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GENES REQUIRED FOR IN SITU SEDIMENT SURVIVAL OF *SHEWANELLA ONEIDENSIS* MR-1

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GENES REQUIRED FOR IN SITU SEDIMENT SURVIVAL OF *SHEWANELLA ONEIDENSIS* MR-1

A Dissertation APPROVED FOR THE
DEPARTMENT OF BOTANY AND MICROBIOLOGY

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Table of Contents

List of Tables

Chapter 4.

Appendix 1.

List of Figures

Chapter 1.

Chapter 2.

Appendix 1.

Preface

Biological oxidation and reduction of metals are very important global processes. These reactions control the biogeochemical cycling of a variety of elements. From the standpoint of human and environmental health, changing the state of metals can also alter their solubility and subsequently how easily they are dispersed from sources. This becomes a significant problem where toxic metals (e.g., chromium) and radionuclides (e.g., uranium and technetium) are concerned.

As the result of interest in metal cycling and bioremediation applications, several microorganisms capable of metal reduction have been isolated and studied in-depth regarding their physiological responses to metals and regarding their involvement in other biogeochemical processes. Over the years, microbiologists have come to understand mechanisms and have identified some genes/proteins involved in metal respiration by important environmental bacteria, such as *Geobacter* and *Shewanella* species. Even further progress was made when genome sequences appeared for these bacteria, with *Shewanella oneidensis* MR-1 being the first dissimilatory metal-reducing bacteria for which the entire genome was sequenced.

To examine how these organisms behave and respond to stimuli in the environment, and therefore to determine how best they can be incorporated into bioremediation processes, an in situ-based approach is required. Studies conducted in laboratory medium might not capture the effects of some abiotic and biotic factors that are present in the natural environment. The ideal approach would also allow researchers to examine an entire set of genes required for an organism to survive in sediment. In this regard, signature-tagged mutagenesis (STM) is a useful technique that was popularized in

pathogen studies. Since its inception in 1995, STM has been used with 31 pathogenic and commensal bacteria to identify over 1700 bacterial genes involved in virulence. To date, this procedure has not been applied to questions of environmental significance, and the project comprising this thesis accomplished this by examining genes required for sediment survival of *S. oneidensis* MR-1. Although this thesis will not address the following, we also approached this project with the future goal in mind to apply our findings to identification of genes required for survival in radionuclide-contaminated sediments.

In Chapter 1, I examine the ability of *S. oneidensis* MR-1 to survive in sediments, as modeled by microcosms representing various respiratory conditions. I show that MR-1 can persist without any amendments, but that Fe(III)-reducing conditions are not favorable for growth and survival without the addition of an appropriate electron donor/carbon source (e.g., lactate) or an electron shuttle or Fe(III) chelator. This may result from competition with indigenous Fe(III)-reducing bacteria that may be better suited physiologically for reduction of insoluble metals. This work could explain why in past surveys that employed molecular techniques DNA sequences of *Shewanella* species have not been detected frequently in Fe(III)-reducing sediments, while *Geobacter* sequences were dominant in such environments. For bioremediation purposes, it may also be necessary to add lactate to sediments to selectively stimulate *Shewanella*. This chapter was written with Dr. Lee Krumholz in the style required by *Environmental Microbiology*.

In Chapter 2, I present a modified approach for signature-tagged mutagenesis (STM) that takes advantage of microarray technology. In this manner, the process of screening

tagged-mutant pools is more streamlined when compared to earlier STM procedures. This system is applicable to any bacterium that can be randomly mutagenized by the mini-Tn*10* transposon employed in our study. Together with Qingwei Luo, I have demonstrated that this system can be used to identify genes required for survival of MR-1 (a γ–*Proteobacterium*) and also *Desulfovibrio desulfuricans* G20 (a δ–*Proteobacterium*). In future studies, our method can be used on a variety of microorganisms to address many questions of economical and environmental significance, such as which genes are involved in biologically-mediated iron corrosion or in production of a renewable energy source (e.g., H_2). This chapter was written with Qingwei Luo, Dr. Jimmy Ballard, and Dr. Lee Krumholz in the style required by *Applied and Environmental Microbiology*.

In Chapter 3, I present the genes that were identified by our STM method. Some unexpected findings were that phage- and transposon-related genes were found to enhance sediment survival of MR-1. I also describe the involvement of several genes in defensive mechanisms (such as multidrug efflux). These findings provide a unique view into microbial interactions with abiotic and biotic environmental factors. This chapter was written with Qingwei Luo, Dr. Jimmy Ballard, and Dr. Lee Krumholz in the short-form publication style required by *Applied and Environmental Microbiology*.

In Chapter 4, I examine the function of the putative multidrug efflux gene *mexF* that was identified in the STM study that comprises this thesis. This study describes the function of a gene involved in multidrug resistance, a problematic phenomenon that until this time was primarily studied within pathogenic microorganisms. I show that this gene is involved in MR-1 resistance to several antibiotics and that it is not involved in resistance to toxic heavy metals. I also show that this gene is likely regulated by a TetR

family regulatory protein that precedes the *mexEF* operon. This chapter was written with Dr. Lee Krumholz in the style required by the *Journal of Bacteriology*.

Abstract

We examined survival of *Shewanella oneidensis* MR-1 in sediment microcosms under various respiratory conditions. MR-1 was able to maintain its population in aerobic, transitory, and unamended anaerobic sediments; however, under Fe(III)-reducing conditions, the addition of lactate or an electron shuttle/Fe(III) chelator was required for maintenance of inoculum concentration and for growth in some instances. Loss of numbers in the absence of these compounds may result from direct competition with indigenous bacteria, such as *Geobacter* species, that are better suited physiologically for obtaining energy from Fe(III) reduction.

We developed a modified version of the traditional signature-tagged mutagenesis (STM) procedure, incorporating microarray technology to streamline the entire screening process. We also identified a mini-Tn*10* transposon that is capable of randomly mutagenizing model bacteria of the γ– and δ–*Proteobacteria*. This system can therefore be applied to a wide variety of environmentally significant bacteria to answer questions of ecological or economical importance. This is the first study to use STM in studying bacteria of environmental significance.

We identified 47 genes required for sediment survival of MR-1. We validate our STM screening method by identifying genes that would be expected to enhance sediment survival (e.g., chemotaxis genes) or would be required for growth with Fe(III) (i.e., *gspN*, a gene within an operon that was shown previously to be required for Fe(III) reduction). We describe the putative sediment functions of many groups of these genes, and we propose roles for phage- and transposon-related genes, as well as many genes involved in defensive mechanisms, such as multidrug resistance.

We determined the function of one sediment-survival gene, examining *mexF* in greater detail. The protein encoded by *mexF* shares high homology with MexF in pathogenic *Pseudomonas* where it has been shown to confer a multidrug resistance phenotype on its host. We determined that *mexF* in MR-1 also functions in multidrug resistance, although additional substrates, including those that may be found in sediment remain to be elucidated. We propose that similar genes, present in the genomes of both pathogenic and nonpathogenic bacteria, have persisted over time in order to combat natural (such as plant- and bacterial-derived antimicrobials) as well as man-made toxins.

Chapter 1

Factors influencing growth and survival of *Shewanella oneidensis* MR-1 in sediments

Summary

The ability of *Shewanella oneidensis* MR-1 to reduce radionuclides, including technetium and uranium, has generated great interest in its use for bioremediation. Optimization of in situ bioremediation processes requires knowledge of an organism's ability to grow and survive in the natural environment. We assessed survival of MR-1 through growth studies in various sediment systems that simulate environments where MR-1 could theoretically live, based on its respiratory versatility. We examined microcosms simulating aerobic conditions, transition of aerobic to Fe(III)-reducing conditions, and Fe(III)-reducing conditions with amendments, including anthraquinone-2,6-disulfonate (AQDS), nitrilotriacetate (NTA), and lactate. Survival in the presence of natural microbial communities was tested alongside survival under axenic conditions. Under each of the respiratory conditions, MR-1 grew to higher numbers (approximately 10- to 100-fold increase) and generally survived better in sterile sediments than in nonsterile sediments. When no electron donor was added to non-sterile sediments, MR-1 concentrations remained steady or gradually died below the initial inoculum concentration within a week. Based on our growth studies, the non-sterile, Fe(III)-reducing sediment system with lactate added comprised the best growth conditions with minimal amendments for MR-1, where cell numbers increased approximately 10-fold over the inoculum concentration.

Introduction

A long-standing interest exists in regard to survival of bacteria that are introduced into the environment. Many studies have focused on survival of either fecal indicator organisms (Arana *et al.*, 2003) or bacteria with bioprotection/bioremediation potential (Heuer *et al.*, 1995; Amarger, 2002). The latter group often consists of genetically engineered microorganisms (GEMs) that can be used either to enhance degradation of environmental pollutants (Heuer *et al.*, 1995) or to increase agricultural yields through disease protection or improved nutrition (Amarger, 2002). When these exogenous bacteria are introduced into the environment, several scenarios may ensue: the population could die-off, could maintain the inoculum concentration, or could grow and thrive in the new environment. The result is often dependent on both biotic (e.g., competition, predation) and abiotic (e.g., temperature and nutrient availability) factors (Stotzky and Babich, 1984; Scheuerman *et al.*, 1988).

Shewanella species have been isolated from a variety of environments, including lake and marine sediments and water and oil field groundwater (Venkateswaran *et al.*, 1999). Of these, *Shewanella oneidensis* MR-1 is the best characterized strain, having been the focus of many environmental studies on account of its ability to reduce insoluble electron acceptors [Fe(III) and Mn(IV)(Lovley *et al.*, 2004)], and to reduce radionuclides [U(VI) and Tc(VII) (Ganesh *et al.*, 1997; Lloyd *et al.*, 2002)]. *Shewanella* will also grow by reducing Cr(VI), fumarate, nitrate, trimethlyamine *N*-oxide (TMAO), dimethyl sulfoxide (DMSO), sulfite, thiosulfate, elemental sulfur, or oxygen. Such versatility in respiration allows these organisms to live at oxic-anoxic interfaces, such as the surface of lake

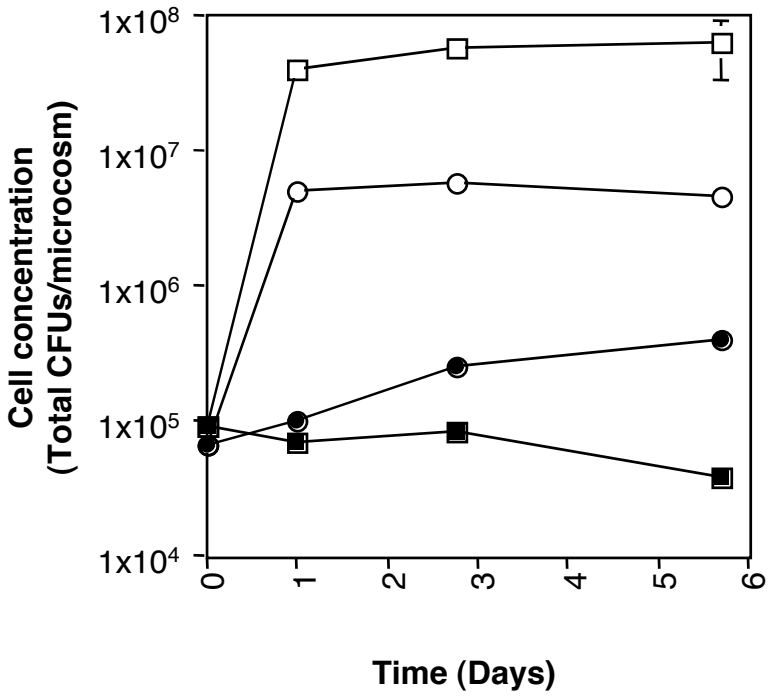
sediments (Myers and Nealson, 1988), and contributes to the idea that *Shewanella* species could be useful for bioremediation.

While studies have thoroughly addressed respiratory capabilities of *Shewanella* and more recently, have delved into new research areas, such as biofilm formation (Thormann *et al.*, 2004; De Vriendt *et al.*, 2005) and protection of steel from corrosion (Dubiel *et al.*, 2002), no studies have examined the survival capacity of this bacterium when inoculated into the natural environment. In this study, we examine growth and short-term survival of *S. oneidensis* MR-1 in sediment microcosms as part of a larger study (Groh *et al.*, 2005) that identifies genes required for in situ survival of this bacterium. We found that MR-1 can survive at initial inoculum concentrations when added to unamended sediments, but that amendments, such as an appropriate electron donor or electron shuttle/chelator for Fe(III) reduction, are required for substantial growth of MR-1. Competition with indigenous microorganisms may drive this requirement for amendments.

Results

Growth in aerobic microcosms. As shown in Fig. 1, over the course of 6 days, there was little growth of MR-1 in non-sterile sediments from either terrestrial or lake sources. In the same sediments that have been sterilized, however, MR-1 reached high numbers rapidly and maintained these numbers over the course of the experiment. Live sediments may lack appropriate electron donor and/or nutrients for growth, while sterile sediments have eliminated the competition, and lysed bacterial cells could provide nutrients for MR-1 growth.

Figure 1. Growth of MR-1 Rf^r Sm^r in terrestrial sediments (circle symbols) and aquatic sediments (square symbols) under aerobic conditions. Filled symbols represent non-sterile sediments, while open symbols are sediments that were sterilized prior to inoculation by MR-1. Data points represent the average of at least duplicate microcosms, and error bars represent standard deviations (too small to appear in most cases).



Growth in transition microcosms. The addition of a small amount of air was meant to simulate the transition from oxic to anoxic conditions, as MR-1 likely encounters in its natural environment. One study showed that aerobic conditions and supplemental levels of tyrosine stimulate melanin production by *Shewanella algae* BrY (Turick *et al.*, 2002). As this compound can serve as an electron shuttle to insoluble Fe(III) (Turick *et al.*, 2002), we hypothesized that its production, or production of similar endogenous electron shuttles would allow MR-1 to compete more effectively for Fe(III). However, in our experiments, addition of oxygen had little effect on growth in non-sterile, Fe(III)-reducing sediments (Fig. 2). Fe(II) concentrations (Table 1), measured at the endpoint for each microcosm condition, were low for sterilized sediments (2 to 3 μmol produced) and higher for non-sterile sediments (19 to 26.5 μmol produced), indicating that MR-1 was not reducing Fe(III) either while the indigenous population was.

To explain these observations, the amount of air added may have been insufficient to stimulate melanin production, and we did not add tyrosine to our microcosms, although only trace amounts of oxygen and tyrosine were proposed requirements for the production of physiologically significant amounts of melanin (Turick *et al.*, 2002). Furthermore, as MR-1 did not grow significantly in non-sterile, aerobic sediments, it may not have grown sufficiently here either for melanin production to occur prior to the complete consumption of oxygen by the system.

Growth in Fe(III)-reducing microcosms. Although MR-1 is capable of using a variety of electron acceptors in the absence of oxygen, we chose to test its ability to survive in sediments under Fe(III)-reducing conditions, as many studies have focused on

Figure 2. Growth of MR-1 Rf^r Sm^r in terrestrial sediments (circle symbols) and aquatic sediments (square symbols) amended with Fe(III) oxyhydroxide and approximately 10% air in the headspace initially. Filled symbols represent non-sterile sediments, while open symbols are sediments that were sterilized prior to inoculation by MR-1. Data points represent the average of at least duplicate microcosms, and error bars represent standard deviations (too small to appear in most cases).

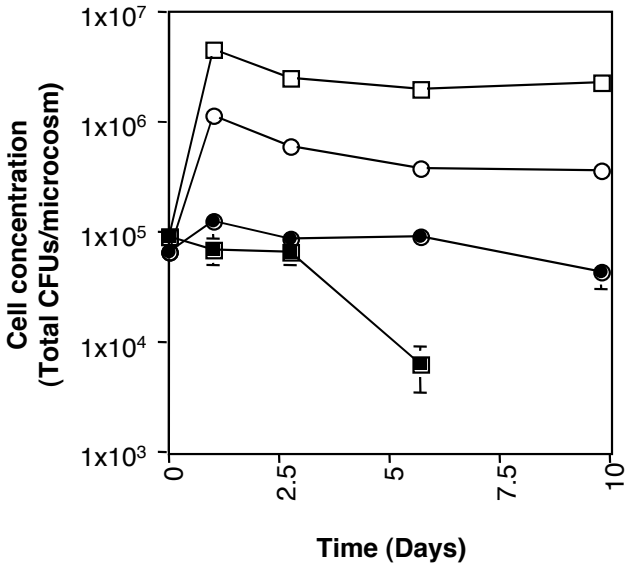


Table 1. Fe(II) concentrations (in μmol) at the endpoint of various microcosm incubations that utilize sediment collected from terrestrial (landfill) or aquatic (Lake Thunderbird) sources in June 2002. The day on which Fe(II) was measured is found in parentheses following the listed sediment conditions. Background Fe(II) concentrations for each condition, measured in sterile uninoculated controls at the same time point, have been subtracted from experimental microcosms to yield Fe(II) concentrations shown below. All of these sediments were amended with Fe(III) oxyhydroxide.

Sediment conditions	Sterile sediments [Fe(II)] in μmol	Non-sterile sediments [Fe(II)] in μmol
Transition conditions (10)		
Terrestrial	1.9±0.11	19.1±5.7
Aquatic	2.9±1.2	26.5±3.4
Lactate-amended (7.5)		
Terrestrial	3.0±0.05	6.8±1.4
Aquatic	6.4±2.2	7.8±1.7
Lactate, AQDS-amended (8)		
Terrestrial	15.5±1.2	30.7±1.8
Aquatic	10.1	17.7±4.2
Lactate, NTA-amended (8)		
Terrestrial	9.3±3.9	10.8±2
Aquatic	10.5±2.3	11.9±2

Fe(III) reduction in *Shewanella* species (Dobbin *et al.*, 1995; Roden and Zachara, 1996; Fredrickson *et al.*, 1998; DiChristina *et al.*, 2002; Dubiel *et al.*, 2002; Haas and Dichristina, 2002; Kostka *et al.*, 2002). Because the sediments we collected were sulfidogenic, we added Fe(III) oxyhydroxide to stimulate Fe(III)-reducing microorganisms.

For the first set of Fe(III)-reducing incubations (Fig. 3), we added H_2 as an electron donor to one set of terrestrial sediments amended with Fe(III) oxyhydroxide. This gas is often produced and consumed in sediments where Fe(III) reduction is coupled to organic matter mineralization (Lovley and Phillips, 1987), and past studies have shown that H_2 is used by *Shewanella* species during Fe(III) reduction (Lovley *et al.*, 1989). Replicate microcosms contained no added H_2. In non-sterile sediments, with or without H_2 added, MR-1 numbers declined rapidly (Fig. 3A). In sterile sediments, MR-1 was able to grow approximately 4 times higher than the initial cell concentration. Fe(II) produced in non-sterile sediments was approximately 15 μmol at peak concentrations, while Fe(II) in sterilized sediments with MR-1 added remained at levels comparable to the sterile, uninoculated control for the duration of the experiment (Fig. 3B).

Studies have shown that *S. algae* BrY requires specific carbon sources (e.g., citrate, fumarate, or malate) for growth with H_2 and Fe(III), while acetate and succinate cannot act in this role (Caccavo *et al.*, 1992). Because an acceptable carbon source may have been absent from the Fe(III)-reducing sediments, or if present, was not in concentrations sufficient for MR-1 to compete with the natural population, yeast extract was added alongside H_2 to non-sterile aquatic sediments to see if we could stimulate MR-1 growth in the non-sterile sediments (Fig. 4). We found that 500 μg of yeast extract led to an

Figure 3. Growth (A) of MR-1 Rf^r Sm^r and Fe(II) (B) produced in terrestrial sediments with Fe(III) oxyhydroxide added. H_2 was added as the electron donor to some microcosms (triangle symbols). Filled symbols represent non-sterile sediments, while open symbols represent sediments that were sterilized prior to inoculation with MR-1. Data points represent the average of at least duplicate microcosms, and error bars represent standard deviations (too small to appear in most cases). The sterile, uninoculated control for Fe(II) is represented by the asterisk symbol in (B).

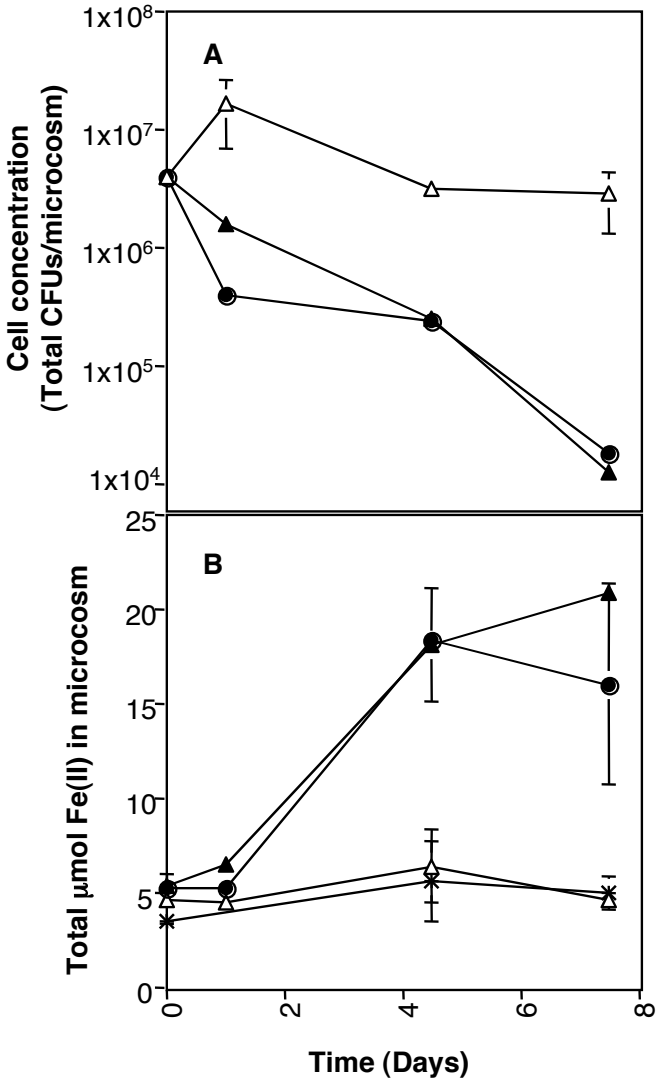
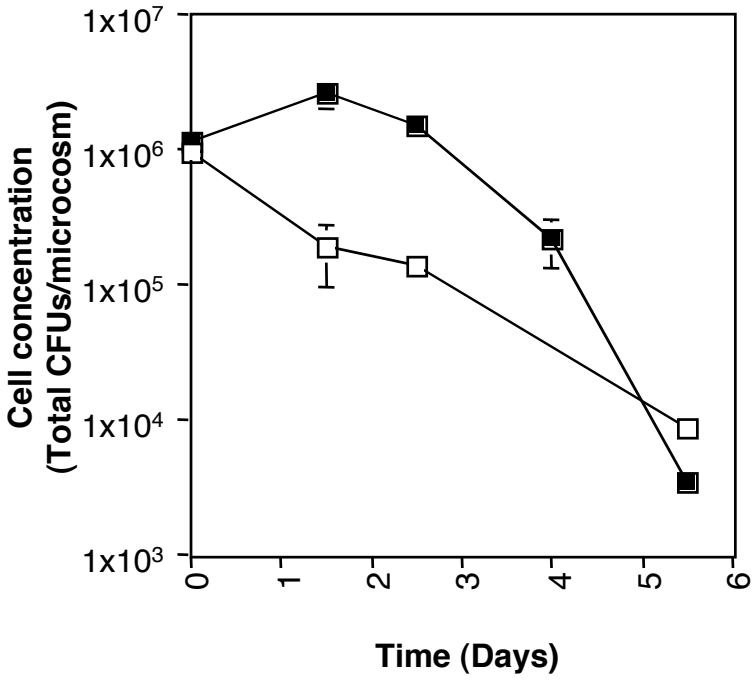


Figure 4. Growth of MR-1 Rf^r Sm^r in non-sterile, aquatic sediments with Fe(III) oxyhydroxide and H_2 added. Filled symbols represent addition of yeast extract, while open symbols are without yeast extract addition. Data points represent the average of at least duplicate microcosms, and error bars represent standard deviations (too small to appear in most cases).



increase in the MR-1 population of approximately 2.5 times; without yeast extract, MR-1 numbers declined rapidly, as in non-sterile terrestrial sediments with or without H_2 added.

We also added an electron donor/carbon source that was more defined than yeast extract. Addition of lactate stimulated growth and survival of MR-1 in non-sterile, Fe(III)-reducing sediments from both lake and terrestrial sources (Fig. 5). In sterile sediments, Fe(II) concentrations measured at 7.5 days were 1.5 to 2 times greater than concentrations observed under transition respiratory conditions (Table 1). In sterile aquatic sediments, Fe(II) was nearly as high as concentrations in non-sterile sediments (Table 1). In our experiments, formate was not suitable as an electron donor for MR-1 growth (Fig. 8), and it appeared to inhibit Fe(III) reduction by indigenous bacteria in non-sterile, Fe(III)-amended sediments [1.1±0.1 μmol Fe(II) produced with formate versus 9.1±0.3 μmol Fe(II) produced with no further amendments]. This also suggests that concentrations used were toxic or that MR-1 required adaptation with formate prior to inoculation into sediments containing formate.

As expected, addition of either AQDS (Fig. 6) or NTA (Fig. 7) was followed by an increase in MR-1 populations of 10- to 1000-fold in both non-sterile and sterile sediments. The increase was more pronounced where AQDS was added to sediments. In sterile sediments with AQDS added, Fe(II) produced was approximately half the concentration produced by non-sterile sediments (Table 1). Fe(II) produced in sediments amended with NTA was approximately the same in both non-sterile and sterilized sediments.

A second sampling and incubations with MR-1 (using landfill sediment collected in March 2005) that were amended with Fe(III) oxyhydroxide and either nothing further,

Figure 5. Growth of MR-1 Rf^r Sm^r in terrestrial sediments (circle symbols) and aquatic sediments (square symbols) amended with lactate and Fe(III) oxyhydroxide. Filled symbols represent non-sterile sediments, while open symbols are sediments that were sterilized prior to inoculation by MR-1. Data points represent the average of at least duplicate microcosms, and error bars represent standard deviations (too small to appear in most cases).

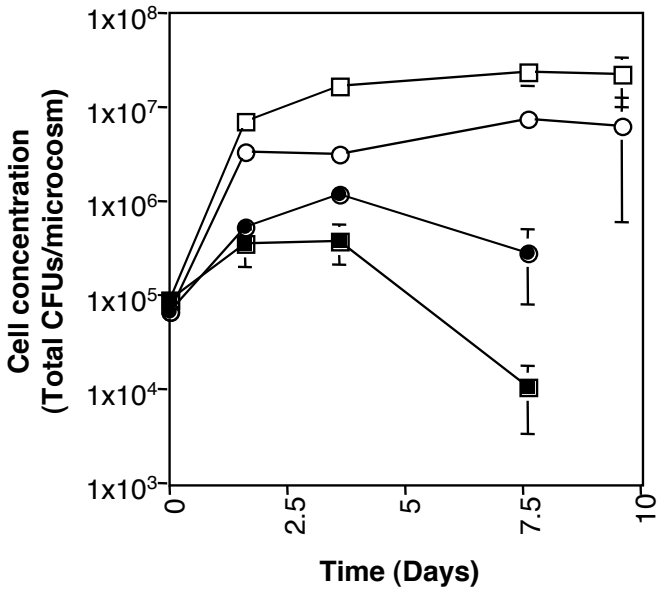


Figure 6. Growth of MR-1 Rf^r Sm^r in terrestrial sediments (circle symbols) and aquatic sediments (square symbols) amended with lactate, Fe(III) oxyhydroxide, and AQDS. Filled symbols represent non-sterile sediments, while open symbols are sediments that were sterilized prior to inoculation by MR-1. Data points represent the average of at least duplicate microcosms, and error bars represent standard deviations (too small to appear in most cases).

