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Bacterial biofilm-community selection during autohydrogenotrophic reduction of nitrate and perchlorate in ion-exchange brine

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Abstract Three hydrogen-based membrane biofilm reactors (MBfR) biologically reduced nitrate and perchlorate in a synthetic ion-exchange (IX) brine. Inocula from different natural saline environments were employed to initiate the three MBfRs. Under high-salinity (3%) conditions, bioreduction of nitrate and perchlorate occurred simultaneously, and the three MBfRs from the different inocula exhibited similar removal fluxes for nitrate and perchlorate. Clone libraries were generated from samples of the biofilms in the three MBfRs and compared to those of their inocula. When H_2 was the sole exogenous electron donor under high-salinity conditions, MBfR-driven community shifts were observed with a similar pattern regardless of inoculum. The following 16S rRNA gene phylogenetic analysis showed the presence of novel perchlorate-reducing bacteria in the salt-tolerant mBfR communities. These findings suggest that autohydrogenotrophic and high-salinity conditions provided strong selective pressure for novel perchlorate-reducing populations in the mBfRs.

Keywords Membrane biofilm reactor (MBfR) · Hydrogen · Ion-exchange (IX) brine · Nitrate · Perchlorate

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Introduction

Perchlorate (ClO_4^-) is a strong oxidizing agent that has been used as a propellant of rockets and in explosives and fireworks (Losi et al. 2002). Because of its high solubility and low reactivity, perchlorate that has been improperly disposed of spreads quickly in surface and subsurface waters and is a particular problem in the southwestern United States (Logan 2001). Perchlorate interferes with the production of thyroid hormones, which are needed for pre- and post-natal growth and development as well as for normal metabolism in adults (USEPA 2007). Recently, the California Department of Public Health set a maximum contaminant level (MCL) of 6 ppb in drinking water (CDPH 2008). The Massachusetts Department of Environmental Protection established a lower regulatory limit of 2 ppb in drinking water (MassDEP 2006).

Advanced water-treatment technologies such as ion-exchange (IX) and reverse osmosis (R/O) processes are effective for removing perchlorate (Batista et al. 2002; Giblin et al. 2002; Gu et al. 2007). However, perchlorate is not detoxified in these processes, but rather accumulates in the IX and R/O brine concentrates. Other anions (e.g., nitrate) exist concomitantly in groundwater, usually at concentrations of one to three orders of magnitude

higher than that of perchlorate (Batista et al. 2002); these anions accumulate in the IX brine at proportionally high concentrations.

Biological reduction is a promising process for detoxifying the IX or R/O concentrates because it transforms the oxyanions to harmless forms (i.e., nitrate to N_2 gas, and perchlorate to chloride ion and water). In the case of IX, the detoxified brine can be reused to regenerate the IX resin (Batista et al. 2002). Biological reduction of nitrate and perchlorate is a well-established process for direct groundwater treatment, particularly by the hydrogen (H_2)-based membrane biofilm reactor (MBfR) (Nerenberg et al. 2002; Rittmann 2006; Chung et al. 2007; Rittmann 2007). In the MBfR, H_2 is the electron donor for autotrophic bacteria that reduce oxidized contaminants. Hydrogen is delivered by diffusion through a bubble-less gas-transfer membrane to a biofilm of H_2-reducing bacteria, which accumulate on the outside wall of the membrane (Rittmann 2006).

The salinity of IX brine ranges from 3% to 6% NaCl, unlike brine produced from the MBfR treatment of freshwater. This creates a high-saline condition that inhibits the growth and accumulation of nitrate or perchlorate-reducing bacteria, with the exception of halophiles, which have special means to balance the osmotic pressure across their membranes and thus adapt well to high salt concentrations (Gingras and Batista 2002). Some halophiles try to balance the ionic strength of their cytoplasm with the saline environment by accumulating compatible solutes; they can use inorganic solutes (Mg^{2+} or K^{+}) or organic solutes (amino acids, glycerol, or glycine betaine) for this purpose. However, this approach consumes energy, which slows their growth rate (Oren 1999).

