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September 27, 2005

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Prof. Brun:

I have enclosed an application packet that contains the requested information regarding the faculty position in systems biology/microbiology in the Department of Biology. I enjoy working with various systems, but the primary focus of my laboratory is microbiology. Currently, my laboratory studies two major models for heavy-metal/radionuclide reduction, *Desulfovibrio vulgaris* and *Shewanella oneidensis*. We are particularly interested in stress response and survival under environmentally relevant conditions (i.e., slow-growth; biofilms), and the cellular impact on biochemical function (i.e., metal-reduction). I have also initiated studies in other novel bacteria, in order to widen our breadth of knowledge on field-relevant organisms (e.g., *Alkaliphilus*, *Anaeromyxobacter, Paenibacillus*) that can reduce heavy metals. In addition, I am interested in mixed communities and the relationship between community structure and biochemical function, and how to use microbial ecology in a predictive manner. My laboratory uses a wide assortment of applications in the fields of physiology, biochemistry, molecular biology, ecology, genomics, and transcriptomics. I also think it is advantageous to view projects from a multi-disciplinary perspective, and I currently collaborate with environmental engineers, geologists, hydrologists, geochemists, and computer scientists.

In my two years as a tenure-track faculty member at MU, 2 manuscripts that included graduate and/or undergraduate MU students have been accepted for publication, 2 are in review, and 2 are in preparation. I have enjoyed my position thus far and have learned a great deal; however, I would like to become part of a larger department to grow and collaborate. Thus, I am extremely interested in your position. I have not informed MU that I have applied; therefore I would like to keep my application confidential. Recommendation letters have been requested to be sent under separate cover. After review of the enclosed materials, please contact me if any additional information is needed. Thank you for your time.

Sincerely,

Matthew W. Fields, Assistant Prof.

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Alkaline Anaerobic Respiration: Isolation and Characterization of a Novel Alkaliphilic and Metal-Reducing Bacterium

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Iron-reducing enrichments were obtained from leachate ponds at the U.S. Borax Company in Boron, Calif. Based on partial small-subunit (SSU) rRNA gene sequences (approximately 500 nucleotides), six isolates shared 98.9% nucleotide identity. As a representative, the isolate QYMF was selected for further analysis. QYMF could be grown with Fe(III)-citrate, Fe(III)-EDTA, Co(III)-EDTA, or Cr(VI) as electron acceptors, and yeast extract and lactate could serve as electron donors. Growth during iron reduction occurred over the pH range of 7.5 to 11.0 (optimum, pH 9.5), a sodium chloride range of 0 to 80 g/liter (optimum, 20 g/liter), and a temperature range of 4 to 45°C (optimum, approximately 35°C), and iron precipitates were formed. QYMF was a strict anaerobe that could be grown in the presence of borax, and the cells were straight rods that produced endospores. Sodium chloride and yeast extract stimulated growth. Phylogenetic analysis of the SSU rRNA gene indicated that the bacterium was a low-G+C gram-positive microorganism and had 96 and 92% nucleotide identity with *Alkaliphilus transvaalensis* and *Alkaliphilus crotonatoxidans*, respectively. The major phospholipid fatty acids were 14:1, 16:1ω7c, and 16:0, which were different from those of other alkaliphiles but similar to those of reported iron-reducing bacteria. The results demonstrated that the isolate might represent a novel metal-reducing alkaliphilic species. The name *Alkaliphilus metalliredigens* sp. nov. is proposed. The isolation and activity of metal-reducing bacteria from borax-contaminated leachate ponds suggest that bioremediation of metal-contaminated alkaline environments may be feasible and have implications for alkaline anaerobic respiration.

Bacterial metal reduction has widened the realm of life-supporting biological reactions (29) and has been implicated as an important biochemical process on early Earth (20, 36, 39). The ability to reduce metals can be exploited for the bioreduction or immobilization of many toxic metals, including cobalt, chromium, uranium, and technetium (6). However, metal reduction in alkaline environments has not been well documented.

Concentrated deposits of boron exist in some arid regions (e.g., Turkey and the United States) and are economically exploited for products including fiberglass, ceramics, glass, laundry bleaches, fire retardants, insecticides, and semiconductors (25, 42). Borax is a common detergent ingredient and is sometimes used as a mild disinfectant. The toxicity is low, but boron is not completely harmless. Boron can potentially affect reproductive organs of males and impair fetal development in pregnant females, as well as be phytotoxic (27, 30). In addition, sodium perborate has been shown to be an in vitro mutagen (34).

Metal-reducing bacteria can be isolated from a variety of habitats, and much work has focused on the metal-reducing bacteria *Shewanella oneidensis* (28) and *Geobacter* spp. (22). Sites contaminated with toxic metals can have drastically different environmental conditions, and the biological reduction of most metals has commonly been studied at circumneutral pH values (36). Little is known about metal reduction under different extreme conditions, and only recently has bacterial Fe(III) reduction been demonstrated under thermophilic (20, 39), psychrophilic (45), or acidic (18) conditions. However, metal reduction under alkaliphilic growth conditions has not been demonstrated. We report the isolation and partial characterization of a novel alkaliphilic microorganism that can reduce metals at pH values up to 11.0 in the presence of elevated salt levels. Phylogenetic analysis of the small-subunit (SSU) rRNA gene indicated that the isolate was a low-G+C, gram-positive bacterium but, in addition to phenotypic differences, had only 96% sequence identity with the closest known relative. The name *Alkaliphilus metalliredigens* sp. nov. is proposed for the microorganism.

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MATERIALS AND METHODS

Site characteristics and water chemistry. Leachate ponds at U.S. Borax Company (Boron, Calif.) were chosen for the study because of alkaline pH. The pond water had prominent blooms of both algae and cyanobacteria at the time of sampling (March 2001). Samples were collected into sterile 50-ml tubes from water in the pond and partially dried soft sediments along the pond embankments. The water samples were filtered onto 0.4-μm-pore-size Gelman filter disks, and supernatants were saved for elemental analyses. Supernatants were

analyzed by inductively coupled plasma mass spectroscopy (Activation Labs Limited).

Bacterial enrichments and isolation. Sediment slurry samples were enriched for microbial growth in the presence of acetate, lactate, and yeast extract and of iron-citrate as the electron acceptor at pH values corresponding to individual samples (pH 8.0 to 9.5). Ferric citrate solution (pH 7.0) was prepared with ferric citrate hydrate, and the pH was modified with NaOH. Anaerobic culture techniques were used throughout the study with anoxic medium in tubes (18 by 150 mm) or serum bottles with butyl rubber stoppers and aluminum crimp seals under an N_2-CO_2 (80:20) atmosphere. All media and solutions were boiled under O_2-free nitrogen gas or an N_2-CO_2 gas mix and dispensed into tubes or bottles under streams of the corresponding gas.

Sediment slurries were diluted 10-fold into anaerobic growth medium with lactate, acetate, and iron-citrate. Cultures were incubated at 20°C, and iron precipitation was observed within 5 days. The medium for enrichments contained the following ingredients: 4.3 mM K_2HPO_4, 9.4 mM $(NH_4)_2SO_4$, 50 mM Tris, 6.1 μM Na_2SeO_4, 4 mM $NaHCO_3$, 7 mM sodium lactate, 7 mM sodium acetate, 15 mM iron-citrate, 0.025 g of yeast extract (Difco) per liter, 10 ml of mineral stock solution (100×), and a headspace of N_2-CO_2 (80:20). The mineral stock contained the following ingredients per liter: 1.5 g of nitrilotriacetic acid, 3.0 g of $MgSO_4$, 0.5 g of $MnSO_4$, 1.0 g of NaCl, 0.1 g of $FeSO_4$, 0.1 g of $CaCl_2$, 0.1 g of $CoCl_2$, 0.13 g of ZnCl, 0.01 g of $CuSO_4$, 0.01 g of $AlK(SO_4)$, 0.01 g of H_3BO_3, 0.03 g of Na_2MoO_4, 0.02 g of $NiCl_2.6H_2O$, and 0.03 g of Na_2WO_4.

The growth medium contained the following ingredients: K_2HPO_4, 5.7 mM; $(NH_4)_2SO_4$, 12.5 mM; NaCl, 327 mM; Na_2CO_3, 19.8 mM; $Na_2B_4O_7$, 10 mM; yeast extract, 0.025 g/liter, 100× mineral solution, 10 ml. The pH was adjusted to approximately 9.5. The headspace was N_2-CO_2 (80:20). Aerobic growth was tested in the above-mentioned medium as well as a medium used for the isolation and growth of different soil microorganisms, MR2A. MR2A contains peptone, tryptone, yeast extract, glucose, pyruvate, starch, and trace minerals (7). A significant decrease in growth rate was not observed up to 25 mM borax, and the samples from the leachate ponds were normally 10 to 15 mM borax. Therefore, the bacterium was cultured in medium with 10 mM borax.

Bacterial isolates were obtained from solid (2% agar) enrichment medium plates in an anaerobic glove bag (Coy Laboratories) with an N_2-H_2 (97%:3%) atmosphere. The enrichment culture was transferred twice before being used as an inoculum for agar plates. Colonies appeared dark brown after growth on Fe(III)-citrate solid medium and became hard. Isolated colonies were transferred to liquid medium with sterile Pasteur pipettes as agar bores after being restreaked at least four times.

Morphology. Bacterial cell morphology was examined under scanning electron microscopy (SEM). Cell cultures (2 ml) were harvested by centrifugation (16,000 × *g*; 5 min), washed in fresh anoxic medium, and centrifuged again (16,000 × *g*; 5 min). Preparation of each sample for SEM, including glutaraldehyde fixation and osmium tetroxide staining, was performed as previously described (1). At least two separately stained and fixed samples were viewed with a JEOL 5500 scanning electron microscope (JEOL Inc., Peabody, Mass.).

Growth studies. Bacterial growth with Fe(III), Co(III), and Cr(VI) was quantified by direct cell counting with epifluorescence microscopy of acridine orange-stained samples and statistical analysis as described previously (9, 15). Inocula were 10% or less of starting culture volumes. Light microscopy was used to visualize wet mounts and acridine orange-stained filters with a Nikon Phase Contrast Optiphot microscope (Nikon). Growth with the humic acid analog 2,6-anthraquinone disulfonate (AQDS) was monitored with a spectrophotometer, and an increase in cell number was monitored via direct counts. All growth studies were done in duplicate, and the error bars in figures indicate standard deviations.

Possible electron donors and acceptors were tested in the Na_2CO_3-buffered medium at pH 9.5 and 20°C and were added from sterile anoxic stock solutions. Lactate (10 mM) and/or yeast extract (0.25 g/liter) was routinely used as the electron donor for testing electron acceptors. Soluble Fe(III) was provided as Fe(III)-citrate or Fe(III)-EDTA (10 mM). Cr(VI) was supplied as K_2CrO_7 (100 μM), and Co(III) was added as Co(III)-EDTA (3 mM). The other tested electron acceptors were used at the concentrations in parentheses: akaganeite (1 mM), manganese oxides (1 mM), trimethylamine oxide (10 mM), dimethyl sulfoxide (10 mM), thiosulfate (10 mM), fumarate (10 mM), and nitrate (1 and 15 mM). Chemicals were purchased from Sigma Chemical Co., except for akaganeite and manganese oxide, which were prepared as previously described (21, 31). The effects of pH, temperature, and sodium chloride levels on the growth of isolate QYMF were determined in the Na_2CO_3-buffered medium by varying the respective parameter. The pH was adjusted by decreasing the Na_2CO_3 levels or by the addition of KOH as previously described (37).

TABLE 1. Nucleotide sequences for PCR primers

Designation	Sequence (5′ to 3′)
FD1	AGA GTT TGA TCC TGG CTC AG
519f	CAG CAG CCG CGG TAA
788f	ATT AGA TAC CCT GGT A
1099f	GCA ACG AGC GCA ACC C
350r	CTG CTG C(gc)(ct) CCC GTA G
529r	CGC GGC TGC TGG CAC
925r	CCG TCA ATT C(ac)T TT(ag) AGT TT
1540R	AAG GAG GTG ATC CAG CC

Chemical and mineralogical analyses. Fe(II) production was monitored with a spectrophotometer by the ferrozine method and acid extraction (21). The decrease in Co(III)-EDTA was monitored using a spectrophotometer at 535 nm (3), and the reduction of AQDS was monitored at 450 nm (24). The decrease in Cr(VI) was determined with a spectrophotometer from H_2SO_4-extractable Cr(VI) with a symdiphenylcarbazide reagent (0.25% acetone) at 540 nm (23). All analyses were done in duplicate.

Precipitates in each sample were collected on a 0.22-μm-pore-size membrane (Millipore; polyvinylidene fluoride) and washed three times with deionized water. The filtered precipitates were dried under an N_2 atmosphere to prevent oxidation. The filtered and dried precipitates were carbon coated with a Ted Pella carbon sputter coater and immediately examined. The morphology, mineralogy, and chemistry of precipitated phases of iron were determined with a JEOL JSM-35CF (JEOL Ltd., Tokyo, Japan) scanning electron microscope with energy-dispersive X-ray analysis (EDX). The mineralogical composition of the precipitated or transformed phases was determined using X-ray diffraction performed on a Scintag (Scintag, Inc., Sunnyvale, Calif.) XDS 2000 diffractometer (40 kV, 35 mV) equipped with Co-Kα radiation (λ = 0.17889 nm).

DNA extraction, amplification, and sequence determination. DNA was isolated with a Promega Wizard kit and the addition of lysostaphin (10 mg/liter; Sigma Chemical Co.) and freeze-thawing according to the manufacturer's instructions. The SSU rRNA genes were amplified from isolates with the FD1 and 1540R primers (Table 1), and PCR products were purified with Montage PCR 96 filter plates (Millipore, Inc.) or treated with ExoSAP-IT (U.S. Biochemical Corporation) according to the manufacturer's instructions. PCR parameters for the amplification of the SSU rRNA gene sequences were described previously (40, 48). DNA sequences were determined with a BigDye Terminator kit (Applied Biosystems) with a 3700 DNA analyzer (Perkin-Elmer) according to the manufacturer's instructions with the SSU rRNA gene-specific primers 350r, 519f, 529r, 788f, 925r, and 1099f (Table 1, *Escherichia coli* designations) and compared with sequences from GenBank.

Raw data were assembled into contiguous sequences and edited with Sequencher software (Gene Codes Corp.). The nearly complete SSU rRNA gene sequences (approximately 1,500 nucleotides) were aligned with ClustalW (38). Phylogenetic analyses of SSU rRNA gene sequences were conducted using MEGA version 2.1 (17). Neighbor-joining and maximum-parsimony phylogenies were constructed from dissimilatory distances and pairwise comparisons and did not differ significantly.

Lipid analysis. Cells (5.0×10^{10}) cultured in Na_2CO_3-buffered medium with yeast extract as an electron donor and 10 mM Fe(III)-EDTA as an electron acceptor were harvested (4,000 × *g*, 30 min), frozen at −70°C, and lyophilized. Lyophilized bacterial cell pellets (approximately 20 mg) were extracted by a modified Bligh and Dyer single-phase organic solvent system that consisted of 142.5 ml of chloroform, methanol, and aqueous 50 mM phosphate buffer (1:2:0.8, vol/vol/vol) as previously described (2, 41). The lipid phase was collected and fractionated on a silicic acid column into neutral lipids, glycolipids, and polar lipids. The polar phospholipid fatty acids were treated using a mild alkaline methanolysis to produce fatty acid methyl esters. Methyl esters were analyzed with an Agilent 6890 series gas chromatograph interfaced to an Agilent 5973 mass selective detector with a 20-m nonpolar column (46). Mass spectra were determined by electron impact at 70 eV. Monounsaturated fatty acid methyl ester double-bond position was determined by gas chromatography-mass spectrometry analysis of the dimethyl disulfide adducts (46).

Nucleotide sequence accession number. The GenBank accession number assigned to the SSU 16S rRNA gene sequence for isolate QYMF is AY137848.

RESULTS

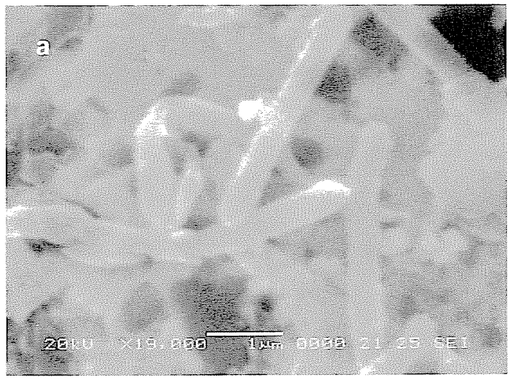
Water chemistry. The hydrogeochemical analysis of water samples from the leachate ponds indicated that Na, Mg, K, and Ca were the major elements, and sodium concentrations ranged from 0.04 to 0.53 M. Boron was between 0.19 and 0.28 M, which corresponded to approximately 2,000 to 3,000 ppm. Iron concentrations were approximately 0.1 to 0.2 mM, chromium ranges were 3.8 to 4.5 μM, and cobalt was present up to concentrations of 0.2 μM. Uranium concentrations ranged from 0.6 to 1.3 μM, arsenic concentrations were approximately 1.7 mM, and selenium was not detected.

Microbial enrichment and isolation. Positive enrichments displayed signs of iron reduction within 5 days. Colonies could be picked and restreaked upon initial appearance, but colonies became hard to the touch of a needle or loop after significant reduction of Fe(III) in the medium. After restreaking, the colonies were transferred to liquid medium via an agar bore from a sterile Pasteur pipette. Seven bacterial isolates were obtained, visualized by phase-contrast microscopy, and compared by partial SSU rRNA gene sequence determination. The seven isolates displayed at least 98.9% sequence identity and had a mean sequence dissimilarity of 0.004% ± 0.002% based on partial (V2-V6 region) SSU rRNA gene sequences (approximately 500 nucleotides). Differences in phenotypic characteristics can sometimes be observed in microorganisms with >98% sequence identity in the SSU rRNA gene, but the isolates had similar metal-reducing capacities. Isolate QYMF is described in detail.

Cell morphology. QYMF cells were straight, with some cells being slightly curved, and had a mean length of 3 to 6 μm and an approximate width of 0.5 μm (Fig. 1). The cells were Gram stain positive. Wet mounts of active cultures visualized with a phase-contrast microscope indicated that the cells were motile, but flagella were not observed by SEM. Exponentially growing cells were often observed as single cells, and few chains were formed as the culture entered stationary phase. As the cells entered stationary-phase growth, terminal endospores were observed (Fig. 1b).

Growth characteristics. Isolate QYMF was a strict anaerobe and did not display aerobic or fermentative growth with glucose, starch, or sucrose. The isolate did not display significant growth in MR2A medium (7) in the presence or absence of oxygen. Growth was not observed in aerobic growth medium. Yeast extract could be utilized as an electron donor in the presence of Co(III), and when yeast extract levels were increased the Co(III) decreased and the cell numbers increased, respectively (Fig. 2). Similar results were observed with Fe(III) (data not shown). However, little growth was observed when the yeast extract concentration was 0.01 g/liter or less without an additional electron donor, and Co(III) was not reduced at a significant amount (Fig. 2 and 3).

Growth was not observed when cells were provided with yeast extract alone, nor was growth observed in the presence of yeast extract and lactate (Fig. 3). Small amounts of yeast extract (i.e., 0.01 g/liter) stimulated growth in the presence of lactate and Co(III), and significant growth was not observed unless the lactate was present (Fig. 2). However, when the yeast extract levels were 0.01 g/liter, little growth was observed compared to that with higher levels of yeast extract. Similar



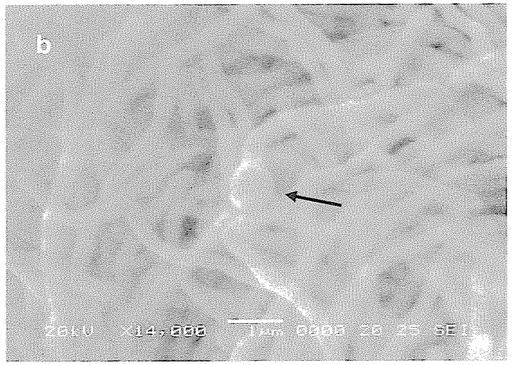


FIG. 1. Scanning electron micrographs of isolate QYMF grown with Fe^{3+}-EDTA at ×17,670 (a) and ×13,020 (b) magnifications. The arrow denotes an endospore. Bars, 1.0 μm.

results were observed with Fe(III) and Cr(VI) (data not shown). Tryptone, but not Casamino Acids, could be substituted for yeast extract. The final pH of the medium was not significantly changed, and significant growth was observed only with the metals Fe(III), Co(III), and Cr(VI).

The isolate could only be grown in the presence of complexed Fe(III) (citrate or EDTA), Cr(VI), and Co(III)-EDTA, as well as the humic acid analog AQDS. Lactate could serve as the electron donor with the metals listed above as electron acceptors, but fumarate, glycine, histidine, alanine, or arginine did not support growth. Nor did the oxidation and reduction of glycine-alanine or arginine-histidine appear to support growth. Growth was not observed with the following electron acceptors: akaganeite, manganese oxides, trimethylamine oxide, dimethyl sulfoxide, thiosulfate, fumarate, and nitrate. Cells could reduce AQDS in the presence of yeast extract, but the addition of AQDS did not promote the reduction of akaganeite.

The temperature range for growth was between 4 and 45°C, and the optimum temperature was approximately 35°C. The generation time was approximately 2.3 h at 35°C and approximately 4 h at 22°C when cells were grown in the presence of

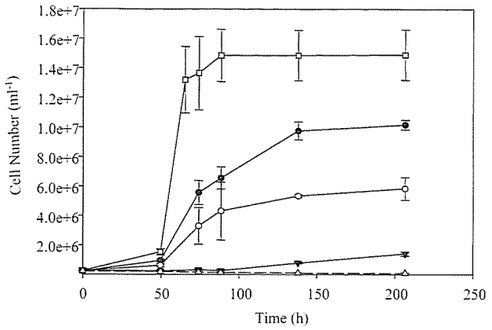


FIG. 2. Cell growth in the presence of Co(III) and 0.25 (□), 0.1 (●), 0.05 (○), or 0.01 (▼) g of yeast extract per liter determined via direct cell counts. The control with yeast extract only (△) did not show significant growth. The medium included 2 g of borate per liter and 20 g of sodium chloride per liter, and the pH value was 9.6. The error bars indicate standard deviations of duplicates.

AQDS (5 mM) at pH 9.5. Little growth was observed above 45°C. When the isolate was grown in the presence of Fe(III)-citrate and yeast extract at 22°C, the pH optimum was approximately 9.5, and growth at pH 7.0 or 11.0 was negligible (Fig. 4a). The salt NaCl was not essential for growth, but the optimal concentration was approximately 20 g/liter (Fig. 4b). The growth rate was decreased threefold when cells were grown in the presence of 80 g/liter, and growth was negligible at 100 g/liter.