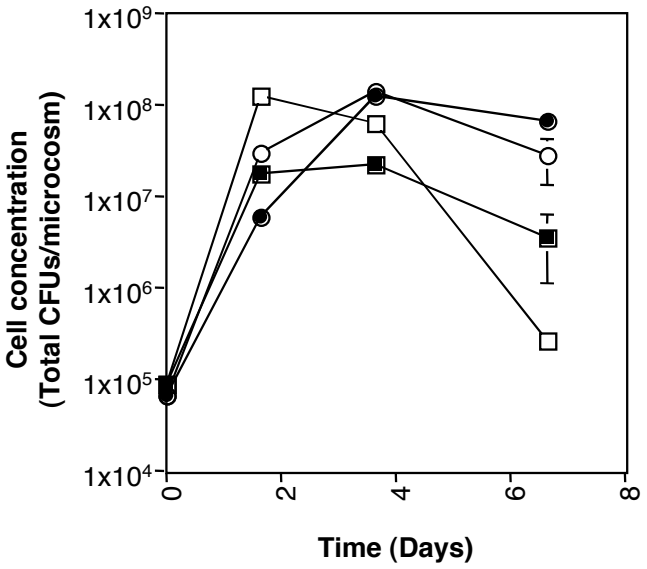
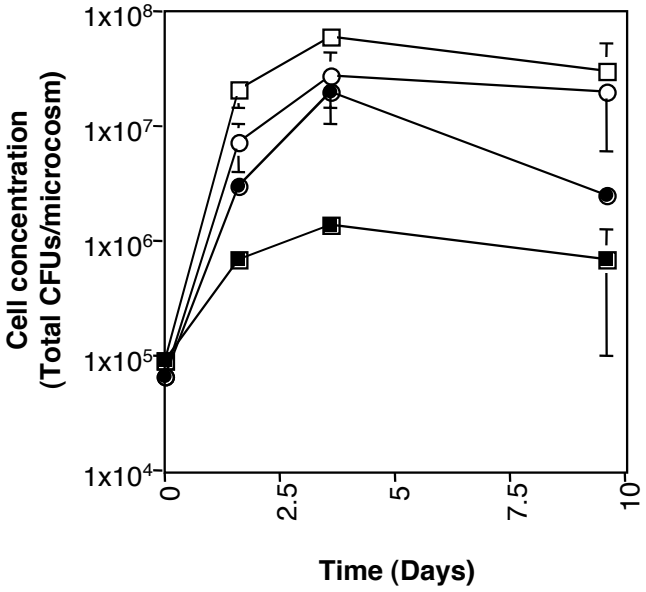


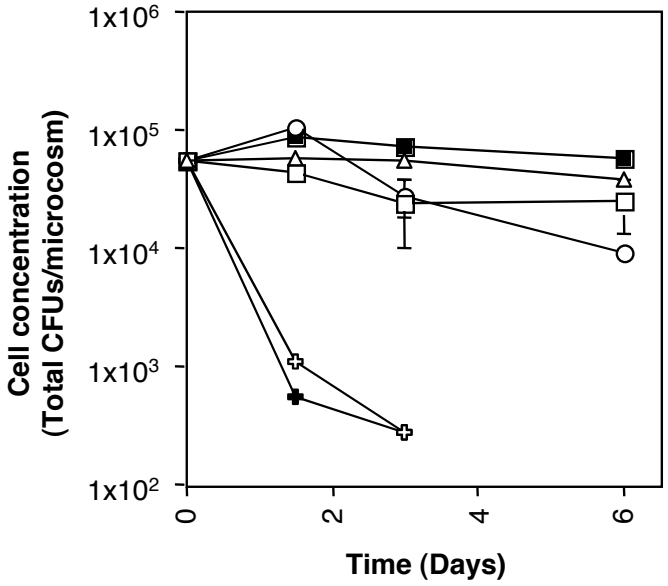
Figure 7. Growth of MR-1 Rf^r Sm^r in terrestrial sediments (circle symbols) and aquatic sediments (square symbols) amended with lactate, Fe(III) oxyhydroxide, and NTA. Filled symbols represent non-sterile sediments, while open symbols are sediments that were sterilized prior to inoculation by MR-1. Data points represent the average of at least duplicate microcosms, and error bars represent standard deviations (too small to appear in most cases).



lactate, or NTA plus lactate gave similar results in both total cell number and Fe(II) production as in experiments with sediment collected in June 2002 (data not shown). Using these sediments, we also examined survival of MR-1 in anoxic sediments with or without added Fe(III) oxyhydroxide and several single amendments (Fig. 8). In the absence of any amendments, MR-1 was able to persist near the inoculum concentration over the course of 6 days; lactate encouraged a slight increase in numbers, and NTA had some effect on growth and also on Fe (II) production when Fe(III) oxyhydroxide was added [17.3±0.5 μmol Fe(II) produced with NTA versus 9.1±0.3 μmol Fe(II) produced with no further amendments].

An explanation for why MR-1 could maintain numbers in unamended sediments but not with added Fe(III) includes cryptic growth and competition with indigenous Fe(III)-reducers. Without available Fe(III) oxyhydroxide or another electron acceptor, presumably indigenous microorganisms and MR-1 could maintain numbers at a certain level through cryptic growth, where starved bacteria die, releasing contents that can support the growth of survivors (Postgate, 1976). When Fe(III) oxyhydroxide was added to sediments, growth of indigenous Fe(III)-reducing bacteria was presumably stimulated; in this situation, MR-1 may be less competitive at using available electron donors, and/or Fe(III) may be too costly, energetically speaking, for MR-1 to compete successfully. Numerous studies have shown that *Shewanella* sequences cannot be detected by molecular techniques within Fe(III)-reducing sediments, whereas Fe(III)-reducing *Geobacter* species dominate these environments (Lovley *et al.*, 2004). Reasons proposed for the dominance of *Geobacter* species were its ability to use a common sediment

Figure 8. Growth of MR-1 Rf^r Sm^r in non-sterile, terrestrial sediments collected in March 2005. Microcosms contain no amendments (triangle symbols), lactate (circle symbols), NTA (square symbols), Fe(III) oxyhydroxide and NTA (filled square symbols), formate (open cross symbols), or Fe(III) oxyhydroxide and formate (filled cross symbols). Data points represent the average of at least duplicate microcosms, and error bars represent standard deviations (too small to appear in most cases).



electron donor (i.e., acetate) and the ability to reduce Fe(III) directly, rather than through the use of energetically costly endogenous electron shuttles (Lovley *et al*., 2004).

Discussion

Of interest to environmental and regulatory agencies is MR-1's ability to use harmful substances as electron acceptors. MR-1 can reduce U(VI) and Tc(VII) to less soluble, and therefore, less toxic forms (Lloyd *et al*., 2002). Because of the utility of MR-1 as a model organism for understanding metal transformations, we tested MR-1's ability to compete with indigenous organisms in sediment microcosms simulating various growth conditions with the goal of further understanding bacterial interactions in sediments.

In all cases where sterile and non-sterile sediments were compared, MR-1 grew substantially better in sterilized sediments, indicating that competition could be one factor limiting growth in sediments. In sterile sediments, it is likely that the indigenous microorganisms that were killed could also serve as electron donor/carbon sources for MR-1 (Postgate, 1976). Blumenroth *et al*. (Blumenroth and Wagner-Dobler, 1998) also found that nonindigenous strains of *Pseudomonas* could survive for up to 70 days at high cell densities (10^7 to 10^9 cells/ml) when inoculated into sterilized sediments, while in non-sterile sediments, inoculants decreased in number immediately after inoculation; however, introduced bacteria will not always grow in sterilized sediment. While some species (e.g., *Sinorhizobium meliloti* and *Agrobacterium tumefaciens*) tested in one study grew at least 100-fold in number in sterile sediments, other species (e.g., *Pseudomonas* sp. 1G and 2K and *Bacillus subtilis*) grew very little or not at all (Acea *et al*., 1988). Such observed differences could be specific to bacterial strain, sediment type, or

sterilization technique. Some studies autoclaved sediments (Blumenroth and Wagner-Dobler, 1998), as we did, whereas others sterilized soil with irradiation (Acea *et al.*, 1988).

It is interesting to note that in our experiments, we found that the condition with no amendments allowed MR-1 to maintain initial inoculum levels. While MR-1 did not increase in number under this condition, it did at least persist well in comparison to other studies where exogenous bacteria were introduced into sediments without amendments and immediately declined in number (Scheuerman *et al.*, 1988; Scanferlato *et al.*, 1989). Whereas climax communities are assumed to resist invasion by exogenous bacteria (Alexander, 1971; Liang *et al.*, 1982), MR-1 survived for at least 6 days when inoculated into non-sterile sediments with no amendments.

Under anaerobic, Fe(III)-reducing conditions, we added some air initially to simulate the transition from oxic to anoxic conditions, a likely scenario encountered by this organism in the lake sediments from which it was isolated (Myers and Nealson, 1988). In these experiments, there was no effect of the added oxygen as measured by growth of MR-1; however, when compared to Fe(III)-only conditions, where MR-1 numbers decreased immediately, the air that was added helped MR-1 to survive in Fe(III)-reducing terrestrial sediments, corroborating the idea that MR-1 survives best at oxic-anoxic interfaces.

From the experiments in aerobic and transition conditions, we hypothesized that the sediments may have lacked available electron donors for growth. To ameliorate this problem, we first added the same concentration of H_2 that stimulated growth of *Desulfovibrio* strains introduced into sulfate-reducing microcosms in other studies within

our laboratory (reference Groh *et al.*, 2005 and unpublished data, Luo and Krumholz). Whereas H_2 did not stimulate growth of MR-1 in non-sterile, Fe(III)-amended sediments, the native populations in these sediments readily reduced Fe(III) whether or not H_2 was present, indicating that those bacteria could use added H_2 or endogenous electron donors. It appears that MR-1 did not use these unknown electron donors or does not use them as efficiently as the native population does. Additionally, an acceptable carbon source may have been absent from the sediments, or if present, was not in concentrations sufficient for MR-1 to compete with the natural population.

Since the ability of MR-1 to compete in natural systems may be directly related to its ability to compete for electron donors, we also tested a well-defined electron donor/carbon source. Lactate has been used as an electron donor in many studies with *Shewanella* species (Fredrickson *et al.*, 1998; Zachara *et al.*, 1998; Zachara *et al.*, 2002). Herein, the addition of lactate stimulated growth and survival of MR-1 in non-sterile sediments from both lake and subsurface sources. It is interesting that with lactate added, the margin narrowed between Fe(II) concentrations measured in sterilized versus non-sterile sediments, and Fe(II) produced in non-sterile sediments with lactate was less than without lactate. This suggests that MR-1 was adapted to using lactate as an electron donor, while the native population either did not use lactate and/or was inhibited by either lactate concentrations employed or by the acetate produced (Steiner and Sauer, 2003). Addition of an appropriate electron donor has been shown to enhance growth and survival of *Pseudomonas* strains when introduced into sediments (Devleigher *et al.*, 1995; Blumenroth and Wagner-Dobler, 1998). Recall also that MR-1 was grown with lactate prior to all sediment inoculations and would be adapted to this substrate. In a

previous study, *Pseudomonas* inoculants that had been adapted to detergent increased by nearly a log unit in detergent-amended sediments, whereas nonadapted inoculants began to die off immediately (Devleigher *et al.*, 1995).

One other consideration, aside from MR-1's need for an electron donor/carbon source, was that the insoluble Fe(III) oxide may have been too costly, energetically speaking, for MR-1 to access, and the sediments may have lacked potential electron shuttles to aid MR-1 in Fe(III) reduction. As mentioned previously, some bacteria, such as *Geobacter* species, may be better suited physiologically for Fe(III) reduction in environments of high competition; while *Geobacter* species reduce Fe(III) oxides by direct contact, *Shewanella* species produce yet unidentified electron shuttles/chelators that are metabolically expensive and may be lost in the environment through diffusion (Lovley *et al.*, 2004). Therefore, we tested Fe(III)-reducing sediments under three conditions: with an Fe(III) chelator alone, with lactate and a synthetic electron shuttle, or with lactate and an Fe(III) chelator. For the shuttle and chelator, respectively, we chose the humic acid analogue, AQDS (Tratnyek and Macalady, 1989), and the Fe(III) chelator, NTA. It is likely that AQDS served to shuttle electrons from MR-1 to the ferrihydrite (Lovley *et al.*, 1998), and NTA makes the Fe(III) more bioavailable by bringing it into solution (Dobbin *et al.*, 1996). The addition of the humic analogue AQDS and the chelator NTA led to substantial increases in MR-1 populations in both non-sterile and sterile sediments (three orders of magnitude increase for AQDS), and the increase was greater than when either lactate or NTA was added alone.

In the absence of electron donor/carbon source or electron shuttles under Fe(III)-reducing conditions, MR-1 may also have lost numbers due to predation, competition, or

phage activity. An immediate decline in numbers of introduced bacteria has been attributed to grazing by protozoa in lake water (Scheuerman *et al.*, 1988; Gurjala and Alexander, 1990). Protozoa (Danso *et al.*, 1975) and antibiotic-producing or lytic microorganisms (Acea *et al.*, 1988) have also been implicated in the decline of bacteria that are introduced into soil. As increased growth rate is one defense against over-grazing by protozoa (Gurjala and Alexander, 1990; Heijnen and van Veen, 1991), microcosm amendments that allowed MR-1 to grow could have prevented substantial population loss by predation. Phage are also known to negatively impact bacterial population size in aquatic (Fuhrman, 1999) and sedimentary environments (Reanney and Marsh, 1973; Keel *et al.*, 2002; Ashelford *et al.*, 2003). Another study suggested that the date of sediment sampling could influence reductions in numbers of introduced bacteria (Blumenroth and Wagner-Dobler, 1998), but we found that time of sampling (June 2002 versus March 2005) did not affect rates of decline of MR-1 populations, but sediment source (terrestrial versus aquatic) did.

One other consideration for decreased numbers in Fe(III)-reducing sediments could be Fe(III) toxicity. We feel that this is unlikely, however, as Fe(III) was considered non-toxic to *P. putida* in one study where high concentrations of chelated Fe(III) (200-260 mM) were required for growth inhibition (Ruggiero *et al.*, 2005). The Fe(III) oxyhydroxide used in our study would be less bioavailable, and therefore less toxic, than in the presence of a chelator. Also, most studies have focused on cytotoxicity of Fe(II), rather than of Fe(III) (Braun, 1997); however, a recent study did find that Fe(III) was toxic to *Salmonella enterica*, *Escherichia coli*, and *Klebsiella pneumoniae* mutants defective in the Fe(III)-responding PmrA/PmrB signal transduction system

(Chamnongpol *et al.*, 2002). In conclusion, however, we believe that the best explanation for the decline of MR-1 numbers in Fe(III)-reducing sediments is from competition with better suited bacteria, such as *Geobacter*, and that electron donors that select for MR-1 (e.g., lactate) can overcome this handicap.

Experimental Procedures

Growth of MR-1 cultures and inoculation into sediment microcosms. *S. oneidensis* MR-1 with spontaneous streptomycin and rifampicin resistance (MR-1 Rf^r Sm^r) was maintained aerobically on standard Luria Broth (LB) medium and was grown anaerobically with a modified lactate-sulfate (LS) medium (Rapp and Wall, 1987) containing 25 mM Fe(III)-citrate in place of sulfate, 50 mM lactate, no bicarbonate or cysteine, and a final pH of 7. This anaerobic Fe(III)-citrate lactate medium was prepared with a N_2 headspace using the techniques of Hungate (Hungate, 1969), as modified by Balch *et al.* (Balch *et al.*, 1979). Vitamin and mineral solutions were added as described previously (Steger *et al.*, 2002), and incubation of MR-1 in all media was conducted at 30°C. A minimal lactate medium was also used for aerobic growth where indicated [amended LS medium without Fe(III)-citrate].

Prior to sediment inoculation, mid-log-phase cells of MR-1 Rf^r Sm^r grown aerobically without shaking in LB medium were transferred into aerobic lactate medium and then through anaerobic Fe(III)-citrate lactate medium. When the brown coloration of the Fe(III)-citrate lactate medium began to clear, indicating that Fe(III) was being utilized and that cells were near the mid-log growth phase (data not shown), cultures were harvested and centrifuged (10,000 X *g*, 10 min) within an anaerobic chamber. The

resulting pellet was washed three times in modified LS anoxic minimal medium buffered with 8.3 mM bicarbonate without addition of the Fe(III), vitamins, trace metals and electron donor. Additionally, 7 mM NaCl and 2 mM $MgCl_2$ replaced Na_2SO_4 and $MgSO_4$, respectively (so as not to stimulate the sulfate-reducing population); KH_2PO_4 was increased to 3.7 mM; NH_4Cl was reduced to 7.5 mM; and $CaCl_2$ was dropped to 0.34 mM to avoid precipitation. After the final wash, cells were resuspended in 1 ml of wash medium, and 100 μl was used as inoculum for sediment microcosms, producing a total concentration of approximately 10^5 to 10^6 cells/microcosm.

Sediment microcosm incubations. Microcosms consisted of 25-ml serum bottles and either 2 g sulfidogenic subsurface sediments [(collected in June 2002, unless indicated otherwise, from sediments underlying a landfill in Norman, OK, a site characterized in previous studies (Beeman and Suflita, 1987, 1990)] or 2 g near-shore lake sediments (collected in June 2002 from Lake Thunderbird, Norman, OK). For aerobic microcosms, sterile serum bottles were capped with aluminum foil after sediment and cells had been added. For anaerobic incubations, bottles were flushed with N_2-CO_2 (4:1), as CO_2 is required to maintain a neutral pH in this system. Amendments were added to obtain the following concentrations in sediments: 500 μg yeast extract, 20 μmol lactate or formate, 200 μmol Fe(III) oxyhydroxide and 4 μmol AQDS or NTA. All amendments were added from anoxic, liquid stock solutions. Amorphous Fe(III) oxyhydroxide was prepared as described previously (Lovley and Phillips, 1986a). For addition of gases to the headspace, 5 ml of H_2 or 2.5 ml of air were added through sterile 0.2 μm syringe filters. For sterile sediments, microcosms were autoclaved twice for 20 min, with a one-day incubation in between treatments, to allow spores to germinate prior

to addition of amendments and MR-1 Rf^r Sm^r.

Recovery of MR-1 from sediment microcosms. For extraction of cells from microcosms, anoxic minimal medium buffer (4 ml) was added to the bottles and they were shaken for 15 minutes. From the 4 ml of buffer, 1 ml was removed, diluted as required in the same buffer, and plated aerobically onto LB plates with 300 μg/ml streptomycin. Plates were incubated at 30°C for approximately 24 hours. To assess levels of Fe(III) reduction, the liquid remaining in the bottles (approximately 3 ml) was acidified by addition of 250 μl 6N HCl and shaken at room temperature for 30 min. Fe(II) was measured from 0.5 ml acidified sample in each bottle using the ferrozine assay (Lovley and Phillips, 1986a, 1986b).

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Chapter 2

A method adapting microarray technology for signature-tagged mutagenesis of *Desulfovibrio desulfuricans* G20 and *Shewanella oneidensis* MR-1 in anaerobic sediment survival experiments

Abstract

Signature-tagged mutagenesis (STM) is a powerful technique that can be used to identify genes expressed by bacteria during exposure to conditions in their natural environments. To date, there have been no reports of studies in which this approach was used to study organisms of environmental, rather than pathogenic, significance. We used a mini-Tn*10* transposon-bearing plasmid, pBSL180, that efficiently and randomly mutagenized *Desulfovibrio desulfuricans* G20 in addition to *Shewanella oneidensis* MR-1. Using these organisms as model sediment-dwelling anaerobic bacteria, we developed a new screening system, modified from former STM procedures, to identify genes that are critical for sediment survival. The screening system uses microarray technology to visualize tags from input and output pools, allowing us to identify those lost during sediment incubations. While the majority of data on survival genes identified will be presented in future papers, we report here on chemotaxis-related genes identified by our STM method in both bacteria in order to validate our method. This system may be applicable to the study of numerous environmental bacteria, allowing us to identify functions and roles of survival genes in various habitats.

Introduction

Microbial survival in a habitat is subject to both biotic and abiotic influences and ultimately depends on the organism's ability to respond to prevailing environmental stresses. This response is believed to involve expression of genes that confer specific "stress response" capabilities on the cells. These functions can involve a variety of biochemical or structural features useful for the microorganism's survival. For example, sediment-dwelling microorganisms can detoxify their surroundings by reducing certain metals to less soluble forms (13, 29, 31, 53). With our current knowledge of the importance of gene expression in response to environmental factors, many essential biological events that occur in situ are most likely missed during studies of laboratory cultures. As well, there has been no way to prove that cell functions observed in the laboratory are important for the organism when it is growing in the natural environment. Recognition of this problem has been pronounced in many fields, but significant progress in addressing this issue has only been made in studies of bacterial pathogens and some commensal organisms (16). This work has allowed for studies of bacterial genes as the organisms are growing within the host. One of the most widely used techniques for identification of genes directly involved with in situ survival of the organism is signature-tagged mutagenesis (STM).

STM requires the generation of oligonucleotide-tagged mutants, the incubation of these mutants in the natural environment, and finally, identification of non-surviving mutants by observing the loss of their corresponding tags using a hybridization approach. The first STM study demonstrated the potential for identifying genes essential for survival of *Salmonella enterica* serovar Typhimurium in the infected host model (mouse) (19). Since this seminal article, increasing numbers of reports appear each year that have

further exploited this approach for studying the in vivo survival of pathogenic microorganisms (8, 14, 16, 26, 27, 34) and commensal bacteria (18, 21). Although the STM procedure has undergone many refinements during these studies (reviewed in reference 26), all reinforce the belief that STM is a powerful approach for screening the genomes of microorganisms for genes that enable bacteria to survive in their natural habitats.

In order to adapt this technique to study bacteria of environmental significance, we chose two model organisms. These are representative of sediment-dwelling anaerobic bacteria that carry out important functions in their environments. Sulfate-reducing bacteria, such as *Desulfovibrio desulfuricans* G20, are involved in the reductive arm of the sulfur cycle and play critical roles in degrading organic compounds in sulfate and organic-rich environments (15, 42). *Shewanella oneidensis* MR-1 is from a genus shown to be abundant in a variety of sedimentary environments, and *Shewanella* species have been used as models for studying Fe(III) (40) and radionuclide [U(VI) and Tc(VII)] transformations (13, 29). With these organisms, difficulty in achieving high efficiency and random transposition with transposon systems similar to those used in prior STM or general mutagenesis studies led us to screen a number of transposon-containing vectors. The availability of microarray technology led us to modify the screening process of the STM procedure, enabling us to mass produce microarray slides with printed tags. The microarray system also enabled us to downsize equipment necessary for carrying out hybridizations. This modified approach to STM allowed us to identify genes critical to the function of organisms of environmental significance, such as anaerobic, sediment-dwelling bacteria.

Materials and Methods

Strains and media. A general outline of the entire STM procedure appears in Fig. 1. A list of strains and plasmids used in this study is given in Table 1. Strain G20 was grown in lactate-sulfate (LS) medium prepared as described by Rapp and Wall (44), using N_2 headspace and vitamin and metal solutions as described elsewhere (50). Prior to autoclaving, the pH was adjusted to 7.2, and after autoclaving, 8 mM bicarbonate and 0.025% cysteine were added from anaerobic stock solutions. For growth of G20 on solid media, LS agar medium (1.5% agar) was prepared, with the addition of 0.005% $PdCl_2$ (rather than cysteine), a catalyst for reduction of the medium by H_2 (contained within the anaerobic chamber). These plates were poured on the bench, and following solidification, the oxidized plates (pink from resazurin redox indicator) were dried overnight in a laminar flow hood and then moved into the anaerobic chamber and reduced overnight. *S. oneidensis* MR-1 was maintained aerobically on standard Luria broth (LB) medium and grown anaerobically with a modified LS medium [25 mM Fe(III)-citrate in place of sulfate, 50 mM lactate, no bicarbonate or cysteine, pH 7]. A minimal lactate medium was also used for aerobic growth where indicated [amended LS medium with Fe(III)-citrate omitted]. For selection of transposon mutants, 175 μg/ml kanamycin was added to agar plates prepared as described above for G20 and 50 μg/ml kanamycin was added to LB plates for MR-1. *Escherichia coli* strains were cultured in LB supplemented with appropriate antibiotics. Additionally, strain β-2155 required 0.05% diaminopimelic acid (DAP) for growth with LB medium. G20 and *E. coli* strains were grown at 37°C, while MR-1 was grown at 30°C. A period of three days was

Figure 1. Overview of STM for G20 and MR-1 sediment survival studies. Although 96 tags were originally designed and moved into *E. coli* β-2155 on pBSL180, only 60 were used for conjugation with G20 and MR-1. This model therefore shows assembly of 60 G20 and MR-1 mutants in each one of 96 "mutant pools."

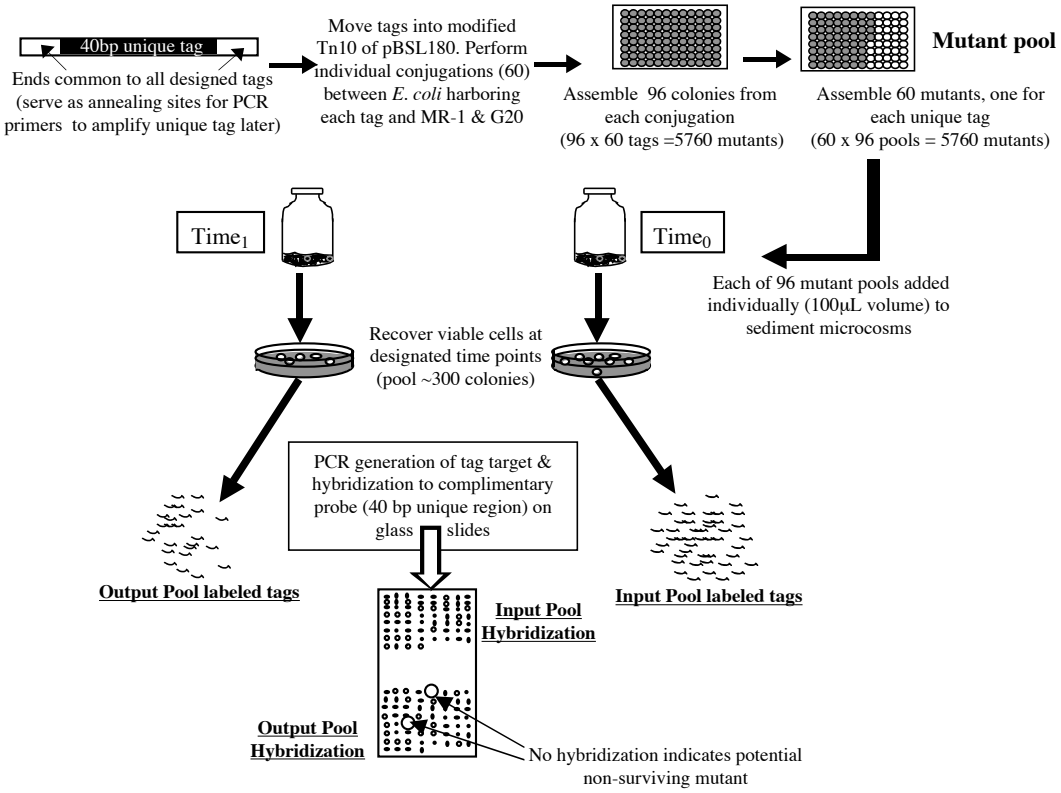


Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant properties[a]	Source or reference
Strains		
Desulfovibrio		
G20	Spontaneous nalidixic-acid resistant G100A (G200) cured of native pBG1	55
$G20_{sediment}$	G20 passed through sediment and selected on nalidixic acid	This study
S. oneidensis		
MR-1	Wild type	38
MR-1 Rf^r Sm^r	Spontaneous Rf^r Sm^r derivative	This study
$MR\text{-}1_{sediment}$	MR-1 Rf^r Sm^r passed through sediments amended with iron oxyhydroxide gel and lactate and selected on streptomycin	This study
E. coli β-2155	K-12 derivative; F'*tra*D36*lacI*^{q} Δ(*lac*Z)M15 *proA*$^+$*B*$^+$/*thr-1004 pro thi strA hsdS* Δ(*lacZ*)M15 Δ*dapA::erm pir::*RP4(::*kan* from SM10)	20
Plasmids		
pRK2096	Tn*7* derivative transposon with Km^r	54
pRK2073	Helper plasmid with Sp^r	24
pRL1058a	Tn*5* transposon with Km^r	57
pQL1058a	CytC promotor inserted in front of Km^r gene in Tn*5* transposon of pRL1058a[b]	This study
pTnMod-RKm	Mini-Tn*5* transposon with Km^r	10
pTnMod-OGm	Mini-Tn*5* transposon with Gm^r	10
pMycoMar	Mariner-based transposon with Km^r	45
pBSL180	Mobilizable suicide vector; modified Tn*10* with Km^r	2

[a]Antibiotic abbreviations: Rf, rifampicin; Sm, streptomycin; Km, kanamycin; Sp, spectinomycin; Gm, gentamycin.

[b]Two PCR primers to regions within pRL1058a transposon were synthesized, each with one half of the *cytC* promotor region from *D. vulgaris* as a tail. Following PCR (to amplify the entire pRL1058a plasmid with the *cytC* promoter inserted) using *Pfu* Turbo DNA polymerase (Stratagene), PCR products were ligated to form pQL1058a. The primers were forward primer 5'TCCCGCTTGGGAAATCCTTAACTTACCTTTGTGAAGGAGGTAGTTCGATCATGATGATTGAACAAGATGGATT3' and reverse primer 5'TGGTATTGTGTCCGCCATGCCGTGTCAAGGAATGGAGCGGGAAAGCCTAGGCGAAACGATCCTCATCCTG3'.

necessary for colonies of G20 to appear on solid media, while 18 h was sufficient to generate 1- to 2-mm-diameter colonies of MR-1.

For construction of our tagged-transposon mutant libraries, we used strains of G20 and MR-1 that had been adapted to sediment conditions. In order to generate these strains, G20 and MR-1 Rf^r Sm^r were incubated individually in sediment microcosms as described in greater detail below (see "Initial sediment survival experiments") and subsequently reisolated at time of peak growth (8 to 9 days for G20 and 3 days for MR-1) based on nalidixic acid (200 μg/ml) resistance for G20 and streptomycin (300 μg/ml) resistance for MR-1. By utilizing such strains (now termed $G20_{sediment}$ and $MR\text{-}1_{sediment}$) that have adjusted to sediment conditions, we hoped to avoid problems with adaptation to environmental conditions and the possibility that important functions may have been suppressed during repeated transfer in laboratory media.

Plasmids were provided by Dianne Newman (pBSL180), Judy Wall (pRK2096, pRK2073, pRL1058a) and Gerben Zylstra (pTnMod-RKm, pTnMod-OGm).

Tag design and screening for cross-hybridization among tags. Based upon tag sequences in a previously described STM system (19), we designed 96 single-stranded DNA tags, each with a 40-nucleotide (nt) variable region flanked by constant arms common to all tags: 5'-CTAGGTACCTACAACCTCAAGCTT-$[NK]_{20}$-AAGCTTGGTTAGAATGGGTACCATG-3', where $[NK]_{20}$ represents the 40-nt variable region; N is A, C, G or T; K is G or T. This design prevented KpnI sites, necessary for later cloning steps, from being incorporated into the tag sequence. Following the individual synthesis of 89-nt single-stranded tags (Integrated DNA Technologies, Inc., Coralville, Iowa), all 96 tags were individually amplified in PCRs using primers P3 and

P5 (homologous to the common arms of all tags) (19), in order to create double-stranded tags for cloning into pBSL180. Primers P2 and P4 (19), internal to primers P3 and P5, were used in later steps described below to PCR amplify the unique region of tags prior to hybridization. Double-stranded tags that had been amplified by primers P3 and P5 were individually digested with KpnI (restriction sites present in the common arms) and ligated individually into KpnI-digested pBSL180. Competent cells of *E. coli* strain CC118 were transformed with the ligation product by using standard electroporation methods. Plasmid isolated with QIAprep spin miniprep kit (Qiagen) from transformed CC118 cells was then used to electroporate *E. coli* strain β–2155.