Despite the challenges of managing osmotic pressure, denitrifying halophiles have been reported in many natural saline environments (Woolard and Irvine 1995; Oren 2001). Halophilic perchlorate-reducing bacteria (PRB) also are found in various natural environments, and most of them oxidize organic substrates (Rikken et al. 1996; Herman and Frankenberger 1999). It has been reported that nitrate and perchlorate can be reduced simultaneously under hypersaline conditions using an organic electron donor (e.g., acetate or ethanol; Giblin et al. 2000; Gingras and Batista 2002; Xu et al. 2003). Although our microbiological knowledge is relatively well established regarding heterotrophic halophiles that are capable of the co-reduction of nitrate and perchlorate, little is known about autotrophic and hydrogen-oxidizing PRB (Zhang et al. 2002). Furthermore, microbial ecology has yet to be explored with regard to microbial selection pressures on autohydrogenotrophic halophiles capable of reducing nitrate and perchlorate, although our previous work (Chung et al. 2007) demonstrated the feasibility of the simultaneous reduction of nitrate and perchlorate in a H_2-based MBfR treating IX brine.

The main objective of this study was to understand how the simultaneous bioreduction of nitrate and perchlorate, using H_2-based MBfR, controlled the characteristics of the microbial communities in autohydrogenotrophic biofilms under hypersaline conditions relevant to treating IX brine. In particular, H_2-based MBfRs were inoculated with microbial inocula taken from three different natural saline environments. After 3 months of MBfR operation with nitrate and perchlorate reductions in 3% salt brine, biofilm samples were taken from the MBfR. The microbial communities were then assayed and compared to their inocula, as well as to one another. This work tests the hypothesis that autotrophic halophiles capable of reducing nitrate and perchlorate are selected when H_2 is the sole electron donor and under saline conditions.

Materials and methods

Synthetic ion-exchange brine water

All media were prepared using ultra-pure water (Purelab Ultra; Elga Labwater, High Wycombe, UK) and research-grade chemicals. In the MBfRs, H_2 gas was delivered through the membrane as the sole exogenous electron donor. Sodium perchlorate and sodium nitrate were added as electron acceptors, and sodium bicarbonate (0.5 g/L) was used as the sole carbon source for autotrophic bacterial growth. The salinity in the medium was approximately 3.0%, primarily from NaCl (30 g/L). $MgCl_2$ was added to maintain a Mg/Na molar ratio of 0.11:1 because magnesium has been shown to increase osmo-tolerance at salinities of 3.0–6.0% (Lin et al. 2007). Macronutrients (grams per liter) included KH_2PO_4 (0.04), K_2HPO_4 (0.05), $CaCl_2{\cdot}2H_2O$ (0.02), and $FeSO_4{\cdot}7H_2O$ (0.02). In addition, a trace nutrient solution was added to the medium (milliliters per liter) and included (milligrams per liter) $CoCl_2{\cdot}6H_2O$ (200), $CuCl_2{\cdot}2H_2O$ (10), H_3BO_3 (300), $MnCl_2{\cdot}2H_2O$ (400), $NiCl_2{\cdot}6H_2O$ (10), $Na_2MoO_4{\cdot}2H_2O$ (30), $Na_2SeO_3{\cdot}5\ H_2O$ (10), $Na_2WO_4{\cdot}2H_2O$ (10), and $ZnSO_4{\cdot}7H_2O$ (100).

Liquid sampling and analysis

All anions were measured by ion chromatography (DX 3000; Dionex, Sunnyvale, CA, USA), equipped with a conductivity detector (CDet 1), an ASRS-ULTRA II suppressor (4 mm), and an automated sampler. For the analysis of perchlorate, an IonPac® AS16 anion analytical column (4 mm×250 mm) and AS 16 guard column were used; the suppressor was set at 193 mA, and isocratic acid was delivered for 15 min as the eluent solution. Nitrate, nitrite, chlorate, and chlorite were measured using an IonPac® AS18 anion analytical column (4 mm×250 mm)

and AG 18 guard column (4 mm×50 mm). The suppressor was set at 87 mA, and a 25-μL sample loop was used. A gradient eluent method was used at flow rate of 1.0 mL/min to separate all peaks (22 mM KOH for 10 min; gradual increase in KOH concentration up to 35 mM between 10 and 12 min; 35 mM KOH between 12 and 18 min). To quantify microbial biomass in effluent, volatile suspended solid (VSS) were measured using a standard condition (550°C for 20 min).

