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Phenotypic and Genotypic Description of *Sedimenticola selenatireducens* Strain CUZ, a Marine (Per)Chlorate-Respiring Gammaproteobacterium, and Its Close Relative the Chlorate-Respiring *Sedimenticola* Strain NSS

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Two (per)chlorate-reducing bacteria, strains CUZ and NSS, were isolated from marine sediments in Berkeley and San Diego, CA, respectively. Strain CUZ respired both perchlorate and chlorate [collectively designated (per)chlorate], while strain NSS respired only chlorate. Phylogenetic analysis classified both strains as close relatives of the gammaproteobacterium *Sedimenticola selenatireducens*. Transmission electron microscopy (TEM) and scanning electron microscopy (SEM) preparations showed the presence of rod-shaped, motile cells containing one polar flagellum. Optimum growth for strain CUZ was observed at 25 to 30°C, pH 7, and 4% NaCl, while strain NSS grew optimally at 37 to 42°C, pH 7.5 to 8, and 1.5 to 2.5% NaCl. Both strains oxidized hydrogen, sulfide, various organic acids, and aromatics, such as benzoate and phenylacetate, as electron donors coupled to oxygen, nitrate, and (per)chlorate or chlorate as electron acceptors. The draft genome of strain CUZ carried the requisite (per)chlorate reduction island (PRI) for (per)chlorate respiration, while that of strain NSS carried the composite chlorate reduction transposon responsible for chlorate metabolism. The PRI of strain CUZ encoded a perchlorate reductase (Pcr), which reduced both perchlorate and chlorate, while the genome of strain NSS included a gene for a distinct chlorate reductase (Clr) that reduced only chlorate. When both (per)chlorate and nitrate were present, (per)chlorate was preferentially utilized if the inoculum was pregrown on (per)chlorate. Historically, (per)chlorate-reducing bacteria (PRB) and chlorate-reducing bacteria (CRB) have been isolated primarily from freshwater, mesophilic environments. This study describes the isolation and characterization of two highly related marine halophiles, one a PRB and the other a CRB, and thus broadens the known phylogenetic and physiological diversity of these unusual metabolisms.

Perchlorate (ClO_4^-) and chlorate (ClO_3^-), collectively designated (per)chlorate, are oxyanions of chlorine that have both natural and anthropogenic sources (1–4). Perchlorate is used extensively in rocket fuel, pyrotechnics, lubricants, and paints (1, 5), while chlorate is used as an herbicide, a defoliant, and a bleaching agent in the paper industry (6, 7). Contamination of groundwater with perchlorate due to human activity is a major environmental concern, since perchlorate inhibits the uptake of iodine by the thyroid gland and may lead to hypothyroidism (8, 9). Chlorate exposure has been correlated with hemolytic anemia as well as with reproductive toxicity (10). The ability of microorganisms to respire (per)chlorate has been exploited recently as an effective method of bioremediation (11–13).

(Per)chlorate-reducing bacteria (PRB) reduce (per)chlorate with the perchlorate reductase enzyme (Pcr), while chlorate-reducing bacteria (CRB) reduce only chlorate, using a distinct chlorate reductase enzyme (Clr) (11, 14–16). The product of both enzymatic reactions, chlorite (ClO_2^-), is detoxified in both PRB and CRB into innocuous chloride and molecular oxygen by the chlorite dismutase enzyme (Cld) (17). The molecular oxygen produced by chlorite disproportionation is either respired or used as a cosubstrate for oxygenases (18).

While a great number of PRB and CRB have been isolated in the past 2 decades, the majority of these isolates were obtained from freshwater, mesophilic, neutral pH environments (11, 19). Exceptions are the thermophiles *Moorella perchloratireducens* (20) and *Archaeoglobus fulgidus* (21) and the halophiles *Arcobacter* sp. strain CAB and *Shewanella algae* ACDC (18, 22). The current study seeks to expand the phylogenetic and physiological diversity of PRB and CRB by characterizing additional isolates obtained from marine environments. Strain CUZ was isolated from sediment in Berkeley, CA, while strain NSS was isolated from hydrocarbon-contaminated sediment in the San Diego Bay, CA. Strain NSS has been mentioned previously in studies of the diversity and evolution of chlorate reduction (6, 23, 24) but has not been char-

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acterized in detail to date. Both strains are most closely related to each other and to the selenate-reducing bacterium *Sedimenticola selenatireducens* AK4OH1 (25). These organisms further represent the first examples of two phylogenetically consonant microorganisms of which one is a PRB and the other a CRB. As such, the study of these strains will provide insight into the independent evolution of (per)chlorate and chlorate metabolisms within a host organism as well as expanding current knowledge regarding the phylogeny and physiology of PRB and CRB.

MATERIALS AND METHODS

Culture conditions, enrichments, and isolations. (i) *Sedimenticola selenatireducens* CUZ. Sediment samples were obtained from a marina in Berkeley, CA (latitude, 37.8269; longitude, −122.3132) using 50-ml Falcon tubes. For enrichment, 1 g of sediment sample was mixed with 9 ml of medium in 30-ml anaerobic tubes (Bellco, Vineland, NJ). Sediment samples had a salinity of ~1.5% and contained no detectable perchlorate. The medium used for enrichment and isolation is described in reference 18. Hydrogen gas (10 ml) and sodium perchlorate (10 mM) were used as the electron donor and electron acceptor, respectively, and 0.1 g/liter yeast extract was added as a carbon source. The enrichment cultures were maintained at 30°C for 2 weeks and were transferred to fresh medium twice prior to isolation. Agar roll tubes with 10 ml hydrogen, 0.1 g/liter yeast extract, and 10 mM perchlorate were used for isolation. For regular culturing postisolation, $MgCl_2 \cdot 6H_2O$ and $CaCl_2 \cdot 2H_2O$ were omitted from the medium.

(ii) *Sedimenticola* strain NSS (formerly "*Dechloromarinus chlorophilus*" NSS). Hydrocarbon-contaminated sediment samples (33 mg polycyclic aromatic hydrocarbon [PAH] per kg sediment) were obtained from the Naval Station site in the San Diego Bay, CA (latitude, 32.6816; longitude, −117.1221) from a depth of 10 m and were sealed under a headspace of N_2 gas using canning jars. For enrichment, 1 g of sediment sample was mixed with 9 ml of medium in 30-ml anaerobic tubes (Bellco, Vineland, NJ). The medium used for enrichment is described in reference 18, except that the medium contained 40 g/liter rather than 30 g/liter NaCl. Sodium acetate (10 mM) and sodium chlorate (10 mM) were used as the electron donor and electron acceptor, respectively. The enrichment cultures were maintained at 30°C and were transferred every 4 days for a total of seven transfers. Anaerobic agar plates amended with acetate (10 mM) and chlorate (10 mM) were used for isolation. The medium used for regular culturing of strain NSS postisolation contained NaCl (20 g/liter), KCl (0.67 g/liter), monosodium PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] (1.6220 g/liter), disodium PIPES (1.7317 g/liter), vitamins (10 ml), minerals (10 ml), and RST minerals (20 ml) as described in references 18 and 31.

Optimum pH, temperature, and salinity. Acetate and perchlorate (for strain CUZ) and lactate and chlorate (for strain NSS) were used to determine the optimum growth temperature, pH, and salinity. The temperatures tested ranged from 15 to 33°C for strain CUZ and from 30 to 56°C for strain NSS. The salinities tested ranged from 0 to 6% (wt/vol) NaCl in 0.5% increments for both strains. For pH, the medium described above was used, except that 10 mM PIPES buffer was substituted for $NaHCO_3$. The medium was adjusted to the desired pH (pH 6, 6.5, 7, or 7.5) by using HCl or NaOH. All analyses were performed on triplicate cultures.

Alternate electron acceptors and electron donors. For the electron donor profile, 10 mM perchlorate (for strain CUZ) or chlorate (for strain NSS) was used as the electron acceptor. The following electron donors were added from sterile aqueous solutions to give the following final concentrations: acetate, 10 mM; propionate, 10 mM; butyrate, 10 mM; isobutyrate, 10 mM; valerate, 10 mM; methanol, 5 mM; ethanol, 5 mM; benzoate, 1 mM; citrate, 10 mM; succinate, 1 mM; lactate, 10 mM; glucose, 10 mM; fructose, 10 mM; yeast extract, 1 g/liter; fumarate, 10 mM; malate, 10 mM; hydrogen, ~50 kPa; catechol, 1 mM; methane, ~25 kPa; protocatechuate, 1 mM; phenylacetate, 1 mM; sulfide, 5 mM. The tubes containing hydrogen and sulfide also contained 0.1 g/liter yeast extract as a carbon source. To determine if strains CUZ and NSS oxidized the tested electron donors coupled to perchlorate or chlorate reduction, samples were taken at the time of inoculation and 15 days later and were analyzed via ion chromatography (see below) for consumption of perchlorate or chlorate, respectively. Each donor was tested in triplicate, and negative controls included a no-donor control and a control with no donor plus 0.1 g/liter yeast extract. Growth on each electron donor was considered positive when consumption of perchlorate or chlorate was observed concomitantly with an increase in turbidity.

For the electron acceptor profile, acetate (10 mM) was used as the sole electron donor and carbon source. The following electron acceptors were added from sterile aqueous stocks at the following concentrations: perchlorate, 10 mM; chlorate, 10 mM; nitrate, 10 mM; nitrite, 2 mM; sulfate, 10 mM; sulfite, 2 mM; thiosulfate, 10 mM; arsenate, 2.5 mM; selenate, 2.5 mM; malate, 10 mM; and oxygen, ~25 kPa. To determine if strains CUZ and NSS reduced the electron acceptors tested, samples were taken at the time of inoculation and 15 days later and were analyzed for consumption of acetate by high-performance liquid chromatography (HPLC) (see below). Each acceptor was tested in triplicate, and experiments included a no-acceptor control. Growth on each acceptor was considered positive when consumption of acetate was observed concomitantly with an increase in turbidity.

To test the preferential utilization of chlorate or nitrate (strains CUZ and NSS) and (per)chlorate or nitrate (strain CUZ) in diauxic medium, cells of strains CUZ and NSS were grown with 10 mM (each) acetate and chlorate or 10 mM (each) acetate and nitrate. Strain CUZ was also grown on 10 mM (each) acetate and perchlorate. The inocula were then transferred to tubes containing 20 mM acetate, 5 mM perchlorate or chlorate, and 5 mM nitrate. The optical density and anion concentration were measured as described below.

Cell growth profiles. Cell growth was measured spectrophotometrically at 600 nm (optical density at 600 nm [OD_{600}]) using a Varian Cary 50 Bio spectrophotometer (Agilent, Santa Clara, CA). All experimental analyses were performed in triplicate.

Analytical methods. Perchlorate concentrations were measured via ion chromatography using a Dionex ICS 2100 system with a Dionex IonPac AS 16 column (4 by 250 mm) (Thermo Fisher Scientific, Sunnyvale, CA) and a 35 mM NaOH mobile phase at a flow rate of 1.0 ml/min. Background conductivity was suppressed with a Dionex ASRS system operating in recycle mode. The suppressor controller was set at 100 mA for analysis, and the injection volume was 25 μl.

Chlorate and nitrate concentrations were measured via ion chromatography using a Dionex ICS 1500 system equipped with a Dionex Ion Pac AS 25 column (4 by 250 mm) with a mobile phase of 36 mM NaOH at a flow rate of 1.0 ml/min. Background conductivity was suppressed with a Dionex ASRS 300 system (4-mm format) in recycle mode. The suppressor controller was set at 90 mA for analysis, and the injection volume was 10 μl.

Acetate concentrations were measured by HPLC (model LC20; Dionex), using a UV-visible (UV-Vis) detector (AD20; Dionex) at a wavelength of 210 nm and a Bio-Rad Aminex HPX-87H column with a mobile phase of 0.016 N H_2SO_4 (flow rate, 0.9 ml/min).

SEM. Silicon wafers were washed with ethanol for 30 s and were air dried. A 20-μl poly-L-lysine drop was placed on the silicon wafers for 1 min and was then withdrawn. The wafers were then rinsed with ultraclean water. A drop of concentrated cells fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer was added to the silicon wafers and was allowed to settle for 1 h. The silicon wafers were then processed as described in reference 18 and were visualized using a Hitachi S-5000 scanning electron microscope (SEM) at 20 kV.

TEM. Flagella were visualized using the negative-stain technique. Formvar- and carbon-coated copper grids (400 mesh) were glow discharged just before use to increase hydrophilicity. A suspension (5 μl) of cells fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer was

deposited on each grid. This was sufficient to cover the grid. After 2 min, the suspension was removed by touching the ragged torn edges of filter paper to the sample until the grid surface was nearly dry. The grid was then washed 3 times with water droplets, and excess water was wicked off using filter paper as described above. A drop of 1% aqueous uranyl acetate was then added to the grid. After 2 min, the grid was dried with filter paper. Samples were examined the same day using a Tecnai transmission electron microscope (TEM) at 120 kV.

Genomic and phylogenetic analysis. Genomic DNA was extracted as described previously (26). The genomic DNA was submitted to Eureka Genomics (Hercules, CA) for Illumina sequencing and partial assembly. The SILVA database and aligner (27) were used to align 16S rRNA gene sequences, and a maximum likelihood phylogenetic tree was constructed with 1,000 bootstrap values using RAxML-HPC (28). The GenBank accession numbers or IMG (Integrated Microbial Genomes) 16S rRNA tags for the microorganisms used in the tree are provided in Table S1 in the supplemental material. *Sulfolobus acidocaldarius* was used as an outgroup.

Nucleotide sequence accession numbers. The genomes of strains CUZ and strain NSS are available on the IMG system of the Joint Genome Institute, and their 16S rRNA gene sequences are additionally available in GenBank (KM192219 and AF170359, respectively).

RESULTS

Enrichment, isolation, and phylogeny. A (per)chlorate-respiring microorganism and a chlorate-respiring microorganism were isolated from sediment collected from a marina in Berkeley, CA, and from a harbor in San Diego Bay, CA, respectively. Phylogenetic analysis of the 16S rRNA gene identified the (per)chlorate-reducing strain from the Berkeley sediment as a gammaproteobacterium (Fig. 1), and its 16S rRNA gene sequence was a 100% match to that of *Sedimenticola selenatireducens* AK4OH1, a selenate reducer isolated from estuarine sediment from the Arthur Kill (New York and New Jersey) (25). Consequently, we identified this PRB as *Sedimenticola selenatireducens* strain CUZ. Despite the high 16S rRNA gene sequence similarity between the two strains, strain AK4OH1 reduced selenate but not (per)chlorate (25), whereas the opposite was true for strain CUZ. Phylogenetic analysis of the chlorate reducer from San Diego Bay sediment also identified this strain as a gammaproteobacterium (Fig. 1) that shared 98% 16S rRNA gene sequence identity with both *S. selenatireducens* AK4OH1 and *S. selenatireducens* CUZ. In previous publications, this isolate was unofficially designated *Dechloromarinus chlorophilus* strain NSS (11, 19, 29), but in light of its phylogenetic relatedness to the *Sedimenticola* species subsequently described formally, we consider NSS to be a strain of the genus *Sedimenticola*. In addition to their relatedness to *S. selenatireducens* AK4OH1, strains CUZ and NSS were both closely related to the thiotaurine-degrading autotroph *Thiotaurens thiomutagens* (30) (97% and 98% 16S rRNA gene sequence similarity, respectively) (Fig. 1) and to marine endosymbionts of tubeworms and bivalves. Given the 16S rRNA gene sequence similarity, future research may ultimately conclude that all *Sedimenticola* and *Thiotaurens* species should be consolidated under a single genus; however, for the purposes of the present study, the proposed names will be used.

Morphology. Both strains are Gram-negative bacteria, with most cells appearing as straight to slightly curved rods. Strain CUZ ranged from 0.75 to 1.2 μm long and from 0.3 to 0.45 μm wide (Fig. 2C), while strain NSS ranged from 1 to 2 μm long and from 0.3 to 0.5 μm wide (Fig. 2D). Both strains were highly motile, and each possessed one polar flagellum (Fig. 2A and B). In contrast to strains CUZ and NSS, *S. selenatireducens* AK4OH1 has been described as a nonmotile rod measuring ~1.5 μm long and 0.5 μm wide (25).

Growth on perchlorate and chlorate. Strain CUZ grew by coupling the oxidation of acetate to the reduction of perchlorate (Fig. 3A) or chlorate. When grown with 10 mM (each) acetate and perchlorate, strain CUZ doubled every 4 h and reached a maximum optical density at 600 nm (OD_{600}) of 0.52 after 32 h. In a 47-h incubation period, 10.6 (±0.3) mM acetate and 5.9 (±0.1) mM perchlorate were consumed, indicating that 4.8 (±0.3) mM acetate (~45%) was assimilated into biomass, which is common for PRB (18, 31). Strain NSS grew by coupling the oxidation of acetate to the reduction of chlorate (Fig. 3B). Unlike strain CUZ, strain NSS cannot reduce perchlorate (data not shown). When grown with 10 mM (each) acetate and chlorate, strain NSS doubled every 3.5 h and reached a maximum OD_{600} of 0.43 after 25 h. In a 29-h incubation period, 9.9 (±0.2) mM acetate and 7.4 (±0.4) mM chlorate were consumed, indicating that 4.4 (±0.4) mM acetate (44%) was assimilated into biomass.

Optimum pH, temperature, and salinity. Both strains were halophilic. Strain CUZ grew optimally at 4% NaCl, pH 7, and 25 to 30°C (see Fig. S1 to S3 in the supplemental material), while strain NSS preferred 1.5 to 2.5% NaCl, pH 7.5 to 8, and 37 to 42°C (see Fig. S1 to S3). Growth was observed at salinities as high as 6% for strain CUZ and 4% for strain NSS (see Fig. S1). As such, both strains CUZ and NSS differ from *S. selenatireducens* AK4OH1, which grew only at salinities up to 2.3% (25). Strain CUZ exhibited optimal growth at lower temperatures than strain NSS; growth of strain CUZ was observed at 15°C but not at temperatures higher than 30°C, while strain NSS grew at 42°C but not at temperatures lower than 30°C (see Fig. S2).

Metabolic diversity. Strains CUZ and NSS utilized a wide variety of electron donors and electron acceptors (Table 1). Both strains oxidized a variety of organic acids and inorganic electron donors, such as hydrogen and sulfide (Table 1). Although sulfide was oxidized, neither strain could grow by this metabolism (data not shown). Both strains respired chlorate, nitrate, and oxygen as terminal electron acceptors, but only strain CUZ respired perchlorate. In comparison, *S. selenatireducens* AK4OH1 utilized nitrate and selenate as electron acceptors, but not (per)chlorate or oxygen (25). Neither strain CUZ nor strain NSS utilized selenate. When provided with acetate (20 mM) as an electron donor and both perchlorate and nitrate (5 mM each) or both chlorate and nitrate (5 mM each) as electron acceptors, strain CUZ utilized (per)chlorate preferentially to nitrate if the inoculum was pregrown on (per)chlorate (Fig. 4A and C). However, if the inoculum was pregrown on nitrate, (per)chlorate and nitrate were reduced concomitantly (Fig. 4B and D). Similarly, when strain NSS was provided with acetate (20 mM) as an electron donor and with both chlorate and nitrate (5 mM each) as electron acceptors, chlorate was utilized preferentially if the inoculum was grown on chlorate (Fig. 4E). If the inoculum was grown on nitrate, simultaneous consumption of chlorate and nitrate was observed (Fig. 4F). Diauxic growth was not observed for either strain (Fig. 4).

This electron acceptor preference profile is unusual among the known PRB (11), suggesting different genetic responses under the various growth conditions. To test this, we compared the transcription of the PRI genes of strain CUZ under aerobic and anaerobic perchlorate- or nitrate-reducing conditions. Differential expression analysis of normalized counts revealed constitutive expression of the *pcrABCD* and *cld* genes (see Fig. S4 in the sup-

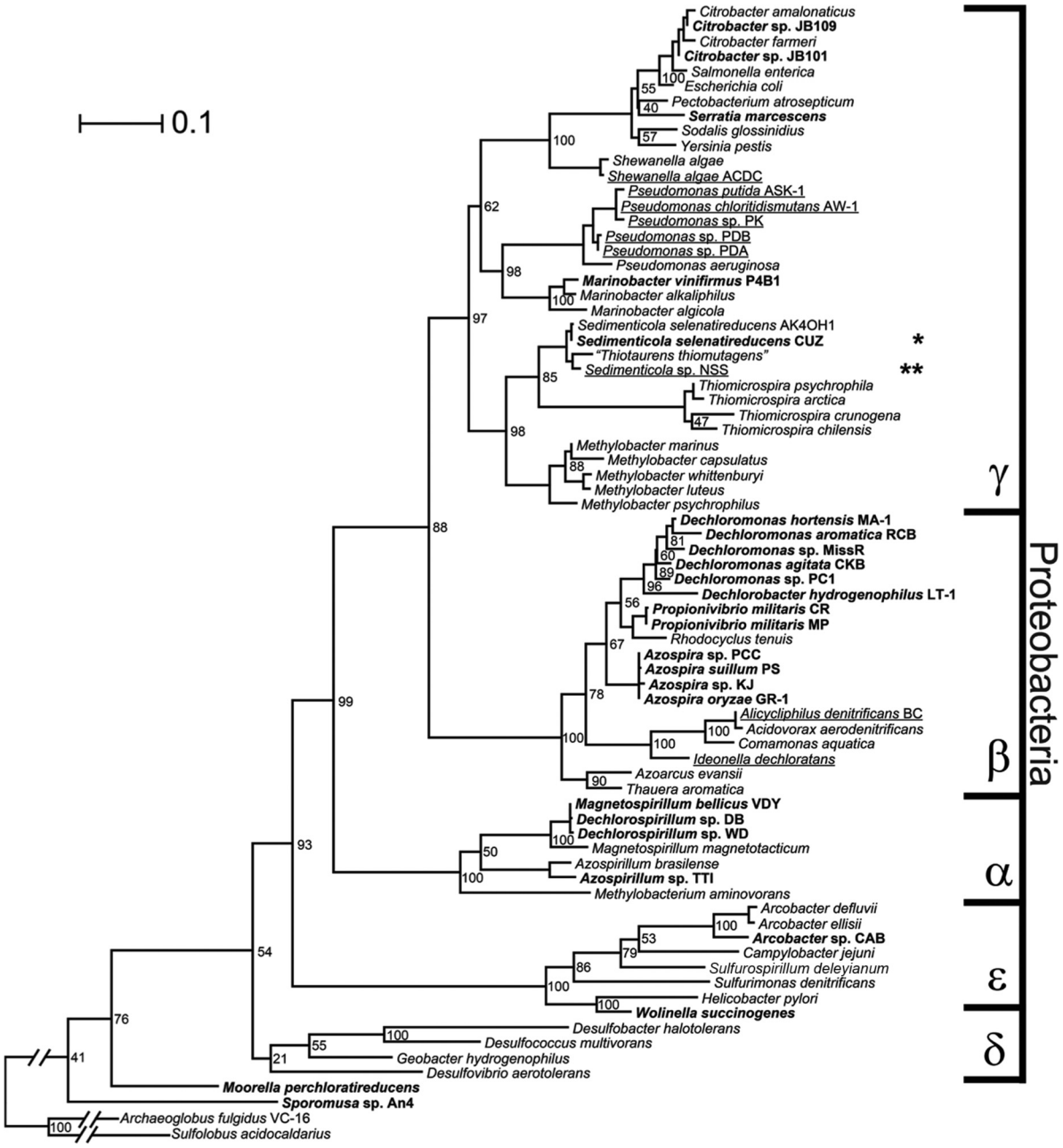

FIG 1 Maximum likelihood tree based on 16S rRNA sequences showing the phylogenetic positions of *S. selenatireducens* CUZ (*) and *Sedimenticola* sp. strain NSS (**), both members of the *Gammaproteobacteria*. PRB are shown in boldface, and CRB are underlined. Bootstrap values are based on 1,000 replications and are shown at the nodes of the tree. Bar, 0.1 expected change per site.

plemental material). This is contrast to the close regulation of PRI expression by the canonical PRB *Azospira suillum* PS and other organisms tested (32).

The genomes of both strains encoded the entire nitrate reduction pathways, including the membrane-bound nitrate reductase Nar (encoded by *narGHJI* [CUZ_01713 to CUZ_01709; NSS_00031830 to NSS_00031860]) and the periplasmic nitrate reductase Nap (encoded by *napFDAGHBC* [CUZ_03010 to CUZ_03004; NSS_00008720 to NSS_00008660]). Also present were genes encoding the nitrite reductase Nir (*nirB* and *nirD* [CUZ_03993 and CUZ_03994; NSS_00004760 and NSS_00004750]), the nitric oxide reductase Nor (*norE* [CUZ_03147; NSS_00018480], *norQ* [CUZ_03149; NSS_00018460], and *norD* [CUZ_03151; NSS_00018440]), and the nitrous oxide reductase Nos (*nosZ* [CUZ_01219; NSS_00015960] and *nosDGHFL* [CUZ_01223 to CUZ_01227; NSS_00015920 to NSS_00015880]).

Both strains degraded aromatic compounds such as benzoate and phenylacetate coupled to the reduction of (per)chlorate, nitrate, and oxygen (Table 1 and data not shown). The genomes of the two strains contained genes for both an anaerobic pathway of benzoate degradation similar to that of *Thauera aromatica* (33, 34)

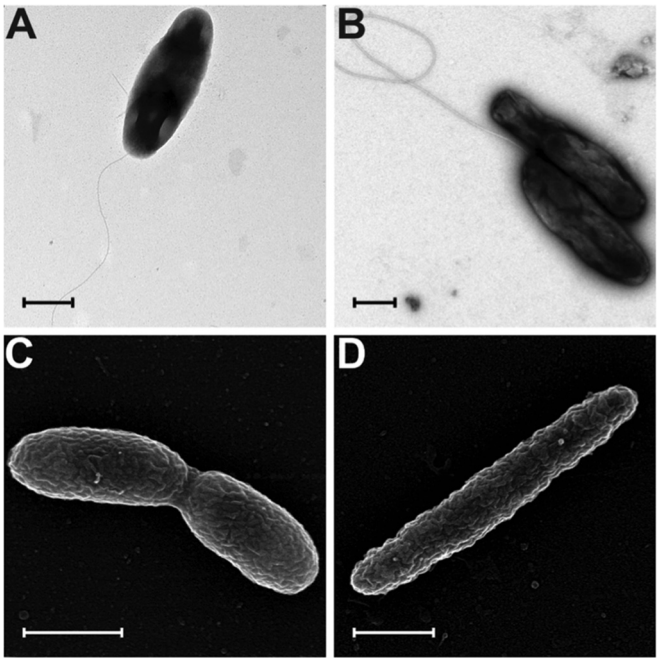

FIG 2 (A and B) Transmission electron microscopy of strains CUZ (A) and NSS (B); (C and D) scanning electron microscopy of strains CUZ (C) and NSS (D). Bars, 0.5 μm.

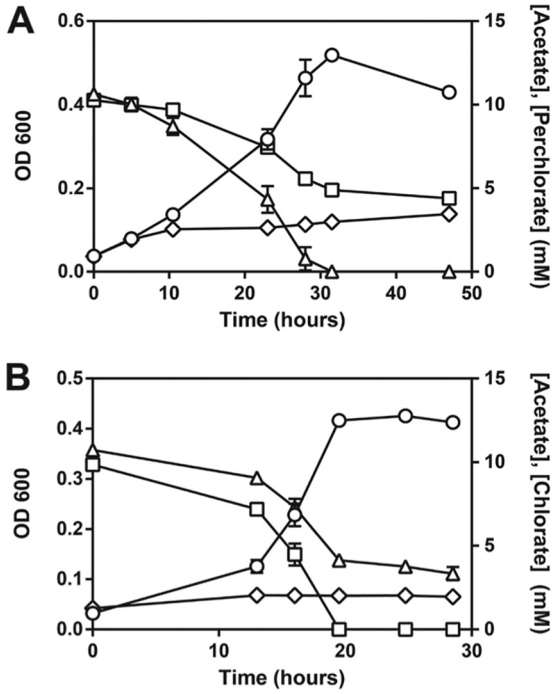

FIG 3 Growth curves of strain CUZ on acetate and perchlorate (A) and of strain NSS on acetate and chlorate (B). Cell density was monitored by OD_{600}. Circles, cell density; triangles, acetate concentration; squares, perchlorate or chlorate concentration; diamonds, cell density for the no-(per)chlorate control. Error bars represent standard deviations of the averages for triplicate samples.

and an aerobic hybrid, epoxide-mediated pathway of benzoate degradation similar to the pathway studied in *Azoarcus evansii* (35, 36). The anaerobic pathway included genes encoding a benzoyl coenzyme A (CoA) reductase (*bcrCBAD* [CUZ_03345 to CUZ_03342; NSS_00026600 to NSS_00026570]), an enoyl-CoA hydratase (*dch* [CUZ_03346; NSS_00026610]), an oxoacyl-CoA hydrolase (*oah* [CUZ_03347; NSS_00026620]), and a hydroxyacyl-CoA dehydrogenase (*had* [CUZ_03348; NSS_00026630]). The aerobic hybrid pathway encoded the beta subunit of the benzoyl-CoA oxygenase (*boxB* [CUZ_03988; NSS_00004800]) as well as the benzoyl-CoA dihydrodiol lyase (*boxC* [CUZ_03987; NSS_00004810]). The benzoate degradation pathways appear to share a benzoate-CoA ligase (*bclA* [CUZ_01337; NSS_00014960]), as reported previously for *T. aromatica* and *Magnetospirillum* sp. strain TS-6 (33). Although BLAST (37) searches for other genes involved in the aerobic hybrid pathway of benzoate degradation yielded several hits for *boxA*, *boxD*, and *boxE* separately from the *boxBC* cluster, it is unclear which, if any, of these genes could be involved (data not shown).

In addition to benzoate, the genomes of CUZ and NSS also included genes encoding both an anaerobic pathway and an aerobic hybrid pathway of phenylacetate oxidation (33). The anaerobic cluster shows similarity to clusters studied in *Rhodopseudomonas palustris* and *Azoarcus* spp. (33) and included genes for the phenylacetate-CoA ligase (*padJ* [CUZ_03796; NSS_00026760]), the phenylacetyl-CoA:acceptor oxidoreductase (*padBCD* [CUZ_03788 to CUZ_03790]; *padBCDE* [NSS_00026680 to NSS_00026710]), and the phenylglyoxylate:NAD^+ oxidoreductase (*padEFGHI* [CUZ_03791 to CUZ_03795]; *padFGHI* [NSS_00026720 to NSS_00026750]). Downstream processing of the benzoyl-CoA intermediate produced during anaerobic phenylacetate degradation would likely proceed using the anaerobic benzoate degradation pathway (*bcr*) described above. In strain NSS and *S. selenatireducens* AK4OH1, the anaerobic benzoate and phenylacetate gene clusters were next to each other, and given the high genome-wide similarities between strains CUZ and AK4OH1 (95%), as well as between strains CUZ and NSS (86%), this is likely also true for strain CUZ. Genes encoding proteins involved in the aerobic hybrid pathway of phenylacetate degradation (33, 38) were also present in the genomes of both strains, and they included the oxepin-CoA hydrolase (*paaZ*), the 2,3-dehydroadipyl-CoA hydratase (*paaF*), the 1,2-epoxyphenylacetyl-CoA isomerase (*paaG*), the 3-hydroxyadipyl-CoA dehydrogenase (*paaH*), the 3-oxoadipyl-CoA thiolase (*paaJ*), and the phenylacetate-CoA ligase (*paaK*) (CUZ_04217 to CUZ_04222; NSS_00036190 to NSS_00036230 and NSS_00036090]). Except for the *paaE* gene (CUZ_04215; NSS_00036170), which is proposed to function as an iron-sulfur oxidoreductase (38), the genes encoding the key oxygenase of the system, phenylacetyl-CoA epoxidase (*paaABCDE*), were missing. Nonetheless, strains CUZ and NSS degraded both benzoate and phenylacetate aerobically and anaerobically, and it remains to be elucidated which of these pathways or combinations of pathways are active under anaerobic, aerobic, or microaerophilic conditions.

Draft genome and (per)chlorate and chlorate reduction genes. The draft genome of strain CUZ was 4.5 Mb and contained 86 contigs ranging from 204 bp to 543 kb. The average contig length was 53 kb, and the GC content was 56.22%. The genome of strain CUZ revealed the presence of a perchlorate reduction island (PRI) that resembled PRIs described previously for other PRB, although it differed from most by the presence of transposases and

TABLE 1 Overview of electron donor and acceptor utilization by strain NSS, strain CUZ, and *S. selenatireducens* AK4OH1[a]

Compound	Concn	Utilization[b] by:		
		Sedimenticola sp. strain NSS	*S. selenatireducens* CUZ	*S. selenatireducens* AK4OH1
Electron donors				
Acetate	10 mM	Y	Y	Y
Benzoate	1 mM	Y	Y	Y
Butyrate	10 mM	Y	Y	ND
Catechol	1 mM	N	N	ND
Citrate	10 mM	N	N	ND
Ethanol	5 mM	N	N	ND
Fructose	10 mM	N	N	ND
Fumarate	10 mM	Y	Y	ND
Glucose	10 mM	N	N	ND
Hydrogen	50 kPa[c]	Y	Y	ND
Isobutyrate	10 mM	Y	Y	ND
Lactate	10 mM	Y	Y	Y
Malate	10 mM	Y	Y	ND
Methane	25 kPa	N	N	ND
Methanol	5 mM	N	N	ND
Phenylacetate	2 mM	Y	Y	ND
Protocatechuate	1 mM	N	Y	ND
Propionate	10 mM	Y	Y	ND
Succinate	1 mM	Y	Y	ND
Sulfide[d]	5 mM	N	N	ND
Valerate	10 mM	Y	Y	ND
Yeast extract	1 g/liter	Y	Y	ND
Electron acceptors				
Arsenate	2.5 mM	N	N	N
Chlorate	10 mM	Y	Y	N
Malate	10 mM	N	N	ND
Nitrate	10 mM	Y	Y	Y
Nitrite	2 mM	N	Y	Y
Oxygen	25 kPa	Y	Y	N
Perchlorate	10 mM	N	Y	N
Selenate	5 mM	N	N	Y
Sulfate	10 mM	N	N	N
Sulfite	2 mM	N	N	ND
Thiosulfate	10 mM	N	N	ND

[a] Electron donors were tested with chlorate or perchlorate as the electron acceptor for strain NSS or CUZ, respectively. Electron acceptors were tested with acetate as the electron donor.
[b] Data for *S. selenatireducens* AK4OH1 were adapted from reference 25. Y, yes; N, no; ND, not determined.
[c] Samples were supplemented with 0.1 g/liter yeast extract.
[d] Although sulfide was oxidized, no growth was observed.

the lack of some of the accessory and regulatory genes that often flank the core Pcr and Cld genes (39). The genes in the CUZ PRI included the previously described genes encoding perchlorate reductase (*pcrABCD* [CUZ_04203 to CUZ_04206]), chlorite dismutase (*cld* [CUZ_04201]), a two-component system composed of a response regulator (*pcrR* [CUZ_04209]) and a histidine kinase sensor (*pcrS* [CUZ_04210]), a PAS domain-containing protein (*pcrP* [CUZ_04211]), and quinol dehydrogenase tetraheme cytochrome *c* (CUZ_04202) (Fig. 5A) (39). However, the *pcrR*, *pcrS*, *pcrP*, and quinol dehydrogenase tetraheme cytochrome *c* genes of strain CUZ were only distantly related to those of the well-studied PRB *A. suillum* PS, and due to their polyphyletic origin (39), it is not known whether or not they are essential for (per)chlorate reduction in strain CUZ (Fig. 5A and data not shown). Notably, the PRI of strain CUZ lacked the conserved promoter for *pcrA* that contains consensus binding sites for the sigma factor RpoN, which is essential for perchlorate reduction in *A. suillum* PS (32). Unlike those of most PRB, the PRI of strain CUZ contained several transposases (Fig. 5A), and as such, its architecture resembled those of the recently published PRB *Arcobacter* sp. strain CAB (18) and other chlorate reducers (29). No genes encoding a separate chlorate reductase were found.

The 3.86-Mb genome of strain NSS contained 49 contigs ranging from 207 bp to 715 kb, with an average length of 79 kb. The GC content was 58.75%. The genome of strain NSS encoded chlorate reduction genes flanked by insertion sequences, forming composite transposons. Composite chlorate reduction transposons have recently been shown to be a common property of CRB and represent mobile genetic units by which chlorate metabolism may be transferred (29). The genome of strain NSS carried the genes necessary for chlorate reduction: those encoding the chlorate reduc-

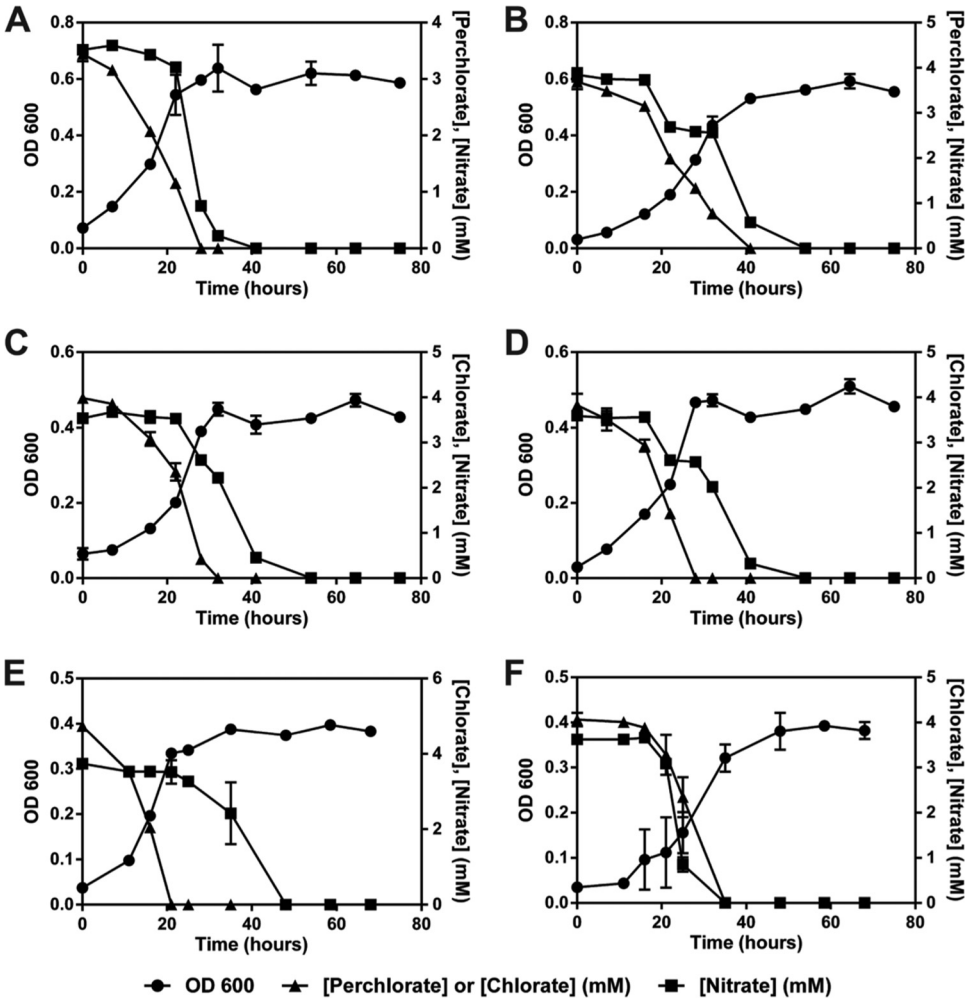

FIG 4 Growth curves of strains CUZ (A to D) and NSS (E and F) in diauxic media containing both nitrate and (per)chlorate. The inoculum was grown with either perchlorate (A), chlorate (C and E), or nitrate (B, D, and F) as the sole electron acceptor. Cell density was monitored by the OD_{600}. ●, cell density; ▲, perchlorate or chlorate concentration; ■, nitrate concentration. Error bars represent standard deviations of the averages for triplicate samples.

tase Clr (*clrABCD* [NSS_00036460 to NSS_00036490]) and the chlorite dismutase Cld (*cld* [NSS_00036440]) (Fig. 5B).

DISCUSSION

The characterization of strains CUZ and NSS contributes to the known physiological diversity of PRB and CRB. To date, only two halophilic marine bacteria that can respire perchlorate or chlorate have been described (18, 22). This study investigated the physiology and phylogeny of two additional halophilic marine (per)chlorate reducers belonging to the class *Gammaproteobacteria*. The high 16S rRNA gene sequence similarity (98%) between the two isolates indicated that they are both members of a single genus, *Sedimenticola*, not previously known for the ability to reduce (per)chlorate. Unexpectedly, these strains also represent the only

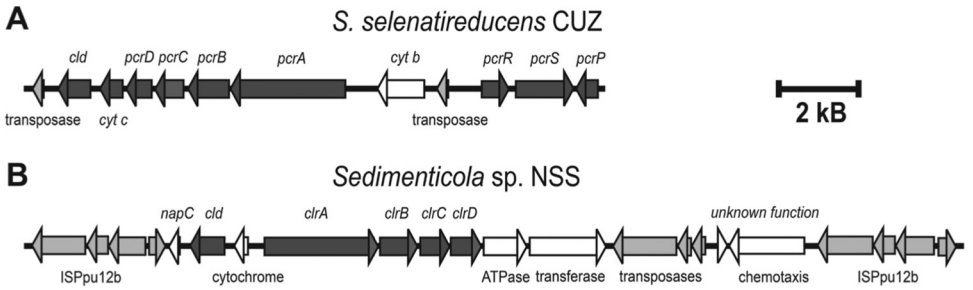

FIG 5 Comparison of genes involved in (per)chlorate reduction for strain CUZ (A) and chlorate reduction for strain NSS (B). Light shaded arrows, transposases and composite transposons; dark shaded arrows, core genes required for (per)chlorate (A) and chlorate (B) reduction.

example of two strains from a single genus of which one is a PRB and the other a CRB. As such, together with the *Sedimenticola* type strain, these organisms provide a unique opportunity to delineate the similarities and differences of the underlying genetics and biochemistry of these metabolic capacities and to distinguish them from the underlying core metabolic capacity of the host organism.

Strain CUZ reduced (per)chlorate, whereas strain NSS reduced only chlorate. This phenotypic disparity is probably due to the specificity of the Pcr and Clr enzymes, respectively. The genome of strain CUZ did not contain a Clr. Similarly, strain NSS did not contain a Pcr. Although both Clr and Pcr belong to the dimethyl sulfoxide reductase family of molybdenum-containing proteins, they belong to distinct clades. ClrA is polyphyletic (29), whereas to the best of our knowledge, PcrA is monophyletic (unpublished results). Given the high 16S rRNA gene sequence similarity between these strains, it is somewhat surprising that they have distinct approaches to chlorate metabolism. However, both the (per)chlorate reduction gene complex (i.e., the PRI) and the chlorate reduction gene complex are associated with transposases (29, 39), supporting mounting evidence that these complexes are transferred horizontally (11). Recent horizontal gene transfer may explain why these closely related species nonetheless have distinct approaches to (per)chlorate metabolism.

Phylogenetically, strains CUZ and NSS are highly related to the selenate-reducing bacterium *S. selenatireducens* AK4OH1 (100% and 98% 16S rRNA gene sequence similarity, respectively), which was isolated from estuarine sediment near Staten Island, NY-NJ (25). Strains AK4OH1, CUZ, and NSS are all halophilic bacteria isolated from marine environments, but they differ metabolically. Strain AK4OH1 reduced selenate but not (per)chlorate. In contrast, strains CUZ and NSS reduced (per)chlorate and chlorate, respectively, but neither reduced selenate (Table 1). The inability of strain AK4OH1 to reduce (per)chlorate is particularly intriguing given the presence in its genome of a PRI similar to that observed in CUZ (see Table S2 in the supplemental material) (Joint Genome Institute, IMG). Overall, 10% of the *pcrA* gene differs between strains CUZ and AK4OH1, whereas the intergenic region upstream of *pcrA* differs only by one single nucleotide polymorphism (SNP), suggesting that the *pcrA* gene is rapidly evolving or adapting to selective pressure. The *pcrA* gene of strain CUZ differs from that of strain AK4OH1 by a C-terminal tail of 14 amino acids (aa) (see Table S2 in the supplemental material). Interestingly, this tail is unique to strain CUZ and a related PRB belonging to the class *Epsilonproteobacteria*, *Arcobacter* sp. strain CAB (18), which was isolated from the same location and is the only other marine PRB isolated to date. The role, if any, of the PcrA 14-aa tail modification remains to be understood. Further studies involving genetics and evolution will aid in determining what changes in the PRI are necessary to confer the ability to reduce (per)chlorate.

Other close relatives of strains CUZ and NSS include the thiotaurine-degrading bacterium *Thiotaurens thiomutagens* (30) and unnamed, poorly studied sulfur-oxidizing marine endosymbionts (Fig. 1 and data not shown). These endosymbionts are thought to grow lithoautotrophically by utilizing sulfide (40). Strains NSS and CUZ both oxidized sulfide but did not grow by this metabolism. It is not known whether strains NSS and CUZ can utilize thiotaurine or whether they can form symbiotic relationships with tubeworms or clams. Because so few isolates of this poorly understood clade of the *Gammaproteobacteria* have been obtained, the isolation and characterization of two novel strains greatly contributes to the known physiology of microorganisms in the group.

The genomes of strains CUZ and NSS included genes for (per)chlorate and chlorate reduction, respectively. In particular, the PRI of strain CUZ adds to the known diversity of the genetics of (per)chlorate reduction. The tail modification of the PcrA enzyme in strain CUZ, which is also present in *Arcobacter* sp. strain CAB, could indicate some adaptive advantage, given that the organisms were isolated from nearly identical environments. Previous work showed that an RpoN-binding motif upstream of *pcrA* was essential for regulating (per)chlorate reduction in *Azospira suillum* PS (32), and such a motif is assumed to have a regulatory role in other (per)chlorate reducers when it is present. Notably, the PRI of strain CUZ lacks an RpoN-binding motif upstream of *pcrA*, suggesting that strain CUZ utilizes a different regulatory circuit. In support of this possibility, transcriptomic data indicated that expression of the PRI in strain CUZ is constitutive (see Fig. S4 in the supplemental material). The genome of strain NSS included chlorate-reducing genes that were 99.9% identical (on the nucleotide level) to those of the chlorate-reducing gammaproteobacterium *Shewanella algae* ACDC, which was isolated from the same environment as strain NSS (29). As reported previously, the presence of composite chlorate reduction transposons in NSS suggests that this metabolism may be transferred horizontally (29).