We screened all 96 tags for cross hybridization prior to mutagenesis of $G20_{sediment}$ and $MR\text{-}1_{sediment}$ with each tagged form of pBSL180. Tagged pBSL180 was first isolated from individual *E. coli* β–2155. We PCR amplified and then labeled tags with Cy5 dye as described in "Colony PCR" and "Target preparation" below, except that here we started with purified plasmid for PCR template and not colonies on agar medium. The 96 tags (approximately 100 ng each) were individually PCR amplified in a 96-well plate. We then pooled well contents (6.25 μl of the 50 μl PCR reaction) from each column (8 tags per column; 12 columns) and each row (12 tags per row; 8 rows) in order to perform 20 separate labeling reactions for 20 hybridizations to microarray slides. Prior to and following the labeling reaction, DNAs from pooled columns and rows were cleaned with Amersham Microspin G-25 columns. Slides were printed with the 40-bp unique region from the 96 tags (as described below), and hybridization of labeled tags and wash conditions were as described in "Hybridization and washing procedures" below. Based on results discussed further below, we eliminated 36 tags from the study due to cross-

hybridization. We continued with the 60 remaining tags (sequences of the 40-nt unique region are found in Table 2).

Generation of mutant libraries. Conjugations were carried out by picking a single colony and transferring to either LB (strain MR-$1_{sediment}$ and each *E. coli* β-2155 containing a uniquely tagged pBSL180) or LS media (strain $G20_{sediment}$). The following manipulations were carried out aerobically with MR-1 or in an anaerobic glovebox (Coy Laboratory Products, Grass Lake, MI) for G20. G20 and MR-1 were grown for 10 to 20 h. *E. coli* β-2155 with one unique tag in pBSL180 was grown overnight, diluted 1:10 in LB without antibiotic selection and grown until the optical density at 600 nm (OD_{600}) was approximately equal to that of the recipient organism (0.5 to 1.0). Volumes of *E. coli* and recipient were centrifuged together (6,000 X *g*, 10 min) at a 1:1 or 1:2 ratio and then resuspended in 100 μl of spent medium from the recipient strain. This mating mixture was placed onto a 0.22-μm filter in the center of an LB-DAP plate for 3 h at 30°C (for MR-1) or an LS plate for 6 h at 37°C (for G20). Cells were washed from the filter by immersion into 1 ml of the medium favored by the recipient (no DAP) in 13- by 100-mm glass tubes. Strain MR-$1_{sediment}$ transposon mutants were washed from the filter and then immediately plated onto LB plates supplemented with kanamycin (no DAP), while strain $G20_{sediment}$ transposon mutants were recovered in liquid LS medium with 200 μg/ml nalidixic acid for 5 h prior to plating onto LS medium with kanamycin. Nalidixic acid was included in LS medium during the recovery in order to kill *E. coli* β-2155, which could grow on LS medium even without addition of DAP. This conjugation procedure was repeated for each of the remaining 59 β-2155 strains harboring a uniquely tagged form of pBSL180.

Table 2. Sequences for 40-nt unique region of each tag

Tag name	Sequence (5' to 3')
A1	ATGTCGTTCGATTGCGAGCTTTCGTGAGCTATATCGCTAG
A2	TGCTATAGCTATTGCGCGTTCGTTGGGTCGTGGCTGAGAG
A3	TTAGCTGGTGAGAGATCTAGACTAGTTGAGCTGGCTATCG
A4	GGATTTCGGGCTTGCTATGGAGCTATGGCTTGCGTTAGCT
A5	GTAGCTTGCGCTAGCGTTCTGGCTTGCGTGAGTTCTAGTG
A6	GGCTTGCGTTCGAGCTTTGGATCTTGAGCTCTAGTTCTCG
A7	GTGGCTAGTTATCGTTAGCGATCTTGATCGAGTTAGTTGG
A8	GGTGCTATTGGGTTATGTGTCGATTGGGTTGGATGTGTGG
A9	GTCGTTAGCGTGCTTGAGCTGTTGGTTGGTAGATTGTGCG
A10	GGCTGGATTGCTAGTTCGTTAGGTCTAGGTTGCTAGGTCG
A12	GTCTAGCTTTGGCTAGGGTGATCGTTGGTTCGTGATTGAG
B1	GGCTGGAGTGCTTGCTTGATGGCTTGAGTTCGCGTTGGGT
B2	TGCTTGATCGGTTGCTAGCTCTCTGGTTCGGTTCTAGCTG
B4	TGCTCGTTGTCGATTGAGCTGGCTTGCGCGGTCTCGATTT
B7	GTCGATCGTGATGTCTGGTGATCGATTTCGATAGCTAGTG
B8	TGGTCTAGCTCGATCTAGCGTTGTATCGAGCTAGCTAGCG
B9	GTATATCTAGATTGCTCTATCGTGATAGCGATCGCTAGTG
B10	TTAGATCTCTGGCTATCGAGATGGCTAGCTAGTGTGGTTT
B11	GGAGCTTTATCGTGATGTCGTTGGATATGGGTCTGTCGAG
B12	TTCGATGTCTAGTTATCTGGCGTTGGATCTCGAGCTTGCT
C5	AGATAGTTCGCTCTTGATCGGGAGCTAGCTATTTATTTAG
C6	ATCGTGATCGATAGTTGGATCGGTCGCTATTTAGTGCGTT
C7	CTTGAGCTATCTATGGGTCTAGCTCTAGGTCGAGTTCGAT
C8	CGTTAGCGCTCTAGATATCGAGTTTGATAGCTAGATCTCT
C10	TTCTTGAGCTCTAGATAGATGTAGCTATGGATCTAGCTCT
C12	CTAGCGATAGGTAGGTCTTTAGATCGGTCTATCGTGCGCT
D2	ATTGCTTTAGCGGTGTATTTCGAGTTCTAGCGTGCTATAG
D3	AGAGATATCGTTAGCGATCTAGCTAGCTCGATCGGTATCG
D4	CTAGATCGTTCGTTAGGTCGTGCTATGGCTATCGTGCTAT
D5	ATTGCTTGAGCTAGAGCTATCGCTATAGCTAGCTATAGCG
D6	CTGTAGGCGGTTATCGATCGCTCGGTAGGTATCGCTTGAT
D7	TTCGTTCTATTGGGATCGTGCTCGCTTTAGCGAGCTAGCT
D8	TTCGATCTAGGGAGTTCGTTAGCGATGTCGATCTAGCGGT
D10	CTTGATTGAGCTCTGTAGCTAGCTCGATCTGTCTAGTGCT
D11	ATCTGTAGCTTTAGCGATCTATCGCTCTAGCGATATGTGT
D12	CGATCTGTCGGTCTAGATCGTTCGATCTAGCGGTCTATCG
E1	CTCTATCGTTGGCGCTATCTAGCGTTCTATCGTTCTATCG
E2	CGGTCTATCGATAGTTATCTCGCTAGGGTTCTAGCGTTAG
E10	GTCGTTAGTTAGGTCGCTGGCGATATCGCGTTCGCTTGCT
E11	TTATCGTTAGGTATCGTTCTAGATATCGGTAGGTATAGCG
E12	AGCTCGTTCGGTAGCGATCGATAGTTCTCGAGTTAGCGAT
F2	CTTTGTGGATCGGGTGGGTTGTCTATTTGTATGTCTATAT

G1	CGCTCGCGGGAGATATCGATAGCGGGATATAGAGCGGGTT
G2	ATTTCTTTGTTTGGGGAGGGTGGGTTGGATAGGGATTGGT
G4	GTTTGTTTCGGGCGGGCTTTATATATGGCGCGCGTTATAG
G5	CGCTTTCTTTAGGGAGGGCGCGCGCTATTTATTTAGAGAG
G6	AGATATATTTAGCGCGCGGGTTTTCGCGCTCTTTATATAG
G7	TGTGTGTTGGAGGGCTTTCTATAGAGCGATAGCTAGAGCT
G8	GGTTGGTTATATATATCTTTATTTCGCGCGGGATTTGGTT
G9	TTTTGGGGAGAGATATCTATCTCTCTTTGGTTGGTTAGAG
G10	GGTTGGTTGTGTATATAGGGATTTCGCGCGGGTTATATCG
G11	TTTGATATCGCGCGATTTCTTTGGTTGGTTAGAGAGATAT
G12	GGGTGGTTATATTTCGTTGGTTAGAGAGGGTTTTAGAGAT
H4	GTTGGTTGAGAGAGATCTCTCTGGTTATTTATTTGTGTTG
H5	ATTTGTTTCTTTCTCTCTATATAGAGAGATTTCTCTGTGT
H6	TTGGTTGGATATGTCGGGCGGGCTCTCTATTTGGGGTTGG
H7	TTGGAGAGATATTTTGGGTTCTTTCTGTTGTGATAGAGAG
H8	GGGGAGGGCGGGTGGGCGGGGGGGTTTTATATGGCGCTCT
H9	CGCTCGCTCGCTATATAGAGAGGGATTTCGCGGGCGATAG
H10	GTGTGGCGCGCTTTATCTATCTAGAGATATATTTGTTTCT

For each tagged pBSL180, we picked 96 random exconjugants to a 96-well plate. With 60 unique tags, we collected 5,760 mutants in total. Mutants were then reassembled so that one mutant (of 96) from each of the original 60 plates was moved to one other plate. The procedure was repeated 96 times so that each new plate contained a pool of 60 uniquely tagged mutants. We had 96 of these mutant pools to screen in sediment per organism (Fig.1). At this stage we also used a random sampling of mutants and standard Southern blotting procedures (3) to determine that single, random transposition events occurred in both G20 and MR-1.

Initial sediment survival experiments. Prior to creation of the transposon mutant libraries, initial sediment survival experiments were carried out for two purposes: to determine when to sacrifice sediment incubations for collection of output pools and to isolate sediment-adapted strains, as described previously. These initial sediment incubations with G20 and MR-1 Rf^r Sm^r were carried out in a manner similar to the mutant pool sediment incubations described below. The only differences were sacrificing replicate microcosms (two bottles per time point for G20 and three bottles per time point for MR-1) at various time points (instead of just at one time point for mutant pools) and selection of recovered cells with solid media containing nalidixic acid (G20) or streptomycin (MR-1). Size of inocula were approximately 10^5 to 10^6 cells for both G20 and MR-1.

With MR-1, we added 200 μmoles amorphous Fe(III) oxyhydroxide, prepared as described previously (33), in order to shift sediment from sulfate-reducing conditions to Fe(III)-reducing conditions (see "Screening of tagged mutants in natural sediments" below for a description of the sediment collected). Because previous work with STM

required some growth of virulent strains in the host (8), we amended MR-1 microcosms with 20 μmol lactate in order to stimulate at least a 10-fold increase in cell numbers. In similar microcosms without addition of lactate, we were unable to achieve this amount of growth (data not shown). To assess levels of Fe(III) reduction, cells (1 ml) were first sampled from sediment incubations (as described below), and then the liquid remaining in the bottles was acidified by addition of 250 μl 6N HCl and shaken at room temperature for 30 min. Fe(II) was measured from 0.5 ml acidified sample in each bottle, using the ferrozine assay (32, 33).

Screening of tagged mutants in natural sediments. Each mutant pool of G20 (60 uniquely tagged mutants) was grown in deep 96-well plates to late log phase (equivalent to OD_{600} in serum tubes of 0.7) with LS medium in an anaerobic glovebox. MR-1 pools were grown in shallow 96-well plates and transferred through LB, minimal lactate (aerobic) and finally modified LS medium (grown in anaerobic chamber). MR-1 pools were grown until the Fe(III)-citrate had cleared in most wells. Experiments were then continued independently for each organism but were maintained in an anaerobic glovebox. The contents of all 60 wells were then pooled. Pooled cells were washed three times in modified LS anoxic minimal media buffered with 8.3 mM bicarbonate without addition of the vitamins, trace metals and electron donor. For G20, Na_2SO_4 was omitted but the solution contained 8.4 mM $MgSO_4$. For MR-1, Fe(III) was omitted; 7 mM NaCl and 2 mM $MgCl_2$ replaced Na_2SO_4 and $MgSO_4$, respectively (so as not to stimulate sulfate-reducing population); KH_2PO_4 was increased to 3.7 mM; NH_4Cl was reduced to 7.5 mM; and $CaCl_2$ was dropped to 0.34 mM to avoid precipitation. Washed cells (100 μl) were inoculated (~10^5 cells) into 30-mL serum bottles containing 2 g sulfidogenic

subsurface sediments from a landfill in Norman, OK, a site characterized in previous studies (4, 5). The initial water content of these sediments ranges from 10% to 25% of the total weight. Bottles were then flushed with N_2-CO_2 (4:1), as CO_2 is required to maintain a neutral pH in this system. MR-1 microcosms were amended with 200 μmol amorphous Fe(III) oxyhydroxide and 20 μmol lactate. One bottle per mutant pool was sacrificed (as described below for output pool) within 3 h, for analysis of tags present in the input pool. A duplicate bottle for each mutant pool was incubated in the dark at room temperature for 8 to 9 days (G20) or 5 to 6 days (MR-1) and then sacrificed.

For extraction of cells, anoxic minimal medium buffer (4 ml) was added to the bottles and they were shaken by hand and vortexed for 15 min. Extracted cells were then diluted in the same buffer and plated onto LS plates with 175 μg/ml kanamycin in the anaerobic glovebox for G20 and aerobically onto LB plates containing 100 μg/ml kanamycin for MR-1 (increased from 50 μg/ml to decrease the background of kanamycin-resistant sediment organisms). Plates were incubated as described in "Strains and media" above.

Colony PCR. Plates from the dilution series were chosen so that one to two plates containing a total of about 300 colonies were used. Colonies were scraped from plates into the same mineral medium used for inoculation of microcosms, and cells were centrifuged for 1 min (12,000 X *g*). The pellet from pooled colonies was resuspended in 200 μl distilled water (dH_2O). A portion (50 μl) was removed to an Eppendorf tube and 1 ml dH_20 was added. The cells were then centrifuged 1 min, and the supernatant was removed. Washing was repeated four times and the final pellet was resuspended in 100 μl dH_2O. This was boiled 5 min, placed on ice for several minutes, and then centrifuged for 2 min. The supernatant (5 μl) was used in a 50-μl PCR reaction containing 1X PCR

buffer; 1.5 mM $MgCl_2$; 0.2 mM each dATP, dCTP, and dGTP; 0.12 mM dTTP; 80 μM aminoallyl-dUTP (Molecular Probes); 0.1 μM each of primers P2 and P4 (19); and 2.5 U Invitrogen Platinum Taq. PCR parameters were 94°C for 4 min, followed by 30 cycles (G20) or 25 cycles (MR-1) of 94°C for 30 sec, 50°C for 30 sec, and 72°C for 30 sec. All PCRs were performed using the GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler. Following amplification, the reaction mixture was purified with an Amersham Microspin G-25 column according to the instructions of the manufacturer. We compared genomic DNA extracted from cell pellets by using the Invitrogen Easy DNA kit as template in tag labeling PCR to that from the boiling extract procedure described above. In every case tested, results were identical (data not shown).

Target preparation. One vial of Cy5 Mono-Reactive Dye Pack (Amersham) was dissolved in 72 μl dimethyl sulfoxide. Aliquots (4.5 μl) were distributed into amber Eppendorf tubes and dried completely in a Labconoco Centrivap (60°C, 45 min). The purified PCR product containing aminoallyl-dUTP was also dried in this manner and resuspended in 6 μl of 0.1 M Na_2CO_3 solution (pH 9). This was added to the dried Cy5 dye and incubated for 1 h at room temperature in the dark. The reaction was stopped by addition of 3 μl of sodium acetate (3 M, pH 4.5) and 41 μl water, and unbound dye was removed with Microspin G-25 columns. Dye incorporation was quantified on a DU530 Life UV/visible spectrophotometer (Beckman Instruments). The labeled PCR product (target) was then dried completely as before and resuspended in 2.5 μl water, 50 μl Roche digoxigenin hybridization solution, and 2.5 μl salmon sperm DNA (Stratagene). Prior to hybridization, the target was heated to 65°C for 5 min and then placed on ice.

Preparation of microarray slides. The 40-nt unique region (Table 2) and its complimentary strand for each of 60 tags were synthesized separately (Invitrogen). Equivalent concentrations of the unique regions and their 40-nt compliments were mixed to create 60 solutions of approximately 3.3-μg/μl final concentration in 3X SSC (1X SSC is 0.15M NaCl plus 0.015 M sodium citrate). A small fraction of all of these solutions was spotted onto FMB Oligo Slides with poly-L-lysine surface chemistry (Full Moon BioSystems Inc.) using a Generation Array III Spotter (Molecular Dynamics), creating 60 spots of approximately 100 μm in diameter (probes). On each slide, the unique tags were printed in duplicate in order to check reproducibility within the hybridization. This duplicate set was also printed on both ends of the slide, enabling us to simultaneously compare input (hybridized on one half of the slide) and output pools (hybridized on the other half of the slide). Spotted slides were stored desiccated until ready for use within 2 months of preparation. Prior to use, slides were rehydrated briefly with steam, immediately dried on a heat block, UV cross-linked, and then blocked using a succinic anhydride blocking solution (http://omrf.ouhsc.edu/~frank/M_Slide_Blocking_Protocol.html).

To demonstrate that the probes were not washed from the slides during hybridizations or washes, we compared rehydrated and UV-cross-linked slides that were stained for 5 min at room temperature with SYTO 61 red-fluorescent nucleic acid stain (Molecular Probes; 1 μl in 50 μl Tris-EDTA buffer spread over spotted probes on each half of slides) either prior to or after hybridization (no labeled target applied with hybridization solution) and washing procedures described below. Slides were washed in Tris-EDTA buffer to remove the excess DNA stain, dried with 95% ethanol, and scanned at 650 nm

using GenePix Pro 5.1 from Axon Instruments to compare the intensities of each stained probe before and after hybridization/washing procedures.

Hybridization and washing procedures. Each slide was placed into a Corning hybridization chamber, and 22I*25 coverslips (Erie Scientific Company) were placed over each half of the slide (one to cover the input pool and one to cover the output pool). Approximately 20 to 25 μl (~100 pmol) of target was added beneath each coverslip and hybridization units were incubated in a waterbath at 37°C overnight. Prior to washing, slides were dipped in dH_2O to remove the coverslips. Following all washing procedures (see below), slides were dried under a gentle stream of nitrogen. Scanning (650 nm) and spot intensity analyses were performed using GenePix Pro 5.1 from Axon Instruments. Background was assessed locally and subtracted from each spot using the companion software. A negative response was set at any signal below 3 times the background level. This cutoff was based on the relative variability of the background.

For washing solutions, we tested a low-stringency (high-salt) procedure and a high-stringency (low-salt) procedure to determine which one resulted in fewer cross hybridizations among the original 96 tags. Comparisons were carried out by pooling columns and rows as described in "Tag design and screening for cross hybridization among tags" above. The low-stringency wash consisted of a series of washes (10 min each at room temperature) with 2X SSC-0.1% SDS, 1X SSC-0.1% SDS, 1X SSC, and 0.5X SSC. For the high-stringency wash (from the Full Moon Oligo Slides protocol), slides were washed in 0.2X SSC-0.2% SDS solution (prewarmed to 55°C) for 30 min on a shaker at room temperature. Slides were then removed from the first wash and dipped twice in 0.2X SSC (also prewarmed to 55°C), and then dipped three times in room

temperature dH_2O. Following analysis of spot intensities, the high-stringency wash was chosen for our STM method (see Results), as fewer tags exhibited cross hybridization with this protocol.

Following analysis of these hybridizations, we attempted to decrease cross-hybridizations further among 36 tags slated for elimination (out of the original 96 tags) by increasing the wash temperature of slides. Twenty pools of tags were prepared as described in the "Tag design and screening for cross hybridization among tags" above. Following hybridizations, replicate slides for each of the 20 pools were washed at 55°C, 60°C, and 65°C using the-high stringency wash solutions.

Confirmation of sediment mutants. Potential nonsurviving mutants were defined as having a signal in the input pool but no signal (below 3 times the background level) in the output pool when hybridizations were analyzed. For G20 studies, mutants were confirmed by following cell number of individual mutants in sediment incubations (carried out exactly as described in "Initial sediment survival experiments" above).

MR-1 mutants were confirmed by subsequent competition experiments in sediment microcosms with parental strain MR-$1_{sediment}$, as performed in previous STM studies (7, 22). These competition experiments were similar to mutant pool incubations, except that now only one transposon mutant and MR-$1_{sediment}$ were inoculated together in a 100-μl volume (~10^3 cells each) of the anoxic minimal medium buffer described above. The inoculum size was lowered from the concentration used in mutant pools (10^5 total cells) to reflect the approximate concentration of one tagged mutant among 59 other tagged mutants. Following extraction of replicate microcosms (in duplicate bottles) with anoxic minimal medium buffer at 0 and 5 days, surviving cells were plated onto LB plus

kanamycin (100 μg/ml) and LB plus streptomycin (300 μg/ml). The transposon mutant was enumerated by colony counts on kanamycin plates, while the number of recovered strain MR-$1_{sediment}$ CFU was the difference between kanamycin (mutant) and streptomycin (total) plate counts. These numbers were used to calculate a Competitive Index (CI), a measure of output ratio of mutant to parent strain/input ratio of mutant to parent strain (7, 22). Where deemed necessary, a CI was also determined in anoxic Fe(III)-citrate laboratory medium for growth of individual potential nonsurvivors competing with strain MR-$1_{sediment}$ (inoculated with ~10^6 to 10^7 cells each, from separate, mid-log-phase aerobic lactate medium cultures). The output ratio in this case was determined by total cell count taken when the brown coloration of Fe(III)-citrate medium was beginning to clear. In previous growth experiments, we monitored cell number (by plating onto LB medium) and Fe(II) accumulation (ferrozine assay described above) in this medium and determined that clearing occurred near mid-log phase of growth and as a result of Fe(III) reduction to Fe(II) (data not shown).

Arbitrary PCR and identification of interrupted genes. Arbitrary PCR was used to determine the DNA sequences of the sites of transposon insertion in confirmed sediment-impaired mutants and was modified from previous procedures (36, 41). Approximately 3 ml of overnight mutant culture was centrifuged and washed, and DNA was extracted using the colony PCR protocol described above. In all PCRs, Expand Long Template PCR system (Roche) was used according to the manufacturer's instructions. For the first round of PCR (25-μl reaction), 5 μl supernatant from the prepared pellet was used with primers Tn10ext (5'GTGTTCCGCTTCCTTTAGCAGC3') and Arb1 (41). Parameters were (i) 95°C for 5 min; (ii) 15 cycles of 95°C for 45 sec, 40°C for 45 sec, and 68°C for

1 min; and (iii) 20 cycles of 95°C for 45 sec, 45°C for 45 sec, and 68°C for 1 min. For the second round of PCR (50-µl reaction), 2 µl of first-round product was used with primers Tn10seq (5'GTCGACGGTATCGATAAGCTTG3') and Arb2 (41). Parameters were (i) 95°C for 5 min; (ii) 15 cycles of 95°C for 45 sec, 45°C for 45 sec, and 68°C for 1 min; and (iii) 15 cycles of 95°C for 45 sec, 50°C for 45 sec, and 68°C for 1 min. The resultant PCR product was purified with the PCR purification kit from Qiagen and sequenced directly using the Tn10seq primer. For identification of the interrupted gene, sequence obtained from arbitrary PCR was compared to the NCBI database using blastn. The genome sequence for MR-1 has been published (17), while annotation completed to date for G20 is available at http://www.ncbi.nlm.nih.gov/genomes/framik.cgi?db=genome&gi=5163.

Results

Transposition system for G20 and MR-1. Wall et al. (54) described several vectors suitable for transposon mutagenesis in *D. desulfuricans* G20. We mutagenized strain G20 and other strains of *Desulfovibrio* with several of these vectors, using both conjugation and electroporation procedures. Although we observed efficient transformation (Table 3) using the Tn*7*-based pRK2096, transposon insertion (as determined by Southern blot hybridization) was not random (data not shown). Subsequent electroporation experiments using Tn*5*-based pRL1058a resulted in low transformation efficiency (4 transformants/ug DNA). Additionally, Southern blot analysis of mutant chromosomal DNA showed only two different insertion patterns (among ten mutants tested) (data not shown). We attempted to improve the efficiency of pRL1058a transposition by inserting the *Desulfovibrio vulgaris* cytochrome *c* promoter in

front of the kanamycin resistance gene of the Tn*5* transposon. This promoter has been used to express different genes in *Desulfovibrio* and *E. coli* (6, 52). Although the transformation efficiency did increase slightly, this modification (plasmid designated as pQL1058a) did not result in random insertions. This was possibly due to transposon instability, since the transposase gene was encoded on the transposon (11).

We also tested the ability of several other vectors to mutagenize G20 using both electroporation and conjugation. This included pTnMod-RKm and pTnMod-OGm, two mini-Tn*5* plasmids that have transposase encoded outside the transposon (10), and pMycoMar, with a mariner transposon (1). In all cases, no transposon mutants were obtained with G20. One commercially available kit with Tn*5* and transposase mix (Epicentre) was also electroporated into G20, but the transformation efficiency was very low (Table 3).

For transposon mutagenesis of MR-1, we obtained a mini-Tn*10* plasmid, pBSL180 (Table 1), whose features include: relatively small size (6.3 kb), R6K origin of replication (requires the π protein for replication), kanamycin resistance gene (*nptII*) and the multiple cloning site of pBluescriptII within the Tn*10*, as well as a mutant ATS Tn*10* transposase gene encoded outside the transposon (2). The R6K origin of replication is critical for use in transposon mutagenesis of MR-1, as many plasmids, including those with origins such as p15A and pMB1, are capable of replicating in *Shewanella* (37; our results; D. Lies, personal communication). The importance of the mutant ATS Tn*10* transposase is that it displays a lower degree of insertion specificity than the wild type Tn*10* transposase, which is known to insert into hot spots (25).

Table 3. Frequency of conjugations and efficiency of electroporations with *Desulfovibrio* G20

Plasmid	Transposon	DNA transfer method[b]	Frequency or efficiency[c]
pRL1058a	Tn*5*	Electroporation	4 transformants /μg
pQL1058a	Tn*5*	Electroporation	6 transformants /μg
pRK2096	Tn*7*	Conjugation	10^{-6}—10^{-5}
pTnMod-OGm	Mini-Tn*5*	Electroporation	0
pTnMod-RKm	Mini-Tn*5*	Electroporation	3 transformants /μg
EZ::TN[a]	Tn*5*	Electroporation	18 transformants /μg
pMycoMar	Mariner	Electroporation	0
pBSL180	Mini-Tn*10*	Conjugation	10^{-6}—10^{-5}

[a]Kit from Epicentre containing a transposon-transposase mixture (not a plasmid).

[b]Electroporation was carried out as previously described (49) with the following modifications. Cells were harvested at late log phase with OD_{600} of 0.6 to 0.7. To maintain osmolarity similar to that of G20, 400 mM sucrose with 1 mM $MgCl_2$ was used as both washing and electroporation buffers. Electroporation was performed in the anaerobic glove box, after which cells were immediately recovered in 400 μl of LS medium.

[c]Frequency represents the number of antibiotic-resistant CFU divided by the number of recipient CFU in conjugation experiments. Efficiency represents transformants per μg of DNA used in electroporation experiments.

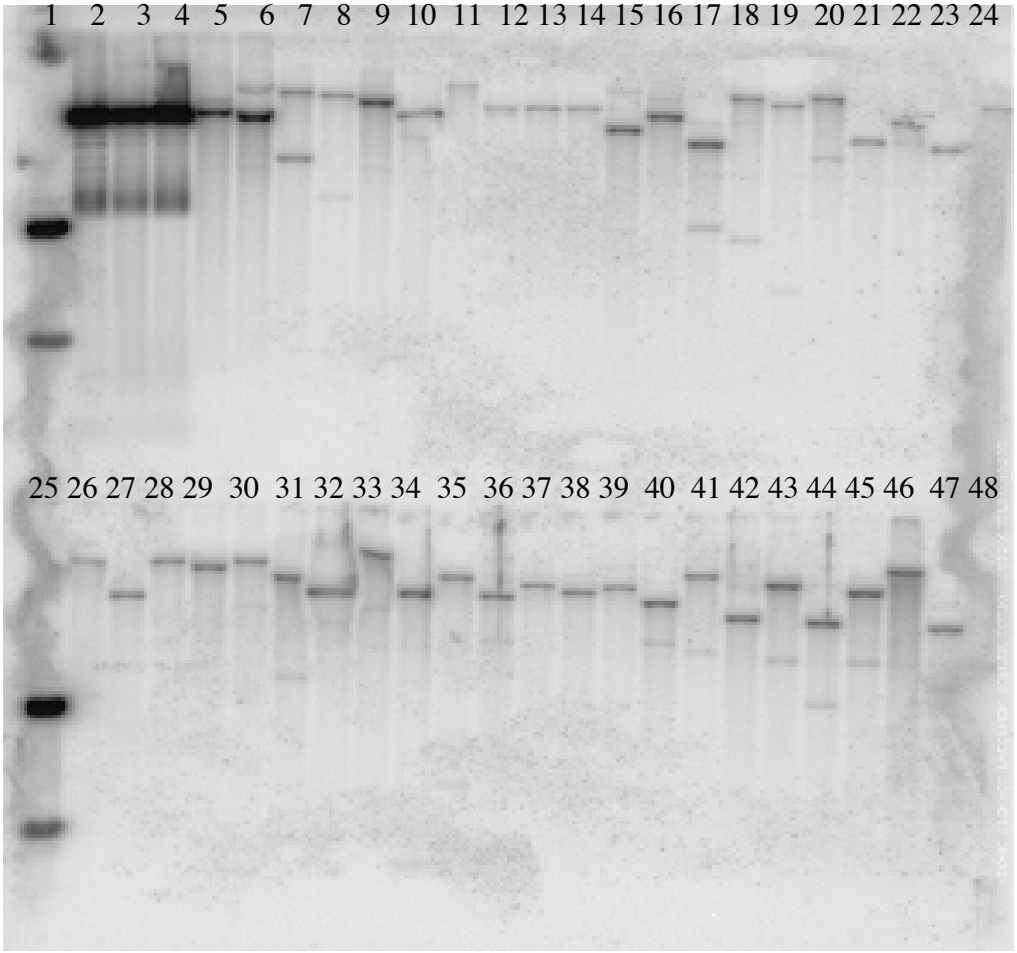
Fortuitously, by conjugating *E. coli* strain β–2155 harboring pBSL180 with G20, we found that the frequency of conjugation was sufficient (Table 3) for us to create STM mutant libraries with this mutagenesis system in both G20 and MR-1. Southern blot analysis confirmed that mutations occurred randomly in the strain G20 chromosome (Fig. 2). Random transposition was previously shown for MR-1 (40) and through a Southern blot performed in our study (data not shown).

