APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 2007, p. 492–498 Vol. 73, No. 2
0099-2240/07/$08.00+0 doi:10.1128/AEM.01086-06

Genes That Enhance the Ecological Fitness of *Shewanella oneidensis* MR-1 in Sediments Reveal the Value of Antibiotic Resistance▽†

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Received 11 May 2006/Accepted 7 November 2006

Environmental bacteria persist in various habitats, yet little is known about the genes that contribute to growth and survival in their respective ecological niches. Signature-tagged mutagenesis (STM) of *Shewanella oneidensis* MR-1 coupled with a screen involving incubations of mutant strains in anoxic aquifer sediments allowed us to identify 47 genes that enhance fitness in sediments. Gene functions inferred from annotations provide us with insight into physiological and ecological processes that environmental bacteria use while growing in sediment ecosystems. Identification of the *mexF* gene and other potential membrane efflux components by STM demonstrated that homologues of multidrug resistance genes present in pathogens are required for sediment fitness of nonpathogenic bacteria. Further studies with a *mexF* deletion mutant demonstrated that the multidrug resistance pump encoded by *mexF* is required for resistance to antibiotics, including chloramphenicol and tetracycline. Chloramphenicol-adapted cultures exhibited mutations in the gene encoding a TetR family regulatory protein, indicating a role for this protein in regulating expression of the *mexEF* operon. The relative importance of *mexF* for sediment fitness suggests that antibiotic efflux may be a required process for bacteria living in sediment systems.

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 molecular to microscopic levels. Some information can be ascertained from studies that monitor microbial communities (33) or dominant respiratory processes (23), 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 environment is often not possible. From an environmental standpoint, an in situ perspective of bacterial fitness 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).

In this study, 47 genes that aid in bacterial ecological fitness in sediments were identified using in situ-based signature-tagged mutagenesis (STM) (12). STM is one technique by which whole genomes are screened for genes that enable bacteria to survive in their natural habitats. Genes involved in general cellular processes of DNA repair, transport, transcriptional regulation, and energy and amino acid metabolism, as well as genes encoding phage-related and transposon-related proteins, were identified as helpful for sediment fitness.

Of all the mutants detected, genes annotated as involved in multidrug resistance (MDR) (31) became the focus of this study. This group was of particular interest given that there has been little research investigating the role of MDR genes in fitness of bacteria in sediment. Efflux pumps encoded by MDR genes have recently garnered attention for their contribution to antibiotic resistance in pathogens. Multidrug transporters appear to be ubiquitous in nature, comprising greater than 10% of all microbial transporters, with the highest concentrations found in soil and plant-associated microbes (30).

The objective of the STM study described herein was to identify genes required for sediment fitness of *Shewanella oneidensis* MR-1. The genes identified might suggest modifications for current bioremediation strategies utilizing *Shewanella* species and/or similar anaerobic bacteria. As early results of this study suggested that antibiotic resistance mechanisms play an essential physiological role in a natural habitat, the focus of the study became the identification of substrates for MexF, a protein encoded by one of the fitness genes.

MATERIALS AND METHODS

Sediment microcosms and STM procedures. Development of our microarray-based STM system, transposon mutagenesis of *Shewanella oneidensis* MR-1, sediment microcosm experiments, and screening for impaired sediment survivors were described previously (12).

Briefly, we constructed a transposon mutant library using *S. oneidensis* MR-$1_{sediment}$, a strain that was inoculated into and recovered from sediment microcosms prior to mutagenesis (12). Sixty MR-$1_{sediment}$ mutants, each containing a uniquely tagged transposon, were assembled per mutant pool. Each pool was first screened in aerobic minimal medium containing lactate and then anaerobic medium containing lactate-Fe(III)-citrate prior to inoculation into sediment microcosms, thus ensuring that sediment attenuation was not attributable to a general growth defect. Microcosms consisted of 25-ml serum bottles and Fe(III)-reducing sediments (2 g anoxic subsurface sediments amended with 200 μmol

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 17 November 2006.

iron oxyhydroxide and lactate [20 μmol] in order to stimulate some growth of MR-1). Sediments underlying a landfill in Norman, OK (3), were collected in June 2002 and stored at 4°C prior to use within 2 months of collection. Lactate was chosen as an electron donor, as the addition of lactate was required to stimulate growth and survival of MR-1 in nonsterile sediments from both lake and subsurface sources (J. L. Groh and L. R. Krumholz, unpublished data).