Experimental set-up and operating conditions

The MBfR systems were the same as those used by Chung et al. (2007). In brief, the main membrane module (working volume=11.7 mL) contained a bundle of 32 hydrophobic hollow-fiber membranes (total surface area=72.6 cm^2; Model MHF 200TL, Mitsubishi Rayon) inside a glass shell. A second membrane module was connected to the main module and contained one membrane fiber that was used as a coupon for biofilm sampling. The system behaved as a completely mixed reactor due to the high recirculation ratio (150:1) afforded by the Masterflex pump (L/S, Cole-Parmer Instrument Company, Vernon Hills, IL, USA).

Three inocula were taken from sediments in natural saline environments: Freeport (FP), TX, USA; the Great Salt Lake (GSL), UT, USA; and the Salton Sea (SS), CA, USA. Detailed information of the areas is described in Van Ginkel et al. (2008). After being vortexed for 1 min, 5 mL of suspension for one inoculum was seeded into each MBfR. For biofilm colonization, the inoculated MBfRs were operated in a semi-batch mode for 2 weeks by replacing 3 mL spent medium with fresh one every second day. After the colonization stage, continuous feeding was enabled by a peristaltic pump (Dynamax RP-1, Rainin Instrument Llc., Oakland, CA, USA). During operation, the H_2 gauge pressure was set at 4.0 psi (0.3 atm), and the influent flow rate was 0.05 mL/min, resulting in a 4-h hydraulic retention time. The influent nitrate concentration was ~250 mg N/L, and the influent perchlorate concentration was ~20 mg/L, maintaining the molar ratio of nitrate/perchlorate at 12.5. The molar ratio of nitrate/perchlorate was used because our preliminary experiment showed microbial perchlorate-reduction activity in the nitrate/perchlorate molar ratio.

Cloning and DNA sequencing analyses

Biomass samples were obtained from three original inocula (day 0) and from the biofilms of their corresponding MBfRs (day 90) to explore changes in the microbial communities in response to MBfR treatments. The coupon fiber in each MBfR was used for biomass sampling; a 2-cm piece was cut from each MBfR, as described by Chung et al. (2007). Biomass samples were immediately put into bead tubes provided by the Ultra Clean Soil DNA Kit (Mo Bio Laboratories, Carlsbad, CA, USA), and genomic DNA was extracted following the manufacturer's protocol.

The 16S rRNA genes in the genomic DNA were amplified by polymerase chain reaction (PCR) using a thermal cycler (Bio-Rad Laboratories, Hercules, CA, USA) with the following steps: initial denaturation at 94°C for 3 min for one cycle; 30 cycles of (a) 94°C for 1 min, (b) 55°C for 30 s, and (c) 72°C for 2 min; and a final extension at 72°C for 5 min with an infinite hold at 4°C. The bacterial 16S rRNA gene was amplified using primers 27F [5′-AGAGTTTGATC(AC)TGGCTCAG-3′] and 1492R [5′ GGTTACCTTGTTACGACTT-3′] (Suzuki and Giovannoni 1996). In one PCR reaction (25 μL), the following reactants were used: 10× PCR buffer, 1.5 mM $MgCl_2$, 200 μM of dNTP (each), 2 μM of 27F primer, 2 μM of 1492R primer, 1 unit of *Taq* polymerase, and 2 μL of each DNA template. All components were purchased from Applied Biological Materials (Vancouver, Canada). After PCR amplification, the sizes of PCR amplicons (~1.4 kb) were ensured using 1% agarose gel electrophoresis. For purification of the PCR amplicons, an Ultra Clean PCR Clean-up Kit (Mo Bio Laboratories) was used according to the manufacturer's instructions.

For 16S rDNA gene cloning, the prepared PCR amplicons were inserted into the pCR4-TOPO cloning vector using a TOPO TA Cloning Kit for Sequencing (Invitrogen, USA). After insertion into each *Escherichia coli* host cell, the individual PCR amplicon in each vector was cloned via the growth of the host cells on a kanamycin-supplemented LB medium. After the vectors containing PCR products were isolated, the 16S rDNA PCR products were sequenced by MACROGEN (Seoul, South Korea). Of the 300 clones used for sequencing, 50 were from each of the three original inocula and their corresponding MBfR-treated biofilm samples.

