related strains biomineralize a single chain of elongated, pseudoprismatic Fe_3O_4-containing magnetosomes oriented along the long axis of the cell (Bazylinski *et al.*, 1988), which sometimes have large gaps between them (Bazylinski *et al.*, 1995). The morphology of the Fe_3O_4 crystals has been described as truncated hexa-octahedrons (Thomas-Keprta *et al.*, 2001) (Fig. 2.3I–K).

5. Strain MMS-1

Cells of this bipolarly flagellated, marine strain are small (1 to 3-μm long) and range in morphology from being bean-shaped to partially helical to fully helical (Bazylinski and Williams, 2007). Little is known about

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crystals; *D. magneticus* (D and E), a sulfate-reducing magnetotactic bacterium, synthesizes bullet-shaped crystals; the magnetotactic coccus, strain MC-1 (G and H), synthesizes elongated pseudohexagonal prismatic crystals; the magnetotactic vibrio, strain MV-1 (I–K), also produces elongated pseudohexagonal prismatic crystals; and the marine magnetotactic spirillum, strain MMS-1 (L–N), forms elongated cubo-octahedral magnetosomes.

this strain. It is capable of chemoorganoheterotrophic and chemolithoautotrophic growth. Thiosulfate is used as an electron donor supporting chemolithoautotrophic growth and certain organic acids for chemoorganoheterotrophic growth (Bazylinski and Williams, 2007). Cells appear to be obligate microaerophiles and no alternate terminal electron acceptors to oxygen have yet been found to support anaerobic growth. Cells are capable of nitrogen fixation and they biomineralize a single chain of elongated, octahedral crystals of Fe_3O_4 in their magnetosomes (Meldrum *et al.*, 1993b) (Fig. 2.3L–N).

III. THE MAGNETOSOME

All magnetotactic bacteria contain magnetosomes, intracellular structures comprising magnetic iron mineral crystals enveloped by a phospholipid bilayer membrane (Gorby *et al.*, 1988; Komeili *et al.*, 2004). The composition, size, and morphology of the magnetosome crystals are important in determining their physical and magnetic properties.

A. Composition of magnetosome crystals

As mentioned earlier, magnetotactic bacteria biomineralize either magnetite (Frankel *et al.*, 1979) or greigite (Mann *et al.*, 1990) within their magnetosomes. Both these minerals are permanently magnetic at ambient temperature. Several other nonmagnetic minerals have been identified in iron sulfide-containing magnetosomes, including mackinawite (tetragonal FeS) and a sphalerite-type cubic FeS that are presumed to be precursors to greigite (Pósfai *et al.*, 1998a,b). Based on transmission electron microscopic observations, electron diffraction, and known iron sulfide chemistry (Berner, 1967, 1970; Lennie *et al.*, 1997; Morse and Rickard, 2004), the likely geochemical pathway scheme for greigite formation in magnetotactic bacteria is cubic FeS → mackinawite (tetragonal FeS) → greigite (Fe_3S_4) as described by Pósfai *et al.* (1998a,b). Orientation relationships between mackinawite and greigite minerals suggest that the cubic close-packed S substructure does not change during the conversion and that only the iron atoms rearrange. Magnetic, monoclinic, pyrrhotite (Fe_7S_8) (Farina *et al.*, 1990), and nonmagnetic iron pyrite (FeS_2) (Mann *et al.*, 1990) have been identified in the MMP but probably represent misinterpretations of electron diffraction patterns. Two types of uncultured bacteria have been found to biomineralize both magnetite and greigite: a group of large marine rod-shaped bacteria (Bazylinski *et al.*, 1993b, 1995; Pósfai *et al.*, 2007) and the MMP (Keim *et al.*, 2007). The mineral composition of the magnetosome appears to be under strict chemical control since cells of several cultured

magnetotactic bacteria synthesize only magnetite, not greigite, even when grown in the presence of hydrogen sulfide (Meldrum *et al.*, 1993a,b).

Generally, magnetite crystals in magnetosomes in cells of cultured and uncultured magnetotactic bacteria are of high chemical purity (Bazylinski and Frankel, 2004). Several studies have shown that iron is not replaced by other transition metal ions in magnetite despite the fact that cells must be grown in the presence of a number of these metals to synthesize active enzymes. The situation may be different for some organisms in natural environments. Trace amounts of titanium were found in magnetite crystals of an uncultured freshwater magnetotactic coccus collected from a wastewater treatment pond (Towe and Moench, 1981) and significant amounts of copper were found in greigite crystals in the MMP collected in a salt marsh (Bazylinski *et al.*, 1993a).

B. Size of magnetosome crystals

Almost all mature magnetite and greigite crystals in magnetosomes fall within a narrow size range of about 35–120 nm when measured along their long axes, although variations exist between species (Bazylinski and Moskowitz, 1997; Bazylinski *et al.*, 1994; Devouard *et al.*, 1998; Frankel *et al.*, 1998). Magnetite and greigite particles in this range are stable single-magnetic domains (SMD) and are permanently magnetic at ambient temperature (Butler and Banerjee, 1975; Diaz-Ricci and Kirschvink, 1992). Grains within the SMD size range are uniformly magnetized, which means their magnetic dipole moment is maximum, that is, equal to the saturation moment M_s. Smaller particles, those less than 30 nm, do not have stable, remanent magnetizations at ambient temperature and are referred to as superparamagnetic particles. Cells initially produce these smaller particles which eventually grow into permanent, SMD-sized crystals (Bazylinski and Frankel, 2000a). Domain walls tending to form multiple domains occur in larger crystals causing them to be nonuniformly magnetized, thereby reducing the remanent magnetization. Thus, by producing SMD particles, magnetotactic bacteria produce the optimum crystal size for a maximum permanent magnetic moment per magnetosome (Bazylinski and Frankel, 2000b).

C. Magnetosome crystal morphologies

Several different morphologies of magnetosome magnetite crystals occur in magnetotactic bacteria but, in general, crystal shape is consistent within cells of a single bacterial species or strain (Bazylinski *et al.*, 1994). Minor variations have been reported to occur in crystals of some species grown under different conditions (Meldrum *et al.*, 1993a). It is not clear whether this applies to greigite crystals since there are no greigite-producing

strains in pure culture to test this. However, much more morphological variation occurs in greigite crystals within cells collected from natural environments, that is several particle morphologies have been observed within a single cell (Pósfai *et al.*, 1998b). Newly formed superparamagnetic crystals have rounded edges and smoother crystal faces which become more defined as the particles mature and increase in size to the SMD size range (Bazylinski and Frankel, 2000b). Three general projected shapes of mature magnetite and greigite particles have been observed in magnetotactic bacteria using transmission electron microscopy (TEM) (Bazylinski and Frankel, 2000a,b). They include: (1) roughly cuboidal (cubo-octahedral) (Balkwill *et al.*, 1980; Mann *et al.*, 1984a), (2) elongated pseudoprismatic (quasi-rectangular in the horizontal plane of projection) (Mann *et al.*, 1984b; Meldrum *et al.*, 1993a,b; Towe and Moench, 1981), and (3) tooth-, bullet- or arrowhead-shaped (anisotropic) (Mann *et al.*, 1987a,b; Thornhill *et al.*, 1994) (Fig. 2.3).

Magnetite crystals from magnetotactic bacteria are considered to be of high structural perfection as determined by a number of techniques, including high-resolution TEM, selected area electron diffraction, and electron tomography. Idealized morphologies of several magnetite crystals of different general shapes have been determined using these techniques (Clemett *et al.*, 2002; Mann *et al.*, 1984a,b, 1987a,b; Meldrum *et al.*, 1993a,b; Thomas-Keprta *et al.*, 2000, 2001). Magnetite and greigite are in the Fd3m space group and have face-centered spinel crystal structures (Palache *et al.*, 1944). Macroscopic crystals of magnetite display habits of the octahedral {1 1 1} form, and rarely dodecahedral {1 1 0} or cubic {1 0 0} form. The idealized habit of magnetite crystals in freshwater *Magnetospirillum* species is cubo-octahedra that is composed of {1 0 0} + {1 1 1} forms (Mann *et al.*, 1984a), with equidimensional development of the six symmetry-related faces of the {1 0 0} form and of the eight symmetry-related faces of the {1 1 1} form. The habits of the nonequidimensional-elongated crystals, like those in the marine vibrio strains MV-1 and MV-2 and the marine coccus strain MC-1, have been described as combinations of {1 0 0}, {1 1 1}, and {1 1 0} forms (Meldrum *et al.*, 1993a,b). In these crystals, the 6, 8, and 12 symmetry-related faces of the respective forms constituting the habits do not develop equally. For example, crystals of strains MV-1, MV-2 (Meldrum *et al.*, 1993a) and MC-1 (Meldrum *et al.*, 1993b) have pseudoprismatic habits elongated along a ⟨1 1 1⟩ axis, with six well-developed (1 1 0) faces parallel to the elongation axis, and capped by (1 1 1) planes perpendicular to the elongation axis. In crystals of strain MV-1 and MV-2, the remaining six (1 1 1) faces form truncations of the end caps, and the remaining six (1 1 0) faces are very small or missing (Thomas-Keprta *et al.*, 2001). In crystals of strain MC-1, truncations at each end consist of three (1 0 0) faces alternating with three (1 1 0) faces. Thus, six (1 1 0) faces are larger and six are smaller, and six (1 1 1) faces are virtually absent in this habit. Only the six (1 0 0)

faces are equidimensional (Meldrum *et al.*, 1993a). The pseudoprismatic pattern of six elongated (1 1 0) faces capped by (1 1 1) faces with differing truncation planes is very common in magnetotactic bacteria that biomineralize nonequidimensional magnetite crystals (Fig. 2.3).

The tooth-, bullet-, and arrowhead-shaped crystals are the most anisotropic of the magnetotactic bacterial magnetite particles. In one uncultured coccoid magnetotactic bacterium, small and large crystals have different habits, suggesting that crystal growth occurs in two stages in which the nascent particles are cubo-octahedra which eventually elongate along a [1 1 1] axis to form a pseudooctahedral prism with alternating (1 1 0) and (1 0 0) faces capped by (1 1 1) faces (Mann *et al.*, 1987a,b).

Although defects such as screw dislocations are rare in magnetosome magnetite crystals, crystal twinning is relatively common (ca. 10% of the crystals in some organisms) with individuals related by rotations of 180° around the [1 1 1] direction parallel to the chain direction and with a common (1 1 1) contact plane (Devouard *et al.*, 1998). Multiple twins have also been observed but are less common.

Greigite crystals in magnetotactic bacteria have same three general morphologies as magnetite (cubo-octahedral, elongated pseudorectangular prismatic, and tooth-shaped (Pósfai *et al.*, 1998b)) that are composed primarily of {1 1 1} and {1 0 0} forms (Heywood *et al.*, 1991). The crystal morphologies of the greigite particles in most uncultured rod-shaped bacteria also appear to be species- and/or strain-specific although this must be proven using pure cultures or with modern molecular biological techniques. However, the MMP contains a combination of greigite crystal morphologies, including pleiomorphic, pseudorectangular prismatic, tooth-shaped, and cubo-octahedral (Pósfai *et al.*, 1998b).

Unlike magnetite crystals in magnetotactic bacteria, individual greigite crystals in these bacteria often exhibit patchy contrast when viewed with the electron microscope (Pósfai *et al.*, 1998b), and instead of having well-defined, distinct facets, greigite crystal surfaces are often rounded and irregular. These defects have been interpreted as resulting from the mackinawite to greigite solid-state conversion process (Pósfai *et al.*, 1998b). Thickness variations and other factors may also contribute to the uneven contrast of these particles.

D. Arrangement of magnetosomes within cells

Magnetosomes are arranged in one or more chains within the cell in most magnetotactic bacteria (Bazylinski and Moskowitz, 1997; Bazylinski *et al.*, 1995) (Fig. 2.3). Several proteins have been shown to be responsible for magnetite magnetosome chain formation in two species of *Magnetospirillum* (discussed in the Section IV.C). In this chain arrangement of the SMD magnetosomes, the magnetic dipole moment of the cell is maximized

because magnetic interactions between the magnetosomes cause each magnetosome moment to spontaneously orient parallel to the others along the chain axis by minimizing the magnetostatic energy (Frankel, 1984). Thus, the total magnetic dipole moment of the chain (and therefore the cell) is the algebraic sum of the moments of the individual SMD crystals in the chain. Results from magnetic measurements (Penninga *et al.*, 1995), magnetic force microscopy (Proksch *et al.*, 1995; Suzuki *et al.*, 1998), and electron holography (Dunin-Borkowski *et al.*, 1998, 2001) have all individually confirmed this notion and clearly show that the chain of magnetosomes in a magnetotactic bacterium functions like a single magnetic dipole and causes the cell to behave similarly. Magnetotaxis results from the magnetic dipole moment imparted by the chain of magnetosomes which cause the cell to passively align along geomagnetic field lines while it swims (Frankel, 1984). Living cells are neither attracted nor pulled toward either geomagnetic pole, and dead cells, like living cells, also align along geomagnetic field lines but do not swim. However, for the magnetosome chain to function as described above, magnetosomes must be anchored in place within the cell as if they were free-floating in the cell, they would likely clump, a condition where the magnetic dipole moment would be significantly reduced from the maximum.

Some uncultured magnetite-producing cocci, greigite-producing, rod-shaped bacteria, and the MMP do not arrange magnetosomes in chains but instead produce a clump of them at one end of the cell or clumps within partial chains (Cox *et al.*, 2002; Heywood *et al.*, 1990; Mann *et al.*, 1990; Moench, 1988; Pósfai *et al.*, 1998a,b). Nevertheless, based on the behavior of the cells in a magnetic field, even these organisms clearly have a net magnetic dipole moment.

E. Biological advantage of magnetotaxis

As stated earlier, the magnetosomes impart a magnetic dipole moment to cells causing them to behave as miniature, motile compass needles that align along the Earth's geomagnetic field lines (Frankel, 1984). Magnetotactic bacteria were originally thought to have one of two magnetic polarities, north- or south-seeking, depending on the magnetic orientation of the cell's magnetic dipole with respect to their direction of motion (Blakemore *et al.*, 1980). The vertical component of the inclined geomagnetic field appeared to select for a dominant polarity in each hemisphere by favoring those cells whose polarity caused them to swim downward toward microaerobic/anaerobic sediments and away from potentially high, toxic concentrations of oxygen in surface waters. This hypothesis appeared to be at least partially true in that it was thought that north-seeking magnetotactic bacteria predominated in the Northern Hemisphere while south-seeking cells predominated in

the Southern Hemisphere (Blakemore *et al.*, 1980). This last notion has been found not to be generally true and the major numbers of some species of magnetotactic bacteria in the Northern Hemisphere have been found to be south-seeking (Simmons *et al.*, 2006). In addition, the isolation and behavior of a polarly magneto-aerotactic bacterium, the coccus strain MC-1, were not consistent with this hypothesis. Cells of MC-1 did not grow at the bottom of culture tubes but formed microaerophilic bands of cells at the OAI (Frankel *et al.*, 1997).

Magnetotaxis has been found to act in conjunction with aerotaxis (magneto-aerotaxis) in the marine, microaerophilic coccus strain MC-1, and *M. magnetotacticum* (Frankel *et al.*, 1997, 2007). Although these bacteria appear to differ in their mechanism of aerotactic response and in the way they use the magnetic field with strain MC-1 using the field as a sense of direction (polar magneto-aerotaxis) and *M. magnetotacticum* using the field as an axis (axial magneto-aerotaxis), they both prefer to be located at the OAI and in this way magneto-aerotaxis works similarly for both organisms (Frankel *et al.*, 1997, 2007). According to the magneto-aerotaxis hypothesis, the direction of migration along the magnetic field is determined by the direction of flagellar rotation (clockwise or counterclockwise), which in turn is determined by the aerotactic response of the cell (Frankel *et al.*, 1997, 2007). The presumed function of magneto-aerotaxis for strain MC-1 and *M. magnetotacticum* is increased efficiently in locating and maintaining position at a preferred oxygen concentration (and perhaps oxidation–reduction potential) at the OAI in vertical oxygen concentration gradients in aquatic habitats (Frankel *et al.*, 1997, 2007) (Fig. 2.1).

IV. CHEMICAL AND MOLECULAR BASIS OF MAGNETOSOME SYNTHESIS

A major focus of research regarding the magnetotactic bacteria since their discovery is the question of how they biomineralize and organize their magnetosomes at the chemical, biochemical, molecular, and genetic level. A good deal of progress has been made in this area mainly because of two developments. The genomes of four magnetotactic bacteria have been at least partially, if not completely, sequenced and annotated and tractable genetic systems have now been devised for two magnetotactic bacteria. Using comparative analysis, the genomic data allowed for the first genome-wide determination of genes involved in magnetite magnetosome biomineralization by magnetotactic bacteria (Grünberg *et al.*, 2001). Together, these developments led to a great deal of recent progress in particularly understanding the molecular basis of magnetite magnetosome chain formation.

Biomineralization of the bacterial magnetosome appears to be a complex process that involves several steps. These including magnetosome vesicle formation, iron uptake by the cell, iron transport into the magnetosome vesicle, and controlled Fe_3O_4 (or Fe_3S_4) biomineralization within the magnetosome vesicle. In *M. magneticum*, it is unclear whether the MM actually represents a true, free-standing vesicle cutoff from the periplasm or a permanent invagination (Komeili, 2007; Komeili *et al.*, 2006) (discussed in more detail later). If the MM structure is a permanent invagination that is not sealed off from the periplasm, then an intricate system for the transport of iron into a vesicle may not be required and iron only need to be transported across the OM. Although most of the biochemical and chemical details of these steps are not known, there is much published speculation particularly on the precipitation of magnetite. One exception is the cellular uptake of iron which has been studied in some detail. Several aspects of iron uptake are known: (1) there is evidence that both Fe(II) and Fe(III) are or can be taken up and used in magnetite synthesis not necessarily simultaneously or by the same bacterium (Matsunaga and Arakaki, 2007; Schüler and Baeuerlein, 1996; Suzuki *et al.*, 2006); (2) iron uptake for magnetite synthesis (as well as the biomineralization of magnetite) appears to occur relatively quickly (Schüler and Baeuerlein, 1998); and (3) there is evidence that siderophores, low molecular weight ligands produced by the cell that generally chelate and solubilize Fe(III) (Neilands, 1995), are involved (Calugay *et al.*, 2003; Dubbels *et al.*, 2004; Paoletti and Blakemore, 1986).

A. Genomics of magnetotactic bacteria

1. Magnetospirillum species

As determined by pulsed-field gel electrophoresis of genomic DNA, the genome of *M. magnetotacticum* strain MS-1 consists of a circular chromosome about 4.3 Mb in size with a possible extrachromosomal structure (Bertani *et al.*, 2001). The genome of this bacterium is partially sequenced and annotated and is available for study at the Joint Genome Institute's website (http://genome.jgi-psf.org/draft_microbes/magma/magma.home.html). *M. magneticum* strain AMB-1 contains a circular chromosome slightly larger than that of *M. magnetotacticum* at 4.97 Mb (Matsunaga *et al.*, 2005). A cryptic plasmid was also detected in this species (Okamura *et al.*, 2003). *M. gryphiswaldense* strain MSR-1 contains a circular chromosome 4.3 Mb in size and also contains a native plasmid (Jogler and Schüler, 2007).

2. The vibrios MV-1 and MV-2

The genomes of strain MV-1 and MV-2 were estimated to be 3.7 and 3.6 Mb in size, respectively. The genomes of both strains consist of a single, circular chromosome and there was no evidence for the presence

of plasmids (Dean and Bazylinski, 1999). The genome of MV-1 has been partially sequenced.

3. The coccus strain MC-1

The genome of strain MC-1 consists of a singular, circular chromosome about 4.5 Mb in size. No evidence was found for the presence of plasmids. The genome sequence of this species has been recently completed (JGI at http://genome.jgi-psf.org/draft_microbes/magm1/magm1.home.html) and annotation is in progress.

The genomes of the three *Magnetospirillum* species and MC-1 have been compared in great detail (Richter *et al.*, 2007). The comparison revealed some new sets of genes that appear to only present in magnetotactic but not other bacteria and therefore might be involved in magnetite magnetosome biomineralization.

B. Genetic systems and manipulations in magnetotactic bacteria

Because of the difficulty in growing magnetotactic bacteria in general, it took many years to develop tractable genetic systems in these organisms. For example, many will not form colonies on agar plates if they are not under a specific oxygen concentration. One of the most powerful and definitive methods for assigning functions to specific magnetosome genes is through single gene knockouts and analysis of the mutant phenotype. Growing the strains on agar plates to obtain single colonies of mutants is therefore a requirement.

Tractable genetic systems have now been developed for *M. gryphiswaldense* strain MSR-1 (Schultheiss and Schüler, 2003) and *M. magneticum* strain AMB-1 (Matsunaga *et al.*, 1992). It is generally easy to detect nonmagnetotactic mutants of magnetotactic bacteria that are unable to produce magnetosomes since magnetite-forming colonies can be easily distinguished by their dark–brown color versus the white-colored colonies of nonmagnetic mutants (Dubbels *et al.*, 2004; Schultheiss and Schüler, 2003). Genome modifications were accomplished by conjugational transfers with conjugation frequencies of 1×10^{0} and 3–4.5 $\times 10^{-3}$ for *M. gryphiswaldense* and *M. magneticum*, respectively (Matsunaga *et al.*, 1992; Schultheiss and Schüler, 2003). Mutants of these strains were generated using transposon mutagenesis (Tn5) as well as broad host range replication (pBBRMCS, IncQ) and suicide vectors (pK19mobsacB, pMB1) (Komeili *et al.*, 2004; Matsunaga *et al.*, 1992; Schultheiss and Schüler, 2003; Schultheiss *et al.*, 2004). The establishment of the genetic system for these strains now allowed for the extrachromosomal expression of genes and the integration of reporter genes like the luciferase or the green fluorescent protein genes (*gfp*) and its derivatives. These techniques have made

it possible to study the subcellular localization of proteins putatively involved in magnetite magnetosome biomineralization (Komeili *et al.*, 2004; Matsunaga *et al.*, 2000a; Nakamura *et al.*, 1995b; Schultheiss *et al.*, 2004). General transposon mutagenesis is random but can generate nonmagnetic mutants that made it possible to identify genes in the genome involved in magnetite biomineralization. Suicide vectors together with genome data now allow for the integration of these vectors at specific genomic sites to generate site-directed gene knockouts to definitively determine the precise roles of specific genes in magnetite magnetosome biomineralization (Komeili *et al.*, 2004, 2006; Pradel *et al.*, 2006; Scheffel *et al.*, 2006).

C. The magnetosome membrane

It was evident from the very beginning of research on the magnetotactic bacteria that the magnetite crystals were enveloped by some type of coating (Balkwill *et al.*, 1980). This coating is now referred to the magnetosome membrane (MM) which encloses magnetite crystals (Gorby *et al.*, 1988; Schüler and Baeuerlein, 1997) in magnetosomes and appears to be the locus of control and regulation of the magnetite biomineralization processes in magnetotactic bacteria.

1. Composition of the MM

Lipid analysis of purified magnetosomes of *M. magnetotacticum* as well as *M. gryphiswaldense* showed that the MM consists of proteins, fatty acids, glycolipids, sulfolipids, and phospholipids (Gorby *et al.*, 1988; Grünberg *et al.*, 2004). The predominant phospholipids were phosphatidylserine, phosphatidylglycerol, and phosphatidylethanolamine. Phospholipids reportedly make up 58–65% of the total lipids of the MM of *M. magneticum*, 50% of that being phosphatidylethanolamine (Nakamura and Matsunaga, 1993). A comparison of the fatty acids of the MM, the cell or cytoplasmic membrane (CM) and the outer membrane (OM) showed that the composition of the MM is similar to the CM but distinct from the OM (Tanaka *et al.*, 2006). The results indicate that the MM is a lipid bilayer membrane. This is striking contrast to other inclusions in prokaryotes which are generally surrounded by a single layer of protein. The results also suggest that the MM originates from the CM.

Magnetite magnetosomes are almost always located adjacent to the CM in *Magnetospirillum* species and for a long time it was not known whether the MM formed by invagination of the CM or by a *de novo* synthesis despite the circumstantial evidence described in the previous paragraph. It was also ambiguous whether magnetosome vesicles are synthesized prior to magnetite biomineralization and exist as empty vesicles in the cell or if the MM envelopes the particle afterward.

An early electron microscopy study showed that cells of *M. magnetotacticum* grown under iron limitation contained empty magnetosome vesicles as well as vesicles with small, immature magnetite crystals (Gorby *et al.*, 1988). Electron cryotomography (ECT) has shown convincingly that the MM is an invagination of the CM rather than a free-standing vesicle in *M. magneticum* (Komeili *et al.*, 2006). These studies also showed that magnetite precipitation occurs after vesicle formation and different stages of magnetite precipitation was observed within MM vesicles. Even cells grown under iron limitation contained empty magnetosome vesicles arranged in a chain engaged to the CM (Komeili *et al.*, 2006). However, only 35% of the magnetosomes examined showed the MM to be an invagination of the CM. MM proteins have been designated the Mam (for magnetosome membrane) or Mms (for magnetic particle membrane specfic) proteins and the respective genes as the *mam* or *mms* genes. This may be due to technical limitations or perhaps the invaginations develop into true vesicles (Komeili, 2007). It also remains to be discussed if this observation is a common characteristic of magnetosomes in all magnetotactic bacteria. In parallel experiments with *M. gryphiswaldense*, Scheffel *et al*. (2006) found empty MM vesicles in cells grown under iron limitation and also found that magnetic cells contain, in addition to filled magnetosome vesicles, many empty MM structures inside the cell.

2. Role of MM proteins and genes

Protein profiles of the MM are distinct from other cell fractions (the cytoplasmic, periplasmic, CM and OM fractions) in *M. magnetotacticum*, *M. gryphiswaldense*, and *M. magneticum* (Gorby *et al.*, 1988; Grünberg *et al.*, 2001; Okamura *et al.*, 2000; Tanaka *et al.*, 2006). In addition, there were also differences in the MM protein profiles between the three species (Grünberg *et al.*, 2004). Thus, it is important to understand that the MM contains proteins that are unique to the MM and that it is likely that these proteins play the key roles in magnetite biomineralization in magnetosomes. Much of the focus of researchers in magnetite biomineralization by magnetotactic bacteria is on these proteins and the genes that encode for them. These proteins are called the Mam (magnetosome membrane) or Mms (magnetic particle membrane specific) proteins (or *mam* or *mms* genes). A list of these proteins and their homologues in different magnetotactic bacteria are shown in Table 2.1.

Identifying the function of MM proteins appears to be the key to understanding magnetosome biomineralization. Presumed functions of MM proteins include iron uptake into the cell and/or the magnetosome vesicles, crystal nucleation and biomineralization of magnetite, and the arrangement of the magnetosomes in the chain configuration. Initially, four specific MM proteins were identified in *M. magnetotacticum* (Gorby *et al.*, 1988), whereas five were found in *M. magneticum* (Okamura *et al.*, 2000).

TABLE 2.1 Magnetosome genes in different magnetotactic bacteria

Gene	MSR-1[a]	MS-1	AMB-1	MC-1	MV-1
mamA	+[b]	+ (*mam22*)[c]	+ (*mms24*)[d]	+	+
mamB	+	+	+	+	+
mamC	+	+ (*mam12*)[e]	+ (*mms13*)[d]	+	+
mamD	+	+	+ (*mms7*)[d]	++	+
mamE	+	+	+	+	+
mamF	+	+	+	+	+
mamG	+	+	+	−	/
mamH	+	+	+	+	/
mamI	+	+	+	+	+
mamJ	+	+	+	−	/
mamK	+	+	+	+	++
mamL	+	+	+	−	+
mamM	+	+	+	+	+
mamN	+	+	+	−	+
mamO	+	+	+	+	+
mamP	+	+	+	+	+
mamQ	+	+	+	+	+
mamR	+	+	+	−	+
mamS	+	+	+	+	+
mamT	+	+	+	+	+
mamU	+	+	+	−	/
mamV	−	+	+	−	/
mamW	+	−	+	−	/
mgI462	+	+	+	−	/
mms6	+	+	+	+	+
mgI459	+	+	+	+	+
mgI458	+	+	+	−	/
mgI457	+	+	+	−	/
mamE/S-like	+	+	+	+	/
mamF-like	+	+	+	+	/
mamH-like	+	+	+	+	/
mamA-like	−	−	−	++	/
mgr4150	+	+	+	−	/
mgr0208	+	+	+	+	/
mgr0207	+	+	+	+	/
mgr0206	+	+	+	+	/
mgr3500	+	+	+	+	/
mgr3499	+	+	+	−	/
mgr3497	+	+	+	+	/
mgr3495	+	−	+	−	/

Subsequent studies revealed an additional 2 MM proteins, Mms13 and Mms7, in *M. magneticum* (Arakaki *et al.*, 2003) while 16 specific MM proteins were identified in *M. gryphiswaldense* (Grünberg *et al.*, 2004). Analysis of the complete genome of *M. magneticum* revealed 78 putative MM proteins, some of which are also present in the CM (Tanaka *et al.*, 2006). In the following paragraphs, we will review what is known about the roles of several MM proteins in magnetosome synthesis.

Using reverse genetics, the first *mam* gene to be cloned was *mam22* of *M. magnetotacticum* (Okuda *et al.*, 1996) and a gene encoding for a homologous protein, *mamA*, was found in *M. gryphiswaldense*, *M. magneticum* (corresponds to *mms24* in this organism), and strain MC-1 (Grünberg *et al.*, 2001; Komeili *et al.*, 2004; Matsunaga *et al.*, 2005). The amino acid sequences of these proteins show high homology to proteins of the tetratricopeptide repeat (TPR) protein family (Okuda *et al.*, 1996). MamA (and Mam22) is thought to be important in protein–protein interactions that might occur in the synthesis of magnetosomes and the magnetosome chain (Okuda and Fukumori, 2001; Okuda *et al.*, 1996) since multiple copies of TPRs are known to form scaffolds within proteins to mediate protein–protein interactions and to coordinate the assembly of proteins into multisubunit complexes (Ponting and Phillips, 1996). A deletion of *mamA* in *M. magneticum* resulted in shorter magnetosome chains and it was concluded that MamA activates magnetosome vesicles (Komeili *et al.*, 2004).

MamB and MamM are present in all *Magnetospirillum* species and strains MC-1 and MV-1 and show strong similarity to heavy metal transporting proteins of the cation diffusion facilitator (CDF) family. An additional MM protein, MamV, also appears to be in this family but is only present in *M. magnetotacticum* and *M. magneticum* and not in other magnetotactic bacteria. Proteins in this family show an unusual degree of size variation, sequence divergence, and polarity, and can catalyze the influx or efflux of metal ions (Paulsen *et al.*, 1997). For this reason, these Mam proteins might be involved in the transportation of the iron into the magnetosome vesicle (Grünberg *et al.*, 2001).

MamE and MamO show sequence similarity to HtrA-like serine proteases and little similarity to each other. The protein product of the *mamP* gene also shows similarity to this group of serine proteases and is present in the same operon as *mamE* and *mamO* but was not identified in the MM. HtrA (also known as DegP) is a heat-shock-induced, envelope-associated

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[a] Organisms: MSR-1, *M. gryphiswaldense*; MS-1, *M. magnetotacticum*; AMB-1, *M. magneticum*; MC-1, strain MC-1, a magnetotactic coccus; MV-1, strain MV-1, a magnetotactic vibrio.

[b] Symbols: +, homologue present in genome; ++, two paralogues in genome; −, homologue absent from genome; /, homologue absent from MV-1 putative magnetosome island (genome sequence of strain MV-1 has not been completed).

[c] From Okuda *et al.* (1996).

[d] From Fukuda *et al.* (2006).

[e] From Taoka *et al.* (2006).

serine protease first found in *Escherichia coli* (Lipinska *et al.*, 1989). HtrA is enzymatically active in the periplasm, where its main role seems to be in the degradation of misfolded proteins (Pallen and Wren, 1997). In addition, these proteases are known to be involved in nondestructive protein processing and modulation of signaling pathways by degrading important regulatory proteins. These proteins are characterized by one or two PDZ-domains (Fanning and Anderson, 1996) and a trypsin-like protease domain. It was speculated that these proteins might function as chaperones in magnetosome formation (Grünberg *et al.*, 2001).

MamN shows some similarity to certain transport proteins, some of which transport protons leading to an idea that this protein might function as a proton pump transporting protons accumulating during magnetite precipitation (Jogler and Schüler, 2007).

MamT contains two possible binding sites for the heme group present in cytochrome *c* and therefore might be involved in redox reactions within the magnetosome vesicle (Grünberg *et al.*, 2004).

The MM proteins MamC [Mms13 in *M. magneticum* (Arakaki *et al.*, 2003), Mam12 in *M. magnetotacticum* (Taoka *et al.*, 2006)], MamD (Mms7), MamF, MamG, MamQ, MamR, and MamS are unique to the magnetotactic bacteria and homologues of these proteins have not been found in nonmagnetotactic bacteria (Grünberg *et al.*, 2004). MamC is an abundant protein in the MM of *M. magnetotacticum* (Taoka *et al.*, 2006), *M. gryphiswaldense* (Grünberg *et al.*, 2001), and strain MV-1 (unpublished data). MamC and MamF are hydrophobic proteins that contain predicted transmembrane helices. MamD and MamG are partially identical and both are similar to the MM protein Mms6 of *M. magneticum*. All three proteins contain large repeating leucine–glycine motifs present in other proteins involved in biomineralization. Mms6 has been shown to affect the crystal morphology of crystals when present during abiotic magnetite precipitation (Arakaki *et al.*, 2003; Prozorov *et al.*, 2007). The specific roles of these proteins in magnetite magnetosome biomineralization are unknown.

A 16-kDa protein that showed GTPase activity, called Mms16, was found in the MM of *M. magneticum* where it was the most abundant of five proteins present (Okamura *et al.*, 2001). Cells grown in the presence of a GTPase inhibitor showed less overall magnetism and produced fewer magnetosomes than in its absence, possibly indicating that GTPase activity is required for magnetosome synthesis. Because small GTPases, such as Sar1p, are known to be essential for the budding reaction in the production of membrane vesicles and vesicle trafficking in eukaryotes (Kirchhausen, 2000), it was thought the Mms16 might play a similar role in the activation of membrane vesicle formation in magnetotactic bacteria. A protein with high sequence similarity to Mms16 was shown to be involved in polyhydroxybutyrate depolymerization in the photosynthetic bacterium *Rhodospirillum rubrum* (Handrick *et al.*, 2004), so there was some

doubt as to whether Mms16 protein was a bona fide MM protein. Schultheiss *et al*. (2005) reported that Mms16 in *M. gryphiswaldense* is a polyhydroxybutyrate body-bound protein that has no GTPase activity. Mms16 and its putative homologue in *M. gryphiswaldense* are not identical however (the protein from *M. magneticum* contains a P-loop that is lacking from that of *M. gryphiswaldense*) and because of this, probably should be considered different proteins (Matsunaga and Arakaki, 2007).

The *mamJ* and *mamK* genes are located within the *mamAB* gene cluster in *Magnetospirillum* species and are cotranscribed (Schübbe *et al.*, 2006). The MamJ protein is a strongly acidic protein with a repeating glutamate-rich section in its central domain (Scheffel *et al.*, 2006) that shows significant homology to certain other acidic proteins (Grünberg *et al.*, 2004). These are characteristics for proteins involved in other biomineralization processes like calcium carbonate biomineralization in shells (Baeuerlein, 2003). Carboxy groups of the acidic amino acids generally have a high affinity for metal ions and because of this, magnetosome proteins with these characteristics have been thought to be involved in the initiation of magnetite crystal nucleation (Arakaki *et al.*, 2003). A role for MamJ in magnetite crystal nucleation has not been shown however. MamK shows some homology to MreB (Schübbe *et al.*, 2003), an actin-like protein that forms cytoskeletal structures in some nonmagnetotactic bacteria (Carballido-Lopez, 2006; Figge *et al.*, 2004; Jones *et al.*, 2001; van den Ent *et al.*, 2001). However, MamK proteins in magnetotactic bacteria are more similar to each other than they are to MreB homologues (Komeili *et al.*, 2006). Fluorescent microscopy studies of a fusion of a fluorescent protein to *mamK* of *M. magneticum* (MamK–mCherry) and an MreB–YFP (yellow fluorescent protein) fusion, coexpressed in *E. coli*, revealed that MreB forms helical structures in contrast to MamK that appeared to make up straight filaments in the same cell. In addition, the straight filaments of MamK are also present in an *E. coli mreB* mutant. This indicates that assembly of the MamK filaments is independent of MreB (Pradel *et al.*, 2006).

Experiments involving gene knockout mutants of *mamJ* (Δ*mamJ*) in *M. gryphiswaldense* and *mamK* (Δ*mamK*) in *M. magneticum* clearly showed that these two gene products are responsible for magnetite magnetosome chain formation in these organisms. Deletion of either *mamJ* or *mamK* leads to disruption of the chains but does not inhibit magnetosome formation. ECT of wild-type (wt) cells of *M. gryphiswaldense* and *M. magneticum* showed that magnetosomes are associated with long filaments running along the CM from the proximal to distal poles (Komeili *et al.*, 2006; Scheffel *et al.*, 2006). In wt cells grown under moderate iron limitation, most of the empty vesicles and partially and completely full magnetosome vesicles were arranged in proximity along this filamentous structure in *M. gryphiswaldense* (Scheffel *et al.*, 2006). In *M. magneticum*, all

empty and fully filled vesicles are arranged along the filaments. The Δ*mamK* mutant of *M. magneticum* did not appear to show MM invaginations or comparable filaments (Komeili *et al.*, 2006). However, in the Δ*mamJ* cells of *M. gryphiswaldense*, filaments are present but empty and filled MM vesicles were dissociated from them (Scheffel *et al.*, 2006). The Δ*mamK* cells had no filaments and magnetosomes were not arranged as a chain but were dispersed in the cytoplasm (Komeili *et al.*, 2006). Complementation of both mutants with *mamJ-EGFP* and *mamK-GFP*, respectively, resulted in restoration of the magnetosome chain attached to magnetosome filaments extending across the cell, following the helical morphology of the cell (Komeili *et al.*, 2006; Scheffel *et al.*, 2006).

3. Organization of the mam and mms genes

Early sequence analyses of clones containing MM protein genes revealed that these genes are in proximity to one another within the genome. The genes *mamA* and *mamB* in *M. gryphiswaldense* are in a collinear order with six other genes that make up the *mamAB* cluster (Grünberg *et al.*, 2001). Another group of MM protein genes, the *mamCD* cluster, simply consists of *mamC* and *mamD*. Similar localizations and arrangements of these genes exist in *M. magnetotacticum* and strain MC-1 (Grünberg *et al.*, 2001).

As more genomic sequence of different magnetotactic bacteria became available, additional genes thought to be involved in the magnetite biomineralization were identified. In *M. gryphiswaldense*, the *mamAB* cluster was actually found to encompass 17 collinear genes on a segment of DNA about 16.4 kb in length. The *mamGFDC* cluster is about 2.1 kb in length and is located about 15-kb upstream of the *mamAB* operon and is composed of four genes. The 3.6-kb *mms6* cluster is located 368-bp upstream of the *mamGFDC* operon and contains five genes (Schübbe *et al.*, 2003). Studies revealed another gene encoding for an MM protein, *mamW*, is not present in these three operons, but is located about 10-kb upstream of the *mms6* operon (Ullrich *et al.*, 2005). All the *mam* and *mms* genes are located on a segment of DNA about 45 kb in length (Fig. 2.4).

The operon-like, collinear organization of the *mamAB*, *mamGFDC*, and *mms6* clusters suggested that they might be transcribed as single long mRNAs. Recent studies involving gene transcriptional analysis confirmed this notion and demonstrated the presence of one long transcript extending over more than 16 kb. The transcription starting points of the *mamAB*, *mamGFDC*, and *mms6*-operons were mapped closely upstream of the first genes in the operons, respectively (Schübbe *et al.*, 2006).

The organization of the magnetosome genes is well conserved in the different, but closely related, *Magnetospirillum* strains. Even high similarities at the protein and DNA levels exist for specific proteins and genes, respectively. The organization and sequence of the magnetosome genes is less conserved in other unrelated magnetotactic strains, including MC-1

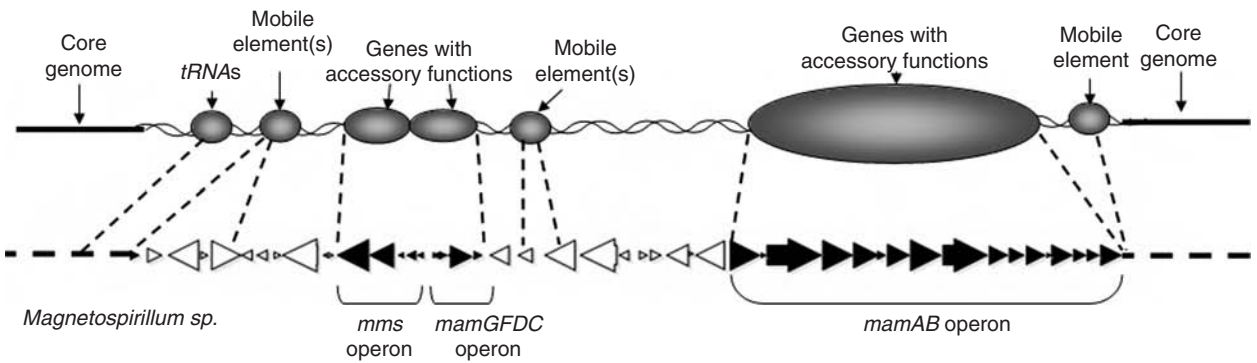

FIGURE 2.4 Structure of the magnetosome gene island (MAI) as present in the genome of species of the genus *Magnetospirillum*. Note that magnetosome genes are in close proximity and make up three operons. The size of this MAI is ~130 kb. Some characteristics of genomic islands including the presence of mobile elements (as integrases and insertion sequences here) and *tRNA* genes that can serve as attachment sides for integrases (three *tRNA* genes are upstream of the *mms* operon) are illustrated as balloons. Genomic islands are reported to be acquired by some organisms through horizontal gene transfer and most have a different G+C content than the core genome.

and the magnetotactic vibrio MV-1 (Schübbe *et al.*, 2003; Ullrich *et al.*, 2005; Bazylinski and Schübbe, unpublished data).

4. Magnetosome genes are clustered within a genomic magnetosome island

The magnetosome genes in every magnetotactic bacteria genome examined showed they are in close proximity. The genomic region that contained the magnetosomes genes in *M. gryphiswaldense* also contains 42 mobile elements as transposases of the insertion sequence type and integrases (Ullrich *et al.*, 2005). These mobile elements are common and important features in genomic islands (Mahillon and Chandler, 1998; Mahillon *et al.*, 1999) (Fig. 2.4). Other features are *tRNA* genes that can serve as attachment sides for integrases (Blum *et al.*, 1994; Reiter and Palm, 1990). The magnetosome gene region in *M. gryphiswaldense* is about 130 kb in size and contains three *tRNA* genes upstream of the *mms* operon. This region shares other characteristics of a genomic island including a slightly different G+C content compared to the rest of the genome as well as containing many hypothetical genes and pseudogenes (Schübbe *et al.*, 2003; Ullrich *et al.*, 2005). Therefore, it is now accepted by many that this region represents a large magnetosome gene island (MAI) which appears to be present in other magnetotactic bacteria (Fukuda *et al.*, 2006; Richter *et al.*, 2007) (Fig. 2.4). Genomic islands are reported to be acquired by a horizontal gene transfer and most have a different G+C content than the core genome (Dobrindt *et al.*, 2004).

The similar organization of the magnetosome operons in different magnetotactic bacterial strains assumes that the magnetosome gene island might have been transferred via horizontal gene transfer to many different types of bacteria. This would explain the great diversity of the group.

The MAI undergoes frequent rearrangements. Spontaneous mutations that lead to a loss of the magnetic phenotype with a frequency of 10^{-2} were observed under starvation conditions in *M. gryphiswaldense* (Ullrich *et al.*, 2005). One spontaneous nonmagnetic mutant, designated *M. gryphiswaldense* strain MSR-1B, was further characterized and showed poorer growth and lower iron uptake compared to the wt strain (Schübbe *et al.*, 2003). Mutants that had lost the ability to synthesize magnetosomes were also observed for other magnetotactic bacteria such as strain MV-1 (Dubbels *et al.*, 2004) and *M. magneticum* (Fukuda *et al.*, 2006; Komeili *et al.*, 2006).

D. Physiological conditions under which magnetite magnetosomes are synthesized

The size and number of magnetosomes in cells of magnetotactic bacteria are strongly dependent on the oxygen and iron concentration of the medium. Oxygen concentration, in particular, influences not only magnetite

biomineralization in all cultured magnetotactic bacteria, but the growth of the bacteria as well. *M. magnetotacticum* grows from 0.1% to 21% O_2 in the headspace of sealed liquid cultures but magnetite magnetosome synthesis was only appreciable between 0.5% and 5% O_2 with maximum magnetite production at 1% O_2 (Blakemore *et al.*, 1985). The presence of nitrate (cells of this species respire with nitrate and denitrify) appeared to stimulate magnetite synthesis. Reduced O_2 concentrations during growth of *M. magneticum* also resulted in increased magnetite magnetosome synthesis as well (Yang *et al.*, 2001b). *M. magneticum* can also biomineralize magnetite under anaerobic conditions when grown with nitrate. In *M. gryphiswaldense*, magnetite magnetosome synthesis was induced below an O_2 concentration of 20 mbar and maximum magnetite synthesis was observed at a pO_2 of 0.25 mbar. An oxygen shift from 20 to 2 mbar O_2 during exponential growth resulted in delayed magnetite production (Heyen and Schüler, 2003). None of the *Magnetospirillum* strains is able to synthesize magnetite under saturated oxygen concentrations (Schüler and Baeuerlein, 1998). These results are consistent with the general fact that magnetite synthesis in magnetotactic bacteria is inhibited by high levels of O_2 and occurs only under microaerobic or anaerobic conditions.

Iron availability is an additional essential factor for magnetite magnetosome biomineralization, and it is clear that magnetotactic bacteria must possess highly efficient mechanisms of iron uptake. Cells of magnetotactic bacteria can consist of >3% iron on a dry weight basis, which is several orders of magnitude higher than nonmagnetotactic bacteria (Blakemore, 1982; Heyen and Schüler, 2003). Iron uptake experiments with *M. gryphiswaldense* showed that cells used even very low amounts of iron (1 μM) for magnetite synthesis, but with decreased magnetism, whereas iron concentration above 20-μM iron only slightly increased cell yield and magnetism (Schüler and Baeuerlein, 1996). An iron shift experiment from iron-limited to iron-sufficient conditions showed no delay in magnetite production in contrast to the oxygen-shift experiment (Heyen and Schüler, 2003). This suggests that there are no chemical intermediates in magnetite biomineralization or that they are unstable and convert to magnetite extremely quickly. The magnetotactic vibrio strain MV-1 continued to biomineralize magnetite and remain magnetotactic when grown anaerobically with N_2O even when iron is limited in the medium. Growth under these conditions showed a decreased growth yield and a lesser number of magnetosomes (Dubbels *et al.*, 2004).

E. Regulation of the expression of magnetosome genes

Recent studies involving transcriptional organization and regulation of the magnetosome gene operons in *M. gryphiswaldense* using an *in vitro* system with MamC showed that these genes are expressed under

conditions where cells do not biomineralize magnetosomes as well as those where they do (Schübbe *et al.*, 2006). In order to determine differences in the expression intensity, a partial genome array was developed for *M. gryphiswaldense* that was validated by real-time polymerase chain reaction (RT-PCR) experiments (Schübbe *et al.*, 2006; Würdemann *et al.*, 2006). In general, results from these experiments revealed a downregulation of the magnetosome genes in nonmagnetic cells under iron limitation, and to a lesser degree during aerobic growth, compared to the expression of these genes in magnetite-forming cells grown microaerobically under iron-sufficient conditions (Schübbe *et al.*, 2006).

In natural environments, most magnetite-producing magnetotactic bacteria are found within or below the microaerobic zone, the OAI (Bazylinski, 1995). Studies in freshwater microcosms showed that significant amounts of soluble Fe(II) are available to magnetotactic bacteria at the OAI (Flies *et al.*, 2005). These observations are consistent with the notion that microaerobic conditions and sufficient iron are required to biomineralize magnetosomes. The results from the gene transcription analyses described above also show that these are the conditions where magnetosome genes show maximum expression (Schübbe *et al.*, 2006).

V. APPLICATIONS OF MAGNETOTACTIC BACTERIA, MAGNETOSOMES, AND MAGNETOSOME CRYSTALS

Cells of magnetotactic bacteria and their magnetic inclusions have novel magnetic, physical, and perhaps optical properties that can and have been exploited in a variety of scientific, commercial, and other applications. The large number of reports on the applications of magnetotactic bacteria is enormous and thus we will only discuss some of the more interesting and significant ones. We direct the reader to reviews devoted to this subject (Lang and Schüler, 2006; Lang *et al.*, 2007; Matsunaga and Arakaki, 2007).

A. Mass cultivation of magnetotactic bacteria

In general, the amount of magnetite and magnetosomes from magnetotactic bacteria is relatively low especially considering the amount needed for specific applications. Thus, in order to produce enough cells, magnetosomes and magnetite crystals for these applications, cells must be grown in very large cultures where the conditions for growth and magnetite synthesis must be optimized.

Mass culture of a magnetotactic bacterium was first described using *M. magneticum*. Cells of this species were grown in a 1000-liter fermentor and the amount of magnetosomes recovered was 2.6 mg per liter of

culture (Matsunaga *et al.*, 1990). Different optimization experiments were conducted in fed-batch cultures of *M. magneticum* that did not result in a higher yield of magnetosomes or cells (Matsunaga *et al.*, 1996, 2000a). Recombinant *M. magneticum* harboring the plasmid pEML was grown in a pH-regulated fed-batch culture system where the addition of fresh nutrients was feedback-controlled as a function of the pH of the culture (Yang *et al.*, 2001a). The magnetosome yield was maximized by adjusting the rate of addition of ferric iron. Feeding ferric quinate at 15.4 μg/min resulted in a magnetosome yield of 7.5 mg/liter which may be the highest reported based on unit volume. Different iron sources and the addition of various nutrients and chemical reducing agents (e.g., L-cysteine, yeast extract, polypeptone) were shown to have a significant effect on magnetosome yield by *M. magneticum* grown in fed-batch culture (Yang *et al.*, 2001b).

A seemingly better control of the growth of *Magnetospirillum* species was achieved using an oxygen-controlled fermentor (Heyen and Schüler, 2003; Lang and Schüler, 2006). Three species were grown using this method, *M. gryphiswaldense*, *M. magnetotacticum*, and *M. magneticum*, and 6.3-, 3.3-, and 2.0-mg magnetite per liter per day were obtained from these species, respectively (Heyen and Schüler, 2003). The use of this system has resulted in the highest yields of cells and magnetite per unit of time reported thus far.

B. Applications of cells of magnetotactic bacteria

North-seeking cells of polar magnetotactic bacteria have been used to determine south magnetic poles in meteorites and rocks containing fine-grained (<1 μm) magnetic minerals (Funaki *et al.*, 1989, 1992). Harasko *et al.* (1993, 1995) investigated the applicability of magnetotactic bacteria for nondestructive domain analysis on soft magnetic materials. Cells of magnetotactic bacteria have also been used in medical applications. For example, they have been introduced to and phagocytized by granulocytes and monocytes which were then magnetically separated (Matsunaga *et al.*, 1989). Since cells of magnetotactic bacteria can be separated magnetically relatively easily, they may have potential in the area of bioremediation. The possibility of using magnetotactic bacteria in the removal of heavy metals and radionucleotides from waste water was discussed (Bahaj *et al.*, 1993, 1998a,b,c). Cells of the sulfate-reducing magnetotactic bacterium, *D. magneticus*, were used in cadmium recovery using magnetic separation (Arakaki *et al.*, 2002). A very recently described application is the trapping of magnetotactic bacteria using a commercial magnetic recording head. This method may be useful in counting magnetotactic bacteria cells in water samples collected from the natural environment or to detect magnetically labeled bacteria or magnetosomes (Krichevsky *et al.*, 2007).

C. Applications of magnetosomes and magnetosome crystals

As described earlier, magnetosomes contain SMD magnetic crystals that have interesting and useful magnetic and physical properties. Moreover, the organic, phospholipid membrane that envelopes the crystals allows for the immobilization of biological molecules such as other proteins or nucleic acids on their surfaces (Fig. 2.5).

Bacterial magnetite magnetosomes have been used in the immobilization of two enzymes, glucose oxidase and uricase, which showed a 40 times higher activity than when immobilized on crystals of artificial magnetite (Matsunaga and Kamiya, 1987). Magnetic antibodies have been devised using bacterial magnetite particles that have proven useful in various fluoroimmunoassays (Matsunaga *et al.*, 1990) involving the detection of allergens (Nakamura and Matsunaga, 1993) and squamous cell carcinoma cells (Matsunaga, 1991) and the quantification of immunoglobulin G (Nakamura *et al.*, 1991). Bacterial magnetite crystals have been used in the detection and removal of cells of *E. coli* with a fluorescein isothiocyanate-conjugated monoclonal antibody immobilized on bacterial magnetite (Nakamura *et al.*, 1993).

Magnetite magnetosomes have been used to detect single nucleotide polymorphism based on fluorescence resonance energy transfer (FRET) technique. Double-stranded labeled DNA synthesized by PCR and immobilized to the magnetosomes hybridizes to target DNA and a fluorescence

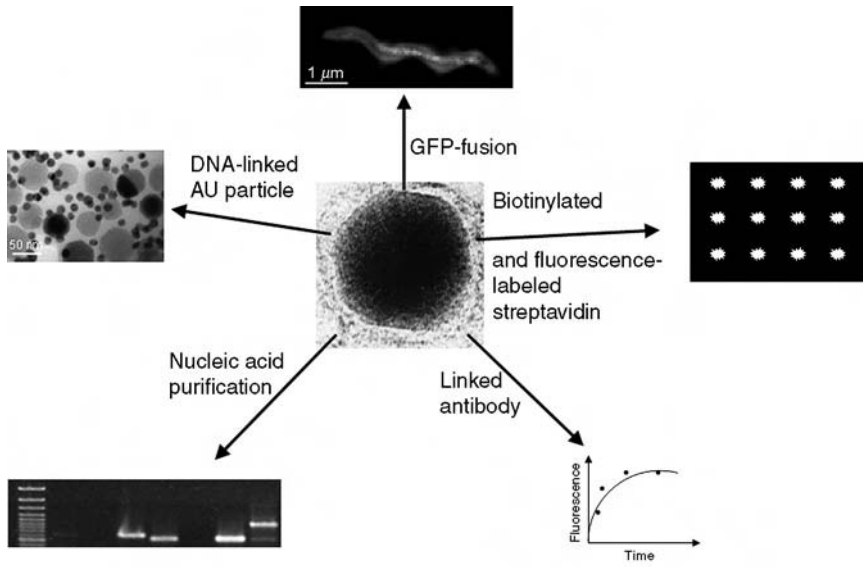

FIGURE 2.5 Representation of some strategies using magnetosomes for commercial, scientific, biomedical, and biotechnological applications. See text for details.

signal is detected (Maruyama *et al.*, 2004; Nakayama *et al.*, 2003; Ota *et al.*, 2003; Tanaka *et al.*, 2003; Yoshino *et al.*, 2003).

Protein displays have been designed using specific MM proteins as anchor molecules for the assembly of foreign proteins on the surface of magnetite magnetosomes. Several MM proteins have been used as anchor proteins, including MagA, MpsA, Mms16, and Mms13 (MamC, Mam12) (Arakaki *et al.*, 2003; Matsunaga and Takeyama, 1998; Matsunaga *et al.*, 1999, 2000b, 2002; Nakamura *et al.*, 1995a,b; Okamura *et al.*, 2001; Yoshino and Matsunaga, 2005, 2006). To investigate the stability of the anchor proteins, they were fused to the chemiluminescent protein luciferase (Matsunaga *et al.*, 2000a, 2002; Yoshino and Matsunaga, 2006). These experiments indicated that the most stable anchor protein is Mms13 (MamC, Mam12), based on the fact that this fusion resulted in 400–1000 times the luminescence activity observed for the Mms16 or the MagA fusions (Yoshino and Matsunaga, 2006).

Magnetosomes have been shown to be useful in the isolation of nucleic acids. Magnetosomes have been modified using compounds such as hyperbranched polyamidoamine dendrimers or amino silanes for the extraction of DNA (Yoza *et al.*, 2002, 2003a,b) An efficient means of isolating mRNA using oligo(dT)-modified magnetosomes has also been described (Sode *et al.*, 1993).

Biotin and other molecules attached to a monolayer-modified substrate were detected by streptavidin immobilized to magnetosomes using a magnetic force microscope indicating that magnetosomes can be used to detect biomolecular interactions in medical and diagnostic analyses (Arakaki *et al.*, 2004). For example, streptavidin-modified magnetosomes have been used for the immobilization of biotin-modified antibodies (Amemiya *et al.*, 2005). Other biomedical applications include the use of magnetosomes in drug delivery (Matsunaga *et al.*, 1997).

As with cells of magnetotactic bacteria, magnetosomes have also been used in highly efficient magnetic cell separation (Kuhara *et al.*, 2004).

Two of the most interesting uses of bacterial magnetite crystals are in geology, paleontology, and astrobiology. Magnetite crystals resembling those present in magnetotactic bacteria living in the present have been found in ancient and modern sediments (Chang and Kirschvink, 1989; Chang *et al.*, 1989) and in the Martian meteorite ALH84001 (Thomas-Keprta *et al.*, 2000, 2001, 2002), and have been referred to as ''magnetofossils,'' indicating the past presence of magnetotactic bacteria in these materials. The presence of these crystals in Martian meteorite ALH84001 sparked great controversy and debate since the implication was that the bacterial life had existed on ancient Mars (Buseck *et al.*, 2001; McKay *et al.*, 1996; Thomas-Keprta *et al.*, 2000, 2001, 2002). In turn, this debate illustrated the need for and the ability to recognize reliable prokaryotic fossils (Bazylinski and Frankel, 2003).

This second use of magnetosomes in geology and paleomagnetism is not a deliberate one but important nonetheless. Bacterial magnetite has been shown to be an important carrier, sometimes the primary carrier, of magnetic remanence in some oceanic and lake sediments (Kim *et al.*, 2005; Oldfield and Wu, 2000; Snowball, 1994; Snowball *et al.*, 2002). It is this fine grained magnetic material that records the Earth's magnetic field at the time it was deposited. Using isotopic dating and other technologies, researchers can determine approximately when the sediments were deposited and track changes in the magnetic field, which in turn provides information about the origin of the geomagnetic field and properties of the deep Earth, history of plate motions and magnetic reversals, and even magnetic proxy records of paleoenvironmental and paleoclimate (Evans and Heller, 2003).

