PHYSIOLOGY OF DIMETHYLSULFONIOPROPIONATE METABOLISM IN A MODEL MARINE ROSEOBACTER, *Silicibacter pomeroyi*

by

JAMES R. HENRIKSEN

(Under the direction of William B. Whitman)

ABSTRACT

Dimethylsulfoniopropionate (DMSP) is a ubiquitous marine compound whose degradation is important in carbon and sulfur cycles and influences global climate due to its degradation product dimethyl sulfide (DMS). *Silicibacter pomeroyi*, a member of the a marine Roseobacter clade, is a model system for the study of DMSP degradation. *S. pomeroyi* can cleave DMSP to DMS and carry out demethylation to methanethiol (MeSH), as well as degrade both these compounds. Differential display proteomics was used to find proteins whose abundance increased when chemostat cultures of *S. pomeroyi* were grown with DMSP as the sole carbon source. Bioinformatic analysis of these genes and their gene clusters suggested roles in DMSP metabolism. A genetic system was developed for *S. pomeroyi* that enabled gene knockout to confirm the function of these genes.

INDEX WORDS: *Silicibacter pomeroyi*, *Ruegeria pomeroyi*, dimethylsulfoniopropionate, DMSP, roseobacter, dimethyl sulfide, DMS, methanethiol, MeSH, marine, environmental isolate, proteomics, genetic system, physiology, metabolism

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DEDICATION

I dedicate this work to my mother and father and to my wife, Emily. My father is my first and most important mentor. My mother raised me with the strength to accomplish my goals. My wife is my lifelong intellectual and emotional companion, and I could not have compleated this degree without her. All three have taught me what is truly important in life.

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CHAPTER 1

LITERATURE REVIEW

1.1 ABSTRACT

In the 1970s a missing component of the global sulfur cycle was identified as the volatile sulfur compound dimethyl sulfide (DMS) that is released from marine systems. The source of this DMS is dimethylsulfoniopropionate (DMSP) produced by marine plants and algae. While the algae that produce DMSP can also degrade it to DMS, further work showed that bacterial degradation of DMSP also leads to the production of DMS as well as other compounds. While some processes in the DMSP degradation pathway are well characterized in ecological studies, many of the molecular mechanisms have not been elucidated. This review will discuss DMSP's importance in the environment, degradation pathways in natural systems, and mechanisms of degradation in cultured bacteria and the roseobacter clade.

1.2 INTRODUCTION

From the beginning of the discipline, microbial ecology has recognized the importance of microbiological transformation of chemical substances in the environment. Beijerinck was the first to use the term "microoekologie" and described the careful design of conditions in enrichment cultures to select for organisms that were able to carry out a particular chemical transformation (Beijerinck, 1913). Given the staggering number (Whitman *et al.*, 1998) and diversity (Schloss and Handelsman, 2004) of microorganisms on earth and that chemical methods have been established for hundreds of years longer than microbial methods, it is not surprising that a compound was often first detected in the environment and only later identified as a component of microbial metabolism. The degradation of DMSP follows this theme.

1.3 Importance of DMSP in the environment

1.3.1 Volatile sulfur in the global sulfur cycle

The broad outline of our knowledge of the global sulfur cycle was in place by the 1970s. The major geological flux of sulfur from weathering and its accumulation in marine systems was well known. A large flux of volatile sulfur from the sea to the atmosphere was required in order to maintain sulfur in terrestrial systems. This transfer was assumed to be in the form of H_2S. However, the oxidizing conditions of marine surface waters did not suggest a compound as reduced as H_2S would be produced in large quantities.

1.3.2 Climate effect of DMS

In the early 1970s, the invention of more sensitive detectors and analytical methods for gas chromatography led to the discovery that dimethyl sulfide (DMS) was the volatile sulfur compound released from marine systems that completed the sulfur cycle (Lovelock *et al.*, 1972). DMS had been previously identified as a compound of biogenic and potentially microbial origin (Challenger, 1951), but its role in a major step of biogenic recycling in marine systems had not been realized. The source of DMS was identified as an algal osmolyte, dimethylsulfoniopropionate (DMSP) (Dickson and Kirst, 1986).

DMS released from marine systems is a major nonanthropogenic source of sulfur in the atmosphere. It is estimated that 40 Teragrams/year of DMS are released from marine systems globally (Lomans *et al.*, 2002). DMS is highly malodorous, end even in low concentrations it is a major contributor to the odor of the sea. Taking into account residence times of various sulfur compounds, averaging over all marine systems the contribution of DMS to the atmospheric sulfate burden is 42% (Simó, 2001). For comparison, the only other major natural source of sulfur to the atmosphere, SO_2 released from volcanoes, contributes 18% to the sulfate burden (Stoiber *et al.*, 1987). Anthropogenic sulfur emissions make up almost all of the remainder (Simó, 2001, based on Kettle and Andreae, 2000; Rodhe, 1999).

In the atmosphere, DMS is oxidized to methanesulfonic acid and eventually to particulate sulfate, which then acts as hygroscopic cloud condensation nuclei. Concentrations of DMS in the atmosphere correlate with the cloud condensation nuclei loads (Bates *et al.*, 1987), which suggest a link between DMS production and albedo (the reflection of solar energy by cloud cover). As decrease in solar irradiance of the ocean could reduce the abundance of the algae that produce the DMSP that DMS is formed from, there is the possibility for a biogeochemical and climate feedback.

Increased solar radiation leads to an increase in the abundance of algae that produce DMSP, which could in turn lead to an increase in the amount of DMS formed and released into the atmosphere, which would lead to increased cloud formation and albedo. As an increase in cloud cover would then decrease the abundance of algae, this system would result in a negative feedback loop with DMS acting as an anti-greenhouse gas. This was termed the CLAW hypothesis, after Charlson, Lovelock, Andreae, and Warren, the last names of the authors of the paper where it was first stated (Charlson *et al.*, 1987). Although there were many connections in this proposed feedback loop that were not fully elucidated, it was the first mechanism for large scale feedback loops in ecological systems and could be an example of the role of biology in preserving global homeostasis, as described in the Gaia hypothesis (Lovelock, 1979). For a review of these climatic implications see Simó (2001).

While the idea of biogenic global homeostasis was criticized based on ideas that natural selection (Darwin, 1859) could not lead to a seemingly altruistic emergent behavior (Caldeira, 1989; Dawkins, 1983; Doolittle, 1981), the biogenic effect of DMS on climate is clear. There is a high negative correlation between DMS concentrations and the radiation dose in the open ocean (Vallina and Simó, 2007). Inclusion of the DMS produced from marine systems is essential for modeling the planet's climate (Stefels *et al.*, 2007) and the results of anthropogenic climate change (Watson, 1998). In models, total marine DMS production provides climatic forcing that would result in a 3.8 °C cooling over timespans of a few hundred years (Watson, 1998). However, while recent models have proposed an important role for DMS in global climate, a consensus has yet

to be reached on the climate forcing of this system (Gunson *et al.*, 2006; Kloster *et al.*, 2007). In addition, the connection between the production of DMSP by algae and DMS concentrations is more complicated, involving both abiotic parameters and a strong microbiological control of the products produced from DMSP (Simó, 2001).

1.4 Degradation pathways of DMSP in the environment

1.4.1 Sources of DMSP

DMSP is an organic sulfur compound produced by many marine algae, dinoflagellates, some diatoms, and a few species of plants, including the common salt marsh cordgrass *Spartina alterniflora* (Visscher *et al.*, 1994), some sugarcanes, and the beach daisy *Wollastonia biflora* (Stefels, 2000). DMSP may function as an osmoprotectant (Dickson and Kirst, 1986), an antioxidant (Sunda *et al.*, 2002), a mechanism to remove reduced compounds (Stefels, 2000), or a precursor for compounds used to discourage algal grazing (Wolfe *et al.*, 1997). Bacterial symbionts and commensals are attracted to their host algae by DMSP (Miller *et al.*, 2004), and it may act as a chemical signal of high productivity to reef fishes, who are also attracted by this compound (DeBose *et al.*, 2008). Dissolved DMSP, synthesized by algae, is ubiquitous in marine environments.

1.4.2 Eukaryotic synthesis of DMSP

The role and importance of DMSP in marine algae was reviewed by Giordano *et al.* (2005). The pathway for DMSP synthesis is still being elucidated, but a general overview has been provided by Stefels (2000). The ultimate source of the sulfur moiety in DMSP is SO_4^{2-}, which is fixed by the APS/PAPS system, reduced to sulphite and then to sulfide in the chloroplasts, incorporated into cysteine, and modified to methionine by the transsulfuration pathway (Stefels, 2000, and references therein). Methionine is then converted to DMSP by one of three different pathways that seem to have evolved independently in two eukaryotic plant groups and in marine algae. The algal pathway has been observed in macroalgae, planktonic algae and diatoms (Gage *et al.*, 1997; Hanson *et al.*, 1994; Kocsis *et al.*, 1998). There may be an additional pathway in other

macroalgae and dinoflagellates (Uchida *et al.*, 1996), although this is under active debate (Stefels, 2000). Common characteristics of these pathways include the methylation of sulfur by AdoMet, removal of the amine group of a precursor amino acid, and oxidation and rearrangements in the carbon backbone (Stefels, 2000). The production of DMSP is an energetically expensive process, requiring eight electrons to reduce the sulfur moiety. However, it is a major component of the dry weight of algae that produce it, reaching up to 2 M intracellularly in some dinoflagellate species, and typically comprising 50–99% of the total cellular sulfur in marine algae (Keller *et al.*, 1999).

1.4.3 Environmental concentrations of DMSP

There are many different parameters that affect the concentration of DMSP in marine systems, including algal growth conditions (van Duyl *et al.*, 1998), viral lysis, and algal grazing (Wolfe *et al.*, 1997). Cell lysis is a particularly important parameter, as the release of DMSP from algae and plants is occurs primarily by cell lysis, with very little exuded from undamaged cells. DMSP concentrations in marine waters ranges from typical levels of about 10 nM up to 1 µM during algal blooms (Simó *et al.*, 2000; van Duyl *et al.*, 1998), and are highly variable spatially and temporally on the scale of days (Simó and Pedros-Alio, 1999b) and seasons (Ledyard and Dacey, 1996). In oligotrophic marine systems, which contain many compounds at low concentrations, DMSP may have highest concentration of any single carbon source. DMSP is not found at significant concentrations in freshwater, although its role in a eutrophic freshwater lake environment has been reported (Ginzburg *et al.*, 1998). The activity of DMSP metabolism parallels the salinity of freshwater, brackish, and saline waters (de Souza and Yoch, 1996a). High DMSP concentrations and the major elements of the DMSP degradative pathway have also been observed in a hypersaline but landlocked lake (Diaz and Taylor, 1996).

1.4.4 Uptake by bacteria

Many bacteria bioaccumulate DMSP in the cytoplasm, where it functions as an osmoprotectant (Chambers *et al.*, 1987). In marine environmental samples amended with [^{35}S]DMSP, almost all of

the DMSP was rapidly accumulated intracellularly by microorganisms, but over time the amount of intracellular untransformed DMSP observed decreased, and only 16% of the intracellular untransformed DMSP remained after two days (Kiene and Linn, 2000b). There are indications from competitive uptake kinetics in marine samples that DMSP could be transported into cells by a glycine betaine uptake system, which has been described genetically (Kiene *et al.*, 1998). However, it is unclear what steps of DMSP degradation take place intracellularly or if there are different cellular locations in different organisms (Yoch *et al.*, 1997).

1.4.5 Rates of DMSP degradation

The factors influencing DMS release from seawater are numerous (for a review see Yoch, 2002) and include physical parameters such as wind speed, water layer mixing (Simó and Pedros-Alio, 1999a), and sedimentation (Osinga *et al.*, 1996). While these abiotic factors are important, there are many others that control the flux of compounds through the DMSP degradation pathway. DMSP consumption and well as DMS production and consumption are the results of a complex microbial food web (Simó *et al.*, 2002). The flux of DMSP is high in most marine systems, with rate measurements indicating that pools turn over in less than 1 day. From the total amount of DMSP consumed and inhibition studies of DMS consumption with chloroform, turnover times for DMS of 0.2–1 day were estimated (Vila-Costa *et al.*, 2006b).Very little of the DMSP cleaved to DMS is released, indicating similar rates of DMS consumption and production (Simó *et al.*, 2000). During an algal bloom, the rates of DMSP degradation and DMS consumption changed over two orders of magnitude over 24 hours, and the change in these rates was due to biological activity (van Duyl *et al.*, 1998)

1.4.6 Pathways of DMSP degradation

There are multiple pathways for the breakdown of DMSP in marine systems (see figure 1.1, reviewed in Kiene *et al.*, 2000; Yoch, 2002). While DMSP can degrade spontaneously in sea water, the rate is too slow to account for the formation of DMS (Dacey and Blough, 1987). Algae that

produce DMSP can usually degrade it to dimethyl sulfide (DMS) extracellularly via a cleavage pathway (section 1.4.7), although this is not the fate of most DMSP released. Many marine bacteria also cleave DMSP (section 1.4.8), but are also able to degrade DMSP by a separate demethylation pathway (section 1.4.9). This demethylation route does not lead to DMS production, but is important in oxic and anoxic marine waters. Anoxic microbial degradation of DMSP also occurs (section 1.4.10), but the remainder of this section will focus on degradation of DMSP and its products DMS (in section 1.4.11) and methanethiol (MeSH, in section 1.4.13) in oxic environments.

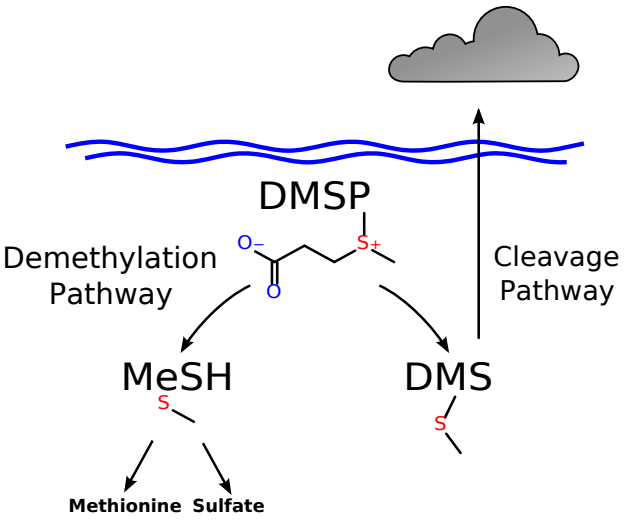

Figure 1.1: Major pathways for DMSP degradation in the environment

1.4.7 Algal cleavage of DMSP

Many algae that produce DMSP cleave it to acrylate and DMS (Challenger, 1959). This activity was also described in a salt marsh fungus (Bacic and Yoch, 1998).

$$\underset{\text{DMSP}}{C_5H_{10}O_2S} \longrightarrow \underset{\text{acrylate}}{C_3H_3O_2^-} + \underset{\text{DMS}}{C_2H_6S} + H^+$$

1.4.8 Bacterial cleavage of DMSP

Many bacteria can produce DMS from DMSP (Dacey and Blough, 1987; Kiene, 1990). This reaction was assumed to be catalyzed by a enzyme similar to the algal DMSP lyase, but other mech-

anisms have been proposed recently (see section 1.6.1). While some DMS is formed by algal cleavage in marine systems, bacterial production of DMS is a critical process (Kiene, 1996), and the abundance of heterotrophic bacteria is highly correlated with the amount of DMS that is vented from the sea (Simó *et al.*, 2002).

1.4.9 Demethylation and demethiolation of DMSP

The alternative to the cleavage pathway is the demethylation pathway, where methanethiol is formed from DMSP (Diaz and Taylor, 1996; Visscher and Taylor, 1994). This is a solely bacterial process. Intermediate steps in this pathway are the demethylation of DMSP to produce methylmercaptopropionate (MMPA), which is then demethiolalated to form methanethiol (MeSH).

$$\underset{\text{DMSP}}{C_5H_{10}O_2S} + \underset{\text{carrier}}{X{-}H} \longrightarrow \underset{\text{MMPA}}{C_4H_7O_2S^-} + \underset{\text{methylated carrier}}{X{-}CH_3} + H^+$$

$$\underset{\text{MMPA}}{C_4H_7O_2S^-} \longrightarrow \underset{\text{acrylate}}{C_3H_3O_2^-} + \underset{\text{MeSH}}{CH_4S}$$

DMS is not formed by the demethylation pathway. Many types of bacteria can demethiolate DMSP to MeSH, which under most conditions is the major pathway of DMSP degradation (Zubkov *et al.*, 2001). Thus, the demethylation pathway is an important control of the amount of DMS released because it provides an alternative fate for DMSP. The bacterial population and their DMSP degradation capabilities have a significant effect on the rate and end products of marine DMSP degradation (Kiene, 1996; Kiene and Linn, 2000a). MeSH also is rapidly use by many marine bacteria.

Most probable number dilution experiments indicated that the population of bacteria that produces MeSH only slightly overlaps with the population that produces DMS (Visscher *et al.*, 1992). However, one third of environmental isolates in the roseobacter clade (see 1.7.1) were found to carry out both pathways (González *et al.*, 1999).

The sequential demethylation of DMSP to mercaptopropionate (MPA) and desulfurization to H_2S and acrylate has also been observed (Taylor and Gilchrist, 1991; Taylor and Visscher, 1996).

$$\underset{\text{MMPA}}{C_4H_7O_2S^-} + \underset{\text{carrier}}{X{-}H} \longrightarrow \underset{\text{MPA}}{C_3H_4O_2S^-} + \underset{\text{methyl carrier}}{X{-}CH_3} + H^+$$

$$\underset{\text{MPA}}{C_3H_4O_2S^-} + H^+ \longrightarrow \underset{\text{acrylate}}{C_3H_3O_2^-} + \underset{\text{sulfite}}{H_2S}$$

While it has been observed in one aerobic isolate, these activities are more common in obligately anaerobic methanogens and sulfate reducing bacteria (Taylor and Visscher, 1996). In addition, MeSH production from the alternative pathway for MMPA degradation is observed at higher rates in oxic environments. Therefore, this route of degradation is not thought to be a major contributor in oxic marine waters, but it could be significant under anoxic conditions.

1.4.10 Anoxic degradation of DMSP

In anoxic marine sediments, DMSP can be cleaved to DMS, which is demethylated to MeSH and then consumed. Demethylation of DMSP to MMPA and MPA also occurs, but little MeSH is released by demethiolation of MMPA to MeSH (Kiene and Taylor, 1988). A DMSP lyase has been characterized from a sulfate-reducing bacterium (van der Maarel *et al.*, 1996), and tetrahydrofolate was found to be a methyl carrier for the demethylation of DMSP and MMPA (Jansen and Hansen, 1998, 2001). Even though the enzyme was purified, neither the protein nor gene sequence was determined (Jansen and Hansen, 2000). Homoacetogenic bacteria also anaerobically demethylate DMSP to MMPA, probably to synthesize acetyl-CoA by the Ljungdahl-Wood pathway. However these organisms are not able to grow on or increase biomass with DMSP demethylation (Jansen and Hansen, 2001). The reduction of MeSH and DMS to methane and H_2S is a significant process in anoxic sediments (Kiene and Taylor, 1988; Lomans *et al.*, 1999a), with methanogens carrying out more demethylation than sulfate- or nitrate-reducing bacteria (Lomans *et al.*, 1999b). In anoxic sediment samples, methanogens use the methyl groups directly from DMSP and MMPA, but at a far lower rate than the methyl groups from DMS or MeSH (van der Maarel and Hansen, 1997).

1.4.11 Fate of DMS

DMS is a volatile compound that is often found at supersaturated concentrations in the open ocean (Kiene and Linn, 2000a). The mixed layer depth is a major abiotic determinant of DMS release to the atmosphere (Simó and Pedros-Alio, 1999a). However, biological DMS consumption is 3–430 times more rapid than abiotic release or degradation (Kiene and Bates, 1990). There are multiple pathways in the environment that lead to the consumption of DMS. Small amounts of DMS are transformed to cellular sulfur (Kiene *et al.*, 1999).There is low, but significant biotic flux from DMS to sulfate, potentially via a non-charged and nonvolatile compound that has not been identified (Kiene and Linn, 2000b). DMSO was the major product of DMS consumption above the mixed surface layer in the Sargasso Sea, while sulfate was the major product below the mixed surface layer (del Valle *et al.*, 2007).

1.4.12 Production and consumption of DMSO

One potential sink for DMS is its oxidation to DMSO by marine bacteria and abiotic photooxidation.

$$\underset{\text{DMS}}{\mathrm{C_2H_6S}} + \tfrac{1}{2}\mathrm{O_2} \longleftrightarrow \underset{\text{DMSO}}{\mathrm{C_2H_6OS}}$$

DMSO can be reduced to DMS as an alternative electron acceptor or degraded by other processes. The rates and endpoints of DMSO metabolism in marine systems are currently not well described (Hatton *et al.*, 2004; Lee *et al.*, 1999). DMSO may be produced and released by marine algae, which would require a high rate of DMSO consumption in marine waters (Simó and Vila-Costa, 2006).

1.4.13 Fate of MeSH

Methanethiol formed from the demethylation pathway is readily incorporated into bacterial amino acids, primarily methionine (Kiene *et al.*, 2000). This is an ecologically important process, involving 15–40% of the DMSP derived sulfur (Kiene *et al.*, 1999).

The oxidation state of the sulfur in DMSP is the same as that in methionine. Eight reducing equivalents are necessary to convert sulfate to this oxidation state. In seawater, where sulfate is found at 10^6 times the concentration of DMSP, organisms will preferentially use DMSP as a sulfur source, presumably because the sulfur does not need to be reduced before incorperation (Kiene *et al.*, 1999). Another possible bacterial use of the sulfur moiety of methanethiol or DMS is oxidation to sulfate, with oxidation or assimilation of the methyl groups.

1.5 Fluxes and endpoints of DMSP degradation

1.5.1 Bacterial control

As the open ocean has low concentrations of many different carbon compounds, DMSP is often the single most significant carbon and energy source for bacterial growth (Kiene and Linn, 2000a). During an algal blooms in the North Sea and North Atlantic, DMSP was estimated to provide up to 15% of the carbon and up to 95% of the sulfur required by bacteria (Simó *et al.*, 2002; Zubkov *et al.*, 2001).

The sulfur demand of the heterotrophic bacterial population may determine if DMSP is degraded via the demethylation pathway to MeSH, or via the cleavage pathway to DMS, as MeSH is the primary compound available for incorporation into cell protein (Kiene *et al.*, 1999). The mechanisms and intermediates of these pathways have not been fully elucidated.

1.5.2 Endpoints of DMSP degradation

Using incubations with oceanic and coastal waters amended with ^{35}S radiolabled DMS, MeSH and DMSP, Kiene and Linn (2000b) measured the end-product partitioning of the sulfur moiety (see Figure 1.2). Some DMSP was accumulated intracellularly and maintained unchanged, but most was transformed into other products. About 60% of [^{35}S]DMSP was incorporated into cellular material in coastal waters, probably into methionine via MeSH. In contrast 16% was incorporated in oceanic waters. MeSH that was not incorperated was oxidized to nonvolatile dissolved sulfur, probably SO_4^{2-}. DMS turnover was much slower than the flux from DMSP through MeSH. Small

amounts of the sulfur from DMS were converted to cell protein, but most of it was converted to sulfate, potentially via another nonvolatile compound that was not identified. This compound did not produce a signal on an ion conductivity detector when separated by chromatography (Kiene and Linn, 2000b). By examining size fractionation and chemical fate of [^{35}S]DMSP incubations, Vila-Costa *et al.* (2006a) estimated a release of 10–50% of synthesized DMSP from algae. The estimated fates of the sulfur from this released DMSP were; 20–40% transformation to nonvolatile sulfur (DMSO, sulfate or other compounds), 5–30% assimilation by bacteria (probably into the protein fraction), and 2–5% transformation to DMS.

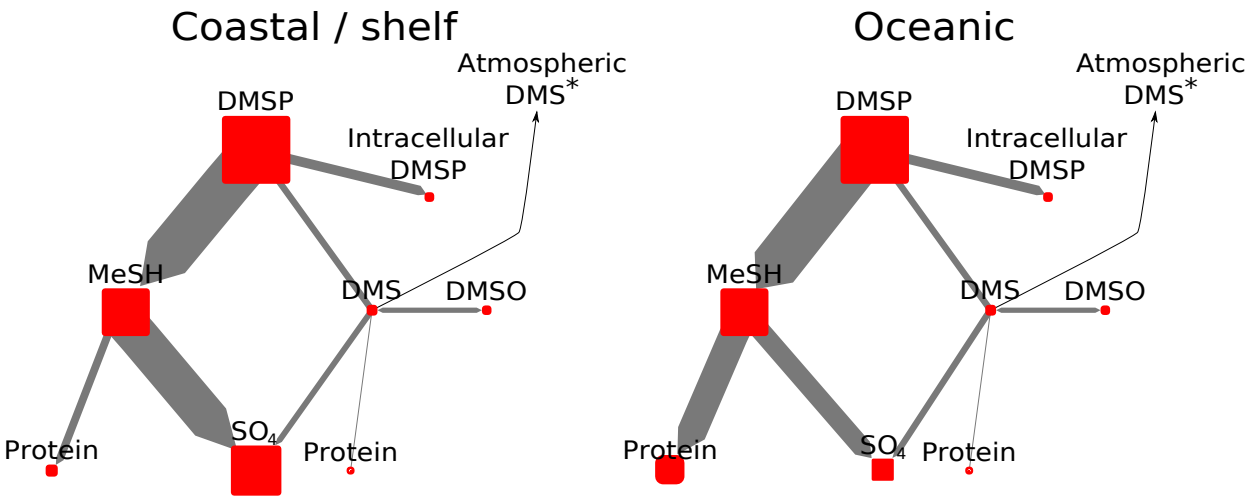

Figure 1.2: DMSP degradation Sankey diagram: Width of lines are proportional to the percentage of DMSP-sulfur estimated to flow through each of the transformations. Figure based on data from Kiene and Linn (2000b).
* DMS release was prevented in these experiments. Rates from other work suggest released DMS ranges from 0–10% of DMSP-sulfur produced (Kiene and Linn, 2000b).

Following incubations of coastal and oceanic samples with [^{35}S]DMS, 70% of DMS was converted to DMSO, 3% was incorporated into macromolecules, and the remainder was oxidized to SO_4^{2-} (Vila-Costa *et al.*, 2006b). When these samples were pre-incubated with DMS, less DMSO was formed. The authors speculate that there was a population of bacteria, perhaps in the roseobacter clade or a *Hypomicrobium*, that converted the DMS to MeSH and then to HS^- by heterotrophic oxidation of the methyl groups. The HS^- was then oxidized to SO_4^{2-} (Vila-Costa *et al.*, 2006b).

Overall, up to 90% of the sulfur in DMSP goes to the demethylation pathway, forming MeSH which is incorporated into cells or degraded to sulfate. Of the DMSP that is cleaved to DMS, 60–99% of the sulfur in DMS is not released, being transformed to sulfate or DMSO.

1.5.3 High variability

The percent of DMSP cleaved to DMS reported in the literature ranges from 9% to 100% (Simó *et al.,* 2000). Some of this variability may be methodological and the result of large errors in estimates, but natural variability is also important. The concentrations of DMSP and other conditions (particularly nutrient availability) vary radically between coastal regions and the open ocean, and the differences in environments may contribute to the poor constraints in the fluxes through this pathway. The flux of DMS could be controlled by different factors under the eutrophic conditions of an algal bloom and the oligotrophic conditions of the Sargasso Sea, where UV dosage was found to be linked to DMS emission (Toole and Siegel, 2004).