After approximately 5 to 6 days of incubation, surviving mutants were isolated from the sediments on Luria-Bertani (LB) spread plates (12). No further colonies were obtained from a second extraction of sediment (data not shown), indicating that all recoverable cells were obtained in the first extraction. This screen competes mutants with endogenous microorganisms in as natural a system as is technically possible for this type of study. Recovery on LB plates incubated aerobically was compared with recovery on 9,10-anthraquinone-2,6-disulfonic acid (AQDS) plates incubated anaerobically, and no differences in colony counts were obtained (data not shown). Microarray hybridization technology (12) was then used to label and visualize the unique tags from mutants in the inoculum and in the pool of colonies isolated from sediment. Attenuated mutants were identified as having tags present in the initial inoculum but absent after a period of sediment incubation.

Mutants found by our hybridization method to be attenuated in sediment fitness were confirmed in sediment competition experiments where individual mutants were incubated with only the *S. oneidensis* MR-$1_{sediment}$ strain. Results in sediment and laboratory medium are reported (see Table S1 in the supplemental material) in terms of a competitive index (CI) as used in previous pathogenic STM studies (5). The CI is a measure of the output ratio of the mutant to parent strain/input ratio of the mutant to parent strain. A CI which is approximately equal to or greater than 1 is not considered impaired in sediment growth.

Arbitrary PCR (12) was used to determine the sequence surrounding the transposon insertion site of impaired sediment mutants (see Table S1 in the supplemental material). A past study using this transposon system with *S. oneidensis* MR-1 (28) and a Southern blot performed in our lab confirmed that insertion of the modified Tn*10* 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 involved in sediment fitness were also analyzed using MicrobesOnline (http://www.microbesonline.org/) (1) and Clusters of Orthologous Groups of proteins (COGs) (www.ncbi.nlm.nih.gov/COG/) (40). In order to identify operons, we used the method of operon prediction (32) on the MicrobesOnline database.

Antibiotic growth curves of strains containing transposon insertions in putative multidrug resistance genes that impact sediment fitness. Growth of strains with transposons in potential MDR transporters was monitored in aerobic LB medium under stationary conditions (no shaking) at 30°C and compared with growth of the parent strain, MR-$1_{sediment}$ (12). Mid-log-phase cultures were used to inoculate triplicate cultures with or without chloramphenicol or tetracycline. For comparison of the *mexF* deletion mutant and wild-type MR-1 (27), strains were grown overnight from single colonies on LB plates streaked from frozen glycerol stocks. These cultures were used as inocula (1:50) for triplicate LB cultures containing chloramphenicol.

In-frame *mexF* deletion mutant construction. In-frame deletion mutagenesis of *mexF* in wild-type MR-1 was as previously described (21, 46). The pDS3.1 plasmid was kindly provided to us by Alex Beliaev (Pacific Northwest National Laboratory). For two separate asymmetric PCRs, 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 noncomplementary to MR-1 genomic DNA but that are complementary 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 and complementary ends. The products of the two asymmetric PCRs were then used in crossover or fusion PCR with the outer primers. Here, the underlined regions of the asymmetric products fuse together to form a single product. This product was gel purified and used in an A-tailing procedure (48) prior to ligation into XcmI-digested pDS3.1.

The recombinant plasmid was then transformed into electrocompetent EC100D *pir*-116 *Escherichia coli* (Epicenter), and white colonies were screened by colony PCR (12) with outer primers. This construct was transformed into *E. coli* strain β-2155 (13), which was then conjugated with wild-type *S. oneidensis* MR-1, as described previously (12). The construct will not replicate in MR-1. FO and RO primers (5′-GCAGAGTGTACTAACCCATTTG-3′ and 5′-GACCATTTCTGGTTCAAATAAG-3′, respectively), designed to bind just outside the outer priming sites, were used with the outer primers in a PCR screen to confirm correct integration in the vector. Sucrose counterselection was then used to select for deletion of the *mexF* gene (21, 46) and confirmed by PCR with FO and RO primers.