Probe preparation for spotting. Complementary oligonucleotide pairs of the unique region for each tag served as the probe (40 bp). In initial experiments, we tested the effect on the hybridization signal of the concentration of probe DNA (10 ng/μl, 300 ng/μl, 670 ng/μl, 3.3 μg/μl) used in spotting. Spots with 3.3 μg/μl of 40-bp DNA produced the strongest hybridization signal. Spot intensities for the remaining concentrations were 2%, 5%, and 32%, respectively, relative to 3.3 μg/μl.

We then compared spotted slides that were incubated with SYTO 61 red-fluorescent nucleic acid stain added either before or after hybridization/washing procedures. We observed that fluorescence from the DNA stain did not change during the hybridization and washing steps (data not shown), indicating that the majority of spotted DNA remained bound to our slides. This also indicated that 3X SSC, commonly used in microarray spotting at the time (47), was sufficient for spotting our double-stranded probes to the glass slide.

Hybridization and washing conditions. Prior to hybridization, blocking of slides was found to be essential to achieve reproducible results, as it allowed for even spreading of target across the spotted slide surface. Comparison of two washing protocols showed that the high-stringency wash yielded the strongest signal with minimal cross

Figure 2. Southern blot analysis of G20 mutants transformed with pBSL180 Tn*10* transposon. pBSL180 was used as a probe. Lanes 1 and 25, 1-kb DNA ladder; lanes 2 to 4, pBSL180 HindIII-digested fragments; lanes 5 to 24 and 26 to 47, HindIII digest of chromosomal DNAs from independent G20 mutants (two bands are expected from Tn*10*); lane 48, HindIII digest of chromosomal DNA of untransformed G20.



hybridization (Fig. 3).

With this chosen washing condition, we investigated whether increasing the washing temperature above room temperature could eliminate the cross hybridization of 36 tags slated for elimination. We found that the chosen increases in wash temperature failed to reduce cross hybridizations and actually decreased spot intensity of all specific hybridizations (Table 4). These 36 tags were eliminated based on these results and those from the cross hybridization screen (by columns and rows as described in Materials and Methods). Sixty of the original 96 synthesized tags remained for mutagenesis of G20 and MR-1 (Table 2).

Initial sediment survival experiments. Initial sediment survival tests were performed to determine how long incubations should proceed before extraction of surviving mutants. From initial sediment survival tests of G20, we found that peak growth (approximately 20-fold over initial inoculum level) was reached after 8 to 9 days incubation at room temperature (Fig. 4a). As we believed that this amount of growth would be sufficient to select nonsurvivors from survivors, STM screens were carried out at this time point for G20. From initial survival tests of MR-1, we found that it was necessary to supply sediments with lactate in order for MR-1 to grow at least 10-fold and to remain at concentrations above the initial inoculum concentration for at least 7 days (Fig. 4b). We then chose to sacrifice MR-1 STM microcosms [amended with Fe(III) oxyhydroxide and lactate] at 5 to 6 days, a time point at which Fe(III) reduction was occurring, as evidenced by Fe(II) accumulation in initial sediment survival experiments (Fig. 4b).

Figure 3. Comparison of (a) high-salt (low-stringency) and (b) low-salt (high-stringency) wash conditions following hybridization of row and column pools (20) for 96 tags. The results shown represent one pooled column (eight tags from eight PCRs in one column of a 96-well plate). The remaining 7 columns and 12 rows were also individually labeled for 19 separate hybridizations with the microarray (spotted with the 96 original tags), but those data are not shown here. The portion of the array displayed is from a region containing the eight complimentary probes for the eight labeled tags used in this example (spots 1 to 4, 7, 8, 13 and 14), in addition to eight other spotted probes (5, 6, 9 to 12, 15, and 16) that are not complimentary to the eight labeled tags added in the hybridization solution. In all 20 hybridizations, cross hybridization of targets to nonspecific probes over the whole array occurred less often for high-stringency conditions (b) than for low-stringency conditions (a). In the example shown in this figure, circles are placed over probes to which nonspecific target has annealed under low-stringency conditions only.

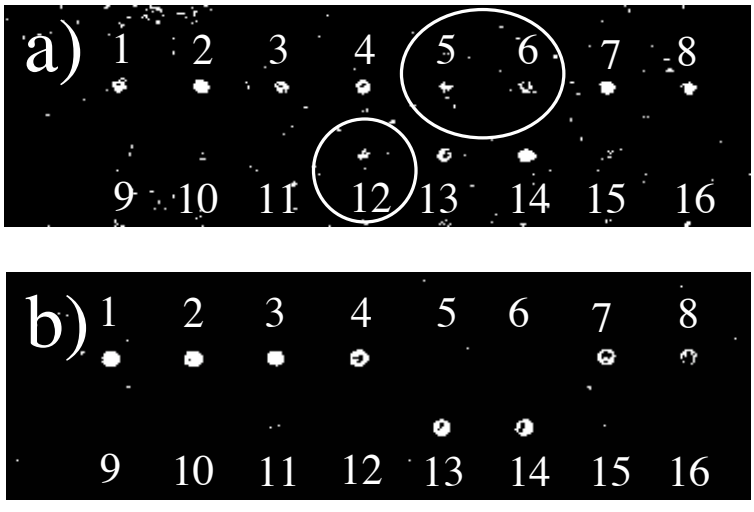
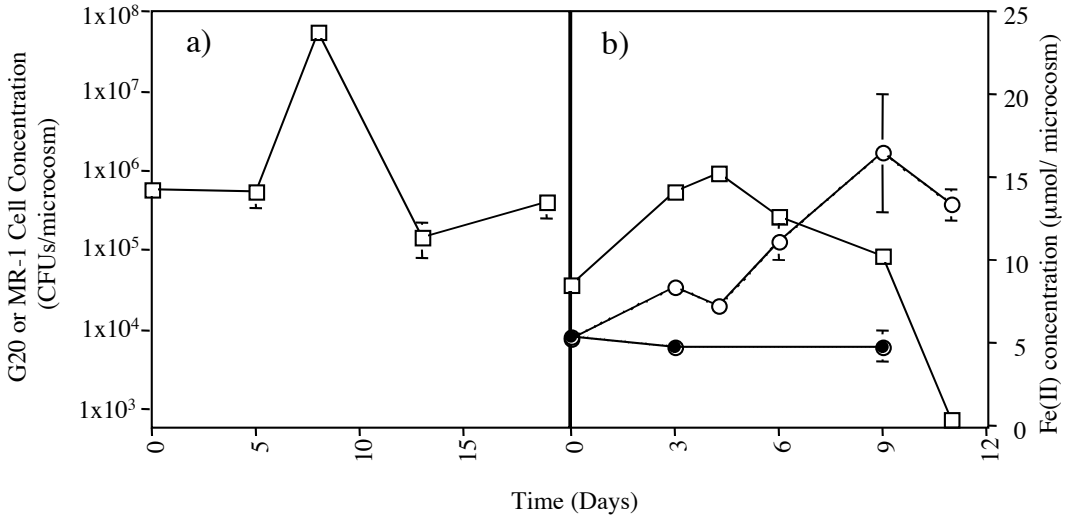


Table 4. Signal intensities of eight tags (from one row of 12 columns and 8 rows tested) bound to their respective complimentary probe as a function of increasing wash temperature.

Temp (°C)		Relative Signal Intensity[a] of tag:							
Preheat	Wash	A1	B1	C1	D1	E1	F1	G1	H1
55	Room	1	1	1	1	1	1	1	1
55	55	0.51	0.44	0.31	0.41	0.34	0.12	0.07	0.07
60	60	0.26	0.25	0.25	0.13	0.17	0.02	0.08	0.1
65	65	0.19	0.21	0.16	0.15	0.03	0.06	0.11	0.12

[a]The number given is the fraction of the observed signal intensity relative to that for the 55°C-preheated wash solution with room temperature washing. For generation of these data, one slide spotted with duplicate arrays was used. The signal was averaged from these two arrays.

Figure 4. Growth curves in subsurface sediments of (a) G20 (data represent averages for two microcosms) and (b) MR-1 (data represent averages for three microcosms). For MR-1, squares represent CFU, open circles represent soluble Fe(II) detected from the same microcosms as CFU, and closed circles represent Fe(II) detected in identical, sterile microcosms. Error bars show standard deviations, but these are too small to appear in most cases.



Screening of tagged mutants in natural sediments. To investigate recovery of tags from the input and output pools, we initially varied the number of colonies pooled for production of labeled target. In these early trials, we compared collections of 300, 700 and 3,500 colonies and found that pooled target from these resulted in similar hybridizations for some mutant pools but that on occasion, pooled target from more than 300 colonies could contribute to weak hybridization signals for mutants that might actually have been impaired in sediment survival. To ensure that we would not miss potentially impaired mutants, we elected to use approximately 300 colonies for recovery in subsequent sediment screens.

With regard to optimization of number of PCR cycles, we observed that 30 PCR cycles with wild-type MR-1 genomic DNA as the template generated unknown labeled target that hybridized weakly to some probe sequences spotted on the glass slide. We cannot explain why this occurred, because wild-type MR-1 does not contain any of the 60 tags and therefore should not generate target that hybridizes with our array, but we did find that using only 25 PCR cycles eliminated this background hybridization.

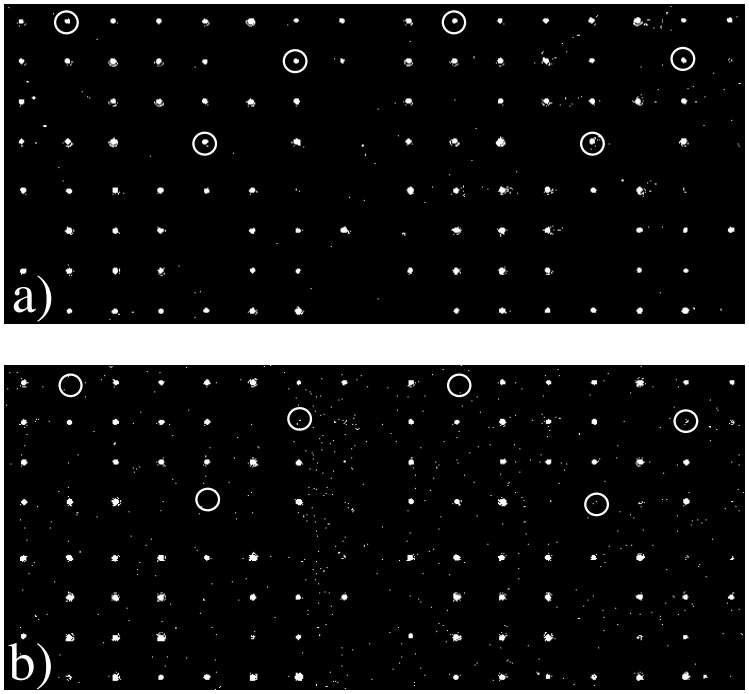
To test the reproducibility of our STM screening procedure, several mutant pools were run through independent replicate sediment microcosms. Target was labeled from 300 colonies collected from each independent microcosm, and hybridizations on separate slides were performed. In all cases, hybridizations identified the same potentially impaired sediment mutants, and the same survivors appeared among all replicates (data not shown). On account of this and the fact that we performed a confirmation step for each potential nonsurvivor to ensure that we had correctly identified mutants impaired in sediment survival, we hybridized DNA from a single microcosm for each of the 96

mutant pools of each organism.

A total of 96 mutant pools (of both G20 and MR-1), containing 60-uniquely tagged mutants in each pool, were grown as described in Materials and Methods, washed, and inoculated into sulfidogenic subsurface sediment microcosms [amended with Fe(III) and lactate for MR-1]. Potential nonsurviving mutants, defined as producing essentially no signal (below 3 times the background level) when hybridized slides were analyzed (a sample input versus output pool hybridization is shown in Fig. 5), were confirmed by subsequent competition experiments. In general, cell numbers for truly impaired MR-1 mutants were 20 to 30% of those for the parent strain when both were recovered after 5 days of incubation in competition sediment microcosms (data not shown). For G20, cell numbers of impaired mutants decreased to 0 to 9% of the initial inoculum. As a comparison, G20 mutants that survived in sediment had individual recoveries above 500% of the inoculum concentration. Of 208 potential sediment-impaired mutants for G20 identified during the first screening, 117 were confirmed by independent reinoculation of each into sediment. Of 86 potential sediment-impaired mutants for MR-1, 56 were confirmed by competition experiments, but 8 of these were also impaired in Fe(III)-citrate growth.

Validation of the methods described herein. In order to validate our method, we present chemotaxis genes that our STM study identified in both G20 and MR-1. For MR-1, the interrupted gene SO2323 had a CI in sediment of 0.1 ± 0.02, confirming its impaired status in sediment. The CI for Fe(III)-citrate growth was not determined for this mutant, as its growth in this medium appeared similar to growth observed for the parent strain prior to inoculation of these strains together into sediment competition

Figure 5. Hybridization results from one mutant pool, comparing (a) input pool hybridization with (b) output pool hybridization following sediment incubation. Circles have been placed around those tags present in the input pool and absent from the output pool. The 60 tags are positioned in four rows and eight columns, and all 60 tags are spotted in duplicate. For the slide shown, 10 tags did not fluoresce in both the input and output pools. As a result of being absent from the input pool, these 10 were not considered impaired mutants in this mutant pool.



experiments. SO2323 encodes a putative methyl-accepting chemotaxis protein (MCP) and starts a multicistronic operon of five genes putatively involved in chemotaxis functions. Similar genes may also be present in other environmental bacteria, as evidenced by protein sequence similarities across entire putative MCPs present in *Magnetococcus* sp. MC-1 and *D. vulgaris* Hildenborough (37 and 32% identity, respectively), while similarity to other bacterial proteins is restricted to the conserved C-terminal half of the protein.

A G20 transposon mutant with a similar gene (VIMMS392990) interrupted was recovered at 4% of the original inoculum concentration when inoculated back into sediment microcosms on its own. This gene is a monocistronic operon. Best similarity to other environmental organisms exists with sequence for MCPs in the NCBI protein database for *D. vulgaris* (designated DcrH) and *Geobacter sulfurreducens* PCA (41 and 44% similarity, respectively), as well as 42% similarity to one protein of *Geobacter metallireducens*. Similarity to protein sequences from these δ-*Proteobacteria* occurs mainly within the C-terminal region of the protein. Interestingly, this protein is orthologous to an MCP of *Vibrio cholerae* and *Shewanella oneidensis* MR-1 across the entire protein, but with a lower degree of homology.

The vast number of genes identified in both organisms cannot be discussed thoroughly in this paper, which focuses on development of a novel STM method for environmental bacteria. We will present our genes, confirmation of said genes (through competition experiments or inoculation of individual mutants back into sediment), and discussions of their potential function in sediment survival in future papers for each organism.

Discussion

This paper is the first report on the use of the STM method with environmental microorganisms. Before we could successfully apply STM to detect genes essential for survival of G20 and MR-1 in their specific niches, we had to satisfy three key requirements of STM: a mutagenesis procedure that provides efficient and random transposition, a model to represent in situ conditions, and a system to screen for transposon mutants impaired in in situ survival. We tried several available transposon systems, including vectors previously described as suitable for transposon mutagenesis in *D. desulfuricans* G20 (54), but found that of all vectors we tested, only the mini-Tn*10* transposon system provided both efficient and random transposition for generation of mutant libraries in both *Desulfovibrio* strain G20 and *Shewanella onenidensis* MR-1 (5,760 mutants each). While this number of mutants did not constitute a saturating screen of either organism's genome (G20 has 3,862 open reading frames [http://img.jgi.doe.gov/pub/main.cgi?page=taxonDetail&taxon_oid=400040000], and MR-1 had 4,758 open reading frames when the sequence was published [17]), we expected 5,760 mutants to reveal many genes necessary for sediment survival. In former STM studies, many genes required for survival were identified, whereas fewer mutants had been screened for bacteria with genome sizes comparable to those of MR-1 and G20 (34).

Just as optimization of the animal host model was important for studying pathogens by STM (reviewed in reference 8), a model sediment system for our environmental microbes had to be optimized, considering parameters such as pool complexity, inoculum concentration, time course for incubations, and recovery of surviving mutants. The

simultaneous use of 60 tagged mutants in our study, a number falling between the 48 (7) and 96 (35) tagged mutants used in pathogenic studies with reusable tags, did not present a pool complexity problem, a condition described in reference 8 in relation to STM pathogen studies. By repeating incubation and hybridization of several pools early into development of our method, we found that the same mutants were consistently identified as impaired. An inoculum size similar to that used in prior STM studies (19, 35) was found to be suitable for our study. The time point at which each organism's mutant pool was extracted was determined from preliminary sediment incubations and occurred following growth of cells, a condition necessary in pathogen STM studies to select virulent strains from avirulent strains (8).

The optimized procedure that we have developed samples fewer colonies from sediment than prior STM studies have used for the host (19, 35). Screening this smaller number ensured that we would not miss potentially impaired mutants, but we had to contend with more false negatives (mutants exhibiting no hybridization signal in the output pool that were subsequently shown to survive in confirmation experiments). The purpose of the secondary screen (competition experiments or inoculation of individual mutants back into sediment) was to identify those mutants that were truly attenuated in sediment survival. Competition experiments with MR-1 showed that impaired mutant cell numbers at 5 days were 20 to 30% of cell numbers for the parent strain. Since 300 colonies would give each tag the chance of being represented five times (if all 60 tagged mutants grew equally and had the same chance of being picked for the 300-colony pool), collection of more colonies could have masked these mutants.

To help reduce the frequency of false negatives and confirmation experiments, at least

duplicate replications of microcosms and microarrays would need to be incorporated into the experimental design. Replicates may also alleviate some problems associated with inherent factors of the STM protocol (e.g., soil heterogeneity and technical issues with PCR and microarrays) that may influence whether or not mutant tags are observed.

Despite the breakthrough made by the original STM method in identification of virulence-related genes in pathogenesis studies, the use of standard dot blotting techniques for pool analysis does not allow for efficient screening of mutants (19). In this respect, the technique was subsequently improved with a PCR-based STM (28). This system uses 12 tags designed and synthesized for specific and optimal PCR detection. Using this method, 12 libraries (one for each unique tag) are obtained, and single mutants from each library are picked to form pools comprised of 12 different mutants. The screening process consists of a separate PCR reaction for each of the 12 tags. Agarose gels obviate the need for hybridizations by showing whether a PCR product is amplified with each tag as a primer paired with a universal primer within the kanamycin resistance gene of the transposon (28). Recently more tags have been designed for use in this PCR-based STM (43), increasing the number of mutants that can be screened at once to 72. This involves the use of additional tags as well as three different miniTn*5*-vectors. Groups of mutants are screened in a similar manner as when there were only 12 tags, but by using multiplex PCR. The disadvantage of this system is that more than one mutagenesis vector may not be available for organisms in which genetic systems are not fully developed (as with many environmentally relevant bacteria). The alternative is to increase the number of PCRs, increasing the cost and the labor involved. For the procedure described in this paper, the addition of more tag sequences to one vector used

to create mutant pools achieves the same result as using multiple vectors to increase the number of uniquely tagged mutants.

A separate method that aimed at more efficient screening of mutants applied high-density oligonucleotide array technology, as developed by Affymetrix (30), to screen double-tagged transposon mutants (22). This technology involves synthesis of oligonucleotides directly on the array using a combination of photolithography and oligonucleotide chemistry, while the method described here utilizes mechanical microspotting (for a more detailed comparison see reference 46). At present, the advantage of our approach is affordability, because STM requires the screening of a large number of mutant pools, dependent on the number of unique tags employed, by individual hybridizations. Furthermore, our method uses only one array per pool, as each slide is spotted with the set of 60 probes on both ends, allowing simultaneous analysis of input and output pools. While the photolithographic approach may provide quantitative fitness data (48, 56), our approach satisfies the original intent of STM and currently does so at lower costs. For STM, quantitative data relevant to survival can also be assessed through studies of each individual impaired mutant that was identified by the screening process, rather than in the screen itself.

A final advantage of our method is that there is no need to digest and purify the 18-bp conserved arms from the labeled tag targets prior to hybridization, because only the 40-bp variable region is immobilized on the array as the probe. The early STM studies required an additional step to remove the invariable arms from the labeled probes ("target" in microarray terminology) prior to blot hybridization with colonies or plasmids containing the tagged transposons (19, 35).

Sequencing of interrupted genes in confirmed mutants from each organism identified MCPs as critical for sediment survival of both G20 and MR-1. While sequence similarity to these chemotaxis proteins exists with other proteins within MR-1 and G20, this similarity is namely confined to C-terminal regions that are conserved across diverse MCPs and not to the variable N-terminal region comprising the periplasmic domain that is involved in recognition of specific attractants and repellants (51). Finding these genes validates our screen in that we expect chemotaxis response proteins to be necessary for bacteria to react to attractants and repellents encountered in their environment in order to compete with surrounding microorganisms. An earlier study has shown that a chemotactic strain of *Pseudomonas fluorescens* survived significantly better in sediment than a nonmotile strain, while both strains had equivalent growth rates in liquid media (23).

At this time we do not know the specific environmental stimuli for the chemotaxis proteins in our survival studies, but both *Desulfovibrio* and *Shewanella* have demonstrated chemotaxis in previous studies. MR-1 displayed chemotaxis to certain electron acceptors, while there was no tactic response to ferric citrate and Mn(IV) oxide (39). In addition, besides a weak response for formate, MR-1 did not display a tactic response to other carbon sources tested (39). Another study on chemotaxis in *Geobacter metallireducens* reported that MR-1 is not motile when grown with Fe(III) oxide in motility plate assays (9). *D. vulgaris* has shown a chemotactic response to oxygen concentration or redox potential of the environment, and chemotaxis may help the cells find an optimal anaerobic environment (12).

In summary, adapting microarray technology to STM enabled us to mass produce

microarray slides spotted with our tags as probe and to downsize equipment necessary for carrying out hybridizations. We created tagged vectors which we demonstrated were suitable for mutagenizing environmentally significant members of the δ- and γ-*Proteobacteria*. This system may be applicable to a variety of environmental bacteria, creating many possibilities for future research areas regarding in situ activities of these bacteria, as in bioremediation and geomicrobiological processes.

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Chapter 3

Bacterial genes that contribute to in situ sediment survival

Abstract

Environmental bacteria persist in various habitats, yet little is known about the genes that contribute to their survival. Identification of 47 survival genes by signature-tagged mutagenesis (STM) of *Shewanella oneidensis* MR-1 provides us with insight into physiological and ecological processes that environmental bacteria use while growing in sediment ecosystems.

Body

Ecology encompasses the study of organisms and their interactions with one another and with the surrounding environment. In the field of microbiology, observations of these relationships are far less direct than studies of macroscopic organisms, as we cannot readily observe, in a traditional sense, in situ processes that occur at microscopic levels. Some information can be ascertained from studies that monitor microbial communities (38) or dominant respiratory processes (32), but in situ molecular scale interactions often elude us. While pure culture studies can monitor physiological and molecular changes (e.g., through microarray and proteomic analyses) in response to defined components of a habitat, extrapolation of laboratory results to the real and complex environment may not be the best approach. From an environmental standpoint, an in situ perspective of bacterial survival would provide a greater understanding of processes that are of fundamental ecological importance, and the knowledge gained can then be applied

toward enhancement of beneficial microbial activities (e.g., bioremediation) or prevention of disruptive activities (e.g., oil well souring).

With this in situ approach in mind, we identified genes critical to survival of a model environmental bacterium in sediment microcosms using a modified version (17) of the traditional signature-tagged mutagenesis (STM) procedure (19). *Shewanella oneidensis* MR-1 (34) was chosen as a model organism representing bacteria of environmental significance based upon its ability to reduce Fe(III) (35), a natural electron acceptor in a variety of anaerobic environments, and radionuclides, such as U(VI) and Tc (VII) (14, 29), rendering this bacterium useful for understanding bioremediation processes. The genome sequence was also available for MR-1 (18) which aided with identification of interrupted genes that appeared to be critical to sediment survival.

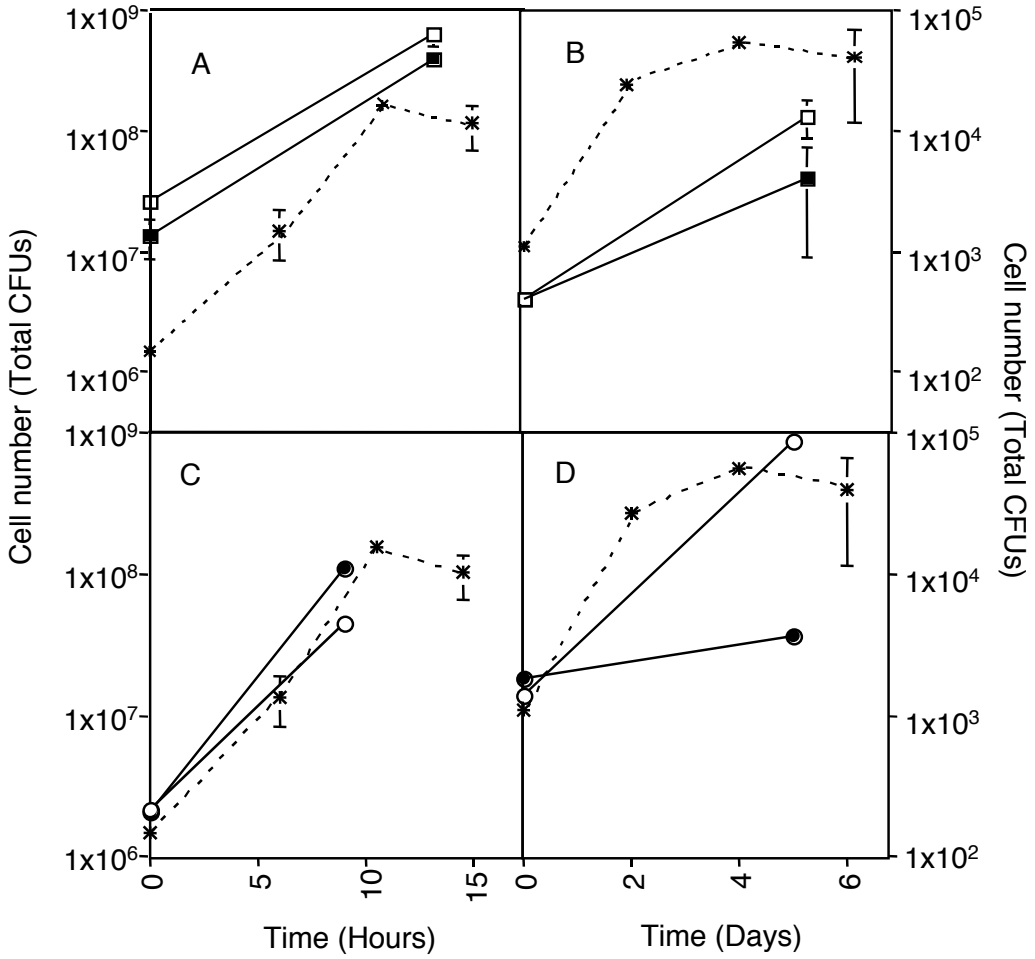
Briefly, we constructed a transposon mutant library using MR-$1_{sediment}$, a strain that was inoculated into and recovered from sediment microcosms prior to mutagenesis (17). Sixty MR-$1_{sediment}$ mutants, each containing a uniquely tagged transposon, were assembled per mutant pool. Each pool was first screened in aerobic minimal lactate medium and then anaerobic lactate-Fe(III)-citrate medium prior to inoculation into sediment microcosms, thus ensuring that sediment attenuation was not attributable to a general growth defect. Microcosms consisted of Fe(III)-reducing sediments (2 g anoxic subsurface sediments amended with 200 μmol Fe(III) oxyhydroxide) and lactate (20 μmol) in order to stimulate some growth of MR-1 (J. L. Groh and L. R. Krumholz, submitted for publication). Approximately 10^5 cells were inoculated into microcosms and incubated for 5 to 6 days, after which surviving mutants were isolated from the sediments. Microarray hybridization technology (17) was then used to identify

attenuated mutants whose tags were present in the initial inoculum and absent after a period of sediment incubation. Mutants identified by our hybridization method to be attenuated in sediment survival were confirmed by individual sediment competition experiments with the MR-$1_{sediment}$ strain. Results of competition experiments are reported in terms of a competitive index (CI), as used in several previous STM studies (9, 23). Fig. 1 graphically illustrates the sediment-impaired status of two representative non-surviving transposon mutants. Some mutants were also identified as impaired in Fe(III)-citrate medium growth.

Arbitrary PCR was then used (17, 36) to determine the sequence surrounding the insertion site of confirmed sediment-attenuated mutants. A past study using this transposon system with MR-1 (35) and a Southern blot performed in our lab confirmed that the insertion of the modified Tn*10* transposon was random and occurred as a single transposition event (data not shown). Sequence similarity searches were conducted with the NCBI public database (http://www.ncbi.nlm.nih.gov/) by employing the blastn (nucleotide) and blastp (protein) algorithms (2). To delineate putative functions, all genes critical to sediment survival were also analyzed using MicrobesOnline (http://www.microbesonline.org/) (1) and Clusters of Orthologous Groups of proteins (COGs, www.ncbi.nlm.nih.gov/COG/) (43-45).