VI. CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

It is clear that the bacterial magnetosome and its magnetic properties have been refined and optimized in the course of evolution by the organisms that synthesize them by controlling the chemical composition, size, and morphology of the magnetosome crystal as well as their position within the cell. The control over the biomineralization and these specific features must be mediated by the protein products of the *mam* and *mms* genes, particularly those located in the MM. Thanks to the relatively recent progress in the development of genetic systems and in genome sequencing of the magnetotactic bacteria, we expect and predict that the next significant discoveries in the magnetotactic bacteria will deal with the determination of the specific functions of these proteins. In turn, once functions of specific magnetosome proteins have been determined, we also expect that genetic modifications will result in much higher yields of magnetosomes and magnetite that should facilitate many more creative commercial and scientific applications for them.

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CHAPTER 3

The Distribution and Diversity of *Euryarchaeota* in Termite Guts

Kevin J. Purdy*

Contents

I. INTRODUCTION

Termites are the dominant invertebrates in tropical ecosystems (Collins, 1983; Eggleton *et al.*, 1996; Wood and Sands, 1978; Wood *et al.*, 1982). Through their consumption and digestion of plant-derived material, they have a major influence on soil structure, plant decomposition, carbon mineralization, and nutrient availability (Bignell and Eggleton, 2000; Lavelle *et al.*, 1997; Lee and Wood, 1971; Lobry de Bruyn and Conacher, 1990; Wood and Johnson, 1986). Studying their ecology and physiology, including the role of symbiotic microbes, is a vital to understanding their role in the global ecosystem.

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Termites are divided into two major groupings: the lower and higher termites (Abe *et al.*, 2000; Inward *et al.*, 2007). The lower termites, which presently consist of six families that all feed on wood or grass, are characterized by relatively simple gut structures and the presence of flagellated protists in their guts. In contrast, the higher termites consist of a single family, the Termitidae, which includes ~70% of all known termite species and are far more abundant than lower termites. Higher termites feed on a wide range of plant material at different stages of decomposition from sound wood to soil (Donovan *et al.*, 2001; Eggleton and Tayasu, 2001). The majority of higher termite species feed on highly humified plant material in soil and are especially diverse and abundant in tropical forest soils (Davies *et al.*, 2003).

Digestion in termites is closely related to gut structure, the physicochemical conditions in different gut regions, and symbiotic microbiota found in their guts (Brauman, 2000; Breznak, 2000; Kane and Mueller, 2002). In particular, methanogenic archaea (methanogens) have been detected in all termite guts that have been studied. The degree to which gut prokaryotes vary between termites is far from clear, but differences in host diet have been correlated with differences in how the microbial community processes hydrogen produced during the fermentation of organic matter. In anaerobic gut regions of both lower and higher termites that feed on wood, bacterial acetogenesis outcompetes methanogenesis for hydrogen, whereas the reverse is true in the guts of soil-feeders (Bignell *et al.*, 1997; Brauman *et al.*, 1992; Tholen and Brune, 1999). In fact the archaeal community in termite guts plays a part not just in termite metabolism but also may have had a role in the evolution and diversification of termites and their ecological success in tropical ecosystems.

II. *EURYARCHAEOTA* IN TERMITE GUTS

The presence of *Euryarchaeota*, and specifically methanogens, in termites from all seven families and all feeding guilds is intriguing. This is especially true as some wood-feeding termites emit little or no methane (Brauman *et al.*, 1992) while in soil-feeding termites methanogenesis can represent as much as 10% of the termite's respiratory effort (Tholen and Brune, 1999). This difference between feeding guilds has led to an effort to understand the community structure and role of methanogens in termite guts. To gain an understanding of the importance and variation in termite gut methanogen communities, it is first necessary to understand the structure of guts within which these methanogens grow.

A. Termite gut structure and metabolism

As termites have evolved from the lower to the higher termites, their guts have become more complex. Termite guts are all tiny but highly effective bioreactors with sharp and constantly maintained gradients of pH, oxygen,

hydrogen, and redox conditions (Brune, 1998). These complex environments are ideal for the degradation of cellulose from wood in wood-feeders and more recalcitrant plant lignocellulose and humics in soil-feeders. The difference between the higher and lower termites is seen clearly in gut structure, pH, and the presence or absence of flagellated protozoa. The classic lower termite gut is a simple structure with a paunch (P3) where almost all of the microbial activity is focused (Fig. 3.1A), while most higher termites have a more complex gut structure with some four major sections (Fig. 3.1B), two of which have high pHs [for a more detailed description see Bignell (1994)]. These reach extremes in the soil-feeding termites where pH of the P3 proctodeal segment can be in excess of pH 11. The high gut pH has been linked to the release of recalcitrant organic matter from soil matrix particles and its chemical decomposition prior to fermentation by gut microflora (Brune, 1998; Brune and Kühl, 1996). Studies using microsensors have indicated that the mixed and P3 segments of *Cubitermes orthognathus* are hydrogen sources while the P4a and b segments are hydrogen sinks. Due to coiling of the guts *in vivo*, these two sections are next to each other and cross-epithelial hydrogen transfer seems to be occurring (Schmitt-Wagner and Brune, 1999). In addition to increased complexity in gut structure and physicochemical conditions, the transition from lower to higher termites is characterized by the loss of the flagellated protozoa. Thus, the lower termites, which are all either wood or grass-feeders, have guts that are

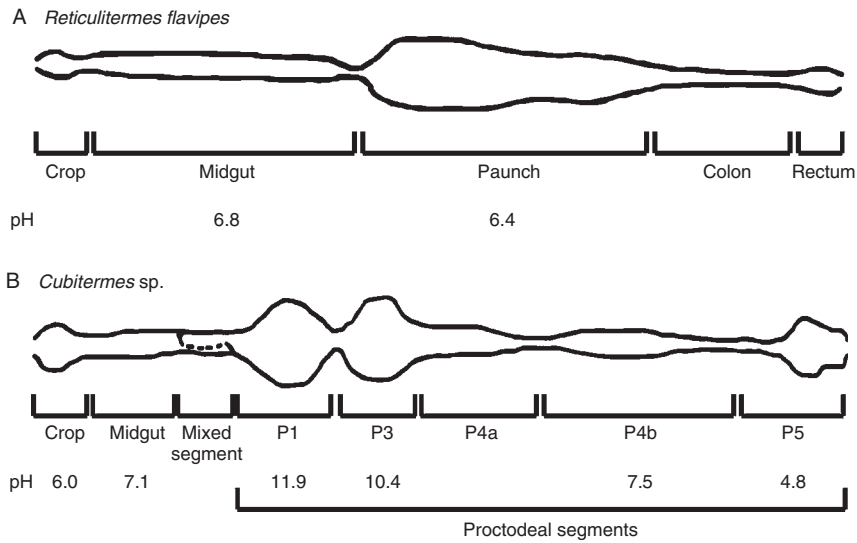

FIGURE 3.1 Diagram of termite gut structure for (A) lower termites (Ebert and Brune, 1997) and (B) soil-feeding higher termites (Friedrich *et al*., 2001). The pH of each segment is as reported by Brune and Kühl (1996) and Brune *et al*. (1995). Diagrams are published with the kind permission from the American Society for Microbiology and Dr A. Brune, MPI, Marburg, Germany.

dominated by flagellated protozoa while the higher termites with their diverse diets from sound wood to true soil-feeding do not.

However, despite the clear differences between lower and higher termite, differences in methane emission rates are related not to phylogeny but to feeding group. Figure 3.2 shows the difference in methane emission from wood-, fungus-, and humus/soil-feeding termites (Brauman *et al.*, 1992). In general, wood-feeding termites emit far less methane than any other termites, while soil-feeding termites emit the most. The explanation for this change in terminal oxidation products in termites revolves around the dominant hydrogen sink process in termite guts. Termite guts, whether lower or higher termites, are essentially minute bioreactors which maintain conditions that favor the degradation of relatively labile or recalcitrant plant material by the termite gut microflora (Brune, 1998). In lower termites and wood-feeding higher termites, hydrogen gas produced by the fermentation of complex organic matter is primarily consumed by homoacetogens that produce acetate which is then absorbed by the host (Breznak and Kane, 1990). However, in soil-feeding termites acetogenesis appears to be very limited (Brauman *et al.*, 1992) and methanogenesis can account for up to 10% of the total respiratory effort of the termite (Tholen and Brune, 1999). Thus, investigating the structure of the methanogen community in termite guts is essential to a deeper understanding of termite function *in situ*.

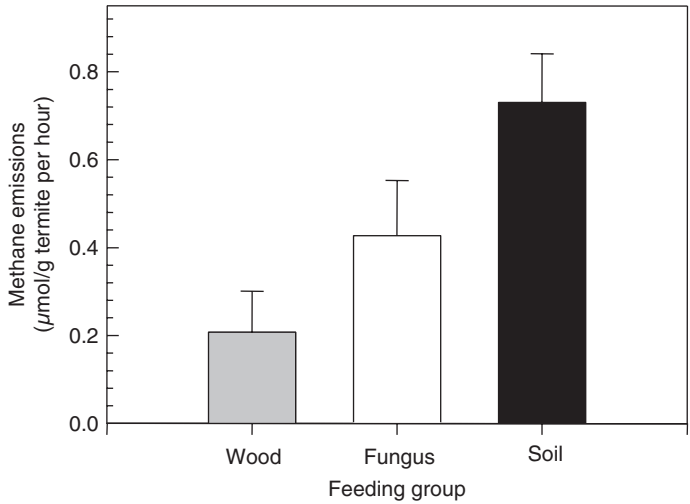

FIGURE 3.2 Methane production rates in different termite-feeding guilds based on data from Brauman *et al.* (1992), excluding measurements taken with exogenously supplied hydrogen.

III. DETECTION OF *EURYARCHAEOTA* IN TERMITE GUTS

A number of studies have investigated *Euryarchaeota* in termite guts, although, to date, there has been no systematic sampling across the termite phylogenetic tree. These studies have identified a range of different methanogens and these are presented in a schematic tree in Fig. 3.3. All of the published data is presented in Table 3.1 and is organized with reference to phylogenetically supported clades shown in Fig. 3.3.

What is clear from this data is that a considerable diversity of *Euryarchaeota* are found in termites. Furthermore, there appears to be a difference between the diversity of *Euryarchaeota* in lower termites compared to higher termites. The schematic tree (Fig. 3.3) which incorporates groups from all of the studies reported to date shows that while *Methanobrevibacter* can be detected in all termites, members of the *Methanomicrobiales* and *Methanosarcinales* are usually only detected in the higher termites. A substantial study using 16S rRNA-targeted oligonucleotide probes detected *Methanobacteriaceae* in all but one termite and *Methanosarcinaceae* in a range of wood-, soil-, and fungus-feeding termites, although there was no indication of which members of these families the signal came from (Brauman *et al.*, 2001). No signal from *Methanomicrobiales* was detected in any termite tested. Additionally, a study by Shinzato *et al.* (1999) reported the detection of a clone related to *Methanocorpusculum* and the presently uncultured *Thermoplasmalates*-related clade (Table 3.1 and Fig. 3.3) in the lower termite *Reticulitermes speratus*. Thus, in general, higher termites, whether soil-feeders or wood-feeders, maintain a more diverse euryarchaeal community in their guts. This observation must be qualified by the fact that a very limited number of termite species have been analyzed in detail. The majority of these studies have been based on very small clone libraries without a significant effort to find any hidden diversity. The two major studies, which have utilized both fingerprinting techniques [Terminal Restriction Fragment Length Polymorphism (T-RFLP)] and clone libraries (Donovan *et al.*, 2004; Friedrich *et al.*, 2001), have detected by far the most diverse communities but both were focused on the soil-feeding genus *Cubitermes*. This suggests that with such limited termite taxon sampling and archaeal community analysis these studies may have underestimated archaeal diversity in termite guts. Even with the limited datasets we have at present, there is much that can be inferred from the *Euryarchaeota* detected in termite guts and from studies on the physicochemical environment and the physical location of detected methanogens.

A. Isolated *Euryarchaeota* from termite guts

Three methanogenic strains have been isolated from the wood-feeding lower termite *R. flavipes* (Leadbetter and Breznak, 1996; Leadbetter *et al.*, 1998) (Table 3.1). The isolation of methanogens related to *Methanobrevibacter*

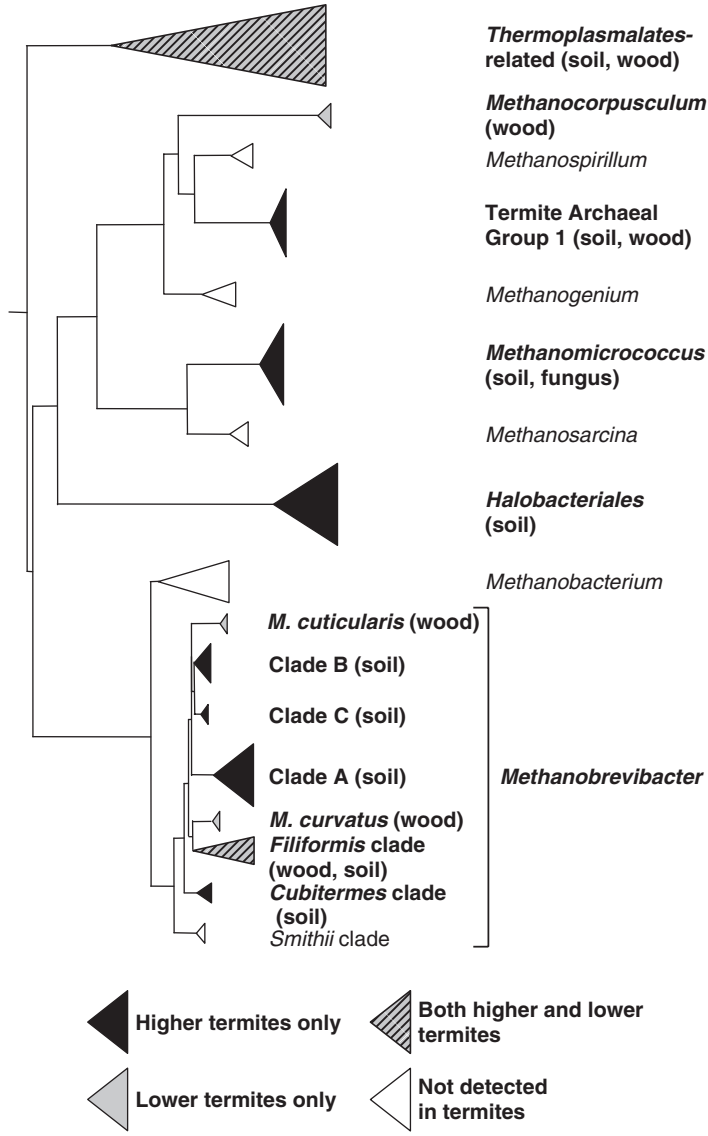

FIGURE 3.3 A schematic phylogenetic tree of euryarchaeal sequences detected in termite guts. Clade names given in bold are those detected in termite guts, the feeding habit of the termites associated with those clades are given in brackets. Tree structure is based on a Logdet/Paralinear distances tree that included sequences from reference taxa and environmental clones (Donovan *et al.*, 2004; Godon *et al.*, 1997; Tajima *et al.*, 2001) giving 981 positions for analysis. Pairwise distances for all alignable sites were calculated using the Logdet/Paralinear distances method using PAUP (Swofford, 1998) as described previously (Purdy *et al.*, 2002) and limited to only variable sites (60%).

TABLE 3.1 Summary of *Euryarchaeota* detected in termite guts

References	Termite species	Methanogen clade (from Fig. 3.3)	Representative clone/ isolate	Accession number
Lower termites				
Leadbetter and Breznak, 1996	*Reticulitermes flavipes*	*Methanobrevibacter*[a]	*M. cuticularis*	U41095
			M. curvatus	U62533
Leadbetter *et al.*, 1998	*R. flavipes*	*Methanobrevibacter filiformis*	*M. filiformis*	U82322
Ohkuma *et al.*, 1995	*R. speratus*	*Methanobrevibacter*	M4	D64027
Ohkuma and Kudo, 1998	*Cryptotermes domesticus*	*Methanobrevibacter*	Cd30	AB008900
Shinzato *et al.*, 1999	*R. speratus*	*Methanobrevibacter*	RS104	AB024040
		M. filiformis	RS301	AB024042
		Methanocorpusculum	RS105	AB024043
		Thermoplasmalates related	RS406	AB024044
Ohkuma *et al.*, 1999	*Hodotermopsis sjostedti*	*Methanobrevibacter*	MHj4	AB009821
Tokura *et al.*, 2000	*R. speratus*	*Methanobrevibacter*	LRsD3	AB026913
			LRsM1	AB026914
		M. filiformis	LRsD2	AB026912
	H. sjostedti	*Methanobrevibacter*	LHD2	AB026920
			LHM8	AB026922
		M. filiformis	HW3	AB026925

(*continued*)

TABLE 3.1 (*continued*)

References	Termite species	Methanogen clade (from Fig. 3.3)	Representative clone/ isolate	Accession number
Higher termites				
Ohkuma *et al.*, 1999	*Pericapritermes*[b] *nitobei*	*Methanobrevibacter* Clade A	MPn19	AB009827
		Methanomicrococcus	MPn1	AB009825
		Termite Archaeal Group 1	MPn4	AB009826
	Nasutitermes takasagoensis[c]	Termite Archaeal Group 1	MNt1	AB009823
	Odontotermes formosanus[d]	*Methanomicrococcus*	MOf1	AB009822
Friedrich *et al.*, 2001	*Cubitermes orthognathus*[b]	*Methanobrevibacter* Clade A	P3-Ar-10	AF293557
		Methanobrevibacter Clade B	P4b-Ar-20	AF293492
		Methanobrevibacter Clade C	P4b-Ar-23	AF293493
			P5-Ar2-16	AF293583
		M. filiformis	P4b-Ar-8	AF293505
		Methanobrevibacter Cubitermes clade	P3-Ar-1	AF293556
			P4b-Ar-14	AF293486
		Methanomicrococcus	P1-Ar-7	AF293519
			P3-Ar-24	AF293569
			P4b-Ar-18	AF293490
		Termite Archaeal Group 1	P3-Ar-30	AF293589
			P4b-Ar-4	AF293501

		Thermoplasmalates-related	P3-Ar-9	AF293578
			P4b-Ar-19	AF293491
			P5-Ar-10	AF293547
Donovan *et al.*, 2004	*Cubitermes fungifaber*[b]	*Methanobrevibacter* Clade A	Gut103-A13	AY487202
		Methanobrevibacter Clade B	Gut103-A08	AY487195
		Methanobrevibacter Clade C	Gut103-A25	AY487200
		Methanobrevibacter *Cubitermes* clade	Gut103-A15	AY487207
		Methanomicrococcus	Gut103-A38	AY487204
		Halobacteriales	Gut103-A24	AY487198
		Termite Archaeal Group 1	Gut103-A11	AY487191

[a] Clones designated ''*Methanobrevibacter*'' were not assigned to monophyletic clades that were strongly supported in a bootstrap analysis of the Logdet tree used in Fig. 3.3.
[b] Soil-feeder.
[c] Wood-feeder.
[d] Fungus-feeder.
Identification is based on sequence position relative to the clades shown in Fig. 3.3. For short sequences or those that targeted a different section of the 16S rRNA gene, identification is based on its phylogenetic inclusion in Arb database tree (Ludwig *et al.*, 1998, 2004).

arboriphilus and *Methanobacterium bryantii* from two *Nasutitermes* higher termite species was reported in a conference abstract (Yang *et al.*, 1985) but there have been no subsequent publications or culture collection deposits to support these claims. The three *R. flavipes* isolates are all *Methanobrevibacter* [*M. cuticularis* DSM 11139, *M. curvatus* DSM 11111, and *M. filiformis* DSM 11501 (Leadbetter and Breznak, 1996; Leadbetter *et al.*, 1998)] that are essentially limited to using H_2/CO_2 as their energy source. Leadbetter and Breznak (1996) determined that some 10% of the cells in the gut of *R. flavipes* were *Methanobrevibacter* and that these were associated with the gut epithelial wall. This was surprising because this region of the gut is exposed to significant amounts of free oxygen (Brune and Friedrich, 2000) that should be toxic to methanogens. However, these two strains, and other *Methanobrevibacter* species, can mediate a small net oxygen consumption, possibly via the activity of a catalase which may help these organisms to survive oxygen exposure (Leadbetter and Breznak, 1996). It has been suggested that methanogens at the gut wall ''mop up'' any hydrogen that is not utilized by acetogens in the main body of the gut (Ebert and Brune, 1997).

B. Uncultured *Euryarchaeota* in lower termite guts

The gut wall was the only site where methanogens could be found in *R. flavipes* (Brune, 1998; Leadbetter and Breznak, 1996), although this was not true in other lower termites (Table 3.1). Using epifluorescence microscopy, Lee *et al.* (1987) showed that several gut protists from *Zootermopsis angusticollis* have exo- and endosymbiotic methanogens that were morphologically similar to *Methanobrevibacter*. Further to this, Messer and Lee (1989) demonstrated that the protozoan *Trichonympha* produced most of the hydrogen in the termite's gut, and methanogenic symbionts in *Trichomitopsis* produced most of the methane in *Z. angusticollis* guts. Interestingly, *Z. angusticollis* appears to produce far more methane than any other wood-feeding termites (Brauman *et al.*, 1992), which may be related to the number of protists that have associated methanogens in their guts which are therefore close to a hydrogen source. Tokura *et al.* (2000) reported that, in *R. speratus* and *Hodotermopsis sjoestedti*, 4–42% of the gut protists had methanogens associated with them. Shinzato *et al.* (1999) cloned a 16S rRNA gene sequence related to *Methanocorpusculum parvum* from the hindgut of *R. speratus*. This is the only report of this genus in termite guts and as *M. parvum* has been detected in anaerobic ciliates (Embley *et al.*, 1992; Finlay *et al.*, 1993), it may also be associated with a protozoa. Thus, it appears that methanogens in lower termites are associated either with the gut epithelium or with protists within the gut. However, it is still unclear what role methanogens play in lower termite nutrition and digestion or how much of the methane derived from lower termites is generated by the protist-associated or the gut wall-associated methanogens.

C. Uncultured *Euryarchaeota* in higher termite guts

Higher termite guts lack the flagellated protozoa that dominate the gut microflora of lower termites, yet can produce more methane. In studies on archaea in higher termite guts, a wider diversity of *Euryarchaeota* have been detected than in lower termites (Table 3.1), including members of the *Methanobacteriales*, *Methanosarcinales*, and the *Methanomicrobiales*. In a very limited analysis, Ohkuma *et al.* (1999) detected members of all three of the above families in the soil-feeder *Pericapritermes nitobei*, members of the *Methanomicrobiales* in the wood-feeder *Nasutitermes takasagoensis*, and *Methanosarcinales* in fungus-grower *Odontotermes formosanus* (Table 3.1).

Two much more substantial surveys of archaeal diversity in soil-feeding termites have, using both T-RFLP and 16S rRNA gene sequences, analyzed the axial distribution of archaea in the gut of the soil-feeder *C. orthognathus* (Friedrich *et al.*, 2001) and the relationship between gut euryarchaeal communities and the termites' food–soil in *C. fungifaber* (Donovan *et al.*, 2004). There was a remarkable degree of similarity in the 16S rRNA gene sequence analyses from these two studies, as can be seen in Table 3.1. Differences between the analyses are discussed in more detail below.

In order to analyze a much wider range of samples that would be possible by 16S rRNA gene sequence analysis, Friedrich *et al*. (2001) and Donovan *et al.* (2004) used T-RFLP as a fingerprinting method. Friedrich *et al.* (2001) used the method as described by Chin *et al.* (1999), which analyzes an 800-bp fragment. This method proved successful at separating a number of archaeal genera but some genera required additional digestion and analysis. A substantial uncut peak was detected which consisted of signal from members of the *Methanosarcinaceae* and *Methanobacteriaceae* which were subsequently separated using a double digest with *TaqI* and *HaeIII*. Donovan *et al.* (2004) focused their analysis by using a euryarchaeal-specific PCR (Munson *et al.*, 1997). T-RFLP analysis of these longer (1050 bp) PCR products was much more effective at distinguishing euryarchaeal genera and clades. While it is difficult to compare across the two exact methods used, there were strong similarities and only small differences between the two studies. Using the clades that are supported by phylogenetic analysis (as shown in Fig. 3.3), the differences in both analysis and community can be seen in Table 3.2. These results indicate that euryarchaeal communities within soil-feeding termite guts may be very stable across species.

The differences between these two studies were similar in both the 16S rRNA gene sequence and T-RFLP analyses. Friedrich *et al.* (2001) detected a substantial number of clones and T-RFLP signal from an uncultured and uncharacterized clade distantly related to the *Thermoplasmalates*, but no clones or T-RFLP signal were detected from this clade by

TABLE 3.2 Summary of terminal restriction fragment length polymorphism (T-RFLP) data of *Euryarchaeota* detected in *Cubitermes* guts (Donovan *et al.*, 2004; Friedrich *et al.*, 2001)

	T-RF size based on method used by		Detected in termite guts by	
Euryarchaeal clade	Friedrich	Donovan	Friedrich	Donovan
Methanobrevibacter Clade C	88	266	Yes	Yes
M. filiformis	611	*794*	Yes	No
Methanobrevibacter A, B, and *Cubitermes* Clades	>800 (uncut)	984–1006	Yes	Yes
Methanomicrococcus	>800 (uncut)	1044[a] (uncut)	Yes	Yes
Halobacteriales[b]	Not detectable[c]	118	No	Yes
Termite Archaeal Group 1	389	568	Yes	Yes
Thermoplasmalates-related	341	*526*	Yes	No
	375	*560*	Yes	No
Methanosaeta[d]	*284*[e]	457	No	Yes

[a] *In silico* analysis suggests a T-RF of 1011 but clones do not cut at this site.
[b] Same T-RF as Rice Cluster I that was only detected in soil (Donovan *et al.*, 2004).
[c] Cut site occurs after reverse primer used by Friedrich *et al.* (2001).
[d] Identified by reference to sequence data and as detected by Chin *et al.* (1999).
[e] T-RF as detected by Chin *et al.* (1999).
Individual Terminal Restriction Fragments (T-RFs, ±2–3 base pairs) have been assigned to the clades shown in Fig. 3.3. T-RFs given in italics were either not detected in the studies quoted or T-RF size are based on *in silico* analysis. Unidentified T-RFs have not been included.

Donovan *et al.* (2004). An analysis of sequences closely related to Friedrich's *Thermoplasmalates*-related clones indicates that the reverse primer used by Donovan *et al.* (2004) would probably not amplify this clade, which may explain the difference in these analyses (K.J.P., unpublished data). This cluster is so distant from the cultured *Thermoplasmalates* that it is not possible to speculate on a possible physiology for this clade, except to say it is unlikely to be acidophilic and thermophilic like the cultured members of *Thermoplasmalates*.

Donovan *et al.* (2004) detected a small number of clones that clustered with the extreme halophiles of the *Halobacteriales* (Oren, 2000). A T-RFLP

signal associated with the *Halobacteriales* clade was only detected in termite guts at one of five distinct African sites studied by Donovan *et al.* (2004). However, a subsequent analysis detected a *Halobacteriales*-related Terminal Restriction Fragment (T-RF) in *C. fungifaber* gut samples taken from four African sites (K. J. P., unpublished data). This would suggest that *Halobacteriales* may represent a minor component of soil-feeding termite gut microflora. The majority of isolated members of the *Halobacteriales* are obligate halophiles, requiring in excess of 8% NaCl in order to grow (Oren, 2000), although there are a few exceptions (Elshahed *et al.*, 2004; Purdy *et al.*, 2004; Savage *et al.*, 2007). It would therefore seem a little strange to find these in termite guts except that termite guts, particularly the very high pH segments, also contain large concentrations of potassium (Brune and Kühl, 1996), which may create conditions that favor the *Halobacteriales*.

Friedrich *et al.* (2001) showed a differential distribution of archaea in the different segments of the gut of *C. orthognathus*. *Methanomicrococcus*-related clones were detected primarily in the alkaline P1 gut segment with *Methanobrevibacter* and Termite Archaeal Group 1 dominant in P3, P4b, and P5 with an increasing proportion of the uncultured *Thermoplasmalates*-related clade in these three segments. This differential distribution does appear to be related to the function of the specific methanogen clades. The majority of methanogenesis occurs in *Cubitermes* species in the P3/P4a and P4b gut sections where the *Methanobrevibacter* and Termite Archaeal Group 1 were dominant. Donovan *et al.*'s analysis suggests that *Methanobrevibacter* and Termite Archaeal Group 1 dominate in whole termite guts. However, their 16S rRNA gene sequence analysis of food–soil detected a large number of clones related to *Methanomicrococcus*, although this was not supported by T-RFLP analysis of the soils. The presence of *Methanomicrococcus* in the mixed segment and P1 gut sections reported by Friedrich *et al.* could be indicative of the presence of a soil-based community in these sections of the gut. This, of course, raises the question of the role soil-based methanogens may play in termite guts. Donovan *et al.* concluded that their data did not support the hypothesis that methanogens in termite guts are derived from their food–soil and data collected from more sites in Africa subsequent to their study supports this (K. J. P., unpublished data). Donovan *et al.*'s data also does not support a purely vertical transmission of gut microflora, with the caveat that there do appear to be specific groups that are found only in termite guts [Termite Archaeal Group 1, *Methanobrevibacter* Clade C, and *Cubitermes* Clade (Table 3.1 and Fig. 3.3)]. This is in line with studies on the bacterial community in termite guts, which suggest a degree of coevolution has occurred between termites and their microflora (Hongoh *et al.*, 2005).

IV. WHY ARE THERE DIFFERENT *EURYARCHAEOTA* IN DIFFERENT TERMITES?

The data that presently exists on the distribution and diversity of *Euryarchaeota* in termite guts is striking, especially as the greatest difference appears to be between wood- and soil-feeding termites. The limited studies that have been performed on wood-feeding termites indicate a euryarchaeal community dominated by *Methanobrevibacter*, while a much more diverse euryarchaeal community is present in soil-feeding termites. If we assume this difference is real rather than a product of poor taxon sampling and limited datasets, then it is pertinent to ask what could be the driver for this difference.

If thermodynamics only are considered, then methanogenesis would be expected to outcompete acetogenesis as hydrogenotrophic methanogens are capable of reducing hydrogen concentrations to a threshold that is too low for acetogenesis to be thermodynamically favorable (Cord-Ruwisch *et al.*, 1988). Clearly, termite evolution has selected for conditions that favor acetogenesis in wood-feeding termites and methanogenesis in soil-feeding termites. Acetogenesis directs energy from hydrogen production back into absorbable carbon that can be used by the termite host which is lost to methane production in soil-feeding termites. This implies that there must be considerable and compelling selective pressures that favor methanogenesis in soil-feeding termites. Soil-feeding termites utilize recalcitrant organic matter in soil, which is more reduced than carbon in sound wood and therefore produces more hydrogen as a waste product during fermentation. In order to maximize energy production and pull these fermentation reactions to completion, this hydrogen must be removed as rapidly as possible. This is most effectively achieved by maintaining as low a hydrogen partial pressure as possible. Acetogenesis, on the other hand, requires a relatively high hydrogen partial pressure to be thermodynamically viable. Thus, it can be hypothesized that in order to utilize the carbon that is present in soil, soil-feeding termites must maintain a lower hydrogen partial pressure than wood-feeding termites. The lower hydrogen threshold that methanogens, and in particular the *Methanomicrobiales*, can maintain compared to acetogens (Cord-Ruwisch *et al.*, 1988) could explain the shift from acetogenesis to methanogenesis in the soil-feeding termites. This is despite the fact that such a shift leads to the effective loss of a significant source of energy (acetate from acetogenesis) for the termite host in soil-feeding termites. The shift from wood- to soil-feeding opened up a vast resource to termites and is clearly the primary reason they have become so important in tropical and subtropical soils.

V. CONCLUSION

The *Euryarchaeota* are a critical component of all termite guts, acting as hydrogen sinks in both lower and higher termites. In lower termites and in the wood- and fungus-feeding higher termites, the role of the methanogens appears to be to mop up trace hydrogen. In the soil-feeding higher termites, methanogenesis lies at the heart of termite nutrition and represents an essential gut process. New analyses of termite evolution suggest that the evolution of the higher termites probably occurred via an externalization of the gut in the fungus-feeding *Macrotermitinae* followed by the evolution of soil-feeding (Inward *et al.*, 2007). It is plausible that a first step in this process would have been the acquisition of a *Methanomicrobiales* strain that had the potential to reduce hydrogen partial pressures to levels that allowed the effective exploitation of soil organic matter. Were this the case then it could be legitimately claimed that the evolution of what has become one of the most important animal families on the planet was dependent on the acquisition of a microbe, in this case a methanogen. Such a hypothesis revolutionizes our understanding of animal–microbe interactions and places microbes at the heart of ecosystem evolution.

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CHAPTER 4

Understanding Microbially Active Biogeochemical Environments

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I. INTRODUCTION

Microbial life accounts for the vast majority of all metabolic and genetic diversity on Earth, and encompasses an overwhelming majority of the Earth's total biomass. Microorganisms survive in almost all environments where it is thermodynamically favorable for them to do so, and niches once considered to be uninhabitable (e.g., hot and cold deserts, hot springs, hypersaline environments, and deep subsurface) are now known to harbor thriving microbial communities (Edwards *et al*., 2000; Nercessian *et al*., 2003; Schippers *et al*., 2005; Smith *et al*., 2006). Within this wide range of habitats, all major groups of microorganisms are represented, including cyanobacteria, bacteria, archaea, microalgae, and fungi, demonstrating the abundance of diversity in the microbial world. Bacteria and archaea, in particular, have been able to adapt to prevailing energy (light or chemical) and nutritional (organic carbon or CO_2) sources, and within this variable energy–nutrition regime they have exploited distinct energy-producing pathways, for example respiration, using a variety of terminal electron acceptors (such as O_2, NO_3^-, SO_4^{2-}, Fe^{3+}). The importance and extent of microbial diversity and metabolism have now captured the attention of the scientific community and as a consequence there is now more interest in assessing biogeochemical ecosystems than at any time in the past.

Until comparatively recently, culture-based bias had been reflected within reported microbial biodiversity due to the small proportion of microbes from natural environments that are culturable. However, the advent of molecular biology has launched a new era in environmental biogeochemistry, enabling a new evaluation of the diversity and importance of geomicrobiological activities such as global elemental and nutrient cycling (Pace and Marsh, 1985; Woese *et al*., 1990). Molecular techniques are valuable tools that can improve our understanding of the structure and nature of microbial communities and provide us with the ability to probe for life in all niches of the biosphere. Rapid progress in genomics has resulted in novel innovations in DNA sequencing capabilities, technologies to monitor gene activities, and statistical and mathematical approaches for analyzing genetic data.

Although knowledge of microbial diversity in geologic systems is evolving, and methods to study diversity are improving, there is still little understanding of the complexity of global geomicrobiological processes or the relationship between biodiversity and biogeochemical function. Perhaps the greatest challenge facing geomicrobiology is linking phylogeny with function, as the most widespread molecular methods, which are based on ribosomal gene analysis, provide extensive information about the taxa present in an environment, but little insight into the functional role of each phylogenetic group. This chapter is intended to give an

overview of the latest findings in the field of geomicrobiology and to provide a discussion on the influence of microbial populations and activities on geologic habitats.

II. AN INTRODUCTION TO THE MOLECULAR MICROBIAL WORLD

The three-domain biological classification system (Fig. 4.1) was introduced by Carl Woese in 1990 (Woese *et al*., 1990). Based on rRNA sequence data, Woese identified 12 major divisions (phyla) in the domain Bacteria, representing almost all major cultured groups of bacteria accumulated during a century of microbiological research. In the years following this breakthrough, culture-independent surveys have identified at least 40 major well-resolved bacterial divisions, indicating that there are ~30 major bacterial divisions with no or very few representatives in culture collections (Hugenholtz *et al*., 1998; Konstantinidis and Tiedje, 2004). Archaea were thought to be present only in extreme habitats such as hot springs, saline lakes, and deep-ocean thermal vents. Culture-independent techniques have revealed that the domain Archaea contains at least 50 distinct phylogenetic groups with 33 from the Euryarchaeota, 13 from Crenarchaeota, 1 each from Korarchaeota, Nanoarchaeota, and the ancient

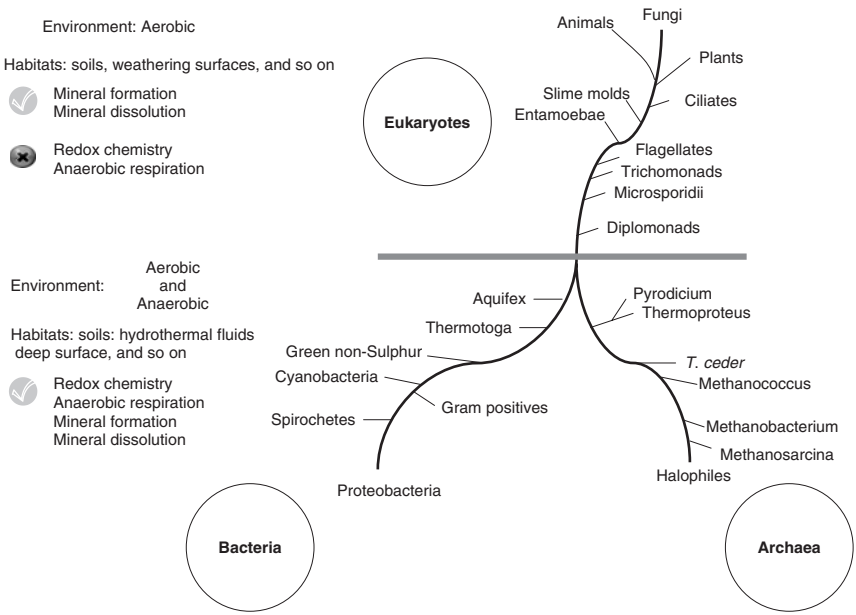

FIGURE 4.1 Basic overview of the tree of life.

archaeal group (AAG) (Huber *et al.*, 2002; Schleper *et al.*, 2005). Among these 50 phylogenetic groups, only 13 have cultured representatives (Schleper *et al.*, 2005). In addition, a diverse range of Archaea from non-extreme habitats, such as gardens and forests, water and sediments in marine and freshwater lakes, has been documented (Bintrim *et al.*, 1997; Buckley *et al.*, 1998; Nicol *et al.*, 2003, 2005). A large number of fungal species have been identified and named, and these species are grouped into five main phyla: Chytridiomycota, Zygomycota, Glomeromycota, Basidiomycota, and Ascomycota. Several recent studies of environmental DNA have identified major groups of unexpected fungal diversity in a variety of environments. Vandenkoornhuyse *et al.* (2002) found 49 unique phylotypes from a library of 200 18S rRNA clones in an analysis of fungal DNA from the roots of the grass *Arrhenatherum elatius*. Only 7 of the 49 were closely related to known sequences (>99% identity), with 5 distinct lineages were reportedly being significantly different from all known fungal sequences.

A. 16S approaches

The use of ribosomal sequences to estimate microbial diversity in environments began with the development of methods to isolate total DNA from the environment, the subsequent cloning of DNA using vectors (such as bacteriophage lambda), and sequencing of clones that hybridized to rRNA probes (Pace and Marsh, 1985). Many types of rRNA sequences not present in culture collections were identified and the later inclusion of gene-specific polymerase chain reaction (PCR) before the cloning step significantly enhanced the method. The phylogenetic analysis of mixed microbial communities in ocean waters was the first application of PCR and led to the discovery of ubiquitous and abundant groups of new microorganisms (Giovannoni *et al.*, 1990). Since then there has been widespread application of PCR-based analyses of rRNA to examine mixed microbial communities in a variety of environments. In addition to redefining the taxonomy and phylogeny of the microbial world, this approach has also revealed the vast extent of microorganisms that exist in all types of geochemical environments (Fig. 4.2). Here we present only an overview of some advances in molecular microbial ecology, and the reader is referred to a growing list of manuscripts dealing more thoroughly with this topic (Hugenholtz *et al.*, 1998; Konstantinidis and Tiedje, 2004; Torsvik *et al.*, 1998; Xu, 2006).

The ability to determine microbial community structure at a high resolution (group, species, and strain) without the need for cultivation has revolutionized our understanding of uncultured microorganisms. Small subunit rRNA genes contain both highly conserved and variable regions which allow phylogenetic relationships at several hierarchical levels to be assessed from comparative sequence analysis. While the

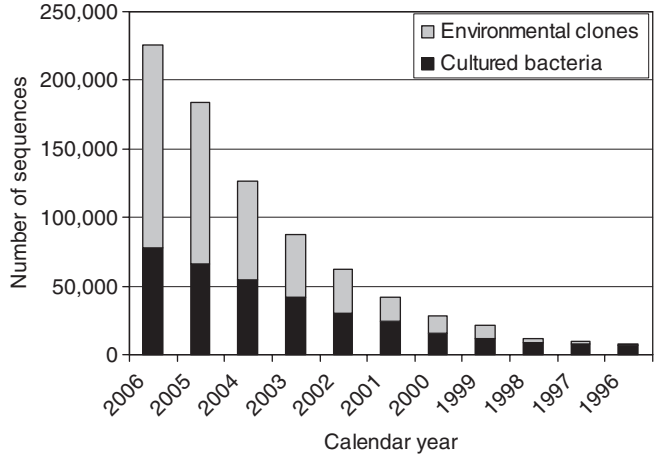

FIGURE 4.2 Cumulative numbers of aligned 16S rRNA gene sequences available for analysis from the Ribosomal Database Project (http://rdp.cme.msu.edu) based on calendar year (2006 figures to May 2006). The numbers of environmental clone sequences are presented in gray and the numbers of sequences from cultivated bacteria are presented in black.

phylogenetic properties and degree of sequence information available make these genes an obvious choice, some caution must be expressed. For example, the heterogeneity of 16S between multiple copies within one species may hinder pattern analysis and interpretation of diversity from clone libraries (McCaig *et al*., 2001). The extent of 16S heterogeneity and resolution also varies between different regions, for example it has been shown that the 16S rRNA gene lacks resolution at the species level (Schmalenberger *et al*., 2001). Despite technical limitations and biases (von Wintzingerode *et al*., 1997), various approaches based on SSU rRNA genes from natural assemblages have proven to be useful in describing the structure of mixed microbial communities. Pattern analysis, or fingerprinting, is carried out by evaluating banding patterns of PCR products on either gel-based or automated sequencer systems. Techniques that use enzymatic digests, such as restriction fragment length polymorphism (RFLP) or single-stranded conformation polymorphism (SSCP) analysis, however produce multiple bands for single species (Stach *et al*., 2001) making community patterns difficult to interpret.

B. rRNA and mRNA

Rapid developments in reverse transcriptase-PCR (RT-PCR) and improvement in RT enzymes have provided opportunities for evaluation of active communities inferred from analysis of rRNA and mRNA.

The detection of mRNA is a definitive indicator of activity, and its detection based on a specific gene sequence can be used to assess gene expression and to determine the response of a particular function to changes in environmental conditions. This approach is limited by the short half-life of mRNA and difficulties in extracting sufficient quantities for analysis before degradation. Another approach assumes that more active and faster growing organisms possess more ribosomes and, consequently, higher levels of rRNA. Therefore, quantification of RNA may be used to indicate levels of activity of different components of a community. Attempts have been made to estimate specific activities based on the basis of *in situ* derived catabolic mRNA levels (Sayler *et al*., 2001). This approach can work well if the level of regulation for the functional gene is understood and detection of mRNA can be correlated specifically with measurement of activity.

C. Recent technological advances

More recently there have been developments in molecular technologies that should ultimately lead to a greater understanding of microbial communities. In particular, microarray technology (high-throughput systems allowing the simultaneous analysis of thousands to hundreds of thousands of genes at the same time) has been developed and evaluated for bacterial detection and microbial community analysis. These arrays include: (1) phylogenetic oligonucleotide arrays containing sequences from rRNA genes of specific groups of organisms, (2) community genome arrays that contain highly specific gene sequences from known cultured microbial species, and (3) functional gene arrays containing the conserved domains of genes involved in specific metabolic pathways, for example the biogeochemical cycling of carbon, nitrogen, sulfate, phosphate, and metals (Zhou, 2003). Preliminary evaluations have suggested that microarrays have enormous potential for the detection, identification, and characterization of microorganisms in natural habitats (Wu *et al*., 2004). Significant challenges still remain in the application of microarray techniques to the analysis of microbes in natural habitats, mainly because microbial communities contain highly heterogeneous groups of organisms with undefined/unknown genomic relationships. The highly skewed distribution of microbial species, the potential of cross-hybridization between closely related species, the genetic variation among strains within species, and the differential efficiencies of isolating DNA from among the species can all bias datasets and influence the interpretation of data.

The study of the collective genomes of an environmental community, or metagenomic technology, has been developed recently for the characterization of entire ecological communities. Studies of metagenomes typically involve cloning fragments of DNA isolated directly from natural

environments, followed by sequencing and functional analysis of the cloned fragments. One of the most extensive microbial metagenomic studies was the shotgun sequencing of microorganisms in the Sargasso Sea in the Atlantic Ocean near Bermuda (Venter *et al*., 2004). This study generated almost 2 million sequence reads and, based on sequence relatedness and unique rRNA gene counts, the analysis has suggested that these DNA fragments were derived from at least 1800 genomic species. Computational analysis of the data identified over 1.2 million potential unique protein-coding genes, a surprisingly large number considering that at the time only about 140,000 protein data entries were available in the Swiss-Prot protein database (Xu, 2006). A more developed tool to understand gene function of microbial species utilizes proteomic technologies to investigate protein–protein interactions (Wilmes and Bond, 2004). As many important biochemical reactions are catalyzed by enzymes, all made up of individual proteins, it seems obvious that we should consider the vast array of microbial proteins in the different ecosystems where they occur. This emerging field of metaproteonomics was first reported in 2004 (Rodriguez-Valera, 2004) where the proteome of a laboratory-scale activated sludge system was extracted and the normal proteonomics approaches then applied (2D-PAGE with excision and characterization of highly expressed spots using time-of-flight mass spectrometry).

III. MICROORGANISMS IN THE ENVIRONMENT

Concomitant with advances in understanding genetic diversity, there has been a growth in the appreciation of prokaryotic diversity at the metabolic level. This diversity clearly defines the prokaryotes as a distinct group from the eukaryotes, and illustrates the strong relationship between prokaryotes and the biogeochemistry of the environment in which they live (Table 4.1). There is a remarkable metabolic diversity that characterizes the prokaryotic world; the ability to utilize inorganic energy sources is found only in the prokaryotes, and the oxidation and reduction of these inorganic compounds form a strong link with their geochemical environment as many of the reactions involve mineral deposition or dissolution (Nealson and Popa, 2005).

A. Microbes and minerals

It is evident that microorganisms play an important role in the weathering of rock surfaces and their constituent minerals. Chemical weathering of rock and mineral substrates may result in the mobilization of essential nutrients (e.g., P, S) and metals (K, Mg, and so on) required for plant and microbial growth, as well as nonessential elements such as Al and Pb

TABLE 4.1 Selected examples of geochemical environments where molecular approaches have been useful in determining microbial population dynamics

Environment	Molecular technique	Specific habitat	Selected references
Surface	Automated ribosomal intergenic spacer analysis (A/RISA)	Weathering silicates	Gleeson *et al.*, 2006
	Denaturing gradient gel electrophoresis (DGGE)/Clone library	Antarctic cold desert	Smith *et al.*, 2006
	Terminal restriction fragment length polymorphism (T-RFLP)/ Clone library construction	Brackish sediment	Banning *et al.*, 2005
Geothermal	Clone library construction	Silica pore waters	Walker *et al.*, 2005
	Hybridizations	Deep-sea hydrothermal vent	Nercessian *et al.*, 2003
Subsurface	Clone library construction	Subsurface cretaceous rock	Kovacik *et al.*, 2006
	T-RFLP	Deep biosphere gold mine	Takai *et al.*, 2001
	Stable isotope probing (SIP)	Cave waters	Hutchens *et al.*, 2004

	Quantitative-polymerase chain reaction (Q-PCR)/Catalyzed reported deposition–fluorescent *in situ* hybridization (CARD-FISH)	Subseafloor biosphere	Schippers *et al*., 2005
	Microarray	Deep biosphere saline waters	Lin *et al*., 2006
Marine	Clone library construction	Evaporative saline pool	Kimura *et al*., 2005
Freshwater	DGGE	Acidic freshwater	Gonzalez-Toril *et al*., 2003
	Clone library construction	Glacial waters	Skidmore *et al*., 2005
Pollutant associated	Clone library construction	Hydrocarbon seeps	Hamamura *et al*., 2005
	Microarrays	Uranium contaminated site	Brodie *et al*., 2006
Acid metal leaching	Metagenomics	Iron mine drainage	Baker *et al*., 2004
	Proteomics	Iron mine drainage	Ram *et al*., 2005

(Burford *et al.*, 2003). Microorganisms influence mineral weathering by increasing mineral dissolution and biomineralization (biologically induced mineralization of the local microenvironment) and alternating mineral surface chemistry and reactivity (Burford *et al.*, 2006; Hochella, 2002). Microorganisms also affect the speciation and distribution of elements and ions by: (1) modulating redox reactions, (2) inducing mineral precipitation, (3) releasing organic and inorganic by-products, and (4) modifying the rates and mechanisms of mineral degradation (Gadd, 2000, 2004, 2007). It has been estimated that 20–30% of rock and mineral weathering is due to biological activity (Wakefield and Jones, 1998). Lithobiotic microbial communities often colonize mineral surfaces forming a biofilm at the mineral–microbe interface, and such biofilms can be found in aquatic, subsoil, and subarial environments. Microbial processes, for example energy and nutrient acquisition, cell adhesion, biofilm formation, and organic matter formation, are also influenced by minerals and their chemistry (Burford *et al.*, 2003). Many bacteria use minerals for respiration (redox-sensitive chemical elements in a mineral are used as a terminal electron acceptors), resulting in reduction (Lovley, 1991). It is clear that, due to active microbial metabolism, habitats change over time, and these changes exert control over the evolution and structure of microbial communities.

B. Silicate minerals

There has been much interest in the weathering of silicate minerals (Bennett *et al.*, 2001; Rogers and Bennett, 2004), the result of which is the formation of new phases (e.g., clays and oxyhydroxide minerals) and altered aqueous and atmospheric geochemistry. Microbes mediate silicate mineral weathering in a number of ways: they may have a direct effect by producing acids, bases, and ligands which differently promote mineral weathering by catalyzing the release of ions to solution, or they may effect a more indirect response by producing compounds such as extracellular polysaccharides that bind particles together, increasing water retention at mineral surfaces and thereby increase the time available for hydrolysis (Bennett *et al.*, 2001). Microbial involvement in surface processes has been considered at a microenvironmental level, with microgeochemical environments being very different from those of bulk solutions, often resulting in localized etching (Fisk *et al.*, 1998; Thorseth *et al.*, 1995). While bacteria have been implicated in the accelerated weathering of minerals, it is not clear if this is simply the coincidental result of microbial metabolism, or if it represents a specific strategy offering the colonizing bacteria a competitive advantage. All microorganisms require elements such as K, Fe, Mg, and so on that can be derived from silicate mineral weathering; however, there are cases whereby colonizing microorganisms do not prefer any particular

naturally favorable site (e.g., a pore or fissure), but rather colonize a substrate on the basis of their own inherent growth patterns (Brehm *et al*., 2005). Mineral dissolution may also be inhibited by the metabolic activities of microorganisms, for example the formation of extracellular polysaccharides that may irreversibly bind to mineral surfaces thus preventing dissolution (Welch and Vandevivere, 1994; Welch *et al*., 1999).

Laboratory studies of silicate weathering have demonstrated that microbes limited by Mg and K produce organic ligands that accelerate dissolution of biotite (Paris *et al*., 1995), while field experiments have shown bacteria preferentially colonizing potassium-rich mineral phases, as well as preferential colonization of apatite grain inclusions (phosphorus-rich) within K-feldspars (Bennett *et al*., 1996). Gleeson *et al.* (2005, 2006) have demonstrated that both bacteria and fungi preferentially colonize different minerals in response to the elemental content of that mineral. It is well understood that quartz is one of the rock forming minerals that is most resistant to weathering (White and Brantley, 2003), and in granitic rocks, quartz grains have a much higher resistance to chemical weathering than many coexisting minerals such as feldspar. However, despite the great chemical and physical resistance of quartz, as well as the lack of obvious nutrition to be derived through its breakdown, some microorganisms have been shown to preferentially colonize quartz (Brehm *et al*., 2005; Gleeson *et al*., 2005). Although the surface distribution of microorganisms may be controlled by mineralogy and the ability of an organism to take advantage of nutrients within mineral structures, surface attachment processes are also likely to be important and may be different for different mineral types. For example, microtopography, surface composition, surface charge, and hydrophobicity may play an integral role in microbial attachment and detachment processes and biofilm formation (Bennett *et al*., 1996).

C. Metals

Metals are known to affect the structure of microbial communities, and key functions of microbes in soil formation such as mineral degradation and dissolution (through microbial excretion of corrosive metabolic products and catalysis of redox reactions in metal transformations) may be inhibited by metal contamination/concentrations (Giller *et al*., 1998). The interactions between metals and microorganisms may also be influenced by the presence of compounds such as inorganic anions, competing cations, and complexing organic matter (Ledin and Pedersen, 1996). All microbes require sources of energy, carbon, and nitrogen for growth and survival, as well as a supply of certain essential elements, including S, P, K, Mg, Ca, and Fe. Although an excess of metals is generally toxic, low levels of certain metallic elements (Cu, Mn, Zn, and so on) are essential to

life, often functioning as cofactors driving enzymatic reactions. Cells need to maintain certain cytoplasmic concentrations of these metals if they are to meet physiological requirements. To this end, microorganisms use a number of mechanisms to maintain the correct equilibrium, including the uptake, chelation, and extrusion of metals (for a comprehensive review, see Silver, 1996). However, high concentrations of some heavy metals can adversely affect the growth, morphology, and biochemical activity of microorganisms, by blocking essential functional groups, displacing essential ions, or modifying the active conformation of biological molecules (Baath *et al*., 1998; Frostegard *et al*., 1993; Hassen *et al*., 1998).

Microbes can passively or actively accumulate metals intracellularly, they can adsorb metals onto functional groups on cell surfaces or on extracellular polymers, and can transform metals from one form to another via alkylation or reduction/oxidation reactions (Ledin and Pedersen, 1996). They also influence metal mobility by producing minerals, such as sulfides, that sequester heavy metals (resulting in low metal solubility), or by producing specific chelating agents that require iron or other essential metals for growth [e.g., iron-chelating siderophores that sequester iron into minerals thereby increasing mineral dissolution rates (Kraemer *et al*., 2005)]. In anaerobic environments, respiration may also promote mineral dissolution (or mineral transformation, depending on the geochemical conditions), for example the reductive dissolution of Fe(III) oxides, which liberates metalloids, such as arsenic, that may be adsorbed to the oxide surfaces (Islam *et al*., 2005).

IV. EXTREME ENVIRONMENTS

Extremophiles are organisms that thrive in environments that lie significantly outside the set of predefined regular conditions (e.g., a temperature significantly above or below 37°C). They are classified further according to the environmental niche required for optimal growth. Extreme environments are often easy to recognize because of the steep geochemical gradients that generally occur at their boundaries. Some examples of extreme environments include low-temperature ice cores (Price, 2000), high-temperature geothermal hot springs (Kimura *et al*., 2005), and deep-sea hydrothermal vents (Nercessian *et al*., 2003). It is not surprising that most organisms that live in extreme environments are prokaryotic due, in most part, to their ability to metabolize almost any available energy source. Many extremophiles are chemotrophic archaea; however, their metabolic diversity extends to members that use an array of energy sources, including hydrogen [e.g., *Pyrolobus fumarii* (hyperthermophile)], reduced S compounds [e.g., *Sulfolobus hyperthermoacidophile Metallosphaera sedula* (thermoacidophile)], and Fe^{2+} [e.g., *Acidithiobacillus ferrooxidans* (acidophile)].

Bacteria, such as *A. ferrooxidans*, tend to be the dominant species in highly acidic ecosystems at middle-range temperatures (such as acid mine drainage (AMD) systems).

A. Microbes in iron- and sulfur-rich environments

AMDs are seminatural environments rich in extremophiles and are created as a result of mining and the exposure of predominantly ferrous iron in pyrite (FeS_2) to the oxygen-rich atmosphere (Baker and Banfield, 2003; Druschel *et al.*, 2004). Iron is one of the most abundant elements in Earth's crust and exists naturally in two oxidative states, ferrous (Fe^{2+}) and ferric (Fe^{3+}). In nature, these two forms cycle as a result of reduction and oxidation by microorganisms and abiotic geochemical processes. The reduction of Fe^{3+} to Fe^{2+} occurs in anoxic environments, with organic compounds in these environments acting as the electron donor. In contrast, the oxidation occurs in oxygenic environment with O_2 as the electron acceptor. Several groups of chemolithotrophic organisms (e.g., *A. ferrooxidans*) actively participate in the oxidation reaction, and thrive in such environments by oxidizing large amounts of ferrous iron (Baker and Banfield, 2003).

Within AMD environments there is continuous cycling of sulfur species, which plays a major role in energy production and the maintenance of the microbial community (Elshahed *et al.*, 2003). The transformation of reduced sulfur (sulfide) to oxidized forms (sulfate) via various intermediate forms represents an important energy-yielding pathway for chemosynthetic microorganisms (Ehrlich, 1996). Sulfur compounds are among the most energy-rich inorganic chemical compounds available to microorganisms. From sulfide to sulfate a total of eight electrons can be exchanged in a stepwise manner to yield not only energy for the organisms but also a wide variety of mineral products, which, in turn, can often undergo redox transformations of their own. Since a wide array of microorganisms is able to oxidize and reduce sulfur, the microbial community structure of sulfur-rich habitats is clearly influenced by the prevalent environmental conditions at a specific site, for example pH; temperature; sulfide, sulfur, or sulfate concentrations; redox conditions; presence of other electron acceptors; light availability; and organic content. Members of the genus *Acidithiobacillus* were the first sulfur-oxidizing isolates from AMD environments, and there have been a large number of publications detailing sulfur cycling by *A. ferrooxidans* (Nordstrom and Southam, 1997 and references therein).