Generation and sequencing of 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 mid-log phase and was then used as inoculum for four independent cultures grown in LB medium with 5 μg/ml chloramphenicol and one culture grown without the antibiotic. After adapting to chloramphenicol (~14 to 24 h following inoculation; optical density at 600 nm of between 0.41 to 0.48), each culture was centrifuged (10,000 × *g*, 10 min) for genomic DNA isolation. Genomic DNA was also isolated from the control culture grown without antibiotics. Primers flanking the gene for the TetR family regulatory protein (SO3494) located directly upstream from the *mexEF* operon were used in PCRs (see Fig. S1 in the supplemental material). The sequence for each primer was 5′-GGCAATTGAGAATTGAGGAGC-3′ and 5′-ATCAATTACCTTTATCGCAATCG-3′. The PCR product of expected size was cloned and sequenced.

Determination of other substrates for *S. oneidensis* 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 (39).

To determine whether ferrihydrite reduction was affected by the *mexF* deletion, wild-type MR-1 and the *mexF* deletion mutant were grown in an anaerobic minimal medium with iron oxyhydroxide (see the supplemental material for preparation), either with or without yeast extract. Cultures were monitored for production of Fe(II) using the ferrozine assay (24).

We also determined the MICs for various other potentially toxic substances (antibiotics, phenolic compounds, and metals) using a modified dilution series method in 96-well microtiter plates (42) inoculated with 10^4 cells/well. The maximum concentration tested for each substance can be found in the supplemental material. Plates were incubated at 30°C and were monitored for turbidity at 24 and 48 h. MICs were identified as the lowest concentrations that inhibited growth. For some drugs, growth curves were performed in a manner similar to growth curves with chloramphenicol to compare the wild type, the *mexF* deletion mutant, and the chloramphenicol-adapted cultures.

RESULTS

Genes that aid the growth of *S. oneidensis* MR-1 in anoxic sediments. Of approximately 5,000 transposon mutants screened in sediments, 47 genes involved in general cellular processes of DNA repair, transport, transcriptional regulation, and energy and amino acid metabolism, as well as genes encoding phage-related and transposon-related proteins were identified as providing fitness in sediments (see Table S1 in the supplemental material). When we consider the functions of these genes, we gain a perspective on cellular processes needed for growth in natural sediments. Several of these processes are considered below in the Discussion section. These 47 genes are among the 60 genes mentioned but not described that validated the modified STM procedure presented as a new method in a previous publication (12). Of the 60 mutants, 5 were excluded from this present discussion because the CI was approximately equal to or greater than 1. Graphical representations of the competition experiments for both the *mexF* and *ccmF-2* transposon mutant demonstrate impaired sediment fitness (Fig. 1). The eight remaining genes (Table 1) 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 (7).

Growth of strains with transposon insertions in putative multidrug resistance genes impacting sediment growth. We first focused our efforts on the genes annotated to be involved