QYMF could reduce Fe(III), Co(III), and Cr(VI) with a concomitant increase in cell number. When Fe(III)-citrate served as the electron acceptor and yeast extract was the electron donor, the growth rate was approximately 0.05 h^{-1} and a brown precipitate was formed (Fig. 5). Complexed Fe(III)-EDTA could also support growth in a similar fashion, but

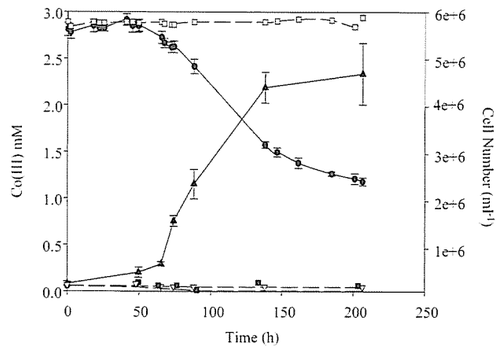
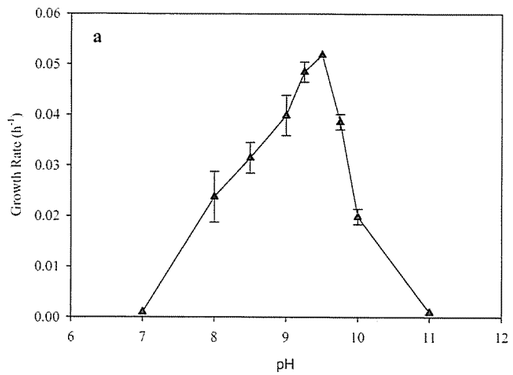


FIG. 3. Cell growth in the presence of Co(III), yeast extract (0.01 g/liter), and lactate (10 mM) determined via a decrease in Co(III) (●) and an increase in cell number (▲). The controls included the following: Co(III) only (□), yeast extract only (▽), and yeast extract plus lactate (■). The medium included 2 g of borate per liter and 20 g of sodium chloride per liter, and the pH value was 9.6. The error bars indicate standard deviations of duplicates.



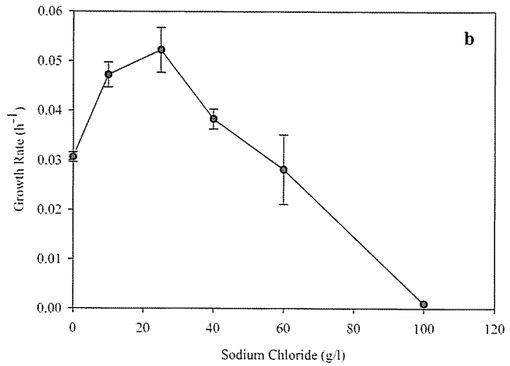


FIG. 4. Relationship between sodium chloride concentration (b) or pH (a) and growth rate when yeast extract and Fe(III)-citrate were provided as electron donor and acceptor, respectively. The borate concentration was 2 g/liter. The pH value was 9.6 for the determination of the pH optimum, and the salt concentration was 20 g/liter for the optimal pH determination. The error bars indicate standard deviations of duplicates. It should be noted that, when sodium chloride was not added, the medium still contained approximately 8 mM sodium from sodium carbonate.

growth resulted in the formation of a white precipitate when the headspace was H_2-CO_2 (80:20). The growth rate in the presence of 4.5 mM Co(III) was more than twofold lower than with 15 mM Fe(III) (Fig. 6), but increased growth rates were observed with lower Co(III)-EDTA concentrations. An increase in cell number was also observed during the reduction of 100 μM Cr(VI) and yeast extract (Fig. 7).

Mineralogy. Cells grown at pH 9.0 with Fe(III)-EDTA as an electron acceptor and yeast extract and lactate as electron donors in medium with a carbonate buffer formed a white precipitate. Analysis with SEM and EDX showed that the precipitated phase contained iron and phosphorus with keg or barrel morphology and a pronounced platy habit (data not shown). X-ray diffraction analysis of the precipitate displayed sharp and intense reflection patterns, and the crystalline precipitates had X-ray diffraction patterns indicative of $Fe_3(PO_4)_2 \cdot 8H_2O$ (vivianite).

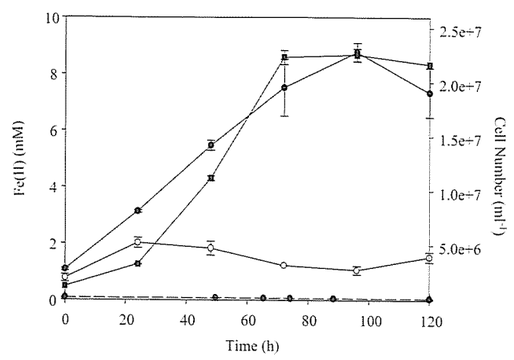


FIG. 5. Production of Fe(II) (●) and increase in cell number (■) when cells were grown with Fe(III), yeast extract, and 20 g of sodium chloride per liter at pH 9.6. The controls included Fe(III) only (○) and yeast extract only (● with dashed line). The error bars indicate standard deviations of duplicates.

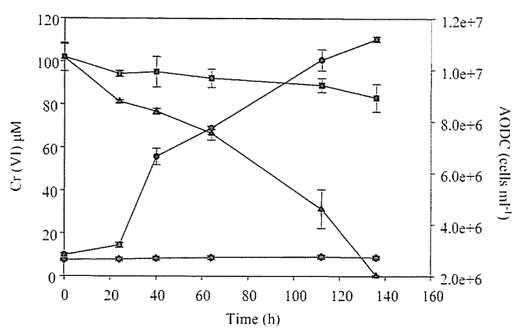


FIG. 7. Decrease in Cr(VI) (▲) and increase in cell number (●) when cells were grown with Cr(VI), yeast extract, and 20 g of sodium chloride per liter at pH 9.6. The controls included Cr(VI) only (■) and yeast extract only (◆). The error bars indicate standard deviations of duplicates.

SSU rRNA gene-based relationships. The SSU rRNA gene was amplified from genomic DNA, and the sequence was determined with multiple primers. The isolate was a low-G+C gram-positive bacterium (Fig. 8). The SSU rRNA gene of the isolate formed a cluster with two other sequences from the recently named genus *Alkaliphilus*. The sequence of isolate QYMF had 96 and 92% nucleotide identity with *Alkaliphilus transvaalensis* and *Alkaliphilus crotonatoxidans*, respectively. Both A. *transvaalensis* and *A. crotonatoxidans* are reported to be strict anaerobes that form endospores (4, 37). The next closest related sequences were from *Clostridium aceticum* and *Clostridium felsineum* (91% identity). The strain QYMF also clustered with *Tindallia magadiensis* and *Natronincola histidinovorans*, two alkaliphilic bacteria isolated from a soda lake in Kenya (14, 47).

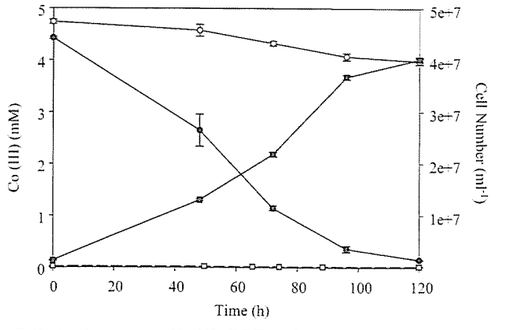


FIG. 6. Decrease in Co(III)-EDTA (●) and increase in cell number (■) when cells were grown with Co(III), yeast extract, and 20 g of sodium chloride per liter at pH 9.6. The controls included Co(III) only (○) and yeast extract only (□). The error bars indicate standard deviations of duplicates.

Polar lipid fatty acid analysis. Lipid extraction and gas chromatography analysis detected the following cellular fatty acids at the given molar percentages: 0.6% i14:0, 14% 14:1, 0.3% 14:1ω5c, 5.4% 14:0, 0.8% br14:1, 0.8% i15:0, 1% a15:0, 3% i15:1, 2.2% 15:1, 1.5% 15:0, 0.6% br15:1, 2.3% 16:1, 27% 16:1ω7c, 1.1% 16:1, 28% 16:0, 0.5% 17:1, 0.8% 17:0, 1.1% 18:1ω9c, 0.4% 18:1ω7c, and 4.1% 18:0. The terminal branched fatty acid, 16:1ω7c; 16:0; and the monounsaturated acid, 14:1, were the most predominant fatty acids, constituting almost 70% of the fatty acid composition. The unbranched saturated acids, 14:0 and 18:0, constituted 9.5% of the total.

DISCUSSION

By definition, strict alkaliphiles should have an optimum pH above 9.0 and display little or no growth at a near-neutral pH of 6.5 (10, 12). Thus, QYMF can be described as an alkaliphile that grew between the pH values of 7.5 and 11. The optimum pH value for growth was approximately 9.5, and this value is similar to the pH values observed with the original samples. The closest known relative, *A. transvaalensis*, could grow in the pH range of 8.5 to 12.5 and had a slightly higher pH optimum of 10.0 (37). The other known *Alkaliphilus* species, *A. crotonatoxidans*, has a pH range of 5.5 to 9.0 but has a pH optimum of 7.5 (4). *T. magadiensis*, an alkaliphilic bacterium isolated from Lake Magadi, Kenya, had a pH growth range of 7.5 to 10.5 and had an optimum of pH 8.5 (47). Another alkaliphile isolated from Lake Magadi, *N. histidinovorans*, had a pH profile more similar to that of strain QYMF and grew at pH values from 8.0 to 10.5 with an optimum of 9.4. However, for both *T. magadiensis* and *N. histidinovorans*, only the fermentation of a limited number of amino acids supported growth with the production of ammonia.

Isolate QYMF had a decreased growth rate when sodium chloride was not added to the medium, and the growth rate was significantly diminished when the salt concentration was >6%. These results suggested that the microorganism was moderately halophilic, and that the sodium chloride requirement and sensitivity determined in pure culture were similar to the measured sodium levels from the leachate ponds (between

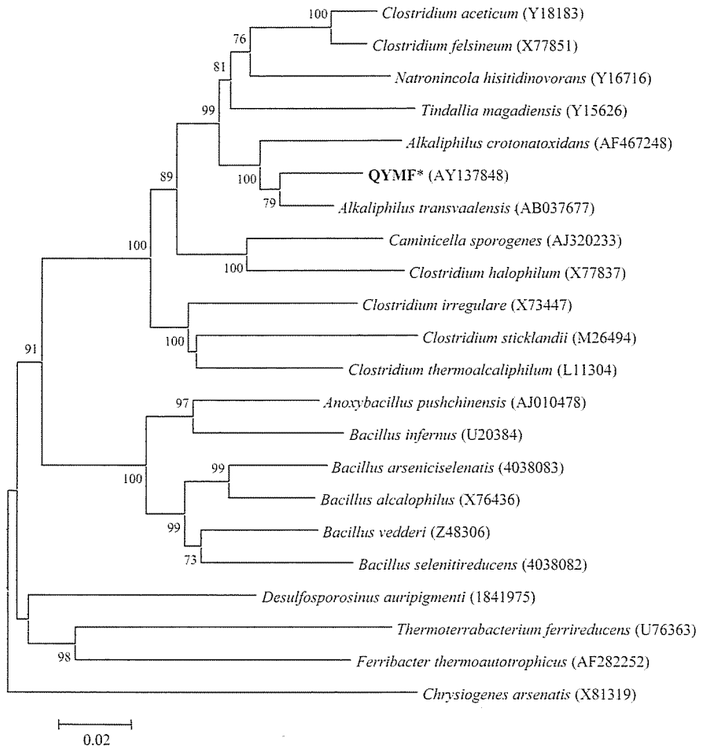


FIG. 8. Phylogenetic relationships between isolate QYMF and other alkaliphilic or metal-reducing bacteria based on nearly complete SSU rRNA gene sequences. Sequences were aligned with ClustalW, and pairwise deletions were used for neighbor joining. Bootstrap (n = 500) values below 50 are not shown, and *Chrysiogenes arsenatis* was used as the outgroup. GenBank accession numbers are in parentheses.

2.0 and 30 g/liter). The alkaliphile *A. transvaalensis* was reported to have an optimal growth rate with 0.5% sea salts and could tolerate 3.3% sea salts; however, values with sodium chloride were not reported for the microorganism (37). The most closely related *Clostridium* spp., *C. aceticum* and *C. felsineum*, are not halophilic; *N. histidinovorans* has a higher salinity optimum of 9% (14), and *T. magadiensis* had a similar range of tolerance between 3 and 6% (47).

The terminal branched fatty acid, 16:1ω7c, and 16:0 were two of the three most predominant lipids, comprising 55% of the fatty acid composition for strain QYMF when cells were grown with Fe(III). The unbranched saturated acids, 14:0 and 18:0, constituted 9.5% of the total and are fatty acids commonly identified in bacteria. The major lipids for the most closely related microorganism, *A. transvaalensis*, were i15:0, 14:0, and 16:0, and this is similar to those observed for thermophilic clostridia (37) as well as *A. crotonatoxidans* (4). The fatty acids i15:0 and i17:0 were not detected in strain QYMF at significant levels, and these results indicated that strain QYMF had a different membrane composition than did the previously observed *Alkaliphilus* spp. The strain QYMF also had a dramatically different lipid profile compared to alkaliphilic *Bacillus* spp., namely, the differences in 14:0, 16:0, and 15:0 derivatives; 16:1ω7c content; and overall total unsaturated lipids (43).

Previous studies have indicated that *Shewanella* species grown in the presence of different substrates have different fatty acid compositions (see discussion in reference 46). The two reported *Alkaliphilus* species were grown with different electron acceptors, and the different growth substrates could have affected the fatty acid composition of the respective cells. Metal reduction in the other two *Alkaliphilus* spp. (*A. transvaalensis* and *A. crotonatoxidans*) has not been reported. Isolate QYMF displayed a lipid profile different from those of other alkaliphilic microorganisms and had a higher percentage of total unsaturated fatty acids, similar to gram-negative microorganisms. Interestingly, the distantly related metal-reducing bacteria *Shewanella algae* and *Geobacter metallireducens* both had levels of 16:1ω7c similar to that of strain QYMF when grown with Fe(III) (46). The results suggested that 16:1ω7c could be a common membrane component observed in metal-reducing bacteria, but further work is needed. The lipid 16:1ω7c has not been reported in other alkaliphilic species;

however, the microorganisms were not or could not be grown with Fe(III).

The majority of metal-reducing bacteria are classified as δ- and γ-*Proteobacteria* and, to a lesser extent, gram-positive bacteria. The closest gram-positive metal reducers were *T. magadiensis* (91%), *Bacillus infernus* (83%), and "*Ferribacter thermoautotrophicus*" (81%) based on SSU rRNA gene sequences. Iron reduction was reported in *T. magadiensis*, but the pH of the medium was not stated, the optimum pH for *T. magadiensis* is only 8.5, and *T. magadiensis* has only 91% SSU rRNA gene sequence identity with isolate QYMF (14). Current data suggest that these other gram-positive bacteria do not reduce metals above pH 9.0 under tested growth conditions, and these results suggested that isolate QYMF was a novel and unique metal-reducing bacterium based on SSU rRNA gene sequence and phenotypes.

Growth of QYMF cells increased with increasing concentrations of yeast extract, and a similar result was observed with *A. transvaalensis* (37). *A. crotonatoxidans* is a strict anaerobe that can utilize yeast extract, peptone, tryptone, and some sugars, but growth was not stimulated in the presence of fumarate, sulfur, and thiosulfate (4). *A. transvaalensis* is also a strict anaerobe that can utilize proteinaceous substrates, and growth was improved with thiosulfate, sulfur, or fumarate (37). Isolate QYMF could utilize yeast extract in the presence of metals, but thiosulfate, fumarate, dimethyl sulfoxide, or sulfate was not used. At the time of sample collection, prominent blooms of algae and cyanobacteria were noted at the leachate ponds, and these might serve as a source of proteinaceous material for strain QYMF and other microbiota. The genus *Bacillus* contains alkaliphilic organisms that can reduce oxyanions (arsenate and/or selenate) (35), but little information exists in the literature regarding anaerobic alkaliphilic respiration with sulfur compounds or metals. The genus *Alkaliphilus* may represent a novel group of anaerobic alkaliphilic bacteria that can utilize proteinaceous material and reduce metals and sulfur compounds.

The growth medium contained phosphate and most likely explained the production of vivianite, as confirmed with EDX. When the buffer was changed from carbonate to Tris or the headspace was changed from nitrogen to hydrogen, the precipitates changed size and morphology, suggesting that variation in the chemical milieu altered the types of observed precipitates. These results indicated that alkaline mineral precipitation may vary according to the dynamics of the chemical environments, but further work is needed to discern the relationships between mineralogy and biochemistry.

Shewanella putrefaciens has been shown to reduce Fe(III)-citrate but not Fe(III)-EDTA (9). The authors suggested the possible importance of ligands to promote bioavailability of Fe(III) from ferrihydrite or other iron oxides in natural habitats (8). Based on the solubility and free energy of ferrihydrite, terrestrial seawater at pH 8.1 can potentially have approximately 20 nM dissolved iron (19). Isolate QYMF could reduce Fe(III)-citrate and Fe(III)-EDTA at significant rates above pH values of 9.0, but insoluble iron was not reduced at elevated pH values. These results suggested that the reduction of insoluble iron was limited by the unavailability of soluble iron at pH values above 9.0; however, further work is needed to characterize the metal-reducing capacity of isolate QYMF and the effects of culture conditions including pH.

Soda lakes, with elevated pH and salinity, are typically located in arid environments and constitute a habitat similar to the borax leachate ponds (12). Both cultivation-based and molecular studies have been done with samples from soda lakes (5, 11, 12), and these environments can contain extreme metabolic diversity within an autonomous microbial community. Zavarzin (44) has argued that continental soda lakes were a possible origin of bacterial diversity. Although present-day soda lakes and other alkaline environments are geologically recent, alkaline habitats have most likely existed since archaean times (11).

In the context of astrobiology, the chemical energy derived from metal redox cycles has been recognized as a plausible source for extraterrestrial microbial life (29, 32, 33). Recently, Kempe and Kazmierczak (13) hypothesized that the Jovian moon Europa had low initial Ca(II) levels that would have promoted biogenesis and that an alkaline saline ocean currently exists (26). The identification of microorganisms from environments possibly analogous to extraterrestrial habitats (e.g., in redox cycles, pH, salinity, and temperature) is ever important (16) and can provide data to improve models for exobiological evolution and detection.

Isolate QYMF is a unique bacterium classified in the recently named *Alkaliphilus* genus and was able to reduce metals in alkaline pH. Future work is needed to assess the possible niches of anaerobic respiring microorganisms in alkaline environments, to more fully understand the role of metal-reducing microorganisms in alkaline environments, and to assess the potential applicability for metal reduction during bioimmobilization of toxic heavy metals. Isolate QYMF was recently selected for whole-genome sequence determination at the Joint Genome Institute, and the genome sequence data will provide insight into the novel and unique lifestyle of this microorganism.

Description of sp. nov. *Alkaliphilus metalliredigens*. *Alkaliphilus metalliredigens* (*metallum*, L., metal; *redigere*, L., to reduce to a specific state or condition). Cells are straight, with some cells being slightly curved, and have a mean length of 3 to 6 μm and an approximate width of 0.5 μm. The cells stain gram positive and form terminal endospores. The strain QYMF is an obligate anaerobe and has a pH optimum of 9.5, a temperature optimum of 35°C, and sodium chloride optimum of approximately 2%. The organism can grow in the presence of 10 mM sodium borate. Yeast extract can be used as an electron donor, and small amounts stimulate growth in the presence of lactate or acetate. The electron donors yeast extract and lactate or acetate can be utilized with a reduction of Fe(III)-citrate or Fe(III)-EDTA. Co(III)-EDTA, Cr(VI), or AQDS can also be reduced. Strain QYMF does not utilize fumarate, nitrate, dimethyl sulfoxide, trimethylamine oxide, thiosulfate, sulfate, glycine, arginine, histidine, or alanine as electron acceptors under the tested conditions. The type strain was isolated from a borax-contaminated alkaline leachate pond in Boron, Calif. The type strain is *A. metalliredigens* strain QYMF.

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Impacts on microbial communities and cultivable isolates from groundwater contaminated with high levels of nitric acid–uranium waste

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Abstract

Microbial communities were characterized at contaminated sites that had elevated levels of nitrate, nickel, aluminum, and uranium (up to 690 mM, 310 μM, 42 mM, and 30 μM, respectively). The bacterial community structure based upon clonal libraries of the SSU rRNA genes (screened clones = 876) was diverse at the background site, but the three acidic samples had decreased diversity and the majority of clones were closely related to *Azoarcus* and *Pseudomonas* species. *Arthrobacter* and *Novosphingobium* sequences were recovered from the background samples but not the acidic sites, and similar pseudomonad populations were present at the background and acidic sites albeit at different relative abundances. Heterologous sequence coverage analyses indicated the microbial communities at the contaminated sites were very similar ($p = 0.001$) but different from the background site. Bacterial isolates ($n = 67$) classified as β-or γ-*Proteobacteria*, high G+C Gram-positive or low G+C Gram-positive were obtained from the background and one contaminated sample, and some of the isolates had less than 95% sequence identity with previously observed microorganisms. Despite variations in nitrate and heavy metal levels and different proximities to the source ponds, the three acidic samples had similar microbial populations. However, the least contaminated site (lowest nitrate and aluminum) had increased diversity compared to the other acidic samples. The results suggested that the combined contamination has decreased the microbial diversity, and *Azoarcus* populations were observed at a drastically increased frequency compared to the background site that had a more even distribution of multiple taxa.

Keywords: Microbial community; SSU rRNA gene; Uranium; Nitrate; Groundwater

1. Introduction

The remediation of mixed wastes and radionuclides is a significant challenge, and bioremediation with microorganisms remains potentially the most cost-effective cleanup technology. However, biodegradation or biotransformation of pollutants in the natural environment is a complex process that depends upon the quantitative and qualitative aspects of the contaminant present, the

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structure and dynamics of the indigenous microbial community, and the geological and chemical conditions at the contaminated sites [1]. In addition, many disturbed sites contain multiple contaminants that can complicate treatment and/or maintenance. The elucidation of individual and community-wide microbial responses to the combination of mixed wastes and varied geochemical conditions is a crucial aspect for the improvement and implementation of bioremediation strategies.

The majority of studies dealing with radionuclide-associated environments have focused on cultivable, activity-based, or RFLP-based assessments of waste piles or repositories of nuclear fuel [2–5]. One study utilized clonal libraries of SSU rRNA gene sequences to characterize microbial diversity associated with naturally radioactive subsurface environments in Gabon, Africa [6]. The biogeochemical processes that occur in groundwater and sediments contaminated with heavy metals and radionuclides can impact the microbial community [7,8], yet little is known about the composition of the indigenous subsurface microbial communities at different sites contaminated with radionuclides. Moreover, even less is known regarding the relationship between the microbial community and the geochemical characteristics of contaminated sites.