In brackish or saline sediments that cycle between oxic and anoxic conditions, the rates and endpoints of DMSP degredation can be different. In a marine marsh, the rates of DMSP-cleavage and demethylation were measured at different DMSP concentrations. Demethylation occurred at a higher rate than cleavage below 26 µM DMSP, but demethylation did not increase at higher concentrations, while the cleavage rate increased linearly with concentration. Therefore the cleavage pathway dominated at concentrations above 26 µM (Jonkers *et al.*, 2000).

Many of these rates are poorly constrained and probably show variability as concentrations change. An understanding of the bacteria responsible, the biochemical mechanism, and the diversity of the genes involved are essential to further understanding of this process.

1.6 Metabolic mechanisms of DMSP degradation in bacteria

Marine bacterial isolates vary in their ability to cleave and demethylate DMSP. Some only carry out reactions in the demethylation pathway, others only carry out reactions in the cleavage pathway,

and some isolates can perform both. Since little is known concerning the regulation of these pathways and many isolates were only tested under one condition, it is possible that organisms displaying one of the two activities might, under other conditions, display the other activity. In bacteria that use both pathways, functional regulation of the two pathways has been observed, but factors that influence this regulation are unknown. One hypothesis is that the sulfur demand of the organism determines the expression of the demethylation pathway (Kiene and Linn, 2000a). The demethylation pathway might be induced in order to produce more MeSH that can be incorporated into methionine, or the cleavage pathway might be suppressed to produce less DMS that cannot be incorporated into methionine. During growth on glucose and 0.0025–10 μM of [^{35}S]DMSP, *Silicibacter pomeroyi* partitioned more ^{35}S into volatile DMS at high DMSP concentrations, and less into cell protein (González *et al.*, 1999). This theory of sulfur demand driving demethylation is supported by similar data from environmental studies (van Duyl *et al.*, 1998).

Many of the steps in the DMSP-cleavage and demethylation pathways are not known. The reactions shown in figure 1.3 have been proposed based on gas chromatographic and NMR detection of intermediates in cultures of different microorganisms, natural assemblages, and environmental samples. Therefore, the pathways may be incomplete or simply wrong. The parts of the pathway that are not well understood include the mechanism of acrylate degradation, the roles of MMPA, MPA and acrylate as intermediates, and the cellular location of these substrates. This section will focus on the mechanisms and genes that are known, focusing on aerobic heterotrophic degradation of DMSP and its products, particularly the sulfur moiety (through DMS and MeSH). The pathways involved in DMSP degradation are also reviewed in the proceedings of the 1995 symposium on DMSP (Kiene, 1996), particularly the chapter by Taylor and Visscher (1996). Other reviews include a thorough coverage of the known biochemistry of DMSP degradation pathways (Bentley and Chasteen, 2004), a review focusing on the degradation of DMS, MeSH, and other volatile sulfur compounds formed by the abiotic oxidation of DMS (de Zwart and Kuenen, 1992; Kelly and Baker, 1990), and a review covering both freshwater and marine as well as aerobic and anaer-

obic degradation of organic sulfur compounds, particularly MMPA and MPA by Lomans *et al.* (2002).

1.6.1 DMSP LYASES

ALGAL ENZYMES

The activity and kinetic properties of Eukaryotic DMSP lyases have been well described (de Souza *et al.*, 1996; Niki *et al.*, 2000; Series *et al.*, 1996; Steinke *et al.*, 1998) since the original isolation by Anderson and Cantoni (1956). DMSP-cleavage is probably localized to the extracellular side of the cellular membrane and is performed by the enzyme dimethylpropiothetin dethiomethylase (E.C. number 4.4.1.3). Even though a cDNA clone of this gene has been isolated (Nishiguchi, 1994), no sequence of the enzyme or its gene has been determined. In some organisms, two DMSP lyases have been isolated (Nishiguchi and Goff, 1995). Antibodies raised against a purified DMSP lyase was shown to cross-react with other algal DMSP lyases (Nishiguchi, 1994).

BACTERIAL ENZYMES

Many DMSP-cleaving bacteria have been isolated, beginning with Kiene (1990). Many *Proteobacteria* isolated from a salt marsh degraded DMSP, but only α-proteobacteria produced DMS in a study by Ansede *et al.* (2001a). Extensive kinetic studies of whole cell DMSP lyase activity was performed by Ledyard and Dacey (1994), who found that DMSP and acrylate induced this activity. A DMSP lyase that stoichiometrically cleaved DMSP to acrylate and DMS and was inhibited by MMPA was characterized from β-proteobacterium *Alcaligenes* strain M3A (de Souza and Yoch, 1995) and γ-proteobacterium *Pseudomonas doudoroffii*. An antibody raised against the *Alcaligenes* lyase crossreacted with both other bacterial and algal DMSP lyases (de Souza, 1996). N-terminal sequences were published from Edman degradation of these purified enzymes (de Souza and Yoch, 1996b). However, no corresponding gene has been found in organisms that exhibit this activity (including *Silicibacter pomeroyi*, the first sequenced organism known to cleave DMSP).

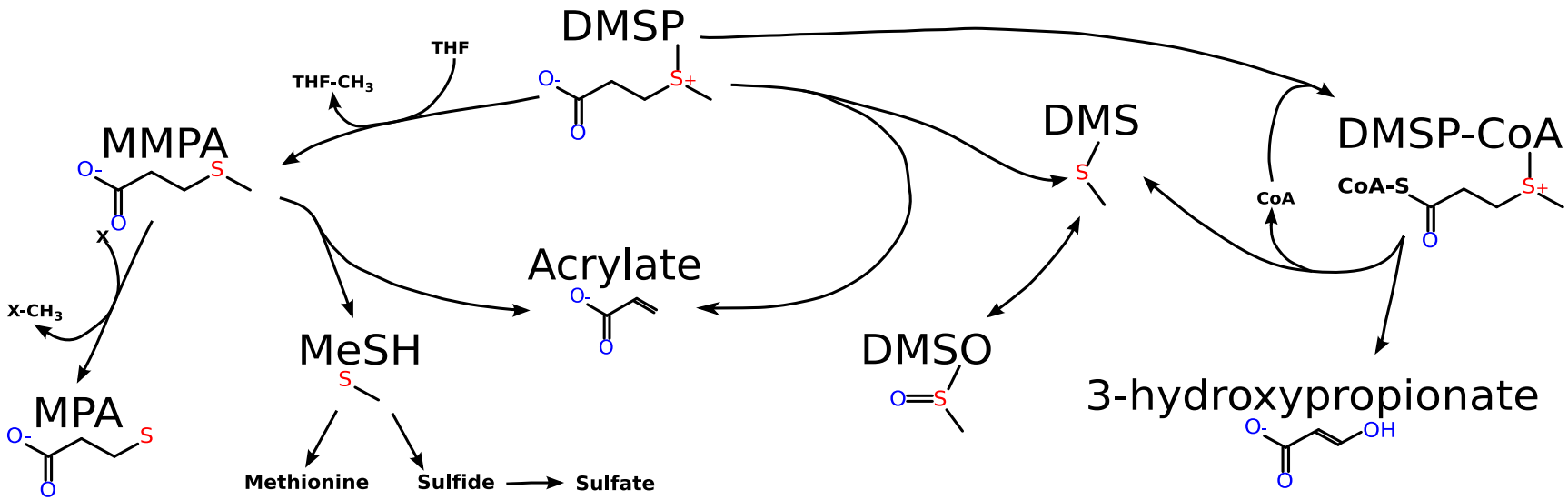

Figure 1.3: DMSP degradation pathways: Activities involved in DMSP degradation pathways.

Moreover, these sequences do not match any in major sequence repositories or metagenomic surveys (Johnston *et al.*, 2008). The cellular locations of these lyase activities were explored, and the DMSP lyase activity was predicted to be extracellular in *Alcaligenes* but not in *Pseudomonas doudoroffii* (Yoch *et al.*, 1997).

dddL

A DMSP lyase from *Sulfitobacter* EE-36 was identified by heterologous expression from a cosmid library in *Rhizobium leguminosarum* strain J391 and screened for DMS production by Curson *et al.* (2008). This cosmid produced barely detectable levels of DMS from DMSP in *E. coli*. To identify the gene, subcloning and screening in *R. leguminosarum* was performed, followed by transposon mutagenesis and subcloning. When expressed in *E. coli* with a native promoter, a single gene, designated *dddL*, was identified which conferred DMSP lyase activity. A knockout of this gene in *Sulfitobacter* EE-36 lost this phenotype. *dddL* has no homologs with known or predicted activity and had no known functional domains. Nine homologs were identified in other (mostly marine) α-proteobacteria, and seven were identified in metagenomic libraries, including the Global Ocean Survey (Rusch *et al.*, 2007). *dddL* cleaves DMSP to acrylate and DMS, as an *E. coli* expressing *dddL* released acrylate into the culture medium (Curson *et al.*, 2008). While *dddL* was the first gene identified that produced DMS and acrylate from DMSP, another mechanism for DMSP-cleavage to DMS had previously been discovered.

dddD

A gene was characterized from a *Marinomonas* strain MWYL1, a γ-proteobacteria closely related to *Alteromonas* that displayed inducible cleavage of DMSP to DMS (Todd *et al.*, 2007). *dddD* was identified by heterologous expression from a fosmid library in *E. coli* screened for DMS production. In the original fragment, four genes (*dddTBCR*) were located upstream and transcribed in the opposite orientation from *dddD*. While *dddD* was sufficient to confer DMSP cleavage activity to

E. coli when supplied with a *lacZ* promoter, *dddR*, a *LysR* family transcriptional regulator, was necessary for expression with the native promoter. Homologs to *dddD* were identified in *Burkholderia cepacia* and *Rhizobium*, and they also conferred cleavage activity when expressed in *E. coli*. These organisms were not previously identified as having DMSP-cleavage activity. While they can not grow on DMSP as a sole carbon source, they do produce DMS from DMSP. A transposon disrupted gene was used to replace the *dddD* in *Marinomonas*. This mutant did not produce DMS from DMSP and was not able to grow on DMSP. The *dddD* gene product is an acyl-CoA transferase. It is composed of two similar domains, each of which has homology to the *caiB* gene of *E. coli*. DddD is predicted to form a coenzyme-A thioester with the carboxyl moiety of DMSP. DMS is hypothesized to spontaneously cleave from the CoA-DMSP product due to the electron withdrawing properties of the thioester or with one or more steps catalyzed nonspecifically by *E. coli* enzymes. This would yield β-hydroxypropionate as opposed to acrylate, as predicted for other DMSP-cleavage pathways (Todd *et al.*, 2007).

DISTRIBUTION OF LYASES

Some DMSP-cleaving organisms only possess *dddL*, while others have only *dddD*. Some organisms have copies of both genes. Despite the unexpected diversity of DMSP lyases, more probably exist. Both the α-proteobacteria *Roseovarius nubinhibens* and the eukaryotic marine algae *Thalassiosira pseudonana* cleaves DMSP to DMS, but their genome sequences do not contain significant homologs to either gene (Curson *et al.*, 2008). Lastly, homologs to these genes are not abundant in marine metagenomic libraries where the activity is widespread. Therefore, it is unlikely that these genes account for a significant amount of this activity in marine systems.

1.6.2 DMSP DEMETHYLATION

In the demethylation pathway, MeSH is formed from DMSP, probably with the single demethylation product methylmercaptopropionate (MMPA) as an intermediate. This metabolism was first observed in pure cultures by Taylor and Gilchrist (1991). They isolated bacteria that generated

MeSH from MMPA or DMSP, and one isolate that did not produce MeSH from MMPA, but demethylated it to MPA. Later, an aerobic methylotroph was isolated that demethylated DMSP to MMPA and then to MPA, as well as demethylated DMS to MeSH and then to H_2S (Visscher and Taylor, 1994).

A demethylase gene was identified by screening a transposon library of *Silicibacter pomeroyi* with Ellman's reagent to detect a mutant unable to produce thiols (Howard *et al.*, 2006). One mutant lacked the initial demethylation activity of MMPA formation. This gene was designated *dmdA* and belonged to the glycine cleavage T-protein family (Pfam PF01571; Howard *et al.*, 2006). The reactions catalyzed by glycine cleavage T-proteins (E.C. number 2.1.2.10) include the demethylation and deamination of their substrates. Homologs to *dmdA* are highly represented in metagenomic libraries, and are found in "*Candidatus* P. ubique", a sequenced representative of the highly abundant SAR11 clade. When the "*Candidatus* P. ubique" *dmdA* gene was expressed in *E. coli* it conferred demethylation activity in cell extracts (Howard *et al.*, 2006).

1.6.3 Acrylate degradation

Acrylate is formed in the cleavage pathway (Kiene, 1990) and is also the most likely product formed in the demethylation pathway after the release of methanethiol (see Figure 1.3). There are a number of possible pathways for acrylate degradation. Three groups of pathways are possible: conversion to lactate, those involving propionyl-CoA as an intermediate, and the methylmalyl-CoA pathway. Acrylate may be degraded via acrylyl-CoA to lactoyl-CoA to lactate, although no organisms are known that use these reactions for growth. Alternate pathways may be via propionate to propionyl-CoA and then by succinate to acrylyl-CoA and lactate, malonic semi-aldehyde to acetyl-CoA and CO_2, malonyl-semialdehyde-CoA to acetyl-CoA and CO_2, or α-hydroxygluterate to lactate and acetate all of which are described in various organisms (Fernández-Briera and Garrido-Pertierra, 1988). A final pathway is via propionate to propionyl-CoA and then by β-hydroxypropionate and malonyl-CoA to acetyl-CoA, but while these enzyme activities are described, no organisms are known that use these reactions for growth. Lastly, acrylate may be

degraded by the ethylmalonyl-CoA pathway that includes β-methylmalyl-CoA and propionyl-CoA and glyoxylate intermediates, which is part of a proposed new pathway for acetate assimilation in the α-proteobacteria (Alber *et al.*, 2006). β-hydroxypropionate formation was identified as an intermediate in DMSP degradation in α- and β-proteobacteria, a reaction that in some organisms appeared to occur extracellularly (Ansede *et al.*, 2001a, 1999, 2001b).

1.6.4 MeSH incorporation to methionine

The entire MeSH molecule can be incorporated into methionine. The enzyme cystathionine γ-synthase (E.C. number 2.5.1.48) is thought to be responsible for this activity (Kiene *et al.*, 1999). This enzyme catalyzes the release of succinate from O-succinyl-L-homoserine and the formation of a C-S bond between cysteine and homoserine to form cystathionine. However, this enzyme has a broad substrate specificity. When methanethiol is the substrate instead of cysteine it will form a C-S bond between methanethiol and homoserine to form methionine (Flavin and Slaughter, 1967). As the sulfur in methionine is at the same redox state as the sulfur in methionine, this reaction would allow the conservation of reducing equivalents during the synthesis of methionine. This may be an obligatory method of methionine synthesis in some organisms, as DMSP was found to be an essential source of reduced sulfur in "*Candidatus* P. ubique" and, by extension, the highly abundant SAR11 clade (Tripp *et al.*, 2008).

1.6.5 MeSH utilization

MeSH (as well as DMS and dimethyldisulfide, see below) are used by autotrophic thiobacilli as a source of energy, by methylotrophic hyphomicrobia as a source of carbon and energy, and by diverse methyl-oxidizing aerobic bacteria as a source of energy (Bentley and Chasteen, 2004; de Zwart and Kuenen, 1992; Kelly and Baker, 1990). MeSH is degraded in *Hyphomicrobium* and *Thiobacillus* by methanethiol oxidase (E.C. number 1.8.3.4), producing formaldehyde and sulfide:

$$\underset{\text{MeSH}}{\mathrm{CH_4S}} + \mathrm{O_2} + \mathrm{H_2O} \longrightarrow \underset{\text{sulfide}}{\mathrm{H_2S}} + \underset{\text{formaldehyde}}{\mathrm{HCHO}} + \mathrm{H_2O_2}$$

The enzyme that catalyzes this reaction was purified from *Rhodococcus rhodochrous* (Kim *et al.*, 2000), but no genes encoding this function are known.

1.6.6 DMDS DEGRADATION

Abiotic oxidation of two MeSH molecules can produce dimethyldisulfide (DMDS), which is malodorous, toxic, and volatile. DMDS degradation was observed in *Pseudomonas fluorescens* strain 76, probably via MeSH. While the organism was not able to grow on DMDS as a sole carbon source, the sulfur moiety was assimilated and served as the sole sulfur source (Ito *et al.*, 2007). *Thiobacillus thioparus* strain E6 also reductivly cleaved DMDS (Smith and Kelly, 1988).

1.6.7 DMS UTILIZATION

Algae that cleave DMSP to DMS may also use DMS by an unknown mechanism (Wolfe *et al.*, 2002). Bacteria and archaea in anaerobic environments demethylated DMS to MeSH using methyltransferases. Aerobes in the genera *Hyphomicrobium* and *Thiobacillus* demethylate DMS to MeSH with a dimethyl sulfide monooxygenase (Bont *et al.*, 1981). These organisms were investigated as a biological control for industrially generated odoriferous DMS. However, the mechanism was not fully elucidated, and no genes were described (Borodina *et al.*, 2000; Kanagawa and Kelly, 1986; Pol *et al.*, 1994; Schafer, 2007; Suylen and Kuenen, 1986)

Visscher and Taylor (1993) proposed another mechanism of DMS degradation in *Thiobacillus* strain ASN-1. It includes a cobalamin methyl carrier and oxidation of folate-bound intermediates without production of free MeSH. The evidence for this pathway included the lack of inhibition by compounds that prevent monooxygenation or oxygenation and an absence of observed catalase activity, necessary to remove the H_2O_2 generated. This mechanism was proposed in a number of methylotrophs (Hoeft *et al.*, 2000).

There are multiple mechanisms for the oxidation of DMS to DMSO. While usually used for the reverse reaction, the widely distributed molybdopterin enzyme, dimethyl sulfoxide reductase (encoded by *dmsABC*), can carry out the oxidation of DMS (reviewed in McCrindle *et al.*,

2005). Dimethyl sulfide dehydrogenases specifically catalyze DMS oxidation in *Rhodovulum sulfidophilum* and are encoded by *ddhABC*. This is a molybdopterin enzyme much like a DMSO reductase (McDevitt *et al.*, 2002). In *Acinetobacter* strain 20B, the *dsoABCDEF* genes cluster was identified. It conferred the ability to oxidize DMS to DMSO and has homology to a multicomponent monooxygenase and phenol hydroxylase (Horinouchi *et al.*, 1997). There have been reports of a transformation of DMS to DMSO, methanesulfonate, formate, formaldehyde, and sulfate in a *Marinobacterium* by a light-dependent oxidation catalyzed by excreted photosensitisers, including riboflavins and FAD (Fuse *et al.*, 2000; Hirano *et al.*, 2003). There are also reports of ammonia monooxygenases that oxidize DMS to DMSO, perhaps as a side reaction (Juliette *et al.*, 1993).

1.6.8 DMSO UTILIZATION

Many organisms have the ability to respire DMSO to DMS. This reaction is so widely distributed, it is used as a marker for bacterial respiration. The molybdopterin enzyme dimethyl sulfoxide reductase, encoded by *dmsABC* catalyzes this reaction (reviewed in McCrindle *et al.*, 2005).

An alternate mechanism for the degradation of DMSO by further oxidation to $DMSO_2$ was proposed based on thermodynamic calculations (Wood, 1981). This chemical reaction was seen in abiotic water samples with chloroperoxidases and H_2O_2 (Taylor and Kiene, 1989) and was a component in a pathway of sulfur assimilation from DMS by *Pseudomonas putida* strain DS1. A transposon mutant was isolated that was not able to catalyze the transformation of $DMSO_2$ to methanesulfonate. This led to the identification of the *sfnEC* genes, which encode a $FMNH_2$ reductase and monooxygenase (Endoh *et al.*, 2003).

METHANESULFONATE UTILIZATION

MeSH is oxidized in the atmosphere to methanesulfonate (MSA, $H_3CSO_3^-$). This acid is a sulfur source for many organisms and a source of carbon and energy for methylotrophic aerobes following oxidation to sulfite and formaldehyde. The gene responsible for this activity is methanesulfonate

monooxygenase. Gene clusters that confer this activity include: *msuD* in *Pseudomonas aeruginosa*, *ssuBACD* in *Bacillus subtilis*, and *ssuEADCB* in *E. coli*. The functional genes in these clusters are all $FMNH_2$-dependent monooxygenases (Bentley and Chasteen, 2004) and have been characterized in *Pseudomonas putida* strain DS1 as *ssuDE* (Endoh *et al.*, 2003). Some methylotrophs have a different system for the utilization of MSA. *msmABCD*, found in *Methylosulfonomonas* and *Marinosulfonomonas*, encode a unique hydroxylase, ferredoxin, and reductase (Kelly and Murrell, 1999).

1.7 Phylogeny of DMSP-utilizing bacteria

The distribution of the DMSP degradation activities in different phylogenetic groups of bacteria has been extensively studied.

[^{35}S]DMSP microautoradiography and FISH blotting has been used to determine the nature of the bacteria incorporating the sulfur moiety of DMSP. α-Proteobacteria were the primary incorporators of DMSP in all conditions, followed by γ-proteobacteria in coastal waters and *Cytophaga-Flavobacter* in both coastal and open ocean waters (Malmstrom *et al.*, 2004b; Vila *et al.*, 2004). The α-proteobacteria were primarily composed of organisms in the roseobacter clade in coastal waters, as 13–43% of DMSP-sulfur incorporating organisms hybridized to a roseobacter specific probe (Vila *et al.*, 2004). By using flow-cell sorting of marine samples amended with [^{35}S]DMSP, Vila-Costa *et al.* (2006a) found heterotrophic bacteria were the highest accumulators of DMSP, followed by *Prochlorococcus* and *Synechococcus*. More recently, [^{35}S]DMSP microautoradiography and FISH blotting has been applied to the α-proteobacterial SAR11 group, and 31–47% of the cells incorporating DMSP hybridized to a probe for SAR11 bacteria. This group is the predominant DMSP accumulator in oligotrophic waters (Malmstrom *et al.*, 2004a).

In an algal bloom in the North Sea, there was a good correlation between the abundance of the roseobacter clade and DMSP concentration (González *et al.*, 2000). In a North Sea algal bloom, the subset of the microbial population that had the highest biomass and *in situ* growth rate was isolated by flow cytometric sorting and was investigated by 16S rRNA gene sequence libraries. Seventy

percent of the genes in the library had roseobacter sequences. The abundance of roseobacters during the algal bloom (quantified by FISH) showed significant correlation to DMSP degradation rates, and there was a single roseobacter clade 16S rRNA gene sequence that made up 24% of the total bacterial population (Zubkov *et al.*, 2001).

In another study, organisms with elevated nucleic acid content (indicating active replication) were isolated by flow-cytometric sorting after DMSP amendment to coastal seawater, and 16S rRNA gene libraries and T-RFLP abundance profiles were used to identify groups that had a response to the DMSP treatment (Mou *et al.*, 2005). There was variability between samples collected from May 2002 – May 2003, but some populations were consistant in their responses to DMSP, particularly sequences in the roseobacter clade, which made up 19–37% of the actively replicating subpopulation. While 28–38% of bacterial sequences could not be identified to a specific group, β-proteobacteria were highly represented in two samples (25–30%), γ-proteobacteria were abundant in another two samples (14–21%) and *Bacteroidetes* made up 35% in one sample (Mou *et al.*, 2005).

To identify organisms that might consume volatile DMS, enrichments inoculated with samples from coastal and oligotrophic marine environments and supplemented with DMS were investigated by 16s rRNA clone library sequencing. The libraries included 55% γ-proteobacteria, 23% α-proteobacteria, and 17% *Bacteroidetes*. The γ-proteobacteria sequences were composed of sequences similar to *Methylophaga* in enrichments from both environments and composed of sequences similar to *Thiomicrospira* from oligotrophic environments. The α-proteobacteria sequences were composed of sequences similar to roseobactera sequences in coastal waters and SAR11 sequences in oligotrophic environments (Vila-Costa *et al.*, 2006b).

While the bacteria accumulating the sulfur moiety of DMSP are well known, phylogenetic distribution of the demethylation, cleavage, and other activities of the DMSP degradation pathways are not fully known and could effect DMS release. While other groups are also involved, the roseobacter clade is linked to environmentally significant amounts of DMSP-sulfur incorporation and DMSP degradation (González *et al.*, 1999). Both pathways of DMSP degradation are

commonly found in isolated members of the roseobacter group. Fifteen isolated strains from the roseobacter clade cleaved DMSP to DMS (only four were isolated using DMSP enrichments), and five formed methanethiol, an intermediate found in the demethylation pathway (González *et al.*, 1999).

1.7.1 The roseobacter clade

A major group of marine heterotrophs, the roseobacter clade, are significant contributors to DMSP degradation in the environment. The roseobacter clade is a monophylogenetic group of strictly marine α-proteobacteria. The first roseobacter member described possessed bacterial chlorophyll and exhibited pink pigmentation (Shiba, 1991), but most members of this group lack these properties (González *et al.*, 2003). They are ubiquitous in marine systems and found in high abundance in seawater (González *et al.*, 1999; González and Moran, 1997). The 16S rRNA gene sequences from the roseobacter clade are the second most common group found in marine 16S rRNA gene libraries (Giovannoni and Rappé, 2000) and comprise 25% or more of the total bacterial sequences (Buchan *et al.*, 2005). They are one of the dominant groups associated with algal blooms (González *et al.*, 2000). Roseobacters are particularly abundant in coastal seawater (González and Moran, 1997) and seem to fill many niches (Buchan *et al.*, 2005). The group has been well reviewed (Buchan *et al.*, 2005; Wagner-Dobler and Biebl, 2006), including reviews focusing on their diversity (Brinkhoff *et al.*, 2008) and genomic analysis (Moran *et al.*, 2007).

Unlike many other abundant groups of marine bacteria, many roseobacters are easily isolated. However, the members of the roseobacter clade grown in pure culture do not capture the diversity of those that are abundant in marine systems. For instance, during an algal bloom, members of the roseobacter clade made up the largest single group, up to 12% of the total bacteria, as enumerated by ROS537 probe FISH vs. DAPI counts. However, only 1% of the bacteria in the bloom were detected with RSB67, a probe that binds to 16S rRNA gene sequences from currently cultured roseobacters (Eilers *et al.*, 2001). The ease of culturing roseobacters compared to other groups of marine bacteria, most of which have not be grown in pure culture, allows experimental approaches

that would otherwise be currently impossible. Environmental roseobacter species are related to organisms that are culturable on defined and rich media, have developed genetic systems, and are amenable to laboratory molecular techniques (Buchan *et al.*, 2000; González *et al.*, 1999). Genes that are found in laboratory strains can be used to discover homologous genes that are important in environmental populations (Buchan *et al.*, 2001). A cultured and sequenced representative, of the roseobacter clade *Silicibacter pomeroyi*, is a model system for both demethylation and cleavage.

1.7.2 *Silicibacter pomeroyi*

ISOLATION

One roseobacter isolate capable of degrading DMSP by both demethylation and cleavage pathways is *Silicibacter pomeroyi*. *S. pomeroyi* was isolated in 1998 from southeastern U.S. seawater enriched with 10 μM DMSP (González *et al.*, 1999) and named after Dr. Lawrence R. Pomeroy for his work in elucidating the "microbial loop" (González *et al.*, 2003).

TAXONOMY

The first *Silicibacter* was isolated from a silica-rich lake (Petursdottir and Kristjansson, 1997). However, this property is not a distinguishing characteristic of bacteria in this genus. It has been proposed that *Silicibacter* should be reclassified into the genus *Rugeria* (Yi *et al.*, 2007), which was proposed by Uchino *et al.* (1998) as a reorganization of marine *Agrobacterium* originally described by Rüger and Hofle (1992). The description of *Ruegeria* has standing in nomenclature over *Silicibacter* because it was validated earlier (IJSEM, 1999a,b). However, further work has suggested that, on the basis of different physiological traits, DNA G+C content, and 16S rRNA gene sequences, that the genera are not synonymous (Muramatsu *et al.*, 2007).