Microbial diversity was measured based upon the 16S rRNA gene sequences from the clones using Richness and the Shannon Index. Richness was calculated by the following formula (Tiquia 2005):

$$\text{Richness} = (S-1)/\log N.$$

The Shannon index (H') was computed by the following equation (Krebs 1998):

$$H' = -\sum_{i=1}^{S} p_i \ln p_i.$$

In these equations, S is the number of species (called species richness), n_i is the number of individuals in each species, and N is the total number of all individuals. The relative abundance of each species (p_i) is calculated as the

proportion of individuals of a given species to the total number of individuals in the community (n_i/N).

For phylogenetic analysis, multiple alignments of the test and reference sequences were conducted using the CLUSTAL-W software (EBI, http://www.ebi.ac.uk/clustalw/). The average size of the aligned sequences was ~700 bp. After parsing (aligning and trimming) the partial 16S rRNA gene sequences, 35 clones for the SS MBfR sample (MBfR SS clones), 15 clones for the FP-MBfR sample (MBfR FP clones), and 25 clones for the GSL MBfR sample (MBfR GSL clones) were obtained. Using the prepared sequences, a phylogenetic tree was constructed with the MEGA4 program (Tamura et al. 2007). For phylogenetic tree inference, the neighbor-joining algorithm was used, and a bootstrapping test (1,000 replicates) was run. For population identification of new sequences, CLASSIFIER software from the Ribosomal Database Project II (http://rdp.cme.msu.edu/) and BLAST from GenBank (http://www.ncbi.nlm.nih.gov/BLAST/) were used.

Nucleotide sequence accession number

The 75 DNA sequences are deposited at the NCBI GenBank database through the Sequin program under the accession number EU923991 to EU924065 (Supplementary material, Table S1).

Results

Removal fluxes of nitrate and perchlorate in MBfRs

Table 1 summarizes the nitrate- and perchlorate-reduction performance of the three MBfRs after 90 days of continuous feeding. After approximately 70 days, effluent nitrate and perchlorate concentrations reached a chemically steady state. The effluent nitrate concentrations ranged from 160 to 177 mg N/L, showing fractional nitrate reductions of 28~34%. Perchlorate concentrations decreased by 1~2 mg/L, and the fractional perchlorate reduction was 7~11%. These indicate that both anionic pollutants could be reduced in the MBfRs.

Nitrate and perchlorate removal fluxes in the MBfRs were calculated using the tabulated data (Table 1) according to:

$$J = \left(S^0 - S\right)Q/A$$

where S^0 and S are the influent and effluent nitrate or perchlorate concentrations (grams per liter), respectively, Q is the volumetric flow rate through the main membrane module (liters per hour), and A is the membrane surface area (square meter). The estimated nitrate removal fluxes ranged between 0.67 and 0.83 g/m^2 day, and the estimated perchlorate removal fluxes ranged between 0.014 and 0.020 g/m^2 day. The nitrate removal flux is approximately 36-fold higher than the perchlorate removal fluxes. Although different inocula were applied to each MBfR, the nitrate and perchlorate removal fluxes were comparable.

Microbial community compositions and diversity

Figure 1 illustrates the microbial community compositions before and after selection by autohydrogenotrophic growth condition as well as exposure to the high-salinity condition in the MBfRs. After 90 days of selection in the hydrogen-fed MBfR, the community structures (at subclass or phylum level) in the MBfR biofilms shifted greatly from the original inocula. Regardless of the inoculum, the alpha- and gamma-subclasses of *Proteobacteria* became the most dominant populations in the nitrate- and perchlorate-reducing biofilm communities. In all the tested MBfRs, the beta-, delta-, and epsilon-subclasses of *Proteobacteria* and *Firmicutes* showed reduced population sizes. *Bacteroidetes* populations increased in the GSL- and FP-inoculated MBfRs, while they showed a reduced population size in the SS-inoculated MBfR. These results indicate that the overall trends in MBfR-driven community shifts exhibit a similar pattern regardless of the inoculum, although the population selection of *Bacteroidetes* by MBfR seems to be somewhat inoculum specific.