The Field Research Center (FRC) is located within the Y-12 Security Complex near Oak Ridge, TN in the Bear Creek Valley, and the site includes 243-acres of a previously disturbed contaminated area. The subsurface at the FRC contains one of the highest concentration plumes of mobile uranium located in the United States, and contains various levels of nitrate, heavy metal, and organic contamination (http://www.esd.ornl.gov/nabirfrc/). Groundwater samples from wells that surround the S-3 ponds were collected, and the ponds were four unlined surface impoundments that received nitric acid/uranium bearing wastes. In order to determine how varying levels of multiple contaminants (e.g., nitrate, metals, radionuclides, low pH) have affected the microbial community structure, three contaminated wells in close proximity to the source ponds as well as a background site were used. In addition, the isolation of microorganisms in relation to nitrate reduction was attempted for the four tested sites.

2. Materials and methods

2.1. Sampling sites

The FRC site includes three areas of contaminated soil and groundwater and an uncontaminated background area that contains soils similar to those found in the contaminated areas. The site is contaminated with nitrate, uranium, heavy metals, and a variety of organic contaminants, and a full description can be located at the FRC website (http://www.esd.ornl.gov/nabirfrc/). Groundwater samples were collected from four wells at the FRC (Table 1). The wells FW-005 and FW-010, located in Area 3, are 32.5 m apart, and are ≈20 m from the former waste pond. Well FW-015 is 27 m from the waste pond embankment in Area 1, and is ≈130 m from the wells in Area 3.

Table 1
The pH, nitrate, uranium, aluminum and nickel levels in the groundwater samples from four characterized FRC groundwater samples

Well	pH	Nitrate (mM)	Uranium (μM)	Nickel (μM)	Aluminum[a] (mM)
FW-300	5.8 ± 0.4	0.04 ± 0.05	ND	0.45 ± 0.5	0.01 ± 0.01
FW-005	4.0 ± 0.2	4.6 ± 2.5	30 ± 4.8	84 ± 1.0	1.74
FW-010	3.5 ± 0.1	694 ± 28	0.8 ± 0.1	310 ± 17	41.5
FW-015	3.5 ± 0.2	154 ± 27	31 ± 2.7	148 ± 2.5	22.9

FW-300 represents the background area. ND, not detected.
[a] Some samples measured once.

The background area is ≈2 km from the contaminated area in the same valley on the Y-12 security complex. The background area lies directly along the geologic strike of the contaminated area and is, therefore, underlain by nearly identical geology, mineralogy, and structure. Contaminants have not been previously or currently disposed at the background area throughout the history of Department of Energy operations (http://www.esd.ornl.gov/nabirfrc/index.html). However, it should be noted, that due to the contamination, the background area is not directly next to the contaminated areas.

Different background wells have very similar geochemical characteristics, and one background site was analyzed with increased coverage. The water table was ≈6 m from the surface, and water was collected from a screened interval below the water table at each of the four wells on the same day. Microbial cell numbers were quantified by direct cell counting using epifluorescence microscopy of acridine-orange stained samples and statistical analysis as described previously [9].

2.2. Water chemistry

Ground water pH was measured at the field site from multi-level wells with a pre-calibrated Yellow Springs Instruments YSI XL6000M multi-parameter probe (Yellow Springs Instruments, CO). Nitrate was analyzed with a Dionex 500 ion chromatograph (Dionex, Sunnyvale, CA) following EPA method 300.0, and TOC was measured by a Shimadzu total organic carbon analyzer (TOC-5000A, Tokyo, Japan) (EPA method 415.1). Nickel and aluminum were analyzed in acidified samples with a Thermo Jarrell Ash inductively coupled plasma PolyScan Iris Spectrometer (EPA reference SW846 ch 3.3), and uranium levels were analyzed with a Perkin–Elmer Elan 6000 ICP-MS (EPA reference 600/R-94-111).

2.3. Bacterial isolation, cultivation, and differentiation

Aliquots were removed from groundwater samples and used to inoculate different media. Denitrifying medium (DM) contained nutrient broth (8 g l^{-1}) and potassium nitrate (1 g l^{-1}), and MR2A medium has been described previously [10]. The pH values (4.0, 5.5, or 6.5) for both media were adjusted with nitric acid. Anaerobic media was prepared by boiling under oxygen-free N_2 gas and sealing the media in roll tubes with butyl stoppers and crimp seals. Samples were incubated in the dark at room temperature. Anaerobic plates were prepared in an anaerobic glove bag (Coy Laboratory Inc.) with a N_2:H_2 atmosphere (95%:5%). Colonies were picked based on differences in morphology, color, and margins.

Isolates were streaked 3–5 times to ensure purity, and then tested for nitrate reduction with inverted Durham tubes and/or Szechrome reagent (Polysciences, Inc.) according to the manufacture's instructions. Bacterial isolates were differentiated with BOX-PCR genomic fingerprinting. Each isolate was transferred to 0.04 M NaOH (50 μl) with a small inoculating loop, and then frozen at −80 °C. The samples were then quickly thawed at 65 °C, and subsequently heated at 96 °C for 10 min. The solution of disrupted cells served as the genomic DNA for BOX-PCR. BOX-PCR was done according to the protocol of Rademaker and de Bruijn [11]. PCR volumes were 25 and 1 μl of template DNA was used. The PCR parameters were as follows: 95 °C for 7 min – 1 cycle; 94 °C for 1 min; 53 °C for 1 min; 65 °C for 8 min – 30 cycles; 65 °C for 16 min – 1 cycle. Samples were observed on 1.5% TAE agarose gels. Gels were analyzed and compared with Molecular Analyst software 1.6 (BioRad, Inc.).

2.4. DNA extraction and purification

Groundwater samples (1–2 l) were collected, and transported to the laboratory in glass, amber bottles. Bacteria were harvested by centrifugation (10,000*g* force, 4 °C for 30 min), and the pellets were stored at −80 °C until used for DNA extraction. The cell pellet was resuspended in a lysis buffer and the cells disrupted with a previously described grinding method [12]. DNA was extracted as previously described [12,13], and the precipitated DNA was purified by gel electrophoresis plus mini-column preparation (Wizard DNA Clean-Up system, Promega) [12].

2.5. PCR amplification and cloning

The nearly complete SSU rRNA genes were amplified in a 9700 Thermal Cycler (Perkin–Elmer) with the primer pair FD1 F (5′ AGA GTT TGA TCC TGG CTC AG 3′) and 1540R (5′ AAG GAG GTG ATC CAG CC 3′) (position 1 and 1540 *Escherichia coli* designations) [14]. The PCR reactions (20 μl) contained 50 mM KCl, 10 mM Tris–HCl pH 9.0 and 0.1% Triton X-100), 1.8 mM $MgCl_2$, 80 ng bovine serum albumin (Boehringer Mannheim, Indianapolis, IN), 0.25 mM 4× dNTPs (USB Corporation, Cleveland, OH), 10 pmol each primer, 2.5 U Taq polymerase, and 1 μl purified DNA (5–10 ng). To minimize PCR-induced artifacts, the optimal number of cycles was determined and five PCR reactions were combined prior to cloning as described previously [15]. The PCR parameters were as follows: 80 °C for 30 s; 94 °C for 2 min; 94 °C for 30 s; 58 °C for 1 min; 72 °C for 1 min, 26 cycles; 72 °C for 7 min. PCR products were analyzed on 1.5% (w/v) TAE agarose gels, and the insert size confirmed.

The combined PCR products were separated by electrophoresis in a low-melting point agarose gel (0.8%), the appropriate band excised, and the DNA extracted with a Wizard Prep Kit (Promega, Madison, WI) according to manufacturer's instructions. Recovered DNA was resuspended in 6 μl ddH_2O, 2 μl was ligated with pCR2.1 vector from a TA-cloning kit, and competent *E. coli* cells were transformed according to the provided protocol (Invitrogen, San Diego, CA).

2.6. Sequence and phylogenetic analysis

PCR products (100 μl) amplified with vector-specific primers via colony PCR were purified with the ArrayIt™ PCR Purification Kit (TeleChem International, Inc., Sunnyvale, CA) or treated with ExoSAP-IT™ (USB Corporation, Cleveland, OH) according to manufacturer's instructions. DNA sequences were determined with a BigDye Terminator kit (Applied Biosystem, Foster City, CA) using a 3700 DNA analyzer (Perkin–Elmer, Wellesley, MA) according to the manufacturer's instructions. DNA sequences were edited with the Sequencher™ software (version 4.0, Gene Codes Corporation, Ann Arbor, MI).

Partial sequences were determined from the purified inserts with the SSU rRNA gene primer 529r, 5′ CGC GGC TGC TGG CAC 3′ (*E. coli* numbering). Unique SSU rDNA clones (⩽97% sequence identity) from each site were identified by direct sequence comparisons, and were designated as OTUs (operational taxonomic unit). The number of sequences determined for each site is listed in Table 3. The unique OTUs were compared to the closest related sequences from GenBank and the RDP, and nearly complete SSU rDNA sequences (n = 82) were determined for representative clones (Figs. 2–5).

The sequences were aligned with ClustalW, and alignments compared with reference sequences from the database [16]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 2.1 [17], and phylogenetic trees were constructed with distance

matrices and the minimum evolution method with a Jukes–Cantor distance model. The initial trees were obtained with the neighbor-joining method and interior branch tests were determined. The presumptive phylogenetic relationships between the clonal sequences are being used as a relative comparison between the observed sequences and not as definitive designation for phylogeny. The nucleic acid sequence accession numbers in GenBank for the bacterial isolates and the unique clonal sequences described in this study are: AY661909–AY662049. The diversity (Shannon–Wiener and Simpson's index) and evenness (equitability) indices were based upon the distribution of unique OTUs obtained from the clonal libraries using equations from Krebs [18].

LIBSHUFF (version 1.2) analysis computes the homologous and heterologous coverage within and between clonal libraries [19]. The analysis estimates the similarity between clonal libraries from two different samples based upon evolutionary distances of all sequences. Thus, the sampled diversity of a community can be directly compared to another community. The predicted coverage of a sampled library is denoted by the homologous coverage, and the heterologous coverage is the observance of a similar sequence in a separate library. The values are reported over a sequence similarity range or evolutionary distance (D) based upon a distance matrix. Analyses were performed according to specified directions given at the LIBSHUFF website (http://www.arches.uga.edu/~whitman/libshuff.html).

3. Results

3.1. Site characteristics

The chemical characteristics were significantly different among the four samples (Table 1), and the wells except background had nitrate levels over the drinking water standards of 10 ppm or 0.16 mM. The TOC (total organic carbon) levels were similar for FW-005 and FW-015 (5.8 mM vs. 5.4 mM, respectively), and were ≈2-fold above background (2.5 mM). Site FW-010 had a TOC value that was ≈6-fold increased compared to background (14.6 mM). The substratum of the sampling sites was similar, and consisted of shale with inner-embedded limestone that has been weathered to clay, silty saprolite. The groundwater flow was in a south–southwest direction from the source ponds towards FW-005, FW-010, and FW-015.

3.2. Bacterial isolates

Bacteria were isolated from FW-300 ($n = 59$) and FW-005 ($n = 9$) with either MR2A medium [10] or nutrient broth amended with nitrate. Isolates were not obtained from FW-010 or FW-015. Microbial isolates with significant sequence identity to predominant clones were not obtained from any of the samples, except for isolate DM-E3 (FW-300) which had 97% sequence identity with clone 005Aa-H03 that accounted for 7% of the sampled diversity from FW-005 (Fig. 3). All isolates from FW-005 could be classified as β-*Proteobacteria*, except 005-J which was related to *Microbacterium*, and could grow at pH values between 4.0 and 7.0 in MR2A medium. All isolates could reduce nitrate in MR2A medium and the closest phylogenetic associations are listed in Table 2.

3.3. Community structure based on partial SSU rRNA sequence comparisons

The collected biomass from the groundwater samples yielded PCR products, and 876 clones were screened via comparison of partial sequences of the V2–V6 region of the SSU rRNA gene sequence (≈400–500 nucleotides at the 5′ end). Between 100 and 320 clones were screened for each sample (Table 3), and rarefaction analyses indicated that the majority of recovered diversity was sampled within 85 analyzed clones for FW-010, ≈135 analyzed clones for FW-005 and FW-015, and 245 clones for FW-300 (Fig. 1). The background, FW-300, displayed the highest diversity compared to the acidic, contaminated sites (Table 3). At the time of sampling, bacterial numbers in FW-300 groundwater were estimated to be $\approx 10^6$ cells ml^{-1} with acridine orange and $\approx 10^5$ cells ml^{-1} in the acidic groundwater.

A significant proportion of the FW-005 clones were *Azoarcus*-like (005C-F11) and comprised almost 40% of the FW-005 library (Table 4). Two different *Pseudomonas*-like sequences (005C-G12 and 005Aa-H03) were also relatively abundant and were 20% and 7% of the library, respectively. Ecological indices indicated that FW-005 had the highest diversity and evenness values of the contaminated sites and 27 OTUs were estimated based on ⩽97% similarity of SSU rRNA gene sequences (Table 3).

The well, FW-010, also had an increased occurrence of *Azoarcus* and *Pseudomonas*-like sequences (010B-F11, 010D-B08, and 010B-E10), and these genera comprised almost 90% of the FW-010 library (Table 4). The nitrate and aluminum levels of FW-010 groundwater were the highest of the sampled sites, and FW-010 had the lowest number of estimated OTUs. An increased proportion (40%) of the FW-010 library appeared to be *Pseudomonas*-like compared to the other acidic samples, and 12 OTUs were less than 2% of the library. The majority of recovered sequences for FW-015 were also closely related to *Azoarcus* and *Pseudomonas*. The *Azoarcus* group accounted for 49% of the library and pseudomonads accounted for ≈25% (Table 4).

Table 2
Bacterial isolates obtained from FW-300 (background) or FW-005

Sample	Percent identity to closest match (%)	Closest match
FW-005		
005-L	99	*Delftia acidovorans* (AF526915)
005-M	97	*Acidovorax* 3DHB1 (AF458096)
005-3	95	*Burkholderia phenazinium* (AB021394)
005-2	94	*Burkholderia phenazinium* (AB021394)
005-D	96	*Pandoraea* G5084 (AF247693)
005-4	96	*Chromobacterium violaceum* 52 (AY117554)
005-J	94	*Microbacterium* VKM (AB042074)
FW-300		
M-A24	90	*Variovorax paradoxus* (AJ420329)
AM-Z2	95	*Burkholderia phenazinium* (AB021394)
DM-R2	88	*Chromobacterium violaceum* 52 (AY117554)
DM-L2	90	*Chromobacterium violaceum* 52 (AY117554)
DM-P3	97	*Chromobacterium violaceum* 52 (AY117554)
AnM-A3	98	*Stenotrophomonas detusculanense* (AF280434)
M-A21a	99	*Stenotrophomonas rhizophila* (AJ293463)
DM-P2	96	Biofilm clone IAGBH3 (AF286178)
DM-N2	95	Biofilm clone IAGBH3 (AF286178)
AnM-E3	96	Biofilm clone IAGBH3 (AF286178)
DM-A3	90	*Serratia proteamaculans* (AJ508694)
DM-D3	92	*Serratia proteamaculans* (AJ508694)
M-12	95	*Acinetobacter johnsonii* (AB099655)
M-3	94	*Acinetobacter johnsonii* (AB099655)
DM-E3	95	*Pseudomonas syringae* (AB001439)
DM-V2	96	*Pseudomonas aeruginosa* (AY162139)
M-A12	93	*Rhizobium galegae* (Z79620)
M-11	93	Clone R10 (AF407686)
M-1	99	Clone ph5Lac302-37 (AY527741)
M-4	96	Clone ph5Lac302-37 (AY527741)

All isolates from FW-005 were isolated with MR2A medium. Isolates from FW-300 designated with a DM were isolated with nitrate amended nutrient broth, and all other isolates from FW-300 were obtained with MR2A.

Table 3
Characteristics and diversity estimates for SSU rRNA gene clones from four FRC groundwater samples

Well	Number of clones[a]	OTU[b]	H′[c]	1/D[d]	Evenness[e]
FW-300	321	79	5.17	22.7	0.97
FW-005	211	27	3.18	4.91	0.82
FW-010	113	19	2.62	3.81	0.77
FW-015	231	34	2.84	3.43	0.73

FW-300 represents the background area.
[a] Number of clones analyzed from each library.
[b] Operational taxonomic units based on unique, partial SSU rRNA gene sequences (⩽97%).
[c] Shannon–Weiner index, higher number represents more diversity.
[d] Reciprocal of Simpson's index, higher number represents more diversity.
[e] As Evenness approaches 1, the population is more evenly distributed.

Table 4
The distribution of observed SSU rRNA gene sequences for FW-005, FW-010, FW-015, and FW-300 based on partial sequence determination

Sample	Clone	Number of clones	Closest match
FW-005	005C-F11	82/211	*Azoarcus* FL05 (AF011330)
	005C-G12	42/211	*Pseudomonas stutzeri* (U65012)
	005Aa-H03	14/211	*Pseudomonas marginalis* (AF364098)
	005Aa-B11	13/211	*Rhizobium giardinii* (U86344)
	005C-G01	8/211	*Wautersia eutropha* (M32021)
	005Aa-C05	6/211	*Stenotrophomonas rhizophila* (AJ293463)
	005Aa-F09	6/211	Unidentified clone (Z93955)
FW-010	010B-F11	51/113	*Azoarcus* FL05 (AF011330)
	010D-B08	37/113	*Pseudomonas stutzeri* (AJ270451)
	010B-E10	14/113	*Pseudomonas marginalis* (AF364098)
	010A-E08	2/113	*Ralstonia metallidurans* CH34 (Y10824)
FW-015	015d-A03	113/231	*Azoarcus* FL05 (AF011330)
	015B-H08	39/231	*Pseudomonas stutzeri* (U65012)
	015C-D08	18/231	*Pseudomonas marginalis* (AF364098)
	015C-F06	7/231	Uncultivated clone (AF072924)
	015E-E03	5/231	*Ralstonia metallidurans* (Y10824)
	015C-F09	5/231	*Acidovorax delafiedii* (AF332187)
FW-300	300I-E04	51/321	*Arthrobacter polychromogenes* (Y80741)
	300A-A08	29/321	*Pseudomonas marginalis* (AF364098)
	300C-C3	25/321	*Novosphingobium subterraneum* (AB025014)
	300I-A04	22/321	*Acidovorax delafiedii* (AF332187)
	300I-E02	19/321	Uncultured clone (AY037565)
	300E2-D10	10/321	*Pseudomonas stutzeri* (U65012)
	300I-F2	6/321	*Propionivibrio limicola* (AJ307983)
	300E2-F01	6/321	*Sphingobium yanoikuyae* (AF509480)
	300A-H04	6/321	*Bdellovibrio bacteriovorus* (AF084850)
	300A-C09	6/321	Unidentified clone (Z93955)

Unique OTUs are at the 97% sequence identity level. Each predominant clone is listed with the number of times a similar sequence was detected in the respective library as well as the closest match from the GenBank database (accession numbers in parentheses).

The background, FW-300, displayed the highest diversity and evenness, and 79 unique OTUs were observed from a clone library of 321 analyzed clones compared to between 19 and 34 unique OTUs at the acidic sites (Table 3). Two *Pseudomonas*-like OTUs were observed at all tested sites, but the occurrence in the background library was reduced compared to the acidic samples (9% vs. 17–33% or 3% vs. 7–9%, respectively). The majority of the observed OTUs from the FW-300 clonal library were unique with respect to the acidic sites.

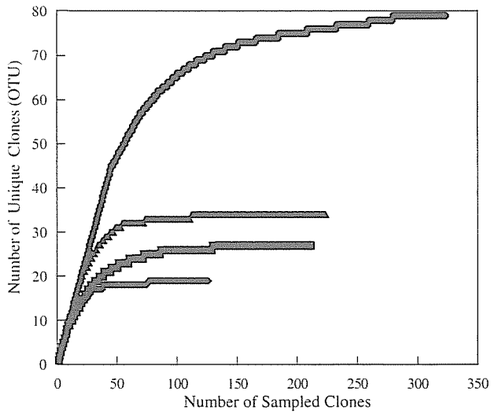

Fig. 1. Number of unique clones versus the number of sampled clones from groundwater samples FW-300 (●), FW-005 (■), FW-010 (◆), and FW-015 (▲).

3.4. LIBSHUFF analyses

The SSU rRNA gene clonal libraries were compared between the sites with differences between coverage curves (LIBSHUFF analysis, version 1.2) as previously described [19]. The acidic samples had the lowest ΔC_{xy} values when compared to each other, and FW-010 and FW-015 were the most similar (Table 5). The majority of difference between the acidic samples was observed at decreased evolutionary distances (>95%). The sites, FW-005 and FW-300, appeared to be the most dissimilar, and the FW-010 and FW-015 also displayed significantly increased dissimilarity with the FW-300 library (3.5 and 5.2, respectively).

3.5. Phylogenetic analysis

The most frequently recovered OTU (005C-F11) for all three acidic samples had 99% sequence identity with *Azoarcus* FL05, and the clonal sequences had 99% sequence identity with one another (Fig. 2). *Azoarcus*-like or *Ralstonia*-like sequences were not observed at the background site, nor were any microbial isolates obtained that had high sequence similarity with these groups. Interestingly, the bacterial isolate, 005-L, from FW-005 had 99% sequence identity with the clone, 005Aa-B06, and 98% sequence identity with *Delftia acidovorans* (Fig. 2).

The majority of pseudomonad sequences grouped with *Pseudomonas stutzeri* and was similar for all sites (although the relative abundances were markedly different). However, the second cluster of pseudomonad sequences grouped with *Pseudomonas marginalis* and *P. rhodesiae*, and was observed at similar frequencies (8–12%) for all four samples. In addition, sequences closely related to the *Enterobacteriaceae* were not identified from any of the clonal libraries, but six bacterial isolates were obtained from FW-300 that could be classified in the *Enterobacteriaceae* (Fig. 3).

Indicative of the increased diversity indices for FW-300, sequences that represented the α-,β-,γ-,δ-*Proteobacteria*, *Acidobacterium*, high G+C, and *Verrucomicrobia* were observed, many were not observed at the acidic samples, and some of the clones were not closely related to previously cultivated microorganisms. Clonal sequences unique to FW-300 had 99% or 96% sequence identity with *Novosphingobium subterraneum* or *Sphingobium yanoikuyae*, respectively, and accounted for a total of 10% of the sampled diversity from the background (Fig. 4). A majority of the novel sequences could be classified as δ-*Proteobacteria*, low G+C, *Acidobacteria* or *Verrucomicrobiales* (Fig. 5).