AMD systems have many microbial niches due to variations in temperature, ionic strength, and pH, and this results in habitats being restricted to a few, specific species. It has been reported using 16S rDNA and fluorescent *in situ* hybridization (FISH) analysis that only a

handful of prokaryotic taxa make up the community in any specific microenvironment within an AMD system (Bond and Banfield, 2001; Druschel *et al*., 2004). This low diversity has also been noted using culture-based approaches (Johnson *et al*., 2001). Metagenomic analyses of a biofilm from an AMD system at Iron Mountain (California) have provided important insights into the microbial community structure in such systems (Tyson *et al*., 2004). From the resulting 78 Mb of sequence obtained, the genomes of the dominant species were constructed. Bioinformatics analyses of the metagome sequence data showed that a *Leptospirillum* group III strain was found to contain genes homologous to those for biological nitrogen fixation. This information subsequently led to the design of a selective isolation strategy that allowed the isolation of this organism (Allen and Banfield, 2005). In addition, genes involved in essential pathways (such as nitrogen and carbon dioxide fixation and iron metabolism) were revealed. A proteomic analysis of this community identified an abundant novel protein, a cytochrome, as an essential component to iron oxidation and AMD formation (Ram *et al*., 2005) However, with the exception of studies that target low-complexity environments such as the acid mine habitat (Tyson *et al*., 2004), the assembly of complete microbial genome from metagenomic data remains a major technical challenge as a result of the immense diversity of many natural samples (Torsvik *et al*., 1998).

In addition to AMD environments, both cold sulfide springs and deep-sea hydrothermal vents are sulfur-rich environments. A study of a cold sulfide spring emanating from a dolomite/gypsum host rock in a temporal climate region showed that sulfate-reducing bacteria living in microbial communities on the solid walls of the rock strata were responsible for reducing the sulfate to H_2S so that the waters emerged were highly charged with this reduced form of sulfur and highly anoxic (Douglas and Douglas, 2000, 2001). Sulfide in the springwater is then oxidized microbially to elemental sulfur by a microbial biofilm, and in the spring mouth itself, sulfur is oxidized by photosynthetic microorganisms (purple sulfur bacteria and green sulfur bacteria) that use the sulfide as an electron donor for photosynthesis, depositing sulfur in elemental form.

Deep-sea hydrothermal vents are important in global biogeochemical cycles as they provide an environment at the seafloor that allows microorganisms to flourish. As hot, acidic, and reduced hydrothermal fluids mix with cold, alkaline, and oxygenated seawater, minerals precipitate to form porous sulfide–sulfate deposits. These environments have been a major source of novel and phylogenetically deeply branched hyperthermophiles, many belonging to the archaeal domain (Takai and Horikoshi, 1999; Takai *et al*., 2001). It has been proposed that fluid pH in the actively venting sulfide structures is generally low (pH < 4.5) (Reysenbach *et al*., 2006), yet no extreme thermoacidophile has been isolated from vent

deposits. Archaea have been found to make up as much as 33–50% of the total microbial community in deep-sea hydrothermal vent environments based on 16S rDNA probing and whole-cell hybridization (Nercessian *et al*., 2003) and are able to occupy the highest temperature niches within the vent environment (Schrenk *et al*., 2003; Takai *et al*., 2001). Studies of microbial communities inhabiting mature hydrothermal vent environments, including those inhabiting *in situ* settling devices, have shown differences in microbial community composition among vents in a single system, as well as temporal changes in the diversity of the microbial community of the order of days (Guezennec *et al*., 1998; Nercessian *et al*., 2003; Reysenbach *et al*., 2006) to years (Huber *et al*., 2002; McCliment *et al*., 2006).

B. Cave systems

Cave environments typically host nonphotosynthetically based communities (Sarbu *et al*., 1994, 1996) and represent an opportunity to examine how chemosynthetic life processes interact with local geology. Usually caves, although poor in organic matter, are rich in redox interfaces, allowing primary growth of chemoautotrophic and ammonium-, nitrite-, sulfur-, manganese-, or iron-oxidizing chemolithoautotrophic bacteria (Northup and Lavoie, 2001). Reduced compounds in cave wall rock may be microbially oxidized to form secondary mineral deposits on top of biofilm surfaces, dissolved rock underneath biofilms, and acidic microenvironmental waters. For example, the metabolic processes of sulfur-iron-, and manganese-oxidizing bacteria (Sarbu *et al*., 1994) can generate considerable acidity, dissolving cave walls and formations (Andrejchuk and Klimchouk, 2001; Engel *et al*., 2004). This leads to the formation of sharp redox boundaries at the microbe–mineral interface as microorganisms use elements from the geologic matrix of the cave wall to produce energy in this organic nutrient-limited environment. These biogenic minerals range from carbonates (moonmilk), silicates, clays, iron, and manganese oxides to sulfur, and saltpeter (potassium nitrate) at scales ranging from microscopic to macroscopic. As a result of such activity, streams only meters apart can have different chemical compositions. One of the most common reactions is the formation of sulfuric acid from sulfide (either atmospheric hydrogen sulfide or cave roof/wall sulfide minerals) by bacteria similar to *Thiobacillus* species (Engel *et al*., 2004). This is the same type of reaction that leads to AMD formation in ore tailings piles (Fortin and Beveridge, 1997; Fortin *et al*., 1995). In caves, sulfuric acid often dissolves carbonate minerals present, widening the passages of limestone caves and liberating elements such as calcium, magnesium, iron, and manganese to be transported to and concentrated in other areas of the cave, usually by microorganisms (Northup and Lavoie, 2001).

C. The deep subsurface

The deep biosphere is estimated to contain a biomass of the same order of magnitude as that of the surface of Earth (Pedersen, 2000). Microbial activity has been detected several hundred meters below the land surface, for example sulfate reduction occurring between sandstone and shales that were deposited during the Cretaceous period (Takai *et al*., 2001) and methanogenesis driven by geothermal waters rich in hydrogen (Chapelle *et al*., 2002). In deep-marine sediments, metabolically active bacterial cells have been quantified in a study that used oligonucleotide probes to target specific active cells and their rRNA. A sizable active bacterial subsurface biosphere was demonstrated, allowing for realistic estimates of cell-specific respiration rates and turnover time for living bacteria in this extreme habitat (Teske, 2005). Schippers *et al.* (2005) also report the use of rRNA to analyze prokaryotic cells of the deep subseafloor biosphere. They used rRNA as a target for a technique known as catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) to directly quantify live cells as defined by the presence of ribosomes, and showed that a large fraction of the subseafloor is inhabited by microorganisms (Schippers *et al*., 2005).

Microbial activity has also been found in even deeper environments such as the gold mines of South Africa, with Takai *et al.* (2001) reporting that South African gold mines harbor novel archael communities distinct from those observed in other environments. These subsurface environments and their microbial communities are of interest, not only because of their unique nature, their lack of direct reliance on solar radiation, and the fact that they may represent a large percentage of the Earth's biosphere, but also because subsurface environments represent the most likely location for life on other planets (Boston *et al*., 1992). Other evidence suggests that a deep crustal biosphere exists beneath both land and sea, reaching ~3 km below the Earth's surface, with oil degradation suggesting that this may be extended to at least 4 km (Head *et al*., 2003).

D. Radioactive environments

Uranium remediation strategies in recent years have focused on containment, and a promising approach to minimizing uranium migration is to catalyze the reduction of soluble U(VI) to the less-soluble U(IV) (Phillips *et al*., 1995). A wide range of bacteria are capable of uranium reduction (Lloyd *et al*., 2002) and it has been shown that the reduction of U(VI) to U(IV) typically coincides with an increase in populations of metal-reducing bacteria such as members of the Geobacteraceae and others within the δ-proteobacteria (Holmes *et al*., 2002; Suzuki *et al*., 2003). Much work on U(VI)-reducing bacteria has been conducted with pure

cultures or enrichments in the laboratory, where it is difficult to reconstruct field conditions. Few studies of known metal reducers capable of U(VI) utilization have been carried out by cultivation-independent techniques with subsurface sediments, and structure–function relationships have not been examined extensively. Brodie *et al*. (2006) have used a high-density oligonucleotide microarray-based approach to analyze the most dynamic groups detected by a 16S microarray. This approach demonstrated that amplicons of known metal-reducing bacteria, such as *Geothrix fermentans* (confirmed by quantitative PCR), and those within the Geobacteraceae were abundant during U(VI) reduction, and did not decline during the U(IV) reoxidation phase. Significantly, it appears that the observed reoxidation of uranium under reducing conditions occurred despite elevated microbial activity and the consistent presence of metal-reducing bacteria.

V. THE ORIGIN OF LIFE ON EARTH, AND BEYOND

In this chapter we have outlined the existence of microbial life in a wide range of extreme environments, ranging from the deep surface, nuclear reactors, hydrothermal vents and springs, AMDs and rivers (such as the Rio Tinto in Spain), areas of high heavy metal concentrations, and polar ice. Understanding the biology of extremophiles will also permit the development of hypotheses regarding the conditions required for the origination and early diversification of life on Earth, and potential habitats for life beyond Earth (on the planet Mars and the Jovian moon Europa). Recent growth in the field of astrobiology has seen the launch of two new journal titles, ''International Journal of Astrobiology'' and ''Astrobiology,'' in 2002 and 2005, respectively, underlining the increasing importance of this area of research.

One of the earliest pieces of evidence of planetary biota is contained in the microfossils of stromatolites (Byerly *et al*., 1986). Extant microbialites are still forming in some environments, in particular warm shallow (marine) waters, and many reviews are available describing stromatolites (Allwood *et al*., 2006; Kempe *et al*., 1991). The living stromatolites of Hamelin Pool in Western Australia are renowned as the most extensive examples on Earth of extant marine stromatolites. Although the biogenic origin of the oldest fossilized stromatolites is under debate (Brasier *et al*., 2002; Schopf *et al*., 2002), the textures and morphological features of modern stromatolites have been considered to resemble closely ancient stromatolite assemblages (Riding, 2000), and thus may represent the oldest examples of life on Earth. Such structures have been preserved in the geologic record, providing information of past geomicrobiological activity and environmental conditions.

VI. CONCLUSIONS

Microorganisms have played a major role in shaping the biological, climatic, geologic, and geochemical evolution of the Earth. Despite the obvious importance of microorganisms to evolution and function of life on Earth, a great deal still remains unknown about how microorganisms interact with each other and with their environment to generate and maintain their vast diversity of species and function. In the past 20 years, however, the application of genomics tools has revolutionized microbial ecological studies and vastly expanded our view of the previously underappreciated microbial world. Microbial species richness and complexity across the entire spectrum of the Earth's environments is enormous, and while it is generally accepted that the vast majority of environmental microorganisms is not culturable [this figure is often cited as being lower than 1% (Torsvik *et al*., 1998)], new techniques allowing for the characterization of microbial community structure at a high-resolution level (group, species, and strain) without the need for cultivation have revolutionized our understanding of the microbial world.

There are many potential practical and intellectual benefits that might arise from the study of geomicrobiology that include: (1) a better understanding of the conditions that result in the dissolution and precipitation of economically important minerals; (2) a potential use for microorganisms in the cleanup of oil spills, toxic mine tailings, and other environmental hazards; (3) the harnessing of microbes to detoxify industrially produced poisons, such as PCBs, that contaminate water, soil, and other parts of the environment; (4) understanding better how microbes are involved in energy production; (5) a greater appreciation for how life survives at nutritional, chemical, and physical extremes; and (6) improving our knowledge of how cell-to-cell communications and microbial community interactions can control global biogeochemical processes, all of which will ultimately lead to a better understanding of Earth's history, including the origin of life and the evolution of global biogeochemical cycles (Welch and Vandevivere, 1994; Welch *et al*., 1999).

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CHAPTER 5

The Scale-Up of Microbial Batch and Fed-Batch Fermentation Processes

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I. INTRODUCTION

Microorganisms are important both for human health and to industry, so the fed-batch cultivation of microbial strains, often overexpressing recombinant or natural proteins, to high cell density has become an increasingly important technique throughout the field of biotechnology, from basic research programs to large-scale pharmaceutical production processes (Hewitt *et al.*, 1999). The scale-up of such a process is usually the final step in any research and development program leading to the large-scale industrial manufacture of such products by fermentation (Einsele, 1978). It is important to understand that the process of scaling-up a fermentation system is frequently governed by a number of important engineering considerations and not simply a matter of increasing culture and vessel volume. Therefore, it is perhaps surprising when the large scale does not perform as the small-scale laboratory process does. It is often observed that the biomass yield and any growth-associated products are often decreased on the scale-up of an aerobic process (Enfors *et al.*, 2001). For *Saccharomyces cerevisiae*, the biomass yield on molasses increased by 7% when the process was scaled-down from 120 m^3 to 10 liter even when a seemingly identical strain, medium, and process were employed (George *et al.*, 1993). In an *Escherichia coli* fed-batch recombinant protein process, the maximum cell density reached was found to be 20% lower when scaling-up from 3 liter to 9 m^3 and the pattern of acetic acid formation had changed. (Bylund *et al.*, 1998). During another study (Enfors *et al.*, 2001), the performance of a recombinant strain of *E. coli* during fed-batch culture was found to vary on scale-up from the laboratory-scale to 10–30 m^3 industrial bioreactors. This included lower biomass yields, recombinant protein accumulation, and surprisingly perhaps a higher cell viability. These findings are typical of those found when scaling-up most fermentation processes, yet only a few mechanisms have been presented that can satisfactorily explain these phenomena.

In this chapter, we will briefly discuss the main engineering considerations involved in fermentation scale-up and then critically review those mechanisms thought to be responsible for any detrimental change in bioprocessing at the larger-scale. Although it addresses mainly *E. coli* fed-batch fermentations, much of the discussion also applies to batch and other single-celled aerobic microbial fermentations too.

II. ENGINEERING CONSIDERATIONS INVOLVED IN SCALE-UP

A. Agitator tasks in the bioreactor

The agitation system in the bioreactor provides the liquid motion that enables many different tasks to be fulfilled. An example of a typical stirred bioreactor is shown in diagrammatic form in Fig. 5.1. It is important to understand the interaction between the fluid motion, the agitator speed, and the power input into the bioreactor and these tasks. It is also necessary to know how a change of scale affects these relationships. Many of these aspects can be studied without carrying out a specific bioprocess and these physical aspects most relevant to bacterial fermentations are listed in Table 5.1. Table 5.2 sets out those aspects that are specific to the organism being grown and will usually be different for each case. The more important of these aspects with respect to scale-up are discussed later.

The physical aspects in Table 5.1 have been discussed extensively for conditions relevant to a wide range of organisms elsewhere (Nienow,

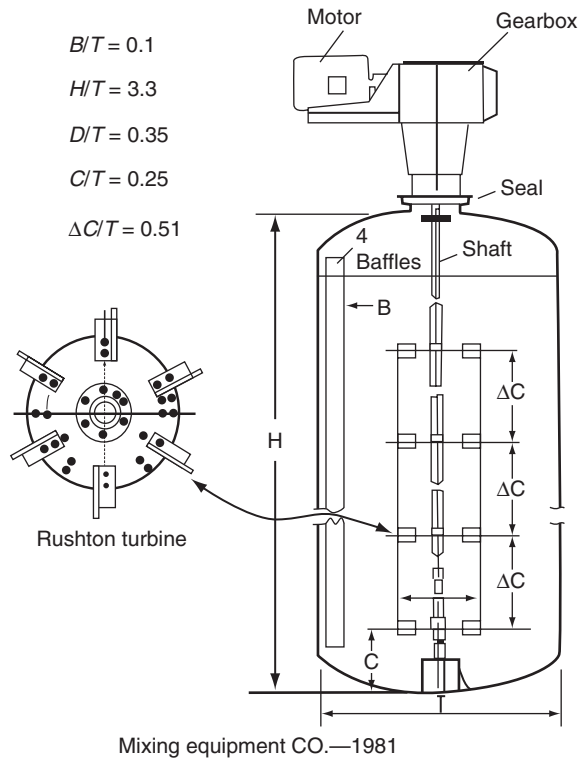

FIGURE 5.1 Schematic representation of multiple Rushton turbine impellers in a fermenter.

TABLE 5.1 Physical aspects of the agitation/agitator requiring consideration (Nienow, 1998)

Mass transfer performance
Heat transfer
Unaerated power draw (or mean specific energy dissipation rate $\bar{\varepsilon}_T$ W/kg)
Aerated power draw (or aerated $(\bar{\varepsilon}_T)_g$ W/kg)
Flow close to the agitator-single phase and air–liquid
Variation in local specific energy dissipation rates ε_T W/kg
Air dispersion capability
Bulk fluid- and air-phase mixing

TABLE 5.2 Biological aspects that are system specific (Nienow, 1998)

Growth and productivity
Nutrient and other additive requirements including oxygen
CO_2 evolution and RQ
Sensitivity to O_2 and CO_2 concentration
pH range and sensitivity
Operating temperature range
Shear sensitivity

1996, 1998; Nienow and Bujalski, 2004). Here, their relevance to microbial fermentations for which the viscosity essentially does not go much higher than that of water is discussed, for example bacteria and yeast. Thus, viscous polysaccharide and filamentous systems are excluded from consideration in this chapter. With such low viscosities, the flow in the fermenter is turbulent from a 5-liter bench bioreactor to the largest scale, that is Reynolds number, $Re = \rho_L N D^2/\mu > \sim 10^4$ where ρ_L is the growth medium density (kg/m^3), μ is its viscosity (Pa s), D is the impeller diameter (m), and N is its speed (rev/s). For scale-up purposes, as long as the flow is turbulent, the actual value of the Reynolds number does not matter and turbulent flow theories can be used to analyze the fluid mechanics in the bioreactors across the scales. The topics listed in Table 5.1 will be considered first for such flows.

1. Mass transfer of oxygen into the broth and carbon dioxide out

The transfer of oxygen into a fermentation broth has been studied since the 1940s when ''submerged fermentations'' were first established. The topic was reviewed by Nienow (2003). The overall oxygen demand of the cells throughout the batch or fed-batch fermentation must be met by

the oxygen transfer rate and the demand increases as long as the number of cells is increasing. Roughly, for every mole of O_2 utilized, 1 mole of CO_2 is produced, that is the respiratory quotient, RQ $\approx$ 1 (Nienow, 2006). Thus, a maximum oxygen transfer rate must be achievable and this rate depends on the mass transfer coefficient, k_La (1/s), and the driving force for mass transfer, ΔC, since

$$\text{OUR} = k_L a \Delta C \tag{5.1}$$

The value of k_La is similar for both O_2 transfer from air to the broth and CO_2 from it. For oxygen transfer, the driving force conceptually is the difference between the oxygen concentration in the air bubbles and that in the broth, which must always be held above the critical dO_2 value throughout the fermenter for the duration of the process. In a similar way, the dCO_2 must be kept below that which will lead to a reduction in fermentation rate or productivity.

It has been shown many times (Nienow, 2003) that in low-viscosity systems, k_La is only dependent on two parameters. These are, first, the total mean specific energy dissipation rate imposed on the system $(\bar{\varepsilon}_T)_g$ (W/kg) and, second, v_s (m/s), the superficial air velocity [=(vvm/60) (volume of broth)/(X-sectional area of the bioreactor]. $(\bar{\varepsilon}_T)_g$ and v_s together must be sufficient to produce the necessary k_La where

$$k_L a = A(\bar{\varepsilon}_T)_g^{\alpha}(v_s)^{\beta} \tag{5.2}$$

This equation applies independently of the impeller type and scale, and α and β are usually about 0.5 ± 0.1 whatever the liquid. On the other hand, A is extremely sensitive to growth medium composition (Nienow, 2003) and the addition of antifoam which lowers k_La or salts which increase it may lead to a 20-fold difference in k_La for the same values of $(\bar{\varepsilon}_T)_g$ and v_s. Typical values of $(\bar{\varepsilon}_T)_g$ are up to $\sim$5 W/kg and for the airflow rate about 1 volume of air per volume of growth medium (vvm). Since the value of k_La is similar for both O_2 and CO_2 transfer, provided scale-up is undertaken at constant vvm (or close to it), the driving force for transfer in of O_2 and transfer out of CO_2 will remain essentially the same across the scales. In this case, since vvm scales with fermenter volume and v_s scales with its cross-sectional area, v_s increases. There is some debate as to whether $(\bar{\varepsilon}_T)_g$ should include a contribution from the sparged air [$\approx v_s g$ where g is the acceleration due to gravity (9.81 m^2/s)], which only becomes significant on scale-up at constant vvm. This approach should also eliminate problems with high dCO_2 on scale-up (Nienow, 2006).

2. Heat transfer

The oxygen uptake rate (OUR, in mol $O_2/m^3/s$) largely determines the metabolic heat release Q (W/m^3) (RQ $\approx$ 1) which is proportional to it (Van't Riet and Tramper, 1991), that is

$$Q \approx 4.6 \times 10^5 \mathrm{OUR} \tag{5.3}$$

This cooling load has to be removed by heat transfer at an equivalent rate given by:

$$Q = UA\Delta\theta \tag{5.4}$$

where U is the overall heat transfer coefficient (which is hardly affected by the agitation conditions), $\Delta\theta$ is the difference between the temperature of the cooling water and the broth temperature (it being critical to control the latter), and A is the heat transfer area available. At the commercial scale, heat transfer is often a problem as Q scales with the volume of the reactor, that is, for geometrically similar systems with T^3 (bioreactor diameter, T m) while cooling surface area scales with T^2. Hence, on the large scale for such systems, cooling coils are often required and sometimes cooling baffles. The inability to meet the cooling requirements at the large scale [especially, e.g., in high cell density (>50 g/liter dry cell weight) fed-batch fermentations] is a very serious problem because it is extremely expensive to resolve.

B. Unaerated power draw *P* (or mean specific energy dissipation rate $\bar{\varepsilon}_T$ W/kg)

These parameters are dependent on the impeller power number, *Po* (dimensionless). *Po* depends on the agitator type, and in the turbulent regime for any one type, it is essentially constant, regardless of the diameter, D (m), relative to the bioreactor diameter, T or the speed, N, and of the bioreactor size (scale) provided geometric similarity is maintained across the scales (Nienow, 1998). The power input, P (W), into the bioreactor imparted by the impeller is given by:

$$P = Po\rho_L N^3 D^5 \tag{5.5}$$

The mean specific energy dissipation rate $(\bar{\varepsilon}_T)$ (W/kg or m^2/s^3) from the impeller is given by:

$$(\bar{\varepsilon}_T) = \frac{P}{\rho_L V} \tag{5.6}$$

where V is the volume of growth medium in the reactor (m^3). The maximum local specific energy dissipation rate $(\varepsilon_T)_{max}$ is close to the impeller, is very high relative to the mean, and depends on the agitator type. Also, the Kolmogoroff or microscale of turbulence, λ_K, which is often considered as an indicator of the potential for mechanical damage to cells (see below for more details) is given by

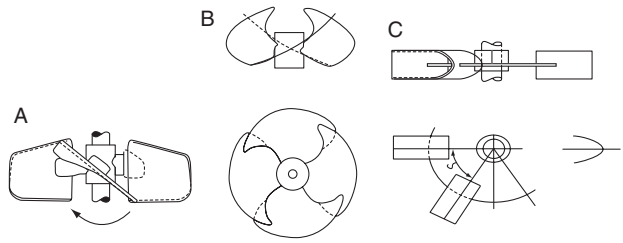

FIGURE 5.2 Newer impellers: (A) "down-pumping, high solidity ratio hydrofoil" Lightnin A315 ($Po = 0.85$); (B) "up-pumping, high solidity ratio hydrofoil" Haywood Tyler B2 ($Po = 0.85$); (C) "hollow blade" Scaba 6SRGT ($Po = 1.5$).

$$\lambda_K = \left(\frac{\varepsilon_T}{\nu^3}\right)^{-1/4} \tag{5.7}$$

where (ε_T) is the local specific energy dissipation rate and ν is the kinematic viscosity, $\approx 10^{-6}$ m^2/s when the cell/medium suspension is water-like.

The traditional impeller for fermentation processes has been the Rushton turbine (Fig. 5.1) and it has a relatively high power number ($\sim$5). It has been clearly shown to have many weaknesses (Nienow, 1996) and it is being superseded by the impellers shown in Fig. 5.2, each of which can be considered an example of a generic type (Nienow, 1996; Nienow and Bujalski, 2004). All these impellers have lower Po values and so can easily replace a Rushton turbine running at the same speed, torque, and power by one of a larger diameter which gives certain processing advantages as set out below.

C. Aerated power draw P_g (or aerated $(\bar{\varepsilon}_T)_g$ W/kg)

Particularly with the Rushton turbine, on aeration at around 1 vvm, the power P_g [and therefore $Po_g (= P_g/\rho_L N^3 D^5)$ and $(\bar{\varepsilon}_T)_g (= P_g/\rho_L V)$] falls significantly, typically by 50%. The relationship with D/T, scale, and impeller speed is very complex and difficult to predict [the commonly used Michel and Miller correlation is dangerously inappropriate (Middleton and Smith, 2003; Nienow, 1998), especially at large scale and with multiple impellers (Nienow, 1998)]. Thus, it is difficult to obtain $(\bar{\varepsilon}_T)_g$, which is a critical requirement for calculating mass transfer on scale-up. Also, it has safety implications since, if airflow is lost, the power drawn by the impeller doubles. Therefore, often a much more powerful motor is installed in order to cope with this possibility. The advantage of the up-pumping, high solidity ratio (SR = plan area of impeller/area of circle swept out by its blade tips) hydrofoil (Fig. 5.2B) and the hollow blade impeller (Fig. 5.2C) is that impellers of both these

types lose very little, if any, power on aeration (Middleton and Smith, 2003; Nienow, 1996; Nienow and Bujalski, 2004). Thus, they finesse the problem of the loss of power found with the Rushton impeller. Finally, it is worth noting that during the earlier stages of a fermentation, lower $(\bar{\varepsilon}_T)_g$ values will suffice for achieving the required O_2 transfer, so a variable speed drive motor for the impeller gives additional flexibility and a reduction in running costs.

D. Flow close to the agitator-single phase and air–liquid

The turbulent flow field close to the agitator depends on its shape and determines its power number and the mechanism by which the air is dispersed, and hence the aerated power draws too. This process is described in detail elsewhere (Middleton and Smith, 2003; Nienow, 1998) and is beyond the scope of this chapter.

E. Variation in local specific energy dissipation rates, ε_TW/kg

The region of $(\varepsilon_T)_{max}$ where mechanical damage due to agitation is most likely to occur is also close to the agitator and $(\varepsilon_T)_{max}/(\bar{\varepsilon}_T) = \Phi$ is similarly dependent on the agitator type. Φ is difficult to determine, leading to a wide range of values being reported, of the order of about 20 to 70 for Rushton turbines (Nienow, 1998) and with similar values for other impellers (Kresta and Brodkey, 2003). The significance of $(\varepsilon_T)_{max}$ for damage to microorganisms is discussed below. Well away from the agitator, Φ is much less than 1. These differences in Φ have important implications for the feed location of nutrients, pH control chemicals, and so on, as discussed below.

F. Air dispersion capability

The flow close to the agitator also determines whether the agitator speed is sufficient to disperse the air. A variety of air dispersion conditions can be usefully identified (Fig. 5.3). If the airflow rate is too high, the airflow dominates the bulk flow pattern and the air is poorly dispersed (Fig. 5.3A). This condition is known as flooding and is to be avoided. For Rushton turbines, the correlation (Nienow, 1998)

$$(Fl_G)_F = 30\left(\frac{D}{T}\right)^{3.5}(Fr)_F \tag{5.8}$$

enables the minimum agitator speed required to prevent flooding, N_F, to be calculated. In this equation, Fr is the dimensionless Froude number, N^2D/g, proportional to the ratio of the inertial to buoyancy forces; and Fl_G is the dimensionless gas flow number, Q_G/ND^3, proportional to the

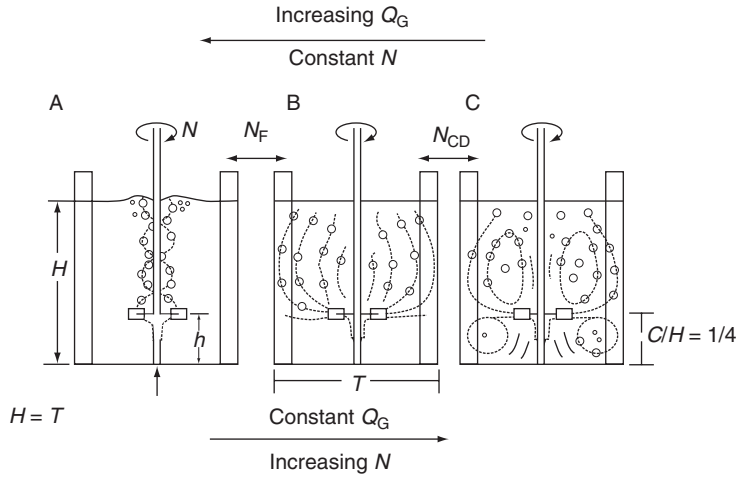

FIGURE 5.3 The flooding-loading-complete dispersion transitions for a Rushton turbine: (A) flooded, (B) loaded, (C) completely dispersed (Nienow, 1998).

ratio of the airflow rate, Q_G (m^3/s), from the sparger to the pumping capacity of the agitator. This correlation also works quite well for hollow blade agitators, but because of their lower *Po*, to draw the same power at the same speed, a larger diameter must be used so that they can handle much more air before flooding (Nienow, 1996, 1998). It can also be shown that on scale-up at constant $\bar{\varepsilon}_T$ and vvm, impellers are much more likely to be flooded. A similar equation, which leads to similar conclusions (Nienow, 1998), applies to the complete dispersion condition, N_{CD} (Fig. 5.3C).

For axial flow hydrofoil impellers, a similar correlation has not been established. However, down-pumping high solidity hydrofoils (Fig. 5.2A), made of a larger diameter as described above, are somewhat similar to the Rushton turbine; while the large diameter up-pumping configurations (Fig. 5.2B) perform similarly to the hollow blade impellers and are significantly better than Rushton turbines, especially as $N_{CD} \approx N_F$ (Nienow and Bujalski, 2004).

G. Bulk fluid- and air-phase mixing

The mixing of the air (gas) phase is important for mass transfer (Nienow, 2003) but is beyond the scope of this chapter. On the other hand, the ability of the agitator to mix the contents of very large (up to 400 m^3) fermenters with multiple impellers compared to the bench scale is the most challenging and important of all the scale-up issues. A measure of this difference is the parameter, the mixing time θ_m (s), which indicates after the addition of a tracer how long it takes to be evenly dispersed throughout the fermenter. For a fermenter containing broth to a height H (m) $= T$,

$$\theta_m = 5.9T^{2/3}(\bar{\varepsilon}_T)^{-1/3}\left(\frac{D}{T}\right)^{-1/3} \tag{5.9}$$

Equation (9) also holds for aerated conditions if $(\bar{\varepsilon}_T)_g$ is used. It implies for fermenters with broth up to an aspect ratio, AR = 1, at constant $(\bar{\varepsilon}_T)_g$, all impellers of the same D/T ratio give the same mixing time which larger D/T ratios can reduce. Most importantly, θ_m increases with (linear scale)$^{2/3}$. For fermenters with AR > 1, with multiple impellers (Nienow, 1998),

$$\theta_m \propto \left(\frac{H}{D}\right)^{2.43} \tag{5.10}$$

Equation (10) indicates the great sensitivity of mixing time to fill height and this increase in θ_m would be even greater with fed-batch fermentations when toward its end, a large portion of the broth often does not experience any direct agitation (Nienow, 2005). The use of multiple high solidity ratio axial flow hydrofoils reduces the mixing time by about a factor of 2 compared to radial flow impellers, and this has led to their use (Nienow, 2005). Unfortunately, in the down-pumping mode (Fig. 5.2A), they lose power (though not by as much as the Rushton impeller) and, more importantly, are prone to two-phase flow instabilities which lead to a large variable loading of the impeller drive motor. These problems are eliminated by the use of up-pumping configurations (Nienow and Bujalski, 2004), which also help reduce foam formation (Boon *et al.*, 2002). The implications for this loss of homogeneity on scale-up and small-scale experiments to mimic it are discussed below. It is also important to consider where additions are made. Although ''final'' mixing time does not depend on where the addition is made, an addition near the impeller in the regions of $\Phi \gg 1$ dramatically reduces the maximum concentration of the additive as it mixes, while an addition onto the top surface where $\Phi \ll 1$ and which is much easier and therefore preferred industrially results in very high local concentrations of additives for some considerable time before they are dissipated (Nienow, 2006). The latter feed position magnifies the lack of homogeneity at the large scale and significantly increases the chances of a different biological performance compared to the small.

H. Main differences across the scales

If these considerations are assessed for the changes that occur across the scale, the following points emerge. First, mass transfer requirements can be met at similar or even lower specific power inputs or $(\bar{\varepsilon}_T)_g$. Thus,

$(\bar{\varepsilon}_T)_{g,max}$ will be the same or less, and since even at the bench scale, the cells are very small compared to the size of the bioreactor, if ''shear damage'' is not an issue at the small scale, then it should not be on scale-up. This aspect is discussed in more detail below. Heat transfer is not an issue provided sufficient area for cooling is provided. However, the mixing time is always very significantly longer and therefore the spatial and temporal homogeneity is generally much worse on scale-up. Again, this aspect is discussed in detail below.

III. PROCESS ENGINEERING CONSIDERATIONS FOR SCALE-UP

A. Fluid mechanical stress or so-called "shear damage"

Anecdotal reference to the damaging effects on cells of fluid mechanical stress or so-called ''shear damage'' is frequently made to explain poor process performance when mechanical agitation and aeration are introduced into a bioreactor as compared to the nonagitated and nonsparged conditions in a shake flask or microtitre plate (Thomas, 1990). Thomas (1990) suggested that cells might be considered to be unaffected by fluid dynamic stresses if they were of a size smaller than the Kolmogoroff microscale of turbulence, λ_K. The microscale of turbulence is related to the local specific energy dissipation rate ε_T by Eq. (5.7). Therefore, if ε_T is 1 W/kg in a water-like medium, $\lambda_K \simeq 30$ μm. However, even though bacterial cells, of size ~1–2 μm, are well below the Kolmogoroff microscale of turbulence, it has been reported that the mean cell volume of two strains of *E. coli* and of two other species of bacteria increased linearly with impeller speed during continuous cultivation with a concomitant increase in intracellular potassium and sodium ion concentration (Wase and Patel, 1985; Wase and Rattwatte, 1985). Toma *et al*. (1991) also studied the effect of mechanical agitation on two species, *Brevibacterium flavium* and *Trichoderma reesei*. In each case, they found that under conditions of high agitation intensity during batch culture, both growth and metabolism were inhibited. They even coined the term ''turbohypobiosis'' to describe this phenomenon and suggested that excessive turbulence may cause this inhibition by damaging the membranes of the cell. However, in these cases, the results are difficult to interpret because any changes in agitation and aeration rate will also affect levels of dissolved oxygen (dO_2) via Eqs. (5.1) and (5.2) and depending on the critical dO_2 value, this parameter may also affect biological performance. Thus, any experimental protocol for investigating the impact of fluid dynamic stress on cell response should be undertaken under steady state (continuous culture) conditions, including the control of dO_2, if the cause of the change

is to be determined conclusively. Therefore, in the cases discussed above, the results were probably based on poor experimental design and their controversial findings may have been due to the lack of controlled dO_2 (Wase and Patel, 1985; Wase and Rattwatte, 1985) or the use of the constantly changing conditions experienced during batch culture (Toma *et al*., 1991).

Studies concerning the impact of agitation and aeration [because animal cells are potentially more easily damaged by bursting bubbles rather than rotating impellers (Nienow, 2006)] on microbial fermentations have been carried out in a stirred tank bioreactor. The bioreactor was operated as a chemostat, with blending of sparged air and nitrogen to control the driving force. Thus, again via Eqs. (5.1) and (5.2), the dO_2 was controlled to a constant value. First, the impact of high levels of agitation and aeration intensity (fluid mechanical stress) on *E. coli* fermentation performance were addressed as measured by standard microbiological techniques and the physiologically sensitive technique of multiparameter flow cytometry (Hewitt and Nebe-von-Caron, 2001, 2004; Hewitt *et al*., 1998). The initial work in glucose-limited continuous culture at the 5-liter scale showed that agitation intensities, expressed as mean specific energy dissipation rates $\bar{\varepsilon}_T$ up to 30 W/kg and aeration rates up to 3 vvm, served only to strip away the outer polysaccharide layer (endotoxin) of the cells but did not lead to any significant change in the physiological response of individual cells, which could lead to a detrimental change in bioprocessing. Estimates of the Kolmogoroff microscale of turbulence based on $\bar{\varepsilon}_T$ at 30 W/kg gives $\lambda_K = 13.5$ μm, well above the size of the cell (∼1–2 μm). Even if the maximum local specific energy dissipation rate is used (∼30 $\bar{\varepsilon}_T$), to estimate λ_K, a value of ∼6 μm is obtained, still greater than the cell size. This agitation intensity is an order of magnitude or more greater than those typically found on the industrial scale, and the range of aeration rates tested was much higher than those normally used, thus eliminating the possibility that damage due to fluid mechanical stresses may occur under the normal range of operating conditions.

Further studies were also undertaken during continuous cultivation with the Gram-positive bacterium *Corynebacterium glutamicum* (Chamsartra *et al*., 2005) with essentially similar results. In this case, it was shown that variations in agitation, aeration rate, or dO_2 concentrations down to ∼1% of saturation do not cause a significant change in physiological response of *C. glutamicum* even though the mean cell size was slightly reduced (Figs. 5.4 and 5.5).

Similar work with the larger (∼7 μm) *S. cerevisiae* showed that under steady state conditions, specific power inputs in the range 0.04–5 kW/m^3 ($\lambda_K = 16$ μm) were found to have little effect on either cellular morphology or physiology even though at the upper end of the agitation range there was a small, but transient measurable effect on cell division

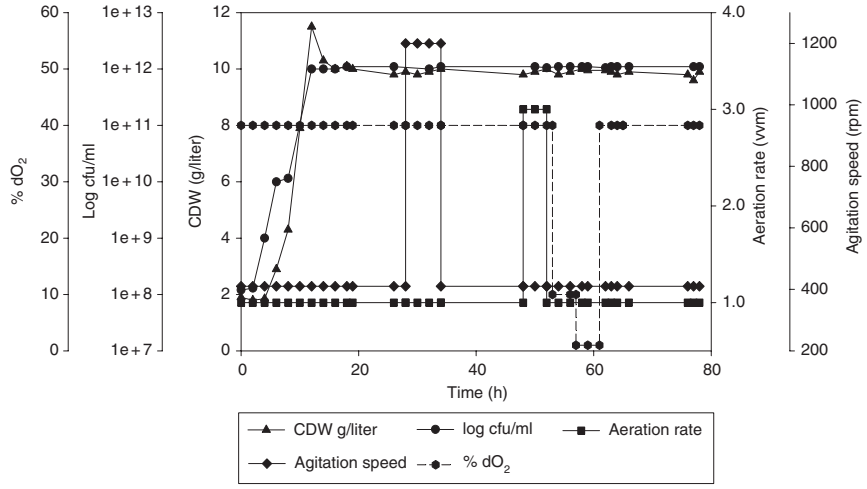

FIGURE 5.4 Operating parameters [agitator speed (rpm), aeration rate (vvm), $\%dO_2$] during continuous cultivation of *C. glutamicum* MCNB 10025 and the resulting $OD_{600\ nm}$, CDW g/liter, and cfu/ml (Chamsartra *et al*., 2005).

(Boswell *et al.*, 2003). This was probably because budding cells may be more susceptible to hydrodynamic stress or that as a cell increases in size during division (~10–12 μm), it approaches the scale at which the Kolmogoroff microscale of turbulence may have an effect. Since the microscale of turbulence decreases with increasing power input and impeller speed, it is expected that such an effect is more likely at high impeller speeds. With this system, at the highest impeller speed used, the microscale is less than 20 μm, that is within the range that might interact with budding yeast cells. Therefore, this work indicates that the potentially deleterious effects of high agitation rates can again be discounted, provided $\bar{\varepsilon}_T < \sim 5.0\ kW/m^3$, for propagation cultures.

All three of these studies concluded that any change in the biological behavior of nonfilamentous microbial cells within the $\bar{\varepsilon}_T$ range representing the normal operating window for mechanical agitation found in bioreactors as compared to the relatively gentle behavior found in shake flasks (Buchs *et al*., 2001) is not due to fluid dynamic stresses, whether arising from agitator-generated turbulence or bursting bubbles. In all cases, any changes in biological performance were only found under the most extreme of agitation intensities at values far above the normal operating range required to satisfy the mass transfer requirements. A review of the issues involved in large scale, free suspension animal cell culture in stirred bioreactors reached essentially the same conclusions (Nienow, 2006).

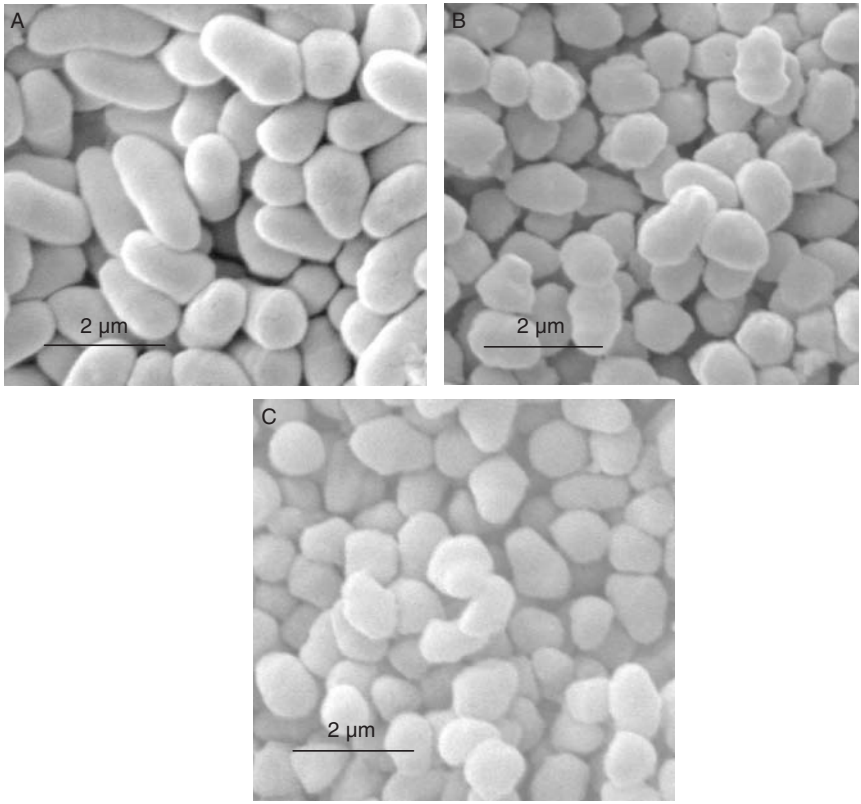

FIGURE 5.5 Scanning electron micrographs of samples taken from different conditions of "fluid mechanical stress" during continuous cultivation: (A) cells grown under "standard" operating conditions after 15 hour [stirred at 410 rpm ($\bar{\varepsilon}_T = 1$ W/kg), 1 vvm, 40% dO_2]; (B) cells from a sample taken during growth under high-intensity agitation at 33 hour [stirred at 1200 rpm ($\bar{\varepsilon}_T = 20$ W/kg), 1 vvm, 40% dO_2]; and (C) cells from a sample taken during growth under high aeration rates at 51 hour (stirred at 410 rpm ($\bar{\varepsilon}_T = 1$ W/kg), 3 vvm, 40% dO_2) (Chamsartra *et al*., 2005).

It is also worth noting that the work reported above was carried out with Rushton turbines, so-called high shear impellers. Even so damage was not found. It is also now understood that many of the so-called low shear impellers have higher values of $(\varepsilon_T)_{max}/(\bar{\varepsilon}_T) = \Phi$ than Rushton turbines. The concept of ''low shear impellers,'' a description which is intended to imply that they cause less damage to cells than other impellers, is essentially a manufacturer sales pitch (Simmons *et al.*, 2007). Overall, since all nonfilamentous cell types have been demonstrated experimentally not to be ''damaged'' by the fluid mechanical stresses found in bioreactors, an alternative explanation for any detrimental change in bioprocessing performance at the large scale must be found.

B. Operational constraints at the large scale

The fed-batch, high cell density cultivation of microbial strains is the preferred industrial method for increasing the volumetric productivity of such bacterial products as nucleic acids (Elsworth *et al*., 1968), amino acids (Forberg and Haggstrom, 1987), and heterologous recombinant proteins (Riesenberg and Schulz, 1991). The salient feature of this type of process is the continuous feed of a concentrated growth-limiting substrate, usually the carbon source, characterized by an ever-increasing level of energy limitation and an ever decreasing specific growth rate. This type of feeding regime avoids problems associated with catabolic regulation, oxygen limitation, and heat generation that can occur during unlimited batch processes (Minihane and Brown, 1986). Importantly, the build up of toxic concentrations of metabolic by-products via so-called ''overflow'' metabolic routes can also be avoided. Overflow metabolism has been reported for *S. cerevisiae* (George *et al*., 1993) as well as for *E. coli* and occurs at glucose concentrations above ~30 mg/liter. For *E. coli*, an accumulation of an inhibitory concentration of acetic acid occurs via the redirection of acetyl CoA from the Krebs cycle, during fast aerobic growth when a rapidly metabolizable carbon source, such as glucose, is available in excess (Andersson *et al*., 1996). For *S. cerevisiae*, overflow metabolism is known as the ''Crabtree effect'' and the inhibitory by-product is ethanol but produced in a similar way to acetate in *E. coli.* In batch fermentation, overflow metabolism can be avoided by the use of a slowly metabolizable carbon source such as glycerol (Elsworth *et al*., 1968), but the preferred method is the use of a fed-batch process where growth can easily be controlled by substrate feed rate (see Lee, 1996 for a comprehensive review).

Although the optimal position for the addition of any feed in order to ensure its subsequent rapid dispersal is in the region near to the impeller of $(\varepsilon_T)_{max}$, which leads to the rapid reduction of the high concentrations in the feed toward the desired mean value (Nienow, 1998, 2006), most large-scale industrial processes still use surface additions [because of concern for contamination, pipe blockage, mechanical stability, and so on (Nienow, 1998)]. Further, bioreactor configurations have traditionally been designed to satisfy oxygen mass transfer using radial flow Rushton turbines, with the inherent assumption that they were well mixed or if not, it was not important. Indeed, such a view is easy to understand as online measurements and control actions appear to show near to steady state conditions or slow progressive changes in those parameters being measured.

Around the mid-1980s, Kossen and coworkers using the concept of ''regime analysis,'' where the rate of oxygen uptake is compared with that of oxygen transfer and the level of dO_2, suggested that, at the large scale, differences in dO_2 would be found in batch fermentations (Manfredini *et al*., 1983; Oosterhuis *et al*., 1985). Sometime later in the early 1990s,

Enfors and coworkers came to similar conclusions for fed-batch fermentations with respect to the nutrient feed (George *et al*., 1993). At around this time, it was shown that replacing Rushton turbines by high solidity ratio hydrofoil impellers that enhanced bulk mixing (spatial homogeneity) improved fermentation performance (Buckland *et al*., 1988).

However, it has been established experimentally that spatial and temporal chemical gradients exist in large-scale fed-batch bioreactors (Xu *et al*., 1999) where additions of a concentrated, often viscous, carbon source at a single point onto the top surface of the growth medium means that mixing times are high [$>\sim$50 s even at the 20 m^3 scale (Vrabel *et al*., 2000)]. Studies using computational fluid dynamics (CFD) based on large eddy simulation (LES) also showed that considerable glucose gradients could be expected, even when a standard 500-g/liter glucose solution was fed to the liquid surface in a 22 m^3 bioreactor fitted with four Rushton turbines (Enfors *et al*., 2001). Such studies also showed that the region (compartment) around the top impeller would have a much higher glucose concentration when compared with the bulk (remainder) of the vessel (Fig. 5.6). The use of LES also shows the temporal as well as the spatial concentration fluctuations of the glucose concentration in the vicinity of the feed point. Indeed, this was confirmed experimentally showing that cells were frequently exposed to peak glucose concentrations several times higher than the mean in the addition zone (Xu *et al*., 1999), and that spatially dependent concentration gradients exist in large-scale fed-batch fermentation processes with a declining glucose concentration found with increasing distance from the feed point (Bylund *et al*., 1998). In laboratory-scale bioreactors on the other hand, where much development work is done, mixing times are low ($<\sim$5 s) and essentially significant temporal or spatial variations in concentration do not exist (Nienow, 1998).

Additionally, at the large scale, any pH controlling action is often based on the point measurement of local pH by a single probe situated adjacent to an impeller and hence in a well-mixed, high ε_T region. By contrast, the controlling agent, like the feed components, is usually added at the poorly mixed surface of the liquid. Additionally, the amount of controlling agent added is not continuous but added as a pulse, the volume of which is largely dependent on biomass concentration and its relative metabolic activity and hence will vary throughout the duration of the process, whether batch or fed-batch. The inherent inertia in such a system can lead to overfeeding of the pH controlling agent and therefore zones of high and low pH, again with temporal fluctuations superimposed on the spatial ones. Indeed, such regions of fluctuating high and low pH have now been measured in 8 m^3 bioreactors for animal cell culture (Langheinrich and Nienow, 1999).

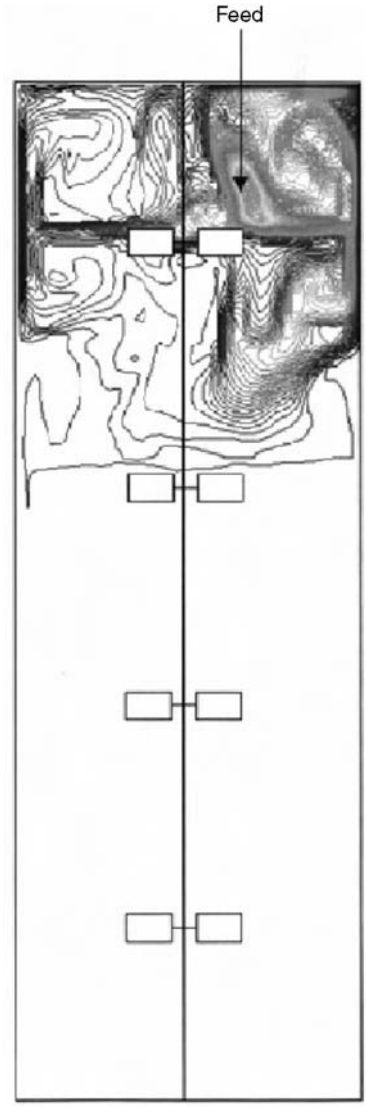

FIGURE 5.6 Large eddy simulation of instantaneous glucose concentration in a 22 m^3 bioreactor fed with a 500-g/liter solution at a rate of 180 liter/hour typically used in large-scale fed-batch processes. Four Rushton turbines and the location of the feed point are indicated. The simulation did not include the microbial consumption of the glucose and can therefore only be used to illustrate mixing efficiency (Enfors *et al*., 2001).

It is our contention that the composition of a cell's microenvironment is a product of the fluid dynamics and a cell's physiological response to it, so cells circulating around a large-scale bioreactor will experience rapidly changing microenvironments. Therefore, knowledge of if/how a cell reacts to such changes is essential if we are to understand the problems associated with bioprocessing on scale-up.

C. The physiological response of cells to the large-scale environment

Complex networks of regulatory systems often known physiologically as the so-called ''stress responses'' are phenomena that have evolved to help microorganisms withstand conditions when their immediate environment becomes suboptimal for growth (Wick and Egli, 2004). However, descriptions and discussions regarding such responses are usually confined to the mainstream microbiological literature. It is only now that it is being understood that the chemical and physical heterogeneities found within a poorly mixed large-scale bioreactor can cause microbial cells to alter their physiology as a response to these environmental stimuli (Enfors, 2004), and that this can have a detrimental effect on bioprocessing. Until recently, any physiological response of microbial cells to changes in environmental conditions within a bioreactor was mostly measured indirectly by measurement of external variables outside the cell. However, recent developments in the so-called ''omics'' analytical technologies have allowed the direct measurement of internal variables within the cell. So it has now been shown that *E. coli* cells respond very quickly to changes in local glucose concentration known to exist within a large-scale bioreactor by the fast transcriptional induction of an alternative set of genes (Schweder *et al.*, 1999). mRNA molecules associated with the expression of stress proteins, sensitive to oxygen limitation, are synthesized rapidly, when a cell passes through a local zone of high glucose concentration (Fig. 5.7). At the 20 m^3 scale, the overall biomass yield was reduced by $\sim$24% as compared to an otherwise identical laboratory-scale process and formate accumulated to 50 mg/liter. In this case, it was concluded that a high local glucose concentration induced local oxygen limitation, so that acetate synthesis was not due to overflow metabolism but due to mixed acid synthesis through fermentative metabolic pathways. Fermentative metabolism in *E. coli* differs in comparison to overflow metabolism in that formate, d-lactate, succinate, and ethanol are produced in addition to acetate under anaerobic conditions. However, both acetate and d-lactate are reassimilated much more quickly than formate when *E. coli* cells reenter an oxygen-sufficient zone, leading to an accumulation of formate in the culture medium. It was concluded that the repeated synthesis and consumption of these mixed acids in response

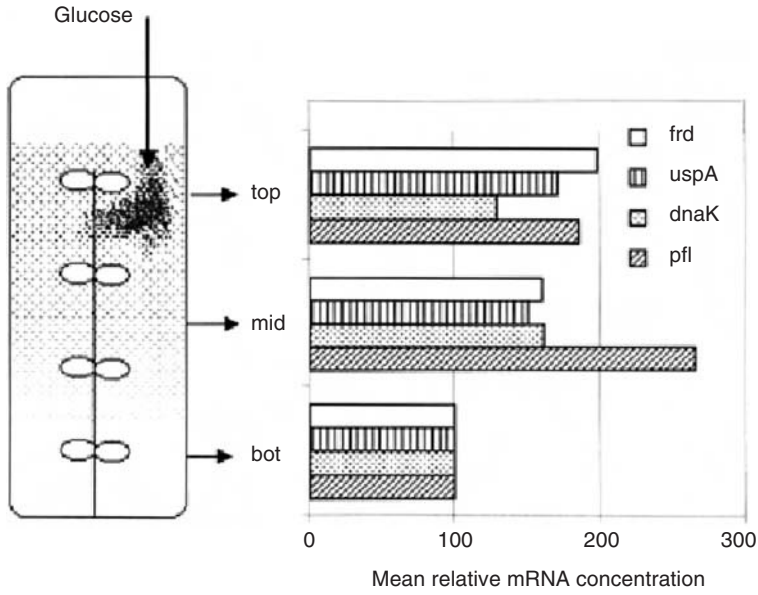

FIGURE 5.7 Analysis of mRNA concentrations of four stress-sensitive genes at three levels of the 22 m^3 fed-batch culture of *E. coli*. The concentrations averaged from quadruplicate samples were normalized to 100% at the bottom port (Enfors *et al*., 2001).

to a cells exposure to oxygen-sufficient/oxygen-deficient zones was responsible for the lower biomass yields experienced at the large scale (Xu *et al*., 1999).

Proteomic and metabolomic techniques can also be used which can reveal posttranscriptional or posttranslational events in cells, which cannot be revealed by transcriptomics alone. Using such sensitive molecular biological techniques, investigations have shown that under such poorly mixed conditions, *E. coli* cells may induce one of a number of interlinked regulatory stress response pathways characterized by rapid increases in the concentration of certain intracellular signaling molecules, such as ppGpp and cAMP, as well as the induction of alternative sigma factors such as RpoS (Hoffman and Rinas, 2004; Schweder and Hecker, 2004). These changes often result in the transcription and expression of a number of stress proteins, the consequences of which can include the inhibition of DNA replication initiation, a reduction in rRNA synthesis, and protein production. In addition, glycolytic activity, DNA metabolism, and the synthesis of structural components may be greatly reduced allowing cells to survive conditions suboptimal for continued growth leading inevitably to low biomass and product yields at the large scale.

D. Small-scale experimental simulation models of the large scale

It has long been the goal of biochemical engineers to be able to quantitatively model and predict large-scale process performance from data obtained from small-scale laboratory experiments. However, in general, mathematical models that are currently used to predict biomass production and protein synthesis during the scale-up of laboratory-scale fermentation processes (Andersson *et al*., 1996) make two basic assumptions. First, that throughout the course of a fermentation, a bacterial population is homogenous with respect to its physiological state and its ability to divide. Second, that the physiological state of a bacterial population is independent of the scale of cultivation. Such assumptions have now largely been shown to be invalid (Hewitt and Nebe-von-Caron, 2001, 2004). The latter would still hold true if the large and the small scale had identical process conditions but for the reasons discussed earlier this is very rarely the case. Techniques such as multiparameter flow cytometry that make measurements on individual cells have now shown unequivocally that different physiological subpopulations exist and evolve throughout the course of many microbial fermentation processes (Enfors *et al*., 2001; Hewitt *et al*., 1999, 2000; Lopes da Silva *et al*., 2005; Onyeaka *et al*., 2003; Reis *et al*., 2005; Sundström *et al*., 2004). For example, it was shown that during a 40 hour, 5 liter laboratory-scale fed-batch process to grow *E. coli* W3110 to high cell density (>50 g/liter dry cell weight), there was a progressive change in cell physiological state with respect to cytoplasmic membrane potential and permeability (Fig. 5.8). With ~16% of the population characterized as being dead at the end of the process and ~5% being in a fluctuating dormant state throughout (Hewitt *et al*., 1999). Other work has demonstrated a catastrophic drop (to ~0) in the number of cfu/ml in the middle of a fed-batch recombinant fermentation, while all other measurements showed that the majority of the cells were viable and metabolizing as normal (Sundström *et al*., 2004). Therefore, this type of study casts doubt on the use of mathematical models with the above assumptions for the reliable prediction of biomass production and product yield on scale-up. So the only definitive way of finding out how an industrial process is going to perform remains to carry out actual large-scale trials of the final process which are often difficult and expensive to carry out. Additionally, when the results differ from the bench scale, they are often difficult to interpret. Therefore, equipment and techniques that allow large-scale studies to be simulated at small scale have become important research tools.