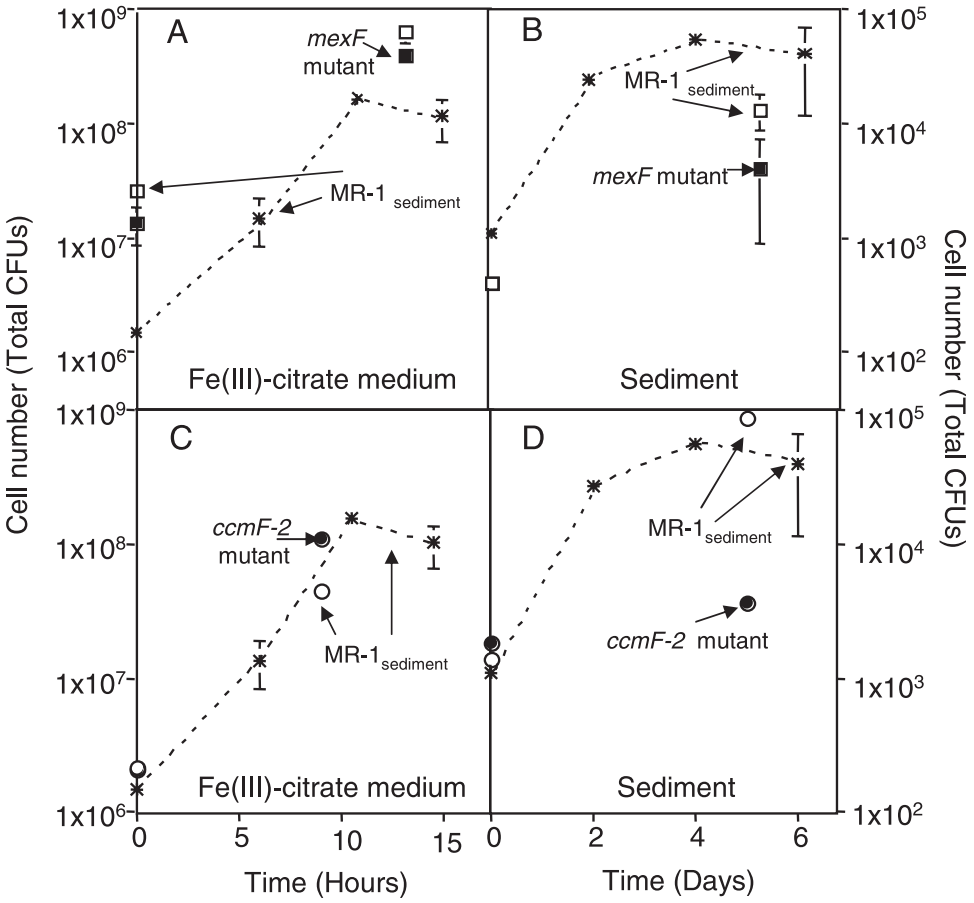

FIG. 1. Competition experiments between the *S. oneidensis* MR-$1_{sediment}$ strain and the *mexF* transposon mutant or the *ccmF-2* transposon mutant carried out in lactate-Fe(III)-citrate medium (A and C) and in sediment microcosms (B and D). In competition experiments, each transposon mutant and the parent strain were grown together. The dashed line and asterisks show growth of the MR-$1_{sediment}$ strain incubated alone. The latter results are shown in each panel to facilitate the comparison between the wild-type growth alone relative to the *mexF* mutant and to the *ccmF-2* mutant. Error bars represent standard deviations but are too small to be visible in most cases.

in multidrug resistance (MDR). While it is known that MDR genes are important in pathogen fitness (14) and that sediments can harbor opportunistic pathogens (11), there has been little research on the role of MDR genes in survival of sediment-dwelling bacteria. The *mexF* transposon mutant was shown to be impaired at 5 μg/ml chloramphenicol, the highest concentration tested (Fig. 2 shows the *mexF* in-frame deletion mutant). Growth of the *mexF* transposon mutant in tetracycline was identical to the parent strain at 0.25 μg/ml and slightly impaired at 0.5 and 1 μg/ml (data not shown). The

TABLE 1. List of interrupted genes that led to attenuation in both sediment and lactate-Fe(III)-citrate medium and competitive index for each gene under each condition

Gene product (gene name, if available)	TIGR locus	Competitive index in:	
		Sediment microcosm	Lactate-Fe(III)-citrate medium
General secretion protein (*gspN*)	SO0176[a]	0.28 ± 0.27	0.2 ± 0.12
Argininosuccinate synthase (*argG*)	SO0278[a]	0.061 ± 0.03	0.69 ± 0.2
Argininosuccinate lyase (*argH*)	SO0279[a]	0.036 ± 0.03	0.39 ± 0.01
Argininosuccinate lyase (*argH*)	SO0279[a]	0.073 ± 0.04	0.3 ± 0.16
Argininosuccinate lyase (*argH*)	SO0279[a]	0.009 ± 0.01	ND[b]
Methyltetrathydrofolate-homocysteine methyltransferase (*metH*)	SO1030	0.66 ± 0.38	ND
Glyceraldehyde 3-phosphate dehydrogenase (*gapA-3*)	SO2347	0.23 ± 0.01	ND
Phosphate acetyltransferase	SO2916[a]	0.011	0.003
Asparagine synthetase B, glutamine-hydrolyzing (*asnB-1*)	SO2767	0.68 ± 0.22	ND
Threonine synthase (*thrC*)	SO3413[a]	0.017 ± 0.016	ND

[a] The gene is predicted to be located within an operon.
[b] ND, not determined. Competition experiments in growth medium were deemed unnecessary, as poor growth of the mutant was observed in medium prior to sediment inoculation.