A high G+C OTU represented by 300I-E04 constituted 16% of the sampled diversity from FW-300 and had 98% sequence identity with *Arthrobacter polychromogenes* (Fig. 5). Few clonal sequences that could be classified as low G+C were obtained from any of the samples, but unique microorganisms were isolated from FW-300 with MR2A medium. Isolates M-1-300 and M-4-300 were most closely related to FRC sediment clone ph5Lac302-37 (GenBank, AY527741) and had 98% and 96% sequence identity, respectively.

4. Discussion

PCR-based cloning approaches can be used to assess diversity and community structure, and can be a valuable tool for microbial community analyses [20]. However, the abundance, dominance, or diversity may not be completely epitomized by clone distribution; therefore, the measurements and indices are used for relative comparisons [20]. The formation of chimeras, heteroduplexes, and mutations can be intrinsic to PCR amplification [15], and protocols were followed to minimize PCR amplification-induced artifacts as described in Section 2. Multiple studies exist in the literature that employ SSU rRNA gene clonal libraries, and for the present study 97% sequence identity was used for the designation of unique OTUs at the species level. It should also be noted that the cloning and sequencing of SSU rRNA sequences could vastly underestimate the number of bacterial species, but can provide information on some of the more significant species present in a given environment [21,22].

Previous studies have shown that mixed microbial communities can differ significantly between attached and unattached cells with respect to biochemical capacity, cell division, and exopolymer production [23]. Microbial populations and communities within groundwater and sediment from the same sampling site could

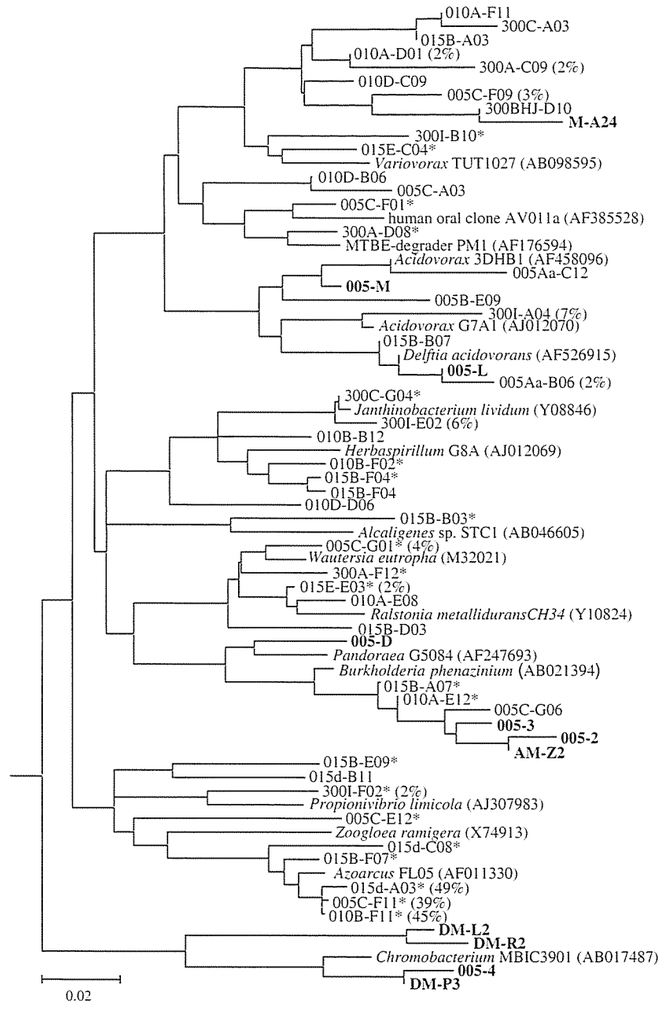

Fig. 2. Phylogenetic relationships of unique cloned sequences and bacterial isolates from the FRC and reference sequences from GenBank classified in the β-*Proteobacteria*. Minimum evolution phylogenies were constructed in MEGA (version 2.1; http://megasoftware.net) with Jukes–Cantor distance model. The sub-tree for the β-*Proteobacteria* is shown and *Methanosarcina mazei* was the outgroup. The percentage (%) represents the abundance of predominant clones from the sampled diversity. Sequences identified as numbers and letters are FRC clones (i.e., 015C-F06, a clone from FW-015), and clones for which nearly complete sequences were determined are denoted with an asterisk. Sequences in bold are from FRC bacterial isolates. The accession numbers for reference sequence are listed in the figure (e.g., *Azoarcus* FL05 (AF011330)), and nomenclature was based upon most recent release of taxonomic outline [31].

be different, and the groundwater populations at the sampled FRC sites might be different from populations associated with the sediment. However, sequences most closely related to *P. marginalis* or *P. rhodesiae* were recently reported from acetate and iron enrichments from background sediments [24]. In addition, Petrie et al. [24] reported low G+C Gram-positive sequences from sediment enrichments that were closely related to our isolates, M-1-300 and M-4-300. The iron-reducing enrichments had either glycerol or acetate and sediments from the background or a contaminated site. Future work includes the assessment of the microbial community structure for different FRC sediments.

The nitrate levels in different groundwater samples from a mill-tailings disposal site (Shiprock, NM) were between 0.002 and 126 mM, and a predominance of

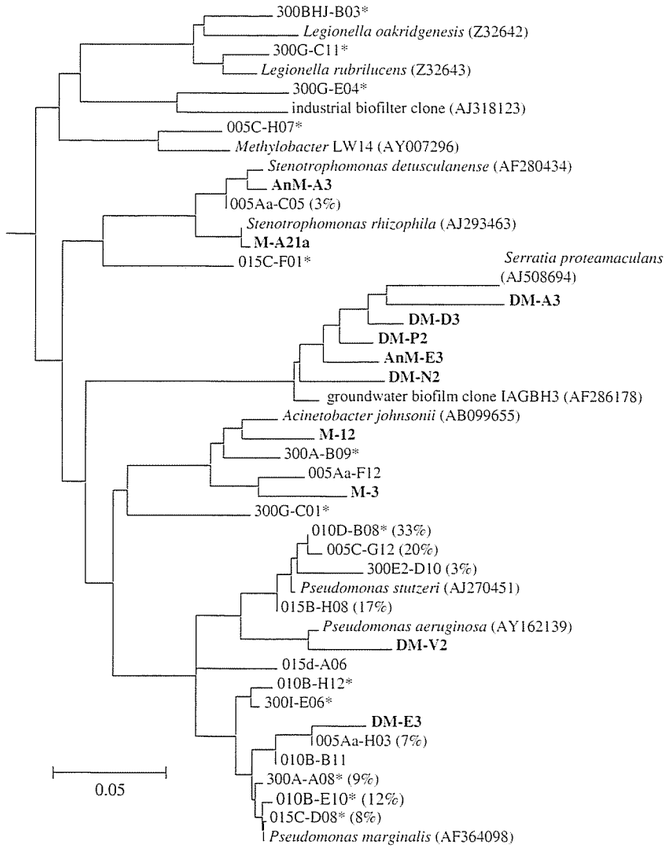

Fig. 3. Phylogenetic relationships of unique cloned sequences and bacterial isolates from the FRC and reference sequences from GenBank classified in the γ-*Proteobacteria*. The tree was constructed as described in the legend of Fig. 2. The sub-tree for the γ-*Proteobacteria* is shown and *Methanosarcina mazei* was the outgroup. Sequences identified as numbers and letters are FRC clones, and clones for which nearly complete sequences were determined are denoted with an asterisk. Sequences in bold are from FRC bacterial isolates. The percentage (%) represents the abundance of the clone in the respective library. The accession numbers for the reference sequences are listed in the tree, and nomenclature was based upon most recent release of taxonomic outline [31].

the ammonia-oxidizing bacterium, *Nitrosomonas*, was observed over *Nitrosospira* [25]. Based on SSU rRNA gene clonal libraries, *Nitrosomonas* or *Nitrosospira* were not detected as significant populations in the FRC groundwater, and this result may be due to the acidic nature of the contaminated samples. Recent results from *amo*A (ammonia monooxygenase) clonal libraries of the same samples suggested the presence of *amo*A-like sequences from *Nitrosomonas* and *Nitrosospira* (unpublished results); however, specific SSU rRNA primers or probes were not tested.

The three acidic sites tested at the FRC had increased levels of nitrate, heavy metals, and aluminum, and the clonal libraries indicated that all three were predominated by β-*Proteobacteria* related to *Azoarcus*. A predominant sequence type identified in a groundwater plume contaminated with coal-tar was closely related to the genus *Azoarcus* [26], however, the presumptive *Azoarcus* sequences from the FRC groundwater could represent different microorganisms. *Azoarcus* species were shown previously to be denitrifiers capable of anaerobic degradation of organic contaminants [12,27], and organic volatile concentrations (including toluene) are between 5 $\mu g\,l^{-1}$ and 10 $mg\,l^{-1}$ across the FRC. Anaerobic enrichments inoculated with acidic, FRC groundwater were positive for toluene degradation and nitrate removal, but isolates were not obtained (data not shown). The results indicated that denitrifying *Azoarcus* spp. capable of anaerobic degradation of organic pollutants may be present at the three acidic sites,

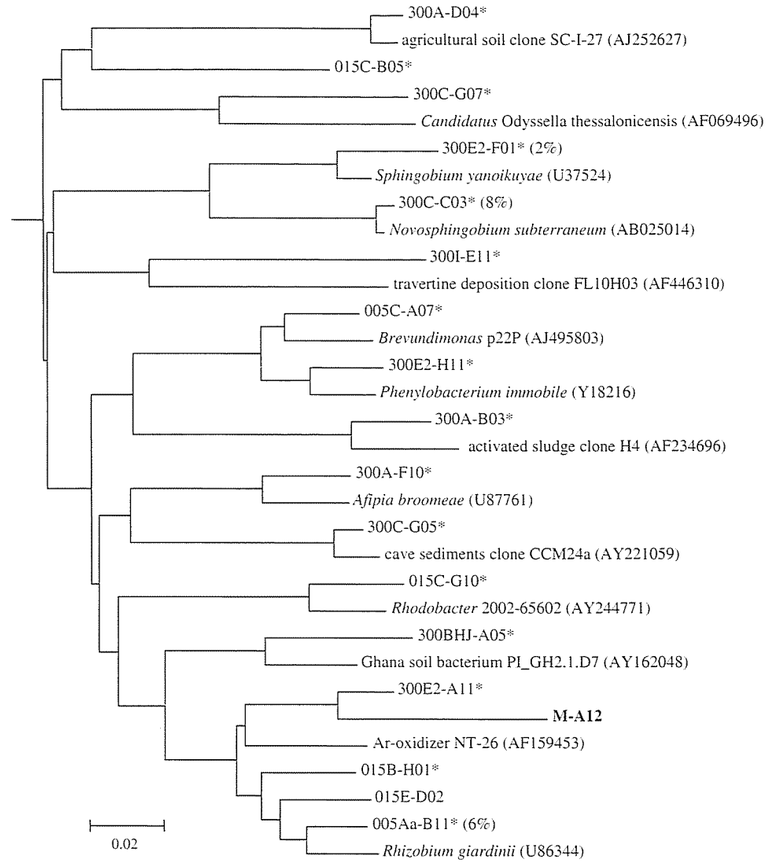

Fig. 4. Phylogenetic relationships of unique cloned sequences and bacterial isolates from the FRC and reference sequences from GenBank classified in the α-*Proteobacteria*. The sub-tree for the α-*Proteobacteria* is shown and *Methanosarcina mazei* was the outgroup. The tree was constructed as described in the legend of Fig. 2. The percentage (%) represents the abundance of the clone in the respective library. Sequences identified as numbers and letters are FRC clones, and clones for which nearly complete sequences were determined are denoted with an asterisk. Sequences in bold are from FRC bacterial isolates. The accession numbers for the reference sequences are listed in the tree, and nomenclature was based upon most recent release of taxonomic outline [31].

but further work is needed to confirm the existence of these microorganisms as well as any biochemical activity. Current work includes the metagenomic sequence determination for the FW-010 groundwater community in order to obtain the nearly complete genomes from the predicted 20 phylotypes.

Similar *Pseudomonas* OTUs were observed at the background and acidic sites, but these populations were decreased 5–11-fold at the background site. The second *Pseudomonas* OTU (*P. marginalis*) was observed at similar frequencies for FW-300, FW-005, FW-010, and FW-015 (9%, 7%, 12%, and 8%, respectively). The results suggested that similar *Pseudomonas* populations were present at all sites but at different proportions of the sampled diversity. The only other γ-*proteobacterial* clone sequence observed at a significant level was closely related to *Stenotrophomonas* DFK5, and accounted for 3% of the sampled FW-005 diversity. Interestingly, *Stenotrophomonas* DFK5 was reported to be isolated from heavy water (GenBank, AB045277). The data suggested that the conditions at the acidic sites have caused the pseudomonad populations to shift, but it is difficult to ascertain the relative contributions of decreased diversity and increased contaminant load for the relative distribution of similar populations.

Novosphingobium- and *Arthrobacter*-like sequences accounted for a majority of the recovered diversity at the background site and not the contaminated sites.

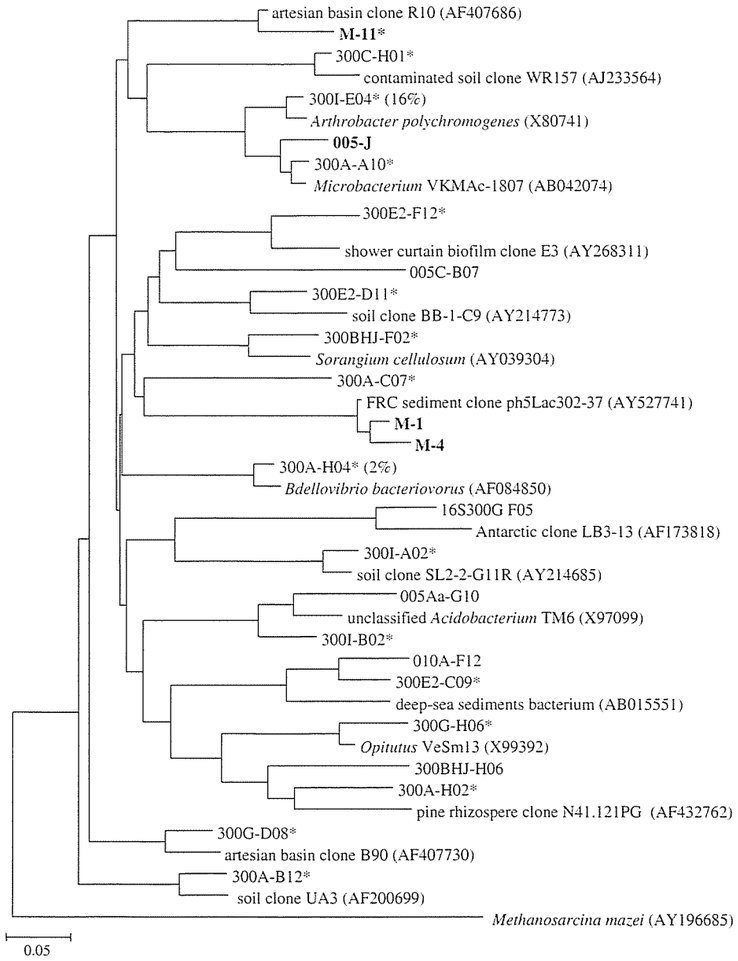

Fig. 5. Phylogenetic relationships of unique cloned sequences and bacterial isolates from the FRC and reference sequences from GenBank classified in the δ-*Proteobacteria*, *Acidobacteria*, high G+C, and low G+C microorganisms. *Methanosarcina mazei* was used as the outgroup. The tree was constructed as described in the legend of Fig. 2. The percentage (%) represents the abundance of the clone in the respective library. Sequences identified as numbers and letters are FRC clones, and clones for which nearly complete sequences were determined are denoted with an asterisk. Sequences in bold are from FRC bacterial isolates. The accession numbers for the reference sequences are listed in the tree, and nomenclature was based upon most recent release of taxonomic outline [31].

The data suggested that *Novosphingobium* and *Arthrobacter* species might not tolerate increased nitrate, increased metals, and/or acidic pH. However, isolates closely related to *Novosphingobium* and *Arthrobacter* were not obtained and could not be tested for sensitivity. FW-005 isolates were evaluated for the ability to grow at different pH values in MR2A medium, and some of the tested isolates (e.g., 005-2, 005-3, 005-L) could grow at pH values down to 4.0 and tolerate nickel concentrations up to 10 mg l^{-1} (data not shown).

Few of the isolates had greater than 97% sequence identity with observed clones from the same sample, and previous studies have reported differences between cultivable and molecular assessments of the same community [28,29]. A previous study by Pedersen et al. [6] described microbial populations from groundwater at a naturally radioactive subsurface. The pH values ranged from ≈5.0 to 7.0, but metal and anion levels were not reported. Samples were analyzed from five wells and β-*Proteobacteria* were a predominant group in most

Table 5
LIBSHUFF analyses of the groundwater communities based on SSU rRNA clonal libraries

Sample comparison	Clones (n_x)	Clones (n_y)	ΔC_{xy}	P value
FW-005 vs. FW-010	211	113	0.63	0.001
FW-005 vs. FW-015	211	231	0.61	0.001
FW-005 vs. FW-300	211	321	8.61	0.001
FW-010 vs. FW-015	113	231	0.44	0.001
FW-010 vs. FW-300	113	321	3.52	0.001
FW-015 vs. FW-300	231	321	5.18	0.001

The respective clone number for each sample is given by n_x and n_y, and ΔC_{xy} represents the difference in coverage of the two sequence libraries (an increased ΔC_{xy} represents greater dissimilarity between the given communities). The software for the analysis was used according to specified directions (http://www.arches.uga.edu/~whitman/libshuff.html).

of the tested wells [6]. The occurrence of similar sequences might be more of an attribute of groundwater itself and not the contamination. Extensive geochemical data was not reported for the Oklo site [6], but the FRC groundwater is most likely very different due to the combination of low pH and multiple contaminants.

Direct comparison of the entire sequence libraries between the sites (LIBSHUFF analysis) indicated that the contaminated samples were significantly different from the background site, and that the acidic sites were significantly similar. In addition, bacterial isolates were only obtained from one acidic site, and FW-005 had the lowest measured aluminum and nitrate levels. However, linear relationships were not observed between the diversity indices (i.e., OTUs, $1/D$, ΔC_{xy}) and the tested geochemical parameters. Further work is needed to identify the key factors that affect microbial community structure and relative abundances at the FRC, as well as major metabolic activities in situ.

Our previous analysis of the geochemical data for the different sites indicated that nitrate was a major factor, but similar *nir*K and *nir*S gene sequences accounted for a majority of the diversity at the background and acidic samples [30]. Based upon SSU rRNA gene sequences, the only similar, significant OTUs between background and the acidic samples were the pseudomonads. However, it is possible that different genera at the FRC have similar *nir*K and/or *nir*S genes. Preliminary results with some of the isolates indicate that the microorganisms can denitrify FRC groundwater amended with electron donors after the pH has been adjusted. Further work is underway to characterize the denitrification capacity of these organisms in the presence of heavy metals, and evaluate the effects of pH on indigenous microorganisms.

In summary, the results showed that: (i) groundwater community structure was affected in a similar fashion for the contaminated samples compared to the background site (diversity decreased with increasing nitrate and aluminum); (ii) *Arthrobacter* and *Novosphingobium* spp. predominated the background sample but were not detected at the acidic sites; (iii) novel sequences (genus and species level) from isolates and clones were identified in contaminated, subsurface groundwater (high nitrate, acidic pH); (iv) similar pseudomonad populations were present at the background and acidic sites, but at different relative abundances; (v) similar *Azoarcus* spp. predominated the acidic samples. Despite variations in nitrate and heavy metal levels and different proximities to the source ponds (different flow paths), the three acidic samples had similar microbial communities. However, the acidic sample with the reduced nitrate and aluminum levels had an increased diversity index compared to the other acidic samples. Future work is needed to characterize the associations between environmental conditions and the microbial communities, to elucidate the response of microorganisms and communities to relevant stresses, and better understand biotic and abiotic factors on functional dynamics.

Acknowledgments

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Statement of Research

My graduate, postdoctoral, and current work has exposed me to different facets of experimental science in environmental microbiology, including physiology, biochemistry, molecular biology, ecology, molecular ecology, genomics, transcriptomics, and biological engineering. During my graduate studies with rumen microorganisms, I developed a more keen appreciation for anaerobic life, microbial communities, and the underlying relationships for functional diversity and stability. During my time at ORNL, I gained experience in molecular microbial ecology, genomics, and transcriptomics. In particular, I was fortunate to work and form collaborations with interdisciplinary teams involved with bioremediation and cell function at environmentally relevant conditions.

Past Research

Graduate work: One of the goals of my graduate work was to identify the role of a CMCase from the predominant rumen bacterium, *Prevotella bryantii*. I identified the *P. bryantii* CMCase as a β-glucanase, and demonstrated that the glucanase and CMCase activities of mixed ruminal bacteria were highly correlated. However, the ability of rumen isolates to utilize cellulose was not correlated with glucanase activity. These data suggested that the extracellular CMCases of ruminal bacteria were involved in β-glucan utilization, and not cellulose. This work demonstrated the environmental importance and possible function of the glucanase. *P. bryantii* represses glucanase expression in the presence of glucose, but not mannose. *P. bryantii* does not have PTS activity and has very low levels of cAMP (common regulation mechanisms); therefore, the common mechanisms of catabolite repression could not explain regulation in this bacterium.

I then focused on the regulation of carbohydrate utilization in *P. bryantii*, but because a genetic system does not exist, biochemical and physiological methods were used. The kinetics of growth and carbohydrate transport were characterized for two sugars, as well as the kinetics of sugar phosphorylation. It appeared that a single protein phosphorylated both glucose and mannose, and the glucomannokinase was purified. The N-terminal amino acid sequence contained a consensus ATP-binding motif, and the predicted amino acid sequence had significant homology with the glucokinase sequences from Gram-positive bacteria. The Gram-positive glucokinases have been shown to have a regulatory role in catabolite repression, and data indicated that the *P. bryantii* glucomannokinase was directly or indirectly involved in catabolite repression.

Prevotella are abundant species in the diverse rumen ecosystem, can utilize multiple carbohydrates and polysaccharides, but little is known about the regulation systems of these bacteria. One of my main goals or interests lies in the identification of predominant microorganisms in a given environment, and the study of how these microorganisms survive and compete. Our data suggested that *P. bryantii* sensed the rate of carbohydrate consumption, and not the carbohydrate itself *per se*. This type of regulation system would be beneficial in an environment such as the rumen where simple sugars (possible repressors) are short-lived, and a mix of more complex carbohydrates are more readily available. The genomes of closely related organisms (i.e., *Prevotella intermedia*, *Porphyromonas gingivalis*, and *Bacteroides* spp.) have been or are currently being sequenced. Preliminary comparisons with these organisms suggest that similar sugar kinases exist, and that sequences with similarity to transport and regulatory proteins are located in the same region of the chromosome.