For simulating the phenomenon of poor spatial and temporal homogeneity at the large scale, a technique used is to divide the large-scale reactor into two compartments, first by Kossens and coworkers (Oosterhuis

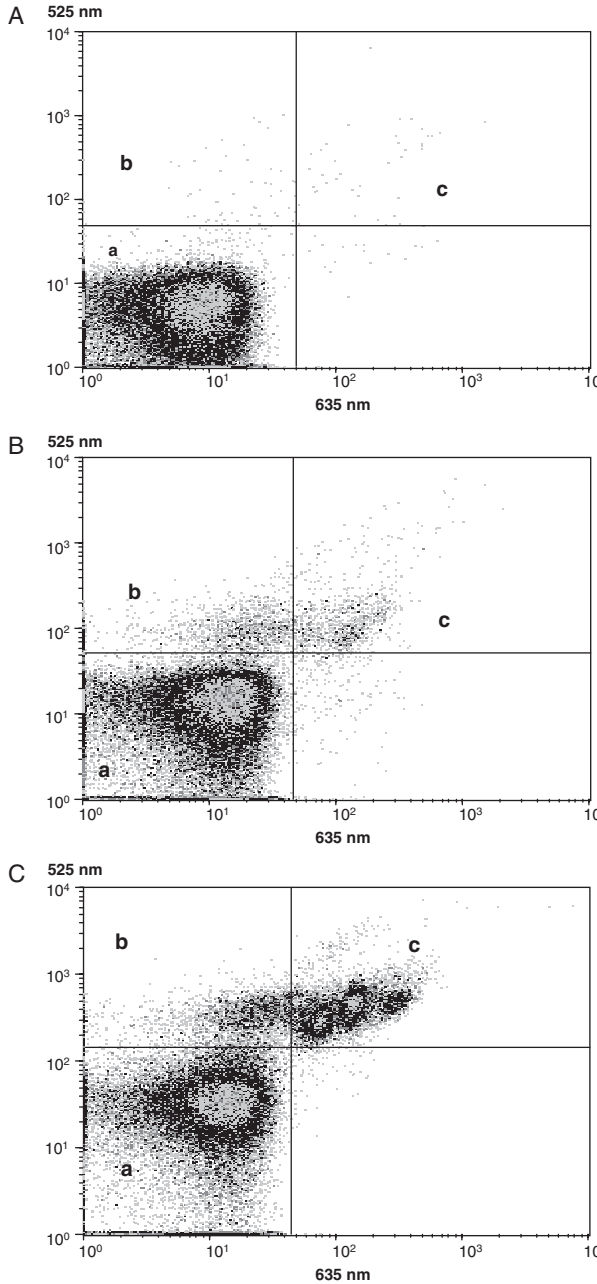

FIGURE 5.8 Density plots of cell samples taken at times 5 hour (A), 16 hour (B), and 36 hour (C) during a high cell density fed-batch fermentation with *E. coli* W3110 stained with propidium iodide (635 nm) and bis-oxanol (575 nm) and analyzed using multiparameter

et al., 1985) and later by Enfors and coworkers (George *et al*., 1993). The conditions established in each compartment depend on the type of poor mixing situation on the large scale that is to be simulated. For fed-batch fermentations and pH control heterogeneities associated with feed streams, these two compartments can represent an addition zone where the most severe pH and nutrient concentration gradients exist and the bulk region where the system can be considered to be essentially well mixed, at a much lower concentration with respect to the nutrient feed and pH. The relative size of the addition zone in the simulation should be of the order of size of the region in which higher concentrations exist at the large scale. The size of this region may be estimated by intuition, by flow visualization (again on the small scale), or by CFD (Enfors *et al*., 2001). Typically, in the small-scale simulation, the addition zone is represented by a relatively small plug flow reactor (PFR) and the bulk region by a stirred tank reactor (STR) (Amanullah *et al*., 2003). The volumetric ratio between these two reactors is equal to the estimated ratio of the addition zone to the bulk region in the large-scale reactor with the rate of circulation between them related to the circulation time of cells in the broth at the large scale due to agitation.

A similar approach is to use two stirred vessels side by side (STR–STR). This method has been particularly used to simulate dO_2 inhomogeneities associated with the slow rate of mixing compared to oxygen utilization. In this case, the volume of the well-oxygenated region is typically made of the order of 25% of the poorly oxygenated region. The STR–STR and STR–PFR have been compared for batch fermentations of *Bacillus subtilis* with respect to pH and dO_2 fluctuations (Amanullah *et al*., 2003). In both cases, significant differences were found compared to the well-mixed case, which depended on the relative size of the two zones and the recirculation rate between them. However, it is not possible to say which is the best technique and both are only rather crude approximations of the real variations actually seen by the cells at the large scale.

E. Results from small-scale experimental trials of large-scale *E. coli* fed-batch processes

1. Experimental setup

Recently, we have carried out a number of small-scale simulation studies of large-scale fed-batch fermentations with *E. coli*. For this work a two-compartment reactor system (Hewitt *et al*., 2000, 2007; Onyeaka *et al*., 2003)

flow cytometry. Three main subpopulations of cells can be distinguished, corresponding to healthy cells (a), no staining, cells with no cytoplasmic membrane potential (b), stained with bis-oxanol; and cells with permeablized cytoplasmic membranes (c), stained with both propidium iodide and bis-oxanol (Hewitt *et al*., 1999).

was used (Fig. 5.9). This system consisted of an STR (5-liter fermenter) linked in series to a PFR. The STR consisted of a 5-liter cylindrical glass bioreactor (162-mm diameter × 300-mm total height), with an initial working volume of 2.5 liter rising to 4 liter at the end of the fermentation. The vessel was fitted with two 82-mm, six-bladed radial flow paddle-type impellers which were 80 mm apart, with the lower impeller situated 80 mm above the bottom of the vessel. The vessel was also fitted with three equally spaced baffles, width 15 mm. The PFR was made up of a glass cylinder consisting of five equally sized sections each containing a set of removable stainless steel static mixer modules, each with 24 individual mixer elements to give a total of 120 mixing elements and a liquid volume of 544 ml (~14–22% of the total working volume). These elements were included in order to reduce radial concentration gradients, to enhance oxygen transfer (where appropriate), and to encourage plug flow. Provision was made so that either the pH controlling agent, or the substrate, or air, or all three could be introduced at the inlet of the PFR as well as into the STR. Medium was pumped through the PFR (the residence time in the PFR could be varied between 60 and 110 s) via a short length of silicone tubing. The PFR was thermally insulated along its length to avoid temperature gradients. All additions were made at 90° to the PFR flow, again in order to minimize disturbances, via energy input, to the plug flow characteristics of the reactor (George *et al*., 1993). Large-scale fermentations were carried out in a 30 m^3 cylindrical stainless steel bioreactor (2090-mm diameter × 9590-mm total height), with an initial working volume of 20 m^3 rising to 22 m^3 at the end of the

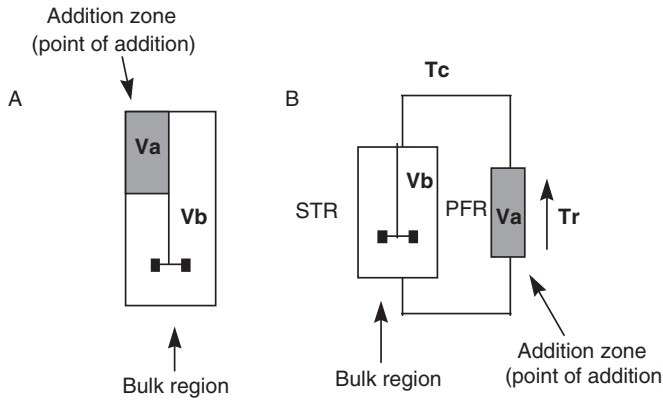

FIGURE 5.9 Experimental setup for scale-down simulation studies. (A) Large-scale STR. Va is the addition zone where the most extreme concentration gradients are known to exist and Vb is the bulk region which can be considered to be well mixed. (B) Scale-down simulation equipment. Here, Va is represented by a 0.544-liter PFR and Vb is represented by a 4-liter STR (Hewitt *et al*., 2000).

fermentation. The vessel was fitted with four Rushton turbines (diameter 690 mm) which were 1460 mm apart with the lower impeller 1110 mm above the bottom of the vessel. The vessel was also fitted with four baffles 90° apart, width 170 mm. Laboratory-scale fermentations were started as batch cultures and an exponential feeding profile was calculated in order to maintain the growth rate below 0.3 per hour from the following equation:

$$F = \left(\frac{1}{s}\right)\left(\frac{\mu}{Y_{xs}} + m\right)X_o e^{\mu t} \tag{5.11}$$

where F is the feed rate (liter/hour), s is the substrate concentration in the feed solution (g/liter), μ is the required specific growth rate (per hour), Y_{xs} is the maximum biomass yield on the limiting substrate (g/g), X_o is the total amount of biomass (g) at the start of feeding, m is the maintenance coefficient (g/g/hour), and t is the time after feeding commences (hour). Exponential feeding was started when the initial glucose had been exhausted. When the DOT had fallen to the 20% saturation level in the STR in all the small-scale cases, the feed rate was held constant for the remainder of the experiment. Large-scale fermentations were started as batch cultures and an exponential feed profile calculated as above was started 1 hour after inoculation and continued for 8.5 hour. This corresponded to a final feed rate of 180 liter/hour which was continued for a further 2.5 hour. This was then reduced to 170 liter/hour for the remainder of the fermentation. For all fermentations, a synthetic medium (Hewitt *et al*., 1999, 2000) was used and they all ran for ~40 hour.

2. Experimental results

First (Table 5.3), we compared the results obtained from a small-scale, well-mixed, 5-liter *E. coli* W3110 fed-batch fermentation with those found from carrying out a similar fermentation at the 20 m^3 scale where the pH and dO_2 were controlled at the same values (Hewitt *et al*., 2000). However, at the 20 m^3 scale, at similar levels of agitation intensity (expressed as W/kg), mixing times are much longer compared to the small scale [Eqs. (5.9) and (5.10), and for results in this 20 m^3 bioreactor, Vrabel *et al*., 2000]. Thus, the spatial and temporal heterogeneity of the environment in the vessel increases and locally higher glucose concentrations are found near the addition point with concomitant lower dissolved oxygen concentrations (Enfors *et al*., 2001). Differences in pH were also observed between the region close to the addition point of the pH controlling agent and the bulk environment where pH is often measured. At the 20 m^3 scale, counterintuitively, relatively very few dead cells (as measured by flow cytometry) were found (<0.5%) but the level of biomass was significantly less compared to the 5-liter scale (32 g/liter

TABLE 5.3 Experimental protocol and summary of the results for the effect of dO_2, glucose, and pH fluctuations on fed-batch fermentations with *E. coli* W3110 (data from Onyeaka *et al.*, 2003)

Experiment	Glucose feed point	NH_4 feed point	Final viability (%)[b]	Final dry cell weight (g/liter)	Air feed to PFR at 1 vvm[a]	Residence time in PFR (s)
Well-mixed 5 liter	STR	STR	84	55	–	–
PFR1	STR	STR	95	38	Yes	50
PFR2	PFR	STR	97	52	Yes	50
PFR3	PFR	STR	89	50	Yes	25
PFR4	PFR	STR	>99	37	No	50
PFR5	PFR	PFR	99	32	No	~50
PFR6	PFR	PFR	94	16	No	~110
PFR7	PFR	PFR/STR	97	24	No	~110
Large scale	STR	STR	>99	35	–	–

[a] 1 vvm with respect to the PFR.
[b] With respect to cytoplasmic membrane permeability.
In all cases the STR was also sparged at 1 vvm and agitated to keep dO_2 in it >20%, a dO_2 level which was also maintained everywhere in the well-mixed 5 liter and in the large-scale fermentation at the dO_2 electrode.

compared to 53 g/liter). These differences in cell biomass and viability were ascribed to the physiological effect on the cells of the combination of the three main heterogeneities, viz., dO_2, glucose, and pH that occur simultaneously at the large scale but not at all at the small scale.

In support of this explanation, the poor mixing was mimicked on the small scale by using the STR–PFR scale-down experimental model (Table 5.4), the PFR representing the poorly mixed addition zone and the STR representing the well-mixed zone in the region of the impeller. The ratio of the size of the PFR to the STR and the circulation time between them was based on the results from large-scale physical mixing time trials (Vrabel *et al*., 2000). This scale-down configuration gave very similar results to those found at the large scale when all three major heterogeneities, dO_2, glucose (Hewitt *et al*., 2000), and pH (Onyeaka *et al*., 2003) were introduced into the PFR simultaneously (Table 5.3). From all of these studies, it was concluded that the STR–PFR scale-down model enabled the impact of the inherently poorer mixing found in large-scale fermenters to be satisfactorily mimicked at the bench scale.

However, these studies were carried out using the untransformed wild-type bacterial strain, *E. coli* W3110. So the work did not take into account the additional physiological stress imposed on a cell by having to synthesize a foreign protein when studying process performance on scale-up. It is known that the synthesis of foreign proteins at high concentrations exerts a severe metabolic stress on the host cell (Borth *et al*., 1998; Lewis *et al*., 2004). Therefore, it was decided to carry out a similar study with a recombinant *E. coli* BL21 (MSD3735) which contains a plasmid coding for an isopropyl thiogalactopyranoside (IPTG)-inducible, model mammalian protein, AP50. This recombinant system is further complicated because under normal growth conditions as used here, the

TABLE 5.4 Summary of the scale-down conditions for all of the PFR simulation studies (Onyeaka *et al*., 2003)

PFR1—a region of low glucose: pH ~ 7
PFR2—a region of high glucose/dO_2: pH ~ 7
PFR3—a region of high glucose/dO_2 but experienced for a shorter time: pH ~ 7
PFR4—a region of high glucose/low dO_2: pH ~ 7
PFR5—a region of high glucose/low dO_2: pH > 7
PFR6—as PFR5 but for a longer time
PFR7—as PFR6 initially and after 28 hour reverting to PFR4
PFR8—a region of low glucose: pH ~ 7
PFR9—a region of high glucose/low dO_2: pH ~ 7
PFR10—a region of high glucose/low dO_2: pH > 7

protein is misfolded, accumulating in the form of insoluble, biologically inactive inclusion bodies in the cytosol of the cell. Relatively low values of agitation intensity $\bar{\varepsilon}_T$ (~1 W/kg) and aeration rate (~1 vvm) were used so that it could be realistically assumed that none of the observed effects on biomass or viability could be ascribed to fluid mechanical stresses in the system. In this way, the effect of any physiological or physical stress imposed by the synthesis of AP50 in addition to that imposed by scale of operation could be investigated (Hewitt *et al*., 2007).

It is clear that the expression of AP50 as insoluble inclusion bodies exerts a profound physiological stress on the host cell during high cell density fed-batch cultures, and that the extent of these effects are dependent on which combinations of the three major spatial heterogeneities associated with large-scale bioprocessing (pH, glucose, and dissolved oxygen concentration) are imposed (Tables 5.4 and 5.5). However, the detrimental effect of AP50 production on viability and physiological response can be reduced by the introduction of a spatial or temporal chemical heterogeneity, the extent of which is again dependent on the number and type of heterogeneities imposed. This result is again probably related to the induction of the one of

TABLE 5.5 Experimental protocol and summary of the results for the effect of dO_2, glucose, and pH fluctuations on fed-batch fermentations of the recombinant *E. coli* strain BL21 (MSD3735) (Hewitt *et al*., 2007)

Experiment	Glucose feed point	NH_4 feed point	Final viability (%)[a]	Final dry cell weight (g/liter)
Well-mixed 5 liter not induced	STR	STR	95.1	48
Well-mixed 5 liter induced $OD_{550\ nm}$ ~ 15	STR	STR	75	18
PFR8 not induced	STR	STR	100	36
PFR8 induced $OD_{550\ nm}$ ~ 15	STR	STR	100	15
PFR9 not induced	PFR	STR	99.9	41
PFR9 induced $OD_{550\ nm}$ ~15	PFR	STR	82.9	14
PFR10 not induced	PFR	PFR	100	34
PFR10 induced $OD_{550\ nm}$ ~ 15	PFR	PFR	82.5	14

[a] With respect to cytoplasmic membrane permeability.
In all cases, the PFR was unaerated while the STR was sparged at 1 vvm and agitated to keep dO_2 in it >20%. The residence time in the PFR was 60 s.

the interlinked regulatory so-called ''stress responses'' by a proportion of the cells as they pass through the chemically heterogeneous zone of the PFR, such that the resultant dormant cells have a reduced capability for AP50 production and are hence protected from the associated physical or physiological stresses (Hewitt *et al*., 2007).

In the earlier work, simulating at the small scale the impact of such heterogeneities on the large-scale performance of a fed-batch fermentation, data from an equivalent 20 m^3 commercial fermenter were available for comparison. For this work, such commercial scale data are not available. Therefore, it is not possible to say which of the three scale-down configurations best mimics performance at the large scale.

IV. CONCLUSIONS AND FUTURE PERSPECTIVE

The scale-up of single-celled aerobic microbial fermentation processes is complicated, and unpredictable process performance can result. However, this is not due to the introduction of fluid dynamic generated stresses (or so-called ''shear damage''), whether arising from agitator generated turbulence or bursting bubbles, rather it is because the large-scale fed-batch bioreactor provides a very dynamic environment with large spatial and temporal heterogeneities. Such environmental heterogeneities can induce multiple physiological responses in cells. These responses consume energy and resources such that biomass concentration as well as product yields can be reduced. These phenomena are not observed in well-mixed homogeneous laboratory-scale reactors where much process development is done and their effects are difficult to model mathematically. Actual large-scale trials are expensive to carry out and often not available to the small business or university. Therefore, the ability to obtain data on how a recombinant laboratory process may perform at the large scale, dependent on feeding regime employed or controlling action taken, is invaluable for any detailed and informed development program. From the work discussed here, it is clear that the scale-down two-compartment model can be used to study the impact of any range or combination of potential heterogeneities known to exist at the large scale at relatively low cost. Unfortunately, such experiments cannot give precise predictions because the spatial and temporal heterogeneities are only a rather crude approximation of the real ones found at the large scale. It is probable that with increasingly sophisticated CFD becoming available, knowledge of the detailed concentration fields on the large scale will become available (Schütze and Hengstler, 2006). However, such information will be of limited use until there is a much increased knowledge of either how cells will respond to such an environment or how such conditions can be mimicked on the small scale. The authors believe that for the foreseeable future, the experimental modeling approach set out here or a variant on it offers the best way forward.

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CHAPTER 6

Production of Recombinant Proteins in *Bacillus subtilis*

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I. INTRODUCTION

The classical way of protein purification starting from a large number of cells producing the protein using its authentic expression signals has been replaced by recombinant technology, where the protein of interest is overproduced in a regulated way. This often means that the recombinant gene is fused to a controllable promoter, which is activated by addition of an inducer to initiate transcription. In most cases, the inducer is a small molecule that is either taken up by the cells or diffuses through the cytoplasmic membrane such as isopropyl thiogalactose (IPTG), xylose, glycine. Alternatively, the inducer can be a stress factor such as a sudden increase or decrease in the growth temperature. Any expression system should guarantee a tight regulation of the promoter that means a very low transcription rate in the absence of the inducer and a high expression rate after addition of the inducer, resulting in the high-level synthesis of the recombinant protein accumulating to 10–30% of the total cellular protein.

Overproduction of recombinant proteins is always a two-step process starting with a growth regimen to obtain a high cell density followed by the expression phase. First, cells are grown into the appropriate medium into the appropriate growth phase (with bacteria normally into the mid- or late-exponential growth phase) and then cells will be induced. Production of recombinant proteins is carried out in different cell types starting with *Escherichia coli*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and other species such as *Pichia pastoris* and *P. methanolica*, filamentous fungi, insect, and Chinese hamster ovary cells (Baneyx, 2004); recombinant proteins are even produced in the milk of mammals termed gene pharming (Wall *et al.*, 1997). The organism used depends on the protein to be purified. If the recombinant protein does not contain any modification, *E. coli* is often the first choice. If the protein contains modifications, appropriate eukaryotic systems have to be used.

B. subtilis is an attractive host for protein engineering because of the following reasons: (1) it is nonpathogenic; (2) it secretes proteins efficiently, especially those originating from Gram-positive bacteria up to

20 g/liter; (3) it does not produce any endotoxin; (4) it has been granted a GRAS (generally regarded as safe) status by the Food and Drug Administration (FDA); (5) it has no significant bias in codon usage; and (6) a great deal of vital information concerning its transcription and translation mechanisms, genetic manipulation, and large-scale fermentation has been acquired (Harwood, 1992; Meima *et al.*, 2004; Schallmey *et al.*, 2004; Westers *et al.*, 2004; Wong, 1995). However, it is known that secretion of heterologous proteins in *B. subtilis* is usually low (Li *et al.*, 2004), and production of high-value human proteins for pharmaceutical applications remains a major challenge, since most medical applications require intact proteins with both authentic primary sequences and properly folded three-dimensional structures.

II. VECTOR SYSTEMS

Basically, three different vector systems have been developed (Bron, 1990): autonomously replicating plasmid vectors, integrative vectors, and bacteriophage vectors. Based on their mode of replication, plasmid vectors can be divided into two groups. The first group replicates according to the rolling circle mechanism (RCM) and the second uses the theta mechanism. Most small plasmids (smaller than ~12 kb) from Gram-positive bacteria replicate via the RCM, while larger plasmids use the theta mechanism (Bron *et al.*, 1991; Ehrlich *et al.*, 1991). A major distinction between the two modes of replication is the generation of single-stranded (ss) DNA intermediates by RCM plasmids (see below). Due to sometimes low transformation frequencies using *B. subtilis* as primary host, it is often more convenient to carry out the initial cloning steps with a shuttle plasmid in *E. coli* and then transfer the recombinant plasmids to *B. subtilis*. Shuttle plasmids contain two different origins of replication; one able to drive replication in *E. coli* and the second in another host, here *B. subtilis*. Integrative vectors allow insertion of the gene of interest into the *B. subtilis* chromosome, and most bacteriophage vectors are based on the temperate phage ϕ105 (Errington, 1993).

A. Rolling circle-type replication vectors

1. Natural rolling circle-type plasmids

A number of small plasmids have been observed to accumulate ssDNA during replication (Gruss and Ehrlich, 1989). Several of these plasmids were studied in detail and shown to replicate by a RCM (Sozhamannan *et al.*, 1990; te Riele *et al.*, 1986). The RCM requires two replication origins, one called ''plus'' and is used for the synthesis of the ss replication intermediates, while the other, termed ''minus,'' is used for the conversion of the ss intermediates into a mature double-stranded molecule. Minus origins are noncoding, highly palindromic sequences, usually

about 200–300 bp, which function only in one orientation. In their absence, plasmid ssDNA accumulates. Initiation of replication at the two origins does not occur simultaneously and therefore results in the accumulation of the ssDNA replication intermediate (Novick, 1989).

The four plasmids pUB110, pC194, pE194, and pT181 were initially identified in *Staphylococcus aureus*. While pUB110 specifies resistance to kanamycin and neomycin and has a copy number of 30–50 per chromosome (Lacey and Chopra, 1974), pC194 codes for chloramphenicol resistance and is maintained at a copy number of about 15 (Iordanescu *et al.*, 1978). The third plasmid, pE194, confers resistance to macrolide-lincosamide-streptogramin B (MLS) antibiotics and is present in ~10 copies per chromosome (Iordanescu, 1976). Most importantly, pE194 is naturally temperature-sensitive for replication (Repts phenotype) and does not replicate above 45 °C in *B. subtilis*. When cells containing pE194 were grown on erythromycin-containing media at 50 °C, erythromycin-resistant cells were selected in which pE194 was found to be integrated into the bacterial chromosome at a variety of sites (Hofemeister *et al.*, 1983). The fourth plasmid, pT181, is similar to many other tetracycline-resistant plasmids, for example pT127 and pSN1 (Iordanescu, 1976), with a copy number of around 20 copies per chromosome. Plasmid pTA1015 belongs to the group of cryptic plasmids isolated from *B. subtilis* strains (Uozumi *et al.*, 1980).

Two types of plasmid instability have been described, *segregational instability* involving loss of the entire plasmid population from a cell and *structural instability* involving the loss, rearrangement, or acquisition of DNA sequences by the plasmid. Loss of the whole plasmid is the consequence of unequal segregation during cell division: all copies segregate into one of the two daughter cells. While low-copy number plasmids are stably maintained by partitioning functions ensuring their accurate segregation at cell division, high-copy number plasmids are segregated randomly at cell division. One important reason for segregational instability of high-copy number plasmids is their tendency to form multimeric forms (in particular of RCM plasmids). The mechanism that controls the copy number of a plasmid ensures, on the average, a fixed number of plasmid origins per chromosome. Therefore, cells containing multimeric plasmids have the same number of plasmid origins but fewer plasmid molecules, leading to a greater instability. Structural instability often leads to deletion formation caused by erroneous replication termination (Michel and Ehrlich, 1986) and aberrant nicking-closing events (Ballester *et al.*, 1989) mediated by the replication proteins of the plasmids. Topoisomerase-like activities were also implicated in illegitimate recombination (Lopez *et al.*, 1984). Yet another important source for deletion formation is based on recombination between short direct repeats stimulated by the RCM (Bron *et al.*, 1991; Jannière and Ehrlich, 1987). Here, 9-bp

direct repeats (and sometimes direct repeats as short as 4 bp) are sufficient for deletion formation and create a major source for structural instability in recombinant plasmids. While the vector plasmids by themselves are normally stable, cloning of a DNA fragment can introduce direct repeats, where one repeat is located within the vector and the other within the insert.

2. Cloning vectors derived from rolling circle-type plasmids

In many cloning experiments, the natural plasmids shown in Table 6.1 have been directly used as vectors. Since these plasmids do not replicate in *E. coli*, several shuttle vectors have been constructed and important ones are listed in Table 6.2. Besides cloning vectors, several expression vectors allowing intra- and extracellular production of recombinant proteins are available. They contain both constitutive and inducible promoters and the coding regions of signal sequences derived from different genes coding for extracellular proteins.

B. Theta-type replication vectors

1. Natural theta-type replication plasmids

Plasmids which do not create ssDNA intermediates belong to the other class and replicate in the host through a theta-type intermediate, and these are present in low copy numbers and are structurally stable. The known prokaryotic theta plasmids can be classified into at least five groups A–E (Bruand *et al.*, 1993; Meijer *et al.*, 1995), where two of these groups incorporate plasmids from Gram-positive bacteria (Table 6.3). One class concerns the broad host range plasmid pAMβ1 of *Enterococcus faecalis* (Bruand *et al.*, 1991) and the highly related streptococcal plasmids pIP501 of *Streptococcus agalactiae* (Brantl and Behnke, 1992) and pSM19035 of *Streptococcus pyogenes* (Tanaka and Koshikawa, 1977; Tanaka and Ogura, 1998). The replication region of pLS32 can support replication of a DNA fragment as large as 310 kb without gross DNA rearrangement (Itaya and Tanaka, 1997), and even of the entire chromosome of *B. subtilis* via bidirectional replication (Hassan *et al.*, 1997). pBS72 has been isolated as a naturally occurring plasmid in an undomesticated strain of *B. subtilis* of the territory of Belarus with a copy number of 6 per chromosome (Titok *et al.*, 2003).

2. Cloning vectors derived from theta-type replication plasmids

Several cloning vectors have been constructed based on theta-type replication vectors. All these vectors are shuttle vectors which allow the cloning and verification steps in *E. coli*, and the final recombinant plasmid is then shuttled into *B. subtilis* (Table 6.4). Cloning vectors are based on the replication functions of pAMβ1 and pTB19 active in *B. subtilis* and the

TABLE 6.1 Important rolling circle-type replication plasmids

Plasmid	Marker	Size (bp)	Original host	References
pUB110	Kanamycin	4548	*Staphylococcus aureus*	Lacey and Chopra, 1974
pC194	Chloramphenicol	2906	*Staphylococcus aureus*	Iordanescu *et al.*, 1978
pE194	Erythromycin	3728	*Staphylococcus aureus*	Iordanescu, 1976
pE194-cop6	Erythromycin	3728	*Staphylococcus aureus*	Weisblum *et al.*, 1979
pT181	Tetracycline	~4500	*Staphylococcus aureus*	Iordanescu, 1976
pTA1015	Cryptic[a]	5807	*Bacillus subtilis*	Meijer *et al.*, 1998
pTA1060	Cryptic	~8600	*Bacillus subtilis*	Uozumi *et al.*, 1980

[a] Cryptic plasmids do not confer a selectable phenotype.

TABLE 6.2 Important cloning shuttle vectors based on rolling circle-type replication plasmids

Plasmid	Origins of replication	Size (kb)	Marker	Vector type	References
pEB10	ori_{pUB110} ori_{pBR322}	8.9	Ap, Km[a]	Cloning vector	Bron *et al.*, 1988
pBE20	ori_{p194} ori_{pBR322}	5771	Cm, Ap[a]	Cloning vector	Nagarajan *et al.*, 1992
pBE60	ori_{pUB110} ori_{pBR322}	7430	Km, Ap	Cloning vector	Nagarajan *et al.*, 1992
pE18	$ori_{pE194\text{-}cop6}$ ori_{pUC18}	3.8	Em, Ap	Cloning vector	Wu *et al.*, 1998
pHV14	ori_{p194} ori_{pBR322}	4.6	Ap, Cm	Cloning vector	Ehrlich, 1978
pLB5	ori_{pUB110} ori_{pBR322}	5.8	Ap, Cm, Km	Cloning vector	Bron and Luxen, 1985
pRB373	ori_{pUB110} ori_{pBR322}	5800	Km, Ap	Cloning vector	Brückner, 1992
pUB18	ori_{pUB110} ori_{pUC18}	3.6	Km, Ap	Cloning vector	Wong *et al.*, 1988
pUB19	ori_{pUB110} ori_{pUC18}	3.3	Km, Ap	Cloning vector	Wu and Wong, 1999
pWB980	ori_{pUB110} ori_{pUC18}	3772	Km, Ap	Secretion vector	Wu and Wong, 1999
pHB201	$ori_{pTA1060}$ ori_{pUC19}	6593	Cm, Km	Cloning vector	Bron *et al.*, 1998
pHPS9	$ori_{pTA1060}$ ori_{pBR322}	5.6	Cm, Em	Cloning vector with *lacZ*α	Haima *et al.*, 1990
pHP13	$ori_{pTA1060}$ ori_{pBR322}	4.9	Cm, Em	Cloning vector	Haima *et al.*, 1987

[a] Ap, ampicillin; Cm, chloramphenicol; Em, erythromycin; Km, kanamycin.

TABLE 6.3 Natural theta-type replication plasmids

Plasmid	Marker	Size (kb)	Original host/function	References
pAM1	Em[a]	26.5	*Enterococcus faecalis*	Clewell *et al.*, 1974
pBS72	cryptic	3081	*Bacillus subtilis*	Titok *et al.*, 2003
pIP404	Cm, Tc	54	*Clostridium perfringens*	Brefort *et al.*, 1977
pIP501	Cm, Em	30	*Streptococcus agalactiae*	Horodniceanu *et al.*, 1976
pLS20	cryptic[b]	5.3	*Bacillus subtilis* var. *natto*	Tanaka and Koshikawa, 1977
pLS32	cryptic	7228	*Bacillus subtilis* var. *natto*	Tanaka and Ogura, 1998
pSM19035		27	*Streptococcus pyogenes*	Ceglowski *et al.*, 1993
pTB19	Tc, Km	26	*Bacillus stearothermophilus*	Imanaka *et al.*, 1981

[a] Cm, chloramphenicol; Em, erythromycin; Km, kanamycin, Tc, tetracycline.
[b] Cryptic plasmids do not confer a selectable phenotype.

TABLE 6.4 Important vectors derived from theta-type replication plasmids

Plasmid designation	Origins of replication	Vector type	References
pHV1431 pHV1432	$ori_{pAM\beta1}$ ori_{pBR322}	Cloning vector	Jannière *et al.*, 1990
pHV1436	ori_{pTB19} ori_{pBR322}	Cloning vector	Jannière *et al.*, 1990
pHCMC02	ori_{pBS72} ori_{pBR322}	Expression vector; P_{lepA}	Nguyen *et al.*, 2005
pHCMC04	ori_{pBS72} ori_{pBR322}	Expression vector; P_{xylA}	Nguyen *et al.*, 2005
pHCMC05	ori_{pBS72} ori_{pBR322}	Expression vector; P_{IPTG}	Nguyen *et al.*, 2005
pMTLBS72	ori_{pBS72} ori_{pBR322}	Cloning vector	Titok *et al.*, 2003
pNDH33	ori_{pBS72} ori_{pBR322}	Expression vector; P_{grac}	Phan *et al.*, 2006
pTRKH2	$ori_{pAM\beta1}$ ori_{p15A}	Cloning vector	O'Sullivan and Klaenhammer, 1993

replicons of pBR322 of *E. coli*. A series of expression vectors have been developed in my group based on the newly discovered cryptic *B. subtilis* plasmid pBS72.

C. Integrative vectors

An alternative method to avoid the problem of the instability of recombinant plasmids in *B. subtilis* is to use integrative plasmids allowing for the ectopic insertion of cloned genes into sites of the chromosome. Besides conferring stability, integration of genes into the chromosome offers additional advantages. Integration ensures that normally a single copy of the transgene is present, which can be maintained in the same copy number even in the absence of selection (Vazquez-Cruz *et al.*, 1996). The presence of a single copy of the transgene can be important for gene regulation. Such integrative plasmids are usually based on an *E. coli* replicon (mostly pBR322 or one of its derivatives) and carry an antibiotic-resistant marker gene that can be selected in *B. subtilis*, and DNA sequences homologous to the *B. subtilis* chromosome. All these integration plasmids contain DNA sequences of a nonessential gene which are split by at least one or more unique restriction sites allowing cloning of any gene. Some of these vectors contain promoters ensuring constitutive or regulated expression in *B. subtilis*, or a reporter gene such as *lacZ* allowing construction of transcriptional fusions. After transferring into *B. subtilis* cells, in most cases, the whole plasmid is integrated into the chromosome using one of the two halves of the nonessential gene via a Campbell-type of recombination. At a low frequency, only the DNA sequences sandwiched between the two halves of the gene are transferred to the chromosome by double homologous recombination. There are two possibilities to distinguish between these two events. First, one can screen for the loss of the function of the disrupted nonessential gene. Second, one can disrupt the gene by insertion of an antibiotic resistance gene within the chromosome and use that strain as a recipient during the transformation process. When a double crossover event has happened, the antibiotic resistance gene is lost, which can be screened for on appropriate selective agar plates.

The most commonly used vectors are those allowing integration at the *amyE* locus (Shimotsu and Henner, 1986). The inserted genes disrupt *amyE*, preventing the production of α-amylase. Important vectors are listed in Table 6.5. Other integration vectors allow for the insertion of genes at the *thrC* locus, resulting in threonine auxotrophy (Guérout-Fleury *et al.*, 1996), at the *lacA* locus coding for a β-galactosidase (Härtl *et al.*, 2001) and three new genes, namely *pyrD*, *gltA*, and *sacA* (Middleton and Hofmeister, 2004). Only vectors pAX01 and pA-*spac* contain

TABLE 6.5 Integration sites and important vectors

Gene function	Vector(s)	References
amyE α-amylase	pDH32	Shimotsu and Henner, 1986
	pDG271	Antoniewski *et al.*, 1990
	pDG1661, pDG1662, pDG1728	Guérout-Fleury *et al.*, 1996
	pMLK83	Karow and Piggot, 1995
	pDL, pDK	Yuan and Wong, 1995a
trpC tryptophan biosynthesis	pDG1663, 1664, 1729, 1731	Guérout-Fleury *et al.*, 1996
lacA β-galactosidase	pAX01, pA-spac	Härtl *et al.*, 2001
pyrD pyrimidine biosynthesis	pPyr-Cm, pPyr-Kan	Middleton and Hofmeister, 2004
gltA glutamate biosynthesis	pGlt-Cm, pGlt-Kan	Middleton and Hofmeister, 2004
sacA levansucrase	pSac-Cm, pSac-Kan	Middleton and Hofmeister, 2004

inducible promoters (xylose and IPTG, respectively) to allow regulatable expression from the chromosome (Table 6.5).

It is also possible to increase the copy number of the recombinant gene within the chromosome by gene amplification. It has been shown that a construct consisting of a genetic marker flanked by directly repeated sequences can be amplified in the *B. subtilis* chromosome (Albertini and Galizzi, 1985; Jannière *et al.*, 1985; Sargent and Bennett, 1985; Young, 1984). The amplified structure consists of tandemly repeated amplification units, which comprise the marker and one of the flanking direct repeats. A maximum of 20–50 copies of the amplification unit per chromosome could be selected for when an antibiotic resistance marker was used (Jannière *et al.*, 1985). Amplification was relatively stable on growth of cells under nonselective conditions, and each copy of an amplified gene was expressed with equal efficiency. Furthermore, it was demonstrated that gene amplification can be stimulated by induction of replication of a plasmid integrated into the *B. subtilis* chromosome (Petit *et al.*, 1992). The system relies on the plasmid pE194, temperature-sensitive for replication, which was stably integrated into the chromosome. An amplification unit, comprising an antibiotic resistance marker flanked by directly repeated

sequences, was placed next to the integrated plasmid. Activation of pE194 replication led to multiplication of the amplification unit (Petit *et al.*, 1992). Gene amplification may be used to obtain enhanced expression of recombinant genes. This has been demonstrated elegantly by placing the glucanase A gene of *Clostridium thermocellum* into an amplification unit where amplification was driven by replication of the nearby pE194 resulting in hypersecretion of the cellulose (Petit *et al.*, 1990).

D. Bacteriophage vectors

Two temperate phages, ϕ105 and SPβ, have proved to be particularly useful for cloning and gene manipulation in *B. subtilis* (Errington, 1993). ϕ105 is similar to *E. coli* phage λ in terms of its size (39.3 kb), morphology, and genome organization. After infection, its DNA circularizes via ss cohesive termini (7-base long), which is able to integrate at a unique chromosomal attachment site near *pheA*. Phage SPβ is much larger (120 kb), but less is known about its general organization.

There are two different approaches for cloning using ϕ105 vectors: direct infection and prophage transformation. In the first approach, the DNA fragments are directly inserted into the ϕ105 vector followed by transfection into *B. subtilis* cells. The second approach takes advantage of the efficient transformation of *B. subtilis* with linear DNA fragments. Restriction fragments carrying the transgene are ligated to fragments of the vector DNA. The resultant linear concatemeric DNA is introduced into a host strain carrying a ϕ105 prophage, where homologous recombination can result in the insertion of the transgene into the prophage genome. The transformation efficiency is significantly enhanced by using prophages carrying an *ind cts-52* double mutation and plasmids containing defined regions of homology with the prophage. Several ϕ105-based vectors allowing the insertion of foreign DNA have been described (East and Errington, 1989; Errington, 1984; Jones and Errington, 1987). In addition, expression vectors have been developed allowing the high-level production of recombinant proteins such as ϕ105MU209 (Thornewell *et al.*, 1993) and ϕ105MU331 (Leung and Errington, 1995). These expression vectors not only provide inducible transcription of transgenes, but also prevent lysis of the host cell. Using ϕ105MU331 as a cloning vehicle, the protective antigen of *B. anthracis* has been secreted into the culture supernatant at $\sim$2 mg/liter (Baillie *et al.*, 1998) and a β-lactamase inhibitory protein at 3.6 mg/liter (Liu *et al.*, 2004). In this vector, transcription is initiated by a temperature upshift resulting in inactivation of the temperature-sensitive *cts-52* repressor. The same phage vector has also been used to ensure expression of two different genes forming an artificial operon (Chan *et al.*, 2002). In another approach, two transgenes were expressed that had been inserted into two

different replicons, one into the ϕ105MU331 prophage and another into a pUB110-based vector (Ho and Lim, 2003).

ϕ105J70 carries the *cts-52* allele facilitating synchronous induction when the temperature is raised, and the *cat* gene allowing selection of lysogenic cells on chloramphenicol-containing plates. Furthermore, it is defective in cell lysis due to the removal of the gene coding for the holin protein causing lesions in the cytoplasmic membrane (Errington, 1984). In the ϕ105MU622 derivative, the *cat* gene has been replaced by the *aphA-3* gene allowing selection on kanamycin-containing plates. Furthermore, genes can be fused to a promoter which is expressed during lytic development (Gibson and Errington, 1992).

In principle, a prophage-based expression system has several advantages over plasmid systems. First, the prophage offers stability in the absence of selective pressure, since the phage DNA is covalently inserted in single copy into the host chromosome. Second, the lysogenic state also involves strong repression of phage transcription, hence expression of potentially toxic and destabilizing genes can be minimized during the growth phase. Third, on prophage induction, strong promoters are activated, and expression is facilitated by phage DNA replication, resulting in a rapid increase in copy number. Fourth, the potential problem of cell lysis for secreted proteins can be avoided by the incorporation of deletion mutants that prevent cell lysis. In the system described here, the gene coding for a holin protein causing lesions in the cytoplasmic membrane is inactivated.

III. EXPRESSION SYSTEMS

Several expression systems have been used to overproduce heterologous polypeptides in different species of *Bacillus*. This goal has mainly been achieved by the combination of strong but regulatable promoters, a variety of translation/secretion signals, and transcription terminators. All these factors have been utilized in different genetic backgrounds and under a variety of growth conditions.

A. Promoter systems

For high-level gene expression and recombinant protein production it will be necessary to use strong promoters, preferably nonartificially activated by inexpensive inducers. Based on the induction mechanism, promoters can be classified into three categories: inducer-specific promoters, growth phase- and stress-specific promoters, and autoinducible promoters. Inducer-dependent promoters are the most widely used ones.

1. Inducer-specific promoters

Important inducer-specific promoters are under the negative control of a transcriptional repressor, which binds to an operator sequence usually located immediately downstream of the promoter. To obtain expression of the recombinant gene, cells are grown to the mid- or late-log phase followed by addition of the inducer. Some expression cassettes provide, in addition, a strong Shine-Dalgarno (SD) sequence to enhance expression of the recombinant gene. Furthermore, other expression units are equipped with the coding region for a signal sequence, which is recognized by one of the three different secretion pathways (see below). In principle, the four different modules promoter, operator, SD sequence, and signal sequence are interchangeable and allow the assembly of an optimal expression unit.

Most promoters are activated by one of two inducers, either IPTG or xylose. These inducers bind to the LacI or the XylR repressor, respectively, and cause the dissociation from their cognate operators. The very first inducible promoter for *B. subtilis* has been designated as *spac* (Yansura and Henner, 1984). Here, a promoter of the *B. subtilis* phage SPO-1 has been fused to the *E. coli lac* operator resulting in *spac*. Furthermore, the *E. coli lacI* gene coding for the LacI repressor has been engineered in such a way that it can be expressed in *B. subtilis*. Induction of P_{spac} occurs by addition of IPTG. In another system, the *E. coli lac* repressor-based expression system has been functionally implemented in *B. subtilis* using a two-plasmid system. While one plasmid carries the constitutively expressed *lacI* gene (pREP4), the second compatible plasmid is equipped with the strong promoter P_{N25} fused to a synthetic *lac* operator (p602/22) (Le Grice, 1990). Beside this two-plasmid system, pREP9 has been constructed carrying both elements. During subsequent years, a variety of expression systems have been constructed based on this expression system (Härtl *et al.*, 2001; Nguyen *et al.*, 2005; Phan *et al.*, 2006; Vagner *et al.*, 1998). Three disadvantages of the IPTG-inducible system have to be mentioned: (1) IPTG is expensive and toxic and therefore not suitable for large-scale fermentations; (2) the P_{spac} promoter is not strong enough for large-scale protein production; (3) the control of the promoter is not tight, resulting in the synthesis of small amounts of the recombinant protein even in the absence of IPTG. The first problem can be solved by introducing the *lacY* gene coding for the lactose permease, and a mutant *lacZ* gene able to convert lactose into allolactose but not able to degrade it to glucose and galactose. Expression of both genes in *B. subtilis* will allow the addition of the inexpensive lactose as inducer to the culture medium. The second problem has been mainly eliminated by the construction of the improved P_{grac} promoter allowing a high level of protein production (Phan *et al.*, 2006).

The second promoter system is based on xylose as inducer. The genes involved in the degradation of xylose are under the negative control of the XylR repressor encoded by the *xylR* gene. The first published system is based on the *B. subtilis* xylose-inducible promoter–operator elements (Gärtner *et al.*, 1992). Another system is derived from a comparable system of *B. megaterium* and allows integration of the expression cassette at the *amyE* locus (Kim *et al.*, 1996). Here, induction of expression is normally realized by addition of xylose up to 0.1–2%, resulting in an induction factor of up to 200. This system is not sensitive to general catabolite repression but is sensitive to glucose repression (Kim *et al.*, 1996). The third system uses citrate as inducer. The *citM* gene of *B. subtilis* codes for a secondary transporter for Mg–citrate complexes and is under the positive control of the CitST two-component signal transduction system (Yamamoto *et al.*, 2000). Induction of the system occurs by addition of 2-mM citrate and has been used for the synthesis of a number of proteins (Fukushima *et al.*, 2002).

A fourth inducible expression system relies on the promoter region of the *sacB* gene coding for an extracellular levansucrase (Lvs) (Zukowski and Miller, 1986). Production of a class of degradative enzymes, including an intracellular protease and several secreted enzymes such as Lvs and alkaline protease, is controlled at the transcriptional level by the products of the *degS* and *degU* genes (see below for details).

A fifth system uses tetracycline as inducer. The *tet* operator sequence has been placed between the −35 and −10 elements of the *B. subtilis*-derived strong *xyl* promoter. In the presence of a *tetR* repressor gene, this construct is about 100-fold inducible and exhibits high promoter strength. Basal expression is avoided by placing a second *tet* operator downstream of the −10 element, at the expense of reduced inducibility (Geissendorfer and Hillen, 1990). The expression system has been successfully used for the high-level production of a glucose dehydrogenase and human single-chain urokinase-like plasminogen activator (Geissendorfer and Hillen, 1990).

Yet another system is based on the glycine riboswitch. Riboswitches are regulatory elements located within the 5′ untranslated region of some mRNAs (Mandal and Breaker, 2004; Tucker and Breaker, 2005; Winkler and Breaker, 2005). They form secondary structures which serve as binding sites for metabolites, such as vitamins and amino acids, and often control expression of genes involved in the biosynthesis or transport of the metabolite sensed. In bacteria, riboswitches control either transcription elongation or translation initiation. While most metabolites prevent gene expression by interaction with their cognate riboswitch, binding of glycine to its riboswitch leads to transcription attenuation in *B. subtilis* (Mandal *et al.*, 2004). Here, glycine leads to the activation of a tricistronic operon involved in the degradation of this amino acid if present at high

concentrations. Based on this finding, an expression system has been constructed, which can be induced by the addition of glycine (Phan and Schumann, 2007).

2. Growth phase- and stress-specific promoters

Transcriptome analysis has revealed genes that follow a growth-specific expression pattern (Biaudet *et al.*, 1997). While some genes are exclusively transcribed during the exponential growth phase, others become activated when cells enter stationary phase. Expression cassettes derived from these genes can be used to drive expression of recombinant genes, avoiding the addition of any inducer. Two different promoter regions have been used to drive expression of recombinant genes. One of these is based on the promoter of the *rpsF* gene, which is active during exponential growth phase, and the other is based on the *aprE* gene, which is active during stationary phase. The *rpsF* gene is part of the tricistronic operon *rpsF-ssb-rpsR* coding for the ribosomal proteins S6 and S18 and a ssDNA-binding protein (Lindner *et al.*, 2004). The promoter of the *rpsF* operon has been used to obtain high-level production of *β*-toxoid of *C. perfringens* (Nijland *et al.*, 2007).

Another example for a growth phase-regulated gene is *aprE* coding for the subtilisin protease. This gene is induced at the end of the exponential growth, when the maximum biomass is reached which represents an important economical advantage and simplicity for fermentation processes at large industrial scales. The *aprE* promoter is σ^A-dependent and its activity is highly controlled (Valle and Ferrari, 1989). Using *lacZ* as a reporter gene, around 10% of the total recombinant protein could be produced in *B. subtilis* from a single copy integrated into the chromosome (Jan *et al.*, 2001).

B. subtilis ATCC 6633 produces the lantibiotic subtilin, which is subject to quorum-sensing control. The sensor kinase SpaK senses subtilin and transduces the signal to the response regulator SpaR. SpaR∼P binds to *spa* boxes in the promoter regions upstream of several genes and triggers promoter activation (Kleerebezem *et al.*, 2004). Additionally, production of subtilin in *B. subtilis* is controlled by the transition-state sigma factor σ^H (Stein *et al.*, 2002). As a consequence of this dual regulation mechanism, the level of production of subtilin during the early and mid-log phase of growth is relatively low, while high levels of subtilin are produced during the late exponential and transition growth phases (Stein *et al.*, 2002). Based on these observations, the *su*btilin-*r*egulated gene *e*xpression (SURE) system was constructed based on the *spaS* promoter (Bongers *et al.*, 2005).

One of the major drawbacks during high-level production of recombinant proteins in bacteria is the inability of many recombinant proteins to reach their native conformation. One experimental approach to limit aggregation of overproduced proteins consists in cultivating the cells at

low temperatures (Schein and Noteborn, 1988). To ensure high-level production of recombinant proteins at low temperatures, the transgenes can be fused to a cold-shock-inducible promoter. In *B. subtilis*, the *des* gene codes for the enzyme desaturase, which introduces *cis* double bonds into a wide variety of saturated fatty acids (Aguilar *et al.*, 1998). Expression of the *des* gene depends on a two-component signal transduction system, which consists of the sensor kinase DesK and the response regulator DesR (Aguilar *et al.*, 2001). When the sensor kinase senses a temperature downshift through changes in the physical state of the cytoplasmic membrane, it undergoes autophosphorylation with subsequent transfer of the phosphoryl group to the response regulator. Phosphorylated DesR binds to two adjacent DNA-binding sites leading to transcription activation of the *des* promoter (Cybulski *et al.*, 2004). Two expression vectors have been constructed based on the cold-inducible *des* promoter allowing intra- and extracellular synthesis of recombinant proteins (Le and Schumann, 2007).

3. Autoinducible promoters

The *pst* operon of *B. subtilis* is involved in phosphate transport and is strongly induced in response to phosphate starvation (Eymann *et al.*, 1996). The *pst* operon is transcribed from a single promoter and is regulated by the PhoP–PhoR two-component signal transduction system. The expression from the *pst* promoter is induced over 5000-fold on phosphate starvation (Qi *et al.*, 1997). Using a *pst* expression cassette, phytase could be secreted to 2.9 g/liter, representing ~65% of the total extracellular protein at the end of cultivation (Kerovuo *et al.*, 2000). In similar experiments, it could be shown that the promoter region preceding the alkaline phosphatase gene of *B. licheniformis* is tightly regulated by the phosphate concentration in the medium and can be used for heterologous gene expression (Lee *et al.*, 1991). Similar results have been published for the *phoD* promoter of *B. subtilis* which was induced about 2000-fold 12 hour after growth in a low-phosphate medium (Eder *et al.*, 1996).

The second example is the *gsiB* promoter where *gsiB* belongs to sigma-B regulon (Price, 2002). This regulon codes for general stress proteins which are induced after the imposition of a variety of stress factors such as heat shock; salt, acid, or ethanol stress; lack of oxygen or glucose starvation (Hecker and Völker, 1990). The small hydrophilic GsiB protein is synthesized at higher rates and accumulates to a higher level than other σ^B-dependent general stress proteins (Bernhardt *et al.*, 1997). This high-level accumulation of GsiB has been at least partially attributed to the remarkable long half-life of about 20 min of the *gsiB* mRNA (Jürgen *et al.*, 1998). This finding prompted the construction of a plasmid-based expression system, which can be induced by one of the stress factors mentioned above (Nguyen *et al.*, 2005) and has been successfully used for the production of antigens following oral or parenteral delivery to mice

(Paccez *et al.*, 2006). Here, the *gsiB* promoter is induced most probably due to the absence of oxygen within the cells of the animals.

The third autoinducible promoter system is based on the lysine riboswitch. In contrast to the glycine riboswitch, the lysine riboswitch confers transcription termination in the presence of a sufficient amount of lysine in the cytoplasm (Sudarsan *et al.*, 2003). When the level of lysine drops below a threshold value, transcription attenuation occurs, leading to the expression of the downstream *lysC* gene coding for the aspartokinase II α-subunit. Based on this finding, an expression system has been constructed, which leads to autoinduction when the lysine concentration within the growing cells drops below the threshold value (T. T. P. Phan and W. Schumann, to be published).

B. Secretion systems

As *Bacillus* species have a high capacity for secreting their own proteins into the extracellular medium (Ferrari *et al.*, 1993), they are also considered as attractive hosts for producing heterologous secretory proteins. *B. subtilis* is able to secrete several proteins up to 20 g/liter (Schallmey *et al.*, 2004), but it is also known that secretion of heterologous proteins is usually lower (Li *et al.*, 2004). This problem, which applies especially to eukaryotic proteins, can be attributed to a variety of secretion bottlenecks, including poor membrane targeting, inefficient membrane translocation or cell wall passage, slow or incorrect polypeptide chain folding, and degradation (Bolhuis *et al.*, 1999a). Three major pathways allow for translocation of proteins from the cytoplasm to the extracytoplasmic site: (1) the largest number of proteins use the Sec pathway; (2) two proteins have been described so far being translocated through the Tat pathway (Jongbloed *et al.*, 2000, 2004); and (3) an unknown number of proteins is SRP-dependent (Zanen *et al.*, 2006). All proteins being translocated through one of the three pathways are synthesized as preproteins with N-terminal signal peptides that direct them into the appropriate pathway. The signal peptides consist of three distinct domains: a positively charged N-domain, a hydrophobic H-domain, followed by a C-domain that includes the recognition and cleavage site for the signal peptide (Tjalsma *et al.*, 2004). In the case of the Tat pathway, the N-domain contains a twin-arginine motif (RR), and SRP-dependent proteins are enriched in hydrophobic amino acids within the H-domain.

1. Secretion using the Sec system

The various components of the Sec-dependent secretion machinery consist of six classes: (1) cytosolic chaperones, (2) the SecA translocation motor protein, (3) the SecYEG translocation channel, (4) signal peptidases, (5) signal peptide peptidases, and (6) folding factors on the *trans* side of

the cytoplasmic membrane [see excellent review by Tjalsma *et al.* (2000)]. Since secretory proteins destined for the Sec-pathway are first synthesized in the cytoplasm, they have to be kept in a translocation-competent form by cytosolic chaperones. Here, only the trigger factor, a ribosome-bound protein, can maintain the translocation competence of precursor proteins. Although the DnaK and the GroE chaperone machines do not play a general role in keeping precursor protein translocation competent, secretion of antidigoxin single-chain antibody (SCA), which has a tendency to accumulate in inclusion bodies, these chaperones were demonstrated to improve secretion by about 60% through concerted overproduction (Wu *et al.*, 1993). The *B. subtilis* chromosome codes for five different type I signal peptidases, which all can process secretory preproteins, but only SipS and SipT are of major importance (Tjalsma *et al.*, 1998). When complete translocation of the polypeptide chain has occurred, these proteins have to fold into their native conformation at the *trans* side of the membrane to avoid degradation by the multifold of proteases present in this environment. Folding catalysts include peptidyl-prolyl isomerases (PPIases), thiol-disulfide oxidoreductases, and, perhaps, molecular chaperones. Several extracellular proteases are competing with these folding catalysts. Details of these important issues are presented below.

Different secretion vectors have been developed based on various signal sequences combined with their own or foreign promoters. The first secretion vectors described use the α-amylase secretory system (Palva *et al.*, 1983; Taira *et al.*, 1989), and these are reviewed by Sarvas (1986). Other secretion systems are based on protease export systems (Saunders *et al.*, 1987; Schein *et al.*, 1986; Vasantha and Thompson, 1986). Expression from these systems occurs during stationary phase, when most of the secreted enzymes of *B. subtilis*, including the major proteases, are secreted. An alternative secretion system, which is active during the logarithmic growth phase, is based on Lvs system. Lvs is encoded by the *sacB* gene and expressed from a strong sucrose-inducible promoter, which is located within the *sacR* regulatory region (Gay *et al.*, 1983). The Lvs system is subject to a complicated regulation which has been used to obtain expression of recombinant proteins (see below).

2. Secretion using the Tat system

Besides the Sec-pathway, *B. subtilis* codes for a functional Tat pathway, and two substrate proteins have been described so far to depend on this pathway for their secretion, the phosphodiesterase PhoD (Jongbloed *et al.*, 2000) and YwbN (Jongbloed *et al.*, 2004). In contrast to the Sec pathway, the Tat pathway is capable of transporting fully folded proteins across membranes (Palmer *et al.*, 2005; Robinson and Bolhuis, 2001). The name of this pathway has been derived from the fact that it translocates proteins carrying twin-arginine (RR)-signal peptides. In *E. coli*, the key components of

the Tat pathway are the integral membrane proteins TatA, TatB, and TatC. The general view is that TatB and TatC serve in RR-signal peptide recognition, while TatB and TatC in complex with multiple TatA components form a protein-conducting channel (Alami *et al.*, 2003). *B. subtilis* contains two TatC proteins (TatCd and TatCy) and three TatA/TatB-like proteins (TatAd, TatAy, and TatAc) (Jongbloed *et al.*, 2000). It has been shown that the Tat machinery consists of at least two minimal Tat translocases, each composed of one specific TatA and one specific TatC component. While the TatAcCd translocase recognizes the twin-arginine preprotein PhoD, the TatAyCy deals with the YwbN preprotein (Jongbloed *et al.*, 2000, 2004).

The Tat pathway offers the possibility to translocate folded recombinant proteins into the growth medium. In principle, fusing a RR-containing signal peptide to a mature part of a protein should be sufficient to secrete any folded protein into the medium. This has been shown for the tightly folded green fluorescent protein (GFP) in *E. coli*, where the twin-arginine signal peptide of TMAO reductase (TorA) is necessary and sufficient to translocate this eukaryotic protein into the periplasm in its fully active form (Thomas *et al.*, 2001). When GFP was fused to a Sec-dependent signal peptide, it was translocated into the periplasm, too, but remained largely inactive, indicating that GFP has to fold in the cytoplasm. For so far unknown reasons, this did not work with the two identified RR-signal peptides of *B. subtilis*. We fused the RR-signal peptide of YwbN to GFP and observed secretion into the medium, but this occurred also in a *tatAy tatCy* double mutant (R. Puff and W. Schumann, unpublished results). Further experiments have to be carried out to develop the Tat pathway into a system applicable for recombinant proteins. An alternative could be the SRP pathway provided the recombinant proteins can fold on the outside of the cells (see below).

3. Secretion using the SRP system

The signal recognition particle (SRP) delivers membrane proteins and secretory proteins to the Sec translocation channel in the cytoplasmic membrane. SRP is a protein–RNA complex present in all organisms but with different composition (Keenan *et al.*, 2001). In *E. coli*, the SRP consists of a 4.5S RNA and one protein called Fifty-four homologue (Ffh) where its M-domain (methionine rich) is involved in RNA interaction and signal peptide binding. The protein FtsY associates with the plasma membrane and acts as a receptor protein for the SRP complex. Signal sequences of target proteins are specifically recognized by the SRP as they emerge from the ribosome. Signal peptides contain a 9- to 12-residue-long hydrophobic stretch in the middle that adopts an α-helical conformation, and recognition of this hydrophobic stretch occurs through the M-domain of Ffh. This complex subsequently interacts with the membrane-bound receptor FtsY,

which assists in the delivery of the complex to the SecYEG translocation channel in the membrane. It has been published that binding of the signal peptide of AmyQ (α-amylase) to the *B. subtilis* SRP can be enhanced by increasing its hydrophobicity (Zanen *et al.*, 2005). Thus, the *B. subtilis* SRP system is also able to discriminate between signal peptides with relatively high hydrophobicities.