Table 1 Loading rates, concentrations, removal fluxes, and percentages for nitrate and perchlorate in MBfRs (hydrogen gauge pressure=4 psi (0.3 atm) and hydraulic retention time (HRT)=3.9 h) operated with 30 g/L NaCl

Inoculum	Electron acceptor	Influent (mg/L)	Loading rate (g/m^2 day)	Effluent (mg/L)	Flux[a] (g/m^2 day)	Removal (%)
FP	$NO_3^- - N$	245±17	2.4	160±16	0.83	34
GSL	$NO_3^- - N$	245±17	2.4	170±11	0.74	31
SS	$NO_3^- - N$	245±17	2.4	177±12	0.67	28
FP	ClO_4^-	18.4±1.0	0.18	16.4±0.4	0.020	11
GSL	ClO_4^-	18.4±1.0	0.18	16.1±0.4	0.023	13
SS	ClO_4^-	18.4±1.0	0.18	17.1±0.6	0.014	7.4

[a] The fluxes were computed as the mass-per-time rate of substrate removal normalized to the membrane surface area

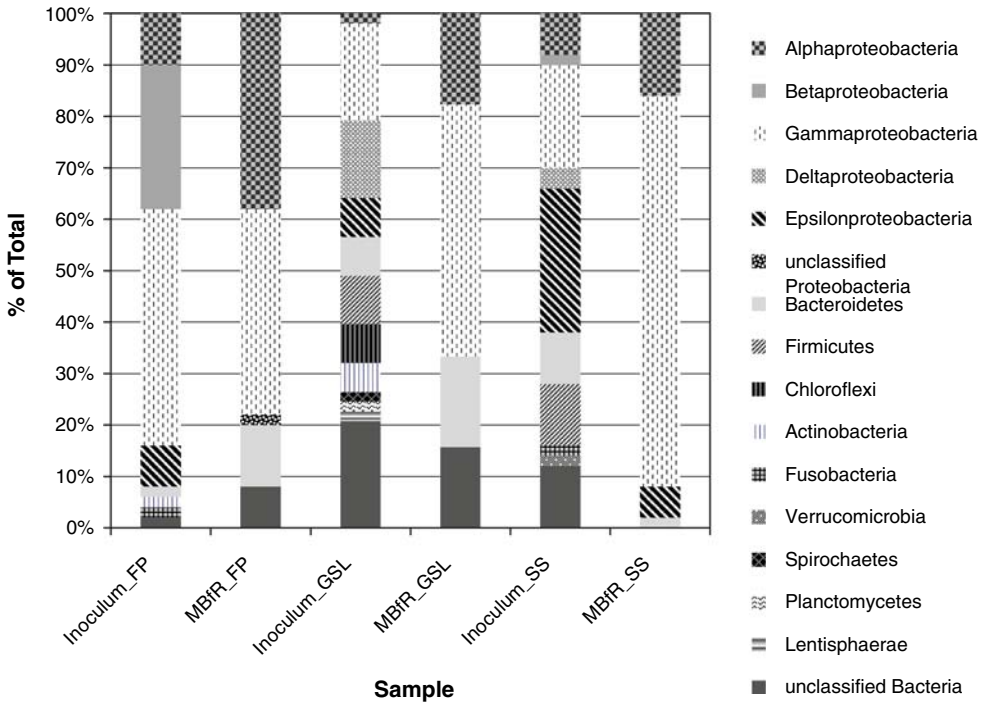

Fig. 1 Microbial community compositions of the original inocula and in the biofilms after 90 days of MBfR operation

In addition, MBfR may have provided effects to reduce the microbial diversity of the biofilm communities. This observation is supported by the Richness and the Shannon Index values for the SS- and GSL-inoculated MBfR communities (Table 2). In the case of the FP-inoculated MBfR community, however, the Richness and the Shannon Index values were not significantly influenced by the MBfR treatment. This may be attributed to the fact that the indigenous bacterial community in the FP inoculum has low microbial diversity, i.e., the Shannon and Richness Index values of the FP inoculum are lower than those of the GSL and SS inocula. The intrinsically low microbial diversity in the FP inoculum could not have been further reduced by the MBfR selection.

Identification of dominant populations

Based on the 16S rRNA gene sequences from the clones, dominant bacterial populations in the hydrogen-utilizing MBfR biofilm communities were identified at the genus level. In the GSL-inoculated MBfR community, unclassified gamma-*Proteobacteria* (35%), uncultured *Bacteroidetes* (17.6%), unclassified *Rhodobacteraceae* (9.8%), and *Marinobacter* (7.8%) were identified as dominant populations. In the SS-inoculated MBfR community, *Marinobacter* (44%), unclassified gamma-*Proteobacteria* (14%), unclassified *Rhodobacteraceae* (14%), *Alcanivorax* (12%), *Halomonas* (6%), and *Sulfuricurvum* (6%) were major populations. In the FP-inoculated MBfR community, *Marinobacter* (20%), unclassified *Rhodobacteracea* (6%), uncultured *bacteroidetes* (13%), *Nitratireductor* (26%), unclassified gamma-*Proteobacteria* (6%), *Alcanivorax* (13%), and *Oceanicola* (13%) were dominant populations. Among the dominant populations, *Marinobacter*, unclassified gamma-*Proteobacteria*, and unclassified *Rhodobacteraceae* were common in all MBfR biofilm communities regardless of the different sources for inoculation.