Postdoctoral/Staff Scientist: In December 2000, I accepted a postdoctoral position through the Oak Ridge Institute of Science and Education at Oak Ridge National Laboratory with the Microbial Genomics group. I immediately became involved with multiple projects, and was offered a position as a staff scientist after 9 months. My goals and interests of microbial communities, predominant microorganisms, and the relationship to individual biochemistry and physiology have been continued at ORNL, although with different environments. One area of focus has been groundwater and sediments contaminated with nitrate, heavy metals, and uranium. Large clonal libraries have been constructed from groundwater samples with varying geochemical characteristics for the SSU 16S rRNA, *nir*K and *nir*S, and *amo*A. When over 1600 SSU rRNA genes were sequenced, the results indicated that the microbial community composition was drastically altered in relation to contaminants, the overall diversity was reduced, and predominant genera could be identified from the different sites.

We have also analyzed the affects on functional genes, such as the key intermediate enzyme for denitrification, nitrite reductases (*nir*K and *nir*S). Unique sequences were identified from different sites, and compared phylogenetically. The distribution of unique sequences was characterized and then compared with different geochemical measurements with components analysis. Particular relationships could be correlated with certain geochemical characteristics, and artificial neural networks (ANNs) are now being used to assist in the identification of other relationships as the datasets become more complex. We have also identified similar *nir* genes in bacterial isolates from the same sites, and have initiated biochemical characterizations.

A denitrifying, fluidized bed reactor (FBR) that contains activated carbon (AC) as a biocarrier will be part of the treatment process for the contaminated field site, and a pilot FBR has been set-up with our collaborators at Stanford University. The FBR was inoculated with an enrichment culture from the field site, and in conjunction with the working parameters of the FBR, molecular analysis of the liquid and biocarrier fractions are being conducted. Clonal libraries for the SSU rRNA/Intergenic Spacer (ITS) region, *nir*K and *nir*S (nitrite reductases), and *dsr*AB (sulfite reductase) were constructed, and confocal microscopy indicated that a biofilm covered the GAC. Recent comparisons have been made for these genes between the biofilm and planktonic communities. The pilot community was used to inoculate the field site reactor, and we plan to use the above information to monitor the reactor.

Current Research

- ***Desulfovibrio vulgaris* Hildenborough**

My lab is interested in general survival of *D. vulgaris* under different environmental settings relevant to conditions that promote and prevent the reduction of heavy metals. I am a Co-PI with VIMSS – the Virtual Institute of Microbial Stress and Survival (http://vimss.lbl.gov)- which studies the mechanisms of stress survival in *D. vulgaris*, *S. oneidensis*, and *Geobacter metallireducens* (important models for bioremediation of heavy metals and radio-nuclides). Current projects include: (i) biofilm formation on different surfaces; (ii) chromium tolerance/reduction; (iii) transcriptomic profiles during different growth conditions (e.g., stasis and Cr-stress)

- ***Shewanella oneidensis* MR-1**

My lab is interested in the environmental signals and cognate sensors that help mediate control over cellular physiology in the context of stress and survival. In particular, we are interested in the gene products used to sense environmental changes in response to oxygen, heavy metals, biofilm formation, and metal reduction. In addition, we are studying mutants in MR-1, and other strains, that are involved in sensing environmental stimuli important to metal-reduction and biofilm formation. Recently, I have initiated experiments to elucidate interactions between MR-1 and nanoparticles (proposal to be submitted in November).

- **Microbial Ecology of Contaminated Groundwater and Sediments**

I collaborate with colleagues at a field site in Oak Ridge, TN, and my lab has been analyzing groundwater and slurry samples from bio-stimulated samples (reactors and sediments) to help identify responsible populations. In addition, we are looking for ways to use sequence data to test ecological theory in order to better understand the interactions between microbial communities and contaminants that promote bioremediation. We are also isolating microorganisms from enrichment cultures that

can reduce metals and sulfate in order to study field-applicable organisms in the laboratory. For instance, we are in the process of isolating a novel *Anaeromyxobacter* species that can reduce metals and halorespire.

- **Metagenomics of Contaminated Groundwater**

The goal of the project is to determine the metagenome of a microbial community from contaminated groundwater that contains nitrate, metals, and radionuclides (http://www.jgi.doe.gov/sequencing/why/CSP2005/groundwater.html). Preliminary data suggests that the highly contaminated groundwater contains only a few species. We are in the process of isolating and characterizing isolates from the same groundwater. Interestingly, the metagenome sequences indicate the presence of a novel, aciduric bacterium.

- ***Alkaliphilus metallireducens***

We isolated a novel bacterium, *Alkaliphilus metalliredigens*, from leachate ponds at borax mines, and the bacterium can reduce different metals at elevated pH and salt levels. The microorganism can also tolerate borate, and current research is to determine the mechanisms of borate resistance and heavy metal reduction. The complete genome sequence has been determined at the Joint Genome Institute (Fields, PI), and is currently being closed at LANL. In addition to the organisms' novelty of alkaliphilic, metal-reduction, we are investigating the possibilities of biological interactions with borates.

- ***Thermoanaerobacter ethanolicus***

We are working with colleagues towards the genome sequence and annotation of a thermophilic iron-reducer from deep subsurface sediments, *Thermoanaerobacter ethanolicus* 39E.

- **Pulsed Subsidy Effects on Microbial Communities**

The cicada project involves collaboration with faculty in the zoology and botany departments at MU. Allochthonous subsidies can have significant effects on ecosystems and food webs, and we are interested in characterizing the microbial responses at the population and community levels to nutrient inputs. In this case, the nutrient input is the natural phenomenon of a periodical cicada emergence. The majority of work on this project is has been conducted by undergraduates.

Future Research

I plan to continue our work with *D. vulgaris* and *S. oneidensis* as model systems for stress response in terms of bioremediation, as well as, begin characterization of other novel isolates (e.g., *Alkaliphilus*, *Anaeromyxobacter*).

D. vulgaris

- Recently, I received new funding to initiate biofilm studies in *D. vulgaris* for the U.S. DOE for which I am the PI. We plan to identify the different genes and pathways involved in growth as biofilms compared to non-adhered cells of *D. vulgaris* grown under sulfate- and metal-reducing conditions via transcriptomics, proteomics, and metabolomics; identify key genes involved in the formation and/or maintenance of *D. vulgaris* biofilms; and define the genes and proteins associated with cellular responses to heavy metals and mixed contaminants of *D. vulgaris* biofilms. This work is in collaboration with colleagues at ORNL, LBNL, and University of Missouri. We have recently purchased a biofilm reactor, and have begun initial growth studies.
- We are currently completing work on stationary-induced genes (global transcriptomic) in *D. vulgaris* over time as the cells experience a decline in electron donor. Interestingly, the *D. vulgaris* genome does not contain an annotated *rpo*S gene, and we are using bioinformatics to try to identify response elements based upon clustering and bi-clustering of global expression data. We are also pursuing carbohydrate levels during growth, and the repercussions for biofilm formation. Interestingly, we observe structures in biofilm cultures, and a graduate student is working towards the identification of these extracellular structures and possible functions.
- We are also characterizing growth physiology in response to Cr(VI), and the possible re-routing of electron flow during the stress response in *D. vulgaris*.
- Characterization of insertion mutants in *D. vulgaris*. We are currently working with mutants in a Co-transporter, histidine kinase, DNA-binding protein, and sensory box proteins. These make good projects for undergraduates in the laboratory for initial characterization work under the guidance of a graduate student.

Example: He, Z., L. Wu, **M.W. Fields** and J. Zhou. 2005. Comparison of microarrays with different probe sizes for monitoring gene expression. Appl. Environ. Microbiol. 71:5154-5162

Clark, M.E., Q. He, Z. He, E. Alm, K. Huang, T.C. Hazen, A. Arkin, J.D. Wall, J. Zhou, and **M.W. Fields**. Temporal transcriptomic analyses of *Desulfovibrio vulgaris* Hildenborough grown under electron acceptor excess conditions. J. Bacteriol. (in preparation)

S. oneidensis

- We have two mutants in *S. oneidensis* that are impaired transitions to anaerobic conditions and cytochrome content. The mutants are in sensory box proteins, and we plan to further characterize the role of these and other sensory box proteins in sensing environmental stimuli and coordinating cellular responses and metabolism.
- We have also initiated biofilm studies in *S. oneidensis*, and the identification of the carbohydrate matrix that is produced. We hope to elucidate the role of the matrix in forming and maintaining biofilms under environmentally relevant conditions.
- We have started studies investigating the cellular responses of *S. oneidensis* to different nanomaterials, and elucidating microbial interactions at the micro-nano scale.
- Characterization of in-frame deletion mutants in *S. oneidensis*. We are currently working with mutants in sensory box proteins and oxygen-sensors. These make good projects for undergraduates in the laboratory for initial characterization work under the guidance of a graduate student.

Example: Liu, Y., W. Gao, L. Wu, X. Liu, T. Yan, **M.W. Fields**, D.K. Thompson, and J. Zhou. 2005. Genomic expression profiling of *Shewanella oneidensis* MR-1 response to sodium salt stress. J. Bacteriol. 187:2501-2507

Kurowski, J., A. Sundararaja , A. Klonowska, T. Yan, D.M. Klingman, M. Duley, J. Zhou, and **M. W. Fields**. Deletion of a multi-domain PAS protein causes pleiotropic effects in *Shewanella oneidensis* MR-1. J. Bacteriol. (in review)

A. metallireducens

- *A. metallireducens* is a novel, alkaliphilic metal-reducer that can also tolerate borates. The genome has been sequenced (JGI) and is in the process of being closed (LANL). I hope to have a fully annotated, closed genome by the end of the year. I plan to characterize metal-acquisition and metal-reduction mechanisms in this novel organism under alkaline conditions. In addition, I plan to investigate possible interactions with borates, due to their biotechnological importance. I remain in contact with colleagues at the U.S. Borax company who have an interest in the work.
- I also have new bacterial enrichments and isolates that originated from samples at the U.S. Borax mining site in CA. I plan to further characterize the isolates and communities from catalogued samples.

Example: Ye, Q., Y.Roh, B.B. Blair, C. Zhang, J. Zhou and **M.W. Fields**. 2004. Isolation and characterization of a novel, alkaliphilic, metal-reducing bacterium, and possible implications for alkaline chemotrophy. Appl. Environ. Microbiol. 70:5595-5602

Microbial Community Interactions

- We plan to further characterize time samples at a contaminated field site that has been bio-stimulated for uranium-reduction. We will determine the community composition and structure in relation to geochemical data, and identify possible populations that can be associated with desired activities.
- We have also been conducting experiments for the enrichment and cultivation of microorganisms able to reduce iron, sulfate, and/or uranium. The enrichments have been partially characterized with respect to reduction rates, as well as some molecular analysis. The enrichments can reduce different metals (including uranium), and the community structure associated with different activities is being assessed.
- I also plan isolation and characterization of isolates from samples in conjunction with metagenomic analyses of contaminated groundwater communities. Again, in hopes, of understanding how environmental conditions impact the structure and function of microbial communities.
- Further characterize groundwater and sediment samples in terms of phylogenetic and functional diversity in the context of geochemical conditions. What are the possible relationships between phylogenetic diversity, functional potential, and habitat conditions for bacterial communities?

Example: Hwang, C., W.-M.Wu, T.J. Gentry, J. Carley, S.L. Carroll, C. Schadt, D. Watson, P.M. Jardine, J. Zhou, R.F. Hickey, C.S. Criddle, and **M.W. Fields**. 2005. Changes in bacterial community structure correlate with initial operating conditions of a field-scale denitrifying fluidized bed reactor. Appl. Microbiol. Biotech. (accepted)

Fields, M.W., C.E. Bagwell, S.L. Carroll, T. Yan, X. Liu, D.B. Watson, P.M. Jaridne, C.S.Criddle, T.C. Hazen, and J. Zhou. Gene sequences as indicators of bacterial community responses to mixed-waste contamination. Environ. Sci. Technol. (in review)

With a re-vitalized appreciation of the important roles of microbial communities and of the practically untapped treasure trove of biochemical diversity and function; microbiology is poised to make great progress in understanding how microbial cells function from a molecular to cellular to community level. The advent of high-throughput methodology and –omics approaches has made a systems level understanding of a microbial cell plausible, and should vastly impact the applied microbial sciences. A systems-level understanding at the cellular, population, and community level would vastly impact our previous notions about how microorganisms function and interact, and would greatly improve predictive capabilities. My laboratory is interested in how microorganisms and communities sense and respond to environmental conditions and adjusts cell physiology and metabolism. The goal is to gain a better understanding of biochemical and cellular processes at different levels of resolution.

Teaching Interests

As a Master's student, I had a Teaching Assistantship, and taught laboratory courses every semester. I taught Botany Lab for non-science majors as well as microbiology laboratories, and during these endeavors I began to learn that teaching should not be analogous with lecturing. As a graduate student at Cornell University, I was asked to assist in a newly funded program - termed the Micro-Ambassador Program. The course had two parts: the first being to develop simple microbiology exercises and teach the background information to undergraduates, and then help the undergraduates incorporate the exercises and content into a teaching plan targeted for a high school classroom. The exciting part was that we actually went into high school classrooms (local schools and one week in Rochester, NY) and the undergraduates became the teachers. I developed a new appreciation for teachers, and I learned a great deal about different teaching and presentation styles.

As a graduate student, I also volunteered for an outreach program, and developed a once-a-week workshop for 6th graders that lasted 8 weeks. The program covered the microbial world, and included simple exercises that the students could perform. I believe that just about any idea or theme can be taught to anyone with patience, simplicity, and proper analogies to which students can relate. Once the basics have been acquired, additional ideas and information can be evaluated in order to increase complexity and gain further understanding.

I was also the teaching assistant for a Bacterial Physiology Laboratory, and in addition to all experiments and lab preparation, I wrote and evaluated tests and worksheets that included considerable interaction with the students. During the lab course, the students also had to develop a unique research project with a testable hypothesis, conduct experiments, and describe the results in a scientific abstract. I was awarded the Outstanding Graduate Teaching award in the Department of Microbiology at Cornell University. From my experience as faculty thus far, I believe that dedicated undergraduates should have access to research possibilities in the lab in order to foster growth as a scientist – the earlier the exposure to the scientific process the better. For graduate students, the teaching experience should include ample opportunities to conduct, analyze, interpret, and communicate science.

I believe that during my doctorate studies, I developed a more keen appreciation and respect for teaching, and a realization of the amount of work it required. However, I also learned of the rewards that could be reaped from such challenging endeavors, and recognized

the importance of good, exciting classroom instruction that did more than reverberated textbook paragraphs. I enjoy a more discussion-oriented style of teaching, where questions within outlined topics can be pursued and evaluated. Hopefully, foundations are started, and discussions stimulate further questions.

In the last two years, I have taught Bacterial Physiology (425/525) and Microbial Ecology (475/575) courses as well as covered microbiology topics in a general biology course. I have learned a great deal about teaching and continue to learn and respond to the students. I now appreciate the importance of content coverage within a time line; to make sure students are presented the basics, but also exposed to facts in the context of a conceptual framework that fosters discovery. My teaching interests would lie in microbiology, prokaryotic physiology/biochemistry, microbial ecology, environmental genomics, anaerobic microbiology, genomics, and applied microbiology. Within my current microbial ecology and physiology courses, I try to incorporate the changing fields of genomics, metabolomics, and bioinformatics.

Microbiology is exciting to me, and I feel fortunate to have the opportunity to conduct scientific research and teach in this field (and I believe that one complements the other). I enjoy sharing this excitement and fervor with students in the classroom, students in the lab (undergraduate and graduate), and colleagues. I particularly enjoy working on interdisciplinary projects for several reasons: (i) I have the opportunity to share my excitement for microbiology; (ii) I am in a learning environment that exposes me to new systems and questions; and (iii) interdisciplinary approaches allow for a better understanding of the system. I think it is important and I try to convey the idea of interdisciplinary science and systems-level understanding in the classroom.

Over the course of my career, I have been involved in instruction of a variety of age groups, including: 6^{th} grade, 8^{th} grade, 9^{th} grade, 12^{th} grade, freshman biology, split-level undergrad/grad, and graduate student journal clubs. Each requires a unique set of techniques and approaches that are geared to the student; however, excitement and approachability are paramount for all situations.

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Empirical Establishment of Oligonucleotide Probe Design Criteria†

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Criteria for the design of gene-specific and group-specific oligonucleotide probes were established experimentally via an oligonucleotide array that contained perfect match (PM) and mismatch probes (50-mers and 70-mers) based upon four genes. The effects of probe-target identity, continuous stretch, mismatch position, and hybridization free energy on specificity were tested. Little hybridization was observed at a probe-target identity of ≤85% for both 50-mer and 70-mer probes. PM signal intensities (33 to 48%) were detected at a probe-target identity of 94% for 50-mer oligonucleotides and 43 to 55% for 70-mer probes at a probe-target identity of 96%. When the effects of sequence identity and continuous stretch were considered independently, a stretch probe (>15 bases) contributed an additional 9% of the PM signal intensity compared to a nonstretch probe (≤15 bases) at the same identity level. Cross-hybridization increased as the length of continuous stretch increased. A 35-base stretch for 50-mer probes or a 50-base stretch for 70-mer probes had approximately 55% of the PM signal. Little cross-hybridization was observed for probes with a minimal binding free energy greater than −30 kcal/mol for 50-mer probes or −40 kcal/mol for 70-mer probes. Based on the experimental results, a set of criteria are suggested for the design of gene-specific and group-specific oligonucleotide probes, and the experimentally established criteria should provide valuable information for new software and algorithms for microarray-based studies.

Microarrays are one of the most powerful technologies currently available for genomic research (6, 7, 9, 12, 17, 19, 23, 28, 32, 34), and various formats and probe types have been developed. Two types of microarrays, DNA arrays and oligonucleotide arrays, are commonly used (29). Oligonucleotide arrays have increased in use because of several advantages, including better specificity, easy construction, and cost efficiency (21, 29). In previous studies that used short oligonucleotide probes, multiple oligonucleotide probe pairs (perfect match and a single-mismatch control) per gene were necessary to detect differential gene expression under different physiological conditions (13, 32). Recent studies indicated that a single 50-mer to 70-mer oligonucleotide per gene could produce comparable hybridization signals obtained with DNA arrays under different experimental conditions (11, 26; Z. He et al., unpublished data). However, a recent study that compared three different microarrays for the same gene set resulted in different sets of genes (15). To achieve specific hybridization, the major challenges are to establish probe design criteria and identify optimal probes for each gene or a group of genes in a sequence database (e.g., whole genomes) in a standardized manner.

Initially, for 50-mer oligonucleotides, Kane et al. (11) suggested that an oligonucleotide probe showing >75% identity with nontargets might cause cross-hybridization. Kane et al. (11) also showed that a 50-mer probe, which had a 15-base, 20-base, or 35-base stretch with nontargets, had approximately 1%, 4%, or 50% of the target signal intensity, respectively. Similar results were observed by Hughes et al. (8) for 60-mer oligonucleotides. Based on sequence identity and/or continuous stretch criteria, a few probe design programs, such as OligoArray (24), OligoWiz (16), and OligoPicker (31), have been developed. In OligoArray 2.0, the oligonucleotide specificity is computed by binding free energy (25). In addition, other factors that influence the specificity and sensitivity of oligonucleotide arrays, such as secondary structures and T_m, have been considered in OligoArray (24), OligoArray 2.0 (25), and OligoPicker (31).

However, many aspects regarding oligonucleotide probe design remain unclear. First, parameters that affect probe specificity have not been extensively investigated in the following aspects: (i) the effects of sequence identity and continuous stretch on cross-hybridization have not been tested separately; (ii) for long oligonucleotide probes, mismatch position has not been studied rigorously or implemented in any available probe design programs; and (iii) the relationship between theoretical free energy and experimental hybridization signal intensity has not been examined extensively. Second, most of the criteria and the respective threshold values have not been determined experimentally. For example, OligoPicker uses a 15-base stretch and a BLAST score of 30.0 as cutoffs for 70-mer oligonucleotide selection (31), and OligoArray 2.0 determines probe specificity for oligonucleotides with varying lengths by predicting secondary structures and computing the thermodynamics of probe hybridization with targets (25). Although Kane et al. (11) suggested that 75 to 85% sequence identity and 15-base continuous stretch should apply to 50-mer oligonucleotide probe design, similar experiments have not been done with 70-mer oligonucleotides, which are also used in microarray-based studies (e.g., whole-genome microarrays). Third, the recognized criteria have not been comprehensively

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compared in any study. A very stringent single criterion may miss truly specific probes and limit the gene coverage. In contrast, the relaxation of a single criterion may produce a significant number of nonspecific probes and decrease the quality of microarrays (X. Li et al., unpublished data). Therefore, the consideration of multiple criteria is essential to eliminate probe candidates with possible cross-hybridization and maintain specific oligonucleotide probes.

In addition, the criteria for group-specific probe design have not been experimentally established. For a group of highly homologous sequences with >90% sequence identity, the selection of gene-specific probes will be difficult. In this case, multiple probes should be considered to represent a group. In addition, direct performance comparisons between commonly used 50-mer and 70-mer oligonucleotide probes for oligonucleotide array construction have not been evaluated. Therefore, in this study, we have experimentally determined the effects of probe-target identity, length of continuous stretch, free energy, and mismatch positions on microarray hybridization specificity. Based on the experimental results, a set of criteria for the design of gene-specific and group-specific 50-mer and 70-mer probes were established.

MATERIALS AND METHODS

Oligonucleotide probe preparations. 50-mer and 70-mer perfect match (PM) oligonucleotide probes were designed with a modified version of the software, PRIMEGENS (33) based on four genes (SO1679, SO1744, SO2680, and SO0848) from the *Shewanella oneidensis* MR-1 genome. The mismatch (MM) probes were generated with a C++ program as follows: based on the PM probes designed above n (n = 3, 5, . . .37), random matches were introduced for each probe to generate MM probes. Three random probes were selected at each level of mismatches. The nucleotide composition (A, T, C, or G) at each mismatched position was randomly assigned. Thus, in total, 45 MM probes were generated for each template with a length of 50 or 70 nucleotides. All designed oligonucleotides were commercially synthesized without modification by MWG Biotech, Inc. (High Point, NC). The concentration of oligonucleotide probes was adjusted to 100 pmol/μl. Detailed information about all designed oligonucleotide probes are listed in Table S1 in the supplemental material.