DMSP DEGRADATION

S. pomeroyi can demethylate DMSP or cleave it to DMS, grow on DMSP as sole carbon source, and incorporate sulfur from DMSP into cell protein (González *et al.*, 1999). It is able to reduce

DMSO to DMS, consume DMS and MeSH (González *et al.*, 1999) and degrade other sulfur-containing compounds (González *et al.*, 2003). *S. pomeroyi* regulates the activity of the cleavage pathway based upon DMSP concentrations and growth stages (González *et al.*, 1999; Kiene and Linn, 2000a). These characteristics make it an excellent model organism in which to study the mechanism and regulation of these activities, particularly in relation to sulfur demand in marine systems.

GENES INVOLVED IN SULFUR TRANSFORMATION

The genome sequence of *S. pomeroyi* consists of a 4.1 megabase circular chromosome and a 0.5 megabase megaplasmid. The megaplasmid may be linear and encodes many genes involved in energy metabolism and transport, as well as two tRNA genes, suggesting that it is a essential part of the genome (Moran *et al.*, 2004). Multiple replicative units are common in the α-proteobacteria. *S. pomeroyi* has more genes involved in transport than any previously sequenced α-proteobacteria, including five systems for transporting glycine betine (*OpuAD*-like genes), which may also transport DMSP, based on uptake competition studies (Kiene *et al.*, 1998).

The gene responsible for DMSP-cleavage in *S. pomeroyi* has not been conclusively identified. *S. pomeroyi* contains a homolog with 41.2% identity and 60.0% similarity to the *dddD* characterized from *Marinomonas*, at locus SPO1703. It may be upregulated during growth on DMSP (Burgmann *et al.*, 2007). *S. pomeroyi* does not contain a close homolog to *dddL*. Two N-terminal fragments of DMSP lyase proteins have been identified from *Pseudomonas* and *Alcaligenes* isolates (de Souza and Yoch, 1995, 1996b), but no corresponding gene could be found in these or other organisms that exhibit this activity (Bacic, 1999), and full length sequences of these genes have never been described. A search in the predicted proteome of *S. pomeroyi* with Protein Prospector (Chalkley *et al.*, 2005) did not find any significant matches. In addition, no significant matches were found using a Blast search optimized for short nearly exact matches (Altschul *et al.*, 1990) and a PSI-Blast search (Altschul *et al.*, 1997) using the consensus positions of the two fragments as the motif region in the *S. pomeroyi* genome.

The *dmdA* gene that catalyzes the first step of the demethylation of DMSP to MMPA was first described in *S. pomeroyi* and is found at locus SPO1913 (Howard *et al.*, 2006). The demethylation of DMSP is a THF-dependent reaction. *dmdA* and genes involved in methyl transfer via THF were upregulated in microarray experiment with DMSP (Burgmann *et al.*, 2007). Upstream of *dmdA* and transcribed in the opposite direction is a GntR family transcriptional regulator. Immediately downstream of *dmdA* is a dehydrogenase. *S. pomeroyi* contains many other genes belonging to the aminomethyltransferase family, which may catalyze demethylation of other compounds in the DMSP degradation pathway. α-Proteobacteria, particularly roseobacters, are among the bacteria that contain the largest number of proteins in this family, and *S. pomeroyi* has 42 genes classified as aminomethyltransferase family genes (Pfam01571).

The enzyme that may be responsible for incorporating MeSH into methionine, methionine γ-lyase (E.C. number 4.4.1.11), is found in the *S. pomeroyi* genome at locus SPOA0318. This gene was upregulated in microarray experiments with growth on DMSP (Burgmann *et al.*, 2007). Unlike other organisms that require uptake of the cofactor for methionine γ-lyase, *S. pomeroyi* has all genes necessary to synthesize pyridoxal phosphate via the *de novo* pathway. Some genes involved in the degradation of products of DMSP cleavage or demethylation (acrylate, DMS, and MeSH) are found in the *S. pomeroyi* genome, but others are not yet identified.

The acrylate formed by the classical cleavage pathway and by the demethylation pathway may be degraded via acrylate-CoA or propionate and propionyl-CoA. While these transformations are catalyzed by various enzymes, *S. pomeroyi* lacks the genes for of many of these pathways. A recently described pathway for acetate assimilation widespread in the α-proteobacteria, the ethylmalonyl-CoA pathway, has propionyl-CoA as a important intermediate (Erb *et al.*, 2007). The known genes in the ethylmalonyl-CoA pathway have high homology to genes in *S. pomeroyi*. In particular, a diagnostic enzyme for this pathway, crotonyl-CoA carboxylase/reductase, from *Rhodobacter sphaeroides* strain 2.4.1 has 93.0% similarity to the predicted gene product of SPO370 in *S. pomeroyi*. Genes that are theorized to catalyze the remaining reactions in the

pathway have high homology to genes of *S. pomeroyi* (Alber *et al.*, 2006; Erb *et al.*, 2007; Meister *et al.*, 2005).

Homologs to the anaerobic dimethyl sulfoxide reductase genes (*dmsABC*) are found in the genome of *S. pomeroyi* at locus SPO3557–SPO3559. Homologs of this enzyme can catalyze the reduction of DMSO or the oxidation of DMS, as well as similar activities for a wide variety of methylated S- and N-compounds. The genes were upregulated during growth on DMSP (Burgmann *et al.*, 2007). However, *S. pomeroyi* was observed to carry out the reduction of DMSO to DMS, but not the reverse reaction (González *et al.*, 1999), which would indicate that this enzyme is not involved in the degradation of DMS. *S. pomeroyi* does not have homologs to known dimethyl sulfide dehydrogenases, dimethyl sulfide monooxygenases or aminomonooxygenases that may catalyze the oxidation of DMS, DMSO, or methanesulfonate. PQQ is an important cofactor for some methylotrophic pathways, including methylamine degradation (Duine and Frank, 1990). While many organisms use this cofactor, not all can synthesize it (including *E. coli*). *S. pomeroyi* carries all the genes for the *de novo* synthesis of PQQ (*pqqABCD* at SPO1504–SPO1501).

Another possible pathway for use of the sulfur moieties from MeSH and DMS is the energy-conserving oxidation to sulfate. This activity is widespread in marine bacteria. *S. pomeroyi* has genes encoding sulfur oxidation (the *sox* cluster reviewed in Friedrich *et al.*, 2005, 2001). *S. pomeroyi* has genes with high homology to the genes of the *sox* system, composed of *soxRSVWXYZABCDF*, *soxG*, and *soxH*, that are found at the loci SPO0989–SPO1001, SPO0788 and SPO2822, respectively (Moran *et al.*, 2004). *S. pomeroyi* also displays high sulfite oxidase activity in enzyme assays following growth with either thiosulfate or sulfite (González *et al.*, 1999). Reduced sulfur in the form of thiosulfate increased the growth yield of *S. pomeroyi* in carbon-limited batch cultures (Moran *et al.*, 2004), indicating that the organism is able to decrease the amount of fixed organic carbon that is respired to CO_2 when using this system. This type of lithoheterotrophic metabolism might be used in DMSP degradation, as reduced sulfur moities might be formed by the degradation of MeSH or DMS. A lithoheterotrophic metabolism is also indicated by the *coxSML* genes for CO oxidation (but not genes for known autotrophic pathways;

Moran *et al.*, 2004). This activity is widespread in marine systems, particularly in the roseobacter clade (Tolli *et al.*, 2006).

A final system for degradation of organic sulfur compounds is found in the genome. *S. pomeroyi* has a novel pathway for the degradation of taurine (a sulfonate) and other organic compounds with an oxidized ($C-SO_3^-$) sulfur moiety (Cook *et al.*, 1998; Denger *et al.*, 2006). This pathway proceeds via cysteate (containing a $-SO_3^-$ and $-NH_3^+$ group) to sulfite (Cook *et al.*, 2006). Key enzymes in this pathway use pyridoxal 5‘-phosphate cofactors. *S. pomeroyi* possesses all genes necessary for the mineralization of the sulfonate taurine (Cook and Denger, 2006; Gorzynska *et al.*, 2006) and can grow on this compound as a sole carbon and energy source (González *et al.*, 2003).

1.8 Importance and Relevance

The degradation pathway of DMSP and the role that microorganisms play in this environmental pathway is an area of active research. It is important in our understanding of global climate and climate regulation, the global sulfur and carbon cycles, and the marine microbial loop (Pomeroy, 1974). Understanding the mechanism of DMSP degradation will add to our knowledge of bacterial biochemical activities, the diversity of genes encoding for these important functions, and the details of the metabolism of this compound.

DMSP is a significant marine carbon and sulfur source and has a large environmental and climatic impact due to DMS release from the oceans. Knowledge of DMS climate feedbacks would improve our ability to model global climate. Bottom up approaches to algal production of DMSP have not led to a better understanding of DMS release. It has become clear that it is the bacterial metabolism and alternative fates of the sulfur moiety that drive much of the degradation of DMSP. It is necessary to understand how the entire marine bacterial community will degrade DMSP to predict the release of DMS. The process is complicated and may rely on whole ecosystem measurements, like reduced sulfur demand by microorganisms, to determine the rates of the different processes that make up the DMSP degradation pathway.

Members of the roseobacter clade appear to be important in these activities in natural systems. *S. pomeroyi* is a sequenced roseobacter species, easily manipulated in pure culture, and will serve as a gateway to understanding other organism's DMSP degradation activities. Since *S. pomeroyi* has both the cleavage and demethylation pathway, it is an interesting system to investigate questions of sulfur demand and regulation. *S. pomeroyi* genome has been sequenced, opening up many approaches for gene discovery.

Many chemical intermediates in the DMSP-degradation pathway have not been confirmed, and the enzymes that carry out many of these processes are not known. Knowledge of conserved genes responsible for particular enzymatic activities would allow the use of molecular techniques to investigate the DMSP degrading properties of marine populations and allow further work on the complex properties that determine DMSP degradation in marine systems. With extended sequencing and metagenomic investigations of the ocean, the staggering number of genes for which there is no known or predicted function has emerged. Identifying new pathways and identifying unknown genes that code for known functions is a major challenge of environmental microbiology. Many of the metabolic processes involved in DMSP degradation are still not known. While recent work has uncovered two DMSP lyase genes, at least one more remains to be found, and none of the available lyases seem numerically important in marine systems, based on the abundance of homologous genes in metagenomic surveys (Howard *et al.*, 2008). Also, the mechanisms of DMS and MeSH degradation in marine systems have not been identified, and further work is necessary to elucidate the role of DMSO in the DMSP-degradation pathway. An understanding of the abundance and phylogenetic distribution of the genes involved in each step of the DMSP degradation pathway would help link DMSP degradation rates and fate to other ecological and biogeochemical parameters, including nutrient limitation and the flux through the microbial loop.

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CHAPTER 2

GROWTH OF *Silicibacter pomeroyi* AND DMSP DEGRADATION

2.1 ABSTRACT

Silicibacter pomeroyi, a member of the a marine roseobacter clade, is a model system for the study of dimethylsulfoniopropionate (DMSP) degradation. *S. pomeroyi* can cleave DMSP to dimethyl sulfide (DMS) and demethylate DMSP to methanethiol (MeSH). While a number of genes involved in sulfur transformations have been found in the sequenced genome of *S. pomeroyi*, many genes involved in the degradation of DMSP are not known. In this work, two important tools for the differential display gene discovery of genes responsible for DMSP degradation were developed: a *Silicibacter* minimal medium to aid in growth studies and a chemostat to provide steady state growth conditions. Growth experiments from these two developments have yielded important insights into the energetics of DMSP degradation.

2.2 INTRODUCTION

Release of volatile organic sulfur (VOS) from DMSP degradation is a critical part of the global sulfur cycle, and heterotrophic marine bacteria, particularly the roseobacter clade, play a critical role in DMSP's compeating cleavage and demethylation degradation pathways. Key enzymes in these pathways have not been identified. One approach to discover these unknown genes is to identify them in a model organism in pure culture. *S. pomeroyi* (Figure 2.1) is a member of the roseobacter clade that was isolated in 1998 from seawater enriched with 10 µM DMSP (González *et al.*, 1999). *S. pomeroyi* can grow on DMSP as sole carbon source, demethylate DMSP to MeSH, cleave DMSP to DMS, incorporate DMSP-sulfur into cellular protein, and catabolize other sulfur-containing compounds (González *et al.*, 2003, 1999). Cleavage and demethylation activity in

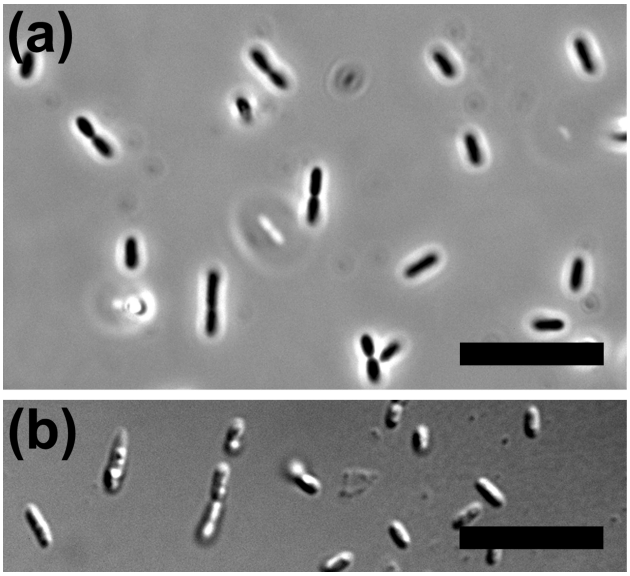

Figure 2.1: *S. pomeroyi* micrographs: a) Phase contrast and b) differential interference contrast light micrographs of *S. pomeroyi*. Bars, 5 µm.

S. pomeroyi vary under different concentrations of DMSP and during different stages of growth (González *et al.*, 1999; Kiene and Linn, 2000), indicating complex regulation of genes in both pathways. With its ability to both cleave and demethylate DMSP, *S. pomeroyi* is an excellent model organism to understand the mechanism and regulation of these activities in marine systems.

2.2.1 Growth of *S. pomeroyi*

Many characteristics of *S. pomeroyi* physiology have been determined in batch growth studies. *S. pomeroyi* has an optimal salt concentration of 100–400 mM NaCl and a temperature range from 10–40 °C (González *et al.*, 2003). In marine basal media (MBM), it is able to grow on acetate, ethanol, DL-β-hydroxybutyrate, glucose, succinate, acrylic acid, acetate, cysteic acid, glycerol, citrate, pyruvate, Casamino acids, L-alanine, L-arginine, L-serine, L-taurine, L-methionine, glycine betaine, and DMSP as the sole source of carbon and energy (González *et al.*, 2003).

During growth on 10 µM DMSP, *S. pomeroyi* produced DMS and MeSH during exponential-phase growth (González *et al.*, 2003). At this concentration of ^{35}S-DMSP, 38% of the radiolabeled sulfur was released as volatiles (mostly DMS), 38% was released as dissolved non-volatile compounds, and only 12% was incorporated into trichloroacetic acid (TCA) insoluble material (González *et al.*, 1999). At low (2.5 nM) concentrations, radiolabeled sulfur from ^{35}S-DMSP was mostly (75%) incorporated into TCA insoluble material, probably protein, while lower amounts were released as SO_4^{2-} (1.3%) and volatile compounds (2%).

While both MeSH and DMS are released by *S. pomeroyi* during growth on DMSP, they are also consumed after longer incubation times and during nutrient depletion. During growth on DMSP, DMS accumulated in the headspace over 24 h and was not consumed as it was being produced. However, when DMS was provided to *S. pomeroyi* in media containing glucose or yeast extract, 55% or 100% of the DMS, respectively, was consumed after 48 h (González *et al.*, 1999). Radiolabled MeSH was completely consumed after 48 h by *S. pomeroyi* in MBM with glucose (González *et al.*, 1999). At low (29 nM) concentrations, radiolabled MeSH was mostly (92%) incorporated into TCA insoluble material, with trace amounts (2%) released as SO_4^{2-}. At higher concentrations (950 nM) 29% was released as dissolved non-volatile compounds, with 67% incorporated into TCA insoluble material (González *et al.*, 1999). *S. pomeroyi* also released MeSH from the degradation of 3-methiol-propionate and 2-ketomethiol-butyrate (González *et al.*, 1999).

Both cleavage and demethylation pathways for DMSP degradation are active when DMSP is present (see Section 5.4.1), but the differential expression of the two pathways is consistant with a shift to demethylation when cells have a higher demand for reduced sulfur compounds (Kiene *et al.*, 1999). If the DMSP degradation pathways are regulated in response to the sulfur demand of the culture, demethylation genes should be expressed at higher levels when other sources of reduced sulfur are not available. Cleavage genes should be expressed at higher levels when the concentration of DMSP exceeds the demand for reduced sulfur. Whether or not this regulatory hypothesis is correct, genes involved in DMSP degradation are induced in the presence of DMSP, and gene expression may change in response to the concentration of DMSP (González *et al.*, 1999). There-

fore, genes for DMSP degradation in *S. pomeroyi* are good candidates for a differential display gene discovery approach. A differential display proteomic approach to detect proteins upregulated with growth on DMSP would allow the identification of genes involved in DMSP degradation. In order to achieve this goal, a defined medium and a method to control for growth effects of different carbon substrates is necessary.

2.3 Materials and methods

2.3.1 Strains and media

Silicibacter pomeroyi DSS-3^T was obtained from Dr. Mary Ann Moran (deposited as DSM 15171 and ATCC 700808) (González *et al.*, 2003). Stock cultures were frozen in 40% glycerol plus Half strength yeast tryptone sea salt media and passed no more than six times. Cultures were grown aerobically at 30 °C in the dark unless otherwise noted.

Half strength yeast tryptone sea salt

Half strength yeast tryptone sea salt media (1/2YTSS) was composed of 4 g tryptone, 2.5 g yeast extract, and 20 g sea salts (Sigma Chemical Co., Saint Louis, MO, USA) in 1 L H_2O. Agar was added at 15 g L^{-1} agar for solid medium (González *et al.*, 1996).

Marine basal medium

Marine basal medium (MBM) was composed of 50 mM NaCl, 12.5 mM $MgSO_4$, 2.5 mM KCl, 2.5 mM $CaCl_2$, 0.24 mM K_2HPO_4, 1.33 mM NH_4Cl, 0.07 mM FeEDTA, 0.07 mM TRIS adjusted with HCl to pH 7.5, 0.24 mM K_2HPO_4, 1.33 mM NH_4Cl (Baumann and Baumann, 1981) and was amended with a 1% vitamin solution (González *et al.*, 1997).

Silicibacter BASAL MEDIUM

Silicibacter basal medium (SBM) was prepared as a mixture of three solutions: pre-basal, pre-salt, and carbon source. Stocks were mixed in the order given below. The pre-salt solution was composed of 580 mL distilled H_2O, 50 mL of 0.2 M KCl, 50 mL of 0.2 M $CaCl_2$, 50 mL of 1 M $MgSO_4$, and 50 mL of 4 M NaCl. This pre-salt solution was sterilized by autoclaving and allowed to cool to 50 °C. The pre-basal solution was composed of: 56.5 mL distilled H_2O, 50 mL of 1 M PIPES at pH 7.5, 20 mL of 50 mM K_2HPO_4, 20 mL of 0.5 M NH_4Cl, 50 mL of 1.36 mM FeEDTA, 1 mL of trace metals mixture, and 2.5 mL of vitamin mix. The trace metals and vitamin mixtures are described below. The pre-basal solution was filter sterilized. DMSP, acetate, or other sources of carbon and energy were prepared in 20 mL of distilled H_2O and sterilized by filtration. The final medium was prepared by combining these three solutions in the following ratio: 780 mL of the pre-salt solution, 200 mL of the pre-basal solution, and 20 mL of the carbon source, resulting in a final concentrations of: 200 mM NaCl, 50 mM $MgSO_4$, 10 mM KCl, 10 mM $CaCl_2$, 50 mM PIPES, 1 mM K_2HPO_4, 10 mM NH_4Cl, 0.07 mM FeEDTA, trace amounts of vitamins and minerals, and a carbon source, usually provided at 5 mM to 50 mM. Agar was added at 1.5% w/v for solid medium.

TRACE MINERALS SOLUTION

The trace metals solution used in SBM was based on that of Whitman *et al.* (1987) and was composed of the following compounds: 7.8 mM nitroloacetic acid, 0.53 mM $MnSO_4$, 0.42 mM $CoCl_2$, 0.35 mM $ZnSO_4$, 0.038 mM $CuSO_4$, 0.11 mM $NiCl_2$, 1.2 mM Na_2SeO_3, 0.41 mM Na_2MoO_4, 0.33 mM Na_2WO_4, and 0.25 mM Na_2SiO_3. It was sterilized by filtration and stored at 4 °C.

VITAMIN SOLUTION

The vitamin solution used in MBM and SBM was composed of the following (all percentages in w/v): 0.002% biotin, 0.002% folic acid, 0.01% pyridoxine-HCl, 0.005% riboflavin, 0.005% thiamine, 0.005% nicotinic acid, 0.005% pantothenic acid, 0.0001% cyanocobalamin, and 0.005% w/v p-aminobenzoic acid. It was sterilized by filtration and stored at 4°C.

2.3.2 CHEMICALS

The MeSH source used as a standard was obtained from a continuous-release Dynacal permeation tube purchased from VICI metronics (Part number 107-050-6000-C35, Santa Clara CA). Crude DMSP was generously provided by Dr. Al Place at COMB. Purified DMSP was purchased from Selact BV (Netherlands) or synthesized using the following protocol (see 2.3.3). All other compounds were purchased from Sigma-Aldrich (Saint Louis, MO) or Difco (Detroit, MI).

2.3.3 DMSP SYNTHESIS

Dimethylsulfoniopropionate (CAS# 4337-33-1, MW 170.6589, M.P. 125 °C) was synthesized using the procedure of Chambers *et al.* (1987) as modified in Steinke *et al.* (1998). DMSP was formed by bubbling gaseous hydrochloric acid through a mixture of DMS and acrylic acid in methylene chloride, and purified by recrystallization from a methanol and ether mixture. This is the reverse of the biologically catalyzed DMSP cleavage reaction, and requires a free H^+ ion, provided by $HCl_{(g)}$:

$$C_3H_3O_2-_{(l)} + C_2H_6S_{(l)} + H^+ \longrightarrow C_5H_{10}O_2S_{(s)}$$

All reagents used were reagent grade and anhydrous (including the DMS). Gaseous hydrogen chloride was generated from the reaction of aqueous hydrochloric acid introduced by capillary tube to the bottom of a dripping funnel filled with sulfuric acid by the method of Conant and Quayle (1943), referencing Sweeney (1917) (see Figure 2.2). In a typical reaction, 10 mL of DMS (the limiting reagent), 2 mL of acrylic acid, and 60 mL of methylene chloride were mixed in a round-bottom flask, and HCl(g) was bubbled through the mixture for 20 min (Figure of 2.2). The resulting white crystals were decanted, collected by filtration in a fritted glass funnel with weak vacuum, and washed with 600 mL of methylene chloride. Recoveries of 97%, as measured by elemental analysis, were routine. This crude DMSP was dissolved in a minimal volume of methanol with gentle

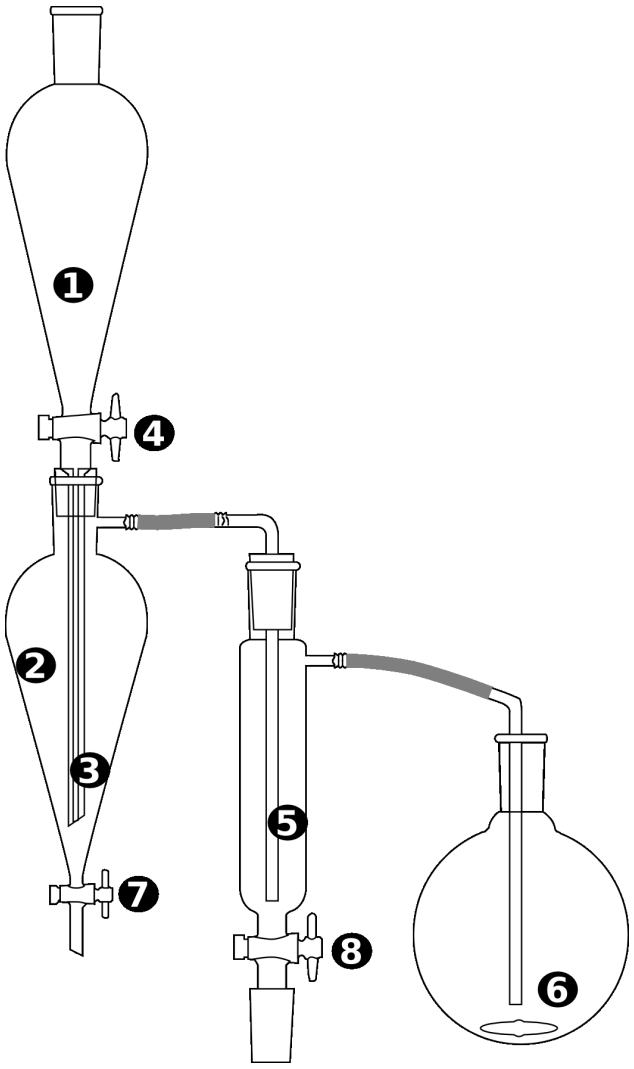

Figure 2.2: DMSP synthesis glassware: Diagram of the glassware for the synthesis of DMSP: 1) 125 mL dripping funnel filled with 100 mL $HCl_{(aq)}$; 2) 500 mL separatory funnel filled with 100 mL H_2SO_4; 3) capillary tube used to introduce $HCl_{(aq)}$ into H_2SO_4 and, by hydrostatic pressure, prevent gas flow back up the tube; 4) valve for controlling the release of $HCl_{(aq)}$; 5) 250 mL column for bubbling through 100 mL H_2SO_4; 6) 250 mL round bottom flask with a Teflon magnetic stir bar and a tube for bubbling with $HCl_{(g)}$; 7) and 8) valves to allow removal of unused acids from apparatus. All tubing was Teflon, all seals were ground glass joints.

heating and stirring. The solution was then cooled, and diethyl ether was added dropwise until crystals formed. Purified DMSP was crystallized at 4 °C and recovered by filtration and washing with 600 mL of methylene chloride. The resulting DMSP was a white fluffy powder and was recovered at routine yields of 68%, as measured by elemental analysis. To confirm the synthesis, elemental analysis for C, H, O, S, and Cl was performed by Atlantic Microlab (Atlanta, GA, USA). Initial synthesis was confirmed by NMR at the Chemical Sciences Magnetic Resonance Facility, UGA. Purities of 98% for the initial crude preparation were common, and the final recrystallized purified preparation was greater than 99.5% DMSP, with less than 0.5% acrylate as measured by either method.

2.3.4 Chemostat design

Two continuous flow chemostats were constructed from glass and operated in parallel (see Figure 2.3 for a diagram of one chemostat). The retention volume for each was 50 mL or 100 mL. Both chemostats were operated aerobically and sparged with room air sterilized by a 0.2 µm high volume filter (Whatman 6702-3600). Air flow was measured and controlled by rotameters (Aalborg 184373-3), and a constant flow of air insured that the chemostats maintained positive pressure. For growth with DMSP and acrylate, air flow was at 20 mL min^{-1}. A Teflon-coated magnetic stir bar was used for mixing, and temperature was controlled by a water pump operating at 30 °C and water-jacketed vessels. All liquid influx and egress was through drip chambers to prevent back-contamination. Back pressure was prevented in the reservoirs by venting with 0.2 µm sterile air filters (Whatman 673-5000). The chemostats were assembled with medium in the reservoirs (or the phase salt solution for growth on SBM), autoclaved for 60 min, attached to air pumps, allowed to cool, and any additional temperature-sensitive components to the medium were added via syringes though butyl rubber stoppers. Tubing was L/S16 Tygon, mated to L/S13 Tygon and passed through a Masterflex L/S peristaltic pump (with pump head 77200-50), which provided controlled flow rates of 0.05 to 10 mL min^{-1}. For growth on DMSP and acrylate, a 0.07 mL min^{-1} dilution rate was used, providing a volumetric retention time of 23.8 h for the 100 mL culture.