Of approximately 5,000 transposon mutants screened in sediments, 47 genes representing general cellular processes of DNA repair, transport, transcriptional regulation, energy and amino acid metabolism, as well as genes encoding phage-related and transposon–related proteins were identified in this study as critical for sediment survival (Table 1). Additionally, transposon insertions into many conserved hypothetical

Figure 1. Competition experiments in lactate-Fe(III)-citrate medium (A and C) and in sediment microcosms (B and D) for the *mexF* transposon mutant (filled squares, A and B) and MR-$1_{sediment}$ (open squares, A and B) or for the *ccmF-2* transposon mutant (filled circles, C and D) and MR-$1_{sediment}$, (open circles, C and D). In competition experiments, each transposon mutant and the parent strain were grown together. The dashed line and asterisks show growth curves conducted independently with MR-$1_{sediment}$ alone. Error bars represent standard deviations, which were too small to appear in most cases.



proteins led to decreased survival of MR-1 in our sediment systems, as did insertions into numerous genes whose function does not fall within a discernible category (Table 1). Many genes were also selected in which transposon interruption led to decreased survival in growth medium prior to inoculation into sediments (Table 2). These genes exhibit decreased fitness in both growth medium and sediment because they are involved in central metabolic processes, such as amino acid synthesis and *gspN*–mediated transport of proteins involved in Fe(III) reduction (12).

In order to demonstrate that these survival genes are ubiquitously found in environmental bacteria, we used the NCBI protein database to identify homologous protein sequences from any of fifty-four environmentally relevant bacteria (percent identity and the fifty-four bacteria are found in Table 3). We found that protein sequences from most of the MR-1 survival genes are shared among genomes of diverse bacteria that play important roles in the environment and are often used in studies relevant to sediment ecology, survival, and bioremediation. In Table 1, we list the number of these sequences found within various Phyla. Although phylogeny does not necessarily equate to function, it is worth noting that similar protein products are found in a variety of environmental bacteria and that most of the genes identified in our study are not solely found within MR-1.

Sequence analysis of the genes identified by STM revealed interesting physiological events necessary for sediment survival of S. *oneidensis* MR-1. Several genes encoding proteins involved in DNA repair enhanced survival in sediment, suggesting that environmental conditions may be mutagenic. Helicases such as RecG have been implicated in the repair of DNA damage induced by weak organic acids (42), and in our

Table 1. Interruption of genes that led to attenuation of MR-1 in Fe(III)-reducing sediment. For competitive indices, in situ refers to competition experiments between each transposon mutant and MR-$1_{sediment}$ in sediment microcosms, while in vitro refers to competition experiments in lactate-Fe(III)-citrate medium. pBLAST analysis (NCBI) was used to identify sequences from 54 environmentally relevant bacteria that contained 20% identity or better to the entire protein sequence (unless indicated otherwise in Table 3) of MR-1 sediment survival genes. The number of similar sequences found for each bacterial group follows in parentheses. The identity of organisms from which sequences were obtained and the exact similarity can be found in Supplementary Table 1.

Gene product (gene designation in parenthesis, if available)	TIGR Locus	In situ Competitive Index	In vitro Competitive Index	Protein sequences with shared similarity from diverse environmental bacteria	COG
Bacteriophage genes					
prophage MuSo2, tail sheath, putative	SO2693‡	0.71±0.024	0.76	α (1), β (1), γ (3), δ (1)	COG4386, Mu-like prophage tail sheath protein gpL
conserved hypothetical	SO1442	0.59±0.35	ND	δ (1)	None
conserved hypothetical	SO1443	0.34±0.05	1.6±0.7	γ (1)	None
AlpA	Intergenic region between SO1441-SO1442	0.33±0.26	ND	β (2), γ (3), δ (2)	COG3311, Predicted transcriptional regulator
conserved hypothetical	SO2660‡	0.18±0.03	ND	β (2), γ (1)	None

DNA repair					
ATP-dependent DNA helicase	SO4364	0.11±0.002	0.57	α (1), β (9), γ (10), δ (2), *Cyanobacteria* (1)	COG1200, RecG-like helicase
RecG helicase	SO0368	0.39±0.05	ND	δ (2), *Planctomycetes* (1)	COG1061, DNA or RNA helicases of superfamily II
helicase	SO2744	0.85±0.38	ND	γ (1)	COG1061, DNA or RNA helicases of superfamily II
Transcriptional regulators					
transcriptional regulator HlyU	SO3538	0.24±0.7	1.9±0.6	α (5), β (6), γ (9),	COG640, Predicted transcriptional regulators
phosphate regulon response regulator PhoB (*phoB*)	SO1558‡	0.054±0.031	0.78±0.31	α (4), β (9), γ (10), δ (4)	COG745, Response regulators consisting of a CheY-like receiver domain and a winged-helix DNA-binding domain
transcriptional regulator IlvY (*ilvY*)	SO4350	0.16±0.07	0.74±0.02	α (1), β (6), γ (7), δ (3), *Firmicutes* (1)	COG583, Transcriptional regulator
transcriptional activator, putative	SO1985‡	0.3±0.25	ND	α (3), γ (3)	COG3806, Transcriptional activator
transcriptional regulator, TetR family	SO1703	0.3	1.3±0.78	α (2), β (3), γ (4), δ (2)	COG1309 Transcriptional regulator
Transport functions					
L-lactate permease (*lldP*)	SO0827[a]	0.3±0.074	ND	α (3), β (4), γ (3), δ (1), *Firmicutes* (3), *Actinobacteria* (1), *Deinococcus-Thermus* (1)	COG1620, L-lactate permease
K+ uptake protein KtrA, putative	SO4281‡	0.76	ND	β (2), γ (5), δ (2), *Firmicutes* (4), *Actinobacteria* (1),	COG569, K+ transport systems, NAD-binding

				Deinococcus-Thermus (1)	component
TonB-dependent receptor C-terminal domain protein	SO0815	0.28±0.14	1.8±0.58	α (2), β (4), γ (3), δ (1)	COG4206, Outer membrane receptor protein, mostly Fe transport
toxin secretion, membrane fusion protein	SOA0050‡	0.43±0.14	ND	α (2), β (3), γ (3), δ (1)	COG0845, Membrane-fusion protein COG1566, Multidrug resistance efflux pump
RND multidrug efflux transporter MexF (*mexF*)	SO3492‡	0.28±0.06	1.3±0.26	α (4), β (10), γ (11), δ (5), *Cyanobacteria* (2), *Chlorobi* (1), *Planctomycetes* (1)	COG0841, Cation-multidrug efflux pump
HlyD family secretion protein	SO0524‡	0.59±0.07	1.3±0.54	α (6), β (2), γ (6), δ (3), *Chlorobi* (1)	COG1566, Multidrug resistance efflux pump
Energy Production-Conversion					
periplasmic Fe hydrogenase, large subunit (*hydA*)	SO3920‡	0.59±0.14	2.2±0.8	α (1), δ (4), *Firmicutes* (2), *Actinobacteria* (1)	COG4624, Iron only hydrogenase large subunit, C-terminal domain
Cyt. c-type biogenesis protein CcmF (*ccmF-2*)	SO0478[b]	0.031	2.7	β (5), γ (8)	COG1138, Cytochrome c biogenesis factor
molybdopterin biosynthesis MoeA protein (*moeA*)	SO0138‡	0.53±0.1	ND	α (2), β (9), γ (6), δ (4)	COG303, Molybdopterin biosynthesis enzyme
S-adenosylmethionine: 2-demethylmenaquinone methyltransferase (*menG-2*)	SO4197[c]	0.73±0.1	ND	α (2), β (2), γ (8), *Deinococcus-Thermus* (1)	COG 0684, Demethylmenaquinone methyltransferase
tryptophan halogenase	SO3513‡	0.315	1.1±0.55	α (1), γ (2)	COG644, Dehydrogenases (flavoproteins)

Amino acid metabolism					
tryptophan synthase, beta subunit (*trpB*)	SO3023‡	0.3±0.26	ND	α (8), β (8), γ (7), δ (3), *Firmicutes* (5), *Cyanobacteria* (1)	COG133, Tryptophan synthase beta chain
anthranilate synthase (*trpE*)	SO3019‡	0.14±0.15	ND	α (5), β (9), γ (8), δ (3), *Firmicutes* (5), *Deinococcus-Thermus* (1), *Cyanobacteria* (1), *Chloroflexi* (1)	COG147, Anthranilate/para-amino-benzoate synthases component I
glutamate synthase, large subunit (*gltB*)	SO1325‡	0.11±0.014	ND	α (9), β (8), γ (10), δ (1), *Firmicutes* (7),*Cyanobacteria* (2), *Deinococcus-Thermus* (1), *Chloroflexi* (1)	COG69, Glutamate synthase domain 2
glutamate synthase, small subunit (*gltD*)	SO1324‡	0.006±0.001	0.53±0.27	α (9), β (8), γ (10), δ (4), *Firmicutes* (7), *Chlorobi* (1), *Deinococcus-Thermus* (1)	COG493 NADPH-dependent glutamate synthase beta chain and related oxidoreductases
2-isopropylmalate synthase (*leuA*)	SO4236‡	0.15±0.015	1.1±0.42	α (5), β (11), γ (6), δ (5), *Firmicutes* (3), *Deinococcus-Thermus* (1), *Cyanobacteria* (2), *Chloroflexi* (1)	COG119, Isopropylmalate-homocitrate-citramalate synthases
Other					
serine protease, subtilase family	SO4539	0.6±0.25	1.036	γ (1), δ (1), *Chloroflexi* (1), *Firmicutes* (4), *Cyanobacteria* (1), *Actinobacteria* (1)	COG1404, Subtilisin-like serine proteases
serine protease, subtilase family	SO4539	0.54±0.23	ND	γ (1), δ (1), *Chloroflexi* (1), *Firmicutes* (4), *Cyanobacteria* (1), *Actinobacteria* (1)	COG1404, Subtilisin-like serine proteases
alpha-glucosidase (*susB*)	SO2450	0.07±0.066	ND	α (1), γ (1), *Planctomycetes* (1)	None
lipoprotein, putative	SO2570[c]	0.11±0.002	ND	α (1), γ (3), *Firmicutes* (1), *Cyanobacteria* (1)	COG4805, Uncharacterized protein conserved in bacteria
methyl-accepting chemotaxis protein	SO2323‡	0.1±0.024	ND	α (1), β (1), δ (6)	COG0840, Methyl-accepting chemotaxis protein

putative methylase	SO1478	0.87±0.12	ND	*Cyanobacteria* (1)	None
cold shock domain protein	SO1648[c]	0.37±0.07	ND	β (9), γ (13), δ (2), *Firmicutes* (4)	COG1278, Cold shock proteins
Transposon-related					
ISSod4, transposase	SO3451[‡]	0.55±0.16	1.1±0.29	γ (1), δ (1)	COG3328, Transposase and inactivated derivatives
ISSod3, transposase	SO2283	0.4±0.28	ND	γ (2)[d]	None
ISSod1, transposase OrfB	SO2169	0.53±0.43	ND	α (1), β (3), γ (4)[d], *Cyanobacteria* (1)	COG2801, Putative transposase
Conserved hypothetical proteins					
	SO1652[‡]	0.4±0.42	ND	γ (2), δ (1), *Firmicutes* (1), *Actinobacteria* (1)	COG1387, Histidinol phosphatase and related hydrolases of the PHP family
	SO1831	0.349	ND	α (1), γ (4)	None
	SO1922	0.53±0.065	ND	γ (2)	COG3097 Uncharacterized bacterial conserved region
	SO2385	0.43±0.16	ND	α (4), β (3), γ (5), *Cyanobacteria* (1)	COG3211 Predicted phosphatase
	SO2827	0.21±0.2	ND	β (3), γ (5), *Planctomycetes* (1), *Deinococcus-Thermus* (1)	None
	SO3331	0.063±0.035	ND	α (5), β (2), γ (2), δ (1), *Cyanobacteria* (1)	COG1192, ATPases involved in chromosome partitioning
	SO4302	0.7±0.22	ND	α (1), β (2), γ (3), δ (3), *Firmicutes* (2)	COG2846, Regulator of cell morphogenesis and NO signaling

Hypothetical proteins					
	SO0753	0.21±0.25	ND	None	None
	SO4435	0.67	ND	None	None

[‡] gene predicted to be within an operon

[a] transposon insertion was 117 bp prior to gene

[b] transposon insertion is 7 bp upstream from potential ribosomal binding site

[c] transposon insertion is within transcription terminator

[d] many paralogs present in MR-1

ND represents not determined; in vitro competition experiments were deemed unnecessary, as mutant growth was similar to growth of the parent strain in medium prior to sediment inoculation

Table 2. Interruption of genes that led to attenuation in both sediment and Fe(III)-citrate lactate medium

Gene product (gene name, if available)	Locus	In situ CI	In vitro CI
General secretion protein (*gspN*)	SO0176‡	0.28±0.27	0.2±0.12
argininosuccinate synthase (*argG*)	SO0278‡	0.061±0.03	0.69±0.2[a]
argininosuccinate lyase (*argH*)	SO0279‡	0.036±0.03	0.39±0.01
argininosuccinate lyase (*argH*)	SO0279‡	0.073±0.04	0.3±0.16
argininosuccinate lyase (*argH*)	SO0279‡	0.009±0.01	ND
methyltetrathydrofolate-homocysteine methyltransferase (*met*H)	SO1030	0.66±0.38	ND
glyceraldehyde 3-phosphate dehydrogenase (*gapA-3*)	SO2347	0.23±0.01	ND
phosphate acetyltransferase	SO2916‡	0.011	0.003
asparagine synthetase B, glutamine-hydrolyzing (*asnB-1*)	SO2767	0.68±0.22	ND
threonine synthase (*thrC*)	SO3413‡	0.017±0.016	ND

ND represents not determined; in vitro competition experiments were deemed unnecessary, as poor growth of the mutant was observed in medium prior to sediment inoculation

[a]although the CI is not too low, we observed poor growth in medium prior to sediment inoculation

‡ gene is predicted to be within an operon

Supplementary Table 1. Relatedness (by percent identity at the amino acid level) of proteins encoded by MR-1 sediment survival genes to proteins found in any of 54 environmentally relevant bacteria. Abbreviations for these 54 bacteria, defined below the table, follow respective percent identities. Reported comparisons have less than 15% gaps and protein lengths are similar, unless indicated otherwise. For some MR-1 sediment survival gene products, we include comparisons to proteins that are from bacterial strains (names spelled out) that do not belong to the 54 in which we were interested. aa represents "amino acids".

TIGR locus	Gene or gene product	NCBI accession number	length (amino acids)	% Identity
SO2693	prophage MuSo2 putative tail sheath	NP_718277	493	41/40/31% Dv, 34% Ps, 36% Pu, 42% Po, 33% Pf, 29% Go
SO1442	conserved hypothetical	NP_717059	489	21% Dd
SO1443	conserved hypothetical	NP_717060	215	28% So
*	Prophage CP4-57 regulatory protein AlpA	*	70	43% Py, 40% Vf, 38% Dp, 35% Rg, 34% Xf, 34% Gs, 34% Rm
SO2660	conserved hypothetical	NP_718244	206	59% So, 47% Bc, 48% Mf, 35% *Anopheles gambiae* str. PEST
SO4364	RecG	NP_719888	688	64% Pp, 61% Ms, 59% Pu, 53% Xf, 52% Ac, 52% Mc, 50% Ne, 50% Rs, 45% Gm, 45% Gs, 62% Il, 58% Pf, 52% Md, 51% Mf, 49% Re, 49% Rg, 49% Az, 48% Da, 49% Td, 47% Po, 57% Py, 41% Sm, 39% Gv
SO0368	helicase	NP_716008	982	43% Pi, 41% Gs, 38% Dp
SO2774	helicase	NP_718328	590	48% Pp
SO3538	*hlyU*	NP_719085	98	73% Pp, 65% Vf, 52% Md, 51% Bj, 50% Si, 48% So, 49% Rp, 47% Pf, 46% Sm, 45% Il, 44% Rm, 43% Py, 51% Ml, 42% Rs, 39% Ac, 43% Ne, 43% Re, 37% Xf, 40% Po, 39% Da

SO1558	PhoB	NP_717171	239	77% Vf, 75% Pp, 65% Pu, 65% Az, 65% Md, 64% Pf, 64% Ac, 60% Mc, 57% Ne, 57% Rs, 56% Xf, 53% Rp, 53% Bj, 52% Ml, 52% Sm, 47% Dd, 66% Td, 63% Mf, 58% Da, 57% Re, 57% Py, 53% Rg, 53% Po, 53% Si, 48% Gm, 47% Gs, 44% Dv
SO4350	IlyV	NP_719874	290	49% Pp, 47% Ms, 44% Dp, 37% Ds, 29% Rs, 23% Dv, 27% Ac, 25% Az, 26% Af, 26% Bj, 27% Ne, 26% Pu, 25% Po, 28% Rm, 26% Oi, 25% Pf, 25% Md, 27% Da
SO1985	transcriptional activator	NP_717592	230	46% Vf, 27% Ml, 29% Rp, 33% Il, 25% Md, 27% Rh
SO1703	transcriptional regulator, TetR family	NP_717314	106	matches are only to the central portion of MR-1 protein (~70 aa), and the following are greater than 200 aa in length: 63% Pp, 54% Vf, 51% Si, 34% Rs, 30% Mc, 40% Da, 37% Gs, 38% Dv, 36% Rp, 40% Az, 40% So
SO0827	L-lactate permease	NP_716458	545	69% Pu, 66% Ac, 63% Pf, 61% Dr, 54% Dv, 45% Rp, 44% Rs, 64% Rm, 62% Mm, 62% Re, 55% Go, 51% Gk, 55% Dh, 41% Rg, 35% St, 38% Bh
SO4281	potassium uptake protein KtrA, putative	NP_719806	214	75% Pp, 62% So, 52% Il, 45% Ac, 36% Bh, 34% Oi, 33% St, 29% Tt, 34% Vf, 33% Dr, 30% Ds, 29% Dd, 27% Po, 34% Gk, 30% Az
SO0815	TonB-dependent receptor C-terminal domain protein	NP_716446	653	28% Rp, 27% Ds, 27% Bj, 23% Pp, 22% So, 21% Ac, 24% Rs, 29% Da, 30% Rg, 25% Po
SOA0050	toxin secretion, membrane fusion protein	NP_720386	415	30% Xf, 23% Rs, 22% Pp, 23% Ml, 21% (over the last ~300 aa of SOA0050 and 446 aa Dd protein) Dd, 21/22% So, 27% Rg, 25% Mm, 22% Mf
SO3492	*mexF*	NP_719041	1047	73% Pu, 72% Ps, 67% Rs, 65% Sm, 65/51/45% Bj, 63/38% Pp, 59/42% Mc, 54/44% Ne, 50/47% Pi, 51/46% Ml, 45/39/35% Dv, 44/43% Gv, 42% Rp, 58/35/34% *Rhodospirillum rubrum*, 39% *Silicibacter pomeroyi* DSS-3, 35% Az, 57/38% Da, 56/39/34% Mf, 59% Po, 37/35/34% Re, 60/37/36/35% Rm, 60/54% Rg, 36% Td, 33% Ac, 37% Il,

				36/35/34% Pf, 34% Py, 35% So, 38% Vf, 34% Xf, 34% Dp, 36% Dd, 37/34% Gm, 35% Gs, 39/35% *Wolinella succinogenes* DSM 1740, 37% uncultured Acidobacterium, 34% Ct, 38/36% *Bacteroides thetaiotaomicron* VPI-548, 44/41% *Rhodopirellula baltica* SH1, 39% *Anabaena variabilis* ATCC29413, 35% Cw
SO0524	HlyD family secretion protein	NP_716160	301	44% Sm, 44% Bj, 40% Mc, 42% Ml, 40% Pf, 41% Pu, 40% Rp, 38% Rs, 37% Gs, 38% Ct, 38% Gm, 36% Ac, 41% Vf, 39% Mm, 36% Pp, 35% Rm, 31% Go, 22% Dd (6% gaps; 414 aa)
SO3920	periplasmic Fe hydrogenase, large subunit	NP_719451	410	49% Dv, 45% Dd, 44% Dp, 44% St, 43% Tt, 41% Rp, 24% Gm, 44% Dh
SO0478	cytochrome c-type biogenesis protein CcmF	NP_716114	685	48% So, 46% Pp, 41% Pf, 45% Mc, 41% Ms, 42% Ne, 41% Pu, 41% Xf, 39% Da, 38% Az, 38% Il, 38% Rm, 36% Rg
SO0138	molybdopterin biosynthesis MoeA protein	NP_715780	417	52% Pp, 48% Rs, 48% Ms, 46% Mc, 42% So, 38% Bj, 40% Pu, 39% Sm, 39% Ds, 50% Vf, 49% Rg, 48% Rm, 48% Mf, 49% Re, 45% Az, 41% Po, 44% Td, 42% Da, 39% Gm, 37% Gs, 34% Dd
SO4197	*men*G-2	NP_719725	161	78% Pp, 66% Ms, 58% Pu, 49% Ac, 43% Rs, 41% So, 43% Mc, 46% Dr, 68% Vf, 56% Pf, 49% Az, 47% Da, 44% Rg
SO3513	tryptophan halogenase	NP_719060	507	34% *Caulobacter crescentus*, 29% *Bdellovibrio bacteriovorus* HD100, 43% Md, 35% Na, 31% Pf
SO3023	tryptophan synthase, beta subunit	NP_718591	396	80% Pp, 79% Vf, 75% Ms, 69% Il, 57% Tt, 53% Bj, 55% Gk, 53% Mm, 54% Si, 56% Ne, 53% Ex, 56% Ds, 53% Oi, 54% Gv, 53% Ml, 54% Sm, 55% Mf, 52% Go, 54% Mc, 55% Re, 55% Td, 54% Dh, 53% Py, 52% Rp, 52% Md, 54% Rm, 53% Gs, 54% Rg, 54% Da, 52% Na, 54% Rs, 53% Dv
SO3019	anthranilate synthase component I	NP_718587	574	54% Pp, 53% Il, 55% Vf, 41% Gs, 40% Mc, 38% Si, 35% Go, 40% Gm, 40% Az, 37% Gk, 39% Dr, 39% Mf, 40% Rh, 34% Pu, 39% Ac, 37% Mm, 39%

				Ne, 39% Td, 32% Da, 38% Na, 37% Oi, 38% Pf, 38% Tt, 38% Dp, 38% Rg, 38% Gv, 37% Rs, 37% Re, 45% Ca, 37% Rm, 37% Xf, 39% Dh, 31% Ex
SO1325	glutamate synthase, large subunit	NP_716945	1482	67% Pp, 61% Il, 58% Ac, 58% Pu, 58% Pf, 58% Md, 56% Py, 56% Td, 52% Xf, 44% Vf, 44% Az, 47% Ds, 44% Bh, 45% Gv, 43% Rs, 43% Bj, 43% Oi, 43% Cw, 44% Dh, 44% Sm, 43% Gk, 43% Rp, 44% Mm, 43% Rm, 43% Re, 44% Mc, 43% Po, 44% Go, 43% Ml, 43% Na, 43% Dr, 43% Rg, 42% Si, 42% Rh, 43% Ex, 45% Ca, 31% Gs, 31% Gm, 42% Mf
SO1324	glutamate synthase, small subunit	NP_716944	468	67% Md, 66% Ac, 66% Pf, 67% Pu, 65% Pp, 64% Py, 63% Il, 62% Td, 58% Xf, 49% Rh, 46% Si, 47% Mm, 45% Go, 37% Dh, 37% Tt, 37% Ct, 37% Gk, 36% Mc, 36% Gs, 36% Ex, 36% Dp, 36% Dd, 37% Rp, 34% Az, 34% Bj, 36% Sm, 34% Bh, 36% Na, 35% Dv, 34% Po, 34% Rs, 33% Rm, 34% Ml, 35% Dr, 33% Da, 34% Rg, 34% Oi, 32% Re, 33% Vf, 45% Ds
SO4236	2-isopropylmalate synthase	NP_719763	522	70% Vf, 69% Pp, 66% Ms, 49% Ne, 51% Mc, 49% Gs, 50% Gm, 50% Mm, 49% Cw, 48% Td, 49% Ds, 47% Rs, 48% Bh, 46% Da, 46% Md, 47% Az, 48% Gk, 46% Rm, 47% Gv, 47% Dv, 46% Dd, 46% Bj, 46% Re, 46% Rp, 47% Po, 47% Dr, 47% Rg, 47% Mf, 45% Oi, 46% Go, 42% Xf, 44% Rh, 43% Si, 43% Ca
SO4539	serine protease, subtilase family	NP_720056	1634	not over the entire MR-1 protein (as it is longer): 33% Ca (820 aa), 37% (1287 aa) & 34% (1300 aa) So, 29% Ds (20% gaps; 1075 aa), 27% Oi (17% gaps; 740 aa), 27% Bh (25% gaps; 757 aa), 23% Gv (15% gaps; 1054 aa), 24% St (15% gaps; 1313 aa), 23% Oi (18% gaps; 1257 aa), 22% *Lactobacillus delbrueckii* subsp. *Lactis* (18% gaps; 1942 aa), 24% *Streptococcus thermophilus* (17% gaps; 1585 aa), 24% Ex (17% gaps; 1239 aa), 24% Tt (24% gaps; 1999 aa)

SO2450	alpha-glucosidase	NP_718040	699	65% Md, 49% Na, 27% Pi, 55%/53% *Xanthomonas campestris*, 45% *Bacteroides thetaiotaomicron* VPI-5482, 44% *Bacteroides fragilis*, 39% *Tannerella forsythensis*
SO2570	lipoprotein, putative	NP_718156	592	31% Gv, 23/21% So, 23% Xf, 40% Dh (317 aa protein that matches the last ~300 aa of SO2570), 21% Il (17% gaps; 622 aa) & 22% (13% gaps; 604 aa), 22% Na (match to last 300 aa of MR-1 protein; 10% gaps; 619 aa)
SO2323	methyl-accepting chemotaxis protein	NP_717913	646	41% Ds, 37% Dv, 36% Dd, 31% Rp, 28% Dp; Last portion: 43% Da (match to last 345 aa out of 541 aa of Da protein), 42% Gs (match to last 310 aa/568 aa), 32% Gm (match to last 439 aa/581 aa)
SO1478	methylase, putative	NP_717094	257	37% Gv
SO1648	cold shock domain family protein	NP_717259	69	83% Pp, 86% Vf, 74% So, 81% Sv, 69% Rg, 74% Il, 68% Ne, 67% Td, 64% Pf, 67% Da, 66% Md, 66% Rs, 66% Az, 64% Re, 62% Mf, 67% Ex, 64% Rm, 64% Py, 66% Oi, 68% Mc, 65% Gk, 59% Mm, 64% Dp, 58% Xf, 53% Ms, 64% Dh, 57% Ac, 64% Gs
SO3451	ISSod4, transposase	NP_719002	80	92% to ISSod1, transposase OrfB, So, 43% Ds
SO2283	ISSod3, transposase	NP_717879	404	99%+ paralogs many in MR-1, 49% Pp
SO2169	ISSod1, transposase OrfB	NP_717769	269	100% many paralogs in MR-1, 40% Rs, 38% Ne, 40% Mc, 41% Gv, 38% Po, 41% Pu, 43% Py, 40% Ml
SO1652	conserved hypothetical protein	NP_717263	252	49% Pp, 49% Tt, 43% Ds, 28% St, 44% Vf
SO1831	conserved hypothetical protein	NP_717439	609	43% Xf, 37/34% So, 29% Pp, 43% Il, 43% Na, 42% uncultured bacterium 561 from Monterey Bay Coastal Ocean Microbial Observatory environmental clone sequencing, 35% conserved hypothetical protein from uncultured marine proteobacterium ANT32C12, 35% uncultured marine proteobacterium ANT8C10

SO1922	conserved hypothetical protein	NP_717529	104	52% Pp, 51% Vf
SO2385	conserved hypothetical protein	NP_717975	685	57% Pu, 35% Sm, 33% Ml, 27% Gv, 44% Il, 40% Py, 40% Rg, 39% Po, 39% Az, 35% Pf, 35% Vf, 33% Si, 37% Rh
SO2827	conserved hypothetical protein	NP_718399	123	Only over first ~80aa of SO2827, as the following are less than 80aa long: 77% Pp, 65% Pi, 61% Pu, 61% Dr, 58% Ac, 68% Vf, 73% Po, 69% Md, 66% Da, 60% Pf
SO3331	conserved hypothetical protein	NP_718885	260	Match is to first 200 aa of SO3331: 70% So (222 aa long), 28% Mc (207 aa long), 28% Bj (212 aa long), 28% Rs (212 aa long), 27% Rp (217 aa long), 22% Sm (238 aa long), 20% Ds (16%gaps/254 aa), 36% Cw (223 aa long), 28% *Chlorobium limicola* (209 aa long), 28% Go (225 aa long), 26% Mm (212 aa long), 25% Po (229 aa long)
SO4302	conserved hypothetical protein	NP_719827	222	40% Gs, 36% Ds, 35% Gm, 35% Rs, 35% Ms, 33% Sm, 36% Gk, 33% Dh, 32% Re, 38% Il, 26% Md
SO0753	hypothetical protein	NP_716384	85	
SO4435	hypothetical protein	NP_719956	86	100% to 35 aa SO3320 (of 173 aa protein) that has following homologs: 58% Gv, 56% Ms, 56% Rs, 50% Rp, 49% Ac

*the transposon insertion in this mutant was 163 bp before SO1442, in an intergenic region based on TIGR annotation of MR-1. Upon further investigation using ORF Finder (NCBI), we determined that a gene with high similarity (53% identity) to the *E. coli* gene encoding AlpA exists in this region between SO1441 and SO1442.