Microarray construction. Oligonucleotide probes prepared in 50% dimethyl sulfoxide (Sigma Chemical Co., MO) were spotted onto SuperAmine glass slides (Telechem International, CA) using a PixSys 5500 robotic printer (Cartesian Technologies Inc., CA). Each probe had two replicates on a single slide. In total, there were 736 spots on the array. After printing, the oligonucleotide probes were fixed onto the slides by UV cross-linking (300 mJ of energy) according to the protocol of the manufacturer (Telechem International, CA).

Synthesis and preparation of artificial target templates. Four 70-mer artificial targets (T1-SO1679, T2-SO1744, T3-SO2680, and T4-SO0848) that were complementary to the 70-mer PM probes were synthesized (Molecular Structure Facility at Michigan State University, East Lansing). The artificial oligonucleotide targets were labeled at the 5′ end with Cy5 (T1-SO1679, T2-SO1744, and T3-SO2680) or Cy3 (T4-SO0848) fluorescent dyes during synthesis.

Genomic DNA extraction, purification, and labeling. Genomic DNA was isolated and purified from *S. oneidensis* MR-1 as described previously (35). The purified genomic DNA was fluorescently labeled by random priming using Klenow fragment of DNA polymerase. Mixture I (35 μl), which contained 500 ng of genomic DNA and 20 μl of random primers (Invitrogen Life Technologies, CA), was heated at 98°C for 3 to 5 min, cooled on ice, and then centrifuged. Mixture II (15 μl), which contained 1 μl of a solution consisting of 5 mM (each) dATP, dGTP, and dTTP and 2.5 mM dCTP, as well as 2 μl (80 U) of Klenow (Invitrogen Life Technologies, CA), and 0.5 μl of Cy3 or Cy5 dye (Amersham BioSciences, United Kingdom) were added to mixture I. A total of 50 μl of labeling reaction solution was incubated for 3 h at 42°C. The labeling reaction was terminated by heating at 98°C for 3 min. The tubes were removed and placed on ice. After a quick centrifugation, the sample was hydrolyzed in 50 mM NaOH at 37°C for 10 min and then neutralized with the same amount of HCl. The labeled cDNA targets were purified immediately using a QIAquick PCR purification column and concentrated in a Savant Speedvac centrifuge (Savant Instruments Inc., Holbrook, NY).

Microarray hybridization, washing, and scanning. Hybridization was conducted in triplicate, and each slide contained two replicate spots of each probe so that six data points for each probe were obtained. The microarrays were hybridized at 45°C overnight in the presence of 50% formamide. The labeled cDNAs were resuspended in 20 to 25 μl of hybridization solution that contained 50% formamide, 1 mM dithiothreitol, 3× saline-sodium citrate, 0.3% sodium dodecyl sulfate, and 0.8 μg/μl of herring sperm DNA (Invitrogen Life Technologies, CA). The sample was incubated at 98°C for 5 min, centrifuged to collect condensation, and kept at 50 to 60°C. The sample was immediately applied to the microarray slide, and hybridization was carried out in a waterproof Corning hybridization chamber (Corning Life Science, NY) submerged in a 45°C water bath in the dark for 16 h. After hybridization, microarray slides were washed according to the protocol of the manufacturer (Telechem International, CA). Microarrays were scanned with a ScanArray 5000 microarray analysis system (Packard BioChip Technologies, MA). Normally, 95 to 100% of laser power and 70 to 80% photomultiplier tube efficiency were selected.

Data analysis and normalization. Scanned images were analyzed using the software ImaGene 5.5 (Biodiscovery Inc., CA). Prior to normalization, negative spots and poor-quality spots were flagged by ImaGene and then removed in Excel. The signal-to-noise ratio (SNR) was also computed for each spot to discriminate true signals from noise. The SNR ratio was calculated as follows: SNR = (signal mean − background mean)/(background standard deviation). A commonly accepted criterion for the minimum signal (threshold) that can be accurately quantified is an SNR of >3.0 (30).

Stretch length and free energy calculation. The BLAST program (5) was used to identify regions with high probe-target identities. A simple Perl program extracts the BLAST output and calculates stretch length using 2.0 bit scores as 1 base perfect match. If more than one stretch was identified in a probe, the longest one was used. A C++ program was used to calculate binding free energy values based on a nearest-neighbor model using established thermodynamic parameters (1–4, 10, 14, 18, 20, 27).

MMPD calculation. The distance of the mismatch in the middle position (*mid*) is set as zero. For each mismatch at position i, each distance (d_i) value is calculated using the formula $d_i = [(mid - i) \cdot (mid - i)]^{1/2}$. The average distance ($D_{avg}$) of a probe to a particular nontarget is the sum of each individual d_i value divided by the number (n) of mismatches, $D_{avg} = (\Sigma d_i)/n$. The maximal mismatch position distance (MMPD) value is obtained by taking the maximal value of all D_{avg} values. The calculation of all MMPD values was performed by a Perl program.

RESULTS

Effects of probe-target identity on signal intensity. Probe-target identity is a crucial factor that determines the specificity of microarray probes, particularly oligonucleotide arrays. To investigate the relationship between probe-target identity and signal intensity, artificial oligonucleotide targets with 0 to 37 mismatches were used. Four artificial targets were mixed equally in different concentrations to determine the optimal concentrations to achieve specific signals and good sensitivity. The experimental results indicated that 10.0 pg or 2.0 pg of synthetically labeled target was needed to achieve appropriate specificity and sensitivity for 50-mer or 70-mer probes, respectively. The experimentally determined optimal concentrations of targets were used in the later studies.

The effects of probe-target identity on relative signal intensity were assessed (Fig. 1). Relative signal intensity was calculated with the signal intensity of each PM probe as 1.0, and all MM probe signals were compared to that of the PM probe. In general, little hybridization was observed for both 50-mer and 70-mer probes with less than 85% identity to the respective targets, whereas the signal intensity increased substantially for probes that had more than 90% identity to the respective targets (Fig. 1). Approximately 33 to 48% of the PM probe signal intensities were detected when probe-target identity in-

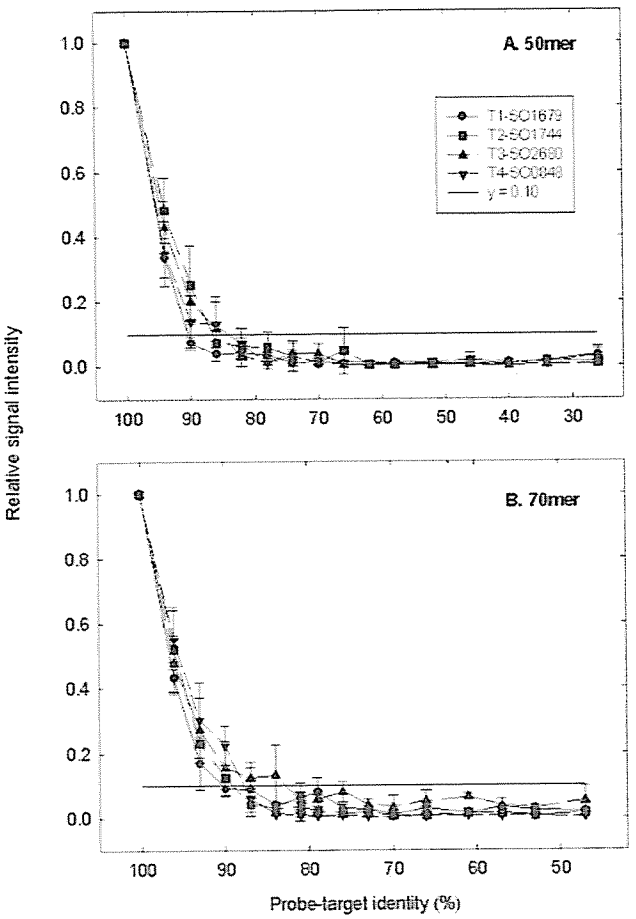

FIG. 1. Relationships between probe-target identity and relative signal intensity. Four artificial oligonucleotides (T1-SO1679, T2-SO1744, T3-SO2680, and T4-SO0848) containing 10 or 2.0 pg of Cy dye (2.5 or 0.5 pg for each target) were hybridized with 50-mer (A) and 70-mer (B) oligonucleotide arrays, respectively, at 45°C in the presence of 50% formamide. Data are presented as the average and standard deviation from six replicate data points. The horizontal line ($y = 0.10$) shows that less than 10% of the PM signal was observed when identity was <85%.

creased to 94% for 50-mer oligonucleotides (Fig. 1A). Similarly, 70-mer probes that showed 96% similarities to the targets detected between 43 and 55% of the PM probe signal intensities (Fig. 1B). In addition, the effect of sequence identity on signal intensity appeared to be sequence dependent. For example, for 70-mer probes, little hybridization (<10% of the PM probe signal intensity) was observed for target gene SO1679 at a probe-target identity of 90%, whereas at the same identity relatively strong signals (approximately 25% of the PM probe signal intensity) were observed for target gene SO0848 (Fig. 1B). Similar results were obtained by Rhee et al. (22) with 50-mer oligonucleotide arrays for genes involved in the biodegradation of organic contaminants. The data suggested that GC content or T_m could not explain the observed phenomenon. For instance, the GC content and T_m for the PM and MM probes from SO0848 were not significantly different from other probes. These results suggested that a gene-specific probe should have an identity of <85% to nontargets and that a group-specific probe should have an identity of >95% within a group and an identity of <85% outside the group under the conditions examined.

Due to the complicated nature of surface hybridization, one would expect low levels of cross-hybridization for the MM probes. A central question is how to distinguish true hybridization signals from nonspecific background noise. One common approach is to determine signal-to-noise ratios. In general, the hybridization of a probe with an SNR less than 3.0 was treated as background noise (30). To translate this to the signal intensity of the MM probes relative to the PM probes, the relationships of relative signal intensities to SNR values were further analyzed for the MM probes with an SNR less than 3.0. On average, 2.5% ± 2.9%, 7.5% ± 2.7%, and 9.5% ± 3.2% of the PM probe signal was detected for the 50-mer MM probes at an SNR between 0 and 3.0, 1.0 and 3.0, and 2.0 and 3.0, respectively. Similarly, an average of 3.5% ± 3.8%, 8.0% ± 4.4%, and 9.0% ± 3.3% of the PM probe signal was observed for the 70-mer MM probes at an SNR between 0 and 3.0, 1.0 and 3.0, and 2.0 and 3.0, respectively (data not shown). The data also suggested that a probe with up to 16% of the PM probe signal had an SNR of <3.0, or that a probe with as low as 8% of the PM signal had an SNR of >3.0. Therefore, in this study, if an MM probe had less than approximately 10% of the PM probe signal, the hybridization signal of the MM probe was considered to be background noise. This value was used in subsequent analyses for the establishment of probe design criteria.

Relationship between a continuous stretch and signal intensity. Probes with a 15-base or shorter stretch to nontargets were treated as nonstretch probes, and probes with a 16-base or longer stretch were treated as stretch probes. All designed probes with more than 90% probe-target identity had long stretches, and all probes with less than 74% probe-target identity did not have long stretches. Therefore, only probes with a probe-target identity between 90% and 76% for 70-mer probes or between 90% and 70% for 50-mer probes were selected for the determination of the effect of long stretches on signal intensity (see Fig. S1 in the supplemental material).

The hybridization intensity for the nonstretch probes increased slightly with the increase of probe-target identity (see Fig. S1 in the supplemental material). For example, 9% and 2% of the PM signal intensities were observed at probe-target identities of 90% and 70%, respectively, for 50-mer probes (see Fig. S1A). In a similar fashion, 4% and 2% of the PM signal intensities were observed at probe-target similarities of 90% and 76%, respectively, for 70-mer probes (see Fig. S1B). For stretch probes, the signal intensity increased as the probe-target identity increased, but the relationship was not linear (see Fig. S1). The highest cross-hybridization signal was observed at a probe-target identity of 78% for 50-mer probes, and the stretch probes had 22% of the PM signal compared to 5% for the nonstretch probes at the same sequence identity (see Fig. S1A). Similarly, the highest cross-hybridization was observed at a sequence identity of 84% for 70-mer probes, and the stretch probes had 17% of the PM signal compared to 4% for the nonstretch probes at the same sequence identity (see Fig. S1B). For the stretch probes, the relationship between signal and identity may also depend on stretch characteristics (the length and the position of a stretch) and other parameters (free energy and MMPD). The results indicated that the

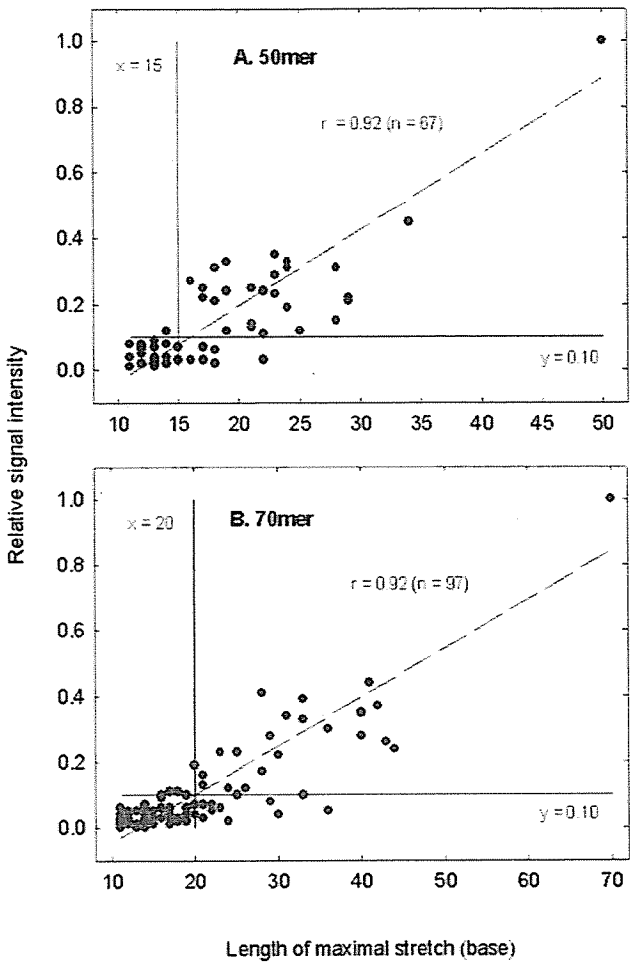

FIG. 2. Relationships between the maximal length of stretch and the relative signal intensity for 50-mer (A) and 70-mer (B) oligonucleotide probes. Only more-than-10-base stretches were considered. The vertical line (x = 15 or 20) and horizontal line (y = 0.10) show that less than 10% of the PM signal was observed when stretch length was <15 or 20 bases.

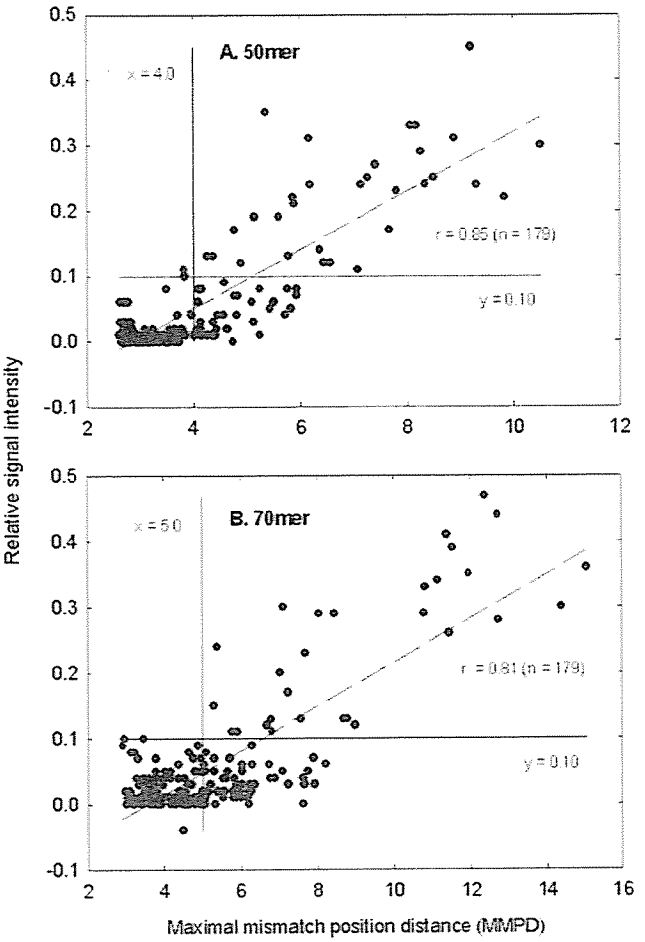

FIG. 3. Relationships between the MMPD and the relative signal intensity for 50-mer (A) and 70-mer (B) oligonucleotide probes. The vertical line (x = 4.0 or 5.0) and horizontal line (y = 0.10) show that less than 10% of the PM signal was observed when MMPD was <4.0 or 5.0.

stretch probes might be responsible for an additional 9% (3 to 17%) of the PM probe signal intensity compared to the non-stretch probes at the same sequence identity. Significant hybridization was not observed even at a probe-target identity of 90% when a probe did not have a 16-base or longer stretch to the nontarget templates (see Fig. S1 in the supplemental material).

To investigate the effects of stretch length on signal intensity, more-than-10-base stretches of a probe with the four artificial targets were calculated and plotted against relative signal intensity, and a linear correlation between stretch length and relative signal intensity was observed with a P value of <0.001 for both 50-mer and 70-mer probes (Fig. 2). For 50-mer probes, little signal intensity (<8% of the PM probe signal) was observed when a probe had a 15-base or shorter stretch. The signal intensity increased when the stretch length was increased. For example, 30% or 55% of the PM probe signal intensity was expected when a probe had a 25- or 35-base stretch, respectively (Fig. 2A). For 70-mer probes, about 3% or 10% of the PM probe signal was detected when a probe had a 15- or 20-base stretch, respectively. When stretch length increased to 35 or 50 bases, the signal intensity reached 32% or 55% of the PM probe signal intensities, respectively (Fig. 2B).

Relationship between mismatch position and signal intensity. When the 50-mer probes were used to examine the relationship between the position of mismatches and relative signal intensity, the MM probes had an average MMPD value of 4.09. As expected, the signal intensity increased as the MMPD increased. Little signal intensity (less than 3% of the PM probe signal) was observed when the MMPD was less than 3.5, but the signal intensity was approximately 10% of the PM probe signal when the MMPD value increased to 5.0. The majority of data points were well fitted to the line (P < 0.001), and this result indicated that hybridization signal intensity was closely correlated to MMPD (Fig. 3A). Similar results were observed for 70-mer probes. For example, the MM probes had an average MMPD value of 5.37. Approximately 1.5% and 8% of the PM signals were observed when the MMPD values were 4.0 and 6.0, respectively (Fig. 3B).

Relationships between free energy and signal intensity. The relationship between the calculated minimal free energy and

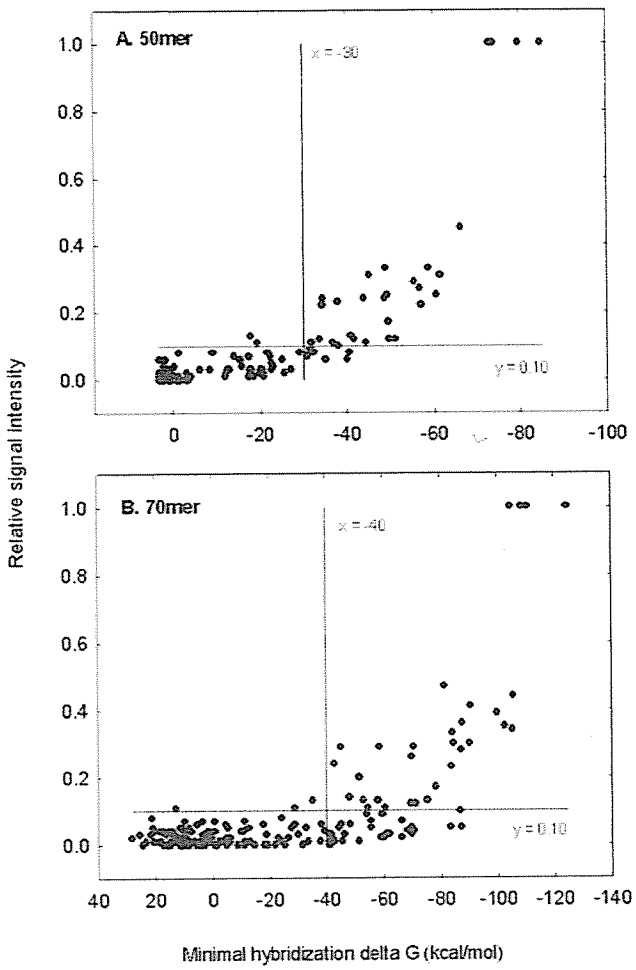

FIG. 4. Relationships between the minimal hybridization free energy and the relative signal intensity for 50-mer (A) and 70-mer (B) oligonucleotide probes. The vertical line ($x = -30$ or $x = -40$) and horizontal line ($y = 0.10$) show that less than 10% of the PM signal was observed when binding free energy was less than −30 or −40 kcal/mol.

relative signal intensity is shown in Fig. 4. The binding free energy of a 50-mer PM probe with the respective target was less than −70 kcal/mol, and hybridization signal intensities decreased as binding free energy increased (absolute values decreased). Approximately 35% of the PM signal was observed when free energy was −60 kcal/mol, and cross-hybridization was almost eliminated when the binding free energy was greater than −30 kcal/mol (Fig. 4A). A 70-mer PM probe had a binding free energy value of less than −100 kcal/mol, and approximately 45% of the PM signal intensity might be observed when the free energy value was −90 kcal/mol; little (<5% of the PM probe signal) cross-hybridization was obtained when the binding free energy value was greater than −40 kcal/mol (Fig. 4B).

Comparison of theoretical prediction and hybridization results. Further comparisons showed that each single criterion (identity, stretch, free energy, or MMPD) was able to identify some common specific probes and could also remove some additional nonspecific probes (data not shown), and these results suggested that the combination of different criteria was needed. To understand how different combinations of criteria affected the probe design outcome, the hybridization data for the MM probes were further examined (see Table S2 in the supplemental material). The threshold values of identity, stretch, free energy, and MMPD were set to 85%, 15 bases, −30 kcal/mol, and 4.0 for 50-mer probes, respectively, and 85%, 20 bases, −40 kcal/mol, and 5.0 for 70-mer probes, respectively. In our experiments, 32 (50-mer) and 36 (70-mer) out of 180 MM probes were experimentally detected to be nonspecific based on SNR ratios. Our results demonstrated that it was difficult to exclude all nonspecific probes based on a single criterion (see Table S2). For example, for 50-mer probes, the best condition was C (free energy of −30 kcal/mol). Based on this criterion, 39 out of 180 MM probes should be excluded, and among the 39 predicted nonspecific probes, 29 were consistent with the experimental results. These data suggested a prediction rate of 74%, and 3 of 32 experimentally proven nonspecific probes were not eliminated by the criterion (misinclusion rate of 9%). In addition, 10 theoretically excluded probes actually did not have significant cross-hybridization signals (misexclusion rate of 26%). The criterion MMPD (D) predicted most nonspecific probes (49) but had the highest mis-excluded rate (45%), the lowest prediction rate (57%), and a mis-included rate of 12% (see Table S2 in the supplemental material).