This finding should have significant practical applications. Since many presecretory proteins are not efficiently translocated in *E. coli*, this failure may be due to premature folding into a translocation-incompetent conformation. It should be possible to target such proteins to the inner membrane at an early stage of their synthesis via the SRP pathway, thereby overcome the problem of misfolding in the cytoplasm by simply increasing the hydrophobicity of their signal peptides.

C. Vectors allowing the addition of tags to recombinant proteins

Different tags have been described which can be covalently added to recombinant proteins, and these serve different purposes. While purification tags allow a single-step purification of recombinant proteins by affinity chromatography, epitope tags provide the proteins with an extension which is recognized by commercially available antibodies; localization tags allow the allocation of proteins to specific cellular compartments. The most important tags are the purification tags, and protein and peptide affinity tags have become highly popular tools for the purification of recombinant proteins and native protein complexes (Terpe, 2003). These tags can provide 100- or even 1000-fold purification from crude extracts without prior steps to remove nucleic acids or other cellular material. In addition, the relatively mild elution conditions employed make purification tags useful for purifying individual proteins and protein complexes. The available protein and affinity tags can be categorized into three classes depending on the nature of the affinity tag and its target. The first class of tags uses peptide or protein fusion that binds to small molecule ligands linked to a solid support. Examples are the hexahistidine tag that binds to immobilized metal (Hochuli *et al.*, 1987), while glutathione *S*-transferase protein fusions bind to glutathione attached to chromatography resins (Smith and Johnson, 1988). In the second class of affinity tags, a peptide tag binds to a protein-binding partner immobilized on chromatography resin. One example is the calmodulin-binding peptide that specifically binds to calmodulin, allowing proteins fused to the peptide to be purified using calmodulin resin (Stofko-Hahn *et al.*, 1992). The third class of epitope affinity tag uses as a binding partner an antibody attached to the resin. Examples are the FLAG

and the cMyc tag, which can be used with anti-FLAG or anti-cMyc antibodies, respectively (Brizzard *et al.*, 1994; Evan *et al.*, 1985).

The first tagging vectors described for *B. subtilis* are pUSH1 and pUSH2 (Schön and Schumann, 1994). Both vectors are based on the *E. coli–B. subtilis* shuttle vector p602/22 carrying the IPTG-inducible promoter P_{N25} (LeGrice, 1990). While pUSH1 allows addition of six histidine residues to the N-terminus of proteins, pUSH2 provides six histidine residues for the C-terminus. Another publication reports on integrative vectors allowing the addition of three different epitope tags, namely those coding for FLAG, hemagglutinin, and cMyc, and three localization tags (GFP, YFP, and CFP) (Kaltwasser *et al.*, 2001). The coding region for these three tags replaced the *lacZ* gene in the pMUTIN2 vector (Vagner *et al.*, 1998). IPTG-inducible vectors have been described providing recombinant proteins with three different tags: His, Strep, and cMyc (Nguyen *et al.*, 2007).

D. DNA elements improving the production of recombinant proteins

The average functional half-live of bacterial mRNAs is around 2 min, but a few transcripts exhibit increased stability up to 30 min. This enhanced half-life is a consequence of either 5′ or 3′ stabilizing elements or of SD sequences.

1. 5′ Stabilizing elements

Two 5′ stabilizer elements have been analyzed in *B. subtilis*, the leader regions of *ermC* and *aprE*. The *erm* family of genes specifies rRNA methylases that confer resistance to MLS antibiotics by reducing the affinity between these antibiotics and ribosomes. Expression of the methylases is induced, for example, by nanomolar concentrations of the macrolide antibiotic erythromycin, and the induction mechanism has been studied in detail with the *ermC* gene, which is carried on the plasmid pE194. The transcript codes for two different proteins, a 19-amino acid leader peptide and the methylase, and both reading frames are preceded by SD sequences. In the absence of erythromycin, the *ermC* mRNA leader sequence is folded in a stable stem-loop structure such that the SD sequence preceding the methylase-coding sequence is unavailable for translation. In the presence of a subinhibitory erythromycin concentration, an antibiotic-bound ribosome stalls while translating the leader peptide, thereby opening the stem-loop to allow ribosome binding at the second SD sequence, resulting in a 20-fold increase in methylase translation. Furthermore, this induction is accompanied by a 15- to 20-fold increase in *ermC* mRNA stability (40-min half-life) (Bechhofer and Dubnau, 1987). It has been suggested that the stalled ribosome

physically protects the mRNA from endonucleolytic cleavage in the leader peptide sequence and it was later shown that the *ermC* transcript is degraded by a ribonucleolytic activity starting at the 5′ end (Bechhofer and Zen, 1989). When the *ermC* leader region was transcriptionally fused to the *cat-86* reporter gene, the hybrid transcript was stabilized both physically and functionally by erythromycin (Sandler and Weisblum, 1989).

When *B. subtilis* cells enter the stationary phase, several new genetic programs are switched on, including one for the production of several extracellular enzymes (Ferrari *et al.*, 1993). One of these enzymes is the protease subtilisin encoded by the *aprE* gene, whose transcript is extremely stable in stationary phase cells, with a half-life of at least 25 min (Resnekov *et al.*, 1990). The 5′ stabilizing element has been identified to include the untranslated leader sequence of the *aprE* mRNA (Hambraeus *et al.*, 2000). A closer inspection of this leader sequence revealed that a 5′ stem-loop and binding of ribosomes are necessary for the stability of the *aprE* leader mRNA (Hambraeus *et al.*, 2002). The stabilizing function of this leader sequence could be transferred to an *aprE-lacZ* fusion mRNA (Hambraeus *et al.*, 2000). A genome-wide survey of mRNA half-lives has identified additional transcripts with extreme stability, suggesting the existence of additional 5′ stabilizers (Hambraeus *et al.*, 2003).

2. 3′ Stabilizing elements

Only one 3′ stabilizing element has been described so far, which is located at the 3′ end of the *cryIIIA* toxin gene of *B. thuringiensis* (Wong and Chang, 1986). This type of regulation by a 3′ stabilizer has been termed retroregulation (Schindler and Echols, 1981). When the *cryIIIA* retroregulator was fused to the distal ends of either penicillinase (*penP*) gene of *B. licheniformis* or the human interleukin-2 cDNA, the half-lives of the mRNAs derived from the fusion genes were increased from 2 to 6 min in both *E. coli* and *B. subtilis* (Wong and Chang, 1986). In contrast, when this sequence was fused to the 3′ end of the *lacZ* reporter gene, it resulted only in a minor increase in the β-galactosidase activity, indicating that *lacZ* mRNA stability was not a limiting step under the conditions tested (Jan *et al.*, 2001).

3. Translation signals

Three elements of the translation initiation region influence the amount of gene product synthesized: the SD sequence, the initiation codon, and the spacer region between these two elements. The SD sequence can exert a dual function by influencing the rate of ribosome assembly and stabilizing the transcript. In contrast to *E. coli*, several mRNAs of *B. subtilis* such as that of phage SP82 RNA (Hue *et al.*, 1995) and the *gsiB* transcript

(Jürgen *et al.*, 1998) and *B. thuringiensis cryIIIA* toxin RNA (termed STAB-SD) (Agaisse and Lereclus, 1996) are stabilized by either binding or stalling of ribosomes near the 5′ end. In all three cases, canonical SD sequences function as stabilizers of the downstream sequences, and ribosome binding might protect the 5′ ends of the transcripts from degradation by nucleases. The phage SP82 stabilizer conferred increased stability to several heterologous mRNAs when inserted at their 5′ ends (Hue *et al.*, 1995). It has been suggested that ribosome binding at or near the 5′ end of the mRNA interferes with a 5′ end-dependent activity such as RNase J1 or/and J2 (Even *et al.*, 2005).

Inspection of the *B. subtilis* genome indicated that the frequency of utilization of AUG, UUG, and GUG as initiation codons is 78%, 13%, and 9%, respectively. After changing the initiation codon of the *aprE* gene GUG to AUG, no significant increase in the amount of the reporter enzyme could be measured (Jan *et al.*, 2001). The same group also investigated the influence of the SD sequence on the yield of the protein synthesis. It has been reported that SD sequences closer to the *B. subtilis* AAGGAGG consensus increased the translation efficiency (Band and Henner, 1984). Again, this effect could not be confirmed with a modified *aprE* SD sequence (Jan *et al.*, 2001). The significance of the spacer region has not yet been analyzed.

IV. TRANSFORMATION SYSTEMS

Several different methods have been published allowing the introduction of genes into *B. subtilis* cells. The first to be described is based on the ability to take up exogenous DNA and incorporate it into the genome. This system is based on natural competence (see Chen and Dubnau, 2004 for review). The low competence exhibited by some *Bacillus* strains led to the development of other strategies, involving protoplast transformation (Trieu-Cuot *et al.*, 1987). The details of most of these methods, together with their advantages and disadvantages, will be described.

A. Natural competence

Many strains of *B. subtilis* are naturally capable of taking up DNA fragments under certain physiological conditions (Chen and Dubnau, 2004). This physiological state is referred to as competence and occurs within utmost 20% of the cells in a culture shortly before the cessation of exponential growth. Competent cells will take up both linear and circular DNA, but the latter will be linearized during uptake (Contente and Dubnau, 1979a). Only one strand of the DNA molecule is efficiently transported into the cytoplasm, while the other strand is degraded into

nucleotides, which are released into the extracellular environment. The incoming ssDNA can be integrated into the bacterial chromosome by a RecA-dependent process that requires sequence homology between the incoming DNA and the bacterial chromosome. Competent cells can be readily generated by growing *B. subtilis* cells in Spizizzen minimal medium (Spizizen, 1958), and competent cells can be frozen at −80 °C (in 15% glycerol) and stored for several months prior to use.

One disadvantage of competent *B. subtilis* cells concerns the low efficiencies obtained in shotgun cloning experiments, which is manifested in low numbers of clones. The reason for this is that monomeric plasmid DNA is virtually inactive in transforming *B. subtilis* and that stable plasmid-containing transformants only result when the donor plasmid is a trimer or higher multimeric form (Canosi *et al.*, 1978). No significant transformation resulted from introduction of monomers or dimers (Mottes *et al.*, 1979). Several strategies have been described to circumvent the problem of the poor recovery of plasmid transformants with monomers, or following ligation. One of them is called rescue of donor plasmid markers by a homologous resident plasmid. Here, the recipient strain carries a homologous plasmid and the recombinant plasmid to be introduced in that strain is linearized within the homologous moiety. After uptake, the incoming linear DNA will recombine with the resident plasmid in a RecA-dependent reaction (Contente and Dubnau, 1979b; Gryczan *et al.*, 1980; Haima *et al.*, 1990). This recombination reaction is precisely analogous to classical transformation using chromosomal markers that are rescued by recombination with the resident chromosome. Another strategy is to use plasmid vectors with internal repeats 260- to 2000-bp long (Michel *et al.*, 1982). Monomers of such plasmids transformed competent cells, and the efficiency of transformation varies with the square of the length of repeats. A third strategy is to use bifunctional (shuttle) vectors that replicate both in *E. coli* and in *B. subtilis*, and this strategy is used in most cases. All cloning steps are carried out in *E. coli*, and the final recombinant plasmid is then used to transform the appropriate *B. subtilis* cells.

B. Protoplasts

Protoplasts are derived from vegetative cells by removing most of the cell wall, and this is accomplished by treatment of the cells with lysozyme. With rod-shaped cells like those of *B. subtilis*, protoplast formation can be followed under the light microscope due to the morphological change into spherical cells. Protoplasts of *B. subtilis* are prepared by lysozyme treatment of cells in media supplemented with osmotic shock stabilizers such a sodium chloride or sucrose. It has been demonstrated that protoplasts of *B. subtilis* cells can be transformed with plasmids in the presence

of polyethylene glycol (Chang and Cohen, 1979). After addition of DNA, protoplasts are resuspended into a regeneration medium which is nutritionally complex. The transformation efficiency of protoplasts by plasmids is much higher than is obtained using competent cells, with an efficiency of 4×10^7 transformants per microgram of supercoiled DNA. The reason for this is that monomeric double-stranded plasmids are taken up during the transformation procedure. It should be mentioned that protoplasts do not survive freezing (Haima *et al.*, 1988).

C. Electrotransformation

Electrotransformation (also called electroporation) is a simple and widely used technique for the transformation of various bacterial species (and eukaryotic cells). This technique uses an electric pulse treatment of cells to induce a membrane potential causing the temporary breakdown of the cell membrane permeation barrier, to allow the entry of DNA into the cell (Tsong, 1992). The optimum electric field strength varies among bacterial species and is usually lower for Gram-positive than for Gram-negative species, since Gram-positive bacterial species are more sensitive to a high field strength treatment (Dower *et al.*, 1992). In general, transformation efficiencies obtained by electroporation of Gram-positive bacteria are relatively low, and improvement of cell survivability will lead to an increase in the efficiency of transformation. Osmoticums, such as sucrose, sorbitol, and glycerol, are often used at around iso-osmotic concentrations as a medium for suspending electrocompetent cells. Addition of sorbitol and mannitol, in the electroporation, growth and recovery media resulted in $\sim 1.4 \times 10^6$ transformants per microgram of DNA (Xue *et al.*, 1999). When plasmid DNAs in the range of 2.9–12.6 kb were electroporated into a *B. subtilis* strain, the transformation efficiency decreased with increasing size of the DNA. But even with the 12.6-kb plasmid, about 2.0×10^3 transformants per microgram of DNA were routinely obtained. A method has been described allowing the electrotransformation of recalcitrant undomesticated strains of *B. subtilis* (Romero *et al.*, 2006).

D. Mobilization from *E. coli* to *B. subtilis*

A vector strategy has been designed that allows transfer by conjugation of recombinant plasmids from *E. coli* to various Gram-positive bacteria, including *B. subtilis* (Trieu-Cuot *et al.*, 1987). The prototype vector, pAT187, consists of (1) the origins of replication of pBR322 and pAMβ1, (2) a kanamycin resistance gene known to be expressed in *E. coli* and *B. subtilis*, and (3) the origin of transfer of the IncP plasmid RK2. pAT187 can be successfully mobilized by filter matings from the *E. coli* strain SM10, carrying RK2 integrated into the chromosome (Simon *et al.*, 1983) to *B. subtilis* cells at

frequencies of about 3×10^{-7}. Another mobilizable vector, pTCV-*lac*, carries the origin of replication of pACYC184 instead of that of pBR322 and a promoter-less *lacZ* gene, allowing construction and analysis of transcriptional fusions (Poyart and Trieu-Cuot, 1997).

V. CHROMOSOMAL MUTATIONS ENHANCING PRODUCTION OF NATIVE INTRA- AND EXTRACELLULAR PROTEINS

A. Molecular chaperones

To produce recombinant proteins intracellularly in a soluble form, several approaches have been developed including the coproduction of molecular chaperones that mediate protein folding, assembly, and secretion. *B. subtilis*, as all bacterial species, codes for two series of general molecular chaperones, the GroE and DnaK machines. The structural genes for these chaperones are organized in two operons, the heptacistronic *dnaK* (*hrcA-grpE-dnaK-dnaJ-orf35-orf28-orf50*) and the *groE* (*groES-groEL*) operon (Homuth *et al.*, 1997; Schmidt *et al.*, 1992). Studies carried out with *E. coli* indicate that these two chaperone machines can act either independently or synergistically in a successive manner to facilitate the proper folding and assembly of recombinant proteins (Fenton and Horwich, 1997; Gragerov *et al.*, 1992; Langer *et al.*, 1992; Nishihara *et al.*, 1998). In *B. subtilis*, the *dnaK* and the *groE* operons are regulated by a common transcriptional repressor, HrcA, the activity of which is modulated by GroE (Mogk *et al.*, 1997; Reischl *et al.*, 2002). Inactivation of *hrcA* results in the constitutive production of the intracellular chaperones from these two operons (Schulz and Schumann, 1996; Yuan and Wong, 1995b). Besides these two intracellular chaperones, the extracellular chaperone PrsA has been shown to increase the production of α-amylase and proteases (Kontinen and Sarvas, 1993). This lipoprotein is believed to mediate protein folding at the late stage of secretion (see below).

The successful secretory production of a biologically active antidigoxin SCA fragment has been reported at a concentration of around 5 mg/liter in a shake flask culture (Wu *et al.*, 1993). Analysis of the distribution of this protein revealed that the secreted fraction represented only 23% of the total SCA fragments produced by the cell. The majority of the recombinant SCA protein (60%) remained insoluble inside the cell. A strain in which the two major intracellular chaperones DnaK and GroE are constitutively expressed reduced the formation of insoluble SCA by 45% and enhanced the secretory production yield by 60% (Wu *et al.*, 1998). If in addition the level of the extracellular chaperone PrsA was increased, a further 2.5-fold increase in the secretory production yield was obtained.

In another approach, inactivation of eight genes coding for extracellular proteases, together with a constitutive high level of the two chaperones, resulted in the secretory production of a fibrin-specific monoclonal antibody at a level of 10–15 mg/liter (Wu *et al.*, 2002b). In a third approach, it could be shown that constitutive expression of the DnaK and GroE chaperones significantly increased the amount of the membrane-attached penicillin-binding protein Pbp4* (encoded by *pbpE*) when overproduced (Phan *et al.*, 2006).

B. Cellular factors affecting extracytoplasmic protein folding and degradation

Three different cellular factors have been described so far influencing the correct and efficient folding of extracellular proteins after successful translocation through the cytoplasmic membrane: the lipoprotein PrsA, the net charge of the cell wall, and thiol-disulfide oxidoreductases. Furthermore, three classes of extracellular proteases can attack recombinant proteins on the *trans* side of the membrane (Sarvas *et al.*, 2004).

1. PrsA

After translocation in their unfolded state, Sec- and SRP-dependent proteins have to fold into their native three-dimensional conformation. While polypeptide chains can fold spontaneously *in vitro*, their folding *in vivo* is frequently assisted by folding catalysts. An important folding catalyst is the lipoprotein PrsA exhibiting homology to peptidyl-prolyl *cis/trans*-isomerases, which is important for protein secretion (Kontinen and Sarvas, 1993; Kontinen *et al.*, 1991). *B. subtilis* strains carrying mutant forms of PrsA show impaired secretion of degradative enzymes (Jacobs *et al.*, 1993; Kontinen and Sarvas, 1993). It has been proposed that PrsA is required to prevent unproductive interactions of unfolded secretory proteins with the cell wall immediately after translocation (Wahlstrom *et al.*, 2003). When the *prsA* gene was expressed from an IPTG-inducible promoter, depletion of PrsA resulted in significant reduction of 32 extracellular proteins (Tjalsma *et al.*, 2004). On the contrary, several model proteins are secreted at enhanced levels when PrsA is overproduced indicating that posttranslational folding is a rate-limiting step in protein secretion (Kontinen and Sarvas, 1993; Vitikainen *et al.*, 2001).

2. The net charge of the cell wall

Besides the cell membrane, the matrix of the cell wall is another factor that affects protein secretion. This matrix consists of a complex heteropolymer of peptidoglycan and covalently linked anionic polymers of teichoic acid or teichuronic acid (Archibald *et al.*, 1993). These anionic polymers, together with membrane-bound lipoteichoic acids, confer a high density

of negative charge to the cell wall. The net negative charge of the wall depends on the degree of D-alanine esterification of teichoic and lipoteichoic acids. Proteins encoded by the *dlt* operon are responsible for the D-alanylation (Perego *et al.*, 1995). In *B. subtilis*, the inactivation of *dlt* and the concomitant increased density of negative charge were found to result in stabilization and enhanced secretion of some mutant proteins (Hyyrylainen *et al.*, 2000). In addition, secretion of pneumolysin was increased 1.5-fold (Vitikainen *et al.*, 2005).

3. Disulfide bond formation

Disulfide bond formation is crucial for the folding and stability of many secreted proteins. Failure to form proper disulfide bonds can lead to protein aggregation and degradation by proteases. Disulfide bonds can be formed spontaneously by molecular oxygen, but this type of random air oxidation reaction is very slow and cannot account for the rapid rates of disulfide bond formation needed by the cell. To accelerate disulfide bond formation, thiol-disulfide oxidoreductases and their cognate quinone oxidoreductases act as folding catalysts (Nakamoto and Bardwell, 2004; Ritz and Beckwith, 2001). In *B. subtilis*, two putative thiol-disulfide oxidoreductases encoded by the genes *bdbA* and *bdbD* and two putative quinone oxidoreductases encoded by the genes *bdbB* and *bdbC* have been identified (Bolhuis *et al.*, 1999b; Dorenbos *et al.*, 2002; Meima *et al.*, 2002). While computer predictions proposed that BdbA and BdbD contain an N-terminal membrane anchor (Tjalsma and Van Dijl, 2005), BdbB and BdbC are integral membrane proteins with four transmembrane segments each (Bolhuis *et al.*, 1999b; Meima *et al.*, 2002). It has been suggested that BdbC and BdbD form a functional pair in which BdbD is a thiol-disulfide oxidoreductase involved in oxidizing the substrate proteins, whereas the BdbC quinone oxidoreductase reoxidizes BdbD (Meima *et al.*, 2002). It has been shown that both enzymes are needed for the posttranslational folding of the pseudopilin ComGC, a critical component in the DNA uptake machinery (Meima *et al.*, 2002), and of the *E. coli* PhoA alkaline phosphatase, when this protein is produced and secreted by *B. subtilis* (Bolhuis *et al.*, 1999b). Both proteins contain intramolecular disulfide bonds that are essential for their activity and stability (Chung *et al.*, 1998; Sone *et al.*, 1997). Furthermore, the peptide antibiotic sublancin 168 contains two disulfide bonds catalyzed by BdbB (Dorenbos *et al.*, 2002).

4. Extracellular proteases

Extracellular proteins unable to fold correctly are prone to degradation by proteases. But even correctly folded heterologous proteins can be degraded by extracellular proteases, which can be found in three different compartments: (1) soluble and localized between the cytoplasmic membrane and the cell wall and within the growth medium, (2) anchored on

the cell wall, and (3) anchored within the cytoplasmic membrane and facing its *trans* side.

Structural genes encoding a total of seven soluble proteases have been cloned and characterized. This started with the characterization of the two major soluble proteases, AprE and NprE, coding for alkaline (subtilisin) and neutral protease, respectively, and the construction of a double-knockout termed DB102 (Kawamura and Doi, 1984). Afterward, the protease-deficient strain WB600 has been constructed with four additional extracellular soluble protease genes inactivated (Wu *et al.*, 1991). These genes code for the extracellular protease (*epr*), the metalloprotease (*mpr*), the bacillopeptidase F (*bpr*), and the neutral protease B (*nprB*) in addition to the two already mentioned proteases. In a further strain, WB800, the genes coding for an additional minor soluble protease (*vpr*) and the cell wall-bound WprA protease (see below) have been deleted (Wu *et al.*, 2002b).

To date, only one cell wall-bound protease, CWBP52, encoded by the *wprA* gene has been described (Margot and Karamata, 1996). Since this serine protease was shown to be involved in the degradation of the *B. licheniformis* α-amylase (Stephenson and Harwood, 1998), it can be assumed that it will attack additional exoproteins. Therefore, a *wprA* knockout has been added to the sevenfold knockout mutant strain for soluble extracellular proteases in strain WB800 as mentioned above (Wu *et al.*, 2002b).

Several proteases have been described to be anchored in the cytoplasmic membrane, where the active site of class I faces the cytoplasm, whereas that of class II is exposed on the *trans* side of the membrane. In principle, both classes can attack and degrade precursor proteins during and after their translocation. Of special interest are two proteases of class II, the expression of which is upregulated after secretion stress. These two proteases are designated HtrA and HtrB, where HtrA has a dual localization. Besides being membrane-associated, some HtrA has been detected in the growth medium, and the physiological relevance of its presence in the medium remains to be shown (Antelmann *et al.*, 2003). Secretion stress is not only provoked by the high-level production of α-amylases (Darmon *et al.*, 2002; Hyyrylainen *et al.*, 2001) but also by mutations in *htrA* or *htrB*, or by exposure of *B. subtilis* to heat suggesting that unfolded proteins represent, directly or indirectly, the stimuli for the *Bacillus* secretion stress response (Hyyrylainen *et al.*, 2005; Noone *et al.*, 2001). Secretion stress is sensed by the CssS–CssR two-component signal transduction system, where the membrane sensor kinase CssS senses stimuli at the membrane–cell wall interface (Hyyrylainen *et al.*, 2001). This information is then transmitted to CssR via phosphorylation. Four genes have been identified as members of the CssRS regulon: *htrA*, *htrB*, *cssR*, and *cssS*, where the latter two form one operon (Darmon *et al.*, 2002;

Hyyrylainen *et al.*, 2001, 2005). In particular, the transcript levels of both *htrA* and *htrB* are significantly enhanced in secretion-stressed cells.

C. Chromosomal mutations enhancing the production of recombinant proteins

Two different chromosomal mutations have been described enhancing the production of recombinant proteins. While one (depletion of the essential protein FtsZ) can be combined with any expression system, the second (*sacU*^{h}) influences expression of a subset of σ^{A}-dependent promoters.

1. The *sacU*^{h} mutation

The *degS–degU* genes of *B. subtilis* code for a two-component signal transduction system, which regulates many cellular processes, including exoenzyme production and competence development (Msadek *et al.*, 1993). While *degS* codes for the sensor kinase, *degU* encodes the response regulator, and phosphorylated DegU triggers transcription of genes coding for degradative enzymes such as the major alkaline protease subtilisin (*aprE*) and Lvs (*sacB*). Certain missense mutations within the *degU* gene, designated *degU*(Hy), that increase the half-life of the phosphorylated from of DegU (DegU∼P) result in the overproduction of secreted enzymes. One of the best characterized mutations is *degU32*(Hy) (Henner *et al.*, 1988). The regulatory region of the *sacB* gene was fused to the *xylE* gene of *Pseudomonas putida* coding for intracellular catechol 2,3-dioxygenase and expressed in a *degU*(Hy) mutant. The recombinant protein represented about 25% of total cellular protein (Zukowski and Miller, 1986). In another experimental approach, endoglucanase A of *C. thermocellum* was secreted in high amounts, again using the *sacB* regulatory region in a *degU*(Hy) background (Joliff *et al.*, 1985).

2. The *ftsZ* mutation

The *ftsZ* gene codes for an essential cell division protein in *B. subtilis* (Beall and Lutkenhaus, 1991, 1992). Its gene product, the FtsZ protein, forms the so-called Z ring at the cell division site, and the Z ring directs subsequent cell division (Addinall *et al.*, 1996; Bi and Lutkenhaus, 1991). FtsZ participates in both vegetative cell division and asymmetric cell division during sporulation. To initiate sporulation in *B. subtilis*, FtsZ switches its localization from medial to polar sites in the dividing cell by the regulation of *minCD* (Bi and Lutkenhaus, 1990; Lee and Price, 1993). When *ftsZ* was placed under the *spac* promoter, cell growth of this mutant and its *β*-galactosidase activity under the *aprE* promoter in the presence of 1-mM IPTG were comparable to the wild-type cells (Park *et al.*, 2005).

When growth occurred with 0.2-mM IPTG, an about 13-fold higher β-galactosidase activity was measured compared to the wild-type strain. While the intracellular level of subtilisin (encoded by *aprE*) was enhanced about 16-fold under these conditions, its extracellular level was only 3 times higher, suggesting one or more bottlenecks during secretion (Park *et al.*, 2005). These results suggest that reducing the amount of FtsZ should be applicable to obtain enhanced production of recombinant proteins when the transgene is fused to the *aprE* promoter.

VI. PRODUCTION OF RECOMBINANT PROTEINS IN *B. SUBTILIS* AND OTHER *BACILLI*

Besides *B. subtilis*, two other *Bacillus* species are used: *B. brevis* and *B. megaterium*.

A. *B. subtilis*

A wide variety of recombinant proteins have been produced in *B. subtilis* cells, both from prokaryotic and from eukaryotic origin (Table 6.7). Expression was achieved either intracellularly or extracellularly using the promoters and signal sequences described above. The first description of using *B. subtilis* cells for the expression of recombinant proteins occurred by Hardy *et al*. (1981) who expressed the hepatitis B core antigen and the major antigen of foot and mouth disease virus intracellularly. The group of Palva developed the first vectors allowing secretion of foreign proteins into the growth medium (Palva *et al.*, 1982). They fused β-lactamase to the signal sequence of an α-amylase derived from *B. amyloliquefaciens*. Based on these secretion vectors, mature human interferon-α2 (IFN-α2) was obtained at 0.5–1 mg/liter (Palva *et al.*, 1983) and pneumolysin of *Streptococcus pneumoniae* at 10 mg/liter of culture medium (Taira *et al.*, 1989). A complete overview of all recombinant proteins produced in *B. subtilis* till 1993 can be found in Simonen and Palva (1993). At best, about 340 mg/liter of recombinant protein have been obtained, which is far away from the 20–30 g/liter reported for homologous proteins. Using the pAMβ1-based expression vector pRBT, up to 200 μg/ml culture *C. perfringens* β-toxoid, corresponding to 25% of total intracellular protein, was obtained (Nijland *et al.*, 2007).

B. *B. brevis*

B. brevis is known to be a harmless inhabitant of soil, milk, and cheese. The major advantage of *B. brevis* over *B. subtilis* is a very low level of extracellular protease activity resulting in stable recombinant proteins secreted into the growth medium. Two different strains are used for the

production of recombinant proteins, *B. brevis* 47 and HPD31. Both strains have been isolated from the soil as protein-hyperproducing cells (up to 30 g/liter of extracellular protein) with little extracellular protease activity (Takagi *et al.*, 1989a; Udaka, 1976). *B. brevis* 31-OK is a mutant derived spontaneously from HPD31, which exhibits little degradation of secreted human growth hormone (Sagiya *et al.*, 1994). The cell wall of *B. brevis* consists of two protein layers, termed the outer and middle wall layer, and a thin peptidoglycan layer. The outer and middle wall layers are each composed of a single protein, outer wall protein (OWP) and middle wall protein (MWP), respectively. During early stationary phase of growth, the protein layers begin shedding concomitantly with a prominent increase in protein secretion (Gruber *et al.*, 1988; Yamagata *et al.*, 1987). During the stationary growth phase, cells continue to synthesize and secrete the cell wall proteins, which do not stay on the cell surface but, instead, accumulate in the medium as extracellular protein up to the 20 g/liter of culture. Based on these unique characteristics of *B. brevis*, host–vector systems have been developed. These consist of the powerful promoter region preceding the operon coding for the two cell wall proteins and contain multiple promoters and the signal peptide-coding sequence (Adachi *et al.*, 1989; Yamagata *et al.*, 1987). One of these expression–secretion vectors, pNU210, allows the production and secretion of recombinant proteins UDAKA (Udaka and Yamagata, 1993). Recombinant proteins produced with *B. brevis* are summarized in Table 6.6.

C. *B. megaterium*

The *B. megaterium* has several advantages over the other recombinant protein production hosts. It does not secrete alkaline proteases and is known for the stable replication and maintenance of plasmids. The strain DSM 319 was improved by deletion of the gene coding for the major extracellular protease NrpM (Wittchen and Meinhardt, 1995). Furthermore, this bacterial species secretes several proteins of commercial interest into the growth medium, including amylases, steroid hydrolases, and penicillin amidase (Vary, 1994). *B. megaterium* has been used to secrete *Leuconostoc mesenteroides* dextransucrase DsrS (Malten *et al.*, 2005a). When the *dsrS* gene was integrated into the chromosome, expression of *sipM*, encoding the signal peptidase responsible for removal of the signal peptide from the dextransucrase precursor protein from a multicopy plasmid, increased the amount of secreted DsrS 3.7-fold (Malten *et al.*, 2005b). Equipped with the esterase LipA signal peptide, up to 1 mg/liter of a His- or Strep-tagged *Lactobacillus reuteri* Lvs was obtained (Malten *et al.*, 2006). Very recently, plasmid systems have been constructed allowing the N- or C-terminal fusion of either the Strep II or the His6 tag to the recombinant protein, in which the tag and the mature protein are

TABLE 6.6 Recombinant proteins produced with *B. brevis*

Species	Protein	Concentration	References
Bacillus licheniformis	α-Amylase	0.7–3.0 g/liter	Yamagata *et al*., 1987
Bacillus stearothermophilus	α-Amylase	ni[a]	Takagi *et al*., 1989b
Human	Epidermal growth factor	240 mg/liter	Yamagata *et al*., 1989
Human	Epidermal growth factor	>1 g/liter	Ebisu *et al*., 1996
Swine	Pepsinogen	11 mg/liter	Takao *et al*., 1989
Human	Salivary α-amylase	60 mg/liter	Konishi *et al*., 1990
Clostridium thermosulfurogens	β-Amylase	0.3–1.6 g/liter	Mizukami *et al*., 1992
Vibrio cholarae	Cholera toxin B-subunit	1.4 g/liter	Ichikawa *et al*., 1993
Fish	Tuna growth hormone	240 mg/liter	Sagiya *et al*., 1994
Human	Single-chain insulin	12.3 mg/liter	Koh *et al*., 2000
Single-chain variable fragment (scFv)		10 mg/liter	Shiroza *et al*., 2001
Human	Interleukin-2	0.12 g/liter	Takimura *et al*., 1997
scFv		10 mg/liter	Shiroza *et al*., 2003

[a] ni, not indicated.

separated by cleavage sites recognized by *Tobacco etch virus* or factor Xa proteases allowing removal of the tag. Up to 9 mg/liter of GFP could be purified from shake flask cultures (Biedendieck *et al.*, 2007). Recently, a self-disruptive *B. megaterium* strain was constructed (Hori *et al.*, 2002). A gene cassette carrying the lysis system of a *B. licheniformis* phage, the genes coding for holin and endolysin, was fused to a xylose-inducible promoter that is subject to glucose repression. Cells were grown in the presence of glucose and xylose, and when the glucose concentration approached zero, self-disruption was spontaneously induced, thereby releasing the recombinant product (Hori *et al.*, 2002).

VII. CONCLUSIONS

B. subtilis is being used both for intra- and extracellular production of recombinant proteins since 1981 (Hardy *et al.*, 1981), and a wealth of information has been accumulated since the first report. The two major problems, structural instability of one class of vector plasmids and extracellular proteases, have significantly been improved through the use of plasmids largely devoid of instability and through the construction of an eightfold proteases knockout strain (Wu *et al.*, 2002b). Can *B. subtilis* compete with *E. coli* in the production of recombinant proteins? What are the advantages and disadvantages? The major advantage of *E. coli* is that more scientists are used to work with this bacterial species rather than with *B. subtilis*. The major disadvantage of *E. coli* is the presence of LPS especially in those cases where recombinant proteins are produced for human therapeutic use. Efficient expression systems are available for both species, allowing high levels of intracellular protein production. Recently, we succeeded in creating an expression system where up to 43% of the total cellular protein consists of the recombinant protein (D. H. Nguyen, T. T. P. Phan, and W. Schumann, to be published). A still open question is whether one of the two species is less prone to the production of inclusion bodies. Formation of protein aggregates can be, at least partially, prevented in *B. subtilis* by the constitutive high-level production of the major chaperone families, DnaK and GroEL (Wu *et al.*, 1998). This can be accomplished by simply deleting a repressor gene, which controls both operons. In *E. coli*, both operons are under positive control together with many other genes including those coding for ATP-dependent proteases (Yura *et al.*, 2000). Therefore, overproduction of both chaperone systems requires their cloning and fusion to an inducible promoter (Nishihara *et al.*, 1998). To come to a final conclusion, two or three different proteins forming inclusion bodies in *E. coli* have to be tested in *B. subtilis*.

The major advantage of *B. subtilis* over *E. coli* is its ability to secrete proteins directly into the growth medium. With this bacterium, up to

TABLE 6.7 Recombinant proteins produced with *B. subtilis*

Donor species	Protein	Concentration	References
Hepatitis B virus	Core antigen	ni[a]	Hardy *et al.*, 1981
Foot-and-mouth disease	Major antigen	ni	Hardy *et al.*, 1981
Rat	Proinsulin	7–10 μg/liter	Mosbach *et al.*, 1983
Bacillus licheniformis	β-Lactamase	20 mg/liter	Palva *et al.*, 1982
Human	Interferon-α2	0.5–1 mg/liter	Palva *et al.*, 1983
Human	Serum albumin	ni	Saunders *et al.*, 1987
Human	Interferon-α2	30–60 mg/liter	Schein *et al.*, 1986
Human	Growth hormone	50–210 mg/liter	Honjo *et al.*, 1987
Plant	Prothaumatin	1 mg/liter	Illingworth *et al.*, 1988
Bordetella pertussis	Pertussis toxin subunit S1	100 mg/liter	Runeberg-Nyman *et al.*, 1987
Human	Atrial natriuretic α-factor	500 μg/liter	Wang *et al.*, 1988
Bacillus stearothermophilus	Pullulanase	ni	Kuriki *et al.*, 1988
Streptococcus pneumoniae	Pneumolysin	10 mg/liter	Taira *et al.*, 1989
Human	Tissue plasminogen activator	20 mg/liter	Wang *et al.*, 1989
Bovine	RNase A	1–5 mg/liter	Vasantha and Filpula, 1989
Bordetella pertussis	Pertussis toxin subunit S1-S5	variable	Saris *et al.*, 1990

Bordetella pertussis	Pertussis toxin subunit S1-S5	S4: 500 mg/liter	Himanen *et al.*, 1990
Clostridium thermocellum	Endoglucanase A	ni	Petit *et al.*, 1990
Escherichia coli	OmpA	100 mg/liter	Puohiniemi *et al.*, 1991
Human	Interleukin-1β	40 mg/liter	Bellini *et al.*, 1991
Neisseria meningitidis	Class 1 outer membrane protein	ni	Nurminen *et al.*, 1992
Antidigoxin single-chain antibody		ni	Wu *et al.*, 1993
Streptomyces avidinii	Streptavidin	30–50 mg/liter	Nagarajan *et al.*, 1993
Human	Granzyme K	ni	Babé *et al.*, 1998
Staphylococcus aureus	Staphylokinase	337 mg/liter	Kim *et al.*, 2007
Bacillus licheniformis	Phytase	ni	Tye *et al.*, 2002
Streptomyces avidinii	Streptavidin	94 mg/liter	Wu *et al.*, 2002a
Chlamydia pneumoniae	Several proteins	ni	Airaksinen *et al.*, 2003
Human	Proinsulin	1 g/liter	Olmos-Soto and Contreras-Flores, 2003
Human	Interleukin-3	100 mg/liter	Westers *et al.*, 2005
Clostridium perfringens	β-Toxoid	20 mg/liter	Nijland *et al.*, 2007

[a] ni, not indicated.

about 30 g/liter of homologous proteins have been reported, but only few milligram amounts of recombinant proteins have been obtained (Table 6.7). This offers a huge potential for further research to identify and improve bottlenecks of the secretion process. Two major open questions to be solved in the near future and related to secretion are the analysis of the SRP and Tat pathways and formation of disulfide bonds in recombinant proteins. Secretion via the well-studied Sec pathway requires the polypeptide chains to be present in a translocation-competent form, and the failure to do so could be one reason for the relatively low yield of some secreted proteins. Alternative secretion routes are the SRP and the Tat pathways. In contrast to *E. coli*, very little is known about the SRP secretion pathway. Based on the rudimentary information, a signal sequence with a high amount of hydrophobic amino acids should be recognized by the SRP and targeted to the SecYEG translocon, where cotranslational secretion occurs. If this will really hold true for *B. subtilis* as well, fusion of the appropriate signal sequence could prevent the problem of premature folding of the polypeptide chain in the cytoplasm and thereby increase the yield of the secreted protein. The Tat pathway could be problematic for secretion of heterologous proteins. So far, fusion of one of the two known signal peptides to heterologous proteins did not target these to the Tat translocon, suggesting that amino acid sequences from the mature part may play a role.

The second open question refers to the formation of disulfide bonds on the *trans* side. Although two pairs of oxidoreductases have been identified, both active on the outside of the membrane, their contribution to the formation of disulfide bonds in heterologous proteins are largely unknown. Here, one or two model proteins with several disulfide bonds, such as the human tissue plasminogen activator (Qiu *et al.*, 1998), should be analyzed and used to optimize the system. Perhaps, it is even possible to engineer *B. subtilis* cells in such a way that they allow intracellular disulfide bond formation as described for *E. coli* (Bessette *et al.*, 1999).

Besides *B. subtilis*, two other *Bacillus* species have been (*B. brevis*) and are being used (*B. megaterium*) for the production of recombinant proteins. For both Bacilli, strains exist largely devoid of extracellular proteases which will stabilize secreted recombinant proteins. But a major drawback in using one of these species is the lack of knowledge of their complete genome, which would allow the identification of those genes involved in secretion of heterologous proteins. This lack of knowledge makes it very difficult to engineer these species in such a way to allow the development of hypersecreters.

In summary, the two challenging tasks for the near future in developing *B. subtilis* cells as a true alternative to *E. coli* will rely on its ability to reduce intracellular aggregation of recombinant proteins and, more importantly, to convert *B. subtilis* cells into hypersecreters.

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CHAPTER 7

Quorum Sensing: Fact, Fiction, and Everything in Between

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Contents

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I. PREFACE

Prior to 1994, quorum sensing was commonly referred to as ''autoinduction'' (Fuqua *et al.*, 1994; Nealson *et al.*, 1970). Autoinduction was originally described for the Gram-negative marine organism *Vibrio fischeri* in early 1970s. The series of experiments conducted by Kempner and Hanson (1968) revealed induction of bioluminescence in freshly inoculated *V. fischeri*. The culture luminated in response to media that was previously conditioned by the same bacterial strain. Nealson *et al.* (1970) were the first to propose that the autoinduction of luminescence in *V. fischeri* occurs on the transcriptional level and that the process is regulated by extracellularly secreted components (Eberhard, 1972).

The term ''quorum sensing'' was introduced by Dr. Steven Winans in 1994, who was putting together one of the first review articles on autoinduction in bacteria. Somehow, the word ''autoinducer,'' a term used to describe the small diffusible molecules involved in the process, just did not seem right to the young professor. Part of that dislike was due to common confusion of the term ''autoinduction'' with ''autoregulation'' (Fuqua *et al.*, 1994). Also, the cross-species induction of the bioluminescence had been reported by Greenberg *et al*. (1979) so by 1994, the term ''autoinduction'' became somewhat inaccurate. Winans was determined to come up with a new name that was innovative, descriptive, and most importantly, catchy. Assisted by his literary-minded brother-in-law, Dr. Winans generated dozens of possible terms including ''gridlockins,'' ''communiolins,'' and ''quoromones.'' None of the terms themselves became popular, but the notion of a quorum was accepted by Winan's colleagues and eventually made it into the title of the chapter. The term

''quorum sensing'' spread like wildfire, making its way into virtually every paper involving autoinduction written afterward.

II. INTRODUCTION

During our investigation of the quorum-sensing processes in *Listeria monocytogenes*, we stumbled on one assay that simply refused to offer meaningful results. This generally accepted assay was designed for the detection of the ''universal'' cell-to-cell signaling molecule, AI-2. A meticulous search through the literature led us to a long-forgotten study that was published in the early 1970s and overlooked in a number of recent publications. Locating this study was crucial for our research project (Turovskiy and Chikindas, 2006), but more importantly, this find propelled us to investigate other ambiguous aspects related to current quorum-sensing theories.

Although defined as a cell density-dependent process, quorum sensing is commonly considered to be ''more than just a numbers game'' (Xavier and Bassler, 2003) and is seen as being synonymous with bacterial communication. Words such as language (Taga *et al.*, 2001) and behavior are frequently used to depict this process in literature. Clever and witty quorum-sensing manuscript titles such as ''Mob psychology'' and ''Bacterial social engagements'' shift the process even further into a social realm.

The appeal of all these allegories is understandable, as is the enthusiasm of the researchers who make these comparisons; however, scientific theories cannot survive solely due to their appeal. When asking questions of current theories, it is best to go back to the roots of their inception. Hence, in the following chapter, we review quorum-sensing data that led not only to the formation of commonly accepted theories but also to conflicting theories which, for one reason or another, never became popular, and finally those that are currently falling apart from the lack of supporting evidence. All in all, the purpose of this chapter is the search for a better understanding of the phenomenon known as quorum sensing.

III. THE BASICS OF MICROBIAL LINGUISTICS

A. Autoinducers: The language of prokaryotic communication

Quorum sensing (QS) was originally described in the Gram-negative marine organism, *V. fischeri*. This bacterium can inhabit light organs of some marine animals, such as *Euprymna scolopes*, with the cell density often reaching 10^{10}–10^{11} CFU/ml (Fuqua *et al.*, 1994). The microorganism bioluminates in these symbiotic associations (Lupp *et al.*, 2003), but not in

a planktonic state where its cell density is below 10^2 CFU/ml (Fuqua *et al.*, 1994). The bioluminescence results from the enzymatic reaction driven by luciferase and apparently expression of the *V. fischeri*'s luciferase gene is regulated through QS (Winans and Bassler, 2002). Only two proteins, LuxI and LuxR, are involved in this regulatory pathway. LuxI catalyzes synthesis of 3-oxo-C6-HSL, which diffuses freely from each cell and can accumulate in the environment if the bacterial population density is high enough. This acylated homoserine lactone (AHL) can be detected by a sensor kinase protein, LuxR, which also has a response regulator domain. LuxR binds the promoter of *lux* operon, thus inducing the expression of *V. fischeri*'s luciferase as well as LuxI and LuxR (Fig. 7.1).

LuxR/I-type systems were identified in more than 70 Gram-negative species (Henke and Bassler, 2004a). Each LuxR-type sensor protein is very specific at detecting a particular AHL signaling molecule. The AHL-type pheromones differ in their acyl chain length, degree of saturation, and the substituent located on the third carbon (Fig. 7.2). Although it was originally believed that AHLs diffuse passively through cellular membranes, the long-chain molecules are actively transported via efflux and influx pumps (Smith *et al.*, 2004). The LuxR/I-type QS systems are known to regulate elastase and rhamnolipid (virulence factors) production in *Pseudomonas aeruginosa*, exoenzyme (another virulence factor) and antibiotic production in *Erwinia carotovora*, bioluminescence in *V. fischeri*, and also pigment/antibiotic production in *Serratia liquefaciens* (Henke and Bassler, 2004a).

Interestingly, *Salmonella enterica* and *Escherichia coli* have a LuxR homologue (SdiS), but lack any genes homologous to *luxI*. There is also no evidence that either of these two organisms produce AHLs (Smith *et al.*, 2004). It is thought that *S. enterica* is capable of responding to AHLs produced by other enteric bacteria through expression of *rck* operon and several other genes, presumably for protection against the host's defenses (Henke and Bassler, 2004a). Some systems unrelated but similar to LuxR/I QS were identified in a few Gram-negative organisms, namely AinS from *V. fischeri*, and HdtS from *P. fluorescens* (Bassler, 2002).

Gram-positive species predominantly utilize small posttranslationally modified peptides for cell-to-cell signaling. These peptide autoinducers are exported via ATP-binding cassette (ABC)-type transporters (Fig. 7.3). The genes encoding the precursor peptide, membrane-bound sensor kinase protein, and the transporter machinery are usually located in a single gene cluster (Bassler, 2002). The regulation of gene expression by peptide pheromones is achieved through a two-component signal transduction system. This system will be illustrated in Section III.B using the example of the *nis* operon. The peptide-type autoinducers are known to regulate competence and sporulation processes in *Bacillus subtilis*,

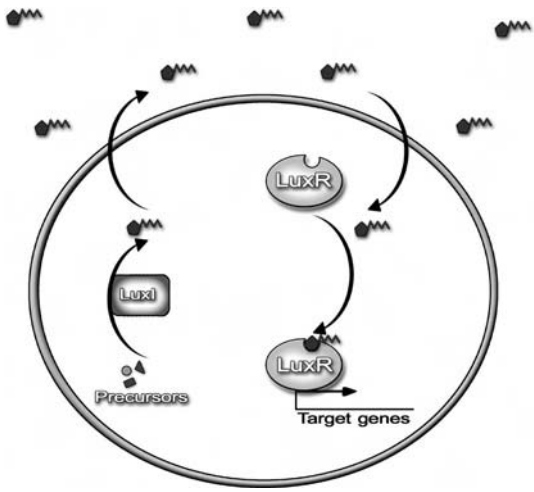

FIGURE 7.1 The LuxI/R families of proteins are commonly involved in synthesis and detection of the QS signaling molecules in Gram-negative bacteria.

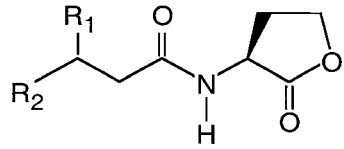

FIGURE 7.2 AHL-type autoinducers differ in the structure of their acyl carbon chain.

virulence and biofilm formation in *Staphylococcus aureus*, and nisin production in *Lactococcus lactis*.

B. Autoinducers with antimicrobial activity

Kaufmann *et al*. (2005) observed that *N*-(3-oxododecanoyl) homoserine lactone, one of the autoinducers used by *P. aeruginosa*, is also an effective bactericidal agent. The autoinducer itself and the corresponding product derived from a spontaneous Clasen-like condensation (Kaufmann *et al*., 2005), 3-(1-hydroxydecylidene)-5-(2-hydroxyethyl) pyrrolidine-2,4-dione, were effective against all tested Gram-positive bacteria. On the other hand, *P. aeruginosa* as well as other Gram-negative bacteria were not affected by either of these two compounds. The bactericidal property of *N*-(3-oxododecanoyl) homoserine lactone was detected at concentration ranges which are typical for this compound in *P. aeruginosa* biofilms (Kaufmann *et al*., 2005). The authors speculate that other known

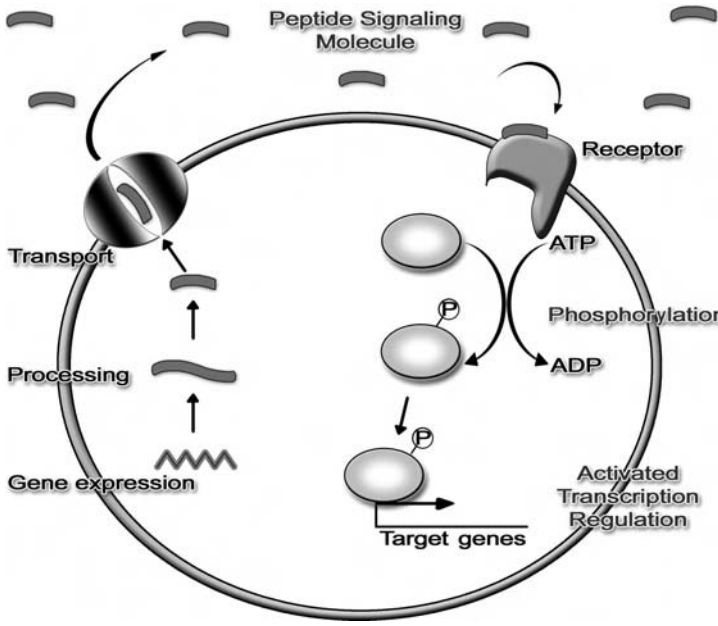

FIGURE 7.3 Quorum sensing in Gram-positive bacteria is typically mediated by oligopeptides, which are modified from the precursors prior to being effluxed from the cell.

autoinducers may also perform additional biological functions. For instance, the well-known group of autoinducers with potent antimicrobial activity is the class I bacteriocins, the so-called lantibiotics (Kleerebezem, 2004).

Bacteriocins are small antimicrobial molecules of proteinaceous nature, which are produced ribosomally by virtually all bacterial species to control other microorganisms competing for the same ecological niche (Klaenhammer, 1993). Generally, these molecules have a narrow range of activity that is usually restricted to Gram-positive species, closely related to the producer strain.

The observation that some microorganisms produce bacteriocins in a cell density-dependent manner led to the discovery of QS involvement in the synthesis of these peptides. Originally, it was noticed that, when diluted in fresh media, some strains would stop producing bacteriocins. However, the synthesis would resume if filter-sterilized spent medium from the same strain was added (Eijsink *et al.*, 2002).

Class I bacteriocins (lantibiotics) undergo extensive posttranslational modification prior to being secreted. This class of antimicrobial peptides (AMPs) is produced by lactic acid bacteria (LAB). Lantibiotics have unusual

amino acids, such as dehydroalanine and dehydrobutyrine as well as thioether bridges called (β-methyl) lanthionines (Kleerebezem, 2004), and are generally hydrophobic. These molecules are known for their broad range of activity, stability to heat, and inherent safety, which makes them excellent candidates as food preservatives (Kleerebezem, 2004).

Biosynthesis of at least some of the lantibiotics is quorum-sensing dependent. Nisin from *L. lactis* and subtilin from *B. subtilis* are structurally very similar. The regulatory machineries for the synthesis of these two molecules have a lot of similarities as well (Kleerebezem, 2004).

All genes necessary for nisin production are arranged in a single gene cluster (Kleerebezem, 2004). The expression of *nisABTCIP* is regulated by the P_{nisA} promoter. This operon includes the structural gene for nisin precursor peptide (*nisA*) and genes necessary for maturation (*nisB*, *nisC*, *nisP*), export of (*nisT*) and immunity to nisin (*nisI*). The regulatory genes (*nisRK*) and the rest of the immunity genes (*nisFEG*) are under the control of P_{nisR} and P_{nisF}, respectively. NisK and NisR constitute a two-component signal transduction system. When a mature nisin molecule binds to NisK, the signal is transduced to a response regulator NisR, which subsequently is able to bind to P_{nisA} and P_{nisF} (Fig. 7.4). The promoter for *nisRK* is not responsive to nisin (Kleerebezem, 2004). Subtilin has almost identical regulatory system with minor differences. Both nisin and subtilin are true pheromones with the antimicrobial properties.

Class IIa bacteriocins are small, heat-stable molecules with a highly conserved YGNGV consensus motif in the N-terminus (Hechard and Sahl, 2002). They are also known as anti-listeria bacteriocions (due to their high activity against foodborne pathogen *L. monocytogenes*) or pediocin-like bacteriocins (named after the first well-studied class IIa bacteriocin). These molecules are synthesized as prepeptides and are processed during translocation across the membrane. The synthesis of many class IIa bacteriocins, such as plantaricin from *Lactobacillus plantarum*, is regulated by peptide pheromones. It was reported that some of these pheromones may have antimicrobial activity themselves (Eijsink *et al.*, 2002), but it is insignificant in comparison with the activity of the actual bacteriocin. The signal transduction is conveyed via a standard two-component system (in some publications, this system is addressed as a three-component system because of the structural gene for pheromone itself) (Eijsink *et al.*, 2002).

The QS regulation of sakacin K was studied in detail (Brurberg *et al.*, 1997). The bacteriocin-like pheromone binds to a sensor kinase protein that activates the appropriate response regulator. The response regulator interacts with promoters upstream of the regulatory and transport operons as well as with the promoter of the structural gene itself. The bacteriocin's structural gene is under stringent control of this system, while the regulatory and transport genes appear to be less responsive to the

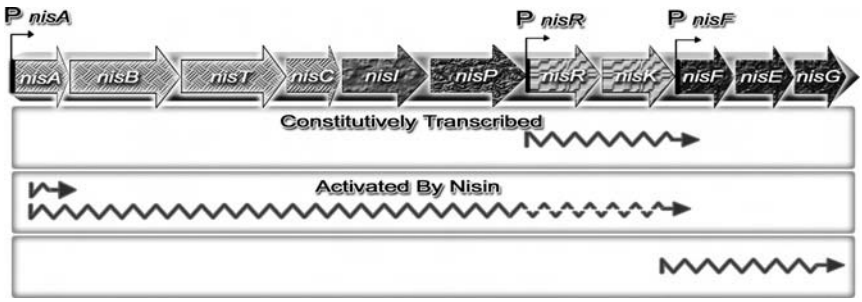

FIGURE 7.4 Transcription of the nisin gene cluster is regulated through QS. Genes coding for NisR and NisK are constitutively transcribed. Although regulated by P_{nisR}, *nisRK* can also be transcribed together with *nisABTCIP*; polycystronic mRNA containing *nisABTCIPRK* has been reported (Kleerebezem, 2004). In contrast to P_{nisR}, P_{nisA} and P_{nisF} are induced by extracellular nisin molecules. Nisin binds to the membrane-associated kinase, NisK, inducing the phosphorylation of the response regulator protein, NisR. The activated NisR induces the transcription of the transcriptional units driven by P_{nisA} and P_{nisF} (*nisA*, *nisABTCIP*, *nisABTCIPRK*, and *nisFEG*).

regulation because the pheromone is exported by the very same ABC transporter (Eijsink *et al.*, 2002).

C. Multiple quorum-sensing systems: Integrating the sensory information

A number of prokaryotes utilize multiple QS systems. The ''sensory'' information collected through these systems has to be integrated for targeted gene expression. Multiple QS systems can share a single genetic regulon; they can target sets of overlapping genes or regulate seemingly unrelated genetic clusters.

P. aeruginosa has two LuxI/R-type QS systems, LasI/R and RhlI/R. These systems work in parallel, but some of the genes targeted by LasR overlap with the genes targeted by RhlR. One of these overlapping genes is *rhlI*. As a consequence, RhlR/I system is turned on not only by accumulation of RhlR's cognate C4-HSL but also by the activation of LasR/I system (Henke and Bassler, 2004a). The result of interaction between *rhl* and *las* signaling pathways is a sequential gene expression. The genes targeted by RhlR are expressed after the genes targeted by LasR. According to Henke and Bassler (2004a) the sequential gene expression is needed for proper maturation of biofilms or successful infection process.

Either of the two peptide pheromones from *B. subtilis*, ComX and CSF, can stimulate the expression of its target genes while repressing the genes targeted by the second peptide (Henke and Bassler, 2004a). This mode of regulation assures that the two sets of genes will not be expressed at

the same time. ComX controls the genes necessary for genetic competence, while CSF targets genetic apparatus necessary for sporulation. According to Henke and Bassler (2004a), such stringent regulation is employed since genetic competence and sporulation are two mutually exclusive physiological states for a bacterium.