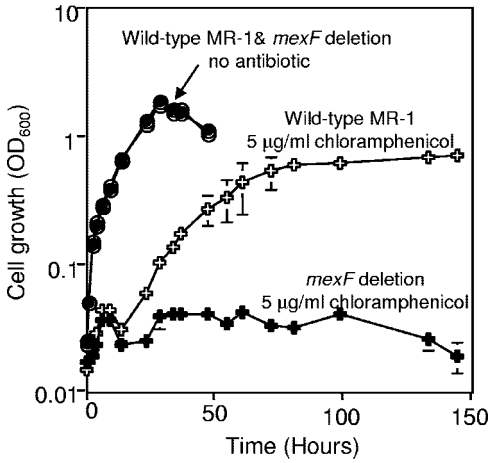

FIG. 2. Growth of wild-type *S. oneidensis* MR-1 and the *mexF* deletion mutant in LB alone or in LB with 5 μg/ml chloramphenicol. No difference was observed between the wild type and the *mexF* deletion mutant at 0.5 and 1.5 μg/ml chloramphenicol (not shown). Data shown represent the averages of three cultures. Error bars represent standard deviations. OD_{600}, optical density at 600 nm.

TetR family regulator (SO1703), the HlyD family protein (SO0524), and the toxin secretion protein (SOA0050), all putatively involved in drug efflux, grew similarly to the parent strain, $MR\text{-}1_{sediment}$ in 0.5, 1.5, and 2.5 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 the latter three mutants.

Growth of wild-type MR-1 was initially delayed in 5 μg/ml chloramphenicol (Fig. 2), suggesting that a genetic variant may have arisen. We hypothesized that this "adapted" variant contains an inactivating mutation in the annotated repressor that precedes the *mexEF* operon (SO3494, TetR family repressor; see Fig. S1 in the supplemental material). TetR family repressors have been implicated in control of multidrug resistance systems in *Pseudomonas aeruginosa* (6). Furthermore, we found that this chloramphenicol-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. 3). When MICs were performed with chloramphenicol, the adapted strain grew in chloramphenicol up to nearly 50 μg/ml (see Table S3 in the supplemental material).

TABLE 2. Mutations present in chloramphenicol-adapted *S. oneidensis* MR-1 in the gene encoding the TetR family regulatory protein (SO3494) that precedes the *mexEF* operon[a]

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

[a] SO3494 was PCR amplified from four independent chloramphenicol-adapted wild-type MR-1 cultures (called A, B, C, and D). Three clones for each of the four cultures were sequenced to give results shown above.

Sequencing potential mutations in the gene encoding the TetR family regulatory protein (SO3494) that precedes the *mexEF* operon. Gene sequence for the TetR family protein in the control culture (wild-type *S. oneidensis* MR-1 that was grown in LB for the same length of time as the wild-type chloramphenicol-adapted strains) did not contain any mutations compared to the MR-1 genome sequence deposited in the NCBI database. Various mutations were evident in this gene, however, for all cultures adapted to 5 μg/ml chloramphenicol. Table 2 shows the mutations observed in all clones that were sequenced from among the four, independent chloramphenicol-adapted cultures. Mutations led to either premature terminator codons or a frameshift.