Table 2 Diversity indices in the original inocula and biofilm samples from hydrogen-based MBfR

Diversity indices	Original inocula			MBfR biofilm sample		
	FP	GSL	SS	FP	GSL	SS
Shannon index	2.16	3.02	2.79	2.44	1.93	1.72
Richness (S)	16	28	23	15	11	9

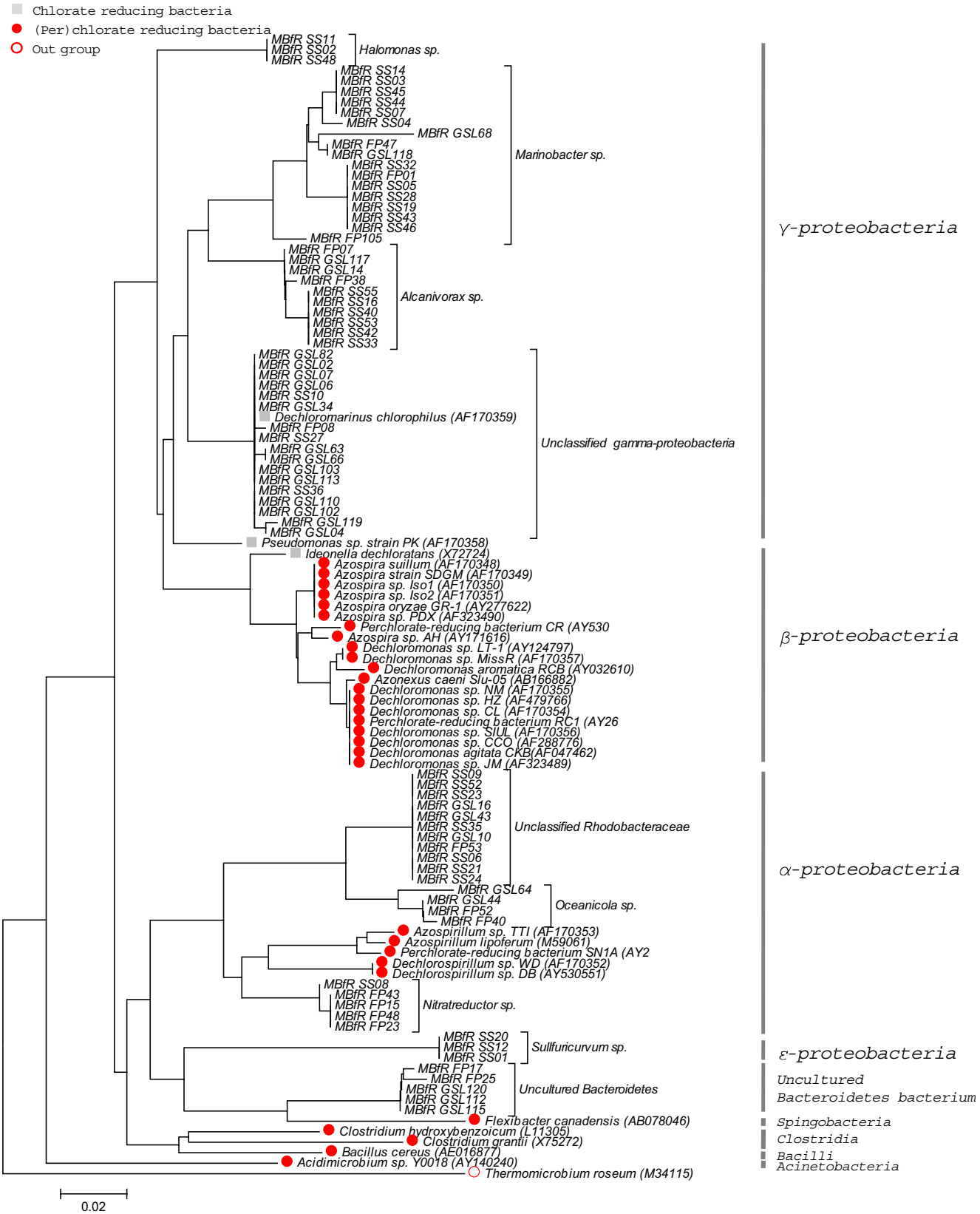

Fig. 2 Distribution of 16S rRNA gene sequences from known perchlorate- and chlorate-reducing isolates and from clones of this work. The *bars on the right side* indicate class-level identifications. The *scale bar at the bottom left* indicates the distance of a 2% sequence divergence

Phylogenetic analysis

Phylogenetic analysis was performed with a subset of the dominant 16S rRNA clones, and the results are summarized in Fig. 2. Genera of bacterial species in the MBfR clones were identified as *Halomonas*, *Marinobacter*, *Alcanivorax*, *Oceanicola*, *Nitratireductor*, and *Sulfuricurvum*. However, genera of the "unclassified gamma-*Proteobacteria* and *Rhodobacteraceae*" clones and the "uncultured *Bacteroidetes*" were not identified. Their sequence divergences from known bacterial species are greater than 7%. This indicates that the unclassified and uncultured populations belong to novel genera.

The phylogenetic analysis also showed that the dominant bacterial species from this work are evolutionarily distant from previously known perchlorate-reducing isolates (Fig. 2). In the clone libraries, no bacterial species belonging to the beta-subclass of *Proteobacteria* were detected, while the majority of known perchlorate-reducing bacteria belong to the subclass of *Proteobacteria* (Coates and Achenbach 2004). In addition, the MBfR clones showed at least 7% sequence divergence from other known perchlorate-reducing species belonging to *Flexibacter*, *Clostridium*, *Bacillus*, *Acidimicrobium*, *Azospirillum*, and *Dechlorospirillum* genera. Among the MBfR clones belonging to the gamma-subclass of *Proteobacteria*, no previously known perchlorate-reducing isolates were detected, while some of the unclassified gamma-Proteobacteria clones are close to chlorate-reducing *Dechloromarinus chlorophilus* species (accession no. AF170359) that are known not to reduce perchlorate (Coates and Achenbach 2004). This suggests that novel perchlorate-reducing isolates were detected in the clone libraries from the hydrogen-fed MBfRs.

Discussion

The negative effect of highly saline conditions (greater than 2% NaCl) on perchlorate-reducing bacteria has been documented in literature (Coats and Achenbach 2004). In the MBfR studied here, increasing salinity also slowed the rates of reduction of nitrate and perchlorate (Van Ginkel et al. 2008). Nevertheless, the hydrogen-utilizing MBfRs exhibited simultaneous reduction of nitrate and perchlorate in highly saline conditions (3%) that are relevant to treatment of ion-exchange brine. A previous study with hydrogen-fed MBfRs (Chung et al. 2007) demonstrated that simultaneous reduction of nitrate and perchlorate by hydrogen-utilizing MBfRs is feasible in highly saline conditions. The nitrate removal fluxes of the MBfRs in this study were approximately one order of magnitude greater than those reported in the previous study. A detailed characterization of the kinetics for nitrate and perchlorate reduction in these and other hydrogen-utilizing MBfRs is given in Van Ginkel et al. (2008). In brief, for the MBfRs studied here, while nitrate fluxes were roughly first order with respect to H_2 pressure, they were not strongly affected by nitrate concentration in the ranges tested. Because perchlorate and nitrate reductions competed for the common electron donor, H_2, perchlorate fluxes declined with higher nitrate loading.