The QS regulon of *V. harveyi* is controlled by three separate QS systems. Each of these three systems has a distinct autoinducer synthase and a specific hybrid sensor histidine kinase protein. HAI-1 (harveyi AI-1) and AI-2 were discovered more than a decade ago (Bassler *et al.*, 1994). *V. harveyi*'s AI-1 is *N*-(3-hydroxybutanoyl) homoserine lactone (HSL), which is synthesized by LuxLM. This protein is not related to *V. fischeri*'s LuxI but it performs a similar function (Federle and Bassler, 2003). *N*-(3-hydroxybutanoyl) homoserine lactone binds its specific sensor protein LuxN.

LuxS is required for the biosynthesis of AI-2, which is 3A-methyl-5, 6-dihydro-furo(2,3-D)(1,3,2)dioxaborole-2,2,6,6A-tetraol. AI-2 is detected by a soluble periplasmic protein LuxP consequently leading to activation of a hybrid two-component sensor kinase response regulator protein, LuxQ.

The third QS system in *V. harveyi* was discovered more recently. The still uncharacterized autoinducer CAI-1 is synthesized by CqsA and detected by its cognate sensor CqsS. The Cqs system was first characterized in *V. cholerae* (Henke and Bassler, 2004a), which is where the name for this system comes from (cholerae quorum sensing).

The rest of the signaling cascade in *V. harveyi* is shared by all the systems (Fig. 7.5). LuxN, LuxQ, and CqsS dephosphorylate the shared phosphotransferase LuxU, which indirectly activates the response regulator LuxR.

In *V. harveyi*, QS is known to regulate bioluminescence, type III secretion, and metalloprotease production (Henke and Bassler, 2004a). The expression of all identified genes in *V. harveyi* QS regulon is regulated exclusively via the Lux circuit. The activation of each system seems to have an additive effect on the regulation of gene expression (Mok *et al.*, 2003).

In the foodborne pathogen *V. cholerae*, AI-2 and CAI-1 downregulate the expression of virulence factors like cholera toxin and toxin-coregulated pilus, as well as the expression of 70 other virulence-related genes. Most of these genes are required for the attachment of the pathogen to intestinal epithelial cells (Federle and Bassler, 2003). AI-2 and CAI-1 also downregulate the expression of genes responsible for biofilm formation, while upregulating the expression of Hap protease, the enzyme facilitating the detachment of *V. cholerae* cells from the intestinal walls (Federle and Bassler, 2003). The analysis of the *V. cholerae* QS regulon suggests that at high cell densities, this parasite tends to abandon its host and reenter the environment, possibly due to nutrient depletion.

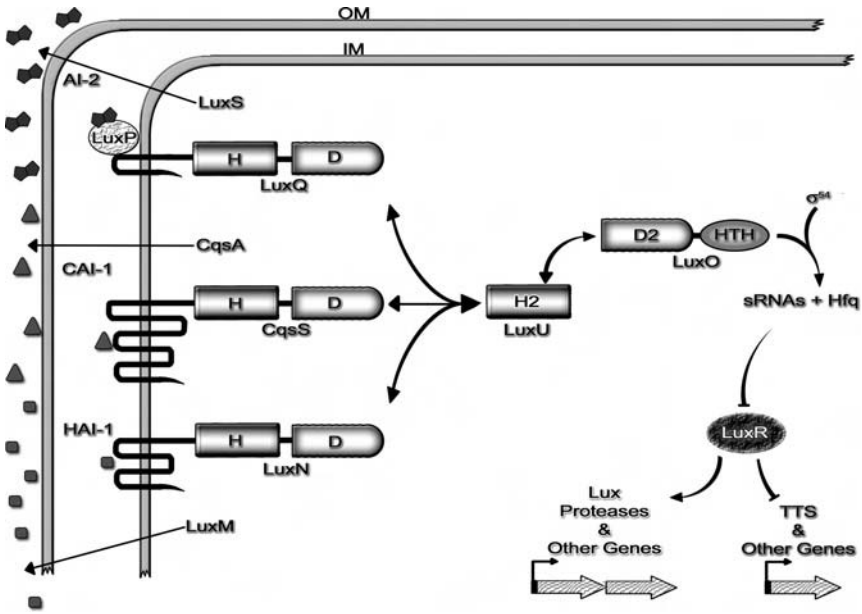

FIGURE 7.5 Multiple QS systems of *V. harveyi* regulate the expression of their target genes via a common response regulator protein, LuxR. The three signal transduction pathways that link the QS sensor proteins to the target genes converge via a shared phosphotransferase, LuxU.

D. The "Environment Sensing" theory: So much for social engagements of bacteria!

Autoinducer-mediated regulation of gene expression is well established in many species of bacteria, that is *V. harveyi*, *V. fischeri*, *P. aeruginosa* (Bassler, 2002; Bassler *et al.*, 1993; Lupp *et al.*, 2003). The benefits of this process are commonly explained in terms of a concerted response on a population level (Henke and Bassler, 2004a; Winans and Bassler, 2002). In fact, the very term ''quorum sensing'' implies a population density-dependent process (Fuqua *et al.*, 1994). Although this cooperative explanation is appealing to many, it may not be the most accurate description of the phenomenon.

The extracellular concentration of autoinducers may reflect population density of a microorganism *in vitro*, due to mixing of the producer cells within the constraints of a vessel. However, the situation is likely to be different *in vivo*, where the concentration of a secreted autoinducer may also depend on the diffusion and flow properties of the environment. In many natural habitats of bacteria, these properties are fluctuating and therefore quite unpredictable, that is soils before and after the rain or tooth enamel before and after consumption of a beverage. Consequently, laboratory conditions cannot simply be extrapolated *in vivo*.

Alternatively, autoinducers may be used by individual bacterial cells to sense the flow dynamics of their immediate environment, as opposed to the population density. This ''Environment Sensing'' theory was proposed by Redfield (2002) but has been overlooked by most researchers thus far. Redfield (2002) theorized that the environment or the diffusion sensing may allow bacteria to prevent wasteful synthesis of extracellularly secreted substances such as bacteriocins, siderophores, exoenzymes, and other effector molecules. These molecules increase nutrient availability for their producers, provided that they remain close to the cell. For example, bacteria break down extracellular macromolecules through the use of the secreted enzymes such as proteases, cellulases, pectinases, collagenases, and chitinases (Redfield, 2002). The success of this process largely depends on the properties of the surroundings (Fig. 7.6). High flow rates can wash the exoenzymes and the products of their digestive

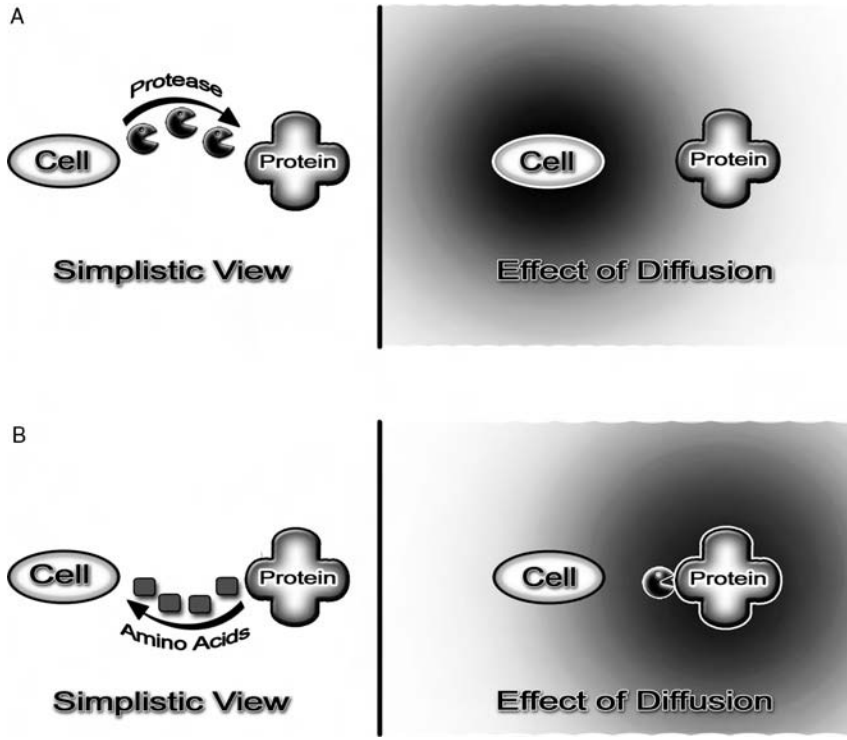

FIGURE 7.6 Secreted exoenzymes are commonly employed by bacterial cells to break down macromolecules. In contrast to the simplistic view (left), the success of the extracellular digestion largely depends on diffusion and flow in the vicinity of the cell (right). This figure was reproduced with minor changes from Redfield (2002) with the permission of the author and the publisher.

reactions away from the producer cell, rendering the extracellular digestion process ineffective. Relatively small metabolic burden is associated with autoinducer synthesis (Keller and Surette, 2006; Redfield, 2002). The restricted diffusion and mixing in the immediate environment of the single cell, a property that is essential for the effectiveness of the secreted products, can be sensed by the cell through the extracellular accumulation of autoinducers. The fact that QS-regulated genes most commonly encode extracellular products and the proteins necessary for their posttranslational modification and secretion (Kleerebezem, 2004; Redfield, 2002) supports the ''Environment Sensing'' theory behind the autoinduction (QS) phenomenon.

The direct benefits obtained by the individual cells through the flow dynamics ''awareness'' may favorably account for at least the initial steps of the evolution of autoinduction pathways. According to Redfield (2002), the synchronized population-wide response (i.e., QS, as it is commonly defined) may simply be a side effect of the Environment Sensing. The variety of the autoinducer-regulated processes is vast; however, the fundamental function of these processes and the driving force behind their evolution may still be elusive. We speculate that in the near future, perception of QS in the scientific community will shift more toward the Environment Sensing theory.

IV. LOST IN TRANSLATION

A. AI-2: The most talked about molecule in the field

In the past two decades, enormous scientific resources have been invested into the search to better understand the elusive molecule known as AI-2. We are aware of at least six independent microarray studies aimed at investigating global transcriptional response of various microorganisms to AI-2-mediated QS (DeLisa *et al.*, 2001; Joyce *et al.*, 2004; Merritt *et al.*, 2005; Ren *et al.*, 2004; Yuan *et al.*, 2005; Zhou *et al.*, 2003). Knockout mutations of *luxS* (AI-2 synthase) have been constructed and characterized in about two dozen bacterial species. In most of these cases, the *luxS* orthologue has been cloned and its functionality has been confirmed. The structures of at least five LuxS orthologues have been determined through resolution of the protein's x-ray diffraction patterns (Das *et al.*, 2001; Hilgers and Ludwig, 2001; Lewis *et al.*, 2001; Rajan *et al.*, 2005; Ruzheinikov *et al.*, 2001).

The molecular structure of AI-2 has been revealed through X-ray crystallography when the molecule is cocrystallized with its two known cognate sensor proteins (LuxP and LsrB). Originally discovered in *V. harveyi*, AI-2 is now known to be produced by more than 70 bacterial

species and more than 50 *luxS* (AI-2 synthase) homologues were identified in sequenced bacterial genomes (Sun *et al.*, 2004). Although still a matter of debate, AI-2-mediated QS is thought to regulate the expression of numerous phenotypes in various bacterial species. Among many other traits, AI-2 is thought to regulate motility in *Campylobacter jejuni*, biosynthesis of the antibiotic carbapenem in *Photorhabdus luminescens*, and the expression of virulence factors in *Streptococcus pyogenes* (Xavier and Bassler, 2003). AI-2 has been proposed to be and is widely accepted in the scientific community as the universal cell-to-cell signal in prokaryotic microorganisms.

This famous, or rather ''infamous,'' molecule and its parental gene (*luxS*) were given a dedicated section in at least 12 review articles. At least three review articles have been solely committed to the discussion of AI-2-mediated QS (De Keersmaecker *et al.*, 2006; Vendeville *et al.*, 2005; Xavier and Bassler, 2003). Finally, being a subject of controversy, *luxS*/AI-2 inevitably has its own devoted section in this chapter as well.

The publications dedicated to arguably the most talked about molecule in the field are purposely reviewed more or less in a chronological order in the following sections. This arrangement gives the reader a chance to follow the events that led to the formation and subsequent decline of the cross-species communication paradigm.

B. The early years of research: AI-2 goes interspecies

It all began with a publication by Greenberg *et al.* (1979), which reported bioluminescence in *V. harveyi* in response to application of culture fluids from several nonluminous bacterial species. A decade later, one of the *V. harveyi* autoinducers (AI-1) had been identified as *N*-3-hydroxybutanoyl homoserine lactone, which belonged to the same class of molecules as the previously identified *N*-3-oxo-hexanoyl-HSL from *V. fischeri*. Surprisingly, the enzymes responsible for synthesis and detection of the AI-1 (LuxM and LuxN, respectively) do not belong to the LuxI/R family of proteins (Bassler *et al.*, 1993). The identification of the system components required for the AI-1-mediated QS in *V. harvey* led researchers to believe that an additional, still unidentified autoinducer was utilized by the microorganism in the cell-to-cell signaling processes (Bassler *et al.*, 1993).

Even before the key components of the AI-2-mediated QS system were characterized, the autoinducer could be detected using a constructed *V. harveyi* mutant, BB170 (Δ*luxN*), as a reporter strain. This *Vibrio* strain was used by Bassler *et al.* (1997) to illustrate that cross-species induction of luminescence in *V. harveyi* is triggered by AI-2. As a result, AI-2 received the esteemed title of ''interspecies communication signal'' (Bassler *et al.*, 1997).

The actual structure of AI-2 was determined when the molecule was serendipitously crystallized in a complex with its cognate receptor protein, LuxP (Chen *et al.*, 2002; Ringe, 2002). As the structure of *V. harveyi*'s LuxP was being resolved through x-ray crystallography, a ligand, identified as AI-2, has been noticed in the cleft between the two LuxP domains. Based on the electron density analysis, Chen *et al.* (2002) proposed the structure of AI-2 as a furanosyl borate diester. The involvement of the Boron atom was also confirmed by NMR spectroscopy and electrospray ionization mass spectrometry (ESI-MS) (Chen *et al.*, 2002).

As the sequence of the AI-2 synthase (LuxS) became available, homologues of the gene coding for this enzyme were identified across various bacterial species (Bassler, 2002; Schauder *et al.*, 2001). Armed with the *luxS* sequence and an easy AI-2 detection assay, researchers were compelled to investigate AI-2-mediated QS in non-*Vibrio* species.

The simplest of these early studies (see references in Table 7.1) included the construction of *luxS* deletion mutants in the studied microorganisms and subsequent confirmation of the LuxS functionality as an AI-2 synthase. The functionality of the *luxS* homologue was typically confirmed by cloning the gene into *E. coli* DH5 (AI-2 strain). The phenotypical changes of *luxS*-null mutations were typically attributed to AI-2/*luxS*-mediated QS. In fact, that is how most of the contemporary evidence for the function of AI-2 as a QS signal has been generated. Table 7.1 lists a few studies that were conducted using the methods just described.

Phenotype rescue was often attempted by reintroduction of *luxS* back into a knockout mutant. As a rule of thumb, reintroduction of *luxS* under the influence of its original promoter would rescue the mutant phenotype (Table 7.2).

Occasionally cell-free culture fluids from known AI-2 producers were used to rescue the mutant phenotypes (Table 7.3). It is important to note that the rescue of the phenotype with the cell-free culture media is an extremely simple procedure. However, on many occasions it has not been reported at all (Tables 7.1 and 7.2). The procedure was successful in the rescue of some phenotypes (Table 7.3; Sperandio *et al.*, 1999); none-theless, these results were questioned after the case of EHEC as described in Section IV.C.

C. The pivotal case of EHEC

Without a doubt, the most extensive study of *luxS*-dependent QS in non-*Vibrio* species has been conducted in *E. coli*. Both commensal and pathogenic strains have been investigated with regard to the presence of this QS system (Challan *et al.*, 2006; DeLisa *et al.*, 2001; Sperandio *et al.*, 2001). In fact, *E. coli* DH5α, which is a *luxS*$^{-}$ strain, is commonly used for cloning

TABLE 7.1 The simplest studies of the AI-2-mediated QS in non-*Vibrio* species involved characterization of *luxS*-null mutants (based on the review by Vendeville *et al.* (2005))

Species name	Knockout mutant phenotype	References
Streptococcus pyogenes	Elevated SLS hemolytic activity Reduced proteolytic activity Media specific growth defect	Lyon *et al.*, 2001
Helicobacter pylori	Thick biofilms	Cole *et al.*, 2004
Serratia 39006	Decreased carbapenem production	Coulthurst *et al.*, 2004
Campylobacter jejuni	Reduced: Motility Autoagglutination *flaA* transcription	Jeon *et al.*, 2003
Porphyromonas gingivalis	Deficiency in: Exoproteases Rgp/Kgp Haemagglutinin activity	Burgess *et al.*, 2002

TABLE 7.2 Generally the *luxS* mutant phenotype could be successfully rescued through gene complementation (based on the review by Vendeville *et al.* (2005))

Species name	Knockout mutant phenotype	Phenotype rescue by gene complementation	References
Streptococcus gordonii	Downregulated *gtfG, fruA, lacD*	ND	McNab *et al.*, 2003
	Defect in formation of mixed species biofilms	Successful	
Neisseria meningitides	Attenuated *in vivo*	Successful	Winzer *et al.*, 2002b

TABLE 7.3 Some studies reported successful phenotype rescue by the AI-2-containing conditioned media (based on the review by Vendeville *et al.* (2005))

Species name	*luxS*-null mutant phenotype	Phenotype rescue by		References
		Gene complementation	Conditioned media	
Shigella flexneri	Deficiency in *virB* expression	ND	Partial	Day and Maurelli, 2001
Clostridium perfringens	Deficiency in:			
	Toxin production	ND	Successful	Ohtani *et al.*, 2002
	pfoA mRNA	Partial	Successful	
Serratia marcescens 274	Impaired virulence	ND	ND	Coulthurst *et al.*, 2004
	Deficiency in:			
	Prodigiosin production	Successful	Successful	
	Hemolytic activity	Successful	ND	

and characterization of *luxS* orthologues from various bacterial species (Elvers and Park, 2002; Fong *et al.*, 2001; Winzer *et al.*, 2002b).

At least three research groups have independently conducted studies aimed at investigating the role of AI-2/LuxS in global transcriptional regulation of the *E. coli* genome. The regulation of *lsr* operon in *E. coli* K12 will be described in Section IV.E. This section is mainly concerned with the ''case of'' enterohemorrhagic *E. coli* (EHEC), as the results of these studies had a significant impact on what was to become the accepted methodology in this area of research.

Enterohemorrhagic *E. coli* (EHEC) O157:H7 is a foodborne pathogen notorious for causing outbreaks of bloody diarrhea and hemolytic-uremic syndrome (Sperandio *et al.*, 1999, 2003). The virulence factors of O157:H7 are localized within the chromosomal pathogenicity island known as locus of enterocyte effacement (LEE). The majority of these genes are arranged in five consecutive polycistronic operons, *LEE1–LEE4* and *tir*. *LEE2*, *LEE3* and *tir* are regulated in a cascade manner by Ler, the transcriptional activator encoded in *LEE1* (Sperandio *et al.*, 1999, 2003). The involvement of AI-2-mediated QS in regulation of LEE genes in EHEC has been meticulously investigated in the past years (Sperandio *et al.*, 1999, 2001, 2003). In the study conducted by Sperandio *et al.* (1999), various strains of *E. coli* were transformed with *lacZ* reporter fusions constructed under the control of the predicted *LEE1–LEE4* and *tir* promoters so that their QS activation patterns could be investigated in different backgrounds. Media conditioned by strains of *E. coli* that contained functional *luxS* (including the *luxS*-complemented DH5α) directly activated *LEE1* and *LEE2* promoters while inducing *LEE3* and *tir* through Ler. Conversely, the medium conditioned by DH5α (*luxS*$^{-}$) did not have any effect on the transcription of LEE genes. These results led the authors to the conclusion that AI-2 was the compound responsible for induction of these virulence genes in EHEC.

In a later study, gene array approach was used by Sperandio *et al.* (2001) to determine the actual extent of transcriptional regulation in EHEC 86–24 that is mediated through the AI-2-dependent QS. Two sets of radioactively labeled cDNA that were derived from EHEC 86–24 and its isogenic *luxS* mutant, respectively, were hybridized to an *E. coli* K-12 gene array, which was subsequently scanned and analyzed for differences in pixel intensity. The data analysis revealed that roughly 10% the genome is differentially transcribed in the *luxS* mutant in comparison to its parental strain, thus indicating that QS may play a role in global regulation of EHEC gene expression (Sperandio *et al.*, 2001).

An independent microarray study conducted by DeLisa *et al.* (2001) and published the very same year as Sperandio *et al.* (2001) seemed to confirm the tremendous impact of AI-2 on global transcriptional regulation in *E. coli*. The study investigated transcriptional response of the

E. coli W3110 *luxS*-null mutant to extracellularly added AI-2. Medium conditioned by the *E. coli* AI-2 producer strain, W3110, was used as the source of AI-2, and the medium conditioned by the W3110 *luxS*-null mutant was used as a corresponding negative control. RNA isolated from the cells exposed to these conditioned media was used for hybridization with the DNA microarray and for subsequent transcriptome analysis. DeLisa *et al.* (2001) reported that roughly 6% of the *E. coli* genome was transcriptionally regulated through the AI-2-dependent QS, even though criteria for significance in this study was less stringent than the criteria used by Sperandio *et al.* (2001).

The results of these two microarray studies further elevated the standing of AI-2 to the status of global regulator, at least for *E. coli* strains (Sperandio *et al.*, 2001). Then, a short article written by Winzer *et al.* (2002a) completely changed everything. Winzer *et al.* (2002a) dug through the literature that was published almost four decades ago and stumbled on the fact that LuxS had been described previously as a ''RH cleavage enzyme'' that is involved in important physiological processes of some bacteria (Duerre and Miller, 1966; Miller and Duerre, 1968). The implication of this discovery is that *luxS*-null mutant phenotypes described for a number of prokaryotic species (Tables 7.1–7.3) may be caused by the altered physiology of the cells and not by QS. As a result, the phenotype rescue procedures using conditioned media preparations of AI-2 should be interpreted with great caution as well, at least in the case of *E. coli* strains. For example, the media conditioned by the *E. coli* O157:H7 and the corresponding *luxS* mutant are likely to differ tremendously in composition (Winzer *et al.*, 2002a) because the two strains have 10% of the genome experiencing altered expression (Sperandio *et al.*, 2001).

Synthetic AI-2 was not available at the time the studies conducted by DeLisa *et al.* (2001) and Sperandio *et al.* (2001) took place, and that is why cell-free culture fluids (conditioned media) had been utilized by the researchers as a supply of AI-2 (Sperandio *et al.* 2003). The later studies conducted with the purified and the synthetic AI-2, however, clearly indicated that AI-2 cannot induce virulence (LEE) genes in EHEC (Sperandio *et al.*, 2003). Apparently, the induction of LEE genes by the culture fluids of *luxS*$^{+}$ *E. coli* strains that had been reported by Sperandio *et al.* (1999) was due to an unrelated and as-yet unidentified compound, termed AI-3. The *luxS*-knockout mutation affects biosynthesis of AI-3 through the shift of cellular metabolic processes which are related to the physiological role of *luxS* (Walters *et al.*, 2006). AI-3, in turn, has been proposed to be the ''true'' interspecies and possibly even an interkingdom communication signal (Sperandio *et al.*, 2003). The subject of AI-3 will not be critically reviewed in this chapter, as the research in the area is still in its infancy (Clarke *et al.*, 2006; Reading and Sperandio, 2006; Walters *et al.*, 2006). It is worth pointing out, however, that the notion of interkingdom

communication is rather difficult to comprehend from the evolutionary perspective (Winzer and Williams, 2003). Most significantly, the study by Sperandio *et al.* (2003) illustrated that the conditioned media approach that has been used to study AI-2-mediated QS in bacteria may result in conclusions that are inaccurate. Additionally, in the case of EHEC, it had clearly been demonstrated that many phenotypes of *luxS*-null mutants result from the alteration in the metabolism of the cells (Walters *et al.*, 2006). The reported metabolic function of *luxS*, and the data collected from the EHEC studies that completely supported this new finding, changed the course of AI-2-related research.

D. The role of *luxS* in cell physiology: Activated methyl cycle

The activated methyl group is required for a number of essential cellular processes in both prokaryotes and eukaryotes. *S*-adenosyl methionine (SAM) is the major methyl donor of the cell (Winzer *et al.*, 2002a). SAM-dependent methylation of DNA, RNA, proteins, and certain metabolites is carried out by dedicated transmethylases with the formation of *S*-adenosylhomocysteine (SAH) that serves as a feedback inhibitor for SAM-dependent methyltransferases (Winzer *et al.*, 2002a). The molecule is highly toxic and it is being recycled by cells via two major pathways.

Some phyla of the Bacteria kingdom and all of the Archaea and Eukarya kingdoms utilize a one-step detoxification pathway (Sun *et al.*, 2004) that involves SahH. This enzyme converts SAH into homocysteine and adenosine (Fig. 7.7). Phylogenic distribution of this pathway implies its ancient origins (Sun *et al.*, 2004).

The alternative, two-step pathway for detoxification of SAH is employed by some species of γ-, β-, and ε-proteobacteria and by all Firmicutes. The first step of the pathway is the conversion of SAH into adenine and *S*-ribosyl homocysteine (SRH), a reaction catalyzed by Pfs. In the second step, SRH is converted into homocysteine and DPD (the precursor for AI-2) by LuxS. The cycle is completed as homocysteine is converted into methionine and subsequently activated back into SAM (Fig. 7.7). DPD formed in the reaction catalyzed by LuxS is rather unstable. The molecule exists in equilibrium with numerous furanones that are formed from spontaneous cyclization (Fig. 7.8). One of this furanones can react with borate to cause the formation of AI-2. Since AI-2 synthesis is tightly linked to important metabolic processes of a cell, theoretically, this molecule could be used to gauge not only the density of a population but also its metabolic state (Xavier and Bassler, 2003). Xavier and Bassler (2003) have argued that AI-2 production has indeed been the driving force behind the evolution of the two-step SAH recycling pathway.

The fact that perfectly viable *luxS*-null mutants have been constructed for numerous bacterial species allows for speculation that the reaction

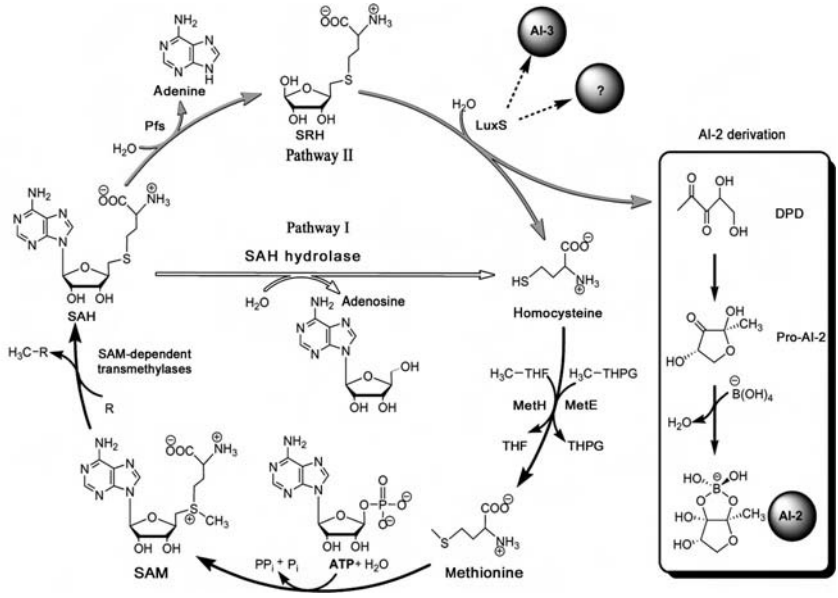

FIGURE 7.7 The two major schemes of the AMC. The white and gray arrows depict alternative biochemical pathways for the recovery of homocysteine from the toxic SAH. This figure was reproduced with minor changes from Sun *et al.* (2004) with the permission of the author and the publisher.

catalyzed by Pfs is sufficient for the recycling of toxic SAH (Xavier and Bassler, 2003). Recent studies indicate that this is not exactly the case. Elevated levels of SAH were detected in the culture fluids of the *luxS*-null mutant constructed from *L. monocytogenes* EGD-e (Challan *et al.*, 2006). Nevertheless, this mutant strain has not been compromised in its ability to grow in the planktonic state.

Theoretically, a *luxS*-null mutant accumulates SRH, while homocysteine is depleted from its cytoplasm. The mutant cells are thought to compensate for the homocysteine deficiency through synthesis of the molecule from oxaloacetate (Kaper and Sperandio, 2005; Reading and Sperandio, 2006). Homocysteine is used for the *de novo* synthesis of methionine, while oxaloacetate along with l-glutamate is used for aspartate synthesis (Reading and Sperandio, 2006; Walters *et al.*, 2006). The global effects of these metabolic shifts are rather difficult to predict as the phenotypes exhibited by *luxS*-null mutants may be caused by a combination of the disturbances in both, QS and metabolic processes of a cell. Generally, discrimination between the possible role of *luxS* in QS and the role of this gene in the central metabolism of a cell proved to be rather challenging (De Keersmaecker *et al.*, 2006).

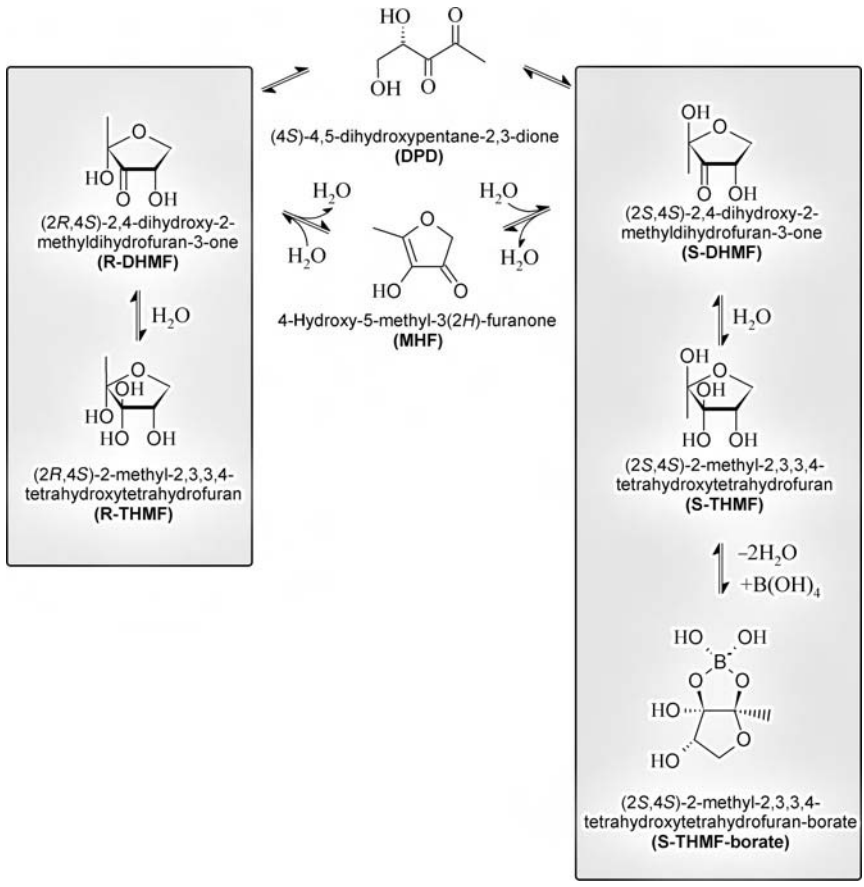

FIGURE 7.8 A series of compounds formed from the spontaneous cyclization of DPD is currently referred to as "AI-2." *S*-THMF-borate, the original AI-2, was recognized as a ligand for LuxP in *V. harvei*. As the LsrB ligand, *R*-THMF, was identified, the term "AI-2" had to be broadened to include other derivatives of DPD shown in the figure.

The effect of the activated methyl cycle (AMC) disruption in some microorganisms can be assessed through evaluation of indirect evidence. For example, despite numerous studies that were aimed at investigation of AI-2-mediated QS in *E. coli*, to date, the genes harbored by the *lsr* operon are the only genes of the microorganism's genome that were shown to be directly regulated by AI-2 (Walters *et al.*, 2006; Xavier and Bassler, 2005). In view of this fact, it is likely that most of the genes influenced by the *luxS*-knockout in *E. coli* are affected by metabolic shifts associated with the disruption of AMC (Sperandio *et al.*, 2001; Walters *et al.*, 2006). Transcriptome analysis conducted by Sperandio *et al.* (2001) has revealed that the *luxS*-null mutant has altered expression of genes

involved in biosynthesis, metabolism, and transport of amino acids; genes involved in biosynthesis and metabolism of nucleotides; as well as genes involved in catabolism of carbon compounds (Sperandio *et al.*, 2001; Walters *et al.*, 2006). A few simple but effective experimental approaches have also been employed by the researchers to account for the pleiotropy of *luxS*. For example, the effects of the growth medium supplemented with AMC intermediates or aspartate on a *luxS*-null mutant phenotypes have been investigated for *L. monocytogenes*, *S. enterica serovar typhimurium*, and *E. coli* (Challan *et al.*, 2006; Miller *et al.*, 2004; Walters *et al.*, 2006). Also, attempts were made to compliment the phenotype of the EHEC *luxS* mutant by transforming the cells with the plasmid containing functional *sahH*. Presumably, the recombinant cells would metabolically ''bypass'' the dysfunctional components of the AMC (Walters *et al.*, 2006); the procedure was successful at restoring the mutant's transcription of the *LEE1* promoter to the wild-type levels. Finally, the phenotype rescue attempts were conducted by growing the mutants in coculture with their parental strains so that both populations are exposed to the same signaling molecules (Challan *et al.*, 2006; Doherty *et al.*, 2006).

Some evidence for the significance of LuxS in the central metabolism of a cell has been obtained through the comparative genomic analysis. The study conducted by Sun *et al.* (2004) involved analysis of 138 fully sequenced genomes. The reciprocal best hit strategy was utilized to search for genes that are orthologous to the key players in the AMC as the function of orthologues is likely to be conserved (Sun *et al.*, 2004). Results of this study indicate that roughly 20% of the investigated organisms lack the set of genes necessary for conversion of SAH into homocysteine (*sahH* or *pfs*/*luxS*). Most of these organisms, however, are either symbionts or parasites that may rely on their host for metabolic processes such as the ''handling'' of SAH (Sun *et al.*, 2004).

With only a few exceptions, bacteria that have the ability to convert SAH into homocysteine also have the necessary enzymes to regenerate this intermediate back into SAM, thus completing the cycle. In contrast, the cognate sensor protein for AI-2 (LuxP) and the key components of the signal transduction circuitry triggered by binding of AI-2 to LuxP (LuxQ and LuxU) seem to be restricted to the *Vibrio* species (Sun *et al.*, 2004). In fact, the lack of AI-2 receptors in non-*Vibrios* has been for years the major missing link in the theory regarding AI-2 being the ''the universal'' signaling molecule.

E. *lsr* operon: The missing link. . . is still missing

It is unclear whether DPD or any of its derivatives (Fig. 7.8) passes across bacterial cytoplasmic membrane through passive diffusion, as these molecules are highly polar (Bassler, personal communication). AI-2 can

exert its ''signaling effect'' by binding to membrane-associated receptors which are unrelated to *Vibrio*'s LuxP. AI-2 can also enter the cell via active transport and the signal transduction then can be initiated from within the cell. Prior to 2001, however, LuxP was the only protein reported to bind AI-2. There is a possibility that a knockout mutation of an as-yet-unidentified AI-2 receptor can be lethal for the microorganism, making it difficult to identify the protein via genetic screening (Bassler, personal communication). Nevertheless, the vigorous search for the AI-2-regulated genes led researchers to the discovery of LsrB, a protein which was later shown to directly interact with AI-2.

The study was triggered by the observation that *S. typhimurium*, along with *E. coli* and a few other microorganisms, seem to degrade their extracellularly excreted AI-2 at the onset of the stationary growth phase. The LuxS of *S. typhimurium* is capable of producing AI-2 and the molecule can be detected in this organism's culture fluids all throughout the exponential growth phase. However, AI-2 levels seem to diminish as the population transcends into stationary growth phase (Surette and Bassler, 1998; Taga *et al.*, 2001, 2003).

Taga *et al.* (2001) screened 11000 random insertion mutants and identified eight genes which were differentially transcribed in *S. typhimurium* 14028 and in its isogenic *luxS*-null mutant SS007. One of the identified genes, *metE*, is induced by homocysteine and, consequently, it was ruled out as a true target of AI-2 regulation (Taga *et al.*, 2001, 2003). The remaining seven genes were located in a single operon *lsrACDBFGE* (*lsr* for *luxS*-regulated) that was shown to be activated by extracellularly added synthetic AI-2 (Taga *et al.*, 2001, 2003). The *lsr* operon has also been characterized in *E. coli* (Xavier and Bassler, 2005), and has identical gene arrangement to the *lsr* operon in *S. typhimurium* except for *lsrE.*

The first four genes of the *lsr* operon code for the ABC-type transporter highly homologous to the Rbs transport apparatus. The *rbs* operon that has mostly been studied in *E. coli* (Taga *et al.*, 2001) harbors genes involved in transport and phosphorylation of ribose and an identical set of genes has been identified in *S. typhimurium*. Similar to ribose, AI-2 is phosphorylated as it enters the cell through its transporter. The transcription of *lsrACDBFGE* is induced by interaction of the phosphorylated AI-2 (AI-2-P) with the repressor protein, LsrR. Genes coding for the transcriptional regulator and cytoplasmic kinase, *lsrR* and *lsrK*, respectively, transcribed divergently of *lsrACDBFGE*.

The ultimate fate of the phosphorylated AI-2 is still unclear, although it has been suggested to be similar to the fate of pentose sugars; AI-2-P is possibly converted to DHAP and then channeled in to a glycolytic pathway (Xavier and Bassler, 2005). It is known that the enzymes coded by *lsrG* and *lsrF* are involved in the reactions that lead to degradation of AI-2-P. However, the products of these enzymatic reactions are yet to be identified (Taga *et al.*, 2003).

Analogous to some operons coding for sugar transporters (i.e., *mal* in *E. coli*), the activation of *lsr* is dependent on cAMP-CAP (Xavier and Bassler, 2005). The accumulation of glycerol-3-phosphate in the cytoplasm of mutant *E. coli* cells led to the repression of *lsr* through the cAMP-CAP-dependent mechanism. The *lsr* operon is also repressed by dihydroxyacetone phosphate (DHAP). The repression of *lsr* by this molecule is independent of cAMP-CAP, and it may be facilitated through the direct interaction of DHAP with *lsr* operon repressor, LsrR.

The structure of the AI-2 molecule in complex with its periplasmic binding protein, LsrB, has been determined through x-ray crystallography (Miller *et al.*, 2004). Surprisingly, AI-2 bound to LsrB appeared to be (2*R*,4*S*)-2-methyl-2,3,3,4-tetrahydroxytetrahydrofuran (*R*-THMF) instead of the familiar furanosyl diester borate. The discrepancy in the ''appearance'' of AI-2(s) has been explained in terms of boron availability. Boron is abundant in marine environment where *Vibrios* reside, but it is scarce in the intestines where *E. coli* and *S. typhimurium* typically dwell (Waters and Bassler, 2005). It is worth noticing that the chemical structure of *R*-THMF is very similar to the structure of D-ribose (Fig. 7.9). Additionally, LsrB shares a significant structural homology with the ribose-binding protein (RBP) from *E. coli* and galactose-binding protein from *S. typhimurium* (Miller *et al.*, 2004). There is also evidence that AI-2 in *E. coli* is being reinternalized through an alternative low-affinity transporter, possibly through Rbs (Taga *et al.*, 2003; Vendeville *et al.*, 2005).

One theory is that analogous to acetate, DPD is not a preferable source of carbon; however, it can be utilized during a period of starvation

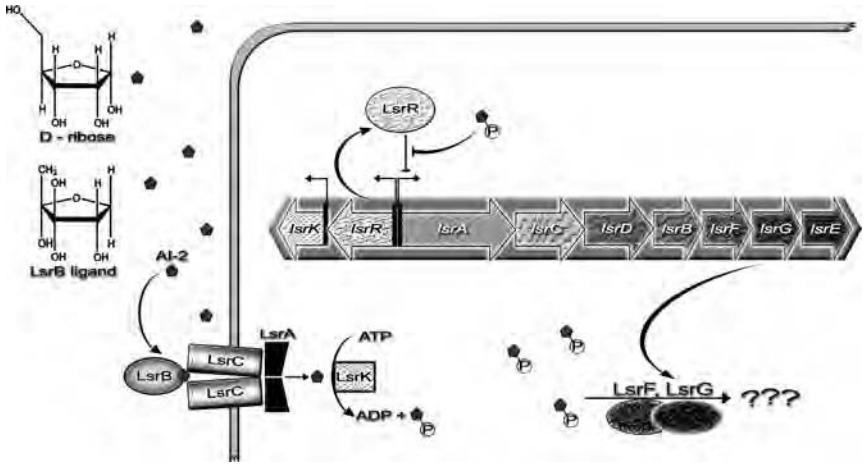

FIGURE 7.9 The *lsr* operon of *E. coli* and *S. typhimurium* is activated by extracellular AI-2. The genes harbored by *lsr* encode proteins necessary for reinternalization, processing, and possibly degradation of AI-2 (Vendeville *et al.*, 2005).

(Winans, 2002). In the absence of glucose, the molecule is reinternalized via the Lsr transporter (Surette and Bassler, 1998; Taga *et al.*, 2001) and presumably metabolized as a last resort. Alternatively, the function of *lsr* is sometimes interpreted in terms of QS. Faithful advocates of the ''microbial Esperanto'' theory currently view AI-2 as a group of molecules derived from DPD (Rickard *et al.*, 2006). Accordingly, microorganisms such as *E. coli* and *S. typhimurium* sequester AI-2 to interfere with the ''social activities'' of the competing bacteria. While this interpretation may be reasonable for those who accept the proposed role of AI-2 as the universal autoinducer, it may not be as obvious for those who reject it or just feel it is unsubstantiated.

F. Multilingual bacteria: Another look at the role of interspecies communication in *V. harveyi*

The role of ''interspecies communication'' in *Vibrio* species is far from being clear. It is commonly believed that AI-1 is used by *V. harveyi* to count ''thy self,'' while AI-2 is used to count potential competitors (Bassler, 2002; Bassler *et al.*, 1997; Waters and Bassler, 2006). Utilization of a two-autoinducer QS system can create four distinct input states for a cell: no autoinducers, AI-1 only, AI-2 only, and both AI-1 and AI-2 (Bassler *et al.*, 1997; Mok *et al.*, 2003). Each of these states theoretically could trigger a unique mode of gene expression. Bioluminescence data supported this hypothesis and the later discovery of the third signal used by *V. harveyi*, CAI-1, implied a possibility of the eight input states (Henke and Bassler, 2004b). However, the study conducted by Mok *et al.* (2003) has indicated that the common notion regarding the function of AI-2 in *V. harveyi* is likely to be mistaken.

The purpose of the study conducted by Mok *et al.* (2003) was the identification of novel AI-2-controlled genes and characterization of their control by the two autoinducers that were known at that time, AI-1 and AI-2. *V. harveyi* MM30 (*luxS*-null mutant, cannot produce AI-2) was subjected to random insertion mutagenesis using Mini-Mu*lacZ* transposon. Ten AI-2-controlled genes were identified through the screen of 6500 of such insertion mutants. LuxLM (AI-1 synthase) was then disabled in each fusion strain by in-frame deletion of *luxLM* on the chromosome. As a result, the 10 engineered strains would not synthesize endogenous AI-1 and AI-2. Transcription level of each fusion in response to the externally added autoinducer(s) was monitored through β-galactosidase assay. Strikingly, all the quorum-sensing-controlled target genes identified by that time in *V. harveyi* appeared to be regulated by both AI-1 and AI-2. This regulation takes place exclusively through the Lux circuit (Fig. 7.5) (Mok *et al.*, 2003). Most importantly, ''*V. harveyi* quorum-sensing circuit . . . discriminates between conditions in which both autoinducers are present and all other conditions''

(Mok *et al.*, 2003). This binary mode of regulation can easily be achieved with a single autoinducer. The multiple QS systems of *V. harveyi* may allow this microorganism to distinguish between the environments and to express genes accordingly. The ability of the *V. harvei*'s QS circuitry to only distinguish the coincidence of the autoinducers from other possible input states is rather difficult to explain in terms of ''intraspecies and interspecies communication''. On the other hand, multiple autoinducers with different diffusive properties (as in the case of AI-1 and AI-2) can provide the cell with the information about its immediate environment (Redfield, 2002). Mok *et al.* (2003) speculates that some natural habitats of *V. harveyi* may be prone to accumulate both autoinducers. The light organ of a host that harbors *V. harveyi* is likely to be an environment that favors accumulation of all the autoinducers.

G. The recent years: Research involving synthetic AI-2

Synthetic AI-2 was not available for the earlier studies described in Sections IV.B and IV.C (Sperandio *et al.*, 2003). Researchers had to rely on culture fluids from AI-2 producers for the supply of this substance. The culture fluids, however, may contain numerous metabolic by-products and/or unidentified autoinducers (Sperandio *et al.*, 2003; Winzer *et al.*, 2002a). Inaccurate results can easily be obtained by this spent media-based approach, as has been illustrated by the case of EHEC. Several procedures (De Keersmaecker *et al.*, 2005; Meijler *et al.*, 2004; Semmelhack *et al.*, 2005) are now available for the *in vitro* synthesis of DPD (precursor of AI-2). This section goes over some of the recent studies that involved synthetic or purified AI-2.

Challan *et al.* (2006) have investigated the role of AI-2-mediated QS in attachment of *L. monocytogenes* EGD-e cells during biofilm formation. The *luxS*-knockout mutant that was constructed from the EGD-e strain produced denser biofilms than its parental strain. Interestingly, extracellularly added AI-2 did not have any effect on the number of the attached cells, while SRH affected the biofilm density for both the wild type and the mutant strain. Additionally, SRH (substrate for LuxS) was shown to accumulate in the culture fluids of the constructed knockout mutant. As a result, Challan *et al.* (2006) came to the conclusion that the mutant phenotype was due to the accumulation of SRH and not due to the disrupted quorum sensing.

Biofilms formed by the *Lactobacillus reuteri* 100–23 were similarly affected by *luxS*-knockout mutation. The mutant cells produced thicker biofilms on plastic surfaces *in vitro* and on epithelial surfaces in an animal model (Tannock *et al.*, 2005). Additionally, the intracellular ATP content of the planktonic mutant cells was 35% lower than the ATP content of the parental strain. *In vivo* competition experiments were used to test the ecological performance of the *luxS* mutant. Although inoculated at

the same level, the *luxS* mutant strain was outcompeted by other strains of the same species (Tannock *et al.*, 2005). According to the authors, it is unclear whether QS has anything to do with this observation. Additionally, *in vitro* biofilm phenotype of the mutant strain could not be rescued by addition of the concentrated AI-2 preparation.

Competition experiments with rather elegant design were also conducted by Doherty *et al.* (2006) in the study of the *luxS* function in *S. aureus*. The constructed *luxS*-null mutant did not have any obvious defects when grown in a rich growth medium (LB medium). In particular, the traits associated with the virulence of *S. aureus*, such as synthesis of hemolysins and extracellular proteases as well as biofilm formation, were not affected by the *luxS* knockout. Conversely, the growth of the *luxS* mutant was compromised under the sulfur-limited conditions. The mutant strain did not grow well in chemically defined medium that contained 5-μM cysteine as a sulfur source. Doherty *et al.* (2006) have reasoned that cells with disrupted AMC (*luxS*-null mutants) increasingly rely on methionine uptake from the surroundings and this shift in metabolism, as opposed to QS defect, is responsible for the growth phenotype of the *S. aureus luxS*-null mutant. The hypothesis was tested through a competition experiment, which involved growth of the *luxS* mutant and its parental strain in a coculture under the sulfur-limiting conditions. The relative population sizes of the two strains following 24 hour of growth have indicated that the mutants were significantly outcompeted by the wild-type cells. Doherty *et al.* (2006) have argued that cells grown in a coculture have same pool of autoinducers; the fact that the growth defect of the *luxS*-null mutants was not relieved under these conditions indicates that the phenotype is not due to QS.

Rather unexpected results were obtained through two independent studies of AI-2-mediated QS in *Neisseria meningitidis*. The *luxS* mutant constructed from *N. meningitidis* MC58 did not exhibit abnormalities in growth kinetics (Schauder *et al.*, 2005). Proteomics analysis conducted by Schauder *et al.* (2005) revealed a lack of any major cellular response by the *luxS*-null mutant to synthetic AI-2. Essentially the same conclusion had been reached through a microarray study of the *luxS* mutant constructed from *N. meningitidis* Z2491. Dove *et al.* (2003) reported that the mutant did not exhibit any concerted transcriptional response to the added AI-2 in the form of culture fluid from the wild-type strain. Doherty *et al.* (2006) suggested that AMC may contribute differently to methionine biosynthesis in various species of bacteria. This variation may explain the vast differences between the transcriptional responses of the *luxS* mutants of *E. coli* and *N. meningitidis* to the media conditioned by their parental strains.

Functional *luxS* driven by its native promoter has been shown to be required for the formation of mature biofilms by *S. typhimurium* SL1344 (De Keersmaecker *et al.*, 2005). The biofilm formation defect of the *luxS*-null mutant could not be rescued by synthetic AI-2. Furthermore, the

phenotype was not restored through supplementation of the biofilm medium with AMC intermediates: methionine, cysteine, or SAM. Quite the opposite, SAM actually amplified the effects of the original knockout. Surprisingly, *luxS* fused with the strong constitutive *nptII* promoter failed to rescue the biofilm phenotype when the gene was introduced into the mutant. However, when the mutant was complimented with *luxS* driven by its original promoter, the biofilm characteristics were restored to the wild-type level. The results of this study emphasize the significance of the *luxS* regulation and the possible effects of this regulation on the smooth running of AMC in *S. typhimurium.*

We are aware of one intriguing study in which a *luxS* mutant phenotype was successfully rescued by supplementing the culture media with the synthetic AI-2 (Rickard *et al.*, 2006). The focus of the study was the formation of mixed biofilms by the two commensal species of oral microflora, *Streptococcus oralis* 34 and *Actinomyces naeslundii* T14V. The biofilms investigated in this study were grown in the reusable flow cells with the saliva being pumped through them. Rickard *et al.* (2006) reported that the dual-species biofilm cannot be formed by *A. naeslundii* T14V and the *luxS* mutant constructed from *S. oralis* 34. The biofilm growth was reestablished as the saliva passed through the flow cells was supplemented with the synthetic AI-2 at concentrations 0.08–0.8 nM (Rickard *et al.*, 2006).

The results of the phenotype rescue studies reviewed in this section are summarized in Table 7.4. Most of the rescue attempts involving purified or synthetic AI-2 were unsuccessful. At the same time, some rather atypical results were obtained in the study conducted by Duan *et al.* (2003). Duan *et al.* (2003) demonstrated that synthetic AI-2 can modulate gene expression in *P. aeruginosa*. The study involved transcriptional analysis of the promoters for 21 virulence factors in the pathogen's genome. Six of these genes were induced by supplementing the *P. aeruginosa* culture with synthetic AI-2 (Duan *et al.*, 2003). This study is unique in that *P. aeruginosa* does not have a *luxS* homologue in its genome, and therefore is unable to produce its own AI-2. One hypothesis is that this pathogen responds to AI-2 produced by commensal microflora in the lungs of a cystic fibrosis patient and activates its virulence based on their presence (Duan *et al.*, 2003).

DPD (the precursor to AI-2) is a common by-product of bacterial metabolism (Sun *et al.*, 2004; Winzer *et al.*, 2002a; Xavier and Bassler, 2003), so it is quite feasible that the microorganisms may have evolved pathways that utilize this molecule and its derivatives as a ''cue'' for the expression of certain genes.

The widespread occurrence of AI-2 is the main reason why this suspected ''universal autoinducer'' historically received a great deal of attention in the scientific community (Winzer *et al.*, 2002a). Most studies involving purified or the synthetic AI-2, however, indicate that QS mediated by this autoinducer may not be as widespread as was originally

TABLE 7.4 Synthetic AI-2 has been utilized in recent AI-2-related studies

Species name	*luxS*-null mutant phenotype	Phenotype rescue	
		Gene complementation	Synthetic AI-2
Staphylococcus aureus	Reduced ability to compete under sulfur-limiting conditions	Successful	Not successful
Listeria monocytogenes	Denser biofilms	Successful	Not successful, phenotype is due SRH
Salmonella typhimurium	Cannot form mature biofilms	Successful only with the *luxS* driven by pCMPG5664	Not successful
Neisseria meningitides	No major effect (proteomics and microarray studies)	Not applicable	No effect
Lactobacillus reuteri	Thicker biofilms, lower ATP content	Successful	Not successful
Streptococcus oralis	Defect in mixed biofilm formation	Successful	Successful

thought (Doherty *et al.*, 2006; Vendeville *et al.*, 2005). The actual signal transduction pathways linking the interaction of the AI-2 molecule and its cognate sensor protein(s) to the expression of the target genes have only been established in *V. harveyi*, *V. cholerae*, *S. typhimurium*, and *E. coli* (Xavier and Bassler, 2005). With that in mind, the biological role of AI-2-mediated gene regulation in *S. typhimurium* and *E. coli* is still a matter of debate since the only known functions of the target genes (*lsrACDBFGE*) are the uptake, phosphorylation, and degradation of AI-2 (Doherty *et al.*, 2006; Xavier and Bassler, 2005). The fact that the transcription of *lsr* is repressed by the common metabolic intermediates G3P and DHAP (Xavier and Bassler, 2005) supports the hypothesis that these two microorganisms reinternalize AI-2 simply to metabolize it in the absence of a preferred carbon source. Much of the early evidence for the AI-2-mediated QS in non-*Vibrio* strains has to be reevaluated using the purified compound; as of now, the majority of this evidence may be considered indirect or incomplete due to the complications associated with the involvement of *luxS* in the AMC (Doherty *et al.*, 2006). It is quite possible that outside the *Vibrio* genus, few organisms (if any) utilize AI-2 for genuine QS (Sun *et al.*, 2004).

H. AI-2 in foods: A few words about the currently accepted AI-2 detection assay

''The currently accepted AI-2 detection assay'' (Rickard *et al.*, 2006) was first described by Bassler *et al.* (1993). The assay is based on the ability of *V. harveyi* cells at certain cell densities to bioluminate in response to externally added AI-2. Interestingly enough, the intensity of the bioluminescent response of the wild-type *V. harveyi* to AI-1 is much higher than its response intensity to AI-2 (Bassler *et al.*, 1997). In fact, AI-2 could not be detected in the culture fluids of some (now known) AI-2 producers using the wild-type *V. harveyi* as a reporter strain. Experiments were also conducted with Δ*luxS* Δ*luxM* double mutant strain, MM77, which is unable to synthesize its own AI-1 and AI-2 but is able to respond to externally added autoinducers. These experiments revealed a 100-fold greater intensity of bioluminescence in response to AI-1 as compared to AI-2 (Mok *et al.*, 2003).

The currently accepted and widely used AI-2 detection assay utilizes the *luxN*-null mutant, BB170, as a reporter strain. This strain is not deficient in autoinducer synthesis, but presumably it cannot respond to AI-1 due to the lack of functional AI-1 receptor, LuxN.

The assay is initiated by mixing the reporter cells with the sample in question (10% v/v). At lower cell densities (10^4–10^6 CFU/ml) the reporter cells respond to externally added AI-2 because the level of endogenously

produced autoinducers has not reached the critical point for the bioluminescent response to take place. The mixture is usually incubated for 3–6 hours, with bioluminescence readings and cell counts taken approximately every 30 min. Noninoculated growth medium is usually used as a negative control, while the culture fluids from wild-type *V. harveyi* strain BB120 are used as a positive control. AI-2-like activity is expressed in relative bioluminescence units (light intensity is normalized to either positive or negative controls) per cell of the reporter strain.

However, there are hidden obstacles to using the AI-2 assay. Back in the early 1970s, it was discovered that even a small quantity of glucose inhibits bioluminescence in *V. harveyi* species (Nealson *et al.*, 1972). It was proposed that this inhibition happens on the transcriptional level of the *Vibrio*'s luciferase biosynthesis, through a catabolite repression mechanism. The ''glucose factor'' was once again brought to the attention of the scientific community by De Keersmaecker and Vanderleyden (2003). Nonetheless, in many reported studies this fact was not taken into account (Lu *et al.*, 2004, 2005; Zhao *et al.*, 2006). Finally, it has been reported that in concentrations below inhibitory levels for bioluminescence of *V. harveyi* BB170, glucose effectively induces the bioluminescent response and therefore may cause false positive results. Glucose and perhaps other sugars tend to support the growth of *V. harveyi* to a much better extent than glycerol, the carbon source in autoinducer bioassay (AB) medium (Turovskiy and Chikindas, 2006). Therefore, during the incubation time (3–6 hours), the culture of the indicator cells with added glucose may reach the critical cell density for QS to take place through endogenously produced AI-2 (*V. harveyi* BB170 has functional *luxS*, and therefore can produce AI-2).

The three AI-2-related studies (Cloak *et al.*, 2002; Lu *et al.*, 2004; Zhao *et al.*, 2006) are of particular interest, as the data in these food-related applications are likely to be affected by glucose. The study by Cloak *et al.* (2002) was aimed at characterization of the AI-2 production by microorganisms in foods. *Campylobacter coli*, *S. typhimurium*, and *E. coli* O157:H7 were inoculated into chicken broth, milk, and apple juice. These microorganisms were grown at various temperatures and each spent medium was assayed for AI-2 presence. The highest AI-2-like activity was observed after 24 hour of growth for microorganisms grown at either 25°C or 37°C in milk and chicken broth. After 48 hour of growth, AI-2-like activity virtually disappeared. Not much AI-2 was produced during the growth at 4°C and ''no notable AI-2 activity was evident in apple juice with any of the organisms examined under any of the conditions tested'' (Cloak *et al.*, 2002). Apple juice is known for high glucose content and this is what probably inhibits bioluminescence in *V. harveyi* BB170, and consequently, the detection of AI-2.