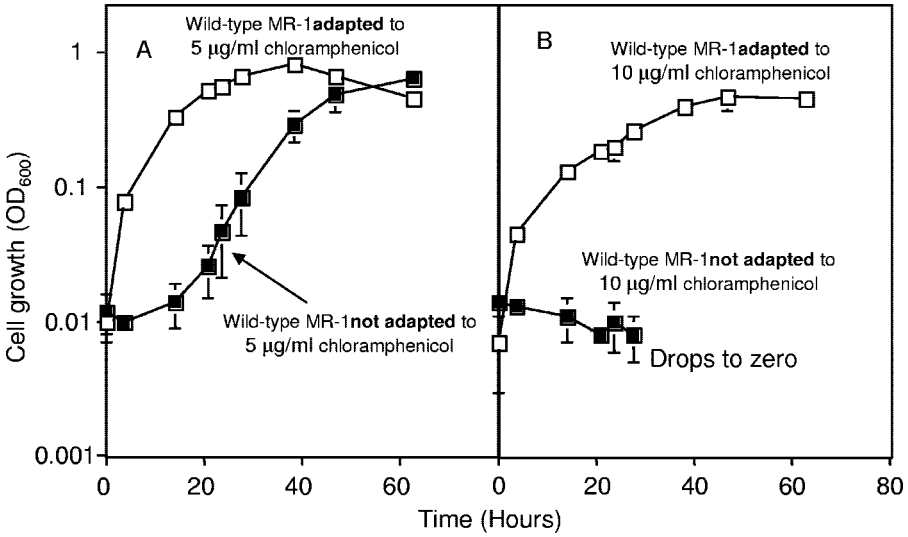

FIG. 3. Growth of wild-type MR-1 that is adapted to chloramphenicol versus growth of wild-type MR-1 that has not been exposed to chloramphenicol. Concentrations tested include 5 (A) and 10 (B) μg/ml chloramphenicol. Data shown represent the averages of three cultures. Error bars represent standard deviations. OD_{600}, optical density at 600 nm.

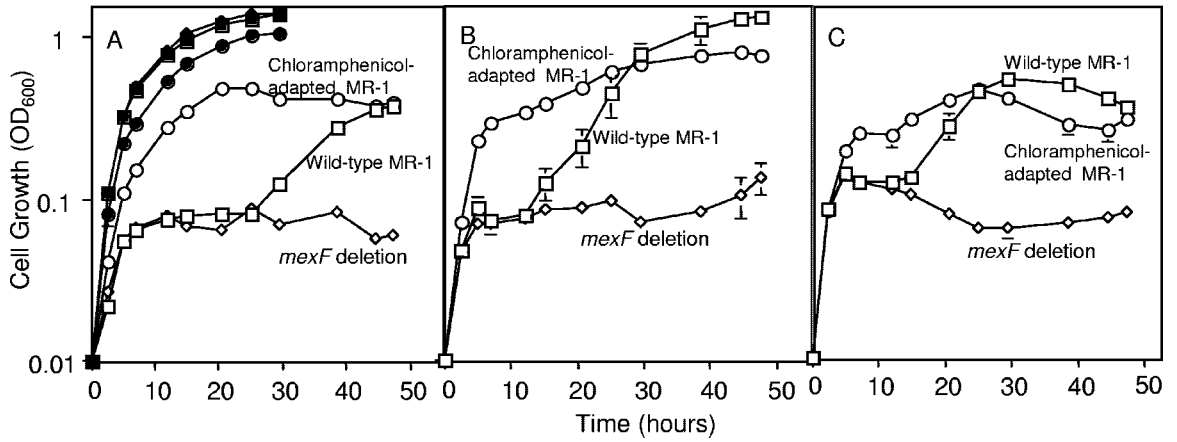

FIG. 4. Growth of wild-type MR-1 (squares), chloramphenicol-adapted MR-1 (circles), and the *mexF* deletion mutant (diamonds) in the presence of other antibiotics. These graphs show growth in LB medium alone (filled symbols in panel A) or LB containing 1.2 mg/ml lincomycin (open symbols in panel A), 0.6 μg/ml nalidixic acid (B), or 1.5 μg/ml norfloxacin (C). Data shown represent the averages of three cultures, and error bars represent standard deviations. OD_{600}, optical density at 600 nm.

An issue of concern is that other unknown mutations (e.g., ribosomal mutations) and/or induction of other multidrug efflux pumps present in *S. oneidensis* MR-1 produced the adapted phenotype in wild-type MR-1 (8). If this were the case, such effects should also have occurred in the *mexF* deletion mutant; however, this mutant never adapted to any of the antibiotics tested. These results suggest but do not confirm in the absence of a complementation experiment that adaptation to chloramphenicol results from a loss of the putative TetR regulatory protein.