An appropriate combination of the criteria (F, G, and H) could exclude all nonspecific probes verified by experiments. In this case, F and H had the same results for theoretically predicted nonspecific probe number (47), prediction rate (68%), and mis-included rate (0%), but H had a lower mis-excluded rate (30%) than F (38%), and these results indicated that a relaxation of one or more criteria may exclude fewer qualified candidates without an effect on specificity. The combination of criteria G had more nonspecific probes (57) predicted, lower prediction rate (56%), and higher mis-excluded rate (44%) than F or H, although it also had all experimentally verified nonspecific probes excluded (see Table S2 in the supplemental material). It appears that the MMPD criterion did not help the probe selection in combination G but did increase the number of mis-excluded probes compared to combination F. The combination of identity and stretch criteria (E) did not exclude all experimentally verified nonspecific probes (see Table S2). The results suggested that an appropriate combination of three criteria (identity, stretch, and free energy) was able to select specific oligonucleotide probes. Similar results were observed for 70-mer probes (see Table S2).

DISCUSSION

Specificity of oligonucleotide probes. Specificity is the most important parameter to evaluate performance of oligonucleotide arrays. The specificity of oligonucleotide arrays is commonly evaluated by the PM/MM method based on sequence identity (21). Due to the complicated hybridization dynamics of oligonucleotides and target sequences, other parameters should also be considered. In this study, the effects of four factors (sequence identity, continuous stretch, free energy, and mismatch position) on oligonucleotide specificity have been determined.

The relationship of hybridization signal intensity to probe-

target identity was recently examined using artificial targets. With a 50-mer oligonucleotide microarray containing 763 probes for genes involved in nitrogen cycling and sulfate reduction, Tiquia et al. (29) showed that with hybridization conditions of 50°C, the oligonucleotide microarray hybridizations could differentiate sequences with <86% identity, whereas at 55°C, sequences with <90% identity could be differentiated. With a 50-mer oligonucleotide microarray that contained 1,662 probes for genes involved in contaminant degradation, Rhee et al. (22) showed that under hybridization conditions of 50°C and 50% formamide, the 50-mer microarray hybridization was able to differentiate sequences with <88% identities. In general, the results in this study were consistent with those observations.

Kane et al. (11) showed that a 50-mer probe that had a 15-base or longer stretch with nontargets could cause significant cross-hybridization, and we observed similar results. This feature should be included in the development and design of oligonucleotides probes. A gene-specific probe should keep a stretch as short as possible with other nontargets, and a group-specific probe should have a common stretch as long as possible within a group but as short as possible outside a group. Our results indicated that a 50-mer gene-specific probe should not have 16-base or longer stretches with nontargets, and the stretch length may be extended up to 20 bases for 70-mer probes. For group-specific probes, the length of stretches may be set as 35 bases for 50-mer probes and 50 bases for 70-mer probes so that each member in a group should have relatively uniform and strong signal intensity. By theoretical calculations, the signal intensity of each group member is able to reach approximately 55% of the PM signal for both 50-mer and 70-mer oligonucleotides under the above conditions. If more than 80% of the PM signal is expected, the common stretch length should be increased to 45 bases for 50-mer and 65 for 70-mer oligonucleotides.

Previous studies (13, 21) suggested that a short probe with a single mismatch in the middle could be more easily discriminated than a probe with a mismatch at other positions. For long oligonucleotide probes, we showed that the relative signal intensity is correlated with MMPD, and this result suggested that a probe with mismatches located closer to the middle position of nontargets should have higher specificity. In oligonucleotide probe design, the MMPD value may help choose the best probes from a probe candidate pool by minimizing MMPD values for gene-specific probes, or maximizing MMPD values within a group for group-specific probes. In addition, MMPD could be used to select gene-specific probes, and the suggested values for 50-mer and 70-mer probes are 4.0 and 5.0, respectively.

The length of stretches is dependent on mismatch positions. To achieve high specificity, stretches should be kept to a minimum and should be evenly distributed within an oligonucleotide probe, particularly when multiple mismatches are present. This does not completely agree with the MMPD criterion that requires mismatch positions to be as close to the middle of the probe as possible. Our results indicated that continuous stretches should be used as an essential criterion and that the MMPD value would be better suited for the optimization process.

Rouillard et al. (25) suggested that the computation of the oligonucleotide specificity might be more accurate using the thermodynamic parameters (binding free energy) rather than sequence identities. In this study, the minimal binding free energy was calculated, and the results indicated that binding free energy was more sensitive than sequence identity, most likely because the matches, mismatches, adjacent nucleotides, and interactions between the oligonucleotide probe and targets or nontargets were considered. Actually, at probe-target identity of 75% or greater, minimal free energy is closely correlated to probe-target identity (data not shown). Free energy can be considered as one of the primary oligonucleotide design criteria for the selection of gene-specific or group-specific probes. Our results indicated that −30 kcal/mol can be set as the threshold for the selection of 50-mer gene-specific probes and −60 kcal/mol for 50-mer group-specific probes. The cutoff values of free energy should be −40 and −90 kcal/mol for the selection of gene-specific and group-specific 70-mer probes, respectively.

TABLE 1. Summary of essential probe design criteria for 50-mer and 70-mer oligonucleotides

Probe type and parameter	50-mer value	70-mer value
Gene-specific probe		
Max. identity with nontargets	85%	85%
Max. stretch length with nontargets	15 bases	20 bases
Min. binding energy with nontargets	−30 kcal/mol	−40 kcal/mol
Max. no. of self-binding nucleotides	8	8
Group-specific probe		
Min. identity within the group	96%	96%
Min. stretch length within the group	35 bases	50 bases
Max. binding energy within the group	−60 kcal/mol	−90 kcal/mol
Max. no. of self-binding nucleotides	8	8

A combination of multiple criteria may exclude fewer qualified probe candidates without a significant effect on specificity. The exclusion of all nonspecific probes with a single criterion (identity, stretch, free energy, and MMPD) would be difficult, and a possible solution would be to increase the criterion stringency until all experimental verified nonspecific probes were excluded. However, this would also exclude a large portion of specific probes and lead to a low gene coverage. A polyphasic approach would improve and standardize probe design, but the appropriate combination of multiple criteria for the design of specific probes and the exclusion of nonspecific probes has not been previously tested. In this study, the comparison of theoretical predictions and experimental hybridization results indicated that a combination of identity (85%), stretch (15 bases), and free energy (−30 kcal/mol) was able to exclude all nonspecific probes for 50-mer probes and a combination of identity (85%), stretch (20 bases), and free energy (−40 kcal/mol) for 70-mer probes. The results also suggested that the relaxation of one or more criteria may exclude all truly nonspecific probes and keep more qualified probe candidates.

Essential criteria for oligonucleotide probe design. Based on our results, a set of essential criteria are suggested for gene-specific and group-specific oligonucleotide probe design (Table 1). First, all criteria must be comprehensively considered, and a single parameter was not stringent enough for the exclusion of all nonspecific probes. For example, a probe-target

identity of 75% was suggested as a potential cutoff value (11), but our data suggested a sequence identity of 85% under the tested conditions. This value is particularly important for the design of probes for groups of highly homologous sequences, such as the construction of functional gene arrays (22, 29). Based on our results, specific hybridization can be obtained for probes without long stretches or low free energy even if the probe-target identity is 85 to 90%, and these results agree with previous experiments (22, 29).

In many probe design programs, the sequence identity is the most influential criterion, and stretch or free energy is rarely considered. Therefore, potential cross-hybridization could occur due to long stretch and/or low free energy problems even though a probe meets the identity criterion. This problem can be avoided when the proposed strategies are used (see Table S3 in the supplemental material). The "good" probes that meet all criteria have low signals and low SNR (<3.0) values, and the sequence identities are as high as 86% for 50-mer probes (e.g., S2-07-p1) and 87% for 70-mer probes (e.g., S3-09-p2). Other probes with significant hybridization signals and SNR of >3.0 were excluded by a single criterion (i.e., stretch or free energy) or by both criteria, even when the identity was set to 90%. However, it should be noted that some probes (e.g., S2-07-p3 and S3-09-p3) with low hybridization signals and low SNR values were excluded by free energy or stretch, and these results suggested that the establishment of universal probe design criteria is still a challenge. Further investigations of the probe-target interactions on glass microarray slides are needed.

For group-specific probes, all sequences must have high identity and a common long stretch within a group. Our experimental results showed that approximately 50% of the PM signals could be obtained at probe-target identities of 96% for 50-mer and 70-mer oligonucleotides. Therefore, for the group-specific probe design, the probe-target identity should be as high (98% to 100%) as possible. A group-specific probe should also have a minimal number of continuous stretches (35 bases for 50-mer and 50 bases for 70-mer probes) and a maximal binding free energy (−60 kcal/mol for 50-mer and −90 for 70-mer probes) within the group. It should also be noted that the maximal nucleotide number for self-binding should be considered to avoid strong secondary structures of designed probes (set to 8, as previously described by Wang and Seed [31]). In addition, other criteria such as GC content, T_m, and sequence complexity also need to be considered. Since GC content varies among different organisms, an oligonucleotide design tool should evaluate all sequences in the data set and determine T_m values that fall into a narrow range to ensure quantitative comparison of gene expression. Those parameters should be used as filters for excluding probe candidates.

Finally, the criteria set in Table 1 are generally conservative. The parameters may be cautiously relaxed when more probes or a high gene coverage rate is needed. For example, based on our experimental results, identity may be increased to 90% for both 50-mer and 70-mer probes, and free energy may be set up to −40 kcal/mol for 50-mer or −50 kcal/mol for 70-mer oligonucleotides if approximately 10% of the PM signal intensity is allowed and the SNR is generally below 3.0, but further relaxation of the criteria would need to be experimentally reevaluated. In addition, for fellow researchers to access the detailed data for various analyses, theoretically calculated values of parameters, such as sequence identity, maximal stretch length, minimal free energy, and MMPD, and experimentally determined hybridization results, such as relative signal intensity (percentage of the perfect match probe signals) and signal-to-noise ratios are summarized in Table S4 in the supplemental material.

Selection of optimal oligonucleotides. Two approaches were considered for the selection of the best 50-mer or 70-mer oligonucleotide probes. One is to rank three essential criteria (identity, continuous stretch, and free energy) in different orders. For example, identity is considered first, then stretch, and then free energy. In this situation, probes with lowest identity to other sequences were usually obtained. Stretch and free energy were then considered in order if some probes had the same identity. The other approach was to assign each criterion (maybe other parameters as well) a weight, and all probe candidates were ranked by a final score for each gene or group of genes. In addition, the optimal probes could be selected based on the MMPD value. In this case, the best gene-specific probe should have the smallest MMPD value for each gene, and the best group-specific probe should have the largest MMPD value for a group of genes.

In summary, we investigated the effects of probe-target identity, continuous stretch, mismatch position, and free energy on the design of 50-mer and 70-mer probes and then experimentally compared the designed probes. The results produced weighted parameters for a polyphasic approach for oligonucleotide probe design and will facilitate the establishment of the criteria for gene-specific and group-specific oligonucleotide probes. Experimentally tested criteria are needed for the development of software and algorithms for oligonucleotide probe design. We are currently developing new algorithms and software to select optimal oligonucleotide probes for functional gene arrays as well as whole-genome microarrays.

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NOTES

Transcriptome Analysis of *Shewanella oneidensis* MR-1 in Response to Elevated Salt Conditions

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Whole-genomic expression patterns were examined in *Shewanella oneidensis* cells exposed to elevated sodium chloride. Genes involved in Na^+ extrusion and glutamate biosynthesis were significantly up-regulated, and the majority of chemotaxis/motility-related genes were significantly down-regulated. The data also suggested an important role for metabolic adjustment in salt stress adaptation in *S. oneidensis*.

Shewanella species inhabit diverse environments, including spoiled food (11) and infected animals (35), deep-sea and freshwater lake sediments (8, 45, 54), and oilfield waste sites (44). *Shewanella oneidensis* MR-1, a facultative, gram-negative bacterium, was isolated from sediments of Lake Oneida in New York (32). The bacterium can anaerobically respire numerous organic compounds, including fumarate and dimethyl sulfoxide (28), as well as reduce metals such as Fe(III), Mn(IV), Cr(VI), and U(VI) (22, 29, 32). Because of the respiratory versatility, which may be exploited for immobilization of environmental pollutants (i.e., chromium and uranium) in soil and groundwater, the metal-reducing capabilities of *Shewanella* spp. have been intensively investigated (6, 14, 15, 26, 30, 33, 39).

The MR-1 genome was recently sequenced (16), and some fundamental similarities and disparities between MR-1 and other sequenced bacteria have been observed (16). To experimentally probe the genomic response of *S. oneidensis* to various physiologically relevant environmental stresses, a whole-genome cDNA microarray for MR-1 was constructed in this laboratory. In this study, we used this cDNA microarray to profile transcriptional responses of MR-1 to elevated sodium salt stress. The results indicated that the expression of the genes involved in osmolyte protection, cation efflux/influx, motility, and electron transport were significantly altered.

MR-1 requires a relatively high salt concentration for optimal growth. Many *Shewanella* species have been isolated from marine environments, whereas some, like MR-1, have been isolated from freshwater environments (36, 39). To understand how various salt concentrations impact the growth of *S. oneidensis*, MR-1 cells were cultivated in triplicates in MR2A medium (12) containing different amounts of NaCl (ranging in concentration from 0 to 0.6 M) at 30°C under aerobic conditions (shake flasks, 120 rpm). Growth curves (Fig. 1) indicated that (i) the growth rate increased slightly with additional NaCl levels up to 0.4 M, (ii) cells grown in the presence of 0.4 M NaCl entered stationary-phase growth at a lower optical density (OD) than cells grown in the presence of 0.1 to 0.3 M NaCl, (iii) the growth rate decreased significantly with the addition of 0.5 M NaCl, and (iv) cell growth was drastically reduced in the presence of 0.6 M NaCl. Based on these results, MR-1 cells required NaCl levels between 0.1 to 0.3 M for optimal growth (5.8 to 17.5 g/liter) in aerobic MR2A medium. A slight decrease in overall growth was observed at 0.4 M NaCl; 0.5 M NaCl (29.2 g/liter) reduced the maximum growth rate twofold compared to the maximum growth rate observed at 0.1 to 0.3 M NaCl, and the maximum growth rate at 0.6 M NaCl was reduced over fourfold. For the present study, 0.5 M NaCl was used as a moderate stress for MR-1 cells.

Microarray analysis of salt adaptation response in MR-1. A whole-genome cDNA microarray was constructed and described previously (7, 13, 49). Briefly, gene-specific DNA fragments (<75% homology) were selected as probes with the software PRIMEGENS (52), and the primers were designed to amplify the gene-specific DNA fragments. A total of 4,648 pairs of gene-specific primers were designed based on the known sequences (13, 16) and synthesized. Gene-specific fragments were PCR amplified in 96-well plates 8 to 16 times in 100-μl reaction mixtures, purified, pooled, quantified, and diluted to a minimum concentration of 50 ng/μl. Microarray fabrication, hybridization, and scanning were carried out as described previously (7, 13, 23, 49).

We harvested cells grown in the presence of 0.1 M (control condition) or 0.5 M (salt stress condition) NaCl for analysis. To evaluate biological variations, we extracted total cellular RNA from three sets of independent salt-stressed and control cultures to serve as biological replicates and that were hybridized at least twice for each replicate set by an optimized protocol (7, 13, 23, 49). The ratios of the salt-stressed samples to the con-

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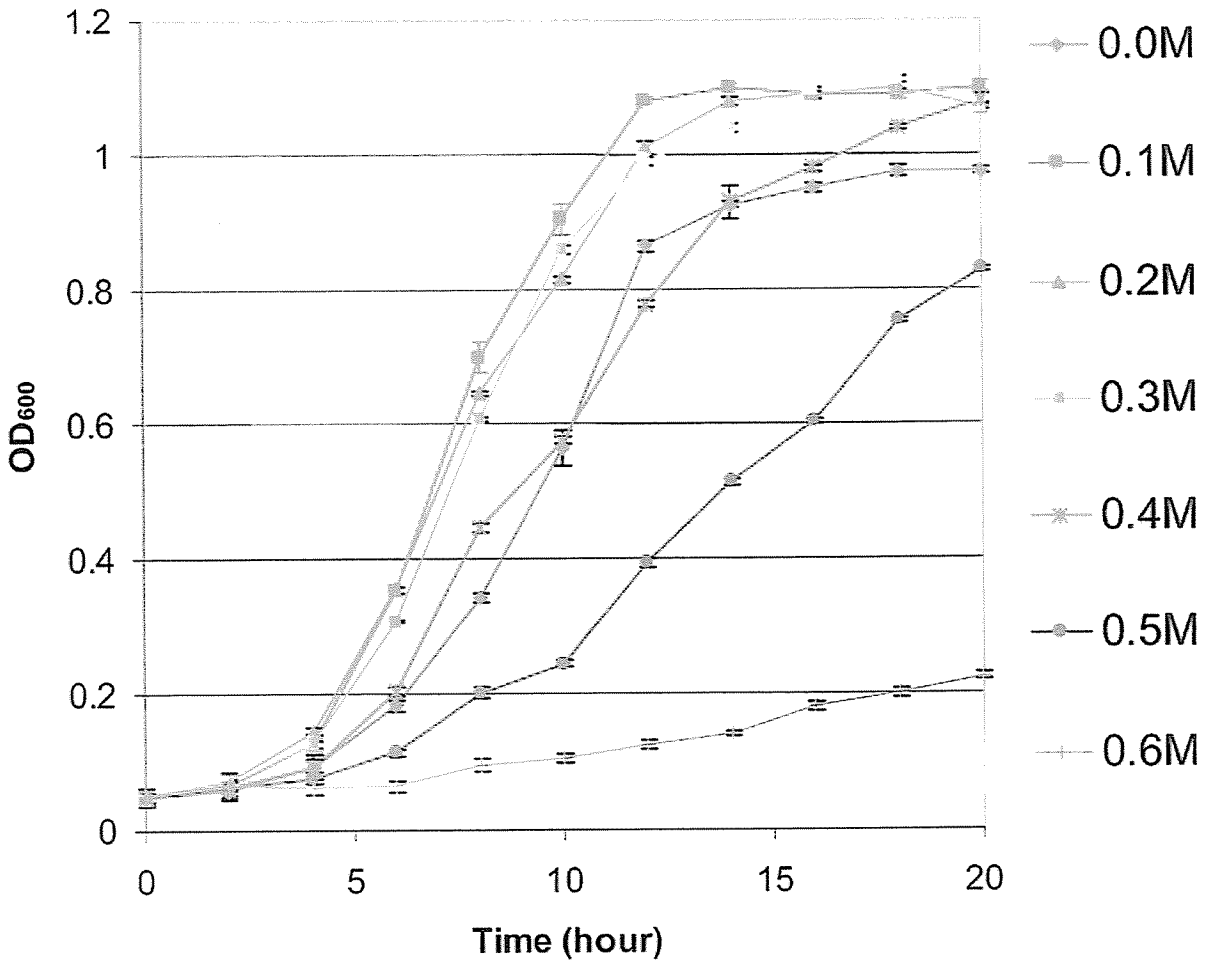

FIG. 1. Relationship between maximum growth rate of MR-1 cells grown in aerobic MR2A medium and increasing levels of sodium chloride.

trol samples for an arrayed gene were normalized by a trimmed geometric mean (48). Data points that were not consistently reproducible and had a disproportionately large effect on the statistical result were removed (23). Student's *t* test was used to identify differentially expressed genes by comparing the means of the normalized and log-transformed control versus salt-treated data with a total of 12 replicates in each set. A significance cutoff for the *t* statistic ($P = 0.05$) of a two-tailed test was chosen, and also required genes with significant changes to show a greater than twofold average change in expression level. As a result, a balance between the number of false negatives and trends supported by concerted changes among multiple genes within the same operon or pathway is achieved. For comparison, we also used the empirical Bayesian method of Lonnstedt and Speed (24) to rank and identify genes with significant changes, and the results are consistent by both methodologies.

The quality of the microarray data was assessed based on a number of criteria. First, expression patterns for genes in the same putative operons were checked. The similarity in gene expression patterns between gene pairs predicted to be in the same operon to that of randomly chosen gene pairs was compared. Consistent with this expectation, we observed that genes within the same operon responded in a similar fashion under salt stress compared to genes randomly selected from the genome. Observed pairwise differences in log ratio expression levels were significantly smaller for the within-operon set (Kolgomorov-Smirnoff test, $D = 0.3925$, $P = < 2.2 \times 10^{-16}$) (37). Second, genes known to function together displayed similar changes in expression levels, as described throughout this article. One example is the consistent down-regulation of flagellar assembly genes (Table 1). Third, expression patterns of well-studied genes were verified (e.g., cation efflux transporters and Na^+/H^+ antiporters; Table 1S, online supplementary data [http://www.esd.ornl.gov/facilities/genomics/pubs/Table1S.xls]). Finally, we selected four predicted open reading frames (ORFs) that displayed significant changes in expression that have not been previously described as osmotic stress response genes in other organisms for real-time quantitative reverse transcription-PCR analysis (23). The expression patterns of the selected genes (*pflA*, *aceA*, *acnA*, and SO3874) were similar to the patterns observed with the microarrays (Table 2S, online supplementary material [http://www.esd.ornl.gov/facilities/genomics/pubs/Table2S.pdf]).