Lu *et al.* (2004), on the other hand, have analyzed a number of foods for the presence of AI-2-like activity and also for the ability to ''interfere'' with the actual AI-2 from *E. coli*. Frozen fish, tomatoes, cantaloupes, tofu, and milk induced high AI-2-like activity in *V. harveyi* BB170. On the other hand, rinses from turkey patties, chicken breast, and homemade cheeses interfered with *V. harveyi*'s response to AI-2. Additionally, the food preservatives sodium acetate at 0.1%, sodium propionate at 0.16%, and sodium benzoate at 0.1% (final concentrations) inhibited *V. harveyi*'s response to AI-2 (Cloak *et al.*, 2002; Lu *et al.*, 2004). The study was conducted in the following manner: The whole fruits and vegetables were swabbed with cotton swabs and then these swabs were soaked in fresh AB medium. Beef and chicken patties were washed and rinsed in AB medium. The medium was then assayed for AI-2 presence. The liquids from frozen fish and tofu packaging were analyzed directly as well as the whole milk samples. Blank AB medium was used as a negative control, and bioluminescence as low as five times higher than a negative control was considered significant. The AI-2-like activity associated with some foods such as frozen fish, tomatoes, and tofu could easily be caused by the presence of very low concentration of glucose in these samples. The bioluminescence assay was conducted in a 96-well plate reader, and final cell counts of *V. harveyi* BB170 were never determined.

The reported ability of propionates, acetates, and benzoates, at conceivable concentrations and neutral pH, to inhibit AI-2-like activity of AI-2 collected from the known AI-2 producer is intriguing. However, the data obtained by this group suggest that the listed compounds may interact with the intracellular signal transduction of *V. harveyi* BB170 and not with the molecule itself. What it means is that these compounds are unlikely to be effective against non-*Vibrio* species, since the signal transduction cascade for AI-2 seem to be restricted to *Vibrio* species only (Sun *et al.*, 2004; Fig. 7.10).

In the study by Zhao *et al.* (2006), BHI medium was used to conduct the AI-2 detection assay instead of the traditional, ''sugar-free'' AB medium. The BHI medium is rich in glucose and other carbohydrates which are funneled into glycolysis and possibly can inhibit luminescence in the *V. harveyi* reporter strain.

The growth of the reporter strain may also be hindered by high acidity of the added sample (De Keersmaecker and Vanderleyden, 2003). Also, there is 30–40% standard deviation associated with the method (Turovskiy and Chikindas, 2006). All the above factors must be considered if reliable data are to be collected using this method. The method may be improved through analysis of the bioluminescence kinetics as opposed to the analysis of the single reading (Y.T. and M.C., unpublished data).

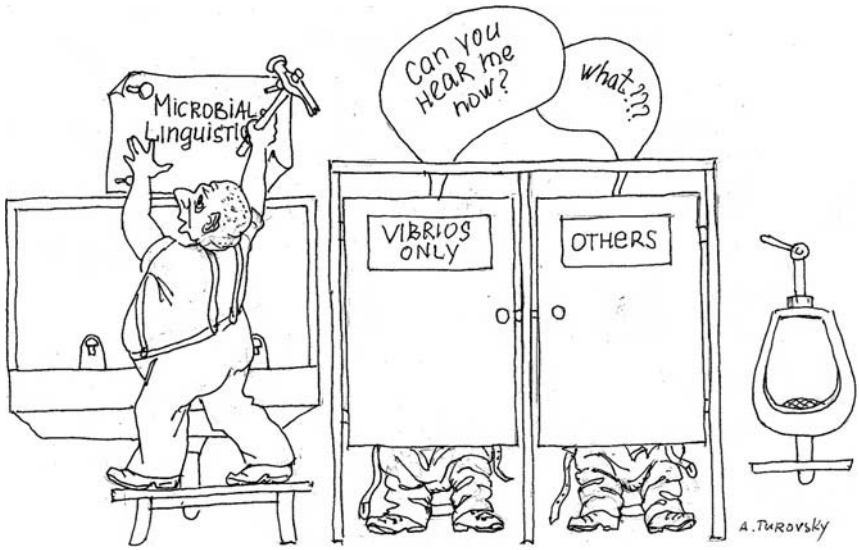

FIGURE 7.10 Quorum sensing: fact, fiction, and everything in between.

V. QUORUM QUENCHING: ALL QUIET ON THE MICROBIAL FRONT

A. Halogenated furanones: The defense system of algae

The process of interference with bacterial quorum sensing is known as quorum quenching and this phenomenon was observed in both prokaryotes and eukaryotes. The Australian macroalga *Delisea pulchra* is known to produce a variety of halogenated furanones. These molecules interfere with AHL-dependent quorum-sensing systems through competitive inhibition at the LuxR-type receptor site. Halogenated furanones also accelerate the turnover time of LuxR family of proteins. This interference is thought to control bacterial biofilm formation on the algae's surface (McLean *et al.*, 2004).

B. AHL lactonases and acylases: Too early to judge

A number of bacteria produce AHL-degrading enzymes known as AHL lactonases and AHL acylases. Lactonases hydrolyze the lactone ring of AI-1-type autoinducers and are found in numerous *Bacillus* species (Zhang, 2003). It was shown that all the tested strains of *B. thuringiensis*, *B. cereus*, and *B. mycoides* were capable of degrading AHLs (Dong *et al.*, 2002).

AHL acylases break the amide linkages of AHLs and were originally discovered in a soil isolate of *Variovorax paradoxus* and later in *Ralstonia* species (Zhang, 2003).

Some enzymes of eukaryotic origin were recognized to have similar activities against AHLs, that is paraoxonases and porcine kidney acylase (I) can degrade AHLs if the conditions are appropriate (Dong and Zhang, 2005). Porcine kidney acylase (I) seems to be widely conserved in eukaryotic organisms; however, the AHL-degrading activity exhibited by this enzyme is largely restricted to alkaline conditions, casting a doubt on the enzyme's *in vivo* role as a quorum quencher (Dong and Zhang, 2005; Xu *et al.*, 2003). Also, paraoxonases encoded by *PON* genes are known to have important physiological functions. Both PON1 and PON2 have antioxidant properties, while PON1 is also involved in degradation of the toxic organophosphate (Billecke *et al.*, 2000; Dong and Zhang, 2005; Draganov *et al.*, 2000).

The *in vivo* function of bacterial AHL-degrading enzymes has also been debated (Roche *et al.*, 2004). Although these enzymes are commonly thought to function as QS disruptors, their primary physiological role may merely be an aspect of the cell's central metabolism. AHLs are abundant in the environment, making it likely for microorganisms to evolve ways to utilize these compounds as metabolites (Roche *et al.*, 2004). It is known that *Variovorax paradoxus* and *Arthrobacter* species are capable of using AHLs as a sole source of energy (Leadbetter and Greenberg, 2000; Park *et al.*, 2003; Roche *et al.*, 2004).

In mixed bacterial communities, enzymatic activities of different microbial species may complement one another (Roche *et al.*, 2004). Thus, species incapable of fully metabolizing AHLs may initiate degradation of these molecules through a lactonase- or acylase-catalyzed reaction, while the coinhabitants of the same niche may use the products of these reactions to complete the breakdown. This argument is supported by the fact that some soil bacteria can use homoserine lactones (products of acylase-catalyzed reaction) as a sole source of carbon (Yang *et al.*, 2006). Roche *et al.* (2004) have also argued that most enzymes capable of deactivating AHLs were identified through specific screens for this deactivation activity. However, catalytic activity of these enzymes *in vivo* may primarily be directed against substrates other than AHLs. At least one identified AHL acylase, PvdQ from *P. aeruginosa*, is known to be implicated in a biochemical pathway unrelated to quorum sensing. The acylase activity exhibited by PvdQ is thought to be directed toward biosynthesis of pyoverdine, a protein involved in iron acquisition (Lamont and Martin, 2003; Ochsner *et al.*, 2002; Roche *et al.*, 2004).

Furthermore, arrangement of the genes coding for some AHL-degrading enzymes often implies their role in central metabolism of a cell. For instance, *Agrobacterium tumefaciens' attM* is harbored by the operon that

also contains homologues of succinate semialdehyde dehydrogenase and alcohol dehydrogenase (Roche *et al.*, 2004). Altogether, due to the lack of conclusive evidence it is too early to designate these enzymes as quorum quenchers.

C. Quorum quenching: Practical applications

Regardless of the function that AHL-degrading enzymes may play *in vivo*, a number of promising applications that involve these enzymes have been reported in the literature. Among the most prominent ones are the two *in vivo* studies of virulence attenuation in *E. carotovora* through interference with the pathogen's quorum-sensing pathways. *E. carotovora* is a plant pathogen and the expression of its virulence factors is known to be controlled through AHL-dependent quorum sensing. *B. thuringiensis*, on the other hand, displays strong AHL lactonase activity (Dong *et al.*, 2004). In one of the experiments, potato slices were dipped into *B. thuringiensis*' liquid culture and afterward inoculated with *E. carotovora*. Negative controls were dipped in sterile water and then inoculated with *E. carotovora* as well. *B. thuringiensis* did not inhibit the growth of the pathogen; however, lesions caused by the pathogen were significantly reduced in size (Dong *et al.*, 2004). The same procedure was repeated with a strain of *B. thuringiensis* which could not produce the lactonase (*aiiA*-null mutant) and resulted in the formation lesions at the sites where *E. carotovora* was inoculated, although these lesions were still less severe than the ones in the negative control. Dong *et al.* (2004) hypothesized that *B. thuringiensis* interfered with *E. carotovora*'s quorum sensing and thus made this pathogen more vulnerable toward the defenses of the potato plant (*Solanum tuberosum*). In another set of experiments, a transgenic *S. tuberosum* was constructed through an *A. tumefaciens*-mediated transformation of *aiiA*. Tubers from these genetically manipulated plants were immune to infection by *E. carotovora* (Dong *et al.*, 2001).

The importance of quorum sensing in the proliferation of pathogens and spoilage organisms in processed foods has not yet been confirmed (Smith *et al.*, 2004). It is difficult to make a definitive conclusion on whether the intervention with QS will make foods safer for consumption and/or if it will extend the products' shelf life. However, it is quite conceivable that quorum-sensing inhibitors (QSIs) will be identified in foods. For example, crude garlic extract contains at least three different compounds capable of interfering with the LuxR/I quorum-sensing system (Persson *et al.*, 2005; Rasmussen *et al.*, 2005). The toluene fraction of this extract was able to interfere with the formation of characteristic mushroom-like structures of *P. aeruginosa* PAO1 biofilms. The biofilms grown in the presence of 2% garlic extract were susceptible to treatment with antibiotics and detergents (Rasmussen *et al.*, 2005).

In another study, Wu *et al.* (2004) have demonstrated that a synthetic QSI, similar in structure to V-30 (produced by *D. pulchra*), can be effective in clearing *P. aeruginosa* lung infections in mice. Supposedly, this compound interferes with the proper formation of the *P. aeruginosa* biofilms, ultimately making the infection more susceptible to the animals' immune response. The assumption has been supported by an *in vitro* study (Wu *et al.*, 2004), which demonstrated that biofilms formed by *P. aeruginosa* in the presence of this QSI have increased susceptibility to tobramycin and SDS.

D. The available screening procedures for quorum-sensing inhibitors

Several biological screening systems for identification of QSIs have been described in the literature. The simplest, and probably the most effective, assay for detection of QSIs is based on the abilities of two naturally occurring microorganisms, *P. aureofaciens* 30–84 and *Chromobacterium violaceum* ATCC 12472, to regulate the expression of their pigmented molecules through QS. The screening procedure is conducted on the agar plates by overlaying samples with soft agar containing either of these two indicator organisms (McLean *et al.*, 2004). Purple-colored violacein is produced by *C. violaceum* and orange-colored phenazine is produced by *P. aureofaciens*. QSIs interfere with production of these pigments and can be easily identified through discoloration of the growing indicator strain. This assay is easy to utilize and it does not require the use of external AHLs.

The recombinant QSI selectors generally have *lux*-type promoter fused with a reporter gene. This fusion is inserted into a vector and then cloned into a model organism. In these recombinant systems, the expression of the reporter gene is under the control of AHL-mediated quorum-sensing processes. AHL antagonists inhibit the expression of the reporter gene and thus can be identified. Rasmussen *et al.* (2005) described the construction of three such recombinant screening systems called QSIS1, QSIS2, and QSIS3. QSIS1, the most successful of the three systems, is established in *E. coli* harboring the pJAB140 plasmid, which contains a *lux* promoter fused with *phlA*. PhlA is toxic for the cells, and in QSIS1, the expression of this protein is dependent on the presence of external AHLs. AHL antagonists allow the indicator cells to grow in the presence of various AHLs. The limitation of this system is that it requires the use of external acyl homoserine lactones.

QSIS2 contains a *sacB* marker fused with a *lasB* promoter and presumably responds to QSIs with a more narrow range of action due to specificity associated with *P. auroginosa*'s LasR/I system (Rasmussen *et al.*, 2005).

This system can be used for selection of antagonists specific to *P. aurogi-nosa* quorum-sensing system. The disadvantage of QSIS2 is that it requires external AHLs and gives false positive results in response to glucose (Rasmussen *et al*., 2005).

The most advanced microtiter plate assay for the selection of QSIs was described by Jafra and van der Wolf (2004). This assay is based on GFP-marked *E. coli*, which fluoresces in response to various external AHLs. QSIs inhibit this fluorescence without affecting the growth of the indicator *E. coli* cells. The advantage of the microtiter plate assay is that it allows for analysis of multiple samples at once, and the fluorescence of *E. coli* cells can be monitored quantitatively. Similar microtiter plate assays which involve mutant *C. violaceum* or *P. aureofaciens* rely on the researcher himself for making a judgment about change of color in the culture of indicator cells (Jafra and van der Wolf, 2004).

VI. THE UPDATE

Recently, Waters and Bassler (2006) identified and characterized many novel QS-regulated genes in *V. harveyi* that were not reported previously by Mok *et al*. (2003). The previously reported genetic screen aimed at recognition of the QS regulon in *V. harveyi* (Mok *et al*. 2003) could have been biased towards the genes modulated by multiple autoinducers, since the procedure was conducted in an AI-2^{-} *Vibrio* strain that was still capable of producing AI-1 and CAI-1.

In contrast, the screen reported by Waters and Bassler (2006) was conducted in an AI-1^{-}/AI-2^{-} background, thus increasing the chance of identifying the genes that are solely controlled by a specific autoinducer. Although most of these newly-identified promoters were only responsive to the simultaneous presence of all three autoinducers (coincidence behavior), some displayed a graded response with detectable alteration in expression triggered individually by AI-1 or AI-2 (Waters and Bassler, 2006).

Still, all the known QS-controlled genes in *V. harveyi* share common regulatory components, a cascade leading to the phosphorylation/diphosphorylationof LuxR. The variation in binding affinities of the LuxR to the QS-regulated promoters is thought to account for the differences in gene expression response.

Although Waters and Bassler (2006) propose that *V. harveyi* QS signals AI-1, CAI-1 and AI-2 are used for intraspecies, intragenera and interspecies communication, respectively, we suggest that multiple QS systems are used by this microorganism to simply assess its immediate environment (IV.F).

VII. CONCLUDING REMARKS

There is no doubt that the practical applications of quorum-sensing research carry enormous potential (Dong and Zhang, 2005; Zhang and Dong, 2004); however, it is crucial to realize that the direction of scientific exploration has always been influenced by the opinions of the scientific community. There are numerous examples in the history of science of appealing yet inaccurate functional theories that directed research onto a path of ambiguity. Our attempt to utilize the relatively simple AI-2 detection assay initiated numerous questions (Turovskiy and Chikindas, 2006), which led us to uncover conflicting theories in every major aspect of quorum-sensing research.

The very function of quorum sensing is not completely clear. The evolution of this process in bacteria could possibly have been driven by the need to sense the flow dynamics of their immediate environment, as opposed to the need for a concerted response. Since multiple factors could influence the direction of development in evolution, both theories could be correct and therefore have a right for coexistence. Conversely, the theory of AI-2-mediated interspecies communication seems likely to remain weak or even fall apart from insufficient supportive results. Data collected over the past decade indicate that in most species of bacteria, the molecule may simply be a metabolic by-product and nothing more. Finally, the function of the AHL-degradative enzymes is still ambiguous. These enzymes may function as quorum quenchers; however, they may also be involved in the central metabolism of a cell.

We end this investigative, possibly controversial review with Einstein's guiding principle behind science itself, ''Never stop asking questions.''

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CHAPTER 8

Rhizobacteria and Plant Sulfur Supply

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I. INTRODUCTION

Sulfur is an absolute requirement for growth of all organisms. Its most important role is in the amino acids, cysteine and methionine, in proteins, where cysteine residues, in particular, are important in determining enzyme structure, and the metal-binding properties of sulfur help catalyze a variety of enzyme reactions. Sulfur is also present in many enzyme cofactors (thiamine, coenzyme A, and biotin). In anaerobic environments, its various redox forms play a crucial part in providing energy for many microorganisms, whereas in plants it is important not just as a constituent of proteins but also as part of several stress responses (Bloem *et al*., 2005). The metal-chelating properties of sulfur in phytochelatins help alleviate heavy metal stress, while glutathione is important in the plant cell's response to both heavy metals and oxidative stress (Meyer and Hell, 2005; Sharma and Dietz, 2006). Sulfur is also important to the plant in responding to pathogen attack, since many defense compounds contain sulfur including glucosinolates and alliins (Brader *et al*., 2006; Jones *et al*., 2004b). Elemental sulfur itself is also used directly by plants to combat fungal infection, being deposited in the xylem parenchyma by a range of different plant species (Cooper and Williams, 2004).

Plants synthesize cysteine from inorganic sulfate, taking it up either from the soil or from the atmosphere via wet or dry deposition (Agrawal, 2003). However, sulfur supply to crops has increasingly become problematic, and sulfur deficiency symptoms have become more frequent, both in Europe and elsewhere (Zhao *et al*., 2006). The introduction of stricter controls on the sulfur content of fuels means that the amount of sulfur released into the atmosphere has fallen considerably (Fowler *et al*., 2005), while inputs into the soil have been reduced by the development of more refined triple superphosphate fertilizers that are low in sulfate. The situation has been exacerbated by simultaneous increases in crop yields due to the development of high yield varieties. The sulfur that is naturally present in soils is largely bound to polymeric organic molecules and is not immediately plant-available. Understanding how crop sulfur deficiency can be remedied therefore requires an understanding of how this organic S pool is transformed by soil microorganisms, and how this sulfur is made available to plants.

In many ways, the problems that plants face in mobilizing organic S from soils are similar to those seen with phosphorus and in part with

nitrogen. The plant's requirement for P is higher than that for S, but the phosphate present in soil is almost entirely in immobile forms, either as organic-bound (such as phytate) or as insoluble inorganic complexes (Richardson, 2001). Plants are able to access this phosphorus largely through interactions with rhizosphere bacteria and with mycorrhizal fungi, which provide extensive extraradical surface area with which to absorb phosphate. The roots and the mycorrhizal hyphae interact with a variety of free-living soil bacteria and fungi that specialize in solubilizing insoluble P minerals by secreting organic acids (Gyaneshwar *et al*., 2002; Richardson, 2001). Nitrogen supply to the plant is also mediated by interactions with microorganisms, either as symbiotic associations with rhizobia or root colonization by free-living nitrogen-fixing organisms, or via interactions with mycorrhizal fungi that can mobilize organic N from the soil organic matter (Hodge *et al*., 2001). There is now increasing evidence that plant assimilation of bound sulfur in the soil is also mediated by specific groups of microorganisms, and this will be described further in this chapter.

II. ASSIMILATION OF SULFUR BY PLANTS

A. Uptake and assimilation of inorganic sulfate

The plant demand for sulfur varies greatly between species and between different stages of development, with higher requirements observed particularly during vegetative growth and for seed development. Sulfur is taken up through the roots primarily as inorganic sulfate and transported via the xylem to target tissues (Fig. 8.1). Assimilation of sulfate to cysteine occurs mainly in the chloroplasts of developing leaves (Wirtz and Droux, 2005), though cysteine/methionine synthesis has also been observed in the roots and in seeds, where it is important in seed maturation (Cairns *et al*., 2006; Hawkesford and De Kok, 2006; Tabe and Droux, 2001). Sulfur can also be assimilated from atmospheric sources, though this seems to play a lesser role (Durenkamp and De Kok, 2004; Yang *et al*., 2006). Plant sulfate assimilation pathways have been studied in detail, and several good reviews of cysteine biosynthesis and its regulation have appeared (Kopriva, 2006; Saito, 2004; Wirtz and Droux, 2005). Excess sulfate taken up by the plant during periods of low demand for reduced sulfur is deposited in vacuoles and remobilized as required. This often results in vacuolar sulfate deposited in older leaves being mobilized to supply younger leaves in subsequent periods of sulfur limitation, and seems to be regulated not just by sulfur stress, but also by nitrogen stress (Hawkesford and De Kok, 2006). A large family of sulfate transporters has been identified and characterized (14 transporters in Arabidopsis).

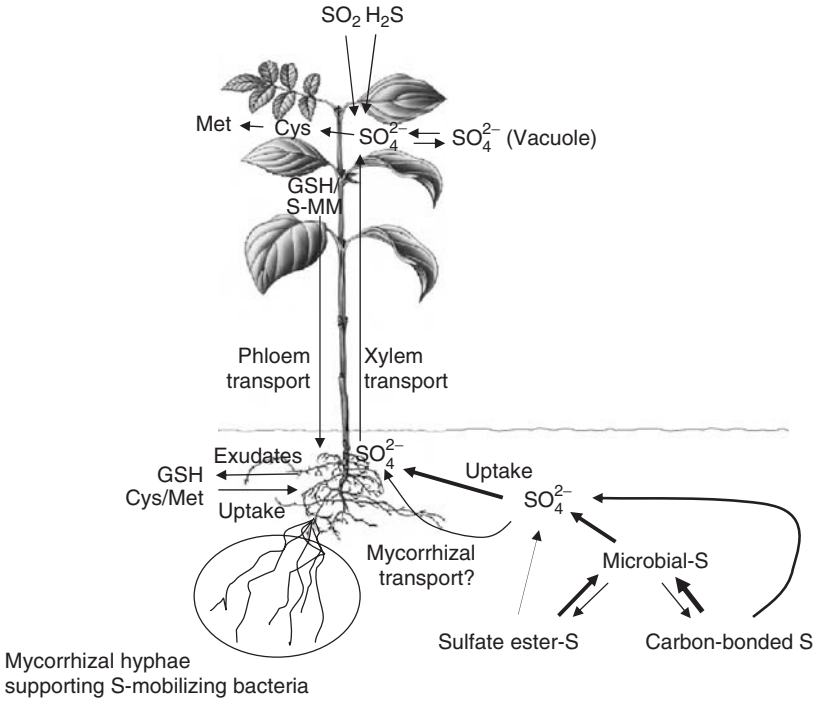

FIGURE 8.1 Overview of sulfur cycling in soil and sulfate assimilation by plants. Cysteine biosynthesis occurs primarily in the leaves using inorganic sulfate transported in the xylem, though some sulfate reduction also occurs in the root (Hawkesford and De Kok, 2006). The sulfur taken up by the root is provided primarily by microbial metabolism of carbon-bound soil sulfur (heavy arrow), especially in the short term. Mycorrhizal hyphae may be involved in the uptake process, but this has not yet been studied in detail, and it is also not yet clear how microbial sulfur is released by bacteria for plant use.

These have been divided into five groups, of which groups I–III play an important role in uptake of sulfate into the roots and transport in and out of xylem and phloem elements, while group IV and V transporters are localized in tonoplast membranes and appear to be involved in sequestration of sulfate in the vacuole (Buchner *et al.*, 2004; Hawkesford, 2003; Hawkesford and De Kok, 2006). These transporters are differentially regulated, and include both high-affinity ($K_m < 10$ μM) and low-affinity ($K_m > 400$ μM) transport systems (Hawkesford, 2003).

Uptake of sulfate into the roots is driven largely by demand, with strong induction of sulfate transport activity under conditions where sulfate is limiting (Buchner *et al.*, 2004). Rapid sulfate uptake is expected to lead to a sulfate depletion zone in the region immediately surrounding

the root, resembling the phosphate depletion zone that has been known for some time (Hinsinger *et al*., 2005; Jones *et al*., 2004a). To examine this, bacterial reporter strains carrying transcriptional *gfp* fusions to bacterial loci that are known to be induced under conditions of sulfate limitation [*ssu, ats, and asf* (Kertesz, 2004)] were introduced into Arabidopsis rhizosphere and gene expression monitored by confocal microscopy (Fig. 8.2).

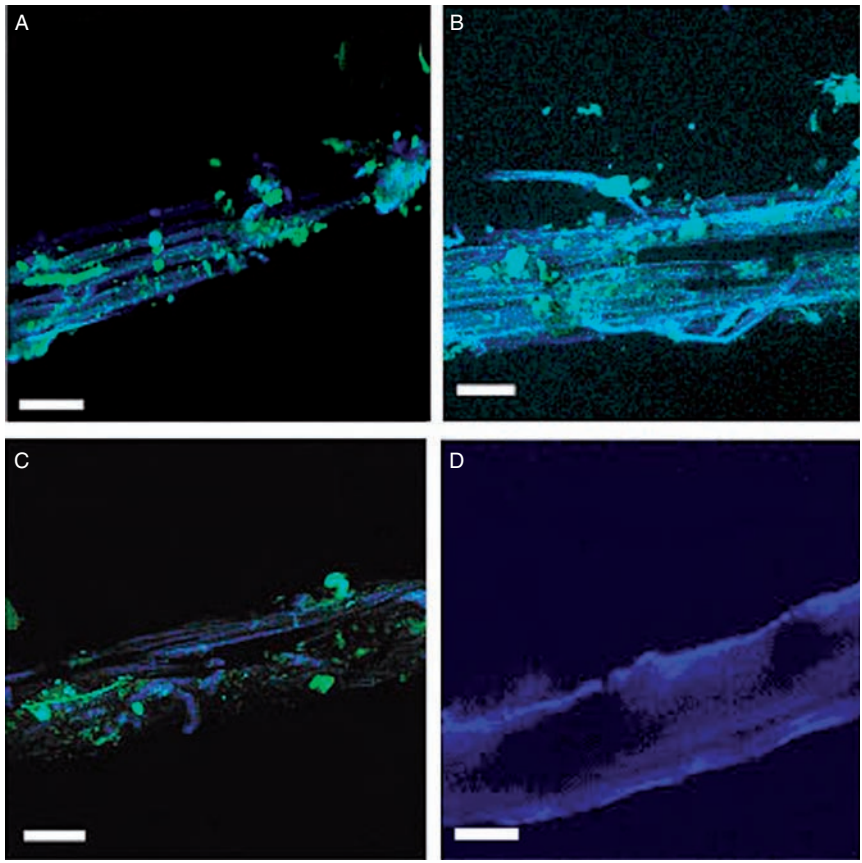

FIGURE 8.2 Rhizosphere expression of bacterial genes related to organosulfur utilization. (A) *asfA::gfp* fusion. (B) *ssuE::gfp* fusion. (C) *atsB::gfp* fusion. (D) Promoterless *gfp* reporter vector with no added insert. Green represents GFP-positive bacteria, and blue is Arabidopsis autofluorescence. Arabidopsis seedlings were inoculated with *P. putida* S-313 carrying a medium-copy *gfp* reporter plasmid and grown for 21 days. Roots were then fixed in 0.1% (w/v) agarose and root sections examined by confocal laser scanning microscopy. All images were taken using a 40× objective (scale bar 50 μm) and contain between 20 and 60 *z* sections with a *z* depth of between 20 and 75 μm.

Expression of each of the promoters studied was induced in the rhizosphere, though it was repressed by inorganic sulfate (100 μM) *in vitro*, confirming that a sulfate-depleted zone indeed exists around the root (Fig. 8.2).

Inorganic sulfate is the main form of sulfur that is transported around the plant, but reduced sulfur is also translocated between tissues via the phloem, primarily as glutathione and *S*-methylmethionine. The levels of these compounds in the phloem can be quite high, with *S*-methylmethionine constituting up to 2% of the free amino acid pool in the phloem and glutathione at nearly the same level (Bourgis *et al*., 1999). A plant glutathione transporter family has been identified (Zhang *et al*., 2004) and other oligopeptide transporters are also known (Stacey *et al*., 2006). Both of these transporter families are active in root tissue, suggesting that some plants may not be entirely reliant on pedospheric sulfate as sulfur source, but may be able to access amino acid sulfur in the form of peptides as well.

B. Amino acids/peptides as a source of plant sulfur

The ability of plants to take up cysteine and methionine derivatives from the soil has been little investigated, but it is important because a significant proportion of soil sulfur is present in this form (Freney *et al*., 1975). However, it is also important for another reason. Plants release large quantities of carbon from their roots as exudates, which largely determines both the biological and physical properties of the rhizosphere. One of the main groups of soluble compounds that are subject to rhizodeposition are amino acids (Jones *et al*., 2004a), though there are few detailed studies on the composition of root exudate under different nutrient conditions. A significant proportion of the amino acids transported around the plant are cysteine and methionine derivatives, and if these are released in exudates, it could represent a severe sulfur loss for the plant. Sulfur-containing amino acids have been reported as components of root exudate in grasses (Bertin *et al*., 2003; Biondini *et al*., 1988; Klein *et al*., 1988), but details are unclear, and NMR studies of exudate composition in cereals do not report any cysteine release (Fan *et al*., 1997, 2001). Release of amino acids into the rhizosphere as part of rhizodeposition has often been regarded as a passive diffusion process over which the plant has little control (Bais *et al*., 2006), but there is now increasing evidence that plants are able to regulate this flux. This may either be direct regulation of release, for compounds that are released in small quantities as signal molecules, or an indirect form of control by up-regulating the recapture of exudate material from the rhizosphere (Farrar *et al*., 2003). This latter path seems to be very important in recovering nitrogen lost to the plant as amino acids. Under axenic conditions, influx and efflux occur simultaneously for a range of amino acids including methionine (though

unfortunately cysteine, methylmethionine, and glutathione were not tested) (Phillips *et al*., 2004). The influx is catalyzed by proton-coupled amino acid transporters in the plant membrane, which are highly efficient—under axenic conditions, the methionine influx/efflux ratio for alfalfa, medicago, wheat, and maize ranged from 3.00 to 6.45, suggesting that very little net methionine would be released into the soil (Phillips *et al*., 2004). However, axenic studies of this nature may be misleading since plants exist in nature together with soil microbes, and these microbes have been shown to compete very efficiently for amino acid nitrogen in the soil. (Bardgett *et al*., 2003; Dunn *et al*., 2006). Amino acids in the rhizosphere have a mean half-life of 1.7 hours, with 34% respired and 66% incorporated into bacterial biomass (Jones, 1999). In a fascinating extension to the above experiment with axenic plants, alfalfa plants were treated with a series of microbial products (phenazine, 2,4-diacetylphloroglucinol, or zearalenone) at ecologically relevant concentrations, and the treated roots were found to display greatly increased levels of net amino acid efflux of 16 amino acids including methionine (200–2600% in 3 hours) (Phillips *et al*., 2004). Rhizosphere bacteria therefore not only compete for released amino acids, but also stimulate net amino acid deposition from the roots directly. It should be noted, however, that this has not yet been shown for sulfur-amino acids specifically.

Most evidence for the uptake of amino acids by plants has been obtained in the context of nitrogen metabolism, and direct studies on uptake of sulfur amino acids are more limited. Exposure of bean seedlings (*Phaseolus vulgaris*) to glutathione *in vitro* showed that these plants can take up the tripeptide through the root and transport it to other tissues (Tausz *et al*., 2004). Uptake of glutathione has also been reported for spruce seedlings (Zellnig *et al*., 2000) and in cultured tobacco cells (Schneider *et al*., 1992). Most interestingly, utilization of glutathione by sessile oak seedlings was observed in the presence of the mycorrhizal fungus (*Laccaria laccata*), and detailed studies showed that the mycorrhizal partner played an essential part in the plant's uptake of both reduced sulfur compounds and inorganic sulfate (Seegmüller and Rennenberg, 2002; Seegmüller *et al*., 1996). Although sulfate is undoubtedly the main sulfur source for plants, more research needs to be done on their utilization of reduced pedospheric sulfur in natural systems.

C. Plant assimilation of oxidized organosulfur

The dominant pathways supplying plants with sulfur are uptake and assimilation of inorganic sulfate, and to a lesser extent uptake of sulfur-containing amino acids, as described above. However, the main forms of sulfur found in soils are bound to organic molecules as sulfonates ($C\text{-}SO_3H$) or sulfate esters ($\text{-}O\text{-}SO_3H$). These sulfur species have been

characterized using specific chemical degradation reactions and by x-ray near-edge spectroscopy (XANES) (Section II). However, little is yet known about the detailed chemical structures of compounds carrying them, and these are presumed to be high molecular weight, humic-derived materials. There has therefore been very little study of how plants can utilize these compounds as sources of sulfur, despite the fact that they dominate the sulfur pool in most soils. Some limited data is available from studies with cell suspension cultures of a range of plants including tomato, Arabidopsis, tobacco, parsley, periwinkle (*Caranthus roseus*), campion (*Silene*), and bentgrass (*Agrostis*) (Wietek and Kertesz, unpublished data). In suspension culture, these cells grew readily in minimal media provided with inorganic sulfate (2 mM), but did not show any growth when the sulfur growth was provided as sulfonates (pentanesulfonate, benzenesulfonate) or an alkylsulfate ester (hexyl sulfate). Some growth was observed with arylsulfates (nitrocatecholsulfate and indolylsulfate), but only with the bentgrass culture, and not with any of the dicot species (Wietek and Kertesz, unpublished data). This monocot growth could be due to arylsulfatase activity in the plant tissue, but related work with sterile wheat plants (Knauff *et al.*, 2003) concluded that arylsulfatase activity in plants derives from endophytic bacteria, and not from the plant itself. The cell culture results suggest that undifferentiated cells do not contain transport and assimilation systems for low molecular weight sulfonates and sulfate esters. However, this does not necessarily reflect the situation with whole plants, and further work is required to establish this clearly. When colonized with specific rhizosphere bacteria, barley plants grew as well with toluenesulfonate as with sulfate as source of sulfur (Schmalenberger and Kertesz, unpublished data), providing evidence for the importance of plant–microbe interactions in plant sulfur supply.

III. MICROBIAL TRANSFORMATIONS OF SULFUR IN SOIL AND RHIZOSPHERE

A. Mineralization and immobilization of soil sulfur

Plants potentially face a significant problem in obtaining sulfur for growth because the pool of inorganic sulfate available for plant uptake from the soil is relatively small, usually making up less than 5% of the total soil sulfur, and the dominant forms of sulfur are bound to organic molecules either as sulfate esters or as directly carbon-bound sulfur. Since these forms of sulfur are not directly bioavailable to plants, they must be converted to an inorganic form before they can be taken up by the plants, a process which is thought to be primarily microbially catalyzed (Kertesz and Mirleau, 2004). The sulfate ester and carbon-bound sulfur pools that

predominate in the soil arise partly from deposition of biological material such as plant residues and animal excreta and partly through immobilization and cycling of sulfur compounds within the soil itself. Leaf litter, for example, contains large quantities of plant sulfolipid from the thylakoid membranes, which is a major component of the sulfur cycle (Harwood and Nicholls, 1979), while animal residues are even higher in organosulfur compounds—sheep urine contains 30% of its sulfur as sulfate esters, while the sulfur in sheep dung is >80% carbon-bound sulfur (Williams and Haynes, 1993). Radiolabeling studies with these sheep residues have shown that the deposited organically bound sulfur persists for a long time in pasture soil, whereas sulfate is rapidly leached (Williams and Haynes, 1993). The organic sulfur in soil is not, however, static in nature, but is rapidly converted into other forms by microbial action. Indeed, most of the organic sulfur in soils arises through biological immobilization of inorganic sulfate to organically bound sulfur, and interconversion of the various organosulfur pools in the soil in a highly dynamic process. Within any given soil, all these processes occur simultaneously (i.e., sulfate immobilization to carbon-bound sulfur and sulfate esters, interconversion of the sulfate ester and carbon-bound S pools, and mineralization of sulfur-containing compounds to sulfate), and a succession of studies have evaluated the relative importance of different sulfur pools and fluxes between these pools in providing plant-available sulfur, starting over 30 years ago (Freney *et al*., 1975).

Most of these studies on soil sulfur composition and sulfur flux have defined the soil sulfur pools operationally by their chemical reactivity. Inorganic sulfate is readily quantified by extraction and ion chromatography, but organosulfur moieties bound to undefined macromolecules in the soil or rhizosphere and in the soil humic fraction are more difficult to differentiate. For this purpose the soil organosulfur pool has traditionally been divided into three fractions that differ in their susceptibility to reducing agents: (1) HI-reducible sulfur, which is thought to be primarily sulfate esters; (2) Raney-Ni-reducible sulfur, thought to be composed of amino acids and peptides; and (3) residual carbon-bonded sulfur, thought to be composed of sulfonates, sulfoxides, and heterocyclic sulfur (Autry and Fitzgerald, 1990; Freney *et al*., 1975; Kertesz and Mirleau, 2004). In several more recent studies, the sulfur content in soils and soil-derived humic substances has been classified according to the sulfur oxidation state, using K-edge XANES (Prietzel *et al*., 2003; Solomon *et al*., 2003; Zhao *et al*., 2006). This method can be performed directly on size-fractionated soils (Mirleau *et al*., 2005), but to reduce the background noise in the XANES spectrum, it has become more usual to use humic extracts (NaOH/NaF)—the method extracts 51–72% of the soil organic matter (Solomon *et al*., 2005; Zhao *et al*., 2006), and gives results that are comparable to those obtained with bulk soils (Solomon *et al*., 2003). Using this

method, a study has broken down the sulfur content of soil humics into five fractions according to the energy of the K-edge absorption (Zhao *et al*., 2006), with two fractions consisting of reduced sulfur (poly-, di-, and monosulfides, thiols, thiophenes), two fractions with intermediate oxidation state consisting of sulfoxides and sulfonates, and one with strongly oxidized sulfur, representing ester sulfates. By using combinations of these fractions, the authors were able to refine their speciation of soil sulfur considerably. Classification of soil S by the XANES method has clear advantages in that it is nondestructive and the sulfur fractions are more defined, but it is a synchrotron-based method, and is therefore less suitable for routine determinations of sulfur speciation in field samples.

Over the past 30 years many studies of the transformations of sulfur in soil to provide plant-available S have looked at incorporation of radiolabeled ^{35}S into soil S fractions determined by the wet chemical methods described above (Freney *et al*., 1975), and at transformations of these fractions. It is therefore of some concern that a detailed study on sulfur speciation in subtropical soils was not able to confirm a close correlation between the results of the wet chemical speciation analysis and those obtained by XANES spectroscopy (Solomon *et al*., 2005), in an examination of both bulk soils and humic extracts. In particular, it appears that the proportion of highly oxidized organic S in the soil (ester sulfate-S) is higher when determined by XANES spectroscopy than by the wet degradation techniques (Prietzel *et al*., 2003; Solomon *et al*., 2005). The discrepancy is probably due to differences in chemical reactivity of different subcomponents of the ''HI-reducible sulfur pool,'' leading to incomplete conversion of this pool to H_2S in the degradation reaction (Prietzel *et al*., 2003; Solomon *et al*., 2003). This has particular relevance for analyses of humic substances, since early publications had proposed that much of the ester sulfate-S was not incorporated into the humic fraction (Fitzgerald, 1978), whereas later determinations suggest that a larger pool of this form of sulfur is in fact present in this fraction (Solomon *et al*., 2005).

Immobilization of sulfate into organic matter has been studied primarily by incorporation of ^{35}S-sulfate either with carrier sulfate or in its absence. The labeled sulfate is incorporated initially into the sulfate ester pool (HI-reducible S), and then slowly transformed into C-bonded S (Ghani *et al*., 1993a). Similar conclusions were reached by measuring overall changes in the soil sulfur pools of pasture soil without labeling (Castellano and Dick, 1991). This immobilization of sulfate is clearly microbially mediated, since it was stimulated by preincubation under moist conditions to stimulate bacterial growth (Ghani *et al*., 1993a). Addition of carbon or nitrogen also promoted sulfate immobilization in pot experiments, with addition of glucose (Dedourge *et al*., 2004; Ghani *et al*., 1993a) or organic acids (Vong *et al*., 2003) stimulating sulfate

immobilization. Addition of model root exudates (sugars, organic and amino acids) also enhanced sulfate immobilization, and though addition of cysteine reversed this on an acid brown soil, no effect of cysteine addition was seen in a calcareous soil (Dedourge *et al*., 2004). The incorporation of sulfate into the organic S pool was dramatically increased by the addition of cellulose as a carbon source (Eriksen, 1997b), and interestingly, the yield and sulfur content of ryegrass grown in cellulose-amended soil was decreased significantly (Eriksen, 1997a). This not only demonstrates that bacteria in the soil can successfully compete with plants for sulfate when adequate carbon is provided, but suggests that the most active species in sulfate immobilization may be cellulolytic bacteria or fungi.

In terms of plant nutrition, however, the critical sulfur dynamic is mineralization of organic S. Early studies (Freney *et al*., 1975) suggested that sulfate ester S and C-bonded S contributed about equally to sulfur mineralization, but other research groups have later reported different conclusions. Other researchers have also found that sulfate ester S (HI-reducible S) rapidly decreases in short-term incubations (Goh and Pamidi, 2003), and indeed it seems likely that sulfate esters might be more sensitive to spontaneous or enzymatic hydrolysis than C-bound S. However, the total S pool in planted soil continued to be depleted even after exhaustion of sulfate ester S, reflecting Freney's earlier conclusion that both pools are implicated in plant nutrition (Freney *et al*., 1975). The extraction protocol used has also been shown to have a major effect on the results obtained (Goh and Pamidi, 2003). Clearer results have been obtained using the nondestructive XANES methods, which have shown a good correlation between S mineralization and the amount of reduced and intermediate-valency sulfur in the soil humic fraction (peptide-S and sulfonate-S), but not with high-valency S (sulfate ester-S) (Solomon *et al*., 2003, 2005; Zhao *et al*., 2006). This confirms earlier conclusions, reached from chemical speciation studies (Ghani *et al*., 1992, 1993b), that showed that almost all the sulfur released in short-term incubation studies derives from C-bonded S. Most interestingly, it also complements a report of plant growth promotion by a *Pseudomonas putida* isolate (Kertesz and Mirleau, 2004), which showed that the ability of the isolate to stimulate plant growth was linked to its ability to desulfurize sulfonates (Section V), but not sulfate esters.

B. Transformations of sulfate esters

Although sulfate esters in soil are now thought to play a less important role than C-bonded S in short-term organosulfur mineralization, they nonetheless constitute up to 60% of the sulfur in soil, especially in agricultural soils (Fitzgerald, 1976). Hydrolysis of these sulfate esters to release sulfate is thought to be primarily catalyzed by sulfatases in the soil, and these have been studied extensively, and are commonly used as

an indicator of soil health and of microbial activity in the soil (Freeman and Nevison, 1999; Taylor *et al.*, 2002). Soil sulfatase enzymes are microbial in origin and not released by plants (Knauff *et al.*, 2003), and are either directly associated with microbial biomass, or secreted (intracellular and extracellular enzymes, respectively) (Klose and Tabatabai, 1999; McGill and Cole, 1981). Traditionally, these two pools of sulfatase activity have been distinguished by measuring enzyme activity before and after plasmolytic treatment, either with toluene or by chloroform fumigation (Klose and Tabatabai, 1999; Tabatabai and Bremner, 1970). However, data obtained with an optimized arylsulfatase assay suggest that toluene addition does not release all the intracellular sulfatase activity (Elsgaard *et al.*, 2002), and it is now known that the sulfatases produced by many soil bacteria (e.g., *Pseudomonas*) are intracellular, usually associated with sulfate ester uptake systems (Kertesz, 1999). This may therefore require some reassessment of the use of arylsulfatase activity as a soil marker. However, because microbial arylsulfatase expression is regulated by sulfur supply (Kertesz, 1999), arylsulfatase activity in the soil is nonetheless a useful marker of S metabolism and is correlated with microbial biomass (Klose and Tabatabai, 1999; Klose *et al.*, 1999; Vong *et al.*, 2003) and with the rate of S immobilization (Vong *et al.*, 2003). The ability to hydrolyze sulfate esters is very widespread in soil bacteria—a selection of bacteria isolated nonselectively from garden soil all expressed arylsulfatase under sulfate-limited conditions (Kertesz and Mirleau, 2004), and studies with sulfatase-negative mutants of *P. putida* showed that expression of sulfatase genes is important for bacterial survival in agricultural soils (Mirleau *et al.*, 2005).

C. Microbial sulfur transformations in nonaerobic soils

Most research on sulfur transformations in the soil has concentrated on aerobic agricultural soils, but sulfur deficiency is increasingly also a problem in rice paddy soils (Zhou *et al.*, 2002), which are flooded and largely anaerobic for a considerable part of the rice growth cycle. During rice growth, the bulk of the sulfur taken up by the plant is derived from the C-bonded S pool, while sulfate ester S is mobilized only later in growth (Zhou *et al.*, 2005). The active bacterial community in paddy soils contains a large proportion of sulfate-reducing bacteria (Lu *et al.*, 2006) and the sulfate content of the soil is therefore reduced, with corresponding increases in sulfide. These sulfide levels are higher in unplanted soils than in planted soils (Wind and Conrad, 1997), since planting increases the redox potential around the roots. Oxidation of elemental sulfur to sulfate and to organosulfur is also faster in planted paddy soils, and in unplanted soils it occurs significantly more slowly than for aerobic soils (Zhou *et al.*, 2002). A study with ^{35}S-labeled straw

has shown that sulfide-S is indeed transformed into plant-available sulfate-S (Zhou *et al*., 2006), but it is not clear whether labeled sulfide was derived via direct sulfide release from the straw or through reduction of sulfate produced as an intermediate product. It is also unclear which organisms catalyze the transformation of C-bonded S to plant-available S under anaerobic conditions. Several groups have reported anaerobic desulfurization of sulfonates as sulfur source by clostridia (Chien *et al*., 1995; Denger *et al*., 1996), and sulfonate dissimilation by a range of other species has been extensively studied (Cook and Denger, 2002; Cook *et al*., 2006). However, a study using ^{13}C-RNA stable isotope probing has shown that although sulfate reducers and clostridia are common in paddy soils early in the rice growth cycle, by 90 days the most active bacteria on the root surface are *β*-proteobacteria, in particular *Azospirillum* and several members of the Comamonadaceae (Lu *et al*., 2006). Results in aerobic agricultural soils have also implicated the Comamonadaceae as important organisms in mobilizing organic S (Schmalenberger and Kertesz, 2007), and it now seems likely that this is also the case in paddy soils, in the aerobic region surrounding the roots.

D. Sulfur transformations by fungi

Fungi play an important role in the rhizosphere both as saprophytic organisms and in direct interaction with the plant as mycorrhizae. Many soil fungi contain arylsulfatase, and there has been considerable early work on these sulfatases in filamentous fungi (Burns and Wynn, 1977; Fitzgerald, 1976; McGuire and Marzluf, 1974). These investigations do not seem to be related to questions of sulfur cycling in the soil or rhizosphere, but as arylsulfatase activities in filamentous fungi are derepressed under sulfate-limited conditions just as in bacteria (Marzluf, 1997), it seems highly likely that they are also important in the mineralization of sulfate esters in the soil. In *Salix* mycorrhizosphere, the levels of arylsulfatase activity were indeed related to the saprotrophic fungi present (Baum and Hrynkiewicz, 2006), and a study of faba bean-colonizing fungi showed that all the fungal isolates obtained had arylsulfatase activity (Omar and Abd-Alla, 2000). By contrast, there is no evidence that fungi catalyze the desulfonation of sulfonic acids. The white rot fungus *Phanerochaete chrysosporium*, for example, catalyzes the transformation of linear alkylbenzenesulfonates exclusively on the side chain, and without desulfurization (Yadav *et al*., 2001), and although sulfonated dyes are extensively used as substrates for fungal laccases and peroxidases, the decolorization observed does not represent desulfonation (Wesenberg *et al*., 2003). However, these fungal enzymes may play an important role in the rhizosphere sulfur cycle nonetheless, since lignolytic degradation of sulfonated humic materials will release monomeric or oligomeric

sulfonates that can be desulfonated by other organisms. Details of these interactions remain to be elucidated in the future.

Mycorrhizal fungi also have the potential to be important in rhizosphere sulfur metabolism, since their extraradical hyphae provide an intimate link between the root and the extended soil environment (hyphosphere). Here again, however, very little research has been carried out. Sulfate uptake and assimilation in the ectomycorrhizal fungus *Laccaria bicolor* has been shown to be up-regulated by sulfate limitation (Mansouri-Bauly *et al*., 2006), and the fungus also harbors a glutathione uptake system that is independent of general peptide transporters (K_m 188 μM). Hyphal growth of the arbuscular mycorrhizal fungus *Glomus intraradices* was stimulated strongly by the addition of MES (2-(*N*-morpholino)-ethanesulfonic acid) (Vilariño *et al*., 1997) to mycorrhized *Trifolium* seedlings, suggesting that the fungus may be able to desulfonate the MES substrate. However, the authors concluded that the fungus is not itself able to access the sulfonate-S directly, and that the observed hyphal stimulation arose from interactions with bacteria that were able to desulfurize MES (Vilariño *et al*., 1997). These synergistic interactions between mycorrhizal fungi and bacteria in the hyphosphere are likely also to be very important in mobilization of naturally occurring organic sulfur, but have not yet been investigated in detail.

IV. FUNCTIONAL SPECIFICITY OF BACTERIA IN SOIL SULFUR TRANSFORMATIONS

Most of the transformation of organically bound sulfur to provide plant-available sulfur is clearly microbially mediated, but little is yet known about the specific microbes that catalyze these reactions. The comparison with phosphorus and nitrogen supply to the plant is a useful one here. Plants gain their fixed nitrogen through interactions with specific groups of microorganisms, with the close symbiosis with rhizobia providing most of the required nitrogen for legumes, while other plants obtain nitrogen through the less intimate association with free-living nitrogen fixers. In the case of phosphorus, most of the P in the soil is sequestered in insoluble, mineral-bound form and is unavailable to plants, but it can be mobilized by specific groups of phosphate-solubilizing microbes (Gyaneshwar *et al*., 2002) and fungi (Bucher, 2007; Whitelaw, 2000) to promote plant growth. It has been noted for some time that cycling of sulfur in soils is dependent on the cropping regime applied to these soils (Castellano and Dick, 1991), and soil enzymes related to sulfur metabolism, such as arylsulfatase, also vary with crop rotation (Knauff *et al*., 2003). The bacterial communities that inhabit the rhizosphere on a given soil are strongly dependent on the nature of the host plant (Appuhn and

Joergensen, 2006)—the variation in sulfur transformations with different cropping patterns may therefore reflect plant-mediated selection for particular microbes that are specialists in the mobilization of organically bound sulfur. Recent work, summarized below, has provided the first evidence of what these might be.

A. Sulfonate desulfurization by rhizosphere bacteria

The ability to desulfurize sulfonates (and in particular aliphatic sulfonates) is widespread in bacteria isolated from soil and rhizosphere. In a survey of random isolates from soil and water environments (King and Quinn, 1997), over 90% of randomly selected (but morphologically variable) strains were able to grow with C2-sulfonates as their sole source of sulfur. No studies have yet been done on the phylogeny of these sulfonate-utilizing species, and so the phylogenetic distribution of desulfonation is still unclarified. The ability to use sulfonates as sulfur source has been best explored in *P. putida* S-313 (Kahnert *et al*., 2000, 2002; Vermeij *et al*., 1999), an isolate of a typical rhizosphere species even though the specific strain studied was not obtained directly from rhizosphere (Zürrer *et al*., 1987). This strain is able to desulfurize not only aliphatic sulfonates such as taurine or isethionate, but also aromatic sulfonates, and its plant-growth promoting properties have been linked to its ability to catalyze the desulfonation of arylsulfonates (Section V). The key enzyme in the desulfonation of both aryl and alkanesulfonates is the monooxygenase SsuD, a member of the extended family of two-component nonflavin-containing reduced flavin-dependent monooxygenases (Valton *et al*., 2006). The SsuD enzyme is a homotetramer that cleaves aliphatic sulfonates to the corresponding aldehydes, releasing sulfite for cysteine biosynthesis [K_m values for pentanesulfonic acid—189 μM, *Escherichia coli* SsuD enzyme (Eichhorn *et al*., 1999); 149 μM, *P. putida* SsuD enzyme (Tralau and Kertesz, unpublished data)]. Unlike SsuD from *E. coli*, the *P. putida* SsuD enzyme is also able to catalyze the release of sulfite from toluenesulfonate, though at a significantly slower rate (Tralau and Kertesz, unpublished data). However, growth of this strain with toluenesulfonate or other arylsulfonates as sulfur source requires not only an intact *ssuD* gene but also the presence of an additional locus containing the *asfAB* genes (Vermeij *et al*., 1999), which encode a reductase/ferredoxin system that is required specifically for cleavage of arylsulfonates and not with aliphatic sulfonates (Vermeij *et al*., 1999). Most probable number analysis suggests that wheat, barley, and *Agrostis* rhizospheres support a population of about 10^5–10^6 toluenesulfonate-desulfurizers per gram root fresh weight (Schmalenberger, unpublished data), and other isolates that can desulfurize toluenesulfonate have been obtained by direct plating of extracts from barley rhizosphere

(Schmalenberger and Kertesz, 2007). Phylogenetically, most of these isolates belong either to the β-proteobacteria (several *Variovorax* isolates, but also strains of *Acidovorax,* and *Polaromonas*) or to the actinobacteria (primarily *Rhodococcus* strains). Most of these isolates contained well-conserved *asfAB* genes, and mutagenesis of the *Variovorax paradoxus asfA* gene confirmed that the *asfA* gene product is also required for aryldesulfonation, just as in *P. putida* S-313 (Schmalenberger and Kertesz, 2007). However, several isolates have also been obtained that do not appear to harbor *asfA*, including a *Delftia* strain, and though they grow with toluenesulfonate as sulfur source, they differ from *P. putida* S-313 in that they do not release cresol as desulfonation product, and presumably use a different desulfonation mechanism. A *Delftia* isolate has previously been reported to cleave arylsulfonates as part of the carbon cycle (Schleheck *et al*., 2004; Schulz *et al*., 2000), and it will be interesting to determine whether it uses the same desulfonation mechanism to provide sulfur for growth.

Most of the organic sulfur present in the soil is bound to high molecular weight humic materials, and it is difficult to see how intracellular enzyme systems like those described above can catalyze soil sulfur cycling. Some initial evidence is now available that rhizosphere bacteria can also utilize polymeric sulfonates, with MPN analysis revealing 10^4–10^5 bacteria per gram root (FW) able to use lignosulfonate as sulfur source (MW about 80,000) (Schmalenberger, unpublished data). This suggests that these bacteria contain either lignolytic activities that can release monomeric or oligomeric sulfonated fragments for uptake into the cell, or that an uncharacterized extracellular sulfonatase activity is present. These possibilities have not yet been examined further, and no isolates have yet been reported that can grow reliably with lignosulfonate as sole sulfur source. It is important to note that the presence of desulfonating bacteria in the rhizosphere does not necessarily imply that these are actively involved in mutualism with the plant. In the complex community of the rhizosphere, it seems most likely that bacteria, fungi, and plants are all involved in transformation of organosulfur, and much remains to be elucidated about the specific roles that different organisms play.

B. Diversity of desulfonation genes in rhizosphere

Research into the role of the *ssuD* and *asfA* gene products for bacterial desulfonation has necessarily concentrated on bacterial species that can be cultivated *in vitro*. Since the vast majority of soil bacterial species cannot (yet) be grown in the laboratory, this work needs to be confirmed and extended by experiments that use molecular techniques to explore the function of the uncultivable portion of the rhizosphere microbial community. An initial approach to assigning desulfonation function to phylogeny within the rhizosphere can be made by assessing the distribution of

the known genes for organosulfur metabolism in the bacterial genome sequences that have currently been determined (457 completed genome sequences at the time of writing). The distribution of plant-associated bacteria within the bacterial kingdom is shown in Table 8.1 (Beattie, 2006), with an indication of the distribution of genome sequences that have been determined. The distribution of *ssuD* homologues is fairly broad, showing that desulfonation is a fairly common ability in bacteria, though it is clear that SsuD homologues are more common in families that have plant-associated members (Table 8.1). SsuD homologues have been defined here by BLAST analysis (Altschul *et al*., 1990), including only homologues for which the encoded protein contains the active site residues reported for *E. coli* SsuD (Eichhorn *et al*., 2002). By contrast, *asfA* homologues have only been found in very few species, and these are almost all plant-associated, including members of the *Azotobacter, Acidovorax, Cupriavidus, Polaramonas, Pseudomonas, Variovorax, Sinorhizobium, Anabaena,* and *Nostoc* genera [(Schmalenberger and Kertesz, 2007) and unpublished results]. The *asfA* genes in these organisms are not all associated with the ability to utilize arylsulfonates, as the *Pseudomonas, Variovorax*, and *Cupriavidus* species grew with toluenesulfonate as sulfur source, but the others did not. Alignment of the AsfA sequences revealed that they clustered into several groups, corresponding to the ability of the host strains to desulfurize arylsulfonates (Schmalenberger and Kertesz, 2007). More importantly, molecular analysis of *asfA* genes obtained from field barley rhizospheres revealed the presence of a considerable diversity of sequences homologous to *asfA*, and these sequences all fell within the group of *asfA* genes obtained from strains that are active in desulfurizing a broad range of sulfonates, including *Pseudomonas* and *Variovorax* (Schmalenberger and Kertesz, 2007). This suggests that the *asfA* gene is widespread in rhizosphere and that it plays an important role in sulfonate utilization. Interestingly, the sequences obtained are all closely related to *asfA* sequences from a small group of β-proteobacteria within the Comamonadaceae family, and this, together with the fact that most of the desulfonating isolates obtained are also from this family (*Variovorax, Acidovorax, Delftia, Polaromonas*), indicates that this group of bacteria may have some degree of specialized function in the rhizosphere related to desulfonation. The exception is the strain in which the *asfA* gene was originally identified, *P. putida* S-313, which is a γ-proteobacterium. However, it is possible that the ability to desulfurize arylsulfonates and arylsulfate esters may have entered this strain by horizontal gene transfer, since the sequenced strain *P. putida* KT2440 does not catalyze these reactions and comparison of the sequenced genome of *P. putida* KT2440 with the regions flanking the *ats* and *asf* loci in strain S-313 suggest that they may have been inserted at the PP210 and PP183 loci, respectively (Schmalenberger and Kertesz, 2007).