Most PRB are capable of reducing nitrate, whereas this metabolism is rare for CRB (11, 41–44). In most cases, a separate nitrate reductase exists for this metabolism, although not all strains can couple nitrate reduction to growth, nor can all strains reduce nitrate completely to nitrogen gas (31, 45). The genomes of strains CUZ and NSS encode separate nitrate reduction pathways, and both are also able to grow by nitrate reduction. These strains preferentially utilize (per)chlorate and chlorate, respectively, before nitrate if the inoculum has been grown on (per)chlorate or chlorate. However, (per)chlorate and nitrate are reduced concomitantly if the inoculum has been grown with nitrate as an electron acceptor. In contrast, other PRB preferentially utilize nitrate or utilize both electron acceptors simultaneously. This is unexpected, because nitrate reduction is thermodynamically slightly less favorable than (per)chlorate reduction (redox potential at pH 7 [$E^{o\prime}$], +746 mV for the couple of NO_3^- and N_2, compared to +797 mV for the biological couple of ClO_4^- and Cl^- and +792 mV for ClO_3^- and and Cl^-) and, to the best of our knowledge, is the only example of a less favorable electron acceptor being preferentially utilized. The PRB *Azospira suillum* PS and *Dechloromonas aromatica* RCB utilize perchlorate only after the complete removal of nitrate (44, 46). *Dechloromonas agitata* CKB, *Marinobacter vinifirmus* P4B1, and strain perc1ace reduced nitrate and perchlorate concomitantly regardless of whether the inoculum was grown on perchlorate or nitrate (41, 44, 47). Further, mixed-culture bed reactors have consistently demonstrated preferential reduction of nitrate over perchlorate in cocontaminated sludge (13, 23). Unlike PRB, most CRB cannot grow by nitrate reduction; however, *Alicycliphilus denitrificans* BC, the one other CRB that can reduce nitrate, differs from strain NSS in that it preferentially utilizes the electron acceptor with which the inoculum was grown (43).

The genomes of both CUZ and NSS contain separate genes encoding the nitrate reductases Nap and Nar, as well as Pcr or Clr, respectively; thus, preferential (per)chlorate or chlorate reduction

cannot be attributed to competitive inhibition where nitrate and (per)chlorate compete for a single binding site. However, it has been shown that Pcr enzymes reduce nitrate, and Nar has been shown to reduce (per)chlorate (15, 16, 24). Preferential utilization of (per)chlorate over nitrate may be genetically controlled via mechanisms that remain to be elucidated. The alternative sigma factor RpoN has been shown to be necessary for perchlorate reduction in *Azospira suillum* strain PS (32), where the promoter region upstream of the *pcrA* gene contains an RpoN-binding motif and PcrR contains an RpoN-interacting domain (32). As noted above, the PRI of strain CUZ lacks the RpoN-binding motif upstream of *pcrA*, and its PcrR lacks an RpoN-interacting domain, suggesting that strain CUZ possesses a regulatory mechanism distinct from that of *A. suillum* PS. Constitutive expression of the PRI of strain CUZ could explain the sequential removal of perchlorate and nitrate when the inoculum was pregrown with perchlorate and the simultaneous removal of perchlorate and nitrate when the inoculum was pregrown with nitrate. A more in-depth comparative analysis of nitrate and (per)chlorate respiration in the diverse PRB and CRB is warranted.

Both strains CUZ and NSS encode genes for aromatic degradation pathways, suggesting further bioremediative applications for these strains. Aromatic compounds are the most abundant class of organic compounds after carbohydrates and are prominent environmental contaminants. Therefore, it is important to understand how these compounds are degraded in nature, especially in anoxic environments (33). Strains CUZ and NSS contain genes involved in the oxidation of benzoate and phenylacetate, both of which are key intermediates in general degradation pathways (33, 35).

Strains CUZ and NSS contain known pathways for both the anaerobic and the epoxide-mediated, aerobic hybrid pathways of benzoate and phenylacetate degradation (33, 35, 36, 38), demonstrating great versatility regardless of electron-accepting conditions. In anoxic environments, PRB and CRB can carry out the aerobic oxidation of aromatic compounds coupled to the reduction of (per)chlorate by utilizing oxygen derived from ClO_2^- dismutation (12, 18, 48), but it is not known whether a fully anaerobic pathway that does not rely on internal oxygen generation can be coupled with (per)chlorate as an electron acceptor. Since strains CUZ and NSS appear to be capable of both anaerobic and microaerophilic oxidation of benzoate and phenylacetate, characterization of this metabolism may help elucidate the regulation of these pathways in response to various environmental conditions. Because the aerobic hybrid pathway is hypothesized to be important in low or fluctuating oxygen concentrations (35), it is conceivable that in anoxic environments, this pathway could be coupled to perchlorate reduction, utilizing the oxygen produced by ClO_2^- dismutation. However, anaerobic pathways for benzoate or phenylacetate degradation could also play an important role, as long as the concentration of oxygen produced by (per)chlorate reduction did not reach levels toxic to the benzoyl-CoA reductase or the phenylglyoxylate oxidoreductase, respectively (49, 50). Due to the presence of genes encoding both anaerobic and aerobic hybrid pathways, strains CUZ and NSS may represent ideal candidates for the study of pathways of aromatic oxidation. (Per)chlorate reduction, and the accompanying low levels of internally generated oxygen, may help reveal how the combination of anaerobic and aerobic hybrid pathways is regulated and whether (per)chlorate reduction can couple effectively to anaerobic pathways.

This study described two closely related halophilic marine gammaproteobacteria capable of metabolizing (per)chlorate. Strains CUZ and NSS share 98% 16S rRNA gene sequence identity, yet one is a PRB and the other a CRB, and each adds to the known physiological and phylogenetic diversity of (per)chlorate reduction. The preferential utilization of (per)chlorate or chlorate over nitrate with (per)chlorate-grown inocula is surprising and suggests a regulatory approach different from that seen in other PRB and CRB. This preference may aid in bioremediation efforts, which have historically been hindered by preferential nitrate reduction in cocontaminated environments (13, 23). Both strains encode both anaerobic and aerobic hybrid pathways for aromatic degradation, providing a unique opportunity for studying the regulation and activity of these pathways coupled to (per)chlorate reduction.

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