Characterization of the response of *S. oneidensis* MR-1 *mexF* to other toxic substances. Reduction of AQDS was identical between the wild-type strain and the *mexF* deletion mutant (data not shown). Furthermore, there was no effect of the *mexF* deletion on growth on iron oxyhydroxide medium [on the basis of quantitation of Fe(II) produced] (data not shown). Additionally, MICs of other metals were similar for 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 $CdCl_2$, $PbCl_2$, and $CoCl_2$. A similar $NaAsO_2$ MIC was reported for strain MR-1 previously (35); the MICs for $CuCl_2$ and Na_2HAsO_4 are similar to values reported for *Pseudomonas putida* and *Deinococcus radiodurans* (34). Finally, the MIC for $HgCl_2$ is a little less than the 500 nM concentration that caused a lag in growth of *Sulfolobus solfataricus* (36). The actual MIC for $PbCl_2$ was probably lower than we report, as some precipitation was observed. Metal loss due to precipitation may also explain why the MICs for $CdCl_2$ and $CoCl_2$ are also high compared to the MICs reported for *P. putida* and *D. radiodurans* (34).

The wild type and the *mexF* deletion mutant exhibited the same MIC patterns for 17 of 18 drugs tested; only for puromycin did the wild type have a higher MIC than the *mexF* deletion mutant (see Table S3 in the supplemental material), but growth curves conducted with 150 and 250 μg/ml puromycin could not confirm inhibition of growth 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 did 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. 4). MICs determined for fulvic acid and other aromatic compounds (ferulic acid, protocatechuic acid, caffeic acid, *p*-hydroxybenzaldehyde, salicylic acid, *trans*-cinnamic acid, and *p*-nitrophenol) were mostly similar for the wild-type strain, the *mexF* deletion mutant, and the chloramphenicol-adapted wild-type culture (see Table S4 in the supplemental material).

DISCUSSION

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. The STM approach we used (12) identified a list of genes whose importance for the in situ fitness of environmental bacteria was previously unknown. Furthermore, these genes are broadly distributed (see Table S2 in the supplemental material), suggesting that many of these genes will likely be important for sediment fitness in many environmental bacteria.

Analysis of these genes revealed several interesting physiological events necessary for *S. oneidensis* MR-1 sediment fitness. Several genes encoding proteins involved in DNA repair are specifically required for growth in sediment, suggesting environmental conditions may be highly mutagenic. Bacteriophage-related, insertion sequence-related, and regulatory gene products may control the expression of yet unidentified genes required for sediment survival mechanisms. Genes encoding products with transport functions could aid in bringing nutrients to the cell or in extrusion of foreign substances or secretion of self-made toxic compounds from within the cell. Energy production genes could encode proteins necessary for utiliza-

tion of sediment resources as opposed to use of laboratory medium components. Protein product from other genes could be required for maneuvering within (methyl-accepting chemotaxis protein) and for modifying components (serine protease and α-glucosidase) of the natural environment to allow *S. oneidensis* MR-1 to locate and grow within an acceptable niche.

To date, the screen likely does not encompass all possible sediment-impaired mutations that can result from mutagenesis of the entire *S. oneidensis* MR-1 genome, although duplicate transposon insertions (within different sites of the gene) were observed for the serine protease gene (see Table S1 in the supplemental material) and *argH* (Table 1). Redundancy of gene function may also have prevented identification of all possible fitness genes. In addition, some tagged transposon insertions could be located within parts of the gene that are not critical to overall function of translated protein.

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 (17). Indeed, one such chemotaxis protein (see Table S1 in the supplemental material) (12) was identified herein. Such a finding helps to validate the sediment screen, while identification of *gspN* (Table 1) validates the 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 *S. putrefaciens* (7).