Abbreviations used for bacteria in table above
Af=*Acidithiobacillus ferrooxidans* (γ-*Proteobacteria*)
Ac=*Acinetobacter* sp. ADP1 (γ-*Proteobacteria*)
Az=*Azoarcus* sp. EbN1 (β-*Proteobacteria*)
Bh=*Bacillus halodurans* C-125 (*Firmicutes*)
Bj=*Bradyrhizobium japonicum* (α–*Proteobacteria)*
Bc=*Burkholderia cepacia* R1808 (β-*Proteobacteria*)
Ct=*Chlorobium tepidum* TLS (*Bacteroidetes*/*Chlorobi*)

Ca=*Chloroflexus aurantiacus* (*Chloroflexi*)
Cw=*Crocosphaera watsonii* WH 8501(*Cyanobacteria*)
Da=*Dechloromonas aromatica* RCB (β-*Proteobacteria*)
Ds=*Desulfuromonas* spp. (δ-*Proteobacteria*)
Dp=*Desulfotalea psychrophila* LSv54 (δ-*Proteobacteria*)
Dh=*Desulfitobacterium hafniense* DCB-2 (*Firmicutes*)
Dd=*Desulfovibrio desulfuricans* G20 (δ-*Proteobacteria*)
Dv=*Desulfovibrio vulgaris* (δ-*Proteobacteria*)
Dr=*Deinococcus radiodurans* (*Deinococcus-Thermus*)
Ex=*Exiguobacterium* sp. 255-15 (*Firmicutes*)
Gk=*Geobacillus kaustophilus* HTA426 (*Firmicutes*)
Gm=*Geobacter metallireducens* (δ-*Proteobacteria*)
Gs=*Geobacter sulfurreducens* PCA (δ-*Proteobacteria*)
Gv=*Gloeobacter violaceus* PCC 7421(*Cyanobacteria*)
Go=*Gluconobacter oxydans* 621H (α-*Proteobacteria*)
Il=*Idiomarina loihiensis* L2TR (γ-*Proteobacteria*)
Mm=*Magnetospirillum magnetotacticum* MS-1 (α-*Proteobacteria*)
Md=*Microbulbifer degradans* 2-40 (γ-*Proteobacteria*)
Ms=*Mannheimia succiniciproducens* MBEL55E (γ-*Proteobacteria*)
Ml=*Mesorhizobium loti* (α-*Proteobacteria*)
Mf=*Methylobacillus flagellatus* KT (β-*Proteobacteria*)
Mc=*Methylococcus capsulatus* str. Bath (γ-*Proteobacteria*)
Na=*Novosphingobium aromaticivorans* DSM 12444 (α–*Proteobacteria*)
Ne=*Nitrosomonas europaea* ATCC 19718 (β-*Proteobacteria*)
Oi=*Oceanobacillus iheyensis* HTE831 (*Firmicutes*)
Pi=*Pirellula* sp. 1 (*Planctomycetes*)
Ps=*Pseudomonas syringae* (γ-*Proteobacteria*)
Pu=*Pseudomonas putida* KT2440 (γ-*Proteobacteria*)
Po=*Polaromonas* strain JS666 (β-*Proteobacteria*)
Pf=*Pseudomonas fluorescens* PfO-1 (γ-*Proteobacteria*)
Pp=*Photobacterium profundum* SS9 (γ-*Proteobacteria*)
Py=*Psychrobacter* sp. 273-4 (γ-*Proteobacteria*)
Re=*Ralstonia eutropha* JMP134 (β-*Proteobacteria*)
Rm=*Ralstonia metallidurans* CH34 (β-*Proteobacteria*)
Rs=*Ralstonia solanacearum* (β-*Proteobacteria*)
Rh=*Rhodobacter sphaeroides* (α-*Proteobacteria*)
Rp=*Rhodopseudomonas palustris* CGA009 (α-*Proteobacteria*)
Rg=*Rubrivivax gelatinosus* PM1 (β-*Proteobacteria*)
Si=*Silicibacter* sp. TM1040 (α-*Proteobacteria*)
Sm=*Sinorhizobium meliloti* 1021 (α-*Proteobacteria*)
So=*Shewanella oneidensis* MR-1 (γ-*Proteobacteria*)
Sv=*Shewanella violacea* (γ-*Proteobacteria*)
St=*Symbiobacterium thermophilum* IAM 14863 (*Actinobacteria*)
Td=*Thiobacillus denitrificans* ATCC 25259 (β-*Proteobacteria*)
Tt=*Thermoanaerobacter tengcongensis* MB4 (*Firmicutes*)
Vf=*Vibrio fischerei* (γ-*Proteobacteria*)
Xf=*Xylella fastidiosa* 9a5c (γ–*Proteobacteria*)

sediment study, weak acids could have accumulated in localized areas as the result of fermentation and/or respiratory processes. Energy production genes could encode proteins necessary for utilization of sediment resources as opposed to use of laboratory medium components. As an example, the *ccmF-2* gene identified in our study is involved in cytochrome *c* maturation, whereby heme is ligated to the apocytochrome (39). Transposon disruptions of *ccmF-1* (encoding protein that is 50% identical to CcmF-2) in another MR-1 study negatively impacted anaerobic respiration and cytochrome *c* production (6). While CcmF-1 was suggested to be the major heme lyase in MR-1 (6), the specific function of CcmF-2 is unknown.

Genes encoding products with transport functions could aid in bringing nutrients (e.g., iron, potassium, and phosphate, as implicated by our study) to the cell or in extrusion of foreign/secretion of self-made toxic compounds from within the cell. Furthermore, the extrusion/secretion function is part of a recurring theme, discussed in the next several paragraphs, where genes involved in various defensive mechanisms were found to enhance sediment survival.

As prior studies suggest, bacteriophage-related genes may defend the host cell from bacteriophage or closely related bacteria. *Spiroplasma citri* resistance to SVTS2 virus was conferred by viral DNA fragments that had integrated into the host's chromosomal DNA (40), a condition known as superinfection immunity (33). A *Rhizobium* strain was reported to produce defective phage particles which had bacteriocidal activity against closely related strains and which resembled the tails of *Escherichia coli* T-even phages (possessing an inner core surrounded by a sheath) (31). Our study identified a prophage tail sheath and several conserved hypothetical proteins (SO1442, 1443, and 2660) with

sequences related to viruses in other bacteria [e.g., CP4-57 cryptic P4-like prophage at min 57 in *E. coli* K12 (26)]. Induction of the lytic cycle of prophage genes also leads to release of lysozyme that could impact other phage-and lysozyme-sensitive bacteria and could provide more nutrients to the lysogen (host bacterium) (33).

Yet another bacteriophage-related gene and several insertion sequence-related genes were identified, and all may regulate transcription of genes in response to specific environmental signals. AlpA, a protein sharing 53% identity to the *E. coli* prophage CP4-57 regulatory protein (26, 46, 47), was implicated in sediment survival through our study. In *E. coli*, this protein regulates production of a protease that degrades aberrant proteins. Insertion elements are known to activate or inactivate nearby genes following transposition (5, 21). One example is where IS*D1* in *Desulfovibrio vulgaris* was found to inactivate a lethal gene (*sacB*) that was introduced into the genome of the sulfate-reducer (13). These previous observations and the identification of several transposon-related genes in our study suggest that random mutagenesis, whereby insertion sequence-related genes respond to sediment stress conditions by altering expression patterns, is one mechanism for increasing a subpopulation's chances of survival in sediment.

Several genes belonging to multidrug resistance (MDR) COGs were identified. These proteins are involved in extrusion of toxins from the cell. The most interesting of these MDR proteins was a gene encoding a protein with 72% identity at the amino acid level to pathogenic *Pseudomonas aeruginosa* PAO1 MexF (27). In *P. aeruginosa*, the MexE-MexF-OprN RND (resistance-nodulation-division) multidrug resistance system consists of a cytoplasmic membrane protein (MexF), an outer membrane protein (OprN) and a membrane fusion protein (MexE) (27). Hyperexpression of MexEF-OprN results in

antibiotic resistance (i.e., chloramphenicol and quinolones) (41). The gene for the HlyD family protein (SO0524) is located in an operon with a major facilitator (MF) superfamily transporter, a periplasmic protein having high similarity to the EmrB/QacA multidrug exporter subfamily (see reference 37 for a review on families of multidrug efflux systems). The TetR family regulatory protein (SO1703) that was identified in our study precedes and likely regulates an operon containing another HlyD family protein (within a multidrug resistance COG) and a permease and ATPase found within the COG for ABC-type multidrug transport systems. Repressors from the TetR family have also been implicated in multidrug resistance systems in *Pseudomonas* (10). The selection of these proteins that are potentially involved in the efflux of toxic substances emphasizes the importance of such genes for survival in sedimentary environments, where synthetic toxins may be present or where natural toxins may be produced by other microorganisms (7, 8, 30, 48-50) and/or from plants [e.g., flavonoids (16) and isoquinoline alkaloids like berberine (28)].

Several more genes identified in our study may be involved in further defenses against sediment stressors. HlyU has been described as a master regulator of virulence expression in pathogenic *Vibrio* (25). HlyU belongs to a family of small transcriptional repressors (e.g., SmtB, CadC, and ArsR) in which metal ion-binding induces a conformational change that prevents interaction of the regulators with DNA (3). The protein may function in regulation of metal ion efflux/detoxification in MR-1, and in a recent study, HlyU was shown to be highly upregulated (6.34- to 14.21-fold increase) when MR-1 is exposed to Co(III), Fe(III), and Mn(IV) (4).

Proteolysis plays a vital role in numerous cell functions, including defense

mechanisms, stress response, and adaptations to changing environments. The closest similarity to bacterial sequences of the subtilase family serine protease (SO4539) identified here is to the recently sequenced *S. amazonensis* SB2B and *Pseudoalteromonas atlantica* T6c (53 and 51% similarity, respectively); similarities also exist over a portion (~1000 to 1300 of MR-1's 1634 amino acids) of smaller proteins found in *Arabidopsis* and tomato plants, as well as *S. baltica* OS155, *Desulfuromonas acetoxidans* DSM 684, *Colwellia psychrerythraea* 34H, *Nocardioides* sp. JS614, *Arthrobacter* sp. FB24, and *Chloroflexus aurantiacus* J-10-fl (ranging from 41 to 49% similarity). Increased expression of one such *Arabidopsis* protease, ARA12 (46% similarity over its 757 amino acids to SO4539), in the presence of jasmonate [compound that induces wounding- and pathogen resistance-response in plants (11)] and cadmium, but not NaCl, suggested that this enzyme may play a role in plant responses toward specific stressors and pathogen stimuli (15). Another study described the upregulation of tomato subtilisin-like protease genes (47% similarity to SO4539 over 754 amino acids) in the presence of salicylic acid, a signaling molecule that induces plant defenses against pathogens (22).

The α-glucosidase produced by a gene identified in our study has some homology to the α-glucosidase of *Tannerella*, one of many asaccharolytic periodontal bacteria known to produce glycosidases. While function of the *Tannerella* α-glucosidase remains unknown, Hughes et al. suggests that the enzyme may modify host glycoproteins as a defense mechanism or as a way to provide nutritional resources to bacteria that can create a beneficial environment for *Tannerella* (20).

Finally, the gene for the toxin secretion, membrane fusion protein (SOA0050) identified here, while also sharing sequence similarities to proteins within the multidrug

resistance COG, is in an operon with SOA0049, a gene annotated as a toxin secretion ABC transporter, ATP-binding subunit/permease protein, putative. This protein is most similar to bacteriocin/lantibiotic exporters with peptidase activity; therefore, the operon may be involved in excretion of compounds with antimicrobial activity toward competitors.

We do not feel that our screen to date encompasses all possible sediment-impaired mutations that can result from mutagenesis of the entire MR-1 genome, although duplicate transposon insertions (within different sites of the gene) were observed for the serine protease gene (Table 1) and for *argH* (Table 2). Falling short of complete saturation is one possible explanation for why all genes that one might expect to be necessary for in situ survival were not identified. An example of genes that one would expect to result from this STM screen might include genes encoding chemotaxis proteins, which have been shown in past studies to aid in sediment survival (24). Indeed, one such chemotaxis protein (Table 1 and reference 17) was identified in our study. Such a finding helps to validate our sediment screen, while identification of *gspN* (Table 2) validates our medium growth screen. The g*spN* gene is within the same operon as g*spE (ferE)*, a gene described previously as being required for dissimilatory Fe(III) reduction in *Shewanella* (12). Other reasons to account for why we may not have identified all genes expected to be necessary for in situ survival are that many genes within MR-1 appear to be redundant. In addition, some tagged-transposon insertions could be located within parts of the gene that are not critical to overall function of translated protein.

With each genomic sequence that becomes available, investigators not only ponder why multitudes of genes are present for which no beneficial function is readily apparent,

but also the degree to which each gene is necessary for the organism's survival. Proteomic and microarray studies are being applied to answer these questions in pure cultures and defined laboratory conditions, but the STM approach we used (17) identified a list of genes whose importance for the in situ survival of environmental bacteria was previously unknown. Furthermore, these genes harbor high degrees of similarity to protein sequence found in other important environmental bacteria in various Phyla. This study also sheds new light on sediment survival, in that many genes, such as transposases and phage-related proteins, which are traditionally overlooked or attributed to general stress responses, seem to play a very significant role. Additionally, *mexF* and several other genes encoding protein products that may be involved in multidrug resistance were shown to enhance survival of a sedimentary bacterium.

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Chapter 4

Multidrug resistance function of the *mexF* gene in *Shewanella oneidensis* MR-1

Abstract

Previously, we reported on genes that enhanced survival of *Shewanella oneidensis* MR-1 in Fe(III)-reducing sediments. Here we investigate the function of sediment-survival genes putatively involved in multidrug resistance. Mutants containing transposon disruptions in genes for the TetR family regulator (SO1703), HlyD family protein (SO0524), MexF (SO3492), and toxin secretion protein (SOA0050) were grown in the presence of tetracycline or chloramphenicol; only growth of the *mexF* transposon mutant was impaired compared to the parent strain. Growth of an in-frame *mexF*-deletion mutant was also significantly impaired by chloramphenicol. Furthermore, it appears that wild type MR-1 becomes adapted to and can resist higher concentrations of chloramphenicol as a result of mutations in the gene encoding a TetR family repressor (SO3494). This gene lies just upstream of the *mexEF* operon in MR-1. In an effort to identify the natural substrate for MexF, we found that growth of the *mexF*-deletion mutant was not impaired relative to the wild type when exposed to either anthraquinone-2,6-disulfonate, sodium dodecylsulfate, ethidium bromide, or to various antibiotics, toxic metals, or organic acids; however, the chloramphenicol-adapted wild type had higher MICs than either the wild type or the *mexF*-deletion mutant with several compounds (e.g., norfloxacin, lincomycin, and nalidixic acid). MexF was subsequently shown to play a role in resistance to high concentrations of these drugs through growth curves

comparing the wild type, chloramphenicol-adapted wild type, and the *mexF*-deletion mutant. While our data demonstrates that *mexF* contributes to multidrug resistance in an environmentally relevant bacterium, additional substrates, and especially those that may be found in sediment, remain to be elucidated.

Introduction

Bacteria of environmental significance are capable of surviving under a variety of conditions, ranging from pristine sediments to highly toxic, contaminated regions of the subsurface. As little was known previously about the genes contributing to the survival of these organisms within the environment, we employed a modified version (15) of signature-tagged mutagenesis (STM) (17) with the model bacterium *Shewanella oneidensis* MR-1 (16, 39). MR-1 was chosen to represent bacteria of environmental significance based upon its ability to reduce Fe(III) (41), a natural electron acceptor found in a variety of anaerobic environments. MR-1 will also reduce radionuclides (31), such as U(VI) and Tc (VII), rendering this bacterium useful for bioremediation. By using the STM system, numerous genes required for bacterial survival in sediments were identified (J.L. Groh, Q. Luo, J.D. Ballard, and L.R. Krumholz, submitted for publication).

Interestingly, a surprising number of survival genes were related to multidrug resistance pumps (MDRs), systems gaining attention in the medical field for contributing to the high incidence of intrinsic and acquired antibiotic resistance of pathogens (see reference 45 for a recent review). These genes encode the TetR family regulator (SO1703), HlyD family protein (SO0524), and MexF (SO3492). Aside from similarities with other *Shewanella* species, the TetR family regulator shares highest similarity with an

uncharacterized *Vibrio vulnificus* AcrR/TetR family transcriptional regulator (70% similarity). It also precedes and likely regulates an operon containing another HlyD family protein (within a multidrug resistance COG) and a permease and ATPase found within the COG for ABC-type multidrug transport systems. Repressors from the TetR family have been implicated in multidrug resistance systems in *Pseudomonas* (10). The HlyD family protein (SO0524) shares 49% similarity with the *Escherichia coli* EmrA that is involved in multidrug resistance (32). It is located in an operon with a major facilitator (MF) superfamily transporter, a periplasmic protein having high similarity to the EmrB/QacA multidrug exporter subfamily (see reference 46 for a review on families of multidrug efflux systems). A toxin secretion protein (SOA0050) was also related to genes within the multidrug resistance Clusters of Orthologous Groups of proteins (COGs, www.ncbi.nlm.nih.gov/COG/) (59-61), but closest similarity is to *E. coli* MchE (44% similarity) that is involved in secretion of microcin H45, an antibiotic peptide (3). SOA0050 is also in an operon with SOA0049, a gene annotated as a toxin secretion ABC transporter, ATP-binding subunit/permease protein, putative. This protein is most similar to bacteriocin/lantibiotic exporters with peptidase activity, such as *E. coli* CvaB (61% similarity) that is involved in colicin V secretion (14); therefore, the operon may be involved in excretion of compounds with antimicrobial activity toward competitors.

Most notable of the MDR genes identified in our sediment-survival study was *mexF*, a gene encoding a protein that shares 72% identity with an MDR component present in pathogenic *Pseudomonas aeruginosa* PAO1 (24). In PAO1, the MexE-MexF-OprN RND (Resistance-Nodulation-cell Division superfamily of transporters) multidrug resistance system consists of a cytoplasmic membrane protein (MexF), an outer

membrane protein (OprN) and a membrane fusion protein (MexE) (24). Hyperexpression of the *mexEF-oprN* operon results in antibiotic resistance (i.e., chloramphenicol and quinolones) for *Pseudomonas* (11).

Proteins of the RND superfamily are divided among several subgroups, of which the most important for Gram-negative bacteria are heavy metal transporters (HME-RND family) or hydrophobic/amphiphilic transporters (HAE-RND family) (64). Aside from export of dyes, detergents, organic solvents and antibiotics, members of the HAE-RND transporter family have also been implicated in the export of endogenous substances [e.g., quorum sensing molecules (25) and pigments (24)]. One study has also shown that an operon encoding proteins with similarity to HAE-RND antibiotic efflux systems contributes to vanadium resistance of *P. aeruginosa* (1).

Within *S. oneidensis* MR-1, homologues of the AcrAB RND system were implicated, but not experimentally shown, to cooperate with TolC in resistance to the humic acid analogue, anthraquinone-2,6-disulfonate (AQDS) (56). TolC is known to interact with multiple drug efflux transporters (e.g., *Escherichia coli* EmrAB) by playing the role of the outer membrane protein (6). Recently it was shown that expression of MR-1 multidrug resistance genes *mexE* (SO3493) and *mexF* (SO3492) of the RND family increased in response to various forms of Co(III), Fe(III), and Mn(IV) (5). RND transporters upregulated by UV radiation in another MR-1 study included an authentic frameshift (SO4328), EmrB/QacA family protein (SO0525; in the same operon as the HlyD family protein identified in our sediment survival study), AcrB/AcrD/AcrF family proteins (SO1923-24), and HlyD family proteins (SO1925, SO4148) (47).

In spite of the insights provided by these MR-1 studies, the natural function of MDR genes in non-pathogens and pathogens alike remains unclear. While MDR systems are involved in the efflux of antibiotics and other toxins (e.g., organic solvents and detergents), this ability may play a role that is secondary to yet unknown natural functions (26, 53, 57). More importantly, alternative physiological roles [e.g., alkali tolerance (28)] may explain why antibiotic resistance can persist without selective pressures. The delineation of these roles may lead to the discovery of novel inhibitors of MDR systems, or at the very least, to a better understanding of the origins and persistence of these efflux proteins (26). In support of the alternative functions hypothesis, our previous results showed that MDR genes play an essential physiological role in a natural habitat (sediment) that, with the exception of organic solvent tolerance of *P. putida* (12, 48), differs from where many prior research efforts have focused (e.g., in host-pathogen interactions that result in selection of antibiotic resistant strains). In this study, we have characterized some MDR genes in an attempt to determine their function.

Materials and Methods

Sediment microcosms and signature-tagged mutagenesis procedures. Development of our STM system utilizing microarray technology, transposon mutagenesis of MR-1, sediment microcosm experiments, screening for impaired sediment survivors, and competition experiments to confirm impairment were described previously (15).

Antibiotic growth curves of strains containing transposon insertions in putative multidrug resistance genes that impact sediment survival. Growth of strains with

transposons in potential MDR transporters was monitored in aerobic Luria-Bertani (LB) medium, under stationary conditions (no shaking) at 30°C and compared with growth of the parent strain, MR-$1_{sediment}$ (15). Mid-log-phase cultures having an optical density measured at 600 nm (OD_{600}) of ~0.5 were used to inoculate 4 ml of medium with or without chloramphenicol or tetracycline. Volumes used for inocula were adjusted so that the starting OD_{600} for all cultures was ~0.03. For comparison of the *mexF*-deletion mutant and wild type MR-1 (39), strains were grown overnight from single colonies on LB plates streaked from glycerol stocks. These cultures were used as inocula (1:50 dilution) for LB containing chloramphenicol. Three culture tubes for each concentration of antibiotic were inoculated from the starter culture of all strains above.

In-frame *mexF*-deletion mutant construction. In-frame deletion mutagenesis of *mexF* in wild type MR-1 was performed according to a previously described protocol (30) that was modified (66) and kindly provided to us by Alex Beliaev, Bree Reed, and David Culley (Pacific Northwest National Laboratory). This research group also provided us with plasmid pDS3.1 (66).

For two separate asymmetric PCR reactions, primer pairs were constructed for sequence flanking *mexF* (mexF5'outer 5'-GTAGATGAGCAAACCTAC-3' used with mexF5'inner 5'-CGTTAGCTGCAGACCTAGGAAAAACTGCGACAACATTCT-3'; mexF3'inner 5'-TTCCTAGGTCTGCAGCTAACGAAGAGGACGCTAGAGGTC-3' used with mexF3'outer 5'-GCAACCATGCGTATATCT-3'). Oligonucleotide sequences that are non-complimentary to MR-1 genomic DNA, but which are complimentary to each other in order to fuse together in a later step, were designed in the inner primers (underlined portions) to create PCR products with 3' staggered ends.

The products of the two asymmetric PCRs were then used in crossover or fusion PCR with the mexF5'outer and mexF3'outer primers. In this PCR reaction, the underlined regions of the asymmetric products fuse together to form a single product. The fusion product of the expected size was gel purified (Qiagen gel purification kit) and used in a 10 μl A-tailing procedure (68). The A-tailing reaction was cleaned with MinElute PCR purification kit (Qiagen), and 50 ng was ligated into 150 ng XcmI digested pDS3.1 (Epicentre Fast-Link DNA ligation kit).

The product was transformed into electrocompetent EC100D pir-116 *E. coli* (Epicentre), and white colonies were screened by colony PCR (15) with mexF5'outer and mexF3'outer primers. This construct was transformed into electrocompetent *E. coli* strain β-2155 (19), which was then conjugated with wild type MR-1, as described previously (15). As pDS3.1 will not replicate in MR-1, FO and RO primers (5'-GCAGAGTGTACTAACCCATTTG-3' and 5'-GACCATTTCTGGTTCAAATAAG-3', respectively), designed just outside the mexF5' and mexF3' outer primers, were used in combination with the mexF5' and mexF3' outer primers in a PCR screen to confirm correct integration of the vector. Sucrose counter-selection was then used to select for strains in which the *mexF* gene was deleted (30, 66), and PCR with FO and RO primers was used for confirmation.

Sequencing potential mutations in the TetR family regulatory protein gene (SO3494) that precedes the *mexEF* operon. A single colony from a glycerol stock of wild type MR-1 was grown in LB medium to an OD_{600} of 0.6 and was then used as inoculum for four independent cultures grown in LB medium with 5 μg/ml chloramphenicol and one culture grown without antibiotic. After each culture adapted to

the chloramphenicol (~14 to 24 h following inoculation), having grown to an OD of approximately 0.41 to 0.48, 4 ml of each were centrifuged (10,000 X *g*, 10 min) for genomic isolation using the Invitrogen Easy DNA kit. Genomic DNA was also isolated from the control culture grown without antibiotics.

PCR was performed using 1 U Phusion High-Fidelity DNA Polymerase (Finnzymes), supplied PCR buffer (final concentration 1X), 0.2 mM deoxynucleoside triphosphate mix, and 0.5 μM forward and reverse primers. Primers were designed to flank the gene for the TetR family regulatory protein (SO3494) located directly upstream from the *mexEF* operon. The sequence for each primer was as follows: 5'-GGCAATTGAGAATTGAGGAGC-3' (TetF primer) and 5'-ATCAATTACCTTTATCGCAATCG-3' (TetR primer). Approximately 200 ng of template (from genomic isolations above) was used for each sample. PCR parameters were 98°C for 1.5 min, followed by 30 cycles of 98°C for 20 sec, 62°C for 30 sec, and 72°C for 30 sec. A final extension was performed at 72°C for 10 min. All PCRs were performed using the GeneAmp PCR System 9700 (Applied Biosystems) thermal cycler.

The PCR product (expected size of 694 bp) for each template was cloned into the pCR4-TOPO vector using the TOPO TA Cloning Kit for Sequencing (Invitrogen). From each cloning reaction, three resultant colonies were grown in LB supplemented with 50 μg/ml kanamycin. Plasmid was isolated from 5 ml of culture using the plasmid isolation kit from Qiagen. Sequencing was performed with the TetF and TetR primers, and resulting sequence was compared to the MR-1 genome sequence in NCBI.

Determination of other substrates for MR-1 MexF. To examine AQDS resistance, wild type MR-1 and the *mexF*-deletion mutant were grown in a 96-well plate containing

200 μl of LB medium and increasing concentrations of AQDS, in a manner similar to that performed previously with a *tolC* mutant of MR-1 (56). Concentrations of AQDS tested ranged from 0.1 mM to 20 mM, and inocula volumes were 1, 5, 10 and 20 μl of aerobic, LB cultures grown to an OD_{600} of approximately 1. The plate was sealed with parafilm and was incubated at 30°C for 15 h.

To determine if ferrihydrite reduction was affected by the *mexF* deletion, wild type MR-1 and the *mexF*-deletion mutant were grown in a minimal medium containing metal solution (12.5 ml/L) (27), 7 mM NaCl, 2 mM $MgCl_2$, 3.7 mM KH_2PO_4, 7.5 mM NH_4Cl, 0.34 mM $CaCl_2$, and 50 mM lactate. In a separate experiment, 0.005% yeast extract was also added. The medium was buffered with 25 mM Hepes and brought to a pH of 7 prior to autoclaving. The techniques of Hungate (22), as modified by Balch et. al (4) were used to prepare the medium anaerobically. Iron oxyhydroxide gel (500 μl), prepared as described previously (36), and 0.1 ml of an amino acid solution containing arginine (20 μg/ml), glutamine (20 μg/ml) and serine (40 μg/ml) were added from anoxic stocks to 10 ml of medium. The second transfers (200 μl of mid-log-phase culture) of wild type MR-1 and the *mexF*-deletion mutant through 10 ml of this medium either with or without yeast extract were monitored for production of Fe(II) using the ferrozine assay (35, 36).

We also determined the MIC for various other potentially toxic drugs using a modified dilution series method in 96-well microtiter plates (63). Cultures in LB were started from glycerol stocks of wild type MR-1, the *mexF*-deletion mutant, and two independent cultures of chloramphenicol-adapted wild type. After 16 h growth at 30°C, appropriate volumes were transferred to 4 ml of fresh LB, resulting in a start OD_{600} of 0.03. After 3 h of growth, appropriate volumes (~10 μl of the *mexF*-deletion mutant and the wild type;

~20 μl of chloramphenicol-adapted cultures) were transferred to 1 ml of LB to obtain a concentration of ~10^3 cells/μl. To inoculate each well, 10 μl of the ~10^3 cells/ul dilution was used (10^4 cells/well). The maximum concentration tested for each drug (contained in 200 μl of LB in the first well of each row) was as follows: 50 μg/ml nalidixic acid, 10 μg/ml tetracycline, 3200 μg/ml trimethoprim, 5.12 μg/ml norfloxacin, 51.2 μg/ml chloramphenicol, 2500 μg/ml carbenicillin, 256 μg/ml spectinomycin, 10240 μg/ml SDS, 256 μg/ml norfloxacin, 256 μg/ml erythromycin, 256 μg/ml puromycin, 800 μg/ml ethidium bromide, 100 mg/ml crystal violet, 2500 μg/ml lincomycin, 10000 μg/ml cholic acid, 2500 μg/ml oleandomycin, 25 μg/ml chlortetracycline, and 100 μg/ml proflavine. Each drug was added to 200 μl of LB in its own well (first column on the microtiter plate). Then 100 μl of this mixture was transferred through subsequent wells in the same row to create a dilution series (100 μl final volume of LB in each well), where following wells in the row contained one-half the concentration of drug in the preceding well. Plates were incubated for 24 h at 30°C. MICs were identified as the lowest concentrations that inhibited growth, and experiments were performed at least twice to confirm results. For some drugs, growth curves were performed, in a manner similar to growth curves with chloramphenicol, to compare wild type, the *mexF*-deletion mutant and the chloramphenicol-adapted cultures.

Using the same MIC dilution series, we also tested some compounds that were structurally similar to chloramphenicol (some of which are derived from plants or are soil-related compounds), in addition to some toxic metals. For these tests, we used the minimal LS medium described previously (15), but concentrations of yeast extract and KH_2PO_4 were decreased to 0.01% and 0.1 mM, respectively, to minimize metal

precipitation. The maximum concentration tested for the organic compounds (contained in the first well of each row) was 15 mM for ferulic acid, protocatechuic acid, caffeic acid, p-hydroxybenzaldehyde, salicylic acid, and trans-cinnamic acid. We used 20 mg/ml of Suwannee River fulvic acid standard (International Humic Substances Society) and 6 mM p-nitrophenol. Stock solutions of the organic compounds (0.5 M) were prepared in ethanol, except for the fulvic acid standard that was dissolved in water. The maximum ethanol concentration in any well was 3%, a concentration shown to be lower than the MIC for ethanol. The pH was adjusted with 0.1 M NaOH so that when stock solutions were added to the medium, the pH of the medium was no less than 6.5. For the metals, the maximum concentration tested was 10 mM $CuSO_4$, 1.6 mM $CuCl_2$, 25 mM Na_2HAsO_4, 10 mM $NaAsO_2$, 400 nM $HgCl_2$, 2 mM $PbCl_2$, 2 mM $CdCl_2$ and 2 mM $CoCl_2$. Metal stock solutions were prepared in water, and the volume of stock solution added did not change the starting pH of the medium.