To the best of our knowledge, this is the first study to report microbial community characteristics of hydrogen-utilizing biofilms that exhibit the simultaneous reduction of nitrate and perchlorate in a high-salinity condition. Considering the similarities among the community structures in the MBfR biofilms (Fig. 1), autohydrogenotrophic growth and the high-salinity condition in the MBfRs seemed to provide strong pressure to select for a particular microbial population—especially the alpha- and gamma-subclasses of *Proteobacteria*—in the nitrate- and perchlorate-reducing biofilm communities. The observed selection of the alpha- and gamma-subclasses of *Proteobacteria* differs from the findings of Nerenberg et al. (2008), wherein perchlorate-reducing *Dechloromonas* (belonging to the beta-subclass of *Proteobacteria)* was selectively enriched in autohydrogenotrophic MBfRs where denitrification and perchlorate degradation occurred in low-salinity conditions. Because high salinity and the inoculum source are the main differences in operational conditions between current and previous works, the population selection of alpha- and gamma-*Proteobacteria* may be attributed mainly to the high-salinity condition and/or the inoculum source rather than the autohydrogenotrophic condition. This explanation is reinforced by other previous reports that alpha- and gamma-subclasses of *Proteobacteria* are generally dominant in marine biofilm environments (Dang and Lovell 2000; Jones et al. 2007; Lee et al. 2008). So far, no isolated microorganism has been shown to grow by perchlorate respiration in salinities greater than 2% (Coates and Achenbach 2004). This is related to the investigation history that most of the known perchlorate-reducing isolates were cultivated in freshwater or low-salinity conditions (Coates and Achenbach 2004). Our findings suggest a potential use of autohydrogenotrophic MBfR with high salinity in cultivating novel perchlorate-reducing bacteria.

Nitrate-reducing halophilic bacteria were selected in the MBfR by the high exposure levels of nitrate (between approximately 160 and 245 mg/L) and salinity (3% NaCl). It is well known that halophilic populations generally have the ability to reduce nitrate (Oren 1999). Most of the genus-identified MBfR clones are likely to be nitrate-reducing halophiles (Fig. 2). *Halomonas*, *Marino-*

bacter, *Sulfuricurvum*, *Oceanicola*, and *Nitratireductor* genera include halophiles and nitrate reducers (Mormile et al. 1999; Martín et al. 2003; Kodama and Watanabe 2004; Labbé et al. 2004; Ying et al. 2007). One exception to this trend is *Alcanivorax*, which is an aerobic obligate marine bacteria able to grow at salinities of 0.46–20.3% NaCl (Golyshin et al. 2003). So far, there has been no report that *Alcanivorax* is able to reduce nitrate. In contrast, this work showed that the increased growth of the *Alcanivorax* species (12% and 13% in the SS- and FP-MBfR communities, respectively, from no detection in the corresponding original inoculation sources) resulted from the denitrifying MBfR experiments. It is not clear how the oxygen-consuming *Alcanivorax* species could grow in the MBfRs where no oxygen was supplied into. A possible explanation for this phenomenon is that oxygen produced from perchlorate reduction could be utilized as the terminal electron acceptor for the *Alcanivorax* growth (Coates and Achenbach 2004).

Among the genus-identified populations, only *Halomonas campisalis* and *Sulfuricurvum kujiense* are known autohydrogenotrophic species (Mormile et al. 1999; Kodama and Watanabe 2004). The other genus-identified populations appear to be heterotrophs (Martín et al. 2003; Labbé et al. 2004; Cho and Giovannoni 2004). It is possible that heterotrophs were supported by utilization of soluble microbial products generated by the autotrophs (de Silva and Rittmann 2000a, b). In the effluents from the MBfRs, microbial biomass was detected (approximately 31–42 mg/L VSS). The existence of detached microbial biomass suggests that soluble microbial products, released from detached biofilm biomass, could have been used as carbon and energy sources for heterotrophs. However, because the concentration of soluble microbial products was not directly measured, it is not clear whether the amount of soluble microbial product was sufficient to support the abundance of heterotrophic bacterial populations in the MBfR communities. Another plausible explanation is the existence of "mixotrophs" that can experience both heterotrophic as well as autotrophic growth (Kuenen and Beudeker 1982; Hoaki et al. 1995; Bowien and Kusian 2002).

The hydrogen-utilizing MBfRs showed simultaneous nitrate and perchlorate reductions in a highly saline condition, which is advantageous for the treatment of ion-exchange brine. Microbial community analysis of the MBfR biofilm samples showed that autohydrogenotrophic and high-salinity conditions selected for bacteria belonging to the alpha- and gamma-subclasses of *Proteobacteria*. The subsequent phylogenetic analysis revealed the existence of novel perchlorate-reducing bacteria in the MBfR communities. Considered together, the findings from this work suggest that autohydrogenotrophic and high-salinity conditions provided strong selective pressure for novel perchlorate-reducing populations in the MBfRs that could remove nitrate and perchlorate from ion-exchange brines.

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