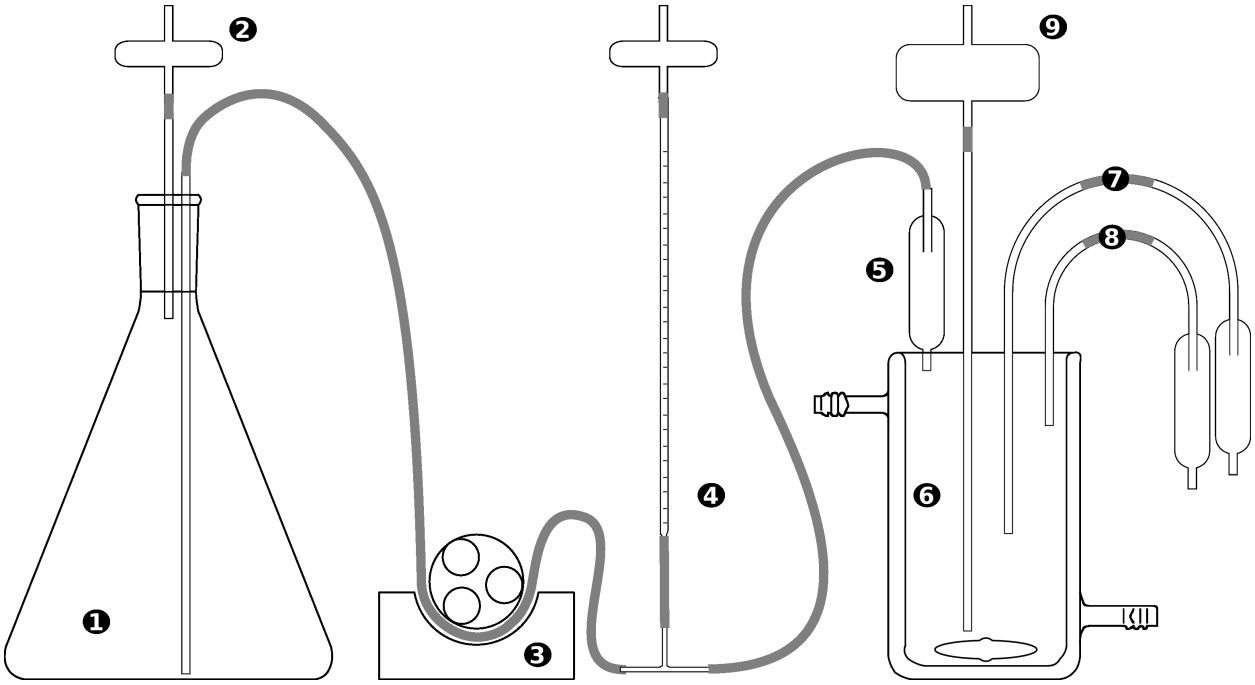

Figure 2.3: Diagram of chemostat: 1) 2 L medium reservoir; 2) 0.2 µm sterile air filters to prevent backpressure in reservoir (); 3) peristaltic pump; 4) in-line pipette equipped with a sterile air filter used to measure flow rates (during normal chemostat operation the tubing to pipette remained crimped and medium flowed directly to chemostat); 5) drip tube to prevent back-contamination; 6) water-jacketed culture vessel with Teflon stir-bar sealed with a rubber stopper; 7) efflux path resulting in 50 mL retention volume (tubing crimped for chemostat retention volume of 100 mL); 8) efflux path resulting in 100 mL retention volume (tubing crimped for chemostat retention volume of 50 mL); 9) high volume air filter for sparging tube.

Rates were measured by an in-line pipette equipped with a sterile air filter as described in Stafford (1986). Initial inoculation was performed though a inlet valve. Collection of overflow in 1 mL increments was used to follow the culture absorbance. Chemostat cultures of *S. pomeroyi* were grown in SBM inoculated with mid-log pre-cultures in the same medium. Steady-state growth was defined as identical absorbance over five volumetric exchanges.

2.4 Results and discussion

2.4.1 *Silicibacter* minimal medium development

The defined medium (Marine Basal Media; Baumann and Baumann, 1981) that was previously used for *S. pomeroyi* growth did not support cell densities high enough for differential proteomic gene discovery experiments or growth rate studies as it limited growth rates to 0.42 day^{-1} (González *et al.*, 2003) and limited final cell yields to low densities. In rich 1/2YTSS medium, *S. pomeroyi* has a doubling time of 1 h with a final absorbance of 1.5 at 600 nm, so these limitations were not an intrinsic property of *S. pomeroyi*. In addition, the effective buffering range of the organic buffer in MBM, TRIS, is 7.5–9 ($pK_a^{30\,^\circ C} = 8$). Therefore, at pH 7.5, the optimum for *S. pomeroyi*, TRIS is a poor buffer for acidic additions to media (Good *et al.*, 1966; Gueffroy, 1975). PIPES has an effective buffering range of 6.1–7.5 ($pK_a^{30\,^\circ C} = 6.7$) and was used instead of TRIS for better acid buffering at pH 7.5 (Good *et al.*, 1966; Gueffroy, 1975). Using the elemental cellular composition determined for *Escherichia coli* (Neidhardt and Umbarger, 1996), MBM media was calculated to be limiting in nitrogen (at an absorbance of 0.365 at 600 nm) and phosphorus (at an absorbance of 0.724 at 600 nm). A new defined minimal medium (SBM) was developed for *S. pomeroyi*, optimized for high growth rates and yield. Sufficient amounts of phosphorus and nitrogen were included, and the salt concentration was designed to be similar to the optimum for *S. pomeroyi*. Using this medium with 20 mM glucose, doubling times of four hours and culture absorbances of 1.8 were routinely observed. This higher growth density was critical for the isolation of sufficient amounts of proteins from cultures for differential display experiments.

2.4.2 *S. pomeroyi* GROWTH IN SBM

S. pomeroyi reached a maximum absorbance of 1.8 at 600 nm in SBM, which exceeded the maximum yield on MBM and 1/2YTSS. However, 1/2YTSS allowed for faster growth than SBM with any of the tested carbon substrates. Growth rates, final absorbances, and cell yields for acetate, acrylate and DMSP are presented in Table 2.1. Selected growth curves are shown in Figure 2.4.

Table 2.1: Characteristics of *S. pomeroyi* growth on SBM with carbon sources from 1 mM to 10 mM.

Substrate	Average Yield (OD_{600} mmol $^{-1}$)	Doubling time (h)
DMSP	0.041	25 [a]
Acrylate	0.037	10 [a]
Acetate	0.037	9

[a] non-logarithmic growth, so maximum doubling time is reported.

Acrylate and DMSP have similar yields and growth rates although growth is linear and not logarithmic. Long lag periods were also observed with acrylate and DMSP. This lag could be alleviated by pre-culture in a SBM with a single carbon substrate. Both DMSP and acrylate were toxic, and inhibited growth at concentrations between 5 mM and 10 mM. While DMSP is a potent osmolyte and is concentrated intracellularly, high extracellular concentrations are not typically experienced by the bacteria. Acrylate or some other intermediate might be toxic when used as the sole substrate for growth. Yields for DMSP or acrylate concentrations above 5 mM were determined by addition of DMSP or acrylate to a culture in 5 mM increments.

Casamino acids, methionine and cysteine supported growth as sole carbon sources. Casamino acid provided the highest growth rate, followed by methionine, with only weak growth on cysteine. *S. pomeroyi* grew well on glucose, acetate, and lactate, and had the shortest observed doubling time on lactate.

2.4.3 STEADY-STATE GROWTH OF *S. pomeroyi* IN SBM

A chemostat was developed to support growth of *S. pomeroyi* on DMSP and other compounds in a defined medium. This allowed the control over the growth rate and densities of *S. pomeroyi*. Ini-

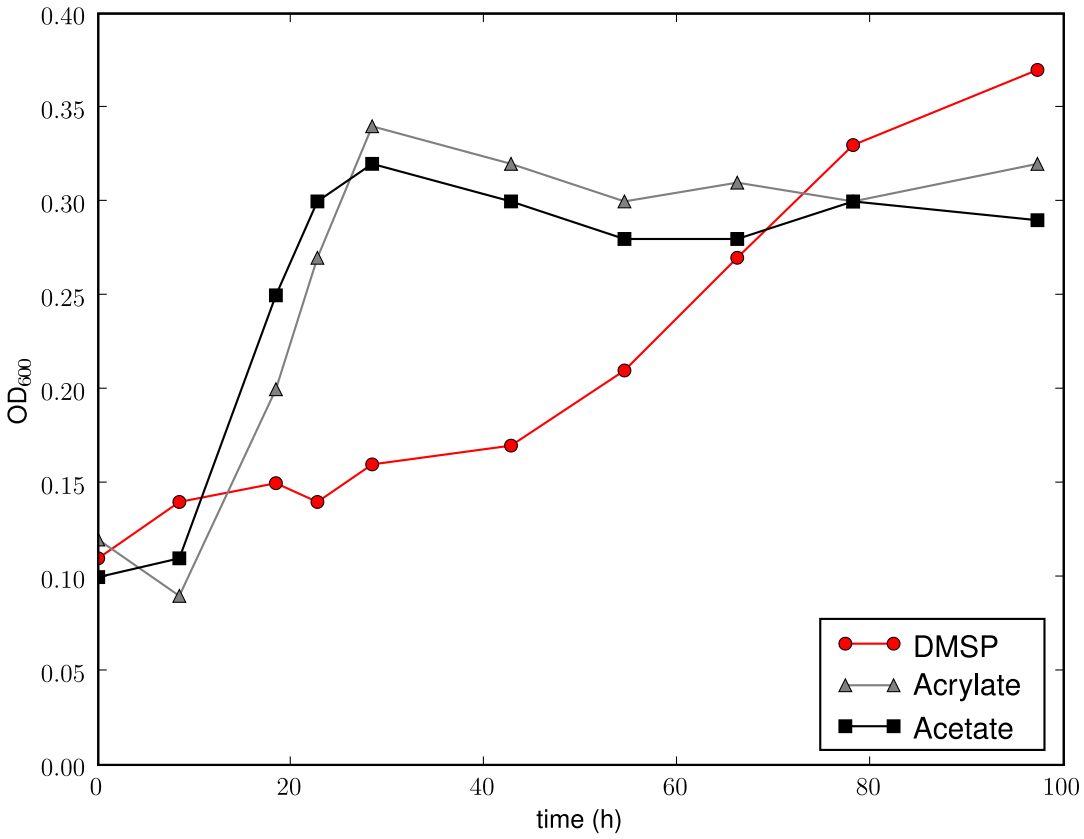

Figure 2.4: Growth of *S. pomeroyi*: Optical density of cultures grown on various compounds as a sole source of carbon and energy in SBM: circle, 5 mM DMSP; triangle, 5 mM acrylate; square, 5 mM acetate

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tially, a nitrogen-limited modification of SBM was tested, but steady state growth was not achieved, and the cultures washed out even at low flow rates (data not shown). A low sulfur medium was also tested, with similar results. Therefore, a carbon-limited chemostat was used, and growth on SBM with DMSP or acetate at a constant cellular density was achived for at least five days (five volumetric exchanges), suggesting maintenance of steady-state conditions. In two parallel carbon-limited chemostats, *S. pomeroyi* was grown with either 3 mM DMSP or 5 mM acetate at a volumetric replacement time of 24 h. The absorbance of outflow was measured at 4 h intervals. The average absorbance at 600 nm for the cultures were 0.248±0.012 for DMSP and 0.260±0.019 for acetate. Therefore the yield was 0.083±0.004 $OD_{600}\,mM^{-1}$ and 0.052±0.003 $OD_{600}\,mM^{-1}$, respectively, assuming that all substrate was consumed.

2.4.4 Energetics of DMSP degradation

Theoretical calculation of the energy available for degradation of a compound can aid in the understanding of it's degradation pathway. Since *S. pomeroyi* is an aerobic heterotroph with the ability to oxidize inorganic sulfur compounds, it is theoretically possible that it carries out the complete oxidation of DMSP ($C_5H_{10}O_2S$) to CO_2 and SO_4^{2-}. With 5 carbons carrying a net oxidation of -4 and the sulfur in the -2 state, the complete oxidation of DMSP to CO_2 and SO_4^{2-} would yield 24 electron equivalents for the carbon and 8 for the sulfur, for a yield of 32 electrons equivalents in total (see Appendix A for calculations). If the sulfur moiety is incorporated into cellular protein, electron equivalents are conserved, as it takes 8 electron equivalents to take SO_4^{2-} (+6) to the redox state of the sulfur in methionine. No net reduction is required to take the sulfur in DMS or MeSH to the same redox state as that in methionine. However, *S. pomeroyi* releases most of the sulfur moiety as volatile gasses, particularly DMS. If the methyl and sulfur moieties of DMSP were released as DMS only 12 reducing equivalents of acrylate would be available. The complete oxidation of acetate yields 8 electron equivalents. Therefore, the ratio of reducing equivalents between acrylate and acetate (1.5) would be observed between DMSP and acetate if DMSP was degraded to DMS and CO_2.

In a carbon-limited chemostat, the ratio of the cellular yield per mol of substrate during growth on DMSP to growth on acetate averaged 1.59$\pm$0.112, close to the expected value of 1.5 for the oxidation of only the acrylate moiety of DMSP. Since the predicted molar ratio for complete oxidation of DMSP to acetate is 4 (from a calculated electron ratio of 32:8), it is unlikely that significant amounts of the reduced sulfur and methyl moieties are oxidized under these conditions. In addition, similar growth yields were observed for batch cultures grown on equimolar concentrations of DMSP or acrylate, supporting this conclusion. These results are consistant with *S. pomeroyi* releasing most of the sulfur from DMSP without oxidation under these growth conditions While *S. pomeroyi* does have the capability of utilizing MeSH and DMS and oxidizing reduced sulfur compounds, these activities do not seem to be significant under these conditions. The small increase above the expected yield could come from: 1) oxidation of small amounts of sulfur, 2) utilization of the methyl group transferred to a carrier in the demethylation pathway, 3) the savings from sulfur assimilation, or 4) compatible solute effects of DMSP.

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CHAPTER 3

PROTEOMIC DISCOVERY AND ANALYSIS OF GENES

3.1 ABSTRACT

Silicibacter pomeroyi, a member of the a marine roseobacter clade, is a model system for the study of dimethylsulfoniopropionate (DMSP) degradation. A differential display proteomic approach was used to identify proteins upregulated with growth on DMSP to identify candidate genes involved in DMSP degradation. *S. pomeroyi* was grown under steady-state conditions in duplicate chemostats with either DMSP or acetate as a sole carbon source. Duplicate samples from each chemostat were collected, and protein was extracted for 2-dimensional differential in-gel electrophoresis. About 1000 protein spots were detected. While 80 protein spots increased in abundance during growth on DMSP, only 25 were sufficiently abundant for trypsin fragment peak profile identification using matrix-assisted laser desorption/ionization (MALDI) time of flight spectroscopy (ToF-MS). Six unique gene products and two gene product paralog pairs were identified with high confidence, including a gamma-glutamyltranspeptidase (at locus SPO0633), a methylmalonate-semialdehyde dehydrogenase (at locus SPO2203), a solute-binding protein (at locus SPO2573), an alcohol dehydrogenase (at locus SPO1914), a formate dehydrogenase (at locus SPOA0272 or SPO3850), and a conserved hypothetical protein (at locus SPOA0269). These gene products and their potentially co-transcribed neighboring gene products may be involved in DMSP degradation.

3.2 INTRODUCTION

Dimethylsulfoniopropionate (DMSP) is an algal osmolyte ubiquitous in marine systems. Bacterial cleavage of dimethylsulfoniopropionate (DMSP) to volatile dimethyl sulfide in marine systems is

an important part of the global sulfur and carbon cycles and a regulator of global climate. An alternate demethylation pathway produces methanethiol that may be incorporated into cellular protein (Kiene *et al.*, 2000). These two pathways have radically different implications for the fate of sulfur in the environment, but many of the enzymes involved remain undescribed. Many aerobic heterotrophic bacteria, including the abundant and ubiquitous marine roseobacter clade, possess one or both of these pathways (Buchan *et al.*, 2005). The roseobacter *Silicibacter pomeroyi* is a model system for both degradation pathways of DMSP. *S. pomeroyi* can cleave DMSP to dimethyl sulfide (DMS), degrade DMS, demethylate DMSP to methanethiol (MeSH), incorporate MeSH into cellular protein, and oxidize MeSH to sulfate (González *et al.*, 1999). While a number of genes involved in sulfur transformations have been found in the sequenced genome of *S. pomeroyi* (Moran *et al.*, 2004), many genes involved in the degradation of DMSP are not known.

One method to identify genes used during growth on a particular substrate is differential display proteomics, where cells are grown with the substrate of interest and reference substrate, and their proteomes are extracted. Next, proteins that are more abundant following growth on the substrate of interest are identified by techniques such as two-dimensional differential in-gel electrophoresis (2D-DIGE). Finally, these proteins are then linked to the genes that code for them, for instance by finding their trypsin-digest peptide fragment pattern using matrix-assisted laser desorption/ionization (MALDI) time of flight mass spectroscopy (ToF-MS). This approach is useful for pathways that are known to be differentially regulated. Using a *Silicibacter* minimal medium that supports high cell densities and a chemostat to provide steady state growth conditions (See Chapter 2), a differential display proteomics approach was used to find genes potentially involved for DMSP degradation in *S. pomeroyi*.

This approach will allow the identification of a subset of the proteins whose abundance increases when *S. pomeroyi* is grown on DMSP compared to an alternate carbon source, and the identification of the genes that code for these proteins. Due to methodological limitations, this approach will only identify a subset of proteins that increase in abundance, and is unlikely to identify membrane-associated and insoluble proteins that do not separate on the 2D gel, proteins

that are resistant to trypsin digestion or have fragments that ionize poorly, and regulated proteins with similar pI and size to constitutively expressed, highly abundant proteins. However, no specific pathway is targeted by this approach — any protein that increases in abundance might be detected. Pathways that might be upregulated include DMSP cleavage and demethylation, MeSH and DMS degradation, MeSH protein incorporation, methyl group utilization, sulfur oxidation, and degradation of the carbon backbone of DMSP.

3.3 Materials and methods

3.3.1 Strains, media, and growth conditions

Silicibacter pomeroyi DSS-3^T (DSM 15171 and ATCC 700808) was grown in *Silicibacter* basal medium (SBM, see Section 2.3.1) at 30 °C unless otherwise noted. For batch-grown conditions, *S. pomeroyi* was grown in 50 mL SBM cultures with either 3 mM DMSP or 5 mM acetate as the sole carbon and energy source in a 250 mL Erlenmeyer flask at 30 °C with shaking.

3.3.2 Chemostat growth

S. pomeroyi was grown in 100 mL chemostats (see Section 2.3.4 for details of construction and operation) in SBM with 3 mM DMSP or 5 mM acetate as the sole carbon and energy source and with water jacket temperature controlled at 30 °C. The chemostat was inoculated with 50 mL of mid-log phase cultures in the same medium. A 0.07 mL min^{-1} medium dilution rate was used, providing a volumetric retention time of 23.8 h for the 100 mL culture. Sterile room air was provided at 20 mL min^{-1}. Cell growth was followed by measurement of the OD_{600} of the chemostat overflow, collected in 1 mL increments. After 5 volumetric exchanges at a constant OD_{600}, a 50 mL sample was removed, and cells collected as described below. After the chemostat was refilled at 0.07 mL min^{-1} and the OD_{600} stabilized for 5 more volumetric exchanges at constant OD_{600}, another 50 mL sample was collected.

3.3.3 Protein extraction

The urea for the lysis buffer was purified by passing 8.5 M urea over a AG501-X8 anion/cation-exchange resin (Bio-Rad) column. Lysis buffer was composed of 8 M urea, 4% CHAPS, and 15 mM TRIS (pH 8.5 at 4 °C). Whole cell proteome extracts were prepared by collecting 50 mL chemostat samples, centrifuging at 12,000 × G for 10 min to collect the cells, washing the cells at 4 °C with deionized, sterile water, resuspending the cells in lysis buffer, and freezing the suspension at -40 °C. Protein was extracted by sonication on ice at 50% power for 30 s six times, centrifuging as before, and centrifuging at 62,000 × G for 1 h. The final supernatant contained the protein extracts and was desalted and concentrated with three exchanges of the lysis buffer using Microcon YM-10 centrifugal filter devices (Millipore). Protein extracts were quantified by the Pierce BCA kit (Smith *et al.*, 1985) using BSA as a standard.

3.3.4 Proteomic identification of peptides with increased abundance

2D-DIGE, gel scanning, statistical analysis, and trypsin digest peak profile protein identification by matrix-assisted laser desorption/ionization (MALDI) time of flight spectroscopy (ToF-MS) was performed using equipment at the Georgia Proteomics Resource Facility and the Medical College of Georgia.

2D-DIGE

Concentrated protein extracts from the two chemostat samples of both the DMSP- and acetate-grown cultures were labled: for each sample, 50 µg total protein was minimally labeled with 200 pmol Cy5 (DMSP-grown cell protein) or Cy3 (acetate-grown cell protein) fluorescent dyes (Amersham Biosciences) at 4 °C for 30 min. A pooled sample composed of 12.5 µg total protein from each protein extract was similarly labeled with Cy2 fluorescent dye. The labeling reactions were quenched with 10 nmol lysine.

One pair of Cy5 labeled DMSP- and Cy3 labeled acetate-grown proteome samples and the pooled Cy2 labeled proteome sample were all combined, and proteins were loaded on two 24 cm

pH 3–10 IPG strips (Amersham Biosciences) cut down to 18 cm to span pH 3–7.5. This pH range was previously shown to capture all of the protein spots that were visable with silver staining. The proteins were separated using the manufacturer recommended voltage program totaling 32,000 cumulative volt-hours in a 5 M urea, 2 M thiourea, 2% CHAPS, 0.5% IPG buffer, and 0.002% bromophenol blue buffer after active rehydration at 30 V. IPG strips were equilibrated in 6 M urea, 2% SDS, 64.8 mM DTT, 30% glycerol, 50 mM TRIS pH 8.8, and 0.002% bromophenol blue for 15 min at room temperature. IPG strips were then equilibrated with the above buffer modified by replacing DTT with iodoacetamide and incubated for 15 min at room temperature. Strips were affixed with molten agar and proteins were separated on 8–15% gradient SDS polyacrylamide gels in an Ettan DALT electrophoresis system (Amersham Biosciences) according to the manufacturers instructions. Gels were fixed in 30% v/v ethanol and 7.5% v/v acetic acid overnight at room temperature and then were imaged using a Typhoon 9400 (Amersham Biosciences). Settings on the photomultiplier tubes were optimized for each laser scan to achieve the broadest dynamic range.

Gel analysis

Gel images were processed using the DeCyder software (Amersham Biosciences). Initial automatic spot selection normalized to background was filtered with the following maximum cutoffs: slope of 1, area of 450, maximum peak of 175, maximum volume of 100,000. Gel images in each fluorescent dye channel were normalized to total protein in the pooled protein sample channel for that gel per the manufacturer's recommended analysis protocol. Spots were aligned between two gels using this mixed standard channel. Differential spot density was statistically analyzed for significance across both replicates based on the student's T and one-way ANOVA tests with a confidence interval of 95%, as implemented by the manufacturer's software. Each statistically significant spot selected for picking was examined manually for potential overlap from neighboring peaks.

Spot picking

Gels were stained with Sypro Ruby (Molecular Probes), destained in 10% v/v methanol and 6% v/v acetic acid for 30 min at room temperature, imaged as above, and matched to the Cy images using DeCyder software (Amersham Biosciences). This scan was used to estimate the quantity of protein found in each spot. The coordinates for the automated picking of spots was based on the Sypro image. In addition, for lower abundance spots a gel with higher protein loads (200 µg of only the protein extracts from the DMSP-grown culture) was run, processed as above, and Sypro stained. Spots were picked in gel plugs with a diameter of 2.0 mm, proteins digested, and peptides extracted and spotted (see below) using a Spot Handling Workstation (Amersham Biosciences) or by hand.

Trypsin digestion

Gel plugs were washed once with distilled deionized water, twice with 50 mM ammonium bicarbonate with 50% v/v methanol for 20 min, and finally with 75% v/v acetonitrile for 20 min at room temperature. Gels were then dried at 40 °C for 10 min. Following reduction with 10 mM DTT in 20 mM ammonium bicarbonate and alkylation in 55 mM iodoacetamide for 30 min, proteins were in-gel digested by incubation with 140 ng of trypsin (sequencing grade; Promega) at 37 °C for 1 h.

MALDI-ToF MS and MS/MS

Peptides were extracted twice with 50% v/v acetonitrile with 0.1% v/v triflouroaceitic acid for 20 min at room temperature. Approximately 25% of the resulting peptides mixtures were spotted with partially saturated α-cyano-4-hydroxy-cinnamic acid (Sigma). MS and MS/MS data were acquired on the 4700 MS-ToF/ToF Proteomics Analyzer (Applied Biosystems) using standard acquisition methods. MS spectra were calibrated using two trypsin autolysis peaks (1045.45 and 2211.096 m/z). MS/MS spectra were calibrated using the instrument default processing method.

PEPTIDE PEAK PROFILE SEARCHING

Peptide peak profiles for each trypsin digestion were filtered for peaks within 0.25 kDa of a predicted human keratin trypsin digestion and matrix fragments. Mass lists for all monoisotopic peptide peak profiles from the chemostat experiments were used to search a custom database built from translations of coding regions of the *S. pomeroyi* genome using Mascot (Matrix Science) with one or fewer missed cleavage sites and 50 ppm mass accuracy. Protein scores over 80 and confidence intervals over 95% were considered good matches, which routinely corresponded to more than eight peptides from the identified protein. In addition, ProteinProspector (Clauser *et al.*, 1999) and Emowse (Pappin *et al.*, 1993) were used to confirm searches. In the initial batch grown culture experiment, Emowse was used for all identifications. Unidentified trypsin digests were analyzed by MS/MS spectra of major peaks using 4700 Proteomics Analyzer MS-ToF/ToF and the resulting peaks analyzed using Data Explorer (Applied Biosystems). Identified proteins were compared with the predicted molecular weight and pI of the original protein spot on the gel.

3.3.5 BIOINFORMATIC ANALYSIS

Tools used to identify genes included the BLAST search engine (Altschul *et al.*, 1990), the Joint Genome Institutes's Integrated Microbial Genomes (Markowitz *et al.*, 2007), and the European Molecular Biology Laboratory's InterProScan (Mulder *et al.*, 2007). Tools used to investigate the roles and pathways of the genes included the Brenda Enzyme Information System (Schomburg *et al.*, 2002), SEED (Overbeek *et al.*, 2005), and MetaCyc (Caspi *et al.*, 2008). Alignments and trees were produced using ClustalW (Thompson *et al.*, 1994) or MUSCLE (Edgar, 2004) followed by Gblocks (Castresana, 2000), PHYLIP (Felsenstein, 1989), and TreeDyn (Chevenet *et al.*, 2006). Alignment of proteins for comparison of conserved or catalytic residues was by the Smith-Waterman algorithm as implemented by EMBOSS (Rice *et al.*, 2000). Operon prediction was by OFS1.2 using a cutoff probability of 0.8 (Westover *et al.*, 2005). Abundance of genes predicted in metagenomic databases was based on the approach of Howard *et al.* (2008) using a 10^{-30} e-value cutoff, and percentages were calculated based on the length-weighted average of single copy genes

(Howard *et al.*, 2008). Sargasso Sea databases were accessed on December 2006 and searched with a local BLAST database and custom scripts written by Shulei Sun. The GOS database was downloaded in April 2007 and BLAST searched with a 10^{-30} e-value cutoff. Reciprocal best BLAST searches for orthologs in roseobacter and "*Candidatus* P. ubique" genomes were performed by a locally installed BLAST database and custom scripts in the Perl programing language with an initial 10^{-30} e-value cutoff.

3.4 Results and discussion

A differential display proteomic approach was used to identify proteins that increased in abundance with growth on DMSP compared to growth on acetate. Acrylate, the presumed common product of both the demethylation and cleavage pathways, would seem to be a good choice for the alternate carbon source in a differential display study, but it has been shown to induce DMS and MeSH production in *S. pomeroyi* (see Section 5.4.1). Therefore, acetate was used to provide a control condition lacking in the production of these gene products. Proteins that are regulated in response to growth on DMSP, including those that carry out the degradation of the 3-carbon backbone of DMSP to central intermediates, are targeted in this approach.

3.4.1 Protein extraction

S. pomeroyi was grown on varying concentrations of DMSP and acetate, and 3 mM DMSP and 5 mM acetate yielded a maximum culture density of approximately 0.25 OD_{600}. The similar growth yields on DMSP and acetate for these concentrations were predicted by electron yield calculations (See Appendix A). Batch grown 100 mL cultures at this density routinely yielded 200 µL of 10 mg mL^{-1} extracted proteins. Washing the cells quickly with distilled water was found to be necessary to reduce salt contamination from the medium. In initial 2D gels, an interfering agent prevented isoelectric focusing. This interfering agent could be removed by TCA precipitation or centrifugation at 62,000 × G for 1 h. In preliminary experiments, a high level of carbamylation was observed as horizontally repeating spots for all proteins. This problem was solved by a

anion/cation-exchange resin wash of the urea used to extract the proteins and keeping proteins at 4 °C during preparation and sonication. The final protocol yielded protein samples at high enough concentrations and free of interfering agents for 2D gel electrophoresis.

3.4.2 Batch grown cell proteomics

The proteome of *S. pomeroyi* batch cultures grown on 3 mM DMSP or 5 mM acetate were extracted and used in a DIGE experiment. The 10 protein spots whose abundance most increased with growth on DMSP were picked and identified by trypsin digest peak profile MALDI ToF-MS. Six gene products were identified (see Table 3.1). While roles for these gene products in the degradation of

Table 3.1: Gene products increased in abundance with batch growth on DMSP.

Abundance Ratio	% matched	Gene locus	Annotation
61.36	50	SPO1508	quinoprotein ethanol dehydrogenase
5.99	30	SPO1914	zinc-binding alcohol dehydrogenase
5.87	23	SPO2814	ABC periplasmic peptide-binding protein
5.49	43	SPO0633	gamma-glutamyltranspeptidase
5.17	24	SPO0326	acetyl-CoA acetyltransferase
10.62	26	SPO0142	acetyl-CoA acetyltransferase

DMSP are possible, variation in 2D spot patterns was seen from experiment to experiment. Additionally, the identified acetyl-CoA acetyltransferase is an enzyme in the energy storage polyhydroxybutarate pathway. This result may indicate that the increase in protein abundance was caused by growth effects due to the slower doubling time on DMSP than acetate and not regulation due to the DMSP substrate. To control for growth effects, chemostat growth with DMSP or acetate as limiting carbon sources were used.

3.4.3 Chemostat growth results

The proteome of duplicate 50 mL samples of chemostat *S. pomeroyi* cultures grown on either 3 mM DMSP or 5 mM acetate were extracted and used in a two-gel DIGE experiment with a pooled reference sample (see Figure 3.1). While statistical significance was weighted for individual pro-

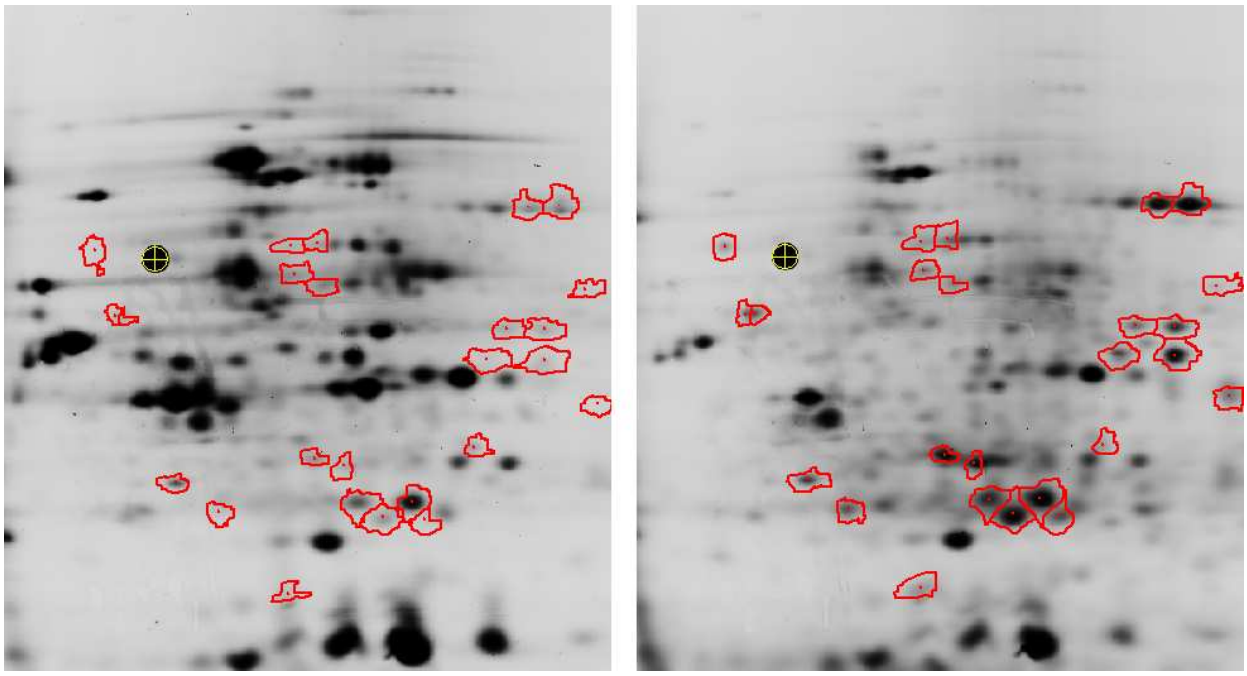

Figure 3.1: 2d gel area of interest: from DIGE scans of the channel from samples grown on acetate (left) and DMSP (right). Spots significantly increased in abundance and selected for identification are outlined. The pI range of this region is roughly 3.5 to 7 (left to right) and the size range is 150 to 20 kDa (top to bottom).

tein detection and abundance, a roughly 2.5-fold increase in abundance was typically statistically significant. Over 1000 filtered protein spots were found on each gel, and 80 were significantly increased in abundance. Of these, 25 spots matched across the two gels were abundant enough (by Sypro staining) to pick for trypsin digest peak profile MALDI ToF-MS (See Table 3.2).

High confidence identification was achieved with 16 of the trypsin digest peak profiles. However, some proteins were identified from multiple plugs from the same gel. This could be due to covalent modification of the protein that caused a shift in the gel or overlap of gel spots and therefore mixing of the proteins. In particular, the plug identified as carrying peptides from the protein at locus SPOA0269 was found in multiple neighboring spots and one at a distant location on the gel, and and may have obscured peptides from other proteins that increased in abundance but were not as ionizable. Peptides matching this gene product were filtered from the trypsin digest peak

profiles, and in one case, the identification of a second protein that had increased in abundance, but obscured by overlapping SPOA0269, was possible. The remaining spots did not contain identifiable peptides of other proteins. In addition to the 6 non-redundant protein spots identified as single gene products, two protein spots were identified as gene products that have close paralogs in the genome, either of which could have produced the protein identified. These paralog pairs produce proteins with high identity, and none of the peptide fragments identified are from regions that were predicted to vary between the paralogs. In total, 8 gene products were identified, two of which could be produced from two loci on the genome (See Table 3.3).

Table 3.2: Identification of proteins increased in abundance following growth on DMSP.

Category	Number
Total protein ORFs predicted in genome	4322
Approximate spots detected in each scan	2000
Approximate filtered spots detected in each scan	1000
Spots aligned across all scans	756
Spots significantly increased in abundance	80
Spots abundant with sypro staining	25
Plugs picked from two gels	32
Non-overlapping plugs	28
Samples with peptide fragments	23
Samples without human keratin fragments	18
Proteins identified with > 90% C.I.	16
Unique proteins identified	6
Pairs of paralogs identified	2

3.4.4 Gene Clusters

Many of the genes found are in clusters of genes that are closely spaced and transcribed in the same orientation (see Table 3.4.4 for a list of these genes). All of the genes except for those at locus SPO3850 and SPO2203 are in clusters of genes that are transcribed on the same orientation and may be co-transcribed. No clear terminator or promoter regions are found between any of the potentially co-transcribed genes (see Figures 3.2 and 3.3). Two of these genes (at loci SPOA0269

Table 3.3: Gene products increased in abundance with chemostat growth on DMSP: The average abundance ratio is reported as the ratio of protein spot area from DMSP grown cells to acetate grown cells across all replicates, the ANOVA score indicates the significance of the increase in abundance of the protein spot, and the confidence interval (CI) indicates the percent probability that the observed trypsin digest peak profile matches this gene product. Both ANOVA score and CI are from the plug with highest identification values. See Section 3.4.6 for details of the identification and annotation.

Avg. abundance ratio	ANOVA	Protein C.I.%	Gene locus	Annotation
2.81	0.01	95.678	SPOA0269	Conserved hypothetical protein
3.04	0.01	99.97	SPO3850	Glutathione-dependent formaldehyde dehydrogenase [a]
3.04	0.01	99.984	SPOA0272	Glutathione-dependent formaldehyde dehydrogenase [a]
3.42	0.03	93.306	SPO2203	Methylmalonate-semialdehyde dehydrogenase
3.5	0.02	98.664	SPO0633	Gamma-glutamyltranspeptidase
5.04	0.01	100	SPO1914	Zinc-binding alcohol dehydrogenase
6.32	0.03	95.151	SPO2573	Periplasmic binding protein
8.94	0.04	99.999	SPO3498	Translation elongation factor [b]
8.94	0.04	99.999	SPO0728	Translation elongation factor [b]
9.06	0.03	100	SPO1625	Serine protease precursor

[a, b] Pairs of orthologs that could not be distinguished.

and SPO1625) are predicted by OFS to be in operons. With the stringency conditions used, OFS is quite conservative in it's operon calls, and there may be other gene clusters that are co-transcribed. One gene is found in an cluster with *dmdA*, a DMSP demethylase (Howard *et al.*, 2006). A trypsin digest peak profile matched both of the paralogs SPOA0269 and SPO3850. It is more likely that SPOA0269 was the gene product that was detected, because SPOA0272 in the same gene cluster was similarly increased in abundance.

3.4.5 Homologs in genomes and metagenomes

Othologs to the genes in these clusters were found using a reciprocal best BLAST hit approach with stringent cut-offs between the genome of the SAR11 member "*Candidatus* P. ubique" and twelve sequenced roseobacters (see Table 3.5). Potential orthologs to many genes in these clusters are found in other roseobacters. Reciprocal best BLAST hits were found for SPOA0272 in all roseobacters, but not for the close paralog SPO3850, indicating that this gene duplication might be unique in *S. pomeroyi* and that SPOA0272 is the more widely conserved gene. Of these roseobacters, all except *Silicibacter sp.* TM1040 (whose production of DMS is unknown), and *Sagittula stellata* E37 and *Sulfitobacter sp.* EE-36 (which do not produce MeSH) produce both MeSH and DMS when grown with DMSP. *Silicibacter sp.* TM1040 and *Roseovarius nubinhibens* ISM produce MMPA from DMSP. This pattern suggests that none of the loci SPO3850, SPOA0268–SPOA0271 are solely sufficient to confer DMS or MeSH production from DMSP. The small number of organisms that only carry out only demethylation or only cleavage makes further predictions based on ortholog patterns difficult.

A metagenomic approach to analysis of the genomes of the roseobacter clade by Moran *et al.* (2007) has made predictions of orthologs that are found in most roseobacters (core roseobacter genes, Table S7 in original publication), orthologs that are specific to the roseobacters (roseobacter specific genes, Table S6 in original publication), and orthologs that have a high association with marine genomes and metagenomes (marine genes, Table S8 in original publication). Many of the genes in DMSP-related clusters are in the core roseobacter group (SPO0635, SPO0726, SPO1914,

Table 3.4: Chromosomal gene clusters: Each cluster of genes contains one or more products (in bold) that increased in abundance with chemostat growth on DMSP. [a]

Avg. abundance ratio	Locus	Annotation
	SPO0632	2-hydroxyacid dehydrogenase family protein
3.50	**SPO0633**	gamma-glutamyltranspeptidase
	SPO0634	FAD-binding oxidoreductase
	SPO0635	aminomethyl transferase family protein
	SPO0725	hypothetical protein
	SPO0726	DNA topoisomerase IV, A subunit
	SPO0727	hypothetical protein
8.94	**SPO0728**	translation elongation factor Tu (tuf)
	SPO1623	Sensor histidine kinase
	SPO1624	DNA-binding response regulator
9.06	**SPO1625**	periplasmic serine protease
	SPO1913	dmdA
5.04	**SPO1914**	zinc-binding alcohol dehydrogenase
3.42	**SPO2203**	methylmalonate-semialdehyde dehydrogenase
	SPO2571	TRAP transporter, putative
	SPO2572	hypothetical protein
6.32	**SPO2573**	extracellular solute-binding protein
8.94	**SPO3498**	translation elongation factor
	SPO3499	translation elongation factor
	SPO3500	ribosomal protein S7
	SPO3501	ribosomal protein S12
3.04	**SPO3850**	glutathione-dependent formaldehyde dehydrogenase
	SPOA0268	transcriptional regulator
2.81	**SPOA0269**	conserved hypothetical protein
	SPOA0270	conserved hypothetical protein
	SPOA0271	methylamine utilization protein
3.04	**SPOA0272**	glutathione-dependent formaldehyde dehydrogenase

[a] The paralog pairs were SPO0663 / SPO0728 and SPO3850 / SPOA0272.

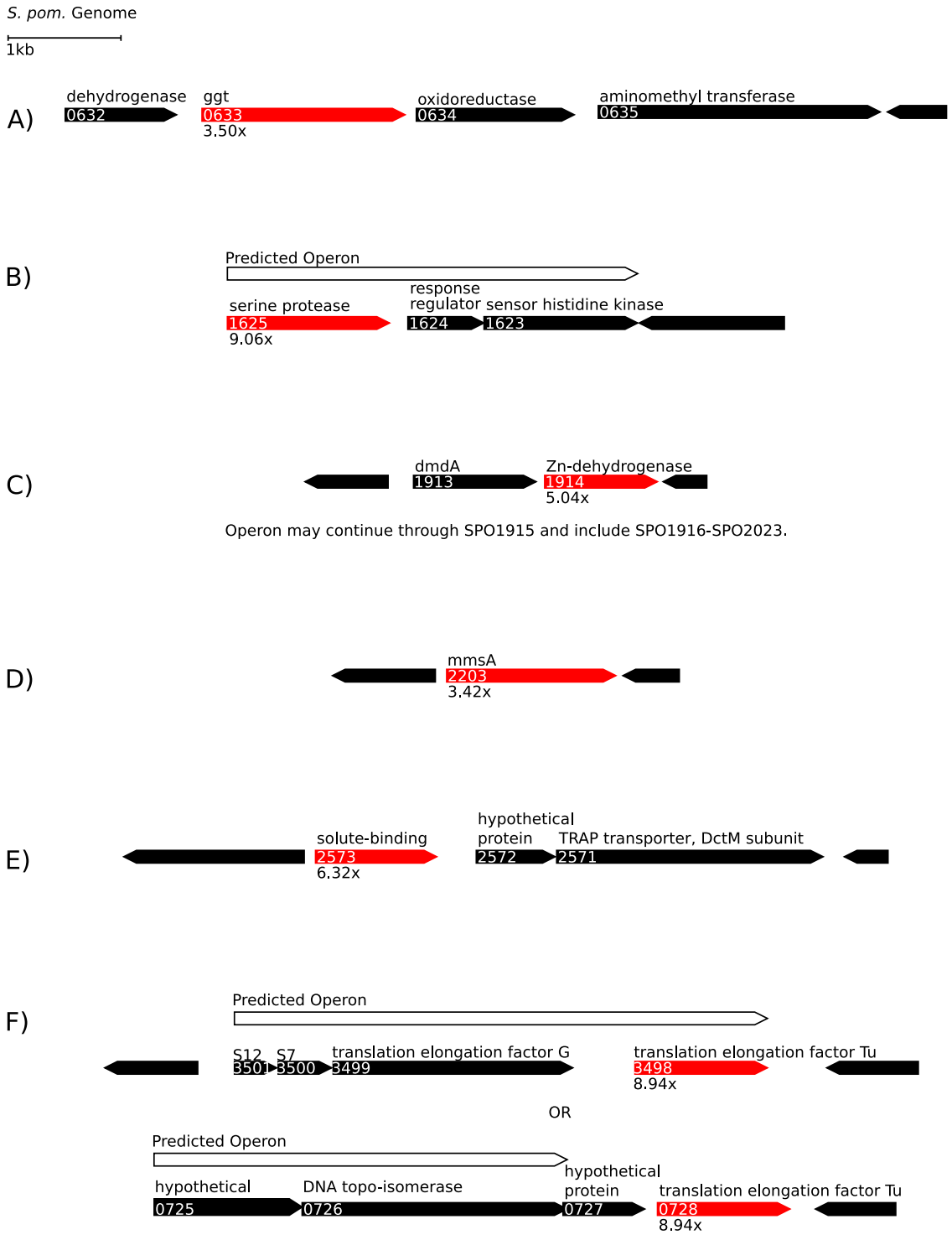

Figure 3.2: Chromosomal gene clusters: Genes coding for a protein increased in abundance with growth on DMSP are shown above the average amount they increased in abundance, OFS predicted operons are illustrated by a outline above genes predicted to be co-transcribed. Due to trypsin digest peak profile ambiguity, either gene cluster in f) could be upregulated.

Table 3.5: Orthologs: Gene clusters with genes (in bold) whose gene product increased in abundance with growth on DMSP. * indicates a reciprocal best BLAST hit to a gene in a sequenced genome of the roseobacter or "*Candidatus* P. ubique": A) "*Candidatus* P. ubique" HTCC1062; B) *Loktanella vestfoldensis* SKA53; C) *Roseovarius sp.* 217; D) *Roseobacter sp.* MED193; E) *Sulfitobacter sp.* EE-36; F) *Roseovarius nubinhibens* ISM; G) *Sulfitobacter sp.* NAS-141; H) *Oceanicola batsensis* HTCC2597; I) *Oceanicola granulosus* HTCC2516; J) *Rhodobacterales bacterium* HTCC2654; K) *Silicibacter sp.* TM1040; L) *Jannaschia sp.* CCS1; M) *Sagittula stellata* E37.

Gene locus	A	B	C	D	E	F	G	H	I	J	K	L	M
SPO0632	*	*	*	*	*	*	*	*		*	*	*	*
SPO0633			*	*	*	*	*	*			*	*	*
SPO0634			*	*	*	*	*	*	*	*	*	*	*
SPO0635	*	*	*	*	*	*	*	*	*	*	*	*	*
SPO0725													
SPO0726		*	*	*	*	*	*	*	*	*	*	*	*
SPO0727			*	*	*	*	*	*		*	*	*	*
SPO0728													
SPO1623			*		*	*	*			*			
SPO1624			*		*	*	*			*			*
SPO1625													
SPO1913	*		*	*		*					*	*	
SPO1914	*	*	*	*	*	*	*	*	*	*	*	*	*
SPO2203	*	*	*	*	*	*	*	*	*	*	*	*	*
SPO2571		*	*	*	*	*	*	*	*	*	*	*	*
SPO2572		*	*	*	*	*	*	*	*		*	*	*
SPO2573		*	*	*	*	*	*	*	*	*	*	*	*
SPO3498	*	*	*	*	*	*	*	*	*	*	*	*	*
SPO3499	*	*	*	*	*	*	*	*	*	*	*	*	*
SPO3500	*	*	*	*	*	*	*	*	*	*	*	*	*
SPO3501	*	*	*	*	*	*	*	*	*	*	*	*	*
SPO3850													
SPOA0268													
SPOA0269													
SPOA0270													
SPOA0271													
SPOA0272		*	*	*	*	*	*	*	*	*	*	*	*

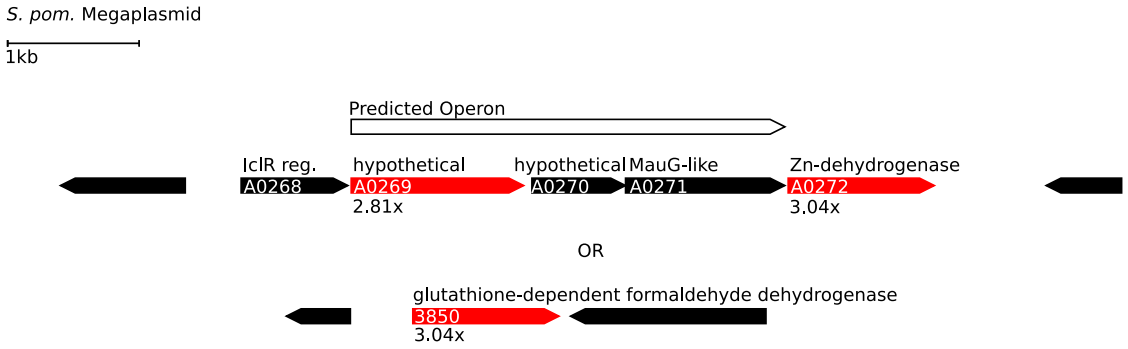

Figure 3.3: Megaplasmid gene clusters: Genes coding for a protein increased in abundance with growth on DMSP are shown above the average amount they increased in abundance, OFS predicted operons are illustrated by an outline above genes predicted to be co-transcribed. Due to trypsin digest peak profile ambiguity, either gene cluster could be upregulated.

SPO2203, SPO2571, SPO2573, SPO3498, SPO3499, SPO3500, SPO3501, and SPOA0272). The genes from the entire cluster of SPO2571–SPO2573 were in the roseobacter specific group. None were found in the marine specific genes. This may indicate that if these genes are involved in degrading a product of DMSP the same genes are found in other organisms outside the roseobacter clade, including non-marine organisms. While the cut-offs used in this study were intended to filter out more distant homologs, close homologs that have similar substrate specificity or those that have high similarity despite catalyzing different reactions could obscure the results.

The abundance of homologs in marine metagenomes can be used to determine how widespread a particular gene is in a population (Howard *et al.*, 2008). Homologs to the genes in these gene clusters were found for the Sargasso Sea metagenomic database (Venter *et al.*, 2004) and the Global Ocean Survey (which includes the Sargasso Sea database; Rusch *et al.*, 2007). Many gene products that increased in abundance with growth on DMSP were highly abundant in these marine metagenomes, including SPO0635, SPO0726, SPO0728, SPO2203, SPO3898 and SPO3899 (see Table 3.6). These genes are likely to be important in the marine environment.

Table 3.6: Homologs in Metagenomes: Gene clusters that contain one or more genes (in bold) whose gene product increased in abundance with chemostat growth on DMSP and the number of homologs and percentage of organisms in the: SS, Sargasso Sea metagenomic database; GOS, Global Ocean Survey database. Percentages over 100 indicates that the gene is more common than single-copy number genes in the metagenome, indicating universal conservation and variability in this enumeration, or multiple homologs in a majority of the organisms sampled.

	SS		GOS	
Locus	Number	Percentage	Number	Percentage
SPO0632	316	28.6	933	19.1
SPO0633	220	19.9	917	18.8
SPO0634	377	34.1	1609	33.0
SPO0635	1020	92.2	4564	93.6
SPO0725	0	0.0	0	0.0
SPO0726	745	67.3	4132	84.7
SPO0727	0	0.0	3	0.1
SPO0728	649	58.7	3984	81.7
SPO1623	11	1.0	17	0.3
SPO1624	206	18.6	259	5.3
SPO1625	514	46.5	2506	51.4
SPO1913	560	50.6	2266	46.5
SPO1914	254	23.0	1077	22.1
SPO2203	1027	92.8	3781	77.5
SPO2571	337	30.5	588	12.1
SPO2572	6	0.5	58	1.2
SPO2573	315	28.5	1121	23.0
SPO3498	649	58.7	3984	81.7
SPO3499	1071	96.8	5169	106.0
SPO3500	369	33.4	1397	28.6
SPO3501	351	31.7	1688	34.6
SPO3850	132	11.9	708	14.5
SPOA0268	0	0.0	7	0.1
SPOA0269	8	0.7	53	1.1
SPOA0270	1	0.1	18	0.4
SPOA0271	12	1.1	89	1.8
SPOA0272	132	11.9	666	13.7

3.4.6 Analysis of function

dmdA gene cluster

The gene at locus SPO1913 is next to a gene whose product increased in abundance with growth on DMSP. SPO1913 is *dmdA* the DMSP demethylase that catalyzes the demethylation of DMSP to MMPA, transferring the methyl group to tetrahydrofolate (Howard *et al.*, 2006). *dmdA* was previously known to be upregulated with growth on low concentrations of DMSP. The chemostat growth conditions provided this condition, and the identification of this gene cluster acts as a proof of principle for the differential display proteomic approach.

The gene product of locus SPO1914 increased in abundance with growth on DMSP and was also seen in batch-grown experiments. It matches Pfam 107, which is composed of NADPH quinone reductases and related Zn-dependent oxidoreductases, and Pfam 8240, which is composed of alcohol dehydrogenases. These families include three major groups of enzymes: alcohol dehydrogenases, NADPH:quinone reductases (recruited in mammals as a structural protein of the eye), and formaldehyde dehydrogenases. While these gene functions are difficult to distinguish on the basis of sequence or even structural similarity (Sulzenbacher *et al.*, 2004), SPO1914 does contain the catalytic domain of alcohol dehydrogenases and a NADH-binding domain (found in all three groups). SPO1914 has higher similarity with both alcohol dehydrogenases and NADPH:quinone reductases than formaldehyde dehydrogenases. The zinc-binding site, which is found in alcohol dehydrogenases but not the NADPH:quinone reductases is not identifiable on the basis of sequence similarity. SPO1914 has 53% identity and 70% similarity to *E. coli* K-12 YhdH, an uncharacterised protein with a solved crystal structure that lacks a bound zinc ion, which suggests that it is a quinone oxidoreductase (Sulzenbacher *et al.*, 2004). All conserved residues predicted to be involved in quinone-binding were found in SPO1914. However, these conserved residues were predicted on the basis of three proteins, none of which have a known quinone substrate. The reaction cleft is too small for many quinones (Sulzenbacher *et al.*, 2004) and may accommodate another type of substrate. Homologs to this gene are universally conserved in genome sequences

of roseobacters and in "*Candidatus* P. ubique" (See Table 3.5), as well as being a core roseobacter gene (Moran *et al.,* 2007). SPO1914 probably reduces NADH with a electron transfer from an unknown quinone or an alcohol. If the electron transfer is from an alcohol, an aldehyde or ketone would be formed. SPO1914 is potentially involved in the oxidation of a degradation product of the DMSP or MMPA carbon backbone.

SPO2203 GENE

The gene product from locus SPO2203 increased in abundance with growth on DMSP. It is not in a predicted operon. SPO2203 was annotated as *mmsA*, a methylmalonate-semialdehyde dehydrogenase (E.C. number 1.2.1.27). It matches protein families Pfam 00171 and TIGRfam 1722, and contains a conserved NADH-binding region and the putative cysteinyl active site of realted enzymes. SPO2203 has 48% identity and 61% similarity to a methylmalonate-semialdehyde dehydrogenase characterized from *Pseudomonas aeruginosa* (Swis-Prot P28810; Bannerjee *et al.*, 1970; Hatter and Sokatch, 1988; Sokatch *et al.,* 1968). It is more similar to this gene and to the *Bacillus subtilis mmsA* than to other aldehyde dehydrogenases with unknown substrate specificity. Methylmalonate-semialdehyde dehydrogenase catalyzes the reaction:

$$\underset{\text{2-methyl-3-oxopropanoate}}{\mathrm{O{=}CHCH(CH_3)COO^-}} + \mathrm{CoA{-}SH} + \mathrm{NAD^+} \longrightarrow \underset{\text{propanoyl-CoA}}{\mathrm{CoA{-}S{-}C(=O)CH_2CH_3}} + \mathrm{CO_2} + \mathrm{NADH}$$

In the rat, *mmsA* was found to also catalyze a similar reaction with the unmethylated compound malonate-semialdehyde ($\mathrm{O{=}CHCHCOO^-}$), forming acetyl-CoA (Goodwin *et al.*, 1989). In addition, with the purified enzyme from *Pseudomonas aeruginosa*, β-mercaptoethanol substituted for the CoA in the reaction. If the 3-carbon backbone of DMSP is oxidized from acrylate to β-hydroxypropionate to malonate-semialdehyde, SPO2203 could catalyze the formation of propanoyl-CoA. However, SPO2203 is also 28% identical and 47% similar to a characterized betaine aldehyde dehydrogenase, involved in the betaine synthesis pathway. This enzyme converts betaine-aldehyde to glycine betaine. If the semi-aldehyde of DMSP, MMPA, or MPA is formed

in the demethylation pathway, SPO2203 could catalyze the formation of the carboxylic acid of these compounds. SPO2203 also has high homology (with 57.3% identity and 73.7% similarity) to *dddC*, a gene in an operon involved in DMSP degradation in *Marinomonas sp.* MWYL1. *dddC* is found in an operon transcribed in the opposite direction from the DMSP cleavage gene *dddD*. SPO2203 is in the core roseobacter genes (Moran *et al.*, 2007). In three sequences from the GOS metagenomic library, homologs to this gene are found immediately up or downstream of *dmdA* homologs. These are all indications of a potential role in DMSP degradation.

SPO0632–SPO0635 GENE CLUSTER

The gene at locus SPO0632 is annotated as a member of the D-isomer specific 2-hydroxyacid dehydrogenase family proteins, based on matches to Pfam 389 and 2826. The conserved catalytic domain and NAD^+-binding regions are conserved in this protein. These proteins include glyoxylate reductase (E.C. number 1.1.1.81, also described as hydroxypyruvate reductase E.C. number 1.1.1.26), which is part of the serine cycle of formaldehyde assimilation. Glyoxylate reductase carries out the reduction of glyoxylate to glycolate, or hydroxypyruvate to glycerate with the oxidation of NADH. *S. pomeroyi* has a gene with high homology to described glyoxylate reductases at locus SPO1564, and this is probably the true glyoxylate reductase. In general, this class of enzymes carry out the formation of alcohols from 2-3 carbon aldehydes or keytones that also have a carboxylic acid at the other side of the molecule. This gene product could be used as part of serine cycle of formaldehyde assimilation for the methyl group of DMSP in the demethylation pathway or to perform the transformation of malonate-semialdehyde to β-hydroxypropionate in the degradation of the carbon backbone of DMSP from either the cleavage or demethylation pathways.

The gene at locus SPO0633 was increased in abundance with growth on DMSP, and was also seen in batch-grown experiments. It was annotated as *ggt*, a gamma-glutamyltranspeptidase (E.C. number 2.3.2.2), based on similarity to the characterized *ggt* from *Bacillus subtilis* and matches to Pfam 1019 and TIGRfam 66. This enzyme catalyzes the transfer of the glutathione glutamyl moiety to either an amino acid, which forms a glutamyl amino acid or peptide, or to water, which

forms glutamate. It is used in the detoxifying gamma-glutamyl cycle. In yeasts it is upregulated during reduced sulfur starvation. *S. pomeroyi* contains two additional homologs to SPO0633 at SPO3411 and SPO0866 (28% and 29% identity, respectivly). However, these two genes are more divergent from characterized *ggt* genes, indicating that SPO0633 may be the functional *ggt*. The SPO0633 gamma-glutamyltranspeptidase may be involved in detoxifying captured formaldehyde or reactive thiols produced by the demethylation pathway.

The gene at locus SPO0634 is an oxidoreductase with clearly conserved FAD-binding domains and similarity to Ipr domains 4113, 6094, 16164, 16166–8, and to Pfam 2913 and 1565, all of which look for conserved regions involved in FAD-binding and surrounding motifs. These gene families encode oxygen-dependent oxidoreductases that catalyze a wide variety of oxidations (often with low substrate specificity), including D-lactate dehydrogenase that catalyzes the oxidation of lactate to pyruvate. In addition, this gene has low similarity to the *glcE* and *glcD* subunits of glycolate oxidase. However *S. pomeroyi* has a predicted operon with genes that have higher similarity to all three subunits of glycolate reductase at SPO3478–SPO3480. SPO0634 is not characterized enough to speculate on its role in DMSP degradation.

The gene at locus SPO0635 has an interesting domain structure. It is a fusion protein, with the amino-terminal domain having high similarity to glycine and D-amino-acid oxidases, and a carboxylic-terminal domain having high similarity to aminomethyltransferases. The amino-terminus domain matches Pfam 1266, which includes the glycine and D-amino-acid oxidases. The carboxylic-terminus domain matches Pfam 1571 and 8669, which includes the glycine T-cleavage proteins that catalyze the transfer of a methyl group to THF. The glycine T-cleavage protein that has highest similarity to this region is *dmdA*, the DMSP demethylase from *S. pomeroyi*.

This gene fusion results in a poor automated annotation and complicates homology searches, as sequences with high similarity to either region will obscure the lower similarity full-length matches. This necessitates an initial screening by conserved domains using NCBI's CDD (Marchler-Bauer *et al.*, 2007). There are characterized enzymes that display the full domain structure of SPO0635 that were identified with CDD pre-screening. Genes with this domain struc-

ture include the dimethylglycine and sarcosine oxidases and dehydrogenases. SPO0635 is more closely grouped in trees of characterized genes with the eukaryotic dimethylglycine oxidases, but there is poor resolution and bootstrap values of some nodes between SPO0635 and the the sarcosine oxidases. Other closely related glycine oxidases catalyze the removal of the methylamine moiety, but the eukaryotic dimethylglycine and sarcosine oxidases catalyze the oxidation of their substrate with the transfer of only the methyl group to THF. When THF is not present with any of the enzymes with this domain structure, formaldehyde is formed instead. Some enzymes with this domain structure carry out the reaction more slowly when THF is present and probably produce formaldehyde *in vivo*. Others transfer the methyl group to tetrahydropteroylpentaglutamate instead of THF. The electron acceptor these types of enzymes can be either $H_2O + O_2$ (which yields H_2O_2 as a product) or the electron transport chain. Molecular oxygen is used in the *Arthrobacter* and *Bacillus* sarcosine oxidases and in the *Arthrobacter* dimethylglycine oxidases. Unknown components of the electron transport chain (substitutable by phenazine methanosulfate) are used in the eukaryotic dimethylglycine and sarcosine oxidases. Coenzyme Q is used by the D-amino-acid oxidase of *E. coli*.

SPO0635 has highest similarity to the enzymes that use the electron transport chain to oxidize dimethylglycine, catalyzing the reactions:

$$\underset{\text{N,N-dimethylglycine}}{H_2O + N(CH_3)_2CH_2COO^-} \longrightarrow \underset{\text{sarcosine}}{NH(CH_3)CH_2COO^-} + 2\,e^- + 2\,H^+ + \underset{\text{formaldehyde}}{O{=}CH_2}$$

$$\underset{\text{N,N-dimethylglycine}}{THF + H_2O + N(CH_3)_2CH_2COO^-} \longrightarrow \underset{\text{sarcosine}}{NH(CH_3)CH_2COO^-} + 2\,e^- + 2\,H^+ + THF{-}CH_3$$

Many of the genes in these families have fairly broad substrate specificity, catalyzing the demethylation of a number of similar compounds. Homologs to this protein form a hetero tetrameric enzyme complex with three other proteins, but these types of oxidases are less similer to SPO0635, and homologs to the other three enzymes of the hetero tetramer are not found in the genome of *S. pomeroyi*. There are 12 homologus genes with the same domain structure in the genome of *S. pomeroyi*. This gene is ubiquitous in roseobacters and highly abundant in marine metagenomes.

This gene could be involved in the demethylation MMPA to MPA with the production of a methylated THF or formaldehyde, depending on the availability of THF *in vivo*.

The gene cluster from SPO0632–SPO0635 is conserved in many roseobacters and "*Candidatus* P. ubique". Immediately preceding this cluster is the divergently transcribed SPO0631, which is a conserved hypothetical protein found in many of the sequenced roseobacters and is possibly involved in regulation. Seven roseobacters, but no sequenced organisms outside of the roseobacters, share this organization of genes. This gene cluster has a number of genes with which could have roles involved with DMSP degradation.

SPOA0268–SPOA0272 GENE CLUSTER AND SPO3850

The gene found at SPOA0268 is a transcriptional regulator of the IclR family, based on similarities to protein family Pfams 1614 and 5471. This type of regulator often regulates glycerol or glyoxylate bypass pathway operons (also termed the acetate operon repressor) in *E. coli* and other proteobacteria. This gene cluster may be repressed by acetate, and therefore would appear to increase in abundance during growth on DMSP. Alternativly, it may have a different regulatory role in *S. pomeroyi*. This gene is well conserved in α-proteobacteria.

The gene product found at SPOA0269 increased in abundance following growth on DMSP. It is incorrectly annotated as a selenium-binding protein, but it is better described as a conserved hypothetical. It has similarity to a gene annotated as a selenium-binding protein (GenBank BAB65016.1) from *Sulfolobus tokodaii* 7 as well as other homologs in eukaryotes and archaea, but the physiological function of these genes are not known. In eukaryotes, there is some indication that they might be involved in changing the redox state of proteins during Golgi maturation. The similarity between these genes and SPOA0269 is not close enough to predict even a general function. However, there are motifs found in SPOA0269 that indicate a potential function. One is similarity to a WD40-like repeat region (Ipro 15943) which form seven-bladed propellers of beta sheets, and a cytochrome CD1 nitrite reductase-like region. Other proteins that have these motifs include a amine dehydrogenase from *Paracoccus denitrificans* (at 32.8% similarity), which

removes an ammonia group from a carbon backbone, and methylamine and dimethylamine dehydrogenases which cleave the C-N bond of these amino-methyl compounds. All of these enzymes have a tryptophan tryptophylquinone (TTQ) cofactor redox center. A gene with high similarity to SPOA0269 is found in the methylotrophic DMS-degrading *Methylococcus capsulatus* strain Bath and in many roseobacters and other α-proteobacteria. In a proteomics screen in *Methylophaga sp.* DMS010 for proteins increased in abundance following growth on DMS, C- and N-terminal sequences matched this gene and a homolog in *Methylococcus capsulatus* strain Bath (Schafer, 2007). *S. pomeroyi* has paralogs to this gene at SPO2378 and SPO1643. SPOA0269, while unannotated, has many indications that it could be involved in the degradation of DMSP. However, there is not enough information available to make a strong prediction of activity. It could catalyze the oxidation and cleavage of methyl groups from the sulfur moiety of DMSP, probably from methanethiol to sulfite and formaldehyde or DMS to methanethiol and formaldehyde, or it could catalyze the oxidation and cleavage of sulfite from 3-mercaptopropionate, the predicted product of a double-demethylation of DMSP.

The gene found at SPOA0270 is a conserved hypothetical with no well defined function. It has motifs that match Ipr domain 12335, which describes a thioredoxin-like fold, and Ipr 3782, which include biogenesis maturation proteins. It has low similarity to proteins involved in maturation of enzymes, redox changes, and loading of metal centers, including enzymes that are involved in the maturation of proteins involved in methylamine degradation. Homologs are found in a number of methylotrophs and some roseobacters. This enzyme may be involved in the maturation of SPOA0269, but there is not enough information to predict this with confidence.

The gene at SPOA0271 has low similarity to *mauG* genes, which encode methylamine utilization genes and the closely related cytochrome-c peroxidases. SPOA0271 has 48.6% similarity and 35.5% identity to the *mauG* of *Methylobacterium extorquens* AM1 (SwissProt Q49128) and matches Pfam 315. Both of the cytochrome-binding sites found in this gene are conserved in SPOA0271. This enzyme is essential for the maturation of the beta subunit of methylamine dehydrogenase and is involved in the synthesis of the tryptophan tryptophylquinone (TTQ) cofactor.

S. pomeroyi contains three paralogs with low similarity (<30% identity) at SPOA0152, SPO0330 (which is more homologous to characterized cytochrome-c peroxidases), and SPO0858. A possible role for this gene product is in the maturation of SPOA0269, perhaps the synthesis of TTQ.

A trypsin digest peak profile of a protein spot with increased abundance following growth on DMSP matched the gene product of both SPOA0272 and SPO3850. The latter does not appear to be co-transcribed. The sequence is very conserved between these two genes, with 97.4% nucleotide identity, and the amino acid sequence differs by a single conserved change, resulting in 99.7% similarity. There is no similarity in the flanking regions or between the upstream regions of SPO3850 and the first gene in the potentialy operon containing SPOA0272. It is more likely that SPOA0269 was the detected gene product as SPOA0272 in the same gene cluster was similarly increased in abundance. Both of these genes are glutathione-dependent formaldehyde dehydrogenases (E.C. number 1.2.1.1) in the family of zinc dependent alcohol dehydrogenases, as identified by matches to protein family Pfam 107 and TIGRfam 2818. The conserved zinc-binding and dehydrogenase regions are conserved in these genes, and they have 84.0% identity and 91.8% similarity to a characterized enzyme from *Rhodobacter sphaeroides* (Barber *et al.*, 1996). The glutathione-dependent formaldehyde dehydrogenases are involved in formaldehyde utilization by catalyzing the oxidation of S-hydroxymethylglutathione that spontaneously forms from the reaction of glutathione and formaldehyde:

$$\underset{\text{formaldehyde}}{\mathrm{CH_2{=}O}} + \underset{\text{glutathione}}{\mathrm{GS{-}H}} \xrightarrow{spont.} \underset{\text{S-hydroxymethylglutathione}}{\mathrm{GS{-}CH_2OH}}$$

$$\underset{\text{S-hydroxymethylglutathione}}{\mathrm{GS{-}CH_2OH}} + \mathrm{NAD^+} \xrightarrow{1.2.1.1} \underset{\text{S-formylglutathione}}{\mathrm{GS{-}CH{=}O}} + \mathrm{NADH} + \mathrm{H^+}$$

This gene was identified as a core roseobacter gene (Moran *et al.*, 2007) and could use formaldehyde generated by the demethylation of MMPA by SPO0635, the reaction with MeSH or DMS potentially catalyzed by SPOA0269, or other formaldehyde generated during the demethylation of DMSP. As formaldehyde is an extremely reactive molecule, many methylotrophs have multiple copies that are differentially regulated. SPOA0269 may be used during growth on DMSP, while

SPO3850 is a more general detoxifying enzyme. The gene region from SPOA0269–SPOA0270 is identified as a conserved region in the SEED database and is found in many methylotrophic organisms and roseobacters. SPOA0269–SPOA0271 is a OFS predicted operon, so these two genes are not likely to be transcribed separately. Functional linkages between SPOA0269, SPOA0270 and SPOA0271 reinforce the prediction of SPOA0269 as a demethylating enzyme involved in methanethiol degradation and SPOA272 as an enzyme involved in the utilization of the resulting formaldehyde.

Other genes and gene clusters

A second trypsin digest peak profile from a protein that increased in abundance matched two paralogs in the genome. SPO0728 and SPO3498 are 100% identical at the nucleotide level, and code for *tuf*, a translation elongation factor in the Tu family. These genes have low similarity to the selenocysteine-specific elongation factor *selB* that is involved in the incorporation of a selenomodified cysteine. SPO0728 is in a gene cluster with two hypothetical proteins and a DNA topoisomerase, while SPO3498 is in a gene cluster with a translation elongation factor G, ribosomal S7 protein, and a ribosomal S12 protein. SPO1625, annotated as a serine protease, also increased in abundance. It contains a PDZ domain, common in sensing proteins. Serine proteases with these domains are often involved in sensing and pre-enzyme activation, regulation and proteolytic activities. SPO1625 is found in a gene cluster with a DNA-binding response regulator. Both SPO0728 / SPO3498 and SPO1625 may be involved in a regulatory cascade that is activated with growth on DMSP compared to growth on acetate.

The gene product of SPO2571 increased in abundance and is annotated as a putative DctM subunit of a TRAP transporter system. It is in a gene cluster with a hypothetical protein and a bacterial extracellular solute-binding protein that has similarity to a periplasmic mannitol-binding protein. These genes are core roseobacter genes (Moran *et al.*, 2007) and may be involved in transport of DMSP or other metabolites.

SPO0728, SPO3498, SPO1625, and SPO2571 are potential regulatory and transport genes that may be involved in DMSP degradation, but none of the genes products in these clusters appear to be catalytic enzymes directly involved in DMSP degradation. The gene clusters containing *dmdA*, SPO2203, SPO0635 and SPOA272 may play a role, either directly or indirectly, in DMSP degradation and are good candidates for further work toward elucidating the genes and physiological activities in this pathway.

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CHAPTER 4

MUTATIONS IN GENES UPREGULATED WITH GROWTH ON DMSP

4.1 ABSTRACT

Silicibacter pomeroyi, a member of the a marine roseobacter clade, is a model system for the study of dimethylsulfoniopropionate (DMSP) degradation. A differential display proteomic approach identified proteins upregulated during growth on DMSP. These proteins are candidates for enzymes involved in DMSP degradation. A genetic system was developed to confirm the function of these proteins. It included: identification of minimum inhibitory concentrations of antibiotics and antibiotic resistance cassettes functional in *S. pomeroyi*; transformation by electroporation with DNA protected by SssI methylase, and construction of gene replacement mutants using suicide vectors. Gene replacement mutants were constructed for SPO0635 and SPOA272. Their genotype was confirmed by PCR amplification and Southern blotting. The phenotypes of mutants in these candidate genes may elucidate undescribed reactions in the globally important DMSP degradation pathway.

4.2 INTRODUCTION

Many methods of gene discovery, including differential display proteomic approaches, show correlation of protein abundance or gene expression with a metabolic state but do not reveal the activity of a gene product. A useful approach for confirmation of function and examination of the metabolic role of a predicted gene product is the generation of mutations in that gene. Methods for the construction of targeted mutations are an important tool for genetic investigation of gene function. The techniques necessary for the development of a genetic system in an organism are identification of functional antibiotics and antibiotic resistance cassettes and development of methods for introducing DNA and inactivating endogenous genes.

A variety of antibiotic cassettes functional in *S. pomeroyi* would aid in genetic manipulation approaches. Ampicillin, tetracycline, and kanamycin resistance genes were used in this study. The ampicillin resistance cassette, *bla*, is a class A serine-β-lactamase. The Tn*7* derived MFS family tetracycline resistance cassette, *tetRA*, is composed of a regulatory gene and a divergently transcribed gene that encodes the tetracycline specific efflux pump. Two kanamycin and neomycin aminoglycoside phosphotransferase resistance cassettes were used: a APH3′IIa *kanR* from Tn5 which is found in pARO181 and pCR2.1 and APH3′Ia *kanR* from Tn903 in EZ-Tn5<Kan2>.

There are multiple methods for transformation that must be empirically tested to develop a genetic system (Mercenier and Chassy, 1988). Electroporation is an established method that can yield high transformation rates in diverse organisms (Lurquin, 1997). Restriction modification endonuclease systems are one barrier against genetic transfer. One approach to combat this problem is to use a methylase enzyme to methylate sites likely to be involved in a restriction modification system. Methylation may also have a role in mediating recombination. Methylation of DNA increases transformation efficiencies up to 4 orders of magnitude in conjugation (Butler and Gotschlich, 1991) and electroporation systems (Donahue *et al.*, 2000). However, this approach can also reduce efficiency if the methylation increases endonuclease activity by a methyl-directed restriction modification system (for example, see Burkhart *et al.*, 1992).

While standard molecular techniques have been optimized for other α-proteobacteria, many of these methods had not been previously tested with *S. pomeroyi*. Relatively few genetic systems that include a gene interuption method are developed for the α-proteobacteria, and almost all depend on mating for the introduction of exogenous DNA. Previous work indicated that the IncP incompatibility group broad host range plasmid pRK415 replicated in *S. pomeroyi* (Buchan *et al.*, 2003; Howard *et al.*, 2006, supplementary materials). The improvement and development of antibiotic cassettes, high-efficiency transformation, and gene interuption will provide a basic genetic system for the conformation of gene function in *S. pomeroyi*.

4.3 MATERIALS AND METHODS

4.3.1 STRAINS, PLASMIDS AND PRIMERS

Strains and plasmids used in this study are listed in Table 4.1. *Escherichia coli* K-12 carrying RP4 was supplied by Dr. Anne Summers. *S. pomeroyi* SAB-3 was supplied by Dr. Alison Buchan. *E. coli* was grown at 37 °C in LB medium unless otherwise noted. Oligonucleotides used in this study are listed in Table 4.2 and were ordered from Integrated DNA Technologies.

Table 4.1: Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
E. coli strains:		Bachmann (1972)
DH5α	cloning strain	Meselson and Yuan (1968)
Top-10	cloning strain	Grant *et al.* (1990)
S17-1	mating helper strain	Simon *et al.* (1983)
K-12 J53(RP4)	mating donor for RP4	Jacoby *et al.* (1976)
S. pomeroyi strains:		
DSS-3	wild type	González *et al.* (2003)
SAB-3	spontaneous RifR^+ mutant	Alison Buchan
41H6	*dmdA*::Ez-Tn<Kan2>	Howard *et al.* (2006)
Δ635	Δ(SPO0635)::*tetRA*	This study
ΔA272	Δ(SPOA0272)::*tetRA*	This study
Plasmids:		
pCR2.1	ColE1 AmpR^+ KanR^+	Invitrogen
RP4	IncP AmpR^+ KanR^+ TetR^+	Datta *et al.* (1971)
pRK415	IncP TetR^+	Keen *et al.* (1988)
pARO181	ColE1 TetR^+	Parke (1990)
pJRH2x635	ColE1 AmpR^+ KanR^+ TetR^+	This study
pJRH2xA272	ColE1 AmpR^+ KanR^+ TetR^+	This study

4.3.2 MOLECULAR METHODS

Standard protocols were used for molecular methods with *E. coli* (Sambrook *et al.*, 2001). PCR amplification for the construction of pJRH2x635 was performed using AccuPrime Taq High

Table 4.2: Oligonucleotides used in this work: bold indicates a restriction site, underline indicates region of sequence identity in SLIC cloning.

Name	Sequence 5′ to 3′
PCRRE-SPO00635-Uf	CCCCC**AAGCTT**TCACCGAAATCACCCTGAAACTGC
PCRRE-SPO0635-Ur	CCCCC**ACTAGT**CTTTGGGATGGTTTATGCCGAAGC
PCRRE-TetR-f	CCCCC**ACTAGT**ACCGTATTACCGCCTTTGAGTGAG
PCRRE-TetR-r	CCCCC**CTCGAG**ACGCTGAGTGCGCTTCAAATCATC
PCRRE-SPO0635-D2f	CCCCC**CTCGAG**AGATGCTCGATCAGCTTTGGGATG
PCRRE-SPO0635-D5r	CCCCC**TCTAGA**GATGAAACCCTATGTCAGCGAG
SLIC-SPOA0272-Uf	CTATGACCATGATTACGCC**AAGCTT**CGCAATGTCTATGGTCTGGGCTTT
SLIC-SPOA0272-Ur	**ACTAGT**TCCGTGGTCTCCTTAGGTCAGAAA
SLIC-SPOA0272-TetR-f	AAGGAGACCACGGA**ACTAGT**ACCGTATTACCGCCTTTGAGTGAG
SLIC-SPOA0272-TetR-r	AGCAGATCCGGGAT**CTCGAG**ACGCTGAGTGCGCTTCAAATCATC
SLIC-SPOA0272-Df	**CTCGAG**ATCCCGGATCTGCTTGCTATGGTT
SLIC-SPOA0272-Dr	GGGCGAATTGGGCCC**TCTAGA**TCCCAATTCCACCATCTCGCTGAT
del-SPOA0272-Uf	AACTGGCAGATATCCAGGCCTTTC
del-SPOA0272-Ur	TTACTGGCACTTCAGGAACAAGCG
del-SPOA0272-Df	TTTGGCATTCTGCATTCACTCGCC
del-SPOA0272-Dr	ATATTGCCATTACCGCCCAACACC
del-SPO0635-Uf	AACGAAACCGGCATCGAAACAGTG
del-SPO0635-Ur	TCGTTGTCAGGAACGTTGAAGGAC
del-SPO0635-Df	ATGATTTGAAGCGCACTCAGCGTC
del-SPO0635-Dr	TCCGATCAACCGCTTTCAGCATTG
int-SPO0635-f	TCAACATGTCCTATGCAACCACCC
int-SPO635-r	TTGACAACGTGCTTGGTAAAGGCG
blot-SPO0635-Uf	TCTGGCGAGCGTACCCTGAAA
blot-SPO0635-Ur	CTTTGGGATGGTTTATGCCGAAGC
blot-SPOA272-Df	ATCCCGGATCTGCTTGCTATGGTT
blot-SPOA272-Dr	TCGAATGACCGAAATGCAACTGCG

Fidelity DNA Polymerase (Invitrogen). All other PCR amplifications were performed using Phusion high-Fidelity polymerase (New England Biolabs). DMSO was added to improve amplification of the SPO0635 downstream region. Plasmids were isolated from *S. pomeroyi* by either the alkaline lysis method (Sambrook *et al.*, 2001) or by using a QIAprep Miniprep kit (Quiagen). Genomic DNA from *S. pomeroyi* was prepared using standard protocols (Sambrook *et al.*, 2001) or the Wizard genomic DNA kit (Promega).

4.3.3 Antibiotic tests

E. coli K-12 J53 carrying the RP4 Birmingham plasmid (Pansegrau *et al.*, 1994) and *E. coli* S17-1 carrying pRK415 were used as conjugation donors in a standard mating protocol (Howard *et al.*, 2006; Sambrook *et al.*, 2001). Mating was performed on filters on fresh 1/2 YTSS plates with *S. pomeroyi* SAB-3 and *S. pomeroyi* 41H6, and transconjugates were selected on 1/2 YTSS plates with rifampicin and kanamycin, respectively. Both *S. pomeroyi* transconjugants, a *S. pomeroyi* EZ-Tn5<Kan2> (Epicenter) insertion mutant (Howard *et al.*, 2006), and *S. pomeroyi* DSS-3 wildtype were grown in 1/2 YTSS with two-fold dilutions of ampicillin, kanamycin, neomycin, and tetracycline from 0.5–256 $\mu g\,mL^{-1}$, and the OD_{600} was measured daily for two weeks.

4.3.4 Construction of pJRH2x635

A shuttle plasmid with regions of sequence identity up- and down-stream of SPO0635 flanking the *tetAR* genes from pRK415 was constructed by sequential PCR amplification and cloning. The upstream region was amplified with primers PCRRE-SPO00635-Uf and PCRRE-SPO0635-Ur, inserted into a self-ligated pCR2.1-TOPO plasmid digested with HindIII and SpeI and selected with blue-white screening. The *tetAR* region of pRK415 was PCR amplified with primers PCRRE-TetR-f and PCRRE-TetR-r, inserted between SpeI and XhoI sites, and selected for with ampicillin and tetracycline. The downstream region was amplified with PCRRE-SPO0635-D2f and PCRRE-SPO0635-D5r and inserted between XhoI and XbaI sites. The resulting plasmid, pJRH2x635 was maintained in *E. coli* TOP-10. The *tetAR* cassette and flanking up- and down-stream regions were

also ligated into pARO181 and pRK415 digested with HindIII and XbaI and maintained in *E. coli* S17-1 for mating experiments.

4.3.5 Construction of pJRH2xA272

A shuttle plasmid with regions of identity to up- and down-stream of SPO0635 flanking the *tetAR* genes from pRK415 was constructed by Simple Ligation Independent Cloning (SLIC) without RecA (Li and Elledge, 2007). Briefly, primers to amplify upstream and downstream regions and *tetAR* were designed with appropriate regions of overlap (SLIC-SPOA0272-Uf and SLIC-SPOA0272-Ur, SLIC-SPOA0272-Df and SLIC-SPOA0272-Dr, SLIC-SPOA0272-TetR-f and SLIC-SPOA0272-TetR-r, respectively). Self-ligated pCR2.1 was cut with HindIII and XbaI, as well as EcoRI to reduce background. After T4 DNA polymerase treatment, the overlapping single strands were annealed, and the reaction transformed into chemically competent DH5α *E. coli* via heat shock (Sambrook *et al.*, 2001).

4.3.6 Electrocompetent *S. pomeroyi*

S. pomeroyi was grown to mid-log growth stage (0.15–0.20 OD_{600}) and chilled at 4 °C for one hour. The cells were harvested by centrifugation at 7,000 $\times$g for 10 min, the supernatant was decanted, and the cells were washed four times with 250 mL, 150 mL, 100 mL, and 2 mL of 4 °C sterile 10% glycerol. After the final wash, 90 µL aliquots of the cell suspension were transferred into microcenterfuge tubes and frozen at -80 °C before use.

4.3.7 Restriction protection

M.SssI CpG methylase (New England Biolabs) was used to methylate 1 µg of DNA according to the manufacturers directions. Protection was checked by digestion with BstUI, a CpG methylation sensitive restriction endonuclease. DNA was isolated using a DNA Clean and Concentrator kit (Zymo Research) and immediately used for electroporation.

4.3.8 ELECTROPORATION OF *S. pomeroyi*

In the optimized protocol, electrocompetent *S. pomeroyi* prepared as above were thawed on ice, mixed with DNA, and immediately electroporated in a 1 cm gap cuvette using a GenePulser II Electroporation system with a PLUS Pulse controller (Bio-Rad) and the following settings: 2.55 kV, 25 µF capacitance, and 200 Ω resistance in parallel. Time constants were 4.7–4.9 ms. When used, TypeOne RE inhibitor (EPICENTRE Biotechnologies) was added immediately before electroporation. The electroporated cells were immediately suspended in 1/2 YTSS at room temperature and transferred to a sterile glass test tube. Cultures were diluted to 0.5 OD_{600}, incubated at 30 °C with shaking and allowed to double (usually within 2–4 hours) and plated on 1/2 YTSS with 20 µg mL^{-1} tetracycline. SssI methylated pJRH2x635 or a linear PCR amplicon of the *tetAR* genes with the SPO0635 flanking regions (amplified from pJRH2x635 with PCRRE-SPO00635-Uf and PCRRE-SPO00635-D5r) were electroporated into electrocompetent *S. pomeroyi* DSS-3 and selected on 1/2 YTSS with 20 µg mL^{-1} tetracycline to generate *S. pomeroyi* Δ635. SssI methylated pJRH2xA272 was electroporated into electrocompetent *S. pomeroyi* DSS-3 and selected on 1/2 YTSS with 20 µg mL^{-1} tetracycline to generate *S. pomeroyi* ΔA272. Mutants were maintained and stored in 20 µg mL^{-1} tetracycline 1/2YTSS media.

4.3.9 CONFIRMATION OF GENE REPLACEMENT

PCR amplifications of genomic DNA extracts with primers that spanned the junction of the flanking region and the antibiotic cassette (del-SPOA0272-Uf and del-SPOA0272-Ur, del-SPOA0272-Df and del-SPOA0272-Dr, del-SPO0635-Uf and del-SPO0635-Ur, del-SPO0635-Df and del-SPO0635-Dr) and with primers internal to SPO0635 (int-SPO0635-f and int-SPO0635-r) were performed. Southern blots (Sambrook *et al.*, 2001) were performed using probes generated by PCR complementary to a region upstream from the SPO636 (using primers blot-SPO0635-Uf and blot-SPO0635-Ur), a region downstream from SPOA0272 (using primers blot-SPOA272-Df and blot-SPOA272-Dr), and a region internal to *tetRA* (using primers PCRRE-TetR-f and PCRRE-

TetR-r). Genomic DNA preparations were digested with SmaI, a restriction endonuclease found in the *tetRA* gene cassette and at locations flanking both disrupted genes.

4.3.10 Maintenance of gene mutants

S. pomeroyi cultures were passed at a 1:500 dilution 5 times in 1/2 YTSS with or without 20 μg mL^{-1} tetracycline and then diluted and plate counted on 1/2 YTSS plates with tetracycline to assess maintenance of the *tetRA* cassette without selection.

4.4 Results and discussion

4.4.1 Antibiotic selection

Initial studies in *S. pomeroyi* were hampered by a lack of stable antibiotic selection and a limited number of functional antibiotic selection cassettes. In particular, during multiple-day growth of *S. pomeroyi* on kanamycin 1/2 YTSS plates (necessitated by long lag times following electroporation), high background growth was observed. In order to identify functional gene cassettes in *S. pomeroyi*, the minimum inhibitory concentration (MIC) were determined for the wild type strain, a strain carrying the RP4 Birmingham plasmid (Pansegrau *et al.*, 1994), a EZ-Tn5<Kan2> (Epicenter) insertion mutant (Howard *et al.*, 2006), and a strain with pRK415. The MICs for wild-type *S. pomeroyi* are: 1 μg mL^{-1} ampicillin, 64 μg mL^{-1} kanamycin, 16 μg mL^{-1} neomycin, and 4 μg mL^{-1} tetracycline in liquid cultures. MIC concentrations were similar on 1/2 YTSS plates. Ampicillin resistance with a MIC of 128 μg mL^{-1} was conferred by RP4, but growth was not observed for 3–6 day after inoculation. This long lag proved problematic for later transformation experiments. Kanamycin and neomycin resistance with a MIC of more than 256 μg mL^{-1} was conferred by EZ-Tn5 and RP4. On 1/2 YTSS plates with kanamycin *S. pomeroyi* DSS-3 wild type showed growth after 5–7 days, but was due to inactivation of kanamycin and not mutation as successive passes on kanamycin plates also displayed this lag in growth. *S. pomeroyi* DSS-3 wild type did not display this growth on neomycin plates. Tetracycline resistance with a MIC of 128 μg mL^{-1} was conferred

by RP4 and pRK415, and growth was observed within 48 hours of plating. As RP4 and pRK415 are low copy number plasmids with four to seven copies per cell, and the MIC for kanamycin was identical for both RP4 and chromosomally integrated kanamycin cassettes, the MIC values for other antibiotic cassettes on plasmids appear to be good predictors of the MIC for chromosomal insertion. The tetracycline cassette from pRK415 was used in further experiments because of the large increase in MIC, the absence of lag in growth, and no observed growth on the wild type after prolonged incubation.

4.4.2 TRANSFORMATION OF *S. pomeroyi*

Initially no transformants were observed in electroporation experiments with pRK415 using standard *E. coli* electroporation protocols (Sambrook *et al.*, 2001). With modification of the washing protocol and an extended incubation of cultures on ice prior to washing (see Section 4.3.6), low levels of transformants were observed (see Table 4.3). In previous work with the EZ-Tn5 trans-

Table 4.3: Shuttle vector pRK415 transformation efficiency.

Treatment	CFU/ µg DNA
E. coli protocol	<1
Optimized protocol	10^2
TypeOne restriction inhibitor	10^3
Prepared from *S. pomeroyi*	10^8
SssI methylation	10^8

posase, it was found that the use of TypeOne restriction inhibitor was necessary for the electroporation of the transposon-transposase complex (Buchan *et al.*, 2003). While the addition of the TypeOne restriction inhibitor improved the transformation efficiency of pRK415 (Table 4.3), which indicated the presence of a restriction-endonuclease system. The *S. pomeroyi* genome encodes 4 putative methylases and 2 predicted endonucleases, including a pair at SPO1048–SPO1049 that are members of the C-5 methylcytosine family (Roberts *et al.*, 2007). *E. coli* DH5α and S17-1 lack the EcoKI restriction system, but have dam, dcm, and EcoKI methylation while *E. coli* TOP-10 lacks

both the EcoKI restriction and methylation genes. DNA prepared from these organisms are methylated at the dam and dcm sites and at the EcoKI site in *E. coli* TOP-10. The dcm methylase is a C-5 methylcytosine methylase, but would not protect DNA from a restriction modification system that was sensitive to methylation at any other C site. SssI methylase, which blocks many C-5 methylcytosine methylases, was used to provide global CpG methylation. This treatment increased the transformation efficiencies to similar levels as pRK415 prepared from *S. pomeroyi*, which was isolated from the rare transformants from earlier electroporation experiments. No transformants were observed with pUC2.1, pUC, pARO181 or any other ColE1 plasmid with SssI protection, making these plasmids useful suicide vectors for *S. pomeroyi*. Transformation efficiencies of 1.4 x 10^8 CFU/ µg were routinely observed for this shuttle vector.

4.4.3 GENE REPLACEMENT IN *S. pomeroyi*

The high rates of transformation were necessary for the development of a functional homologus recombination system, which preliminary results indicated did not occur at high enough rates to be observable with either mating or electroporation. Insertions in pUC2.1 with up to 1,000 bp of identity to 3 different regions on the *S. pomeroyi* chromosome, including one that was known to be non-essential (the full-length SPO01913 *dmdA*) were constructed, but no transformants were ever observed from single-crossing over insertion of this vector by mating or electroporation. Occasionally rare organisms resistant to kanamycin were identified, but PCR amplification of recombination junctions or plasmid regions was not observed, and no ampicilin resistance was observed, indicating that non-specific recombination or spontaneous kanamycin resistance, not homologus recombination, had occurred. No transformants were observed using treatments that have been shown to increase homologous recombination (Hinds *et al.*, 1999), including UV treatment of DNA or recipient cells, NaOH denaturation of DNA, or in different growth phases of the recipient cells (data not shown).

This low rate of recombination despite high transformation rates might be due to the IncPα replicon interfering with chromosomal replication when integrated, which would prevent stable

integration of these vectors (Fenno *et al.*, 1993). In addition, using plasmids with regions of identity to a gene for Campbell insertion of the plasmid and gene interuptions limits the length of the region of identity to less than the length of the gene to be interupted. If longer regions of identity, or the presence of the recombinational hotspot χ site, which occurs on average every 350 - 1200 bp (Karoui *et al.*, 1999) are required, then transformation would not occur. Both issues were addressed by transformation with constructs containing an antibiotic cassette flanked by regions of identity up- and down-stream of a gene to be interupted, so double-crossover homologous recombination would lead to gene replacement.

Constructs with close to 1,000 base pairs of identity up and downstream of SPO0635 did yield transformants, indicating double-crossover gene replacement. Deletion of SPO635 in DSS-3 and 41H6 backgrounds and of SPOA0272 in DSS-3 were constructed, with transformation efficiencies of 10^2–10^3 CFU/ µg DNA. The molar transformation efficiency of double-recombinant replacements of SPO0635 were roughly equivalent for both linear PCR product DNA or circular plasmid DNA, although both required methylation with SssI methylase. Replacements were confirmed by PCR amplification across the up and downstream junctions from genomic DNA preparations. No amplification was seen in the mutant with primers internal to SPO0635, while this was readily observed in the wild type (data not shown). In addition, Southern blots with probes to flanking regions migrated farther in an agarose gel due to the SmaI site introduced upon replacement of the gene with the *tetRA* cassette (See Figure 4.1). These results show that gene replacement, and not a insertion of the plasmid into the chromosome by single homologous recombination has occurred. This bias toward homologous replacement and not integration of the plasmid has been observed in other organisms (Fenno *et al.*, 1993). Tetracycline resistance was maintained for multiple successive passes in without selection, illustrating the stability of the mutation. Using this technique, replacements of many genes of interest can be constructed in *S. pomeroyi*. This is the first gene knockout system described in a roseobacter.

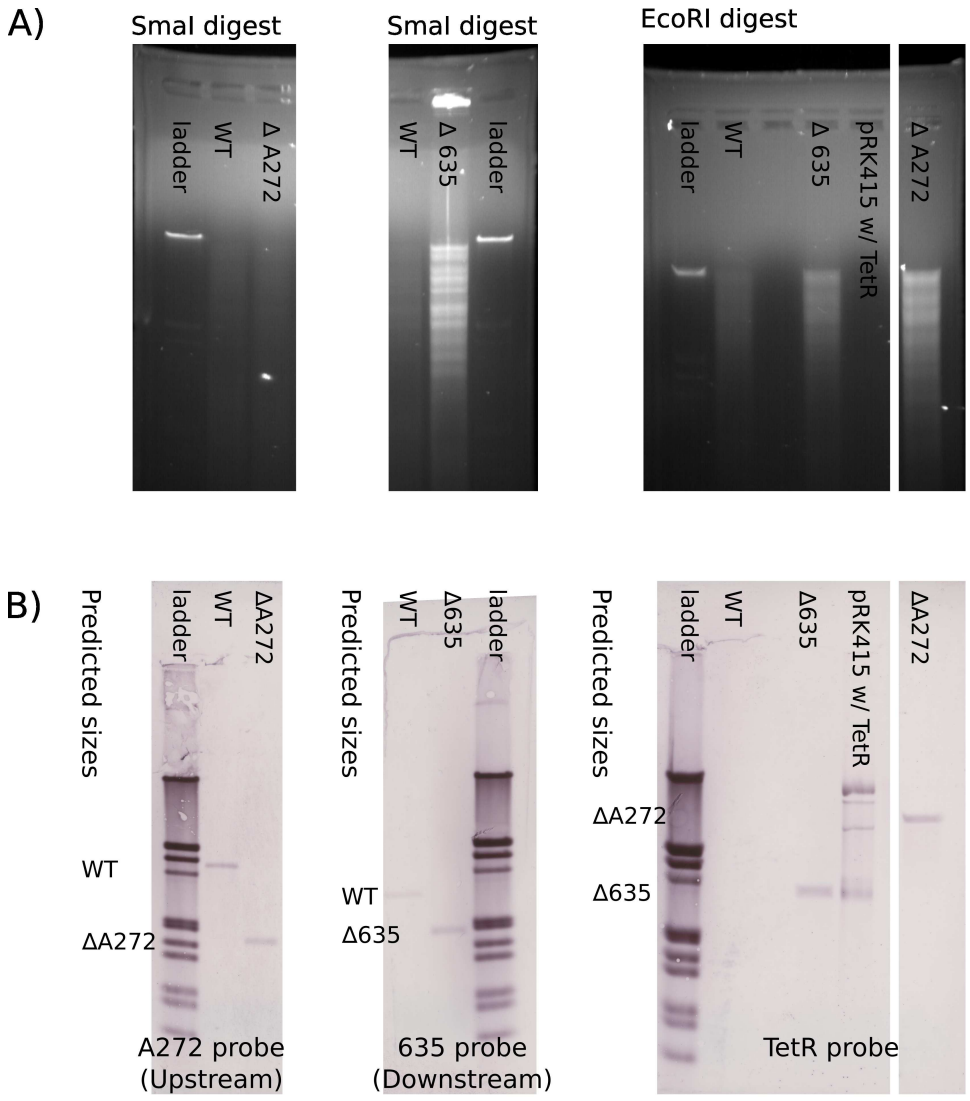

Figure 4.1: Southern blot confirming replacements of SPOA0272 and SPO635 with *tetRA*: A) agarose gels of SmaI digestion of genomic DNA preparations, B) Southern blots with indicated probes and predicted sizes of fragments for wildtype (WT) and deletion mutants indicated. A lack of expected wildtype fragments and the presence of the expected replacement fragments in the mutants indicates double, not single, crossover mutants.

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CHAPTER 5

CHARACTERIZATION OF SPO0635 AND SPOA0272 REPLACEMENT MUTANTS AND METHYLOTROPHY IN *S. pomeroyi*

5.1 ABSTRACT

Silicibacter pomeroyi, a member of the a marine roseobacter clade, is a model system for the study of dimethylsulfoniopropionate (DMSP) degradation. A differential display proteomic approach identified proteins upregulated during growth on DMSP. Gene replacement mutations in two of these genes, SPO0635 and SPOA0272, were constructed. A gas chromatographic system was developed to detect volatile organic sulfur (VOS) in headspace of cultures. No significant differences in VOS production or growth on DMSP were seen in the mutants. Investigations into the possible role of SPOA0272 in energy production from one carbon compounds in *S. pomeroyi* led to the observation of methylotrophic growth on methylamine. This suggests that *S. pomeroyi* expresses the newly proposed ethylmalonyl-CoA pathway with parts of the serine cycle formaldehyde assimilation pathway, which has implications for the fate of DMSP in the marine carbon cycle.

5.2 INTRODUCTION

Dimethylsulfoniopropionate (DMSP) is an algal osmolyte ubiquitous in marine systems. Bacterial cleavage of dimethylsulfoniopropionate to volatile dimethyl sulfide (DMS) is an important part of the global sulfur and carbon cycles and a regulator of global climate. An alternate demethylation pathway produces methanethiol (MeSH) that may be incorporated into cellular protein (Kiene *et al.*, 2000). These two pathways, with their characteristic volatile organic sulfur (VOS) products,

have radically different implications for the fate of sulfur in the environment, but many of the enzymes involved remain undescribed. Many aerobic heterotrophic bacteria, including the abundant and ubiquitous marine roseobacter clade, possess one or both of these pathways (Buchan *et al.*, 2005). The roseobacter *Silicibacter pomeroyi* is a model system for both DMSP degradation pathways. *S. pomeroyi* can cleave DMSP to DMS, degrade DMS, demethylate DMSP to MeSH, incorporate MeSH into cellular protein, and oxidize MeSH to sulfate (González *et al.*, 1999).

Using a *Silicibacter* minimal medium that supports high cell densities and a chemostat to provide steady state growth conditions, a differential display proteomics approach was used to find genes potentially involved in DMSP degradation in *S. pomeroyi* (see Figure 5.1). Two genes were disrupted in *S. pomeroyi* DSS-3, forming the mutants Δ635 and ΔA272 mutants.

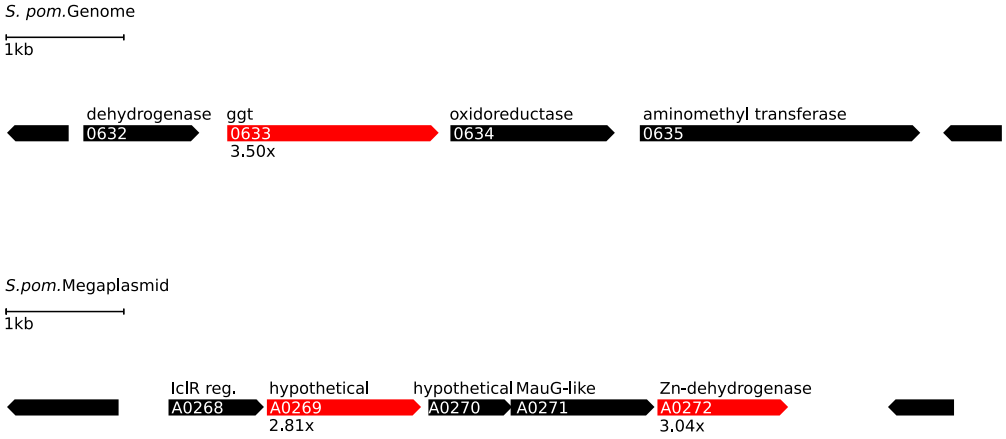

Figure 5.1: Chromosomal gene clusters: containing genes that encode a protein which increased in abundance during growth on DMSP. Genes encoding a protein significantly increased in abundance are indicated in red, and the fold increase in abundance is indicated below each gene.

The gene at locus SPO0635 produces a fusion protein, with the amino-terminal domain having high similarity to glycine and D-amino-acid oxidases and a carboxylic-terminal domain having high similarity to aminomethyltransferases. Known genes with this domain structure catalyze the removal of a methylamine moiety from dimethylglycine or sarcosine. In the presence of tetrahydrofolate (THF) these enzymes form methylene-THF. In the absence of THF, formaldehyde is formed instead. The electron acceptor for these types of enzymes can be either O_2 (which yields H_2O_2) or the electron transport chain.

The gene at locus SPOA0272 is a homolog of glutathione-dependent formaldehyde dehydrogenase. SPOA0272 has high similarity to genes whose products are involved in formaldehyde utilization by catalyzing the oxidation of S-hydroxymethylglutathione. This compound forms spontaneously from the reaction of glutathione with formaldehyde. This activity is found in methylotrophs, where it can be essential for methylotrophy, as well as in non-methylotrophs, where it is used as to detoxify formaldehyde (Barber *et al.*, 1996). There is a paralog to this gene in *S. pomeroyi* at SPO3850. With 97.4% nucleotide identity, the sequence is very conserved between these two genes. In fact, the amino acid sequence differs by a single conserved change, resulting in 99.7% similarity.

In order to investigate the possible role of these genes, growth on various substrates and VOS production by the mutants were investigated.

5.3 Materials and methods

5.3.1 Chemicals

DMSP was synthesized as previously described (Section 2.3.3). MMPA was synthesized by Erinn Howard by alkali treatment of the methyl ester, which is commercially available. The MeSH source used as a standard was obtained from a continuous-release Dynacal permeation tube purchased from VICI metronics (Part number 107-050-6000-C35, Santa Clara CA). All other compounds were purchased from Sigma-Aldrich (Saint Louis, MO) or Difco (Detroit, MI).

5.3.2 Synthesis of MeSH

A protocol to generate MeSH was developed based on Kiene (1991). A buffer composed of sterile water, 0.1 M TRIS pH 8 and 50%v/v propanol was sparged with N_2 gas to remove O_2. Using a N_2 purged syringe and needle, dimethyldisulfide (DMDS, H_3CSSCH_3, Sigma) was diluted to 8.6%v/v in a sealed vial with the anoxic buffer under a N_2 headspace. Using a N_2 purged syringe and needle, tributylphosphine (Sigma), which specifically reduces disulfide bonds (Humphrey and

Potter, 1965; R uegg and Rudinger, 1977), was diluted to 4%v/v in a sealed vial with the anoxic buffer under a N_2 headspace. The strict anoxic conditions were necessary because tributylphosphine reacts violently with oxygen in air. All dilutions and reactions were carried out under a fume hood. The diluted tributylphosphine and DMDS solutions were combined in a 9:1 v/v ratio in a teflon sealed vial filled with N_2, providing a molar excess of tributylphosphine. Yields of MeSH recovered in the headspace were above 85% theoretical yield, with no other VOS detected by GC-FPD.

5.3.3 Analytical methods

GC quantification of volatile sulfur gases

DMDS, DMS and MeSH were measured by GC-FPD using a modification of the method of de Souza and Yoch (1995). Samples were sealed in acid-washed glass vials with butyl Teflon-lined, crimp-seal caps. On-column headspace injections were separated on a 8610C GC (SRI instruments, Torrance, CA) fitted with a Teflon-lined 1.8 m by 0.3175 cm diameter Chromosil 330 column (Supelco, Bellefonte, PA). A flame photometric detector (GC-FPD) was used. The temperature of the column was 60 °C, and the nitrogen carrier gas flow rate was 60 mL min^{-1}. MeSH standards were from a permeation tube that released MeSH at 3.2 µmol min^{-1} in a glass U-tube water-bath operated at 30 °C with constant N_2 gas flow venting through a sampling chamber and bubble flowmeter. DMS standards were generated from alkali hydrolysis of DMSP. DMSP was measured as DMS following alkali hydrolysis with 2M NaOH and agitation at room temperature for one hour. Henry's Law corrections were based on the calculations of Staudinger and Roberts (2001). Quantitative detection limits of 3 ng (70 pmol) MeSH were routinely achieved, with similar detection limits for DMS. Day-to-day variability was less than 10%, with a range of detection of at least three orders of magnitude. Sulfate, methanethiol, dimethyl sulfide (all with a retention time of less than 3 min), and DMDS (with a retention time of 7 min) were well separated with this protocol.

Quantification of DMSP degradation intermediates

Acrylate, methylmercaptopropionate (MMPA), and mercaptopropionate (MPA) were measured by high-performance liquid chromatography (HPLC) based on the method of Ansede *et al.* (1999). Briefly, samples were acidified with sufficient phosphoric acid for a concentration of 0.2%, centrifuged, separated on a Eclipse XDB-C18 2.1 x 150 mm column (Alegent) by isocratic elution with a 2.5% acetonitrile and 0.2% phosphoric acid buffer at 0.75 mL min^{-1}, and detected by absorbance at 214 nm. The detection limit was less than 100 pmol, with a linear responce to 3 nmol. The column was washed with 80% acetonitrile after each separation to remove nonpolar substances. All buffers were HPLC grade, filtered with a 0.2 µM vacuum filter and degassed with helium before use. Standards (Sigma) were prepared in water. The retention times were: 1.96–1.97 min for acrylate, 6.50–6.55 min for MMPA, 26.2–26.19 min for the methyl ester of MMPA, a contaminant from incomplete synthesis of MMPA, 10.42–11.13 min for MPA, with a minor peak found at 3.15–3.16 min that may be a oxidation product of MPA. DMSP was not detected in 1 h runs. With a single batch of running buffer these times do not change more than 2%, but different batches of the running buffer can change the times by as much as 10%.

5.3.4 Strains

Silicibacter pomeroyi DSS-3^T was obtained from Dr. Mary Ann Moran (ATCC 700808; González *et al.*, 2003). Stock cultures were frozen in 40% glycerol plus 1/2YTSS and passed no more than six times. Cultures were grown aerobically at 30 °C with shaking in the dark unless otherwise noted. Strains and plasmids used in this study are listed in Table 5.1.

Table 5.1: *S. pomeroyi* strains used in this study

Strain	Relevant characteristics	Reference or source
S. pomeroyi strains:		
DSS-3	wild type	González *et al.* (2003)
41H6	*dmdA*::Ez-Tn<Kan2>	Howard *et al.* (2006)
Δ635	Δ(SPO0635)::*tetRA*	This work, Chapter 4
ΔA272	Δ(SPOA0272)::*tetRA*	This work, Chapter 4

5.3.5 Media and growth

S. pomeroyi was grown on 1/2YTSS, and pre-cultured in MBM (see Section 2.3.1). For carbon source growth experiments, cells were pregrown in MBM with 1 mM glucose. DMSP and acrylate were added to inoculated cultures in three 1 mM increments, while all other compounds were added at 3 mM. For headspace experiments, Teflon-lined stopper sealed Balch tubes with 10 mL of media and 18 mL of headspace were used. For growth experiments *S. pomeroyi* DSS-3 and Δ635 were grown in MBM media with 1 mM acetate, lactate, acrylate, glucose, MMPA, DMSP, thioglyoxylic acid, methionine, cysteine, spermidine, glycine betine, glyoxylic acid, glutathione, cysteic acid, or taurine. For methyl-group growth experiments, cells were pregrown in 3 mM acetate, inoculated at a 1:500 dilution with 5 or 50 mM methanol, formate, methylamine, or trimethylamine, with and without 3 mM acetate.

5.3.6 Washed cell VOS production from DMSP

S. pomeroyi was grown to an OD_{600} of 0.3 on MBM with 5 mM DMSP (late log phase), collected by centrifugation, and washed twice with an equal volume of MBM. Cells were placed in sterile 9.8 mL crimp-seal bottles and sealed with Teflon-lined stoppers. DMSP was added to a final concentration of 3 mM.

5.4 Results and discussion

Genes that are upregulated during growth on DMSP might be involved in many different cellular processes (see Chapter 3). Pathways that might be upregulated include DMSP cleavage and demethylation, MeSH and DMS degradation, MeSH incorporation into protein, methyl group utilization, sulfur oxidation, and degradation of the carbon backbone of DMSP. To further elucidate the role of the two proteins that were significantly upregulated, the genes SPOA0272 and SPO0635 were replaced in the wild-type strain, yielding strains Δ635 and ΔA272, respectively (see Chapter

4). As neither gene has a downstream gene transcribed in the same direction, polar effects are unlikely.

When grown on 3 mM DMSP, *S. pomeroyi* DSS-3, Δ635, and ΔA272 all grew to an OD_{600} of 0.3 with no significant differences in growth rate. Yield on other carbon sources were investigated, but no significant differences were observed with growth on acetate, acrylate, MMPA or glycine betaine. All three produced DMS and MeSH during growth in batch cultures on 3 mM DMSP, with DMS being the predominate product in the headspace during later growth stages. DMS and MeSH decreased over time in cultures, with no significant difference in the rates between the mutants and the wild type strain (data not shown). However, there was a great deal of variability in VOS production and consumption by different cultures of the same strain and at different points in the growth curve. This could be due to a complex regulatory system and the slow, non-logarithmic growth of *S. pomeroyi* on DMSP.

5.4.1 Volatile sulfur compounds produced by *S. pomeroyi* during growth on DMSP

Washed cell experiments were conducted in order to quantify the production rates of DMS and MeSH. Late-growth phase *S. pomeroyi* DSS-3 cultures produced both DMS and MeSH from DMSP when grown on acetate, acrylate, or DMSP (see Figure 5.2). Trace amounts of DMS were observed in the headspace of DMSP-grown cultures after washing when no additions of DMSP, potentially from intracellular accumulation and carry-over of DMSP (data not shown). The average rate of MeSH and DMS production for acrylate-grown cells was higher than that for DMSP-grown cells, which in turn was higher than acetate-grown cells. The significantly higher rate of MeSH production by cells grown on acrylate might indicate that acrylate induces the demethylation pathway, but does not induce the pathway for the consumption of MeSH, thus resulting in a higher observed rate of MeSH production. Although the doubling time of the cells is 25 hours when grown on DMSP and acrylate, there could be a rapid expression of genes involved in production of VOS,

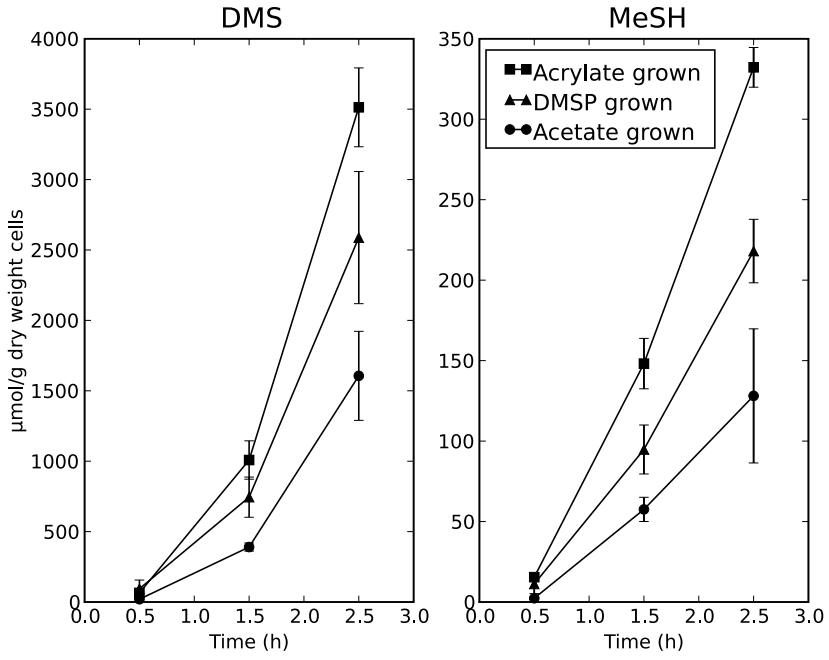

Figure 5.2: Production of VOS from DMSP by *S. pomeroyi* DSS-3: Production of DMS (left) and MeSH (right) from 3 mM DMSP by washed cells grown on ■ acrylate, ▲ DMSP, or ● actate. Error bars are standard deviation of three replicates.

which would interfere with the determination of the rates of activity when grown on other substrates.

S. pomeroyi Δ635 contains a gene replacement of a fusion protein with domains similar to dimethylglycine and sarcosine oxidases and potentially involved in the transfer of a methyl group to THF or the formation of formaldehyde. No significant difference in cell yield was seen between the mutant and wildtype with growth on acetate, lactate, acrylate, glucose, MMPA, DMSP, methionine, spermidine, glycine betine, or glutathione. Neither strain grew on thioglyoxylic acid, cysteine, cysteic acid, or taurine and both showed only weak growth on glyoxylic acid. DMSP grown Δ635 cells were washed, and DMS production from DMSP was measured (see Figure 5.3). No significant difference was seen in the production of VOS. This could be because SPO0635 catalyzes a reaction independent of VOS production. For instance, this gene product might be regulated in the presence

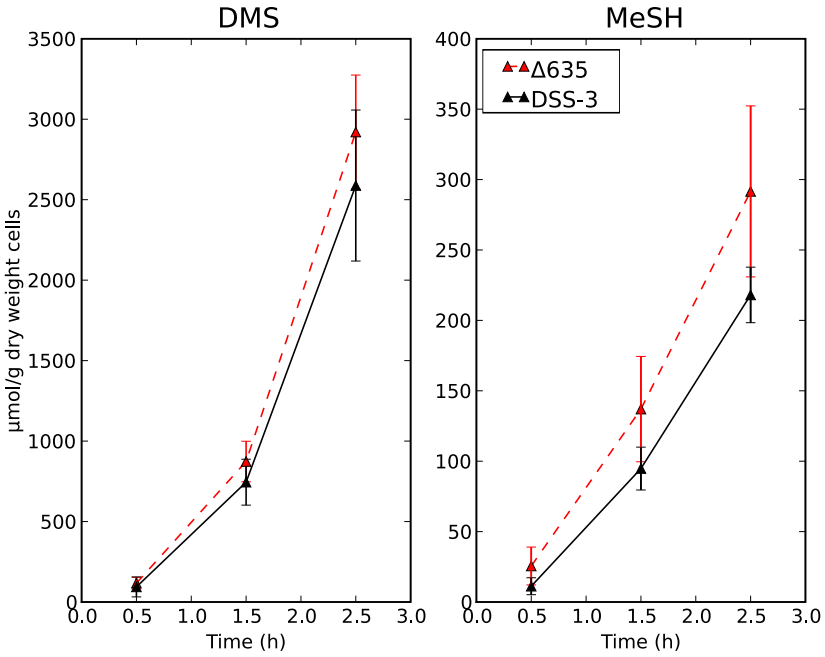

Figure 5.3: Production of VOS from DMSP by *S. pomeroyi* Δ635: Production of DMS (left) and MeSH (right) from DMSP grown washed cells, error bars are standard deviation of three replicates. The final time points are not significantly different (p=0.12) by Student's *t*-test.

of DMSP but not be involved in DMSP degradation, or might be involved in the pathway after VOS production. Alternatively, *S. pomeroyi* may be geneticly redundant for this reaction. The product of a gene (SPO0544) with the same domain structure as SPO0635 is 55% similar and 32% identical to SPO0635. Thus, SPO0635's activity might be complemented by the activity of SPO0544 under these growth conditions.

S. pomeroyi ΔA272 contains a gene replacement of a glutathione-dependent formaldehyde dehydrogenase. The demethylation pathway of DMSP degradation might produce toxic formaldehyde intermediates from the methyl groups and cause the induction of the other glutathione-dependent formaldehyde dehydrogenases in the genome. Therefore, cells were grown on acrylate as well as on DMSP, washed, and the VOS production from added DMSP measured (see Figure 5.4). There were no statistically significant difference in the production of VOS with cells grown

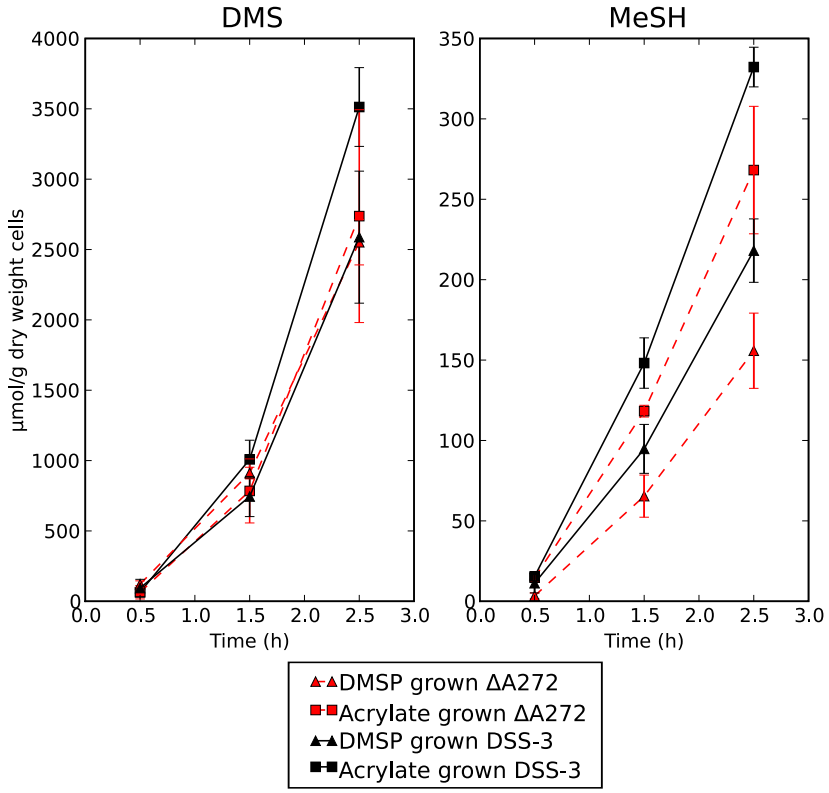

Figure 5.4: Production of VOS from DMSP by *S. pomeroyi* ΔA272: Production of DMS (left) and MeSH (right) from 3 mM DMSP by washed cells grown on ■ acrylate or ▲ DMSP. Error bars are standard deviation of three replicates. The final time points of the acrylate and DMSP grown MeSH values are significantly different by Student's *t*-test (p=0.015 and 0.028, respectively).

on either compound. Perhaps the toxicity of trace amounts of formaldehyde formed in other pathways caused upregulation of the close paralog SPO3850, a non-glutathione formaldehyde oxidation pathway was active, or the formation of formaldehyde did not effect the production of VOS. No significant difference was observed in formaldehyde resistance between the mutant and wild-type as measured by zones of clearing around formaldehyde soaked filters on 1/2YTSS plates (data not shown).

5.4.2 METHYLOTROPHIC GROWTH

During tests for pathways that might generate formaldehyde as an intermediate, it was observed that wild-type *S. pomeroyi* DSS-3 uses one-carbon (1C) methyl compounds for energy and methylamine as a sole source of carbon and energy (see Table 5.2). An increase in cell density above the 0.18 OD_{600} with growth on acetate was observed with additions of methanol, formate, methylamine and trimethylamine. Growth on methylamine as the sole source of carbon and energy indicates that *S. pomeroyi* is a methylotroph. The cultures grown on methylamine were checked by microscopy

Table 5.2: Cell yield of *S. pomeroyi* grown on 1- and 2-carbon compounds: Cultures contain 5 mM of the C1 compounds. Maximimum growth was recorded after 12 days. Values are adverage OD_{600} of duplicates in MBM

1C substrate	C1 compounds alone	+ 5 mM Acetate
No C1	No growth	0.18
Methanol	No growth	0.30
Formate	No growth	0.25
DMSO	No growth	0.15
Methylamine	0.35	0.30
Trimethylamine	No growth	0.30

and displayed the characteristic morphology of *S. pomeroyi* grown on minimal medium. Cultures were grown on 1/2YTSS and exhibited characteristic colonial and cellular morphologies. When sub-cultured, they also displayed the characteristic substrate utilization patterns of *S. pomeroyi*. Multiple passes at low dilution in MBM with methylamine were performed to confirm that the

growth was not due to carry-over of nutrients, and methylotrophic growth was confirmed on a separate source of methylamine.

The genome of *S. pomeroyi* contains methylamine dehydrogenase genes, which oxidize methylamine to formaldehyde, ammonia, and reducing equivalents. *S. pomeroyi* also contains the glutathione-dependent formaldehyde oxidation pathway to formate as well as the glutathione-independent, THF-dependent formaldehyde oxidation pathway to formate. Therefore, even if SPO3850 does not compensate for SPOA0272 in *S. pomeroyi* ΔA272, there is another pathway for formaldehyde oxidation. *S. pomeroyi* contains all necessary subunits of formate dehydrogenase, and could produce energy from the complete oxidation of formate. It was previously shown that *S. pomeroyi* grows by a lithoheterotrophy, oxidizing carbon monoxide and assimilating organic carbon compounds (Moran *et al.*, 2004). However, *S. pomeroyi* was not know to be capable of methylotroph growth.

During the initial annotation of *S. pomeroyi*, many genes in the serine cycle pathway of methylotrophic 1-carbon utilization were identified, including a hydroxypyruvate reductase and L-malyl-CoA lyase that are diagnostic of this pathway. However, several essential genes were not present: there was no isocitrate lyase gene and a gene with only low similarity to glycerate kinase. Recently, the ethylmalonyl-CoA pathway was proposed for *Rhodobacter sphaeroides*, another α-proteobacteria, and *S. pomeroyi* contains genes with high homology to all known genes in this new pathway. In particular, the predicted gene product of SPO370 has 93.0% similarity to a diagnostic enzyme for the ethylmalonyl-CoA pathway, crotonyl-CoA carboxylase/reductase from *Rhodobacter sphaeroides* strain 2.4.1. Genes that are theorized to catalyze the remaining reactions in the pathway have high similarity to genes of *S. pomeroyi* (Alber *et al.*, 2006; Erb *et al.*, 2007; Meister *et al.*, 2005). The ethylmalonyl-CoA pathway would allow isocitrate lyase negative organisms like *S. pomeroyi* to bypass this part of the serine pathway and carry out synthesis of 3- and 4-carbon compounds from formate (Erb *et al.*, 2007; Zarzycki *et al.*, 2008).

During the incorporation of formaldehyde via the serine and ethylmalonyl-CoA pathways, there would be an additional incorporation of four molecules of CO_2 for every three molecules of formaldehyde incorporated into organic carbon molecules.

In marine systems, a significant amount (1–13%) of the total C fixed by autotrophs is incorporated into DMSP (Kiene *et al.*, 2000) and an average of 50–90% of the DMSP is not incorporated into DMS or cell sulfur (Kiene and Linn, 2000). Thus there is the potential for a large amount of chemoautotrophic CO_2 incorporation during the utilization of the two methyl carbons of DMSP. These methyl carbons could be from 0.3–6% of the total C fixed in marine systems, which coresponds to roughly 0.15–3 gigatons of CO_2 incorporated annually. In addition, other sources of methyl groups in marine systems incorporated by serine-cycle methylotrophs could increase this amount. The roseobacter clade is a numerically important group in marine systems. If this pathway of methylotrophy is widespread in this clade, then methylotrophy may play a more important role in marine systems than previously thought (Giovannoni *et al.*, 2008; Sherr and Sherr, 2000).

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APPENDIX A

DMSP DEGRADATION ENERGETICS

A.1 ABSTRACT

A theoretical redox analysis of the degradation of DMSP will aid in the analysis of chemostat growth of *Silicibacter pomeroyi*. Electron equivalents for the complete oxidation and partial oxidation of DMSP (with the release of DMS) and the complete oxidation of acrylate and acetate were calculated. This allowed the comparison of cell yields on acetate and DMSP in chemostat-grown *S. pomeroyi*. The results support complete oxidation of only the acrylate moiety of DMSP during chemostat growth with DMSP as the limiting nutrient.

A.2 COMPOUNDS

The idealized oxidation state of each of the compounds is calculated below, based on the convention that O is -2 and H is +1.

A.2.1 DMSP

Dimethylsulfoniopropionate, also known as: dimethylpropiothetin, DMPT, DMSP, dimethylbetapropiothetin, beta-dimethylsulfoniopropionate, S,S-di-methylbetapropiothetin, 3-(dimethylsulfonio)propanoate, S-dimethylsulfonium propionic acid.

IUPAC Name: 3-dimethylsulfoniopropanoate

Molecular Weight: 134.193 g/mol

Formula: $^{-}C_5H_{10}O_2S^{+}$

Net charge: 0

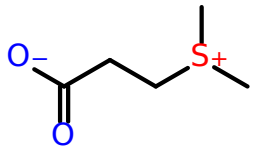

Figure A.1: Structure of DMSP

Element	Avg. State	# Atoms	Total
S	-2	1	-2
O	-2	2	-4
H	$+1$	10	$+10$

Solving for the oxidation state of the carbon atoms:

$$-2 + -4 + 10 + x = 0$$

$$x = -4$$

Element	Avg. State	# Atoms	Total
C	$-\frac{4}{5}$	5	-4

A.2.2 DMS

Dimethyl sulfide, also known as: methyl sulfide (note that this name is more properly a discripter of methanethiol i.e. CH_4S, but is used in the literature and by commercial suppliers to describe this compound).

IUPAC Name: methylsulfanylmethane

Molecular Weight: 62.129 g/mol

Formula: C_2H_6S

Net charge: 0

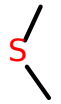

Figure A.2: Structure of DMS

Element	Avg. State	# Atoms	Total
S	-2	1	-2
H	$+1$	6	$+6$

Solving for the oxidation state of the carbon atoms:

$$-2 + 6 + x = 0$$

$$x = -4$$

Element	Avg. State	# Atoms	Total
C	-2	2	-4

A.2.3 METHANETHIOL

Methanethiol, also known as MeSH, methyl-mercaptan, mercaptan C1.

IUPAC Name: methanethiol

Molecular Weight: 48.103 g/mol

Formula: CH_4S

Net charge: 0

Figure A.3: Structure of MeSH

Element	Avg. State	# Atoms	Total
S	-2	1	-2
H	$+1$	4	$+4$

Solving for the oxidation state of the carbon atoms:

$$-2 + 4 + x = 0$$

$$x = -2$$

Element	Avg. State	# Atoms	Total
C	-2	1	-2

A.2.4 ACRYLATE

Acrylate, also known as: acrylic acid, 2-propenoic acid, vinylformic acid, propenoic acid, acroleic acid, propenoate, propene acid, polyacrylate.

IUPAC Name: prop-2-enoic acid

Molecular Weight: 71.055 g/mol

Formula: $C_3H_3O_2^-$

Net charge: -1

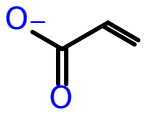

Figure A.4: Structure of Acrylate

Element	Avg. State	# Atoms	Total
O	-2	2	-4
H	$+1$	3	$+3$

Solving for the oxidation state of the carbon atoms:

$$3 + -4 + x = -1$$

$$x = 0$$

Element	Avg. State	# Atoms	Total
C	0	3	0

A.2.5 ACETATE

Acetate, also known as: acetic acid, ethanoic acid, vinegar acid.

IUPAC Name: acetic acid

Molecular Weight: 59.044 g/mol

Formula: $C_2H_3O_2^-$

Net charge: -1

Element	Avg. State	# Atoms	Total
O	-2	2	-4
H	$+1$	3	$+3$

Solving for the oxidation state of the carbon atoms:

$$3 + -4 + x = -1$$

$$x = 0$$

Element	Avg. State	# Atoms	Total
C	0	3	0

A.3 REACTIONS

Balanced reactions were drawn for the complete oxidative mineralization of the following compounds. The oxidation values for carbon and sulfur calculated above were used.

A.3.1 Mineralization of Acrylate

The half reactions:

$$\mathrm{C_3H_3O_2^-} + 4\,\mathrm{H_2O} \longrightarrow 3\,\mathrm{CO_2} + 11\,\mathrm{H^+} + 12\,\mathrm{e^-}$$
$$3\,\mathrm{O_2} + 12\,\mathrm{H^+} + 12\,\mathrm{e^-} \longrightarrow 6\,\mathrm{H_2O}$$

Reaction sum:

$$\mathrm{C_3H_3O_2^-} + 3\,\mathrm{O_2} + \mathrm{H^+} \longrightarrow 3\,\mathrm{CO_2} + 2\,\mathrm{H_2O}$$

In this reaction, 3 carbon atoms at 0 are oxidized to +4 while 2 oxygen molecules are reduced to H_2O for a total transfer of 12 electrons.

A.3.2 Mineralization of DMS

The half reactions:

$$\mathrm{C_2SH_6} + 8\,\mathrm{H_2O} \longrightarrow 2\,\mathrm{CO_2} + \mathrm{SO_4^{2-}} + 22\,\mathrm{H^+} + 20\,\mathrm{e^-}$$
$$5\,\mathrm{O_2} + 20\,\mathrm{H^+} + 20\,\mathrm{e^-} \longrightarrow 10\,\mathrm{H_2O}$$

Reaction sum:

$$\mathrm{C_2SH_6} + 5\,\mathrm{O_2} \longrightarrow 2\,\mathrm{CO_2} + \mathrm{SO_4^{2-}} + 2\,\mathrm{H_2O} + 2\,\mathrm{H^+}$$

In this reaction, 2 carbons are oxidized from -2 to +4 and 1 sulfur atom is oxidized from -2 to +6 while 5 oxygen molecules are reduced to H_2O for a total transfer of 20 electrons.

A.3.3 MINERALIZATION OF DMSP

The half reactions:

$$\mathrm{C_5H_{10}O_2S + 12\,H_2O \longrightarrow 5\,CO_2 + SO_4^{2-} + 34\,H^+ + 32\,e^-}$$
$$\mathrm{8\,O_2 + 32\,H^+ + 32\,e^- \longrightarrow 16\,H_2O}$$

Reaction sum:

$$\mathrm{C_5H_{10}O_2S + 8\,O_2 \longrightarrow 5\,CO_2 + SO_4^{2-} + 4\,H_2O + 2\,H^+}$$

In this reaction, 5 carbons are oxidized from a net of -4 to +4 each and 1 sulfur atom is oxidized from -2 to +6 while 8 oxygen molecules are reduced to H_2O for a total transfer of 32 electrons.

A.3.4 MINERALIZATION OF ACETATE

The half reactions:

$$\mathrm{C_2H_3O_2^- + 2\,H_2O \longrightarrow 2\,CO_2 + 7\,H^+ + 8\,e^-}$$
$$\mathrm{2\,O_2 + 8\,H^+ 8\,e^- \longrightarrow 4\,H_2O}$$

Reaction sum:

$$\mathrm{C_2H_3O_2^- + 2\,O_2 + H^+ \longrightarrow 2\,CO_2 + 2\,H_2O}$$

In this reaction, 2 carbon atoms at 0 are oxidized to +4 while 2 oxygen molecules are reduced to H_2O for a total transfer of 8 electrons.

A.4 Energetics of DMSP degradation

Theoretical calculation of the energy available for degradation of a compound can aid in the understanding of its degradation pathway. Since *S. pomeroyi* is an aerobic heterotroph with the ability to oxidize inorganic sulfur compounds, it is theoretically possible that it carries out the complete oxidation of DMSP ($C_5H_{10}O_2S$) to CO_2 and SO_4^{2-}. With 5 carbons carrying a net oxidation of -4 and the sulfur in -2 state, the complete oxidation of DMSP to CO_2 and SO_4^{2-} would yield 24 electron equivalents for the carbon and 8 for the sulfur, for a yield of 32 electrons equivalents in total. If the sulfur moiety is incorporated into cellular protein, electron equivalents are conserved, as it takes 8 electron equivalents to take SO_4^{2-} (+6) to the redox state of the sulfur in methionine. No net reduction is required to take the sulfur in DMS or MeSH to the same redox state as that in methionine, so 8 electron equivalents would be conserved for each sulfur atom that was incorporated into methionine from DMSP. However, *S. pomeroyi* releases most of the sulfur moiety as volatile gasses, particularly DMS. If the methyl and sulfur moieties of DMSP were released as DMS only 12 reducing equivalents of acrylate would be available. The complete oxidation of acetate would yield 8 electron equivalents. Therefore, the ratio of reducing equivalents between acrylate and acetate (1.5) would be observed between DMSP and acetate if DMSP was degraded to DMS and CO_2.

In a carbon-limited chemostat, the ratio of the cellular yield per mol of substrate during growth on DMSP to growth on acetate averaged 1.59 ± 0.112, close to the expected value of 1.5 for the oxidation of only the acrylate moiety of DMSP. Since the predicted molar ratio for complete oxidation of DMSP to acetate is 4 (from a calculated electron ratio of 32:8), it is unlikely that significant amounts of the reduced sulfur and methyl moieties are oxidized under these conditions, In addition, similar growth yields were observed for batch cultures grown on equimolar concentrations of DMSP or acrylate, supporting this conclusion. These results are consistant with *S. pomeroyi* releasing most of the sulfur from DMSP without oxidation under these growth conditions. While *S. pomeroyi* does have the capability of utilizing MeSH and DMS and oxidizing reduced sulfur compounds, these activities do not seem to be significant under these conditions.

The small increase above the expected yield could come from: 1) oxidation of small amounts of sulfur, 2) utilization of the methyl group transferred to a carrier in the demethylation pathway, 3) the savings from sulfur assimilation, or 4) compatible solute effects of DMSP.

APPENDIX B

S. pomeroyi GENE LOCI

Table B.1: *S. pomeroyi* gene loci and GI assertion numbers

S. pomeroyi gene loci	Protein GI	Gene size (bp)
SPO0632	GI:56677274	987
SPO0633	GI:56677275	1794
SPO0634	GI:56677276	1398
SPO0635	GI:56677277	2508
SPO0725	GI:56677365	1320
SPO0726	GI:56677366	2349
SPO0727	GI:56677367	723
SPO0728	GI:56677368	1176
SPO1623	GI:56678244	1371
SPO1624	GI:56678245	681
SPO1625	GI:56678246	1437
SPO1913	GI:56678524	1095
SPO1914	GI:56678525	993
SPO2203	GI:56678803	1500
SPO2571	GI:56679153	2355
SPO2572	GI:56679154	696
SPO2573	GI:56679155	1086
SPO3498	GI:56680057	1176
SPO3499	GI:56680058	2118
SPO3500	GI:56680059	471
SPO3501	GI:56680060	372
SPO3850	GI:56680398	1113
SPOA0268	GI:56680737	813
SPOA0269	GI:56680738	1311
SPOA0270	GI:56680739	711
SPOA0271	GI:56680740	1215
SPOA0272	GI:56680741	1113