Results

Growth of strains with transposon insertions in putative multidrug resistance genes impacting sediment survival. The *mexF*-transposon mutant was impaired at all chloramphenicol concentrations tested (Fig. 1). For tetracycline, growth of the *mexF*-transposon mutant was identical to the parent strain for 0.25 μg/ml (Fig 2B) and slightly impaired for 0.5 and 1 μg/ml (Fig. 2, C and D). The TetR family regulator (SO1703), the HlyD family protein (SO0524), and the toxin secretion protein (SOA0050) had growth curves that were similar to the parent strain, MR-$1_{sediment}$, in 0.5, 1.5, and 2.5

Figure 1. Growth of MR-$1_{sediment}$ (open symbols) and the STM strain carrying a transposon in *mexF* (filled symbols) in LB only (square symbols), LB plus 0.5 (circle symbols) LB plus 1.5 (triangle symbols), and LB plus 2.5 μg/ml (cross symbols) chloramphenicol. Data shown represent the average of three cultures. Error bars represent standard deviations.

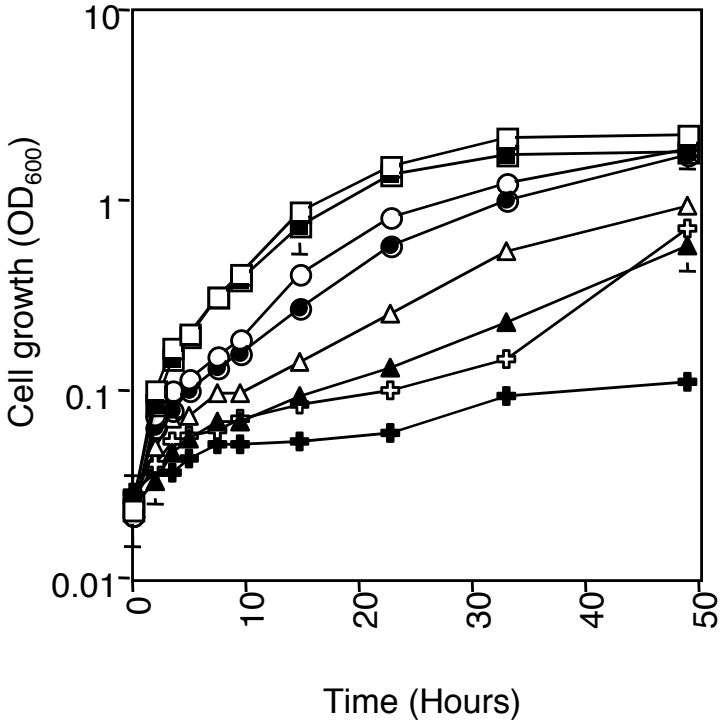
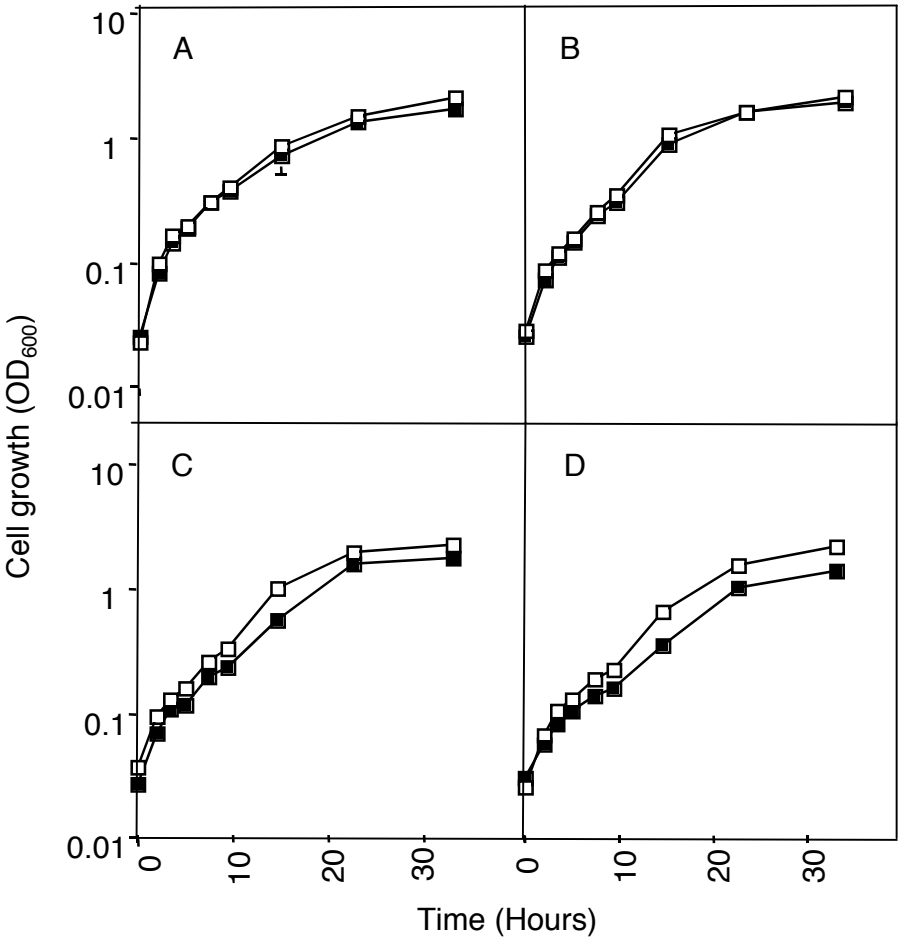


Figure 2. Growth of MR-$1_{sediment}$ (open symbols) and the STM strain carrying a transposon in *mexF* (filled symbols) in LB only (A), LB plus 0.25 (B), LB plus 0.5 (C), and LB plus 1 μg/ml (D) tetracycline. Data shown represent the average of three cultures. Error bars represent standard deviations but are often too small to be seen.



μg/ml chloramphenicol (data not shown) and in 0.25, 0.5, and 1 μg/ml tetracycline (data not shown). No further experiments were performed with these strains.

When we repeated the growth curves in chloramphenicol using the *mexF* in-frame deletion mutant and wild type MR-1, growth at the 0.5 and 1.5 μg/ml concentrations of chloramphenicol was similar (data not shown); however, at 2.5 and 5 μg/ml chloramphenicol, the *mexF*-deletion mutant was impaired (Fig. 3). Additionally, wild type growth was delayed in 5 μg/ml chloramphenicol, suggesting that a genetic variant may have grown up at higher antibiotic concentrations. Furthermore, we found that the adapted strain could grow without a lag in the presence of at least 10 μg/ml chloramphenicol, while the unadapted wild type strain died off rapidly at this concentration (Fig. 4). When MICs were performed with chloramphenicol, the adapted strain grew in up to nearly 50 ug/ml (Table 2).

Sequencing potential mutations in the TetR family regulatory protein gene (SO3494) that precedes the *mexEF* operon. Gene sequence for the TetR family protein in the control (wild type MR-1 that was grown in LB for the same length of time as the wild type strains that eventually became adapted to chloramphenicol) did not contain any mutations when compared to the MR-1 genome sequence deposited in the NCBI database. Various mutations were evident in this gene, however, for all cultures whose genomic DNA was isolated after adaptation to 5 μg/ml chloramphenicol occurred. Table 1 shows the mutations observed in all clones that were sequenced from among the four, independent chloramphenicol-adapted cultures. In general, mutations led to either premature terminator codons or a frameshift.

Figure 3. Growth of wild type MR-1 (open symbols) and the *mexF*-deletion mutant (filled symbols) in the presence of chloramphenicol. No difference was observed between the wild type and the *mexF*-deletion mutant at 0.5 and 1.5 μg/ml chloramphenicol. LB only is represented by circles in (A). Growth curves in 2.5 and 5 μg/ml chloramphenicol are shown in (A) and (B), respectively. Data shown represent the average of three cultures. Error bars represent standard deviations.

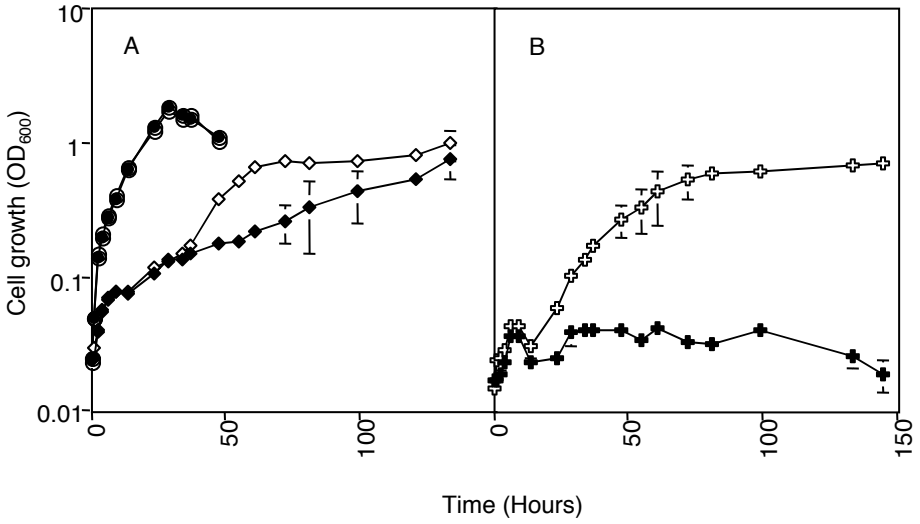


Figure 4. Growth of wild type MR-1 that is adapted to chloramphenicol (open symbols) versus growth of wild type MR-1 that has not been exposed to chloramphenicol (filled symbols). Concentrations tested include 5 (A), 7.5 (B), and 10 (C) μg/ml chloramphenicol. Data shown represent the average of three cultures. Error bars represent standard deviations.

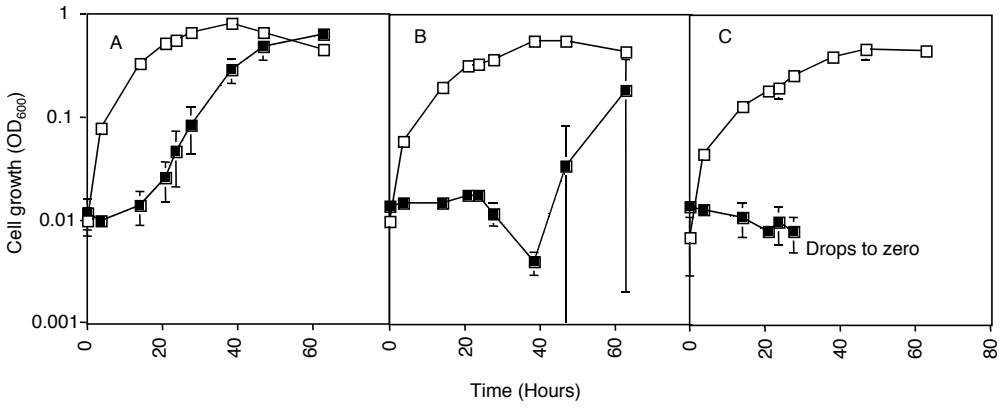


Table 1. Mutations in the gene encoding the TetR family regulatory protein (SO3494) that precedes the *mexEF* operon. These mutations were observed in four independent chloramphenicol-adapted wild type MR-1 cultures (called A, B, C and D). Three clones for each of the four cultures were obtained from PCR amplification of SO3494 and were sequenced to give results shown below.

Sequence change (bp position within gene)	Resultant mutation	Frequency observed	Mutant/Clone
Substitution of T for C (361)	Gln becomes a terminator codon	4	B/1, C/1, C/2, C/3
Substitution of T for G (403)	Glu becomes a terminator codon	1	D/3
Extra G inserted (411)	Frameshift	2	D/1, D/2
G missing (573)	Frameshift	3	A/1, A/2, A/3
8 bp missing (216-223)	Frameshift	3	A/2, B/2, B/3

Characterization of the response of MR-1 *mexF* to other toxins. Incubation of MR-1 in parafilm-sealed 96-well plates allowed anaerobic conditions to develop at the bottom of wells (56). This in turn allowed AQDS reduction to proceed. Reduction of AQDS, indicated by a change in medium color from yellow (uninoculated control) to varying shades of orange and red with increasing initial AQDS concentration, was identical with the wild type strain and the *mexF*-deletion mutant (data not shown).

Regarding metal toxicity/reduction, no difference was observed between wild type MR-1 and the *mexF*-deletion mutant grown on iron oxyhydroxide medium either with or without yeast extract added (based on changing coloration of iron medium and quantitation of Fe(II) produced) (data not shown). Additionally, MICs of other metals were similar between the *mexF*-deletion mutant, the wild type strain, and the chloramphenicol-adapted wild type culture. MICs for all three cultures were as follows: 200 nM $HgCl_2$, 1.25 mM $CuSO_4$, 0.8 mM $CuCl_2$, 0.2 mM Na_2HAsO_4, 0.625 mM $NaAsO_2$, and 1 mM for $CdCl_2$, $PbCl_2$, and $CoCl_2$. A similar $NaAsO_2$ MIC was reported for MR-1 previously (51); the MICs for $CuCl_2$ and Na_2HAsO_4 are similar to values reported for *P. putida* and *Deinococcus radiodurans* (49); and the MIC for $HgCl_2$ is a little less than the 500 nM concentration that caused a lag in growth of *Sulfolobus* (52). The actual MIC for $PbCl_2$ was probably lower than we report as some precipitation was observed after addition of the $PbCl_2$ stock to medium in the first well of the 96-well plate. Precipitation that may have occurred, but which was not directly observed, may also explain why the MICs for $CdCl_2$ and $CoCl_2$ are also quite high compared to MICs reported for *P. putida* and *D. radiodurans* (49).

The wild type and the *mexF*-deletion mutant exhibited the same MIC patterns for 17

of 18 drugs tested; only for puromycin did wild type have a higher MIC than the *mexF*-deletion mutant (Table 2), but growth curves conducted with 150 and 250 μg/ml puromycin could not confirm impairment of the *mexF*-deletion mutant by this antibiotic (data not shown). The two chloramphenicol-adapted wild type cultures exhibited higher MICs than the wild type and the *mexF*-deletion mutant for crystal violet, norfloxacin, lincomycin, nalidixic acid, puromycin, thiamphenicol and carbenicillin. When exposed to concentrations near the MIC of the chloramphenicol-adapted wild type culture, the *mexF*-deletion mutant did not grow as well as either the wild type or the chloramphenicol-adapted wild type culture in medium containing norfloxacin, lincomycin, or nalidixic acid (Fig. 5). The wild type strain was able to grow after a lag period that is similar to its lag in the presence of 5 μg/ml chloramphenicol (Fig. 3B).

MICs determined for fulvic acid and other organic compounds with structures that are similar to chloramphenicol were mostly similar between the wild type strain, the *mexF*-deletion mutant, and the chloramphenicol-adapted wild type culture (Table 3). At 24 hours, the MICs for the chloramphenicol-adapted wild type culture were lower than the wild type and the *mexF*-deletion mutant for the ethanol control, p- hydroxybenzaldehyde, and fulvic acid, but by 48 hours, the MICs were the same for all three MR-1 cultures.

Discussion

While annotation indicated that several genes identified as sediment survival genes encoded protein products with putative roles as components or regulators of multidrug/toxin efflux, we found that only the *mexF* mutant appeared to contribute to

Table 2. MICs of various drugs tested on wild type MR-1, the *mexF*-deletion mutant, and chloramphenicol-adapted wild type cultures. Concentrations listed are in μg/ml. Two independent chloramphenicol-adapted cultures produced the same MICs for all drugs tested by our method.

	Wild type MR-1	*mexF*-deletion mutant	chloramphenicol-adapted wild type
Crystal violet	0.4	0.4	3.125
Ethidium bromide	100	100	100
SDS	5120	5120	5120
Novobiocin	32	32	32
Erythromycin	1	1	1
Puromycin	128	64	>256
Norfloxacin	0.64	0.64	2.56
Lincomycin	625	625	2500
Cholic acid	>10000	>10000	>10000
Oleandomycin	78	78	78
Chlortetracycline	0.78	0.78	0.78
Proflavine	3.125	3.125	3.125
Nalidixic acid	0.195	0.195	0.78
Trimethoprim	25	25	25
Spectinomycin	8	8	8
Chloramphenicol	1.6	1.6	51.2
Carbenicillin	9.75	9.75	19.5
Thiamphenicol	7.8	7.8	>125

Figure 5. Growth of wild type MR-1 (square symbols), wild type MR-1 that is adapted to chloramphenicol (circle symbols), and the *mexF*-deletion mutant (diamond symbols) in LB medium only (A, filled symbols), or LB medium containing 1.2 mg/ml lincomycin (A, open symbols), 0.6 μg/ml nalidixic acid (B) or 1.5 μg/ml norfloxacin (C). Data shown represent the average of three cultures, and error bars represent standard deviations.

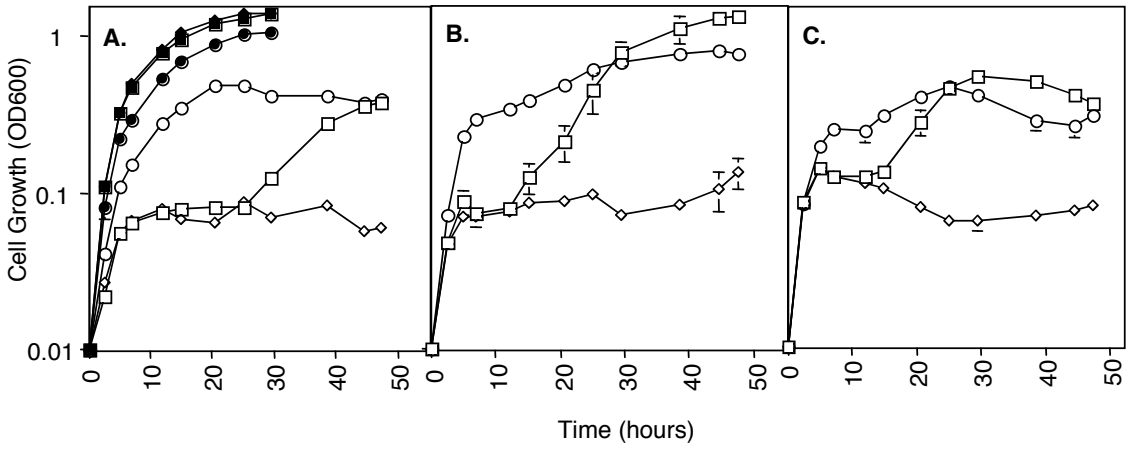


Table 3. MICs of fulvic acid and other compounds structurally related to chloramphenicol that were tested on wild type MR-1, the *mexF*-deletion mutant, and chloramphenicol-adapted wild type cultures. Concentrations listed are in mM, unless otherwise indicated. Two independent chloramphenicol-adapted cultures produced the same MICs for all compounds tested by our method.

	Wild type MR-1	*mexF* deletion mutant	chloramphenicol-adapted wild type
Ferulic acid	15	15	15
Protocatechuic acid	15	15	15
Caffeic acid	7.5	7.5	7.5
Salicylic acid	0.059	0.059	0.059
p-nitrophenol	0.094	0.094	0.094
p-hydroxybenzaldehyde	3.75	3.75	1.9
Fulvic acid	10 mg/ml	10 mg/ml	5 mg/ml
Trans-cinnamic acid	0.94	0.94	0.94
Pthalic acid	>15	>15	>15
Ethanol control	>6%	>6%	6%

tetracycline or chloramphenicol resistance. This finding is not surprising in that while multidrug systems have broad specificities, selectivity still appears to exist and resides with the RND transporter component in the case of Mex pumps (38, 58). Tests with more potential antimicrobials might yield the substrates for the other three putative MDR proteins encoded by genes that enhanced sediment survival of MR-1. Also, we are aware that while the toxin secretion protein (SOA0050) shares sequence similarities to proteins within the multidrug resistance COG, it is most similar to bacteriocin/lantibiotic exporters with peptidase activity and may actually excrete endogenous compounds into the environment to aid in the organism's sediment survival.

After the preliminary antibiotic growth tests, we chose to focus on the *mexF* mutant and on attempts to elucidate potential substrates for the protein encoded by this gene. MexF has been shown to provide the substrate specificity of the multidrug resistance system MexE-MexF-OprN in *Pseudomonas* species (2, 38). Studies indicate that *mexF* contributes to resistance/tolerance of chloramphenicol, trimethoprim, imipenem, fluoroquinolones (e.g., norfloxacin, nalidixic acid) (11, 24), organic solvents (29), dyes (13), and triclosan (9). MexEF-OprN also appears to participate in export of a molecule(s) required for the production of homoserine lactone, a compound involved in quorum-sensing processes (25).

Both the *mexF*-transposon mutant obtained through STM experiments in a prior study (15) and the *mexF*-deletion mutant constructed in this study were significantly impaired when grown in the presence of chloramphenicol. While there was no difference in growth with 0.5 and 1.5 μg/ml chloramphenicol, at 5 μg/ml, the deletion mutant was effectively unable to grow, while wild type MR-1 grew appreciably after a lag period.

The ability of the wild type and the *mexF*-deletion mutant to grow at the lower concentrations may be the result of another system that responds to chloramphenicol. One possible explanation is that MR-1 may be able to acetylate, and thus inactivate, lower concentrations of chloramphenicol by the chloramphenicol acetyltransferase (54, 55) encoded in its genome (SO4299). It is clear, however, that for higher concentrations, the *mexF* gene is required for growth to occur.

The initial delay in growth of wild type with 5 μg/ml chloramphenicol suggests that a mutation, perhaps in a regulatory protein(s), may have been necessary in order for the MexEF system to operate in MR-1. Most of the RND-type efflux systems in *Pseudomonas* are regulated by nearby repressor genes. In the case of the *mexEF-oprN* operon, however, expression is controlled by a positive LysR family regulator encoded by the *mexT* gene (23). It is interesting that this expression results from *mexT* mutations that suppress inactivating *mexT* mutations commonly found in many strains termed wild type *P. aeruginosa* PAO1 (37). In strains that do carry a functional *mexT*, mutation(s) in yet another gene, *mexS*, are vital for the hyperexpression of *mexEF-oprN* that results in resistant phenotypes (57).

The *mexEF* operon in MR-1 does not have a gene upstream encoding product similar to MexT or to MexS, but a TetR family regulator (SO3494) is located just upstream from *mexE*. The chloramphenicol-adapted wild type strain in our study appears to have mutations in this gene that promote the chloramphenicol-resistance profile exhibited in Fig. 3. This protein family binds DNA via a helix-turn-helix motif and includes AcrR, EnvR, QacR, MtrR, and TetC. Many function as repressors that control a cell's response toward hydrophobic antibiotics and detergents (www.sanger.ac.uk/cgi-

bin/Pfam/getacc?PF00440). Deletion of MtrR resulted in increased antibiotic resistance in *Neisseria gonorrhoeae* (44). Expression of another *Pseudomonas* MDR system, MexJK, is also controlled by an upstream negative regulator of the TetR family that is encoded by *mexL*; mutations in MexL render a nonfunctional repressor protein that subsequently allows the expression of MexJK and the occurrence of a triclosan-resistant phenotype in *Pseudomonas* (10).

Additionally, SO3494 shares 56% sequence identity at the amino acid level to a TetR family regulator (RPA3021) in *Rhodopseudomonas palustris* CGA009, and the gene for the *R. palustris* regulator also precedes genes annotated as *mexEF*. Other organisms with a homologous TetR family regulator and nearby genes encoding HlyD family proteins putatively involved in hydrophobic/amphiphilic compounds include *Shewanella putrefaciens* CN32, *S. baltica* OS155, *Pseudoalteromonas atlantica* T6c, *S. denitrificans* OS-217, *S. frigidimarina* NCIMB 400, and *Bradyrhizobium japonicum*. This suggests that many environmental bacteria have MDR genes and associated regulators for controlling production of MDR systems in a manner similar to MR-1's *mexEF* system.

Success with chloramphenicol led us to test more antibiotics and other potentially inhibitory compounds, such as detergents and dyes. We also tested fulvic acids and other compounds that may be found in sediments and that are structurally similar to chloramphenicol. We found that some compounds that serve as substrates for MexF in *Pseudomonas* species (i.e., norfloxacin, nalidixic acid, and chloramphenicol) appear to do the same in MR-1, while others (trimethoprim) do not. Surprisingly, organic compounds that share similar structural features with chloramphenicol did not alter the MIC of the *mexF*-deletion mutant when compared to the wild type. Salicylate, one of these

compounds, has been suggested as a natural substrate for the *Burkholderia cenocepacia* efflux pump that shares 73% and 71% similarity at the amino acid sequence level with MexF in *Pseudomonas* species and MR-1, respectively (40).

Lastly, some heavy metal efflux transporters, such as CzcA in *Alcaligenes eutrophus* (42, 43), belong to the RND superfamily (50, 64). A recent study found that *mexE* and *mexF* expression increased in MR-1 that was exposed to metal electron acceptors versus fumarate (5). We found that the *mexF*-deletion mutant grew as well as wild type MR-1 in Fe(III)-citrate (J.L. Groh, Q. Luo, J.D. Ballard, and L.R. Krumholz, submitted for publication), and in this study, the deletion mutant produced the same amount of Fe(II) from Fe(III) oxyhydroxide as the wild type strain. From our results, it also appears that *mexF* is not involved in reduction/resistance to AQDS, a humic acid analogue, or to fulvic acid itself. Humic acids are prevalent in sediments and may play an important role as electron shuttles for bacterial reduction of insoluble metals, such as Fe(III) (34). As suggested previously, AcrAB, and not MexEF, may interact with TolC to efflux AQDS from the cell (56). It is also worth noting that mutations in TolC, which may interact with MDR inner membrane and fusion protein transporters to achieve efflux of toxic compounds, while shown to be involved in resistance of MR-1 to AQDS toxicity, did not affect reduction of all Fe(III) forms (56). Additionally, a variety of toxic metals appear to have no effect on the *mexF*-deletion mutant when compared to either the wild type MR-1 or to chloramphenicol-adapted wild type culture. This is not too surprising, because the RND family is broad and has several specific branches (64), with MexF belonging to the HAE-RND (hydrophobic and amphiphilic compound efflux) family that exports organic

compounds. To our knowledge, this is the first time MICs for many of these metals have been reported for MR-1.

In conclusion, the true natural substrates for many MDR systems remain unclear (53); however, our initial STM sediment screen indicates that some compounds present in sediment may be used as a substrate by these systems and that these systems are necessary for survival, paralleling studies in pathogens, such as *Pseudomonas* species, where the loss of various Mex transporters decreased its capacity to invade kidney epithelial cells or to induce virulence in mice (20). Similarly, it is still unclear, but interesting as to why both pathogenic and nonpathogenic bacteria contain analogous chromosomally-encoded MDR systems. In the natural environment, as with our sediment studies, these transporters may efflux naturally occurring antimicrobial compounds [e.g., plant exudates (21, 62) and products released by antagonistic bacteria or fungi competing for the same environmental resources or niche (7, 8, 33, 65, 67)], but they may also protect the cell against toxic accumulation of structurally related compounds, such as humic acids (56) and other natural electron shuttles (18). Additionally, the substrate may be intracellular, such as quorum sensing molecules (25) and pigments (e.g., pyocyanin) (24). Although further questions still remain, it is clear that *mexF* in MR-1 is involved in multidrug resistance. Once determined, the elusive answers to questions surrounding MDR systems in general may help to combat antibiotic resistance through increased knowledge of the molecular structures, regulatory mechanisms, and natural substrates of these extraordinary systems (53).

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Appendix 1.

Desulfovibrio sp. genes involved in the respiration of sulfate during metabolism of hydrogen and lactate

Abstract

To develop a better understanding of respiration by sulfate-reducing bacteria, we examined transcriptional control of respiratory genes during growth with lactate or hydrogen as an electron donor. RNA extracts of *Desulfovibrio desulfuricans* subsp. *aestuarii* were analyzed by using random arbitrarily primed PCR. RNA was reverse transcribed under low-stringency conditions with a set of random primers, and candidate cDNAs were cloned, sequenced and characterized by BLAST analysis. Putative differentially expressed transcripts were confirmed by Northern blot analysis. Interestingly, dissimilatory bisulfite reductase was upregulated in the presence of hydrogen. To link these transcriptional changes to the physiology of sulfate-reducing bacteria, sulfide was measured during growth of several strains of *Desulfovibrio* on hydrogen or lactate, and this revealed that hydrogen-grown cells produced more sulfide per unit of cell mass than lactate-grown cells. Transcription of other redox proteins was characterized by Northern blotting to determine whether or not they were also transcribed to higher levels in hydrogen-grown cells. Growth on lactate produced greater transcription of [NiFe] hydrogenase. H_2-grown cells transcribed the adenylylsulfate reductase b subunit and HmcA to higher levels. The results we describe herein provide new insight into the continuing debate over how *Desulfovibrio* species utilize redox

components to generate membrane potential and to channel electrons to sulfate, the final electron acceptor.

Introduction

Sulfate reducing bacteria (SRB) are a diverse group of microorganisms found in a variety of anaerobic environments, and all members possess the ability to use sulfate as a terminal electron acceptor. SRB can use a variety of organic electron donors, including hydrogen, lactate, formate, malate, fumarate, pyruvate, alcohols, and even environmental contaminants (13). While progress has been made in understanding the biochemistry of proteins involved in respiratory processes, to date little is known about how such proteins are utilized by the SRB to gain energy for cell growth.

For years, a debate over the mechanism by which lactate is oxidized in SRB has existed. In 1981, Odom and Peck proposed the hydrogen cycling model for growth on lactate (12). In this model, electrons from lactate are used by a cytoplasmic hydrogenase to generate hydrogen that can diffuse out across the cell membrane to be utilized by periplasmic dehydrogenases. Membrane potential is generated as protons remain in the periplasm while electrons are transferred across the cell membrane to reduce sulfate. When only hydrogen is utilized as an electron donor, it is likely oxidized in the periplasm by hydrogenases, but may use different electron carriers in the reduction of sulfate than electrons generated during lactate oxidation.

We know that lactate dehydrogenase, pyruvate-ferredoxin oxidoreductase, phosphotransacetylase, and acetate kinase convert lactate to acetate and that ATP sulfurylase, pyrophosphatase, adenosine-5'-phosphosulfate (APS) reductase, and bisulfite reductase are responsible for linking electrons produced with sulfate reduction (13).