Interestingly, a surprising number of fitness genes were related to multidrug resistance genes, systems contributing to the incidence of antibiotic resistance in pathogens (for a review, see reference 31). 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 shows the highest similarity with an uncharacterized *Vibrio vulnificus* AcrR/TetR family transcriptional regulator (70% similarity). The HlyD family protein (SO0524) shares 49% similarity with the *E. coli* EmrA that is involved in multidrug resistance (22).

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 *P. aeruginosa* PAO1 (19). This PAO1 MDR protein consists of a cytoplasmic membrane protein (MexF), an outer membrane protein (OprN), and a membrane fusion protein (MexE) (19). Hyperexpression of the *mexEF-oprN* operon results in antibiotic resistance (i.e., chloramphenicol and quinolones) for *P. aeruginosa* (9).

The increased resistance to chloramphenicol observed in adapted MR-$1_{sediment}$ cultures appears to result from a mutation in the nearby gene encoding a TetR family regulatory protein. Most resistance-nodulation-cell division (RND)-type efflux systems are also regulated by nearby genes encoding repressor proteins (6), and the *mexEF-oprN* operon in *Pseudomonas* is no exception but is controlled by a heterologous protein (LysR family activator) encoded by the *mexT* gene (18). The orthologous *acrAB* operon in *E. coli* is controlled by the *acrR* gene (see Fig. S1 in the supplemental material).

The ability of the *mexF* deletion mutant to grow at the lower concentrations of chloramphenicol may be the result of another system aside from that encoded by *mexEF*. *S. oneidensis* MR-1 strains may employ a chloramphenicol acetyltransferase (38) encoded in its genome (SO4299).

Success with chloramphenicol in this study led to testing more antibiotics and other potentially inhibitory compounds. Some compounds that serve as substrates for MexF in *Pseudomonas* species (i.e., norfloxacin, nalidixic acid, and chloramphenicol) (9) appear to do the same in *S. oneidensis* MR-1, while others (trimethoprim) do not. Although some heavy metal efflux transporters, such as CzcA in *Alcaligenes eutrophus* (29) belong to the superfamily of MDR proteins (43), this study does not demonstrate any relationship between MexF and metal resistance. A recent study found that *mexE* and *mexF* expression increased when MR-1 was exposed to metals [Fe(III) or Mn(IV)] as electron acceptors (4); however, no effect of the *mexF* deletion was observed during growth on Fe(III)-citrate or Fe(III) oxyhydroxide.

While the true natural substrates for the Mex system in *Shewanella oneidensis* MR-1 remain unclear, not unlike many MDR systems reported in the literature (37), this study shows that the Mex system in *S. oneidensis* is capable of antibiotic efflux. As this strain was isolated from an environment that was not impacted by the release of pharmaceuticals (27), the Mex system likely prevents toxic accumulation of naturally occurring antibiotics (e.g., plant exudates [15, 41] and antimicrobials released by antagonistic microorganisms competing in the same environmental niche [44, 45]), humic acids (39), or other chemicals that may find their way into the environment (e.g., dyes, detergents, and organic solvents). By doing so, the Mex system increases MR-1 sediment fitness, much like multidrug efflux increases pathogen fitness; loss of MDR transporters has been shown to decrease virulence in *P. aeruginosa* (14).

Because technical issues and short half-lives have limited the utility of direct detection techniques for quantifying antibiotics in soils and sediments (47), previous work has indirectly inferred in situ production of antibiotics largely by demonstrating the presence of cultivatable antibiotic-producing bacteria (10, 16) or the in situ presence of genes coding for antibiotic production (16, 26). A limited group of studies have also shown that antibiotic production mutants are less fit than their antibiotic-producing brethren (25). In situ antibiotic resistance studies have similarly focused on demonstrating the presence of cultivatable antibiotic-resistant bacteria from a number of habitats (20). The significance of this study is that the presence of antibiotic resistance genes (and likely function) is shown to confer an ecological advantage on these microorganisms.

ACKNOWLEDGMENTS

This work was supported by the Environmental Remediation Science Program (ERSP) of the Office of Biological and Environmental Research of the Office of Science, U.S. Department of Energy.

We thank Dianne Newman, Helen Zgurskaya, Keith Poole, Terry Krulwich, and Herbert Schweizer for critical review of portions of the manuscript.

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