Overall genomic expression profile of MR-1 in response to salt stress. The overall genomic expression profiles indicated that the expression of a considerable subset of genes was af-

TABLE 1. Operon organizations and expression ratios of flagellar assembly genes in regions 1, 4, 5, and 6 and chemotaxis genes in regions 2 and 3

Gene ID no.[a]	Name	Putative function	Expression ratio[b]		
			Avg	SD	Sig.
Region 1					
SO1529(−)	*motA-1*	Chemotaxis protein	0.470	0.184	Yes
SO1530(−)	*motB-1*	Chemotaxis protein	0.421	0.109	Yes
Region 2					
SO2120(−)	*cheY-1*	Chemotaxis protein	2.899	1.570	No
SO2121(−)	*cheA-1*	Chemotaxis protein	0.659	0.216	Yes
SO2122(−)	*cheW-1*	Purine-binding chemotaxis protein	0.407	0.222	Yes
SO2123(−)		Methyl-accepting chemotaxis protein	1.004	0.390	No
SO2124(−)	*cheR-1*	Chemotaxis protein methyltransferase	0.934	0.387	No
SO2125(−)	*cheD-1*	Chemotaxis protein	0.865	0.293	No
SO2126(−)	*cheB-1*	Protein-glutamate methylesterase	0.855	0.347	No
Region 3					
SO2317(−)		Methyl-accepting chemotaxis protein	1.295	0.590	No
SO2318(−)	*cheY-2*	Chemotaxis protein	2.085	1.680	No
SO2319		Anti-anti-sigma factor, putative	1.600	1.127	No
SO2320(−)	*cheA-2*	Chemotaxis protein, interruption-N	1.414	1.222	No
SO2321(−)		ISSod4, transposase	No data		
SO2322(−)	*cheA-2*	Chemotaxis protein, interruption-C	3.299	1.611	No
SO2323(−)		Methyl-accepting chemotaxis protein	1.933	1.425	No
SO2324(−)	*cheW-2*	Purine-binding chemotaxis protein	0.875	0.209	Yes
SO2325(−)	*cheR-2*	Chemotaxis protein methyltransferase	1.197	0.618	No
SO2326(−)	*cheD-2*	Chemotaxis protein, putative	1.373	0.666	No
SO2327(−)	*cheB-2*	Protein-glutamate methylesterase	0.713	0.266	Yes
Region 4					
SO3202(−)	*cheW-3*	Purine-binding chemotaxis protein	1.247	0.495	No
SO3203(−)		CheW domain protein	0.987	0.300	No
SO3204		ParA family protein	1.103	0.368	No
SO3205		Hypothetical protein	1.233	0.398	No
SO3206(−)	*cheB-3*	Protein-glutamate methylesterase	1.615	0.717	No
SO3207(+)		Chemotaxis protein	1.226	0.387	No
SO3208(+)	*cheZ*	Chemotaxis protein	1.722	0.908	No
SO3209(+)	*cheY-3*	Chemotaxis protein	1.886	0.940	No
SO3210(+)	*fliA*	RNA polymerase sigma-28 factor	1.052	0.331	No
SO3211(+)	*flhG*	Flagellar biosynthetic protein	0.749	0.141	No
SO3212(+)	*flhF*	Flagellar biosynthetic protein	0.632	0.293	Yes
SO3213(+)	*flhA*	Flagellar biosynthesis protein	0.558	0.149	Yes
Region 5					
SO3936(+)	*motX*	Sodium-type flagellar protein	0.788	0.323	No
Region 6					
SO4286(+)	*motB-2*	Chemotaxis *motB* protein	0.737	0.328	No
SO4287(+)	*motA-2*	Chemotaxis *motA* protein	0.646	0.135	Yes

Gene ID no.	Name	Putative function	Expression ratio		
			Avg	SD	Sig.
Region 4					
SO3214		Hypothetical protein	0.369	0.154	Yes
SO3215(+)	*flhB*	Flagellar biosynthetic protein	0.678	0.237	Yes
SO3216(+)	*fliR*	Flagellar biosynthetic protein	0.439	0.159	Yes
SO3217(+)	*fliQ*	Flagellar biosynthetic protein	0.672	0.252	No
SO3218(+)	*fliP*	Flagellar biosynthetic protein	0.537	0.140	Yes
SO3219(+)	*fliO*	Flagellar protein	0.532	0.210	Yes
SO3220(+)	*fliN*	Flagellar motor switch protein	0.575	0.138	Yes
SO3221(+)	*fliM*	Flagellar motor switch protein	0.716	0.363	No
SO3222(+)	*fliL*	Flagellar protein	0.741	0.156	Yes
SO3223(+)	*fliK*	Flagellar hook-length control protein	0.378	0.244	Yes
SO3224(+)	*fliJ*	Flagellar protein	0.328	0.168	Yes
SO3225(+)	*fliI*	Flagellum-specific ATP synthase	0.577	0.161	Yes
SO3226(+)	*fliH*	Flagellar assembly protein	1.017	0.868	No
SO3227(+)	*fliG*	Flagellar motor switch protein	0.839	0.401	No
SO3228(+)	*fliF*	Flagellar M-ring protein	0.584	0.132	Yes
SO3229(+)	*fliE*	Flagellar hook-basal body complex	0.284	0.127	Yes
SO3230(+)	*flrC*	Flagellar regulatory protein C	0.352	0.137	Yes
SO3231(+)	*flrB*	Flagellar regulatory protein B	0.200	0.073	Yes
SO3232(+)	*flrA*	Flagellar regulatory protein A	0.388	0.150	Yes
SO3233(+)	*fliS*	Flagellar protein	0.527	0.183	Yes
SO3234		Hypothetical protein	0.497	0.273	Yes
SO3235(+)	*fliD*	Flagellar hook-associated protein	0.555	0.304	Yes
S03236(+)	*flaG*	Flagellin	0.345	0.078	Yes
SO3237(+)		Flagellin	0.317	0.097	Yes
SO3238(+)		Flagellin	0.846	0.471	No
SO3239(+)	*flgL*	Flagellar hook-associated protein	0.495	0.226	Yes
SO3241(+)	*flgJ*	Flagellar protein	0.422	0.216	Yes
SO3242(+)	*flgI*	Flagellar P-ring protein	0.383	0.229	Yes
SO3243(+)	*flgH*	Flagellar L-ring protein	0.427	0.170	Yes
SO3244(+)	*flgG*	Flagellar basal-body rod protein	0.389	0.175	Yes
SO3245(+)	*flgF*	Flagellar basal-body rod protein	0.320	0.188	Yes
SO3246		Hypothetical protein	0.434	0.413	No
SO3247(+)	*flgE*	Flagellar hook protein	0.269	0.136	Yes
SO3248(+)	*flgD*	Basal-body rod modification protein	0.276	0.066	Yes
SO3249(+)	*flgC*	Flagellar basal-body rod protein	0.300	0.109	Yes
SO3250(+)	*flgB*	Flagellar basal-body rod protein	0.142	0.053	Yes
SO3251(+)	*cheR-3*	Chemotaxis protein methyltransferase	0.609	0.188	Yes
SO3252(+)	*cheV*	Chemotaxis protein	0.556	0.113	Yes
SO3253(−)		Flagellar basal-body P-ring protein	0.257	0.071	Yes
SO3254(−)	*flgM*	Regulator of flagellin synthesis	0.307	0.164	Yes
SO3255(−)	*flgN*	Flagellar biosynthetic protein	0.190	0.086	Yes

[a] + and − represent the operon location on the positive or negative strand, respectively. Some non-motility-related genes are also included in the table to keep the entity of a complete operon.

[b] The average expression ratio of the salted sample to the control was calculated from 12 replicates together with the respective standard deviation (SD), a $P = 0.05$ standard t test result (significant [Sig.]).

fected during growth in the presence of 0.5 M NaCl. We identified a total of 518 genes (11.2% of the total gene content) as significantly upregulated and 598 genes (13%) as significantly down-regulated by a factor of 2 or more. According to the genome sequence annotations provided by The Institute for Genomic Research (http://www.tigr.org/), the majority of the up-regulated genes fell into the following functional categories: amino acid biosynthesis, protein synthesis, biosynthesis of cofactors, prosthetic groups, energy metabolism, and fatty acid/phospholipid metabolism. A large fraction of the most-highly-down-regulated genes were annotated as chemotaxis-related proteins. Similar to previous studies of microbial stress response (19, 23, 42, 53), changes in the expression of ORFs predicted to be involved with protein biosynthesis seem to play an important role in modulating cellular activities that allow adaptation to environmental stress (Table 1S).

Salt stress activated genes involved in Na^+ efflux and K^+ accumulation. Na^+ extrusion and replacement with K^+ is the primary response of *Escherichia coli* to NaCl stress. To balance the large amounts of cation accumulation, *E. coli* will also accumulate glutamate (46). MR-1 appears to respond similarly to NaCl stress. First, genes encoding K^+ uptake proteins were up-regulated, as well as Na^+ efflux system components that included the Trk K^+ uptake system, Na^+/H^+ antiporters, and Na^+ efflux transporters (Table 1S). As expected, genes (SO1325 and SO4410) putatively involved in glutamate synthesis and a Na^+/glutamate symporter gene (SO2923) were up-regulated in MR-1 by NaCl stress (Table 1S).

Besides the primary response, a secondary response (i.e., the accumulation of compatible osmolytes) may occur when a cell is subjected to salt concentrations of 0.5 M or higher, as observed in *E. coli* (46). Genes that encode the enzymes for trehalose and estoine biosynthesis, however, have not been identified in MR-1, and the corresponding compounds have not been reported. Sequence annotation of the MR-1 genome revealed two operons that contain *proABC* genes encoding enzymes for proline synthesis (SO1121, SO1122, and SO3354), but the expression of these genes was not significantly changed under the salt stress conditions examined (Table 1S). Interestingly, the accumulation of glycine betaine was observed in *S. oneidensis* cells grown in the presence of salted and smoked salmon (20). The authors stated that exogenous choline in the fish was transported and converted to glycine betaine (20). Therefore, MR-1 appears to have the ability to synthesize glycine betaine from choline. Generally, choline is first oxidized to glycine betaine aldehyde by the enzyme choline dehydrognase (BetA) in *E. coli* or by a type III alcohol dehydrogenase (GbsB) in *Bacillus subtilis*. The intermediate glycine betaine aldehyde is then further oxidized to glycine betaine by glycine betaine aldehyde dehydrogenase BetB in *E coli* or GbsA in *B. subtilis* (46). We identified two candidates (SO3496 and SO4480) for aldehyde dehydrogenase, one gene for type II (SO1490) and one gene for type III alcohol dehydrogenase (SO2054), but no candidates for choline dehydrogenase. These candidates, however, may function together to convert choline into glycine betaine in MR-1. The two putative alcohol dehydrogenase genes (SO3498 and SO4480) were slightly but not significantly up-regulated, and the other two aldehyde dehydrogenase genes (SO1490 and SO2054) were significantly down-regulated. It is therefore unlikely that glycine betaine biosynthesis was enhanced under the growth conditions tested.

Up-regulation of respiration-related genes. Microarray analyses indicated that genes involved in both aerobic and anaerobic respiration were significantly up-regulated in salt-stressed MR-1 cells (Fig. 2 and Table 1S). The up-regulated genes involved in aerobic respiration included tricarboxylic acid (TCA) cycle enzymes and ATP synthase (SO4746 to SO4753), and ORFs predicted to play a role in anaerobic respiration included components of fumarate, nitrate, and nitrite reductases. Consistent with the activation of these enzymes, key genes involved in the biosynthesis of such cofactors as molybdopterin (2, 17, 50), heme (55), and menaquinone (31, 41) were also up-regulated (Table 1S). Up-regulated genes reported to be involved in fermentation were also observed, including formate dehydrogenase, quinone-reactive Ni/Fe hydrogenase, and acetate kinase.

Pyruvate can be respired either aerobically through the TCA cycle or anaerobically by formate dehydrogenation and fermentation. The pyruvate formate-lyase encoded by *pflAB* is the key enzyme that catalyzes pyruvate to formate (1), leading to the final products H_2 and CO_2. Significant up-regulation of the *pflAB* genes was observed, suggesting a possible redirection of pyruvate. At the same time, an operon that contained aconitase, methylcitrate synthase, methylisocitrate lyase, and a conserved hypothetical protein was up-regulated 6.7- to 9.1-fold. The apparent up-regulation of both aerobic and anaerobic respiration genes has also been reported for *E. coli* cells exposed to seawater for 20 h (40).

The glyoxylate bypass can reduce NADH production as well as allow a partial TCA to function to generate intermediates for anabolic reactions (e.g., amino acid biosynthesis) without the decarboxylation steps that result in loss of carbon (CO_2). The methylcitric acid pathway can provide additional energy from fatty acid and acetate catabolism. Apparently, the cell needs energy to survive the stress, but the aerobic respiration that can produce more energy may simultaneously generate extra reactive oxygen species as by-products (43), thus resulting in oxidative stress. This effect was observed in the moderately halophilic *Shewanella* sp. strain CN32, which requires 5 to 6% NaCl for optimal growth (4). Up-regulation of anaerobic respiration could help reduce oxidative stress to the cell. In addition, the cells may undergo clumping as a protective response to osmotic stress, as observed in *Azospirillum brasilense* (18) and *Vibrio cholerae* (51), and therefore may experience microaerophilic or anoxic conditions. However, the aggregation of MR-1 cells during salt stress was investigated as previously described (18), and significant aggregation was not detected for either the control or salt-stressed cells (data not shown). Observation of the cells by light microscopy also supported this conclusion. However, the cells were shaken during incubation, and significant clumping might have been prevented. Further work is needed to discern the possible connection between clumping and anaerobic metabolism.

Down-regulation of flagellar assembly genes impacted cell motility. Phylogenetic analysis suggested that *S. oneidensis* flagellar motor proteins were more closely related to the sodium-driven motors in *Vibrio* species than to proton-driven motors. In addition, homologs of the MotAB and MotXY proteins, which are thought to be associated with sodium-driven

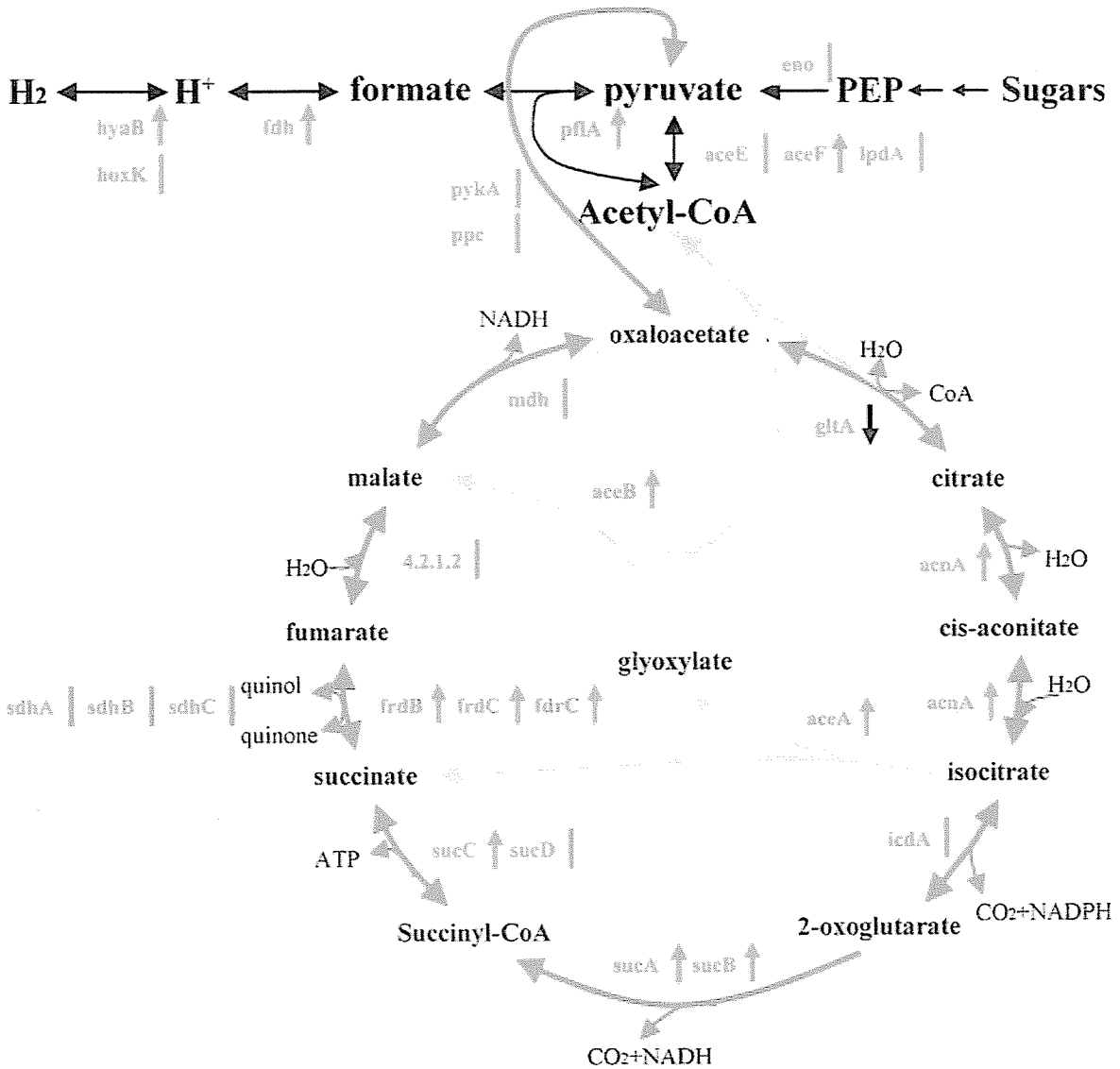

FIG. 2. TCA cycle and associated energy metabolic pathways. On the right side of gene symbols, the blue vertical bars denote no change in expression, whereas red upward arrows and black downward arrows denote significant up- or down-regulation in expression, respectively. Acetyl-CoA, acetyl coenzyme A.

motors, were present in the MR-1 genome (5). Notably, 47 of 49 flagellar assembly genes were repressed by the NaCl stress (Table 1). All flagellar assembly genes are located in region 4 except for the motor-encoding genes (Table 1). Apart from a few methyl-accepting chemotaxis protein (MCP) genes (less than 5% of the total MCP) that are dispersed throughout the genome, almost all chemotaxis-related genes were either significantly down-regulated or unaffected (Table 1 and Table 1S).

To test whether the observed down-regulation of chemotaxis-related protein genes indeed impacted cell motility, cell motility was qualitatively tested with soft agar inoculations. We prepared both solid (1% agar) and semisolid (0.3% agar) MR2A plates in combination with different salt concentrations for motility assessments. Cells (5 μl; OD_{600} = 0.45) were applied to the center of the plate, the plates were cultivated at 30°C for 20 h, and the swarming behavior of the cells was observed. As expected, the cell motility was adversely affected under salt stress even at decreased NaCl concentrations (Fig. 3). These results indicated that down-regulation of flagellar assembly genes caused a decrease in motility, which agrees with previously reported observations for *E. coli* (21), *B. subtilis* (47), and *Salmonella enterica* serovar Typhimurium (34).

Transcriptional regulation of flagellar and chemotaxis genes has been well studied (3, 4, 25) and has been documented in detail for bacteria of the *Enterobacterales* (9), *Bucillaceae* (47), and *Vibrionaceae* (27). Except for 28 MCP genes that are located in different operons, MR-1 has more than 60 flagellar assembly and other chemotaxis genes organized in at least 17 probable operons (Table 1). The operon organization of MR-1 flagellar ORFs most closely resembles that of *V. cholerae*. Maintenance of these large flagellar systems would seem to be

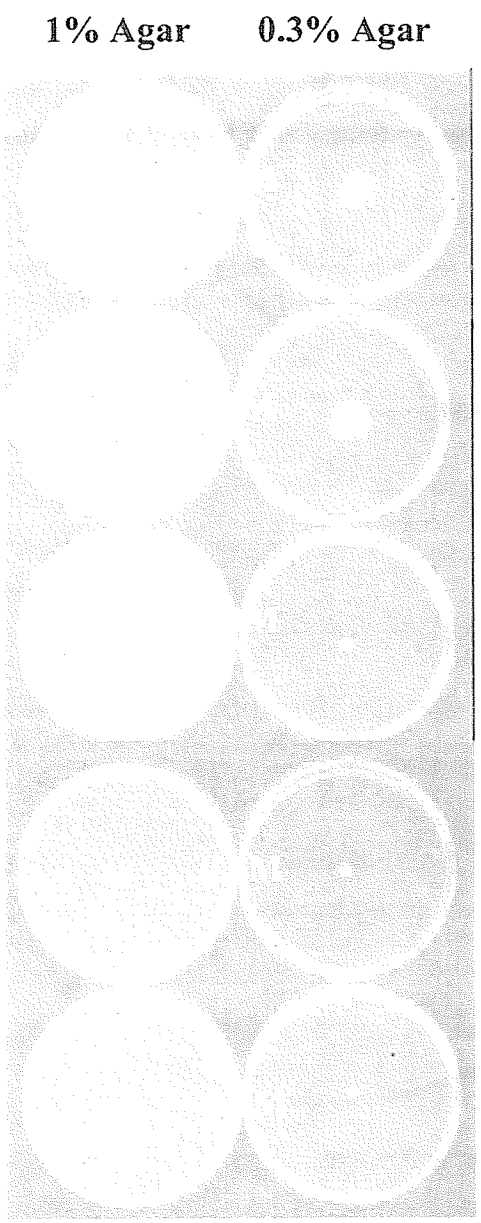

FIG. 3. NaCl at a concentration of 0.3 M or higher completely halts MR-1 cell motility. The solid medium plates (1% agar) are designed to control possible different cell growth rates over varied NaCl concentrations as indicated, whereas the semisolid medium plates (0.3% agar) show the cell ability for motility.

a sizable investment with respect to cellular economy. In *V. cholerae*, the operons constitute a large, coordinately regulated flagelar regulon that is divided into three temporally regulated, hierarchical transcriptional levels: early, middle, and late (27). In *V. cholerae*, FlrA, acting as a σ^{54}-dependent transcription factor, activates transcription of *flrBC*, a two-component signal transduction system. The phosphorylation of FlrC by FlrB is required to activate middle-level flagellar genes (38), which includes most flagellar assembly genes, and *fliA*, which encodes a specialized sigma factor, σ^{28}. σ^{28} activity controls transcription of the late-level genes like the flagellin, motor, and anti-sigma factor genes (27). Salt stress repressed the expression of *flrA* and *flrC*, the master transcriptional regulator genes in MR-1, leading to a complete shutdown of middle- and late-level flagellar assembly genes (Table 1). MR-1 may be similar to *E. coli* in terms of flagellar gene expression regulation, in which a promoter or promoters of the master operon *flhDC* receive a number of global regulatory signals, including the concentration of inorganic salt (9). The simultaneous detection of the whole-genomic expression patterns in response to a specific environmental stress can provide details about the possible connections between components in regulatory networks.

Concluding remarks. The up-regulation of energy metabolism, including electron transport, and down-regulation of flagellar biosythesis in response to elevated salt conditions suggested that MR-1 needs more ATP to pump sodium out of the cell. In addition, an increase in electron transport may directly contribute to the efflux of sodium via the sodium-translocating electron transport complex I. Under high-salt conditions, MR-1 may repress the expression of flagellar genes to conserve energy necessary for sodium transport. The genomic expression profile of MR-1 in response to the sodium salt stress together with comparative genomics analyses indicated that MR-1 resembled responses observed in *V. cholerae*. As with *Vibrio* (10), a majority of *Shewanella* species reside in oceans, costal waters, and estuaries and were therefore more tolerant to sodium salt stress. More genomic similarities of MR-1 to *V. cholerae* clearly outline the connections between environments where the microorganisms naturally reside.

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