Unfortunately, the identity of electron carriers involved in lactate and hydrogen metabolism remains elusive and has prompted a few biochemical and molecular studies in recent years in hopes of better defining this process. Voordouw et al. (18) examined the distribution of cytoplasmic and periplasmic hydrogenases for 22 *Desulfovibrio* species and determined that only the genes for periplasmic [NiFe] hydrogenase were present in all species surveyed. This finding challenged the hydrogen cycling model, which requires SRB to possess a cytoplasmic hydrogenase (possibly a [NiFeSe] hydrogenase) in addition to a periplasmic hydrogenase. Discovery of *hmc* operon in *D. vulgaris* Hildenborough offered a solution as to how electrons in the periplasm could reach the cytoplasmic sulfate reduction enzymes (14, 15). By using antibodies to HmcA and HmcF, expression of *hmc* operon in *D. vulgaris* was found to be highest during growth on hydrogen (8). Deletion of the *hmc* operon in *D. vulgaris* (Hildenborough) impaired growth on hydrogen but not on lactate or pyruvate, confirming the importance of the Hmc complex in electron transport from hydrogen in the periplasm to sulfate in the cytoplasm (4).

This study was originally intended to evaluate the applicability of random arbitrarily primed (RAP-PCR) to environmental bacteria that reduce sulfate, but initial findings led us to probe further into differential expression of SRB redox proteins. We used a combination of RAP-PCR and Northern blotting to identify genes that were differentially transcribed under conditions of growth with either hydrogen or lactate as an electron donor. After the observation that bisulfite reductase was transcribed to higher levels in hydrogen-grown cells than in lactate-grown cells, differential transcription of other

known redox proteins, including [NiFe] hydrogenase and HmcA, was characterized by Northern blotting.

Materials and Methods

Bacterial strains and growth conditions. *Desulfovibrio desulfuricans* Essex 6 (freshwater strain) and *Desulfovibrio desulfuricans* subsp. *aestuarii* (marine strain) were from the culture collection of Dr. Michael McInerney. *Desulfovibrio sp*. strain ASR was obtained from Bradley Tebo at Scripps Institute. Cells were grown in mineral media containing 10 mM Na_2SO_4, 0.2% yeast extract, 0.0001% resazurin as a redox indicator, and vitamins, minerals, and metals solutions prepared as described by Krumholz et al. (9). *Desulfovibrio desulfuricans* subsp. *aestuarii* was used in RAP-PCR experiments, while Essex 6 and ASR were used in a sulfide production assay. For the marine strains, 342 mM NaCl and 14.76 mM $MgCl_2$ were added to the mineral medium. Media were prepared using the techniques of Hungate (6) as modified by Balch et al. (1). The headspace was N_2-CO_2 (4:1). After boiling and cooling, 42 mM sodium bicarbonate and 1 mM sodium sulfide were added from sterile stock solutions. For comparison of hydrogen versus lactate as an electron donor, 25 mM lactate was added to the medium before autoclaving, or 10 ml of H_2 per 10 ml of medium was added to the headspace after autoclaving. Growth curves were performed at 30°C and 37°C, with measurements of optical density at 600 nm taken periodically with a spectrophotometer. Sulfide was quantitated with the dimethyl-phenylene diamine assay (3).

RNA extraction. Total RNA was isolated as described by Shepard and Gilmore (17) with minor modifications to the procedure. Mid-log-phase cells for *D. desulfuricans* strain Essex 6 and late log cells for *D. desulfuricans* subsp. *aestuarii* were harvested by

centrifugation for 5 minutes at 7,000 x *g*. The pellet was resuspended in 1 ml Tri Reagent (Sigma) and transferred to sterile tubes containing 0.5 ml 100-μM-diameter zirconia-silicon beads. Cells were broken in a Mini-Beadbeater (Biospec Products) for 1 min. The supernatant was extracted with 300 μl chloroform and placed on ice for 15 min. After centrifugation for 10 min (12,000 x *g*), nucleic acids in the aqueous phase were precipitated with 750 μl of isopropyl alcohol and placed on ice for 10 min. Following centrifugation and a 75% ethanol wash, pellets were resuspended in 200 μl of diethyl pyrocarbonate (DEPC)-treated water. Contaminating genomic DNA was removed by addition of 22 μl Multi Core Restriction Enzyme Buffer (Promega) and 5 U of RNase-free DNase (Stratagene) with incubation at 37°C for 15 min. Following this treatment, 500 μl phenol-chloroform-isoamyl alcohol was added and centrifuged (12,000 x *g*, 10 min). The aqueous phase was removed, and the phenolic phase was extracted a second time with 250 μl of DEPC-treated water. After centrifugation, this second aqueous phase was mixed with the first, and RNA was precipitated with 1 ml of 100% ethanol for at least 30 min at –80°C. RNA was pelleted by centrifugation (12,000 x *g*, 30 min), washed with 75% ethanol, resuspended in 0.1 mM EDTA, and stored at –80°C. Integrity of RNA was determined by 0.8% agarose electrophoresis in Tris-borate-EDTA buffer, and the concentration was determined by measuring the A_{260}/A_{280} ratio spectrophotometrically (16).

RAP-PCR. RAP-PCR was performed as described by Shepard and Gilmore (17). For each reaction, 14.5 μl containing 1 μg of total RNA, diluted in DEPC-treated water when necessary, was heated to 70°C for 10 min and placed on ice. After a 1-min incubation on ice, 2 μl of reverse transcription buffer (Fisher), 40 U of RNase Block

RNase Inhibitor (Stratagene), a 1.25 mM concentrations of each deoxynucleoside triphosphate, and 1.25 μM concentration of arbitrary primer (Stratagene) were added. After the contents were mixed, the reaction mixture was held at 37°C for 5 min. After equilibration, 25 U of Moloney murine leukemia virus reverse transcriptase (RT) (Stratagene) was added. First-strand cDNA synthesis occurred at 37°C for 1 h, and then the reaction mixture was heated to 90°C for 5 min to inactivate the RT and placed on ice for 10 min. For second-strand synthesis, 10 μl of a 1:10 dilution of first-strand cDNA product was mixed with 39.8 μl of standard PCR mix containing *Taq* buffer without $MgCl_2$ (Sigma), 3 mM $MgCl_2$, a 50 μM concentration of each deoxynucleoside triphosphate, 10 μCi of [α-^{33}P]dCTP, and a 1 μM concentration of the same arbitrary primer used in the first-strand synthesis for a final reaction volume of 50 μl. The reaction mixture was heated to 96°C for 10 min following overlay with 50 μl of light mineral oil. After incubation at 36°C for 15 min, 1 U of *Taq* polymerase (Sigma) was added to each reaction mixture. The reaction continued to equilibrate for another 15 min at 36°C, followed by incubation at 72°C for 5 min. For the remaining 39 cycles of PCR, the following parameters were used: 94°C (1 min), 50°C (1 min), 72°C (2 min), and a final extension at 72°C for 10 min. The reaction mixture was stored at 4°C. Products were resolved on a 6% polyacrylamide gel (Life Technologies) prepared in Tris-borate-EDTA buffer and run at 1,500 V until the xylene cyanol dye migrated to the bottom of the gel. The gel was transferred to 3MW paper (Midwest Scientific, Valley Park, Mo.) and dried under vacuum at 70°C for 45 min. The gel was exposed to Kodak BioMax MR film for 12 h at room temperature. Putative differentially transcribed bands were excised from the gel, eluted from the filter paper with elution buffer (0.5 M ammonium acetate, 10 mM

magnesium acetate, 1 mM EDTA, 0.1% sodium dodecyl sulfate), precipitated with 100% ethanol, and resuspended in 10 μl of sterile water. The cDNA was then reamplified with PCR parameters used in second-strand synthesis and resolved on a 6% polyacrylamide gel with the original RAP-PCR product for comparison to ensure that the correct band was isolated. The candidate PCR product was ligated into pCR4-TOPO vector and transformed into chemically competent One Shot TOP10 *Escherichia coli* (Invitrogen). Plasmid was isolated from 2 ml of liquid cultures of candidate clones grown in Luria-Bertani broth plus ampicillin (50 μg/ml) by using a miniprep plasmid isolation kit (Qiagen) according to the manufacturer's directions. DNA sequencing was carried out at the Oklahoma Medical Research Foundation Core Sequencing Facility. Typically three clones were sequenced per transformation. Candidate inserts ranged in size from 328 to 1,026 bases. GenBank sequence comparison was performed by both nucleotide and protein BLAST searches (19)**.**

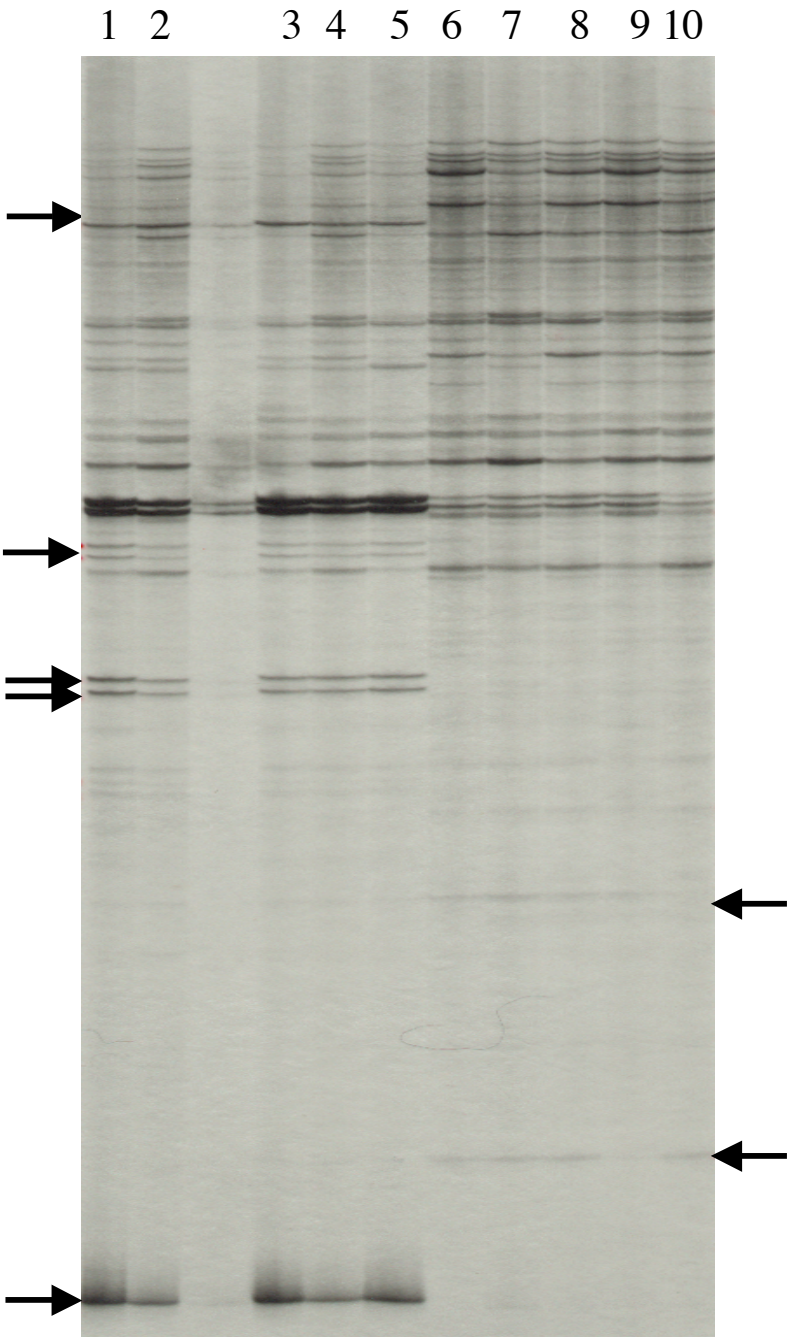
Confirmation of differential gene transcription. RNA probes were prepared from plasmids isolated from RAP-PCR clones. Plasmids were first digested with NotI or PstI to linearize DNA prior to transcription. Linearized DNA (0.2 μg) in RNA polymerase buffer (Ambion); 4 mM (each) ATP, CTP and GTP; and [α-^{32}P]UTP was incubated with either T3 DNA-dependant RNA polymerase or T7 DNA-dependant RNA polymerase (depending on orientation) at 37°C for 60 min. RNase-free DNase (1U) was added and left for an additional 30 min to remove plasmid and template DNAs. The mixture was diluted 20-fold, and unincorporated radiolabeled nucleotide was separated from the probe with a Sephadex G-50 column. At least 10^6 cpm of labeled RNA probe was added to each hybridization mixture. For the RNA blots, 20 μg of total RNA from both growth

conditions was loaded onto a 1.5% agarose gel containing 15% formaldehyde and 1X MOPS [3-(N-morpholino)propanesulfonic acid]. RNA was electrophoresed for 2 h at 150 V with 0.5X MOPS as the running buffer. The gel was transferred overnight with a Turboblotter to Nytran SuPerCharge nylon membranes according to the directions of the manufacturer (Schleicher and Schuell). The membrane was cross-linked to RNA with UV at 125 mJ/cm^2 for 1 min. The membrane was then exposed to a standard prehybridization buffer for 6 h at 65°C and then to hybridization buffer and probe for 12 h at 65°C. Washings were performed with 1X SSPE (0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA; [pH 7.7]) with 0.1% sodium dodecyl sulfate at room temperature (3 times for 5 min each) and at 65°C (3 times for 30 min each). Blots were imaged with a Molecular Dynamics Storm PhosphorImager and a Packard Instant Imager.

Results

RNA extraction and RAP-PCR. The RNA extraction yielded intact RNA which appeared as three bands representing 23S, 16S, and 5S rRNAs following electrophoresis (data not shown). Random primers A3 and A1 (Stratagene) created several RAP-PCR products that were unique to conditions of growth with either hydrogen or lactate (Fig. 1). As expected, a majority of bands were shared by both growth conditions. Products which were unique to either condition occurred reproducibly from at least five independent cultures grown with hydrogen and five independent cultures grown with lactate. Products also did not match bands derived from contaminating genomic DNA in the no-RT control reaction (data not shown). When products were first isolated, re-amplified, and run on a gel again to verify that the correct band had been removed, several bands of various sizes appeared in each lane, suggesting that nearby contaminating bands had

Figure 1. DNA products derived from RAP-PCR of total RNA from five independent cultures of *D. desulfuricans* subsp. *aestuarii* grown with either hydrogen (lanes 1 to 5) or lactate (lanes 6 to 10) as the electron donor. Arrows indicate cDNA products of differentially transcribed mRNA.



been excised in addition to the band of interest. The correct band, determined by comparison to the original RAP-PCR product mix run in an adjacent lane, was once again isolated from this second gel and used in transformation of competent *E. coli* cells.

Sequence identification and confirmation of RAP-PCR bands. Table 1 lists results where cDNA showed sequence similarity in GenBank using nucleotide and protein BLAST searches. Cloned insert sizes ranged from 328 to 1,026 bp. Putative differentially transcribed genes had significant similarity to dissimilatory bisulfite reductase, F_1F_0 ATP synthase and ATP sulfurylase from *D. vulgaris* in hydrogen-grown cells and to genes for *Bacillus subtilis* folate biosynthesis protein, *Clostridium perfringens* molybdopterin biosynthesis protein, and *Campylobacter jejuni* malate dehydrogenase in lactate-grown cells.

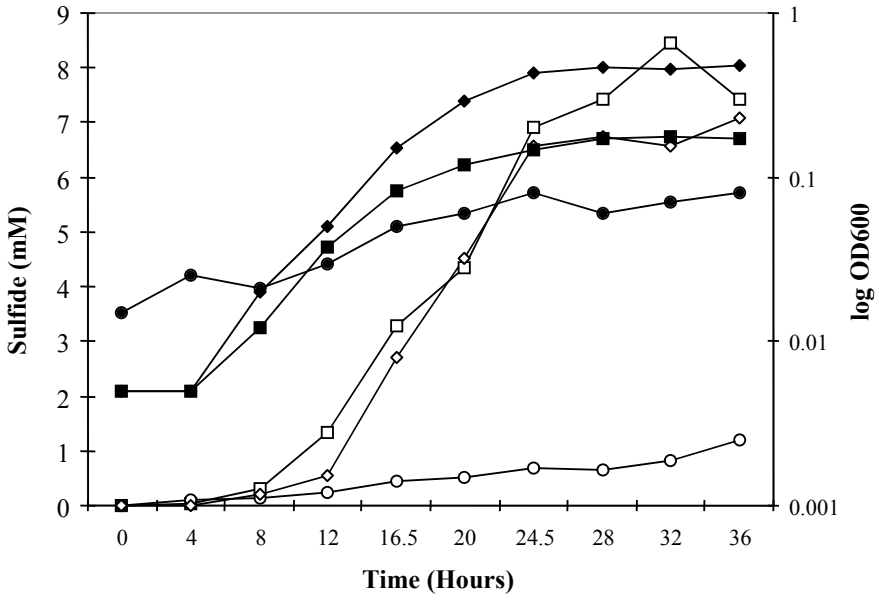
Growth experiments. To provide further support that the bisulfite reductase gene and perhaps other respiratory protein genes were more highly transcribed during growth on hydrogen than on lactate, sulfide assays were performed over the growth curve of several *D. desulfuricans* strains. Figure 2 shows that hydrogen-grown *D. desulfuricans* subsp. *aestuarii* produced more sulfide relative to cell concentration (determined by optical density measurements) than lactate-grown cells, suggesting that transcription of the bisulfite reductase gene could have been increased, in turn increasing expression of the bisulfite reductase protein. Similar results were observed when sulfide production was monitored over a growth curve for *D. desulfuricans* strain Essex 6 and *Desulfovibrio* sp. strain ASR (data not shown). Through these growth curves, yeast extract (0.2%) included in the medium to support growth, appeared to serve as an electron donor to a

Table 1. DNA sequence homology of differentially transcribed bands obtained by the RAP-PCR procedure

Electron donor	Band size (bp)	Similar gene sequence (accession no.)	Lowest-sum probability score	Type of BLAST search	Confirmed by Northern blotting	Increase in trans-cription[a]
Hydrogen	1026	*D. vulgaris* F_1F_0 ATPase subunit b (AB022018)	$8x10^{-24}$	BLAST X	No	---
Lactate	606	*B. subtilis* folate biosynthesis protein (F37854)	$1x10^{-23}$	BLAST X	Yes	104%
Lactate	986	*Campylobacter jejuni* malate dehydrogenase (H81336)	$2x10^{-57}$	BLAST X	Yes	634%
Hydrogen	328	*D. desulfuricans* subsp. *aestuarii* dissimilatory bisulfite reductase (AJ289157)	$1x10^{-168}$	BLAST N	Yes	203%
Hydrogen	507	*Entamoeba histolytica* ATP sulfurylase (AB013399)	$1x10^{-30}$	BLAST X	No	---
Lactate	507	*C. perfringens* molybdopterin biosynthesis protein (BAA76927)	$8x10^{-24}$	BLAST X	Yes	451%

[a]The observed differences between growth conditions with lactate and hydrogen were found to be statistically significant at the 5% level using Student's *t* test.

Figure 2. Sulfide production in *D. desulfuricans* subsp. *aestuarii* throughout growth curve in mineral media with 10 mM sulfate added. Growth was monitored spectrophotometrically (OD_{600}) with either H_2 (filled squares), lactate (filled diamonds) or neither (filled circles) as the electron donor. Sulfide production was monitored by dimethyl-phenylene diamine assay throughout growth on hydrogen (open squares), lactate (open diamonds), or neither electron donor (open circles). Yeast extract (0.2%) was included in all media.



small extent, resulting in the production of some sulfide in the absence of either H_2 or lactate (Fig. 2).

Northern Blotting. In order to confirm differential transcription of genes identified during RAP-PCR, Northern blotting was performed. Total RNA extracts from *D. desulfuricans* subsp. *aestuarii* were incubated with a ^{32}P-labeled RNA probe constructed from the cDNA clone resulting from RAP-PCR. Figure 3 shows the Northern blot for the RAP-PCR band with sequence similarity to dissimilatory bisulfite reductase gene. The results demonstrate that cells grown with hydrogen as the electron donor (lanes 4 to 6) produced higher levels of mRNA for this gene than cells grown without hydrogen. Northern blots also confirmed that malate dehydrogenase, molybdopterin biosynthesis protein and folate biosynthesis protein (2-amino-4-hydroxy-6-hydroxymethyldihydropteridine pyrophosphokinase) were more highly transcribed under growth conditions with lactate (data not shown). We were not able to confirm differential transcription of F_1F_0 ATPase and ATP sulfurylase by Northern blotting.

Differential expression of *D. desulfuricans* strain Essex 6 redox proteins. Differential transcription of bisulfite reductase and growth results showing increased sulfate reduction activity during growth on H_2 led us to examine whether or not mRNAs encoding other proteins involved in redox reactions were also differentially transcribed. Northern blotting was carried out with total RNA isolated from cells grown with either hydrogen or lactate. Probes were synthesized from known gene sequences to strain Essex 6 redox proteins shown in Table 2. Adenylylsulfate reductase b subunit and HmcA mRNAs were more highly transcribed in hydrogen-grown cells, while mRNA for the small subunit of [NiFe] hydrogenase was more highly transcribed in lactate-grown cells.

Figure 3. Northern blot confirmation of differential transcription of dissimilatory bisulfite reductase mRNA. Each lane represents 20 μg of total RNA extracted from three independent cultures of *D. desulfuricans* subsp. *aestuarii* grown with neither electron donor (lanes 1 to 3), with hydrogen (lanes 4 to 6) or with lactate (lanes 7 to 10). The blot was incubated with a ^{32}P-labeled RNA probe constructed from a cDNA clone resulting from RAP-PCR and corresponding to the gene sequence for dissimilatory bisulfite reductase.

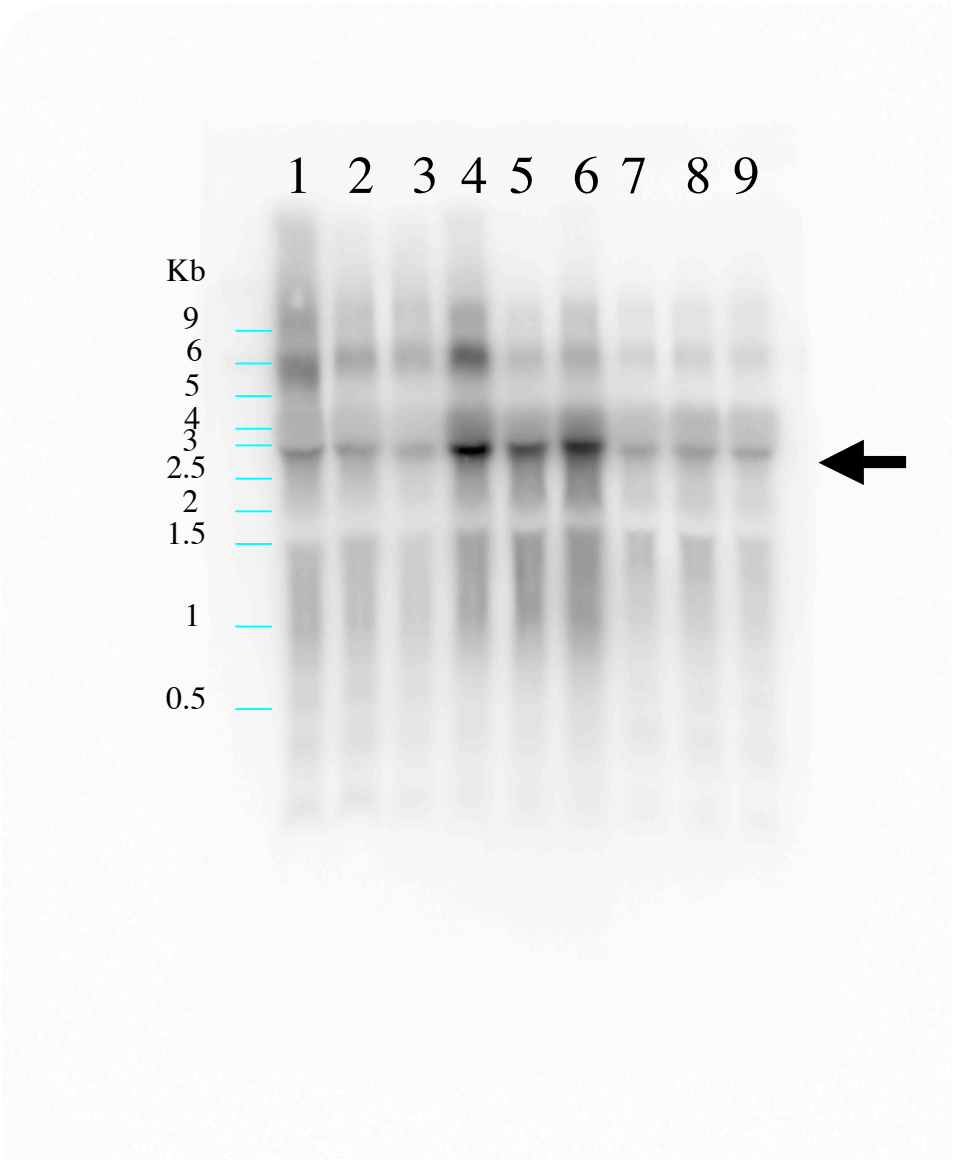


Table 2. Differential transcription of *D. desulfuricans* strain Essex 6 redox proteins with either hydrogen or lactate serving as an electron donor.

Redox Protein (accession no.)	Probe size (bp)	Growth condition of higher transcription	% Increase[a]
Flavodoxin (X59438)	430	None	---
HmcA (AF127653)	820	Hydrogen	84
Ni-Fe hydrogenase small subunit (Af216303)	680	Lactate	132
Adenylylsulfate reductase b subunit (AF226708)	480	Hydrogen	55

[a]The observed differences between growth conditions with lactate and hydrogen were found to be statistically significant at the 5% level using Student's *t* test.

Flavodoxin was not differentially transcribed under the conditions tested.

Discussion

This study was originally intended to evaluate the applicability of RAP-PCR to environmental bacteria that reduce sulfate. The conditions tested were chosen because we were interested in examining mRNA involved in growth on different electron donors. In the past, biochemical and genetic studies with mutants have provided some insight into how *Desulfovibrio* species use these as electron donors. Our study shows that several mRNA species involved in either redox reactions or carbon metabolism are differentially transcribed under these conditions. Sequences for malate dehydrogenase have strong similarity to lactate dehydrogenase (2, 5, 10). Since the sequence for the SRB lactate dehydrogenase is not available in GenBank, the RAP-PCR band that was larger in lactate-grown cells could be from mRNA encoding lactate dehydrogenase, rather than a malate dehydrogenase. The other proteins more highly transcribed during lactate metabolism may come as a result of using lactate as a carbon source as opposed to the CO_2 or yeast extract components utilized by hydrogen-grown cells. Molybdopterin is a component of molybdenum cofactors that participate in redox reactions (11), and its increased transcription in lactate-grown cells may come as a result of increased expression of the redox protein for which it serves as a cofactor. Known molybdopterin-containing enzymes in SRB include aldehyde oxidoreductases, formate dehydrogenase, and nitrate reductase (11). Less-well-characterized molybdenum proteins have been isolated but not classified (11). The genes for these proteins may or may not represent the gene for the redox protein more highly transcribed in lactate-grown *D. desulfuricans*

subsp. *aestuarii*. In the yeast cell *Pichia canadensis,* molybdopterin biosynthesis was found to branch from the folic acid biosynthetic pathway at dihydrohydroxymethylpterin (7). As molybdopterin biosynthesis likely occurs in a similar manner in *D. desulfuricans* subsp. *aestuarii,* increased transcription genes for folic acid and molybdopterin biosynthesis proteins during growth on lactate may be linked.

Increased transcription of the F_1F_0 ATP synthase and ATP sulfurylase during H_2 growth could not be confirmed by Northern blot analysis. However, we did confirm that dissimilatory bisulfite reductase and adenylylsulfate reductase are transcribed to higher levels in hydrogen-grown cells. It is likely that ATP sulfurylase, one of the four cytoplasmic enzymes involved in sulfate reduction by an eight electron transfer, and F_1F_o ATPase were differentially transcribed, but that the Northern blotting was not effective either because of mRNA instability or because of lack of sensitivity. Transcripts with slight levels of differential transcription that RAP-PCR can detect but Northern blots cannot may require more sensitive techniques, such as real-time PCR, for confirmation.

As a follow-up to our findings with dissimilatory bisulfite reductase, we tested whether other redox proteins would be differentially transcribed under conditions of growth with hydrogen. Sequence information for genes encoding several redox proteins was available for *D. desulfuricans* strain Essex 6. Specific primer pairs were designed for flavodoxin, HmcA, [NiFe] hydrogenase small subunit, and adenylylsulfate reductase b subunit. We were unable to amplify these sequences from *D. desulfuricans* subsp. *aestuarii* genomic DNA but were able to amplify and construct probes from Essex 6 DNA. Because the same phenomenon of increased sulfide production during growth on H_2 versus lactate occurred in Essex 6 as in the marine strain, other redox proteins can be

expected to behave in the same manner in *D. desulfuricans* Essex 6 as in *D. desulfuricans* subsp. *aestuarii*. Northern blots with *D. desulfuricans* Essex 6 RNA demonstrated that not all putative electron transport related proteins were transcribed to higher levels when hydrogen was used as an electron donor (Table 2). These results may indicate that certain redox proteins are not involved in hydrogen- or lactate-driven electron transport or that these proteins may not be carrying out rate-limiting reactions. Higher transcription of periplasmic [NiFe] hydrogenase in lactate-grown cells could suggest that this protein plays an important role in metabolism of H_2 or in the production of H_2 by *D. desulfuricans* Essex 6 from lactate, lending support to the hydrogen-cycling model. HmcA expression to higher levels during growth on H_2 was also demonstrated by Keon et al. (8) through Western blotting.

This study helps in revealing the extent to which different redox proteins play a role in either hydrogen or lactate metabolism. Coupled with previous genetic and biochemical studies, these results may help us come to understand the different pathways employed in the metabolism of lactate or hydrogen in these organisms. From the success of this study, we also feel that RAP-PCR can be applied to other questions of environmental significance, such as the identification of genes that are upregulated in the presence of environmental contaminants.

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