TABLE 8.1 Distribution of desulfonation-related genes in bacteria

Phylum	Class	Order/suborder	Genome sequences[a]	plant-associated	SsuD	AsfA
Cyanobacteria		Chroococcales	29			
		Gloeobacteria	1			
		Nostocales	14		X	X
		Oscillatoriales	5			
		Prochlorales	11			
Bacteroidetes/ Chlorobi			44			
Proteobacteria	α-Proteobacteria	Rhodospirillales	19		X	
		Rickettsiales	30			
		Rhodobacterales	43		X	
		Sphingomonadales	17		X	
		Caulobacterales	3		X	
		Rhizobiales	74		X	X
		Parvularculales	1			
	β-Proteobacteria	Burkholderiales	108		X	X
		Hydrogenophilales	1			
		Methylophilales	2		X	
		Neisseriales	10			
		Nitrosomonadales	10			
		Rhodocyclales	5			
		Acidolithiobacillales	3			
		Xanthomonadales	24		X	
		Cardiobacteriales	1			

Firmicutes	γ-Proteobacteria	Thiotrichales	12			
		Chromatiales	5			
		Legionellales	13			
		Methylococcales	1			
		Oceanospirillales	7			
		Pseudomonadales	56		X	X
		Alteromonadales	10			
		Vibrionales	50			
		Aeronomadales	10			
		Enteriobacteriales	194		X	
		Pasteurellales	41			
	δ-Proteobacteria		32			
	ε-Proteobacteria		49			
	Clostridia	Clostridiales	41			
		Thermoananaerobacteriales	1			
		Halanaerobiales	5			
	Mollicutes	Mycoplasmatales	16			
		Entomoplasmatales	10			
		Acholeplasmatales	12			
		Anaeroplasmatales	1			
	Bacilli	Bacillales	183		X	
		Lactobacillales	163			
		Order Actinomycetales				
		Actinomycinaeae	2			
		Micrococcineae	15			
		Corynebacterineae	74		X	X

(*continued*)

TABLE 8.1 (*continued*)

Phylum	Class	Order/suborder	Genome sequences[a]	plant-associated	SsuD	AsfA
Actinobacteria	Actinobacteria	Micromonosporineae	3			
		Propionibacterineae	7			
		Streptomycineae	24		X	
		Streptosporagineae	1			
		Frankineae	6		X	
		Order Bifidomycetales	17			
Planctomycetes			2			
Chlamydiae/ Verrucomicrobiae			16			
Spirochaetes			87			
Fibrobacteres/ Acidobacteria			2			
Fusobacteria			6			
Aquificae			2			
Thermotogae			5			
Deinococcus			13			
Chloroflexi			8		X	
Nitrospiria			4			

[a] Number of sequenced genomes and large plasmids available for each grouping (NCBI).
The presence of the desulfonation genes *asfA* and *ssuD* in different bacterial phylogenetic groupings was determined by BLAST analysis of sequenced microbial genomes. This is compared with the phylogenetic distribution of plant-associated bacteria (shown in gray), as recently reported (Beattie, 2006), and with the number of sequences available for each grouping (genomic sequences and naturally occurring plasmids). Taxonomic classifications within phyla are only shown for those phyla containing plant-associated species.

C. Changes in microbial community with sulfur supply

If certain bacterial families are indeed responsible for a large proportion of the sulfonate cycling in the rhizosphere, then it might be expected that specific bacterial communities might develop in the rhizosphere under defined conditions of sulfur supply, and in particular under conditions of sulfate deficiency. Changes of this nature have been previously observed in a related study on phosphorus supply in which variations in the phosphorus fertilization of chickpea or canola led to significant changes in the bacterial community in the respective rhizospheres (Marschner *et al*., 2004). There has been almost no analysis of the effect of soil sulfur supply on bacterial communities to date, but the first results are now emerging from recent studies carried out on the Broadbalk long-term field experiment at Rothamsted, United Kingdom (Schmalenberger *et al*., 2007). Bacterial communities were analyzed by PCR-DGGE of 16S genes, using DNA isolated from the rhizospheres of field-grown wheat plants grown in plots that had been treated either with NPKS-fertilization or with NPK fertilization without sulfate for the previous 5 years (Schmalenberger *et al*., 2007). Although there was some variation across each plot, cluster analysis showed that distinct bacterial communities had developed in the two plots (Fig. 8.3). The effect on fungal communities was less pronounced, as determined by 18S-PCR DGGE and ITS-DGGE (not shown). Initial sequence analysis of individual bands from within the 16S-DGGE profile suggests that the bands with increased prominence in the sulfate-starved community are derived from *Variovorax* species (Schmalenberger, unpublished data), confirming the results obtained from cultivation-dependent studies.

A more general analysis to link microbial community composition to sulfur function in soil sulfur metabolism has been carried out in tropical plantation soils of various ages (Waldrop *et al*., 2000). Communities were characterized by phospholipid fatty acid analysis (PLFA), and correlated with soil enzyme activities, including sulfatase activity, which is known to respond to sulfate limitation. Sulfatase activities were found to be highly correlated to microbial biomass and to community composition ($p < 0.0001$). Interestingly, whereas changes in phosphatase activity were correlated with those in a wide range of different PLFA, changes in sulfatase activity were correlated ($p < 0.005$) with far fewer biomarkers (Waldrop *et al*., 2000), suggesting specificity in the response to reduced levels of sulfate (and hence increased sulfatase activity). Unfortunately, the PLFA method is not amenable to determination of the individual species or genera associated with this change (Zelles, 1999), and hence putative microbial specialist species were not identified. Different results were obtained in an analysis of maize rhizospheres in which soil enzyme activities and microbial communities were measured at different

FIGURE 8.3 Variation in rhizosphere community with sulfur supply. Total DNA was isolated from the rhizosphere of wheat plants growing on the Broadbalk long-term experiment at Rothamsted, United Kingdom. The bacterial rhizosphere communities were analyzed by PCR-denaturing gradient gel electrophoresis (DGGE) of the V3 region of the 16S rRNA gene (Schmalenberger *et al*., 2007). Replicate samples were analyzed from a field strip that had been fertilized with sulfate (high SO_4) and one with no added sulfate for 5 years (low SO_4). Cluster analysis showed significant differences in community structure due to the different fertilization regime on the two strips (not shown).

distances from the root, in an *in vitro* system (Kandeler *et al*., 2002). In this case, no significant correlation between arylsulfatase activity and community could be determined, suggesting that there may be critical differences in the way microbial communities react to different sulfur conditions in the rhizospheres of different plant species, as previously reported for phosphorus (Marschner *et al*., 2004).

D. Sulfatase genes in rhizosphere

Although several studies have now shown that the main pool of soil organosulfur that is mobilized for plant use is the carbon-bound pool, sulfate esters also provide sulfur for plant growth and there has been a great deal of research on arylsulfatase activities in the rhizosphere. Arylsulfatase activity in the rhizosphere varies greatly with changes in soil type, soil horizons, soil organic matter content, and plant species (Klose and Tabatabai, 1999; Klose *et al*., 1999). These factors are known to be major drivers of microbial community composition in the rhizosphere, and it seems likely that the changes in sulfatase activity observed may be controlled largely by changes in community structure. No detailed studies have been done on the diversity of sulfatase genes in the soil, so it is not yet known whether there are specialist soil microbes that contribute disproportionately to the overall sulfate ester mobilization activity. However, analysis of sequenced microbial genomes (as in Section IV.B) suggests that this is unlikely to be the case as arylsulfatase genes are found in a very broad range of species. These include not only bacteria but also fungi and higher organisms, although plants do not appear to contain sulfatases themselves (Knauff *et al*., 2003). In addition, the ability to express these genes appears to be important for bacterial soil survival, since a mutated *P. putida* strain that was unable to use sulfate esters *in vitro* displayed reduced soil competence (Mirleau *et al*., 2005). Importantly, the so-called arylsulfatases are not specific for aromatic sulfate esters, but catalyze the cleavage of a variety of aromatic and sugar sulfates (Kertesz, 1999).

Three other families of sulfatases have been identified in soil bacteria, and potentially these could also contribute to sulfate ester dynamics in the rhizosphere. The *atsK* gene of *P. putida* S-313 encodes a sulfur-regulated alkylsulfatase (Kahnert and Kertesz, 2000) that catalyzes the dioxygenolytic cleavage of a range of alkyl sulfates. *atsK* homologues are quite frequent in sequenced genomes, but are closely related to taurine dioxygenase homologues (both belong to the α-ketoglutarate-dependent dioxygenase family (Hausinger, 2004), and there is no obvious association of strains containing these genes with soil or plant environments. A second group of alkylsulfatases are the serine-dependent enzymes that have been well characterized biochemically in the past (Dodgson *et al*., 1982), especially in pseudomonads. The first gene sequence for a member of this group has been reported for the *sdsA1* gene of *P. aeruginosa* (Hagelueken *et al*., 2006). It is related to *β*-lactamase proteins, and there is no evidence that it plays a role in soils or rhizosphere. The third enzyme family known to cleave sulfate esters is the arylsulfotransferase group. These enzymes have been largely neglected, but it has been shown that *P. putida* S-313 lacks an arylsulfatase, and its utilization of arylsulfates as sulfur source

requires an arylsulfotransferase (Kahnert *et al.*, 2002). This has some implications for measurements of soil sulfatase activity, since nitrophenol release from nitrophenylsulfate [the standard assay for soil sulfatase activity (Elsgaard *et al.*, 2002; Klose and Tabatabai, 1999)] could well reflect sulfotransferase activity, since phenolic compounds in the soil organic matter could act as sulfate acceptors for the reaction. However, genes related to the *P. putida* arylsulfotransferase gene (*astA*) are not very common, though interestingly there are *astA* homologues in strains of *Shewanella* and *Geobacter*.

E. Influence of mycorrhizal interactions on sulfur supply

As described above, little is known about the molecular details of sulfate ester and sulfonate utilization by fungi. However, mycorrhization of a number of plants has been shown to enhance plant sulfur uptake, suggesting that the fungus–plant interaction and undoubtedly bacterial–fungus–plant interactions are important in this respect. Sulfate uptake in clover and maize was enhanced by the presence of arbuscular mycorrhizae (Banerjee *et al.*, 1999; Gray and Gerdemann, 1973), and the loading of sulfate into the xylem of oak and beech trees was enhanced by ectomycorrhizal associations (Kreuzwieser and Rennenberg, 1998; Seegmüller *et al.*, 1996). Indeed, in the presence of ectomycorrhiza, a study has concluded that woody plant roots (*Pinus banksiana*) are almost entirely dependent on the fungus for sulfate uptake through the fungal mantle surrounding the root tips (Taylor and Peterson, 2005).

It is now generally accepted that mycorrhizal hyphae are heavily colonized by bacterial communities in what is termed the mycorrhizosphere and that the biological interactions between these microbes, the hyphae, and the plant have a significant influence on the chemical and physical properties of the root system (Artursson *et al.*, 2006; Bending *et al.*, 2006). This may relate directly to the supply of nutrients to the plant—in *Medicago sativa*, for example, phosphate-solubilizing bacteria are enriched in the mycorrhizosphere of the arbuscular mycorrhizal fungus *Glomus mosseae* (Toro *et al.*, 1998) and in the ectomycorrhizosphere of Douglas fir P-mobilizing bacteria are also favored (Frey-Klett *et al.*, 2005). To date there have been no direct studies of how the mycorrhizal–bacterial interaction affects the mobilization of organosulfur from the soil and its transport to plants. However, treatment of Scots pine seedlings with lignosulfonate stimulated their mycorrhization by the ectomycorrhizal fungus *Pisolithus tinctorius* (Niemi *et al.*, 2005) in a dose-dependent manner. This is an important observation, since it relates the plant–mycorrhiza interaction to the presence of polymer-linked sulfonates, which are a good model for the high molecular weight sulfonate fraction in soil organic matter. Further research is required to explore this aspect

of rhizosphere sulfur metabolism, but it seems entirely plausible that desulfurization of carbon-bound sulfur in soil is carried out by bacteria interacting with mycorrhizae and that delivery of the released sulfur to many economically important crop and tree species is determined by the presence and extent of arbuscular mycorrhizal (AM) and ectomycorrhizal hyphae in the mycorrhizosphere.

V. PLANT GROWTH PROMOTION AND THE SULFUR CYCLE

The main effects of sulfate limitation for crops are chlorosis, a reduction in yield, and a decrease in the quality of the crop (Schnug and Haneklaus, 1998). Such effects have become increasingly common in recent years as the availability of sulfur from the atmosphere has decreased and crop plants are increasingly dependent on pedosphere sulfate. Increased mobilization of soil organosulfur by inoculation with relevant bacterial species would therefore be expected to lead to increased crop yield. The first evidence for plant growth stimulation as part of the sulfur cycle comes from inoculation with the desulfonating isolate *P. putida* S-313. This organism is able significantly to promote the growth of various plant species, including tomato, Arabidopsis, and barley (Fig. 8.4). Mutants of this strain were generated which contained an insertion in the *asf* locus and were no longer able to grow *in vitro* with arylsulfonates as sulfur source—these strains simultaneously lost the ability to promote the growth of tomato plants (Kertesz and Mirleau, 2004) and Arabidopsis (Schmalenberger and Hodge, unpublished data). A reduction of the tomato plant growth promotion effect was also observed for mutants in the *ssu* locus, which are unable to utilize alkanesulfonates *in vitro*, but no change in PGP-capacity was seen for a mutant in sulfate ester utilization (Kertesz and Mirleau, 2004). This suggests that although the ability to use soil sulfate esters is important in bacterial soil survival (Kahnert *et al*., 2002), the main sulfur pool that is mobilized by bacteria to promote plant growth is the sulfonate-sulfur pool, in agreement with other studies (Ghani *et al*., 1993b; Zhao *et al*., 2006). The plant growth promotion effect was strongest under conditions of increased plant density within a pot, suggesting that localized sulfur exhaustion was occurring (Tétard-Jones *et al*., 2007).

Expression of the genes that are required for desulfurization of both aryl and alkylsulfonates by *P. putida* S-313 are repressed *in vitro* in the presence of excess sulfate (Kahnert *et al*., 2000; Vermeij *et al*., 1999). It is therefore hard to explain why these soil bacteria should release excess sulfur from desulfonation processes in order to supply sulfur to the plant, since the desulfonation process itself is closely regulated by the cell's sulfur requirements. One possible solution to this conundrum is that bacterial sulfur is released into the rhizosphere through predation by

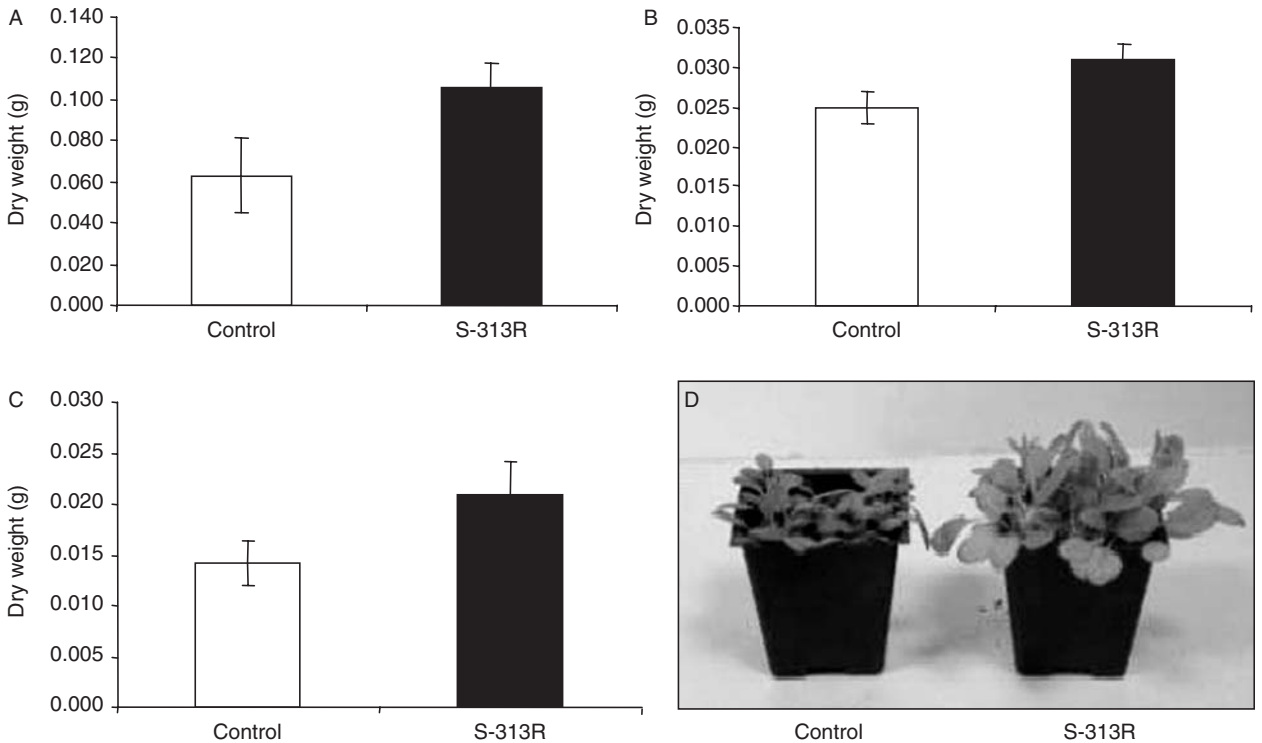

FIGURE 8.4 Growth promotion of different plants by *P. putida* S-313. Seedlings were inoculated with *P. putida* S-313 and grown in unsterilized compost for appropriate times at varying plant densities. Control plants were grown identically, but were not inoculated. (A) Tomato, 5 plants per pot. (B) Barley, 5 plants per pot. (C) Arabidopsis 11 plants per pot. (D) Growth of Arabidopsis in presence or absence of *P. putida* S-313, 14 days.

soil protozoa and subsequent uptake by plants. Protozoan grazing has been shown to have a considerable effect on nitrogen metabolism in the rhizosphere, while also leading to changes in root architecture and hence uptake of nutrients (Bonkowski, 2004; Raynaud *et al*., 2006). A similar mechanism could play a part in sulfur metabolism, but this has not yet been tested. Alternatively, bacterial desulfonation processes may be regulated differently in the rhizosphere than they are *in vitro*. Expression of *asf* and *ssu* promoters from *P. putida* S-313 in the Arabidopsis rhizosphere was examined by construction of transcriptional fusions to the *gfp* gene (Fig. 8.2), and evaluation of GFP fluorescence in rhizo. Similar levels of fluorescence were observed in rhizospheres that were treated with Hoagland's medium and those that were treated with sulfate-enriched Hoagland's medium (500 μM). This suggests that the plant root may be releasing a signal molecule that overrides the bacterially mediated repression of sulfonatase expression in the presence of excess sulfate. The molecular signal involved here has not yet been identified, and the control mechanism clearly needs further investigation.

VI. CONCLUSIONS

It has been established for some time now that much of the sulfur that plants use for growth is derived from the soil organosulfur pool and that the mobilization of this sulfur for assimilation by plants is mediated by the microbial community in the soil and rhizosphere. Recent years have seen some modification of how we understand the details of this microbially mediated sulfur cycling, as technological advances now allow a more defined speciation of the soil sulfur based on molecular spectroscopic studies rather than chemical reactivity. First evidence is that two groups of bacteria (Comamonadaceae and Rhodococci) may play a specialized role in sulfonate cycling in the soil, with the Comamonadaceae being particularly active. Mycorrhizal fungi are also important in providing sulfur to plants, probably in association with bacteria, and protozoa may also play an important role. Our understanding of how soil organosulfur is converted to plant-available sulfur, and how this process is regulated, has made considerable advances in the last few years. Further investigations will expand on this by using a more integrated molecular ecology approach to explore the relevant interactions between plant roots, bacteria, fungi, and protozoa in model ecosystems.

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CHAPTER 9

Antibiotics and Resistance Genes: Influencing the Microbial Ecosystem in the Gut

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I. INTRODUCTION

Genes conferring resistance to antibiotics have been associated with pathogenic bacteria for a long time, and were detected soon after antibiotics first became widely used to treat infections. However, there are an increasing number of publications describing resistance genes harbored by members of the native gut microbiota. This includes the identification of new antibiotic resistance (Ab^R) genes in commensal gut bacteria, for example *tet*(W) (Barbosa *et al.*, 1999), and has consequently led to hypotheses on the role of the gut microbiota as a source of resistance genes potentially transferable to incoming pathogenic bacteria (Salyers *et al.*, 2004; Scott, 2002). Frequencies of gene transfer in the gut are high due to the dense microbial population, and there have been experimental demonstrations of Ab^R gene transfer between pathogenic and commensal bacteria and there is considerable circumstantial evidence from the presence of almost identical genes in diverse bacterial species and genera. Further evidence of the ability of bacterial genomes to adapt to the changing environment is the emergence of resistance to other antibacterial agents (disinfectants and bleaches) (Alekshun and Levy, 1999; Russell, 1999, 2002; Sheldon, 2005) but this will not be discussed further here.

In this chapter, we will summarize the consequences of the widespread use of antibiotics in terms of the prevalence and transfer of bacterial antibiotic resistance genes. We will focus on the gastrointestinal tract (GIT) as a source of bacterial resistance genes and site of gene transfer events, and attempt to address some of the secondary effects antibiotic exposure has on the native gut microbiota. Antibiotic treatment disturbs the normal balance of the gut microbiota, eliminating sensitive bacteria, and this can lead to the development of other specific diseases. We do not presume to include all examples of gene transfer, nor to assess all possible side effects, but aim to highlight some of the more general aspects of antibiotic use that may at times be overlooked.

II. ANTIBIOTIC USE AND THE EMERGENCE OF RESISTANT BACTERIA

Antibiotic selective pressure has certainly contributed to the increased numbers of resistant bacteria, probably due to both a clonal increase of bacteria with a resistant genotype and an increased frequency of gene transfer (Doucet-Populaire *et al.*, 1991; Showsh and Andrews, 1992). In both animal and human medicine, the incidence of Ab^R bacteria correlates to antibiotic treatment (Bergman *et al.*, 2006). The use of antibiotics in the community to treat human illnesses is now discouraged, unless there

is a definite bacterial cause, and could be further reduced by the alternative provision of advice on effective pain relief (Mangione-Smith *et al.*, 2006; van Driel *et al.*, 2006). Antibiotics are poorly absorbed in the gut and a significant amount is excreted unchanged, contributing to the environmental cycling of the selective pressure (Sarmah *et al.*, 2006). Furthermore, Ab^R bacteria in food animals can be detected in meat available for consumer purchase.

Antibiotics are integral to modern farming practice throughout the world and are used in vast amounts (Sarmah *et al.*, 2006), even in countries where their use is regulated. EU legislation (*European Parliament and Council Regulation (EC) No. 1831/2003*), in place since January 2006, has banned the use of all antimicrobial agents in animal husbandry, except as therapeutic agents. The previous legislation preventing the use of ''clinically relevant'' antibiotics as growth promoters in animal husbandry in the EU came into place in 1998. This led to a decline in the use of antibiotics as prophylactic agents, although sales of antimicrobials as therapeutic agents remained largely constant in the United Kingdom between 1998 and 2005 (*Veterinary Medicines Directorate: Report on sales of antimicrobials in the UK in 2005*). In fact, actual sales of both tetracyclines and macrolides increased in the United Kingdom during this period, with tetracyclines accounting for more than half of the annual sales of antibiotics (by tonne).

Limiting the use of antibiotics in animal husbandry does appear to have reduced the carriage of Ab^R bacteria (Witte, 2001), but has not eliminated them. In 1997, prophylactic use of the glycopeptide avoparcin was banned in the EU due to its similarity to vancomycin. Subsequent studies found that carriage of glycopeptide-resistant *Enterococcus faecium* strains had declined (Bager *et al.*, 1999; Klare *et al.*, 1999; Pantosti *et al.*, 1999). One exception was in Denmark where the persistence of glycopeptide resistance *vanA* genes in pigs was attributed to the use of tylosin (now also banned). Tylosin selected for bacteria carrying the macrolide resistance gene *erm*B that was encoded on the same mobile genetic element as the *vanA* gene (Aarestrup, 2000). Generally carriage of antibiotic resistance genes is much lower in Norway (where any antibiotic use is discouraged) than in more intensively reared pig herds elsewhere in Europe where therapeutic antibiotic use persists (Patterson *et al.*, 2007a). Unfortunately, Ab^R bacteria can persist even in the absence of antibiotic selection. Comparison of three farms in the United States indicated that the same *erm*B gene was present in all erythromycin-resistant enterococci, albeit present in only 2% of total enterococcal isolates from farms where tylosin was never used compared to 59% where tylosin was used as a growth promoter (Jackson *et al.*, 2004).

Antimicrobial use has also been linked to the recovery of antibiotic-resistant bacteria from soils (Nwosu, 2001). Soil bacteria that are in contact

with manure or pigsty environments appear to play an important role in the lateral spread of tetracycline and other resistance genes (Agerso and Sandvang, 2005). Spread of untreated animal waste on farmland introduces antibiotic resistance genes originating in bacteria associated with animals and humans to the environment, and leads to the formation of environmental reservoirs of these genes (Jensen *et al.*, 2002) (Fig. 9.1). Analysis of over 750 bacterial isolates from wastewater treatment plants and groundwater wells close to leaking sewers identified resistance to a range of antibiotics (Gallert *et al.*, 2005).

In some cases the occurrence of Ab^R without any antibiotic selective pressure may be the result of coselection of Ab^R determinants together with resistance to other toxic compounds, including detergents, antiseptics, heavy metals (reviewed in Alonso *et al.*, 2001; Baker-Austin *et al.*, 2006). Heavy metal pollution is known to amplify the carriage of Ab^R bacteria in soils, and consequently the complete ecology of a landscape must be considered to appreciate the evolution and dissemination of Ab^R genes (Singer *et al.*, 2006). Even within the human GIT, heavy metal and

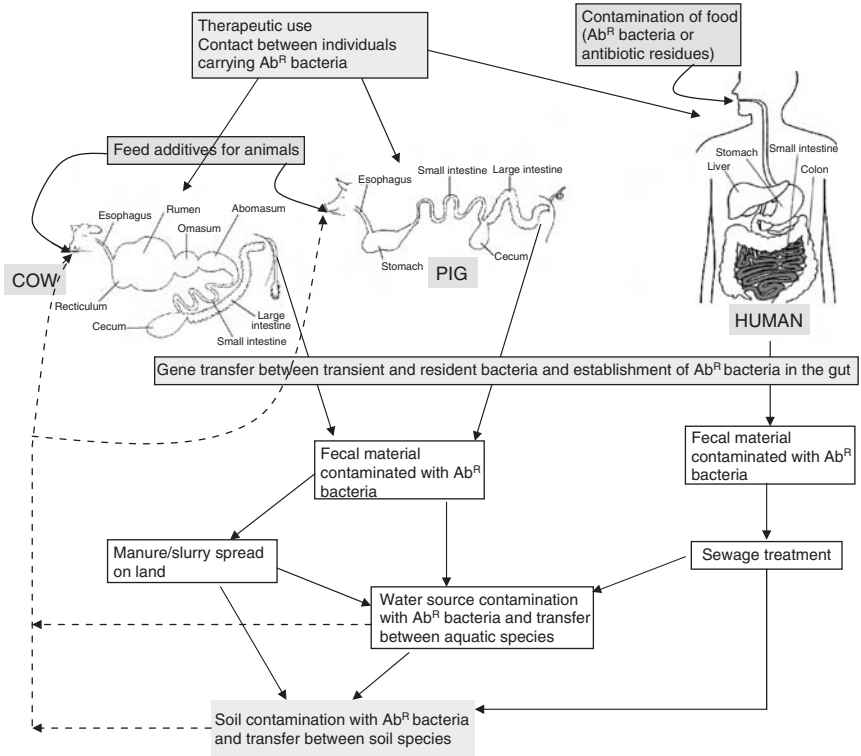

FIGURE 9.1 Schematic representation of antibiotic resistance gene flow in the environment. The cow and pig diagrams—copyright J. A. Moore (with permission).

Ab^R genes can be linked (Davis *et al.*, 2005a,b). This was thought to be a consequence of dental treatment, yet 71% of children with no fillings carried bacteria resistant to mercury and 60% of these bacteria were also resistant to at least one antibiotic (Ready *et al.*, 2003).

Antibiotic resistance genes also disseminate among the human population, and factors other than direct antibiotic exposure can affect the carriage of Ab^R genes and bacteria. Studies on infants that had not been exposed to tetracycline found that 12% of cultivable gut *Escherichia coli* strains were Tc^R, and approximately half of these bacteria were also resistant to another antibiotic (Karami *et al.*, 2006). No difference was found in the incidence of resistant strains between infants who had or had not been treated with other antibiotics, indicating that there was no coselection. Newborn infants up to 3 months old, exclusively breast-fed, who had not been prescribed antibiotics were found to contain some of the same resistance genes as their mothers (Gueimonde *et al.*, 2006). Only a subset of the resistance genes was present, and the prevalence of those predominantly associated with obligately anaerobic bacteria was much lower. This is an example of vertical transfer of resistance, and also implies that the incidence of resistance genes reflects the natural order of bacterial colonization of the GIT in infants (Favier *et al.*, 2003). Sixty-seven percent of individuals living in a remote community in South America, with only limited contact with other societies, carried bacteria resistant to at least one antibiotic (Bartoloni *et al.*, 2004). The resistance genes present were the same as those generally found among individuals exposed to antibiotics. In this case, the resistance genes are predicted to have been acquired by a few individuals from elsewhere, but have been maintained and disseminated through the remote community in the absence of any selective pressure (Pallecchi *et al.*, 2007).

It appears that, once present, Ab^R genes persist in an environment and are hard to eliminate completely. Spread through that environment can be due to horizontal (bacteria:bacteria, person:person, animal:animal) and vertical (mother:offspring) transfer.

III. TRANSFER OF ANTIBIOTIC RESISTANCE GENES BETWEEN BACTERIA

A. Mechanisms of transfer

Gene transfer occurs at high frequencies between both related and unrelated bacteria, and is a driving force for bacterial evolution. Transferable genes may be plasmid encoded, or encoded on mobile pieces of chromosomal DNA. Genes that have been demonstrably transferred between bacteria in laboratory matings include those encoding selective markers

such as antibiotic resistance and virulence (Coburn *et al.*, 2007; Qiu *et al.*, 2006). Metabolic traits can also be transferred between bacteria, and are frequently identified by comparing bacterial genome sequences. For example, the propanediol utilization pathway is presumed to have spread between bacterial species (including *Salmonella* and *Streptococcus*) by horizontal transfer of a large operon encoding the essential genes (Bobik *et al.*, 1999; Xu *et al.*, 2007). Some genes encoded on this operon have also been identified in the human commensal bacterium *Roseburia inulinivorans* (Scott *et al.*, 2006), although the specific arrangement of the genes is different in this bacterium.

The three main mechanisms of gene transfer between bacteria are transformation (uptake of free DNA), transduction (phage-mediated DNA transfer), and conjugation (active DNA transfer between two viable bacterial cells). There is evidence that some of the bacterial species found in the human GIT can uptake and express free foreign DNA (Mercer *et al.*, 1999; Netherwood *et al.*, 2004). The survival time of free DNA under conditions found in the human GIT (Mercer *et al.*, 1999) and rumen (Duggan *et al.*, 2000, 2003) is short but transformation can occur within seconds of DNA release from bacterial cells, and might therefore have an impact on gene transfer between gut bacteria.

Bacteriophages are abundant in the rumen (Klieve and Swain, 1993), and presumably also in the human gut. Thus, they may be important mediators of gene transfer via transduction, but this has not been well studied. One report suggests that transfer of Shiga toxin genes between *E. coli* strains in the gut of houseflies is bacteriophage mediated (Petridis *et al.*, 2006).

Conjugation can occur between both related and unrelated bacterial species and is responsible for the spread of several forms of mobile genetic elements. The transfer of plasmids and conjugative transposons (CTns) between bacteria has been well documented and extensively reviewed, and will not be discussed further here. The reader is directed to articles by Burrus and Waldor (2004), Frost *et al.* (2005), Grohmann *et al.* (2003), Licht and Wilcks (2005), Pembroke *et al.* (2002), Scott (2002), and Whittle *et al.* (2002). In general, transfer of plasmids tends to be species-specific, while CTn transfer leads to the spread, and chromosomal integration, of DNA sequences between related and unrelated bacteria.

Other types of mobile DNA include integrons and insertion sequences (ISs). Integrons are extraordinary DNA elements containing a site-specific DNA recombination system that recognizes and captures mobile gene cassettes (Hall and Collis, 1995). Integrons play an important role in the dissemination of the antibiotic resistance among Gram-negative bacteria (Agerso and Sandvang, 2005; Campbell *et al.*, 2005; Gootz, 2005; Skurnik *et al.*, 2005), but have also been identified in Gram-positive bacteria, including *Corynebacterium glutamicum* (Nesvera *et al.*, 1998), *Enterococcus faecalis* (Clark *et al.*, 1999), various Gram-positive isolates from poultry litters (Nandi *et al.*, 2004), and in Gram-positive *Arthrobacter* spp. and

Corynebacterium spp. isolates from soils in contact with manure or pigsty environments (Agerso and Sandvang, 2005).

Integrons can be divided into two major groups depending on the composition of the gene cassette: the resistance integrons (RI) and super-integrons (SI) (Fluit and Schmitz, 2004; Hall and Stokes, 2004). RIs contain gene cassettes mainly encoding resistance to antibiotics and disinfectants (Paulsen *et al.*, 1993; Sekiguchi *et al.*, 2005) and are located either chromosomally or on plasmids. Over 70 distinct antibiotic resistance gene cassettes have been characterized within RIs (Fluit and Schmitz, 1999; Mazel and Davies, 1999) and up to 7 of these cassettes have even been found simultaneously in a single integron (Naas *et al.*, 2001). SIs are large chromosomally located elements carrying arrays of gene cassettes with a wide selection of adaptive functions, including pathogenicity, metabolic genes, or restriction enzymes.

ISs are small, phenotypically cryptic, genetically compact DNA elements, which usually only encode functions involved in their own mobility (Mahillon and Chandler, 1998; Mahillon *et al.*, 1999; Rice, 2002), although they play an important role in shaping the genomes of their bacterial hosts. Over 500 of these elements have been identified so far, associated with both bacterial chromosomes and also with composite transposons or plasmids. Atypical ISs have been described and are implicated in the spread of many Ab^R genes (Toleman *et al.*, 2006a,b). These common regions (CRs or IS*CR*) can be divided into two groups: those which form complex class 1 integrons (named IS*CR1*) and those that are linked with non-class 1 integrons (IS*CR2–12*) (Walsh, 2006). IS*CR1* is associated with genes encoding resistance to chloramphenicol, trimethoprim, quinolones, and aminoglycosides class C and class A β-lactamases, whereas IS*CR4* flanks the metallo-β-lactamase gene, $bla_{SPM\text{-}1}$. Notably, IS*CRs* are also connected with *Salmonella* genomic islands that harbor pathogenicity functions, and the integrative conjugative element SXT from *Vibrio cholerae* (Toleman *et al.*, 2006a; Walsh, 2006).

B. Why is the gut a good site for gene transfer

The GIT of animals and humans contains a dense and diverse resident microbiota and is continually challenged with incoming food material that may include transient bacterial species. Thus, the potential for gene transfer in this environment is high. It is currently estimated that there are more than 500 different resident bacterial species in the human GIT (Flint *et al.*, 2006; Lay *et al.*, 2005) of which a large proportion (>90%) at present is defined as unculturable (Eckburg *et al.*, 2005), that is they cannot be cultured under the laboratory conditions chosen. Conjugal gene transfer requires two bacterial cells to be in proximity and the biofilms that form in the gut, both on the surface of food particles and on the mucus layer, apparently facilitate this (Licht *et al.*, 1999; Macfarlane and Macfarlane, 2006;

Sorensen *et al.*, 2005). There is evidence that horizontal transfer, even of nonconjugative plasmids, occurs at higher frequencies in biofilms than in liquid cultures between *E. coli* isolates (Maeda *et al.*, 2006). Bacterial conjugation may also influence the development of biofilms since natural conjugative plasmids express factors which can induce the formation of some biofilm communities (Ghigo, 2001; Luo *et al.*, 2005). The high-frequency conjugation system in *Lactococcus lactis* is linked to the cell-clumping phenotype, facilitating the spread of the broad host range, Ab^R plasmid pAMβ1 at higher frequencies than those found for a nonclumping donor strain (Luo *et al.*, 2005). Thus, the biofilms formed by commensal bacteria may act as a reservoir of Ab^R genes and play an important role in their dissemination to other commensal and also to pathogenic bacteria. The exchange of Ab^R genes between commensal and pathogenic bacteria in biofilms could potentially protect antibiotic-sensitive pathogens from eradication, leading to serious medical problems (O'Connell *et al.*, 2006).

Gene transfer has been demonstrated between bacteria normally resident in different ecosystems, and between commensal and pathogenic bacteria. A novel ErmB determinant was transferred between the pathogen *Clostridium difficile* and the rumen commensal anaerobe *Butyrivibrio fibrisolvens* under laboratory conditions (Spigaglia *et al.*, 2005). Similarly, laboratory transfer of tetracycline resistance was possible between unrelated human and rumen commensal bacteria (Melville *et al.*, 2001). *Yersinia pestis*, the bacillus which causes the plague, is an obligate parasite with a complicated life cycle (Titball *et al.*, 2003). The bacterium forms dense aggregates, which resemble bacterial biofilms, in the midgut of its insect vector. Unrelated bacteria cohabiting in the flea digestive tract become incorporated into these aggregates. Ab^R *Y. pestis* strains were isolated from plague patients in Madagascar (Guiyoule *et al.*, 2001), and it was suggested that lateral gene transfer in the flea gut had produced these Ab^R strains (Hinnebusch *et al.*, 2002).

C. *In vivo* demonstrations of resistance gene transfer

Much of the evidence for gene transfer *in vivo* in the gut is circumstantial, based on the discovery of similar genes in unrelated bacterial genera, but there are a number of experimental demonstrations of specific gene transfer events occurring *in vivo* between bacteria in gut ecosystems. Antibiotic selection increases the frequency of gene transfer (Beaber *et al.*, 2004; Hastings *et al.*, 2004), but it is not essential and strains that have acquired resistance determinants can persist without any selective pressure (Licht *et al.*, 2002).

In many cases transfer has been demonstrated between specific bacteria in the gut of gnotobiotic animals. These models simulate the gut ecosystem but enable the addition of large numbers of donor and

recipient bacteria, in a specific order, and permit the recovery of transconjugants without the difficulties of selection against a large background microbiota. Gnotobiotic mouse models were used to demonstrate conjugative transfer of a range of Ab^R determinants at high frequencies between porcine and human strains of *E. faecium* (Moubareck *et al.*, 2003) and of the conjugative transposon Tn*1549* from *Clostridium symbosium* to *E. faecium* and *E. faecalis* (Launay *et al.*, 2006). In the first case, antibiotic selection enhanced the frequency of transfer events and facilitated subsequent colonization by the transconjugants.

Transfer has also been demonstrated in the presence of a background microbiota following ingestion of donor and recipient strains. The *vanA* resistance gene was transferred between *E. faecium* isolates of animal and human origin in the guts of three of six human volunteers (Lester *et al.*, 2006). Although transfer into native gut *E. faecium* strains was not detected, transient colonization of the human gut by animal isolates of *E. faecium* may be long enough for any genes present to be transferred to native human gut species. The *tet*(O) determinant in *Campylobacter jejuni* and *C. coli* was transferred *in vitro* and also in the digestive tract of chickens inoculated with donor and recipient *C. jejuni* strains in the absence of selective pressure (Avrain *et al.*, 2004). A tetracycline resistance determinant transferred between *E. coli* isolates of animal and human origin *in vivo* using mouse and chicken models (Hart *et al.*, 2006). In this case, transfer frequencies increased on administration of tetracycline to the drinking water. An additional antibiotic resistance gene was cotransferred, either simultaneously on the same plasmid or on a second distinct self-transmissible or mobilizable plasmid (Hart *et al.*, 2006). In addition to increasing gene transfer frequencies, oral administration of oxytetracycline to chickens in the water supply on commercial chicken farms is speculated to contribute to the persistence of Tc^R *C. jejuni* isolates. This may be due to the selection of resistant strains in biofilms in the water pipes causing reinfection of subsequent flocks of chickens (Fairchild *et al.*, 2005).

IV. CONSEQUENCES OF ANTIBIOTIC USE

A. Increased carriage of resistant bacteria and resistance genes and the emergence of bacterial strains carrying multiple resistance genes

Antibiotic selection causes an increase in the proportion of resistant bacteria present in the host (as discussed in Section II). One consequence of this is increased shedding of resistant bacteria, and further spread through the population. Animal herds are particularly predisposed to this spread due to contamination of the pasture, or to the proximity of

TABLE 9.1 Incidence of antibiotic resistance in gut bacteria with different exposures to antibiotics

Source of the fecal sample	Ab^R bacteria isolated from feces (%)
Organic pig farm[a]	12–45
Intensive pig farm[a]	79–92
Human not treated with antibiotic[b]	<0.01
Human treated with antibiotic[b]	>99

[a] Data adapted from Patterson *et al.* (2007a).
[b] Data adapted from Scott *et al.* (2000).

the animals when housed inside (Table 9.1, Fig. 9.1). However, Ab^R bacteria also spread through human populations by person-to-person contact (Fig. 9.1). It has been accepted that at least part of the spread of methicillin-resistant *Staphylococcus aureus* (MRSA) in hospitals is due to poor infection control measures or lack of appropriate hand washing; adherence to appropriate guidelines can reduce the spread (Miyachi *et al.*, 2007). The obvious repercussion of any spread of resistant bacterial strains is that the efficacy of the antibiotic ''of choice'' lessens as the incidence of bacterial resistance to that antibiotic rises.

Antibiotic-selective pressure also increases bacterial gene transfer, potentially resulting in the evolution of bacteria carrying several different antibiotic resistance genes. There is a limit to the number of plasmid-encoded resistance genes that can be present in a single bacterium due to size constraints on mobile plasmids and plasmid incompatibility issues that prevent coinfection of a bacterium with several specific types of plasmid. In contrast, incorporation of one type of CTn in a bacterial cell does not seem to preclude association with another. Additionally, in common with integrons (described above), CTns frequently carry several resistance genes conferring resistance to one or more antibiotics. Thus, some of the *Bacteroides* CTns confer resistance to tetracycline [*tet*(Q)] and erythromycin (*erm*F; Salyers *et al.*, 1995) and another putative CTn identified in a Gram-positive *Clostridium* spp. contains at least two tetracycline resistance genes (Scott, Rincon, and Kazimierczak, unpublished data).

B. Evolution of novel forms of resistance genes

Conjugative gene transfer resulting in the coinfection of a single bacterium with two different but related tetracycline resistance genes is also probably the reason for the evolution of mosaic genes. Mosaic forms of *tet*(M) originating from two distinct alleles are thought to have formed by homologous recombination, which may have been driven by selective

pressure (Oggioni *et al.*, 1996). Recombinant forms of tetracycline resistance genes arising from different combinations of progenitor genes have been described (Patterson *et al.*, 2007b; Stanton and Humphrey, 2003; Stanton *et al.*, 2005). Ribosome protection-type tetracycline resistance genes are good candidates for possible recombination events since they are highly conserved with a minimum of 65% identity at the DNA level [see review by Roberts (2005)]. The *tet*(O/W/O) combinations identified in *Megasphaera elsdenii* isolates from swine all conferred greater resistance to tetracycline (and oxytetracycline and chlorotetracycline) than the wild-type *tet*(O) and *tet*(W) genes (Stanton *et al.*, 2004), reinforcing the idea that antibiotic selection resulted in the evolution of the new mosaic resistance genes. Even more complex forms of mosaic genes deriving from three distinct parent genes have been described (Patterson *et al.*, 2007b). One of these, conferring a higher resistance than the others, is composed of six distinct segments, each with >99% sequence identity to the parent gene, *tet*(O/W/32/O/W/O) (Fig. 9.2). These genes were identified in animal fecal samples that had been subjected to intense tetracycline-selective pressure. The mosaic forms of tetracycline resistance genes described in detail to date are all based on *tet*(O), but there is preliminary evidence that

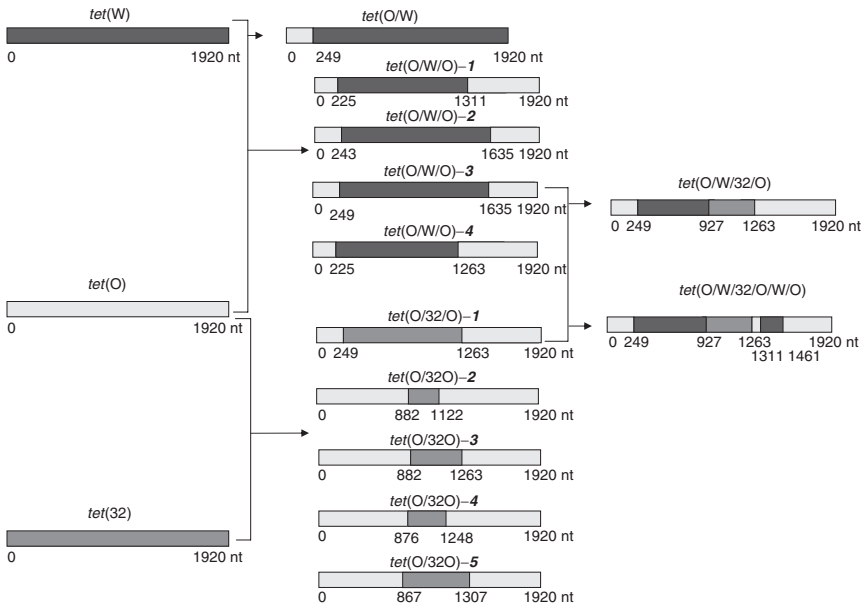

FIGURE 9.2 Hypothetical evolution processes of *tet* mosaic genes. Black—*tet*(W), light gray—*tet*(O), dark gray—*tet*(32). Simple mosaics produced by recombination of two original genes and complex mosaics produced by recombination of three original genes at one or more points. Predicted crossover positions are stated underneath the genes.

other combinations exist by the identification of the 5′-end of a *tet*(W/32–) gene (Patterson *et al.*, 2007b).

C. Impact of antibiotics on the commensal gut microbiota

Antibiotics are generally used to alleviate the symptoms of an illness, and the effects of the antibiotic on the causative bacterial agent have been widely studied. However, antibiotics act not only on pathogenic bacteria but can also affect the normal gut microbiota of patients, causing effects that can persist after the end of antibiotic treatment. In fact, the root of the medical problem of kidney stones appears to lie in the elimination of *Oxalobacter formigenes* (Stewart *et al.*, 2004). This is the only native gut bacterium able to degrade oxalate and it is very susceptible to antibiotics (Duncan *et al.*, 2002).

The knockon effects of antibiotic therapy on the composition of the total commensal microbiota have not been fully investigated, despite knowledge of the susceptibility of many bacterial species to broad-spectrum antibiotics. The gut microbiota of one individual we have studied who had undergone long-term tetracycline therapy was virtually 100% tetracycline resistant (Scott *et al.*, 2000), although detailed molecular profiling revealed that the distribution of the major bacterial groups was normal (Scott and Rincon, unpublished data). Other studies have shown that the diversity of the gut microbiota decreases following antibiotic application, both in infants (Favier *et al.*, 2003; Penders *et al.*, 2006) and in elderly hospital patients (Bartosch *et al.*, 2005).

Any alteration affecting the equilibrium of the gut microbiota can itself result in disease, and antibiotic-associated diarrhea (AAD) is now a medically recognized condition. Temporal changes in the diversity of the gut microbiota, leading to diarrhea, have been illustrated during antibiotic treatment (Young and Schmidt, 2004). Community cases of self-limiting diarrhea could be a result of the effects of changes in the composition of the microbiota on gut bacterial metabolism (Beaugerie and Petit, 2004) or may be due to the establishment of pathogenic bacteria in niches that were previously occupied.

The causative agent in the more serious cases, often occurring in hospitals, is *C. difficile*, which is thought to be able to establish in a niche in the gut created when antibiotic therapy destroys some of the native gut microbiota. Treatment of *C. difficile*-associated diarrhea (CDAD) has historically been with additional antibiotics, but in the past few years, cases have become harder to treat due to the increasing occurrence of antibiotic resistance in *C. difficile* strains (Bartlett, 2006; Noren *et al.*, 2006; Surowiec *et al.*, 2006). Even with successful treatment, 10–20% of patients suffer relapses, probably at least partly due to the persistence of bacterial spores. Alternative antibiotic therapies have been investigated (Musher *et al.*, 2006)

with little additional benefit. Combination treatments with prebiotics (nondigestible food ingredients that specifically promote the growth of beneficial commensal GIT bacteria) and antibiotics may be more effective in providing an actual cure for the diarrhea (Lewis *et al.*, 2005). Presumably, this has the dual effect of killing the infectious bacteria while at the same time stimulating regeneration of the commensal microbiota, thereby preventing reinfection and relapse.

D. Combination therapy: Antibiotics and pro/prebiotics

Bacterial probiotics (live beneficial bacterial supplements) may be given to humans following antimicrobial therapy in order to prevent the gastrointestinal side effects associated with oral administration of antibiotics, as described above.

Trials of probiotic treatments have also been done in critically ill patients in intensive care units (ICUs). These patients are often by necessity dosed with large amounts of antibiotics and also have a considerably altered and reduced food supply. Thus, treatment with synbiotics, a combination of probiotics and prebiotics, could be extremely beneficial (Bengmark, 2002). The results of one of two randomized controlled studies indicated that the addition of a synbiotic formula reduced infection and improved the patients' recovery, ultimately reducing the time in the ICU (Kotzampassi *et al.*, 2006). While the other study reported a favorable alteration in the composition of the gastrointestinal microbiota, no overall clinical benefit was demonstrated (Jain *et al.*, 2004).

Vancomycin and synbiotic therapy (*Bifidobacterium breve, Lactobacillus casei*, and specific galactooligosaccharides) were used successfully to treat a patient with fulminant MRSA enterocolitis. Vancomycin was administered first to eliminate MRSA colonizing the gut intestinal lumen, but subsequent use of synbiotics effectively helped to reestablish the anaerobic bacteria that dominate in the gut microflora, preventing further infection (Kanamori *et al.*, 2003). Thus, probiotics and/or prebiotics should be considered as significant and promising prophylactic agents to prevent the overgrowth of pathogenic bacteria even before the occurrence of severe infections, particularly in compromised patients in ICUs. Daily supplementation with viable probiotic bacteria during and postantibiotic therapy has been shown to reduce disruption to the intestinal microbiota (Plummer *et al.*, 2005).

On a cautionary note, probiotics themselves may sometimes play a role in the spread of Ab^R genes to pathogens, either directly or indirectly via gut commensals. Probiotic strains can be naturally resistant to one or more antibiotics, or they may acquire resistance genes from gut commensal bacteria when coadministered with antibiotics (Courvalin, 2006). The presence of an acquired *tet*(W) gene in several probiotic *Bifidobacterium*

isolates (Masco *et al.*, 2006; Saarela *et al.*, 2007) indicates the necessity for a safety assessment during the selection of new probiotic strains.

It is essential that further studies investigating the efficacy of probiotic, prebiotic, and synbiotic therapy be carried out to confirm whether these types of therapies, in conjunction with antibiotic treatment, can help to alleviate the detrimental nonselective side effects of antibiotics that can adversely affect the status quo of the gut ecosystem. The use of probiotics in particular must be considered very carefully, especially for those probiotic bacteria that are closely related to opportunistic pathogens (Wagner and Balish, 1998).

E. Antibiotics and the early development of the gut microbiota

A study by Favier *et al.* (2003) revealed that the initial colonization of *Bifidobacteria* in infants was linked to the maternal profile, but within 2–3 months unique patterns emerge (Favier *et al.*, 2003). Normally *Bifidobacteria* are the dominant species in the infant gut within 2 weeks, particularly in breast-fed infants, but one baby who had been given antibiotics from birth had still not acquired *Bifidobacteria* species by 3 months of age (Favier *et al.*, 2003). The effect of antibiotics, both before and just after birth, on the development of the infant gut microbiota has been reviewed (Bedford Russell and Murch, 2006). Any early use of antibiotics affects the natural development of the gut microbiota and decreases the numbers of *Bifidobacteria* and *Bacteroides* spp. (Penders *et al.*, 2006). Since the developing microbiota plays a crucial role in the development of the immune system, any treatment that affects the order of colonization can have implications in the development of allergy and immune diseases, within and outside the GI tract, in later life (Kirjavainen and Gibson, 1999; Noverr and Huffnagle, 2004, 2005; Schumann *et al.*, 2005).

Extensive use of antibiotics and other antimicrobial agents, improved standards of hygiene, and widespread vaccination policies have reduced the rate of childhood infections in developed countries. This deprives the immune system of proper microbial stimulation during its maturation, and increases the risk of subsequent development of allergies and autoimmune diseases. This phenomenon is known as the ''hygiene hypothesis'' (Bach, 2005; Prioult and Nagler-Anderson, 2005; Wills-Karp *et al.*, 2001). Exposure to at least one course of antibiotics in the first year of life has been shown to be a risk factor for the development of childhood asthma (Ahn *et al.*, 2005; Marra *et al.*, 2006), although a large-scale study is required to confirm these observations. It is possible that those infants destined to become asthmatic would have required more antibiotic therapy anyway. Exposure to antibiotics through breast-feeding has also been identified as a low risk factor for recurrent wheeze (Kummeling *et al.*, 2007).

Lack of proper development of the immune system due to antibiotic use has also been implicated in the development of atopic eczema in some children (Johnson *et al.*, 2005).

Studies of the health histories of children showed that those with autistic spectrum disorders (ASD) had significantly more ear infections than healthy counterparts, and were consequently exposed to significantly more antibiotics (Niehus and Lord, 2006). It was speculated that the extensive use of broad-spectrum antibiotics, which do not discriminate between pathogenic and commensal bacteria, may disrupt the normal intestinal microbiota facilitating colonization of the intestine by toxin-producing bacteria, like *Clostridium tetani* and *C. perfringens*, and that the toxins produced could affect the brain causing the symptoms of autism (Bolte, 1998; Manev and Manev, 2001). This hypothesis is emotive and to date still unsubstantiated, and much more research is required before any real conclusions can be drawn (Casavant, 2006). For example, the siblings of ASD sufferers also seem to be prescribed antibiotics more frequently than other children, yet do not go on to develop autism (Parracho *et al.*, 2005), illustrating that other factors are almost certainly involved in the development of ASD, possibly including a genetic predisposition (Bailey *et al.*, 1995; Steffenburg *et al.*, 1989).

It is established, however, that autistic children suffer from more gastrointestinal problems than their healthy counterparts. Comparisons of the microbiota of healthy children with ASD children have indicated differences in the numbers of some bacterial groups (Parracho *et al.*, 2005), and this may be a result of repeated antibiotic therapy affecting the normal development and composition of the gut microbiota. Vancomycin was used successfully to treat chronic diarrhea in a group of autistic children, and the level of regressive autism also significantly improved (Sandler *et al.*, 2000). Vancomycin therapy cannot be used for long periods due to the consequences of continual selective pressure on the emergence of resistant strains of bacteria, and the effects on the susceptible commensal GIT bacteria.

V. CONCLUSIONS

The emergence and persistence of Ab^R bacteria is concomitant with the use of antibiotics in clinical therapy and animal husbandry, and only by exercising prudence in the use of antibiotics, can we hope to reduce the levels of resistant bacteria. The problem of bacterial antibiotic resistance is exacerbated by the existence of antibiotic resistance genes on mobile genetic elements that have been shown to transfer between unrelated bacteria, including commensal and pathogenic bacterial isolates. A combination of antibiotic selection pressure and gene transfer has resulted in

the evolution of new mosaic tetracycline resistance genes, one example of the adaptability of bacteria to selective pressures.

A parallel consequence of antibiotic therapy to treat human disease is the effect on the commensal gut bacteria. One of the least serious manifestations of this in ''healthy adults'' is antibiotic-associated diarrhea, but the effects, particularly in children where the gut microbiota is still developing, could be much more serious and considerably more research is required in this area.

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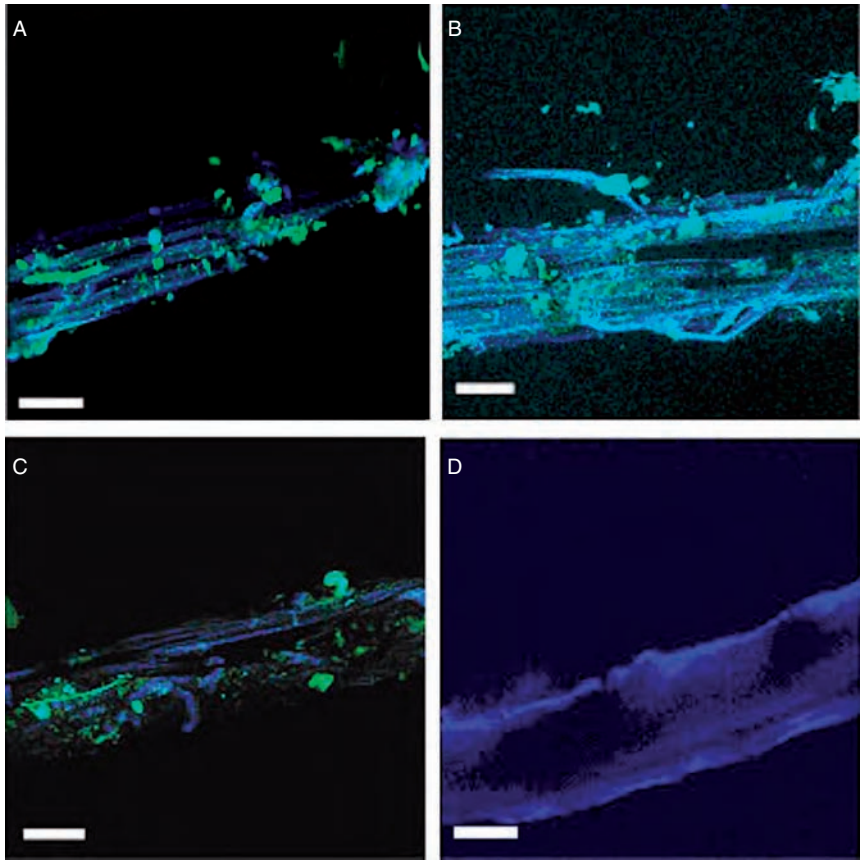

Michael A. Kertesz *et al.*, FIGURE 8.2 Rhizosphere expression of bacterial genes related to organosulfur utilization. (A) *asfA::gfp* fusion. (B) *ssuE::gfp* fusion. (C) *atsB::gfp* fusion. (D) Promoterless *gfp* reporter vector with no added insert. Green represents GFP-positive bacteria, and blue is Arabidopsis autofluorescence. Arabidopsis seedlings were inoculated with *P. putida* S-313 carrying a medium-copy *gfp* reporter plasmid and grown for 21 days. Roots were then fixed in 0.1% (w/v) agarose and root sections examined by confocal laser scanning microscopy. All images were taken using a 40× objective (scale bar 50 μm) and contain between 20 and 60 *z* sections with a *z* depth of between 20 and 75 μm.