CHAPTER 18. ISOLATION AND CHARACTERIZATION OF TWO NOVEL (PER)CHLORATE-REDUCING BACTERIA FROM SWINE WASTE LAGOONS[⊗]

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INTRODUCTION

Microbial chlorate (ClO_3^-) and perchlorate (ClO_4^-) reduction has recently been recognized as an important form of microbial metabolism for the removal of chlorine oxyanion contamination in the environment.[1] Chlorine oxyanions have many industrial applications including use as bleaching agents by the paper and pulp industry,[2,3] as disinfectants and defoliants by the agricultural industry,[4] and as components of explosives and rocket propellants by the aerospace and defense industries.[5] Chlorates can also be formed as a result of ozonation of drinking waters which have been treated with chlorine[6] or photodecomposition of chlorite or chlorine dioxide[7] which are used in addition to chlorine for water disinfection.

Perchlorate has been shown to affect iodide accumulation in the thyroid gland[8] and at concentrations greater than 6 mg kg^{-1} body weight.day^{-1} perchlorate can cause fatal bone marrow disease.[1] In addition, acute haemolysis in animals[9,10] and enzyme damage to human erythrocytes[11] has been associated with both chlorate and chlorite (ClO_2^-). These pollutants also have an effect on aquatic plants and invertebrates,[3,12,13] and are assumed to be the cause of the disappearance of the brown algae, *Fucus vesiculosus,* from the Baltic sea.[14]

It was originally assumed that (per)chlorate reduction in the environment was the result of the activity of nitrate-respirers which coincidentally used chlorine oxyanions in place of nitrate. In support of this, many nitrate respiring bacteria have been shown to be capable of the reduction of chlorate to chlorite, including *Proteus mirabilis*,[15] *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*,[16] however growth was not associated with this metabolism and the chlorite formed as the endproduct was toxic to these organisms.

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The first organism which was shown to couple growth to the reduction of chlorate or perchlorate was recovered as part of an investigation into the biological attenuation of (per)chlorate contamination in the environment.[17] Chloride was produced as the innocuous endproduct of this reductive metabolism. This organism was putatively identified as a *Vibrio* species based on physiology and morphology and was named *Vibrio dechloraticans*. In the last decade, four more dissimilatory (per)chlorate-reducing strains including *Ideonella dechloratans*, *Wolinella succinogenes* strain HAP-1, *Acinetobacter thermotoleranticus*, and strain GR-1 have been isolated from contaminated sediments or wastewater treatment sludge.[18-21] Of these (per)chlorate-reducing isolates, only two, *Ideonella dechloratans* and *Wolinella succinogenes* strain HAP-1, have been characterized both phenotypically and genotypically.[18,19] More recently *Dechlorimonas agitatus* strain CKB was isolated from a paper mill waste sludge as part of a study on the bioremediative potential of (per)chlorate-reducing bacteria.[22-28] Similarly to most of the other known (per)chlorate-reducing isolates, this organism was a facultative anaerobe which coupled chlorate or perchlorate reduction to the oxidation of simple organic acids or alcohols.[22,23] In contrast to the other known isolates, this organism could not grow by nitrate reduction. This further supports the assumption that chlorate reduction and nitrate reduction are two unrelated pathways as suggested recently [18,21] and is in contrast to the suggestions of earlier studies.[29-31]

As part of a study of the ubiquity and diversity of organisms involved in microbial (per)chlorate reduction[27,28,32] we isolated several novel (per)chlorate-reducing organisms from a broad diversity of environments. Here we report on the phenotypic and genotypic characteristics of two of these isolates, *Dechlorisoma suillus* strain PS and *Dechlorospirillum anomalous* strain WD. Both of these organisms were isolated from samples collected from animal waste lagoons on the Southern Illinois University campus Agricultural Research Center.

EXPERIMENTAL

Source of Organisms and Sediments

Dechlorisoma suillus strain PS and *Dechlorospirillum anomolous* strain WD were both isolated from sediment samples freshly collected from the swine waste primary lagoons on the Southern Illinois University campus Agricultural Research Center. Samples were collected in small glass jars (100 mL) which were filled to capacity to reduce exposure to atmospheric O_2 to a minimum. Samples were immediately transported back to the laboratory where they were used within two hours. Enrichment cultures were established by inoculating 1.0 g sediment subsamples into 9 mL of anoxic, defined freshwater medium described below with acetate (10 mM) as the sole electron donor and chlorate (10 mM) as the sole electron acceptor.

Dechlorimonas agitatus was previously isolated in our laboratory[22] and stored as a frozen stock culture. *Rhodocyclus tenuis* was kindly provided from the laboratory culture collection of Dr. Michael T. Madigan. *R. tenuis* was grown phototrophically on a modification of the RCVB medium as described previously.[33] *Duganella zoogloeoides* was purchased from the American Type Culture Collection, Manassas, VA, ATCC# 19544 and was grown aerobically on the recommended yeast-peptone medium (ATCC#1858), unless otherwise noted. The two magnetotactic bacteria, *Magnetospirillum magnetotacticum* strain MS-1, and *Magnetospirillum* species strain AMB-1 were provided by the laboratory of Dr. Dennis Bazylinski. Both strains were grown under microaerobic conditions (1 kPa O_2) in basal media outlined below with acetate as the electron donor and nitrate as the electron acceptor unless otherwise noted.

Media and Culturing Techniques

Standard anaerobic culturing techniques were used throughout[34] unless otherwise specified. Anoxic medium was prepared by boiling under N_2/CO_2 (80/20, vol/vol) to remove dissolved O_2 and dispensed under N_2/CO_2 into anaerobic pressure tubes or serum bottles which were then capped with thick butyl stoppers. The freshwater medium contained (in grams per liter): NH_4Cl (0.25); $NaClO_3$ (1.03); CH_3COONa (1.36); NaH_2PO_4 (0.60); KCl (0.1); $NaHCO_3$ (2.5). Vitamins and trace metals were added (10 mL L^{-1} respectively) from stock solutions. The vitamin stock contained (mg/L): biotin (2), folic acid (2), pyridoxine HCl (10), riboflavin (5), thiamin (5), nicotinic acid (5), pantothenic acid (5), vitamin B_{12} (0.1), *p*-aminobenzoic acid (5), thioctic acid (5). The trace metal stock contained (gL^{-1}): nitrilotriacetic acid (1.5), $MgSO_4$ (3.0), $MnSO_4 \bullet H_2O$ (0.5), NaCl (1.0), $FeSO_4 \bullet 7H_2O$ (0.1), $CaCl_2 \bullet 2H_2O$ (0.1), $CoCl_2 \bullet 6H_2O$ (0.1), ZnCl (0.13), $CuSO_4$ (0.01), $AlK(SO_4)_2 \bullet 12H_2O$ (0.01), H_3BO_2 (0.01), Na_2MoO_4 (0.025), $NiCl_2 \bullet 6H_2O$ (0.024), $Na_2WO_4 \bullet 2H_2O$ (0.025). The pH of the final medium was 6.8-7.0. Electron donors and acceptors were added to the sterile medium from sterile anoxic stocks of the sodium salts. Incubation was done at 30 °C unless otherwise stated.

Solidified media was prepared using the same media components and amending with 2% w/v noble agar (Difco) as previously outlined.[22] The bicarbonate buffer was replaced with 10 mM TES (*N-Tris*[Hydroxymethyl]methyl-2-aminoethanesulfonic acid) buffer, pH 7.0 and the agar plates were poured, streaked and incubated in an anaerobic glove bag with a gas phase of N_2/H_2 (95/5, v/v).

Cell Suspension Preparation

Cells of the various respiratory organisms used in this study were grown in 500 ml volumes anaerobically with acetate as the electron donor and chlorate or nitrate as the electron acceptor as necessary. In the case of *Rhodocyclus tenuis*, cells were grown on RCVB medium phototrophically in the presence of 10 mM sodium chlorate.

After dense growth of all cultures, cells were harvested by centrifugation. Cell pellets were washed twice and resuspended in 1 mL volumes of anoxic bicarbonate buffer (2.5 g L^{-1}) under a headspace of N_2/CO_2 or, in the case of *R. tenuis*, anoxic phosphate buffer (10 mM) under a headspace of N_2.

Cytochromes

As a preliminary investigation into the cytochrome content of *D. suillus* and *D. anomalous*, dithionite-reduced minus air-oxidized difference spectra were obtained on washed, whole-cell suspensions in anoxic bicarbonate buffer. 0.1 mL of the washed cell suspension was diluted into 9.9 mL anoxic bicarbonate buffer and 2 mL aliquots were dispensed into two 3 mL glass cuvettes. One sample was air oxidized by bubbling air through the diluted cell suspension in the cuvette for 1 minute. Crystals of sodium dithionite were added to the second sample. Reduced minus oxidized absorbance spectra were determined using a Spectronic Genesis 5 spectrophotometer over a range of 400 to 700 nm as previously described.[22,27]

Analytical techniques

Acetate concentrations were analyzed by HPLC with UV detection (Shimadzu SPD-10A, Shimadzu Scientific Instruments, Columbia, MD) using a HL-75H^+ cation exchange column (Hamilton #79476, Hamilton Company, Reno, NA). The eluent was 8 mM H_2SO_4 at a flow rate of 0.4 mL min^{-1}. Chlorate, chloride, nitrate and nitrite concentrations were analyzed by ion chromatography with conductivity detection (Shimadzu CDD-6A, Shimadzu Scientific Instruments, Columbia, MD) using a PRP-X100 anion exchange column (Hamilton #79434, Hamilton Company, Reno, NA). Elution was done at 2.0 mL min^{-1} with 4 mM *p*-hydroxybenzoic acid in 2.5% methanol at pH 8.5. Concentrations of N_2

and CO_2 in headspace samples was followed by gas chromatography (Shimadzu GC-8A, Shimadzu Scientific Instruments, Columbia, MD), with thermal conductivity detection. Growth of cultures on soluble electron acceptors was measured by increase in optical density at 600 nm. Molecular oxygen production from chlorite dismutation was detected by an O_2 electrode (YSI, model 5300, Yellow Springs, OH). Concentrations of HCl-extractable Fe(II) were determined colorimetrically by the ferrozine assay.

16S rRNA Gene Sequencing and Phylogenetic Analysis

Cells from 1.5 mL of pure cultures of strain PS and WD were harvested and resuspended in 20 µl sterile water. Chloroform (2 µl) was added and the suspension was boiled for 15 minutes to lyse the cells. Polymerase chain reaction (PCR) was performed to amplify the 16S rDNA sequence with bacterial-specific primers (8F: 5′-AGAGTTTGATCCTGGCTCAG-3′; 1525R: 5′-AAGGAGGTGATCCAGCC-3′). Reagents consisted of 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.2 mM $MgCl_2$, 0.2 mM each dNTP, 75 ng of each primer, 0.5 µlTaq polymerase (Gibco/BRL), and 1 µlof lysed cells in a 50 µlreaction. Amplification parameters were: 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min, and 72 °C for 2 min with a 2-sec auto-extension. The PCR product was electrophoresed on a 1% agarose gel to ensure correct amplification. The DNA was gel-purified on DEAE membrane and extracted as previously described[35] and cycle sequenced (Thermosequenase, Amersham). Sequence entry and analysis was performed with the MacVector 6.0 sequence analysis software program for the Macintosh (Oxford Molecular). Aligned sequences of various 16S rDNAs were downloaded from the Ribosomal Database Project[36] into the computer program SeqApp.[37] Strain PS and strain WD 16S rDNA sequences were manually entered and properly aligned using secondary structure information. Parsimony and bootstrap analysis was performed on a Macintosh G3 using PAUP* 4.0.[38] Bootstrap analysis was conducted on 100 replicates using a heuristic search strategy to assess the confidence level of various clades. 16S rDNA sequences are from the following GenBank accession numbers: *Magnetospirillum gryphiswaldense* (Y10109); *Dechlorospirillum anomalous* str. *WD* (AF170352); *Magnetospirillum magnetotacticum* (M58171); *Magnetospirillum* sp. str. *AMB-1* (D17514); *Phaeospirillum fulvum* (*Rhodospirillum fulvum*, M59065); *Phaeospirillum molischianum* (*Rhodospirillum molischianum*, M59067); *Rhodocyclus tenius* (D16209); *Rhodocyclus purpureus* (M34132); *Dechlorimonas agitatus* str. *CKB* (AF047462); *Dechlorisoma suilla* str. *PS* (AF170348); *Azoarcus evansii* (X77679); *Azoarcus denitrificians* (L33689); *Azoarcus indigens* (L15531); *Thauera selenatis* (X68491); *Duganella zoogloeoides* (*Zoogloea ramigera*, X74913).

RESULTS

Enrichment and Isolation

After seven days incubation, microscopic observation revealed the presence of a variety of rod-shaped bacteria in primary enrichment cultures with acetate as the sole electron donor and chlorate as the sole electron acceptor. These were transferred into fresh, sterile anoxic medium and good growth resulted within 72 hours. After several more transfers into fresh anoxic media, samples from each enrichment culture were streaked onto anaerobic plates with chlorate and acetate as the electron acceptor and donor respectively. Growth on plates, as indicated by the presence of individual visible pink colonies of a consistent morphology, occurred within 10–15 days. Several colonies were picked and inoculated into liquid media. From these active cultures strains PS and WD were isolated.

Cell and Colony Morphology

Strains PS and WD are both gram-negative, complete-oxidizing, non-fermentative facultative anaerobes, although strain WD grows optimally under microaerophilic conditions. Cells of strain PS are rod-shaped, 0.5 μm by 1–2 μm, while strain WD is a spirillum, 0.2 μm x7 μm. Cells of both strains are motile and non-spore forming. Both organisms grow slowly on solid media either aerobically or anaerobically. When grown anaerobically with chlorate as the electron acceptor colonies appear small (0.1–0.5 mm diam.), round, smooth and pink colored. In contrast, when grown aerobically, colonies of both organisms appear white.

Phylogenetic Analysis

Comparative analysis of the 16S rDNA sequence from both strains placed strain PS in the beta subdivision of the Proteobacteria closely related to the phototrophic *Rhodocyclus* species and our previously isolated (per)chlorate-reducer, *Dechlorimonas agitatus* strain CKB (Figure 1).[22]

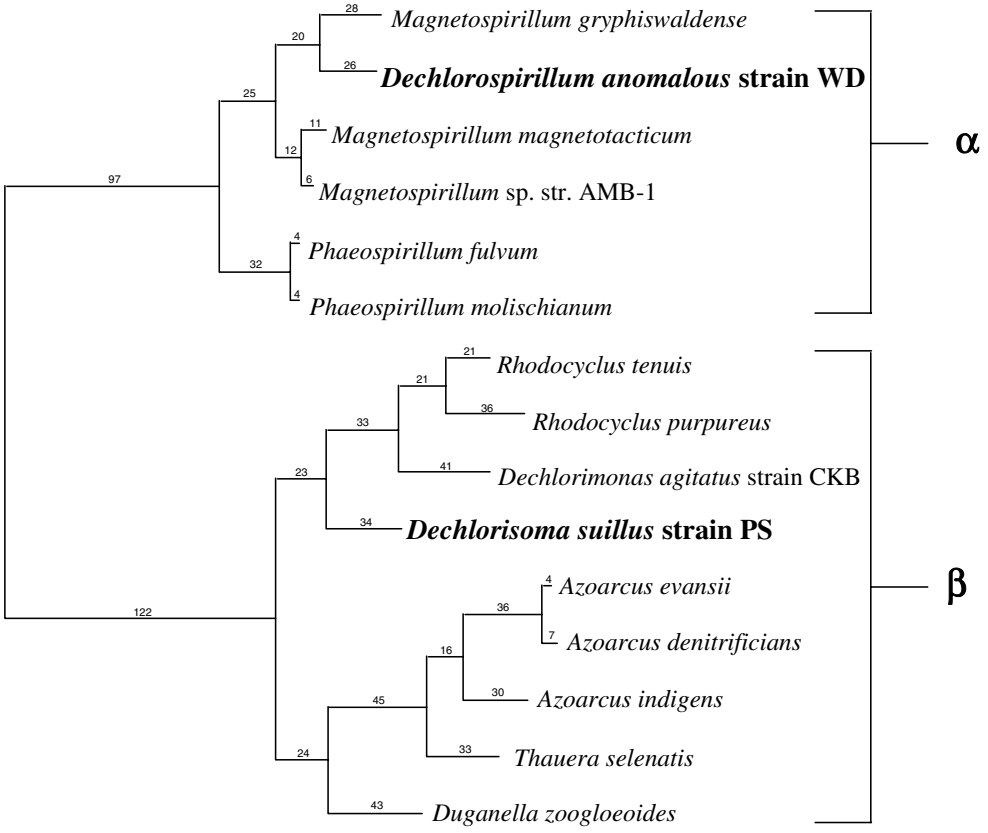

Figure 1.
Phylogenetic tree of the 16S rDNA sequences of strains WD and PS and their closest relatives resulting from a heuristic search using parsimony analysis. Branch length values are indicated. The same topology was obtained using either distance or maximum likelihood and was supported by bootstrap analysis.

Rhodocyclus tenuis is the closest relative (94.4% similarity). Although it is phylogenetically distinct (93.1% similarity), strain PS is physiologically more similar to *Dechlorimonas agitatus* strain CKB which can also grow by (per)chlorate respiration coupled to the oxidation of acetate.In contrast to strain PS, phylogenetic analysis of strain WD placed it in the alpha subdivision of the Proteobacteria, closely related to the magnetotactic *Magnetospirillum* species (Figure 1). *Magnetospirillium gryphiswaldense* is the closest relative. Similarly to strain WD, the *Magnetospirillium* species are facultative microaerophilic spirilla. However, none of the *Magnetospirillium* species tested (*M. gryphiswaldense, M. magnetotacticum* and *Magnetospirillium* strain AMB-1) could couple growth to the reduction of perchlorate or chlorate.

Optimum Growth Conditions

Both strains WD and PS grew over a broad range of environmental conditions. Strain PS grew over a temperature range of 25–42 °C and a pH range of pH 5.0–8.0. Optimum growth was observed at 35 °C and pH 7.2 respectively. Strain WD grew over a temperature range of 25–37 °C and a pH range of pH 6.5–7.5. Optimum growth for strain WD was observed at similar conditions to strain PS i.e. 35 °C and pH 7.2. Neither strain tolerated high concentrations of NaCl and concentrations above 1% were completely inhibitory. Both strains WD and PS grew preferentially in freshwater medium with a sodium chloride concentration of 0%.

Electron Donors and Acceptors

Strains PS and WD have similar metabolic capabilities and both organisms grow by the complete oxidation of acetate coupled to the reduction of chlorate or perchlorate (Fig. 2). In addition to acetate, strains PS and WD also grow on propanoate, butanoate, isobutanoate, pentanoate (valerate),ethyl alcohol, 2-oxo-propanoate (pyruvate), lactate (2-hydroxypropanoate), butanedioate (succinate), hydroxybutanedioate (malate), butenedioate (fumarate) and acid-hydrolyzed casein (casamino acids). Neither strain grew or reduced (per)chlorate with H_2 as the electron donor. Strains PS and WD also did not grow by fermentation in complex, organic rich medium in which the basal medium was amended with yeast extract (5 g L^{-1}), casamino acids (10 g L^{-1}) and glucose (1.8 g L^{-1}). The organisms grew without the addition of vitamins, however, no growth was apparent if the trace element solution was omitted from the media. A wide variety of other potential electron donors did not support growth or (per)chlorate reduction (data not shown).

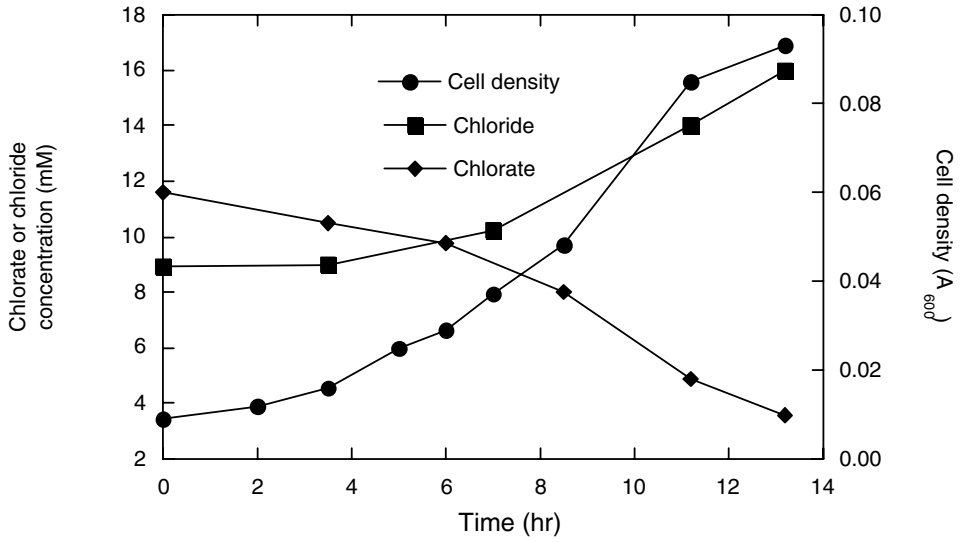

Figure 2. Cell growth and chlorate reduction by strain PS with acetate as the electron donor. Chloride and CO_2 are formed as the innocuous endproducts of this metabolism

With acetate (10 mM) as the electron donor, strain PS grew preferentially with chlorate as the electron acceptor (Figure 3). Strain PS grew in a perchlorate or chlorate concentration range of 1 to 40 mM (Figure 3). Optimum growth was observed at concentrations of 10 mM for both perchlorate and chlorate respectively.

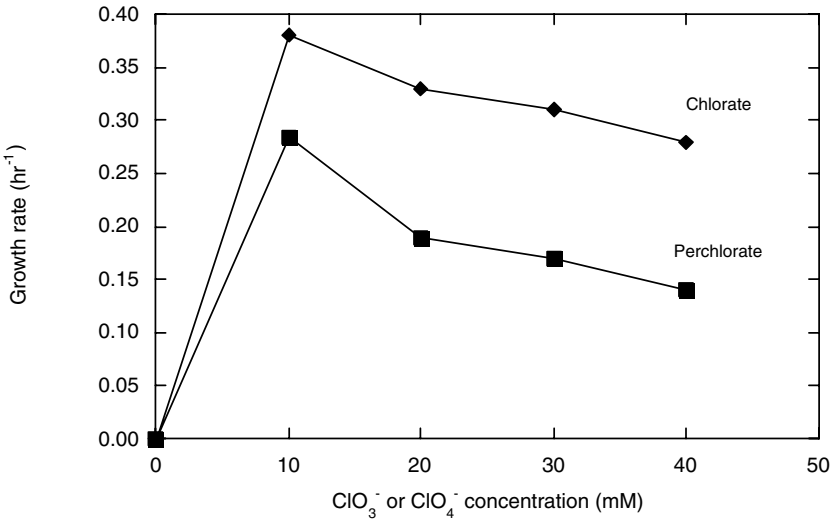

Figure 3. Growth rate of strain PS on different concentrations of chlorate or perchlorate. Chlorate is the preferentially utilized electron acceptor by this organism.

Similarly, strain WD grew preferentially with chlorate as the electron acceptor, although, the growth rate difference between chlorate and perchlorate was not as significant as observed for strain PS (Figure 4). Strain WD grew optimally in chlorate or perchlorate concentrations of 10 mM respectively. However, in contrast to strain PS, strain WD could grow and actively reduce perchlorate concentrations as high as 80 mM. For both strains perchlorate and chlorate were completely reduced to innocuous chloride. The reduction of 8.00 $\pm$ 0.64 mM (mean+standard deviation, n = 3) chlorate resulted in the production of 7.14 $\pm$ 0.71 mM (mean+standard deviation, n = 3) chloride which is in close agreement with the expected values. Chlorite was not detected in the medium during growth.

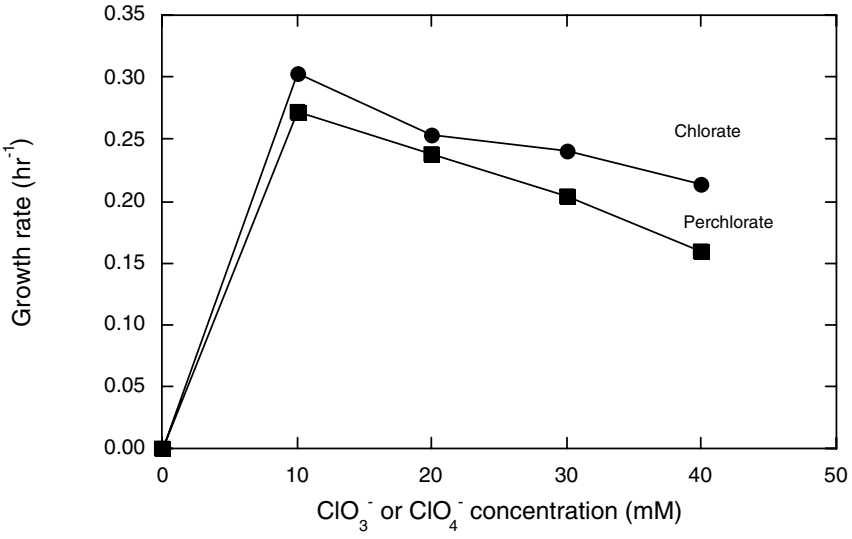

Figure 4. Growth rate of strain WD on different concentrations of chlorate or perchlorate. Although chlorate is the preferentially utilized electron acceptor by this organism, the difference in growth rate on chlorate and perchlorate is not as significant as that observed for strain PS.

In addition to chlorate and perchlorate, both strains can also utilize nitrate as an alternative electron acceptor. In the case of strain PS, N_2 gas is the major endproduct of nitrate reduction and this organism could couple the oxidation of Fe(II) to this reductive metabolism (Figure 5). The endproducts of nitrate respiration were not determined for strain WD.

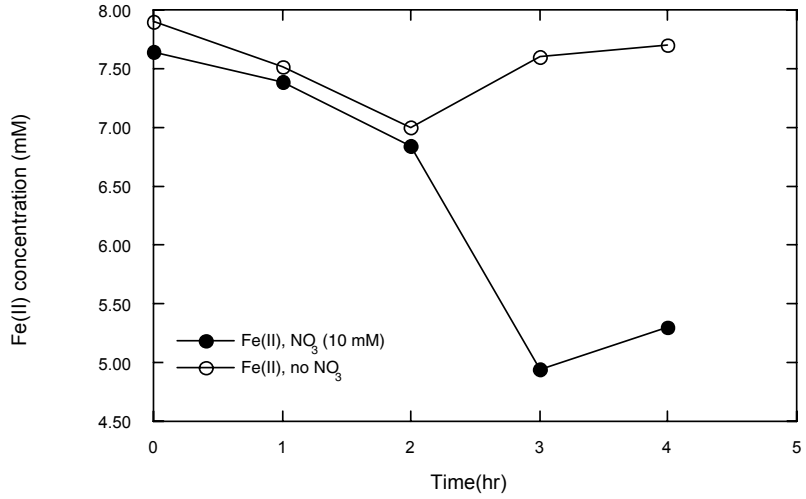

Figure 5. Oxidation of ferrous iron by strain PS coupled to the reduction of nitrate. Insoluble amorphous ferric oxyhydroxide and N_2 are the endproducts of this metabolism.

Chlorite Dismutase Activity

In the presence of chlorite, washed cell suspensions of both strains PS and WD produced O_2. Chlorite was not dismutated in controls in which the cells were omitted or which were heat-treated (Figure 6). O_2 production was rapid and proportional to the initial concentration of chlorite and O_2 production was stoichiometric with initial chlorite concentrations suggesting that chlorite was completely dismutated into Cl^- and O_2 (Figure 6).

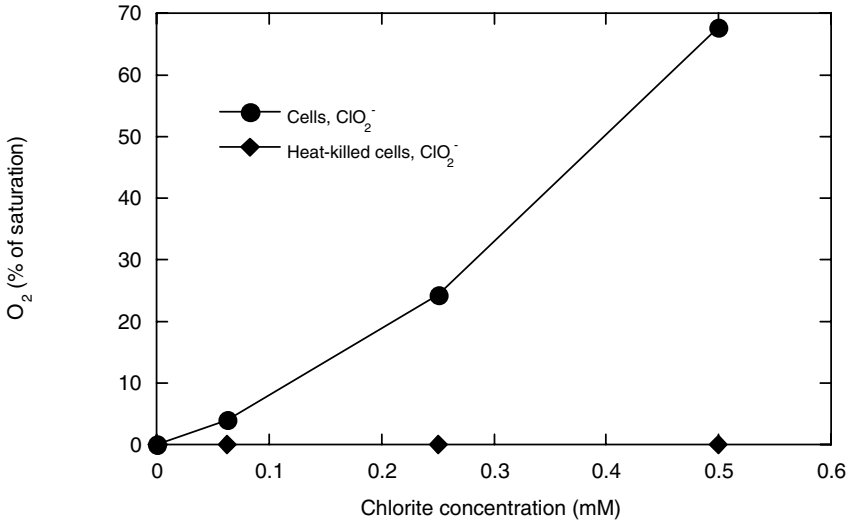

Figure 5. O_2 production from the dismutation of increasing concentrations of chlorite by washed whole cell suspensions of strain PS.

Other anions such as nitrite, hypophosphite, hydrosulfite, or arsenite were not dismutated even after 15 min incubation. If chlorite was added after 15 min., rapid O_2 production and

chlorite dismutation was observed in all cases demonstrating that these compounds were not inhibiting the chlorite dismutase enzyme (data not shown). The non-(per)chlorate-reducing, close relatives of strain PS, (*Rhodocyclus tenuis* and *Duganella zoogloeoides*) did not dismutate chlorite in similar experiments. Similarly, the close relatives of strain WD *(M. magnetotacticum, M. gryphiswaldense* and *Magnetospirillium* strain AMB-1) also did not dismutate chlorite.

DISCUSSION

Two new (per)chlorate-reducing isolates, strains PS and WD, were obtained from sediments collected from swine waste lagoons at the Southern Illinois University Agricultural Research Center. The two organisms differ from each other and from previously described ClRB and strain WD is the first example of a dissimilatory (per)chlorate-reducing organism in the alpha subclass of the Proteobacteria.

To date there are only six other dissimilatory perchlorate-reducing bacteria that have been described in the literature.[18-22,39] Interestingly, these organisms are quite diverse in their physiological characteristics.[18-22,39] Although phylogenetic comparisons can not be made with most of these isolates because the 16S rDNA sequence data is not available, the three isolates that have been genotypically characterized to date are all members of the Proteobacteria. Similarly to strain PS, two of these, *D. agitatus* strain CKB[22] and *Ideonella dechloratans*,[18] belong to the beta subclass of the Proteobacteria. In contrast, the third, *Wollinella succinogenes* strain HAP-1,[19] is a member of the epsilon subclass of the Proteobacteria. In addition, recent studies in our laboratory have resulted in the isolation of (per)chlorate-reducing bacteria that are members of the gamma subclass of the Proteobacteria.[27] These findings demonstrate that microbial (per)chlorate reduction is spread throughout the Proteobacteria and further emphasizes the diversity of microorganisms capable of this novel metabolism. This diversity is unexpected as the only known natural source of perchlorate that has been identified are mineral deposits in Chile and it is assumed that the presence of oxyanions of chlorine in the environment is due to anthropogenic intervention over the last 100 years which represents a relatively short timeframe for such a diverse group of organisms to have evolved this metabolic capability.

Strain PS shows little physiological resemblance to its closest relative, *R. tenuis. R. tenuis* is a purple, non-sulfur photosynthetic organism that cannot respire (per)chlorate or reduce (per)chlorate in cell suspension.[22] In contrast, strain PS is a strictly respiratory, heterotrophic, dissimilatory (per)chlorate-reducer that cannot grow phototrophically. In addition, *R. tenuis* cannot dismutate chlorite in cell suspension. Although *D. agitatus* strain CKB is a more distant relative than *R. tenuis* , there is much greater physiological similarity between this organism and strain PS. Both are capable of coupling the complete oxidation of acetate or other simple organic acids and alcohols to the reduction of chlorate or perchlorate under a broad range of environmental conditions. However, in contrast to strain PS and the previously described (per)chlorate-reducing bacteria, *D. agitatus* strain CKB cannot couple growth to the reduction of nitrate.

In contrast to strain PS, strain WD is a member of the alpha subclass of the Proteobacteria and its closest relatives are the *Magnetospirillum* species. Strain WD is morphologically identical to the known species of this genus, however, there are marked physiological differences between strain WD and its closest relatives. All members of the *Magnetospirillum* genus that have been isolated to date form magnetosomes – an intracellular form of magnetite – when growing microaerophilically on iron-based media which gives these organisms a unique magnetotactic characteristic. In contrast, strain WD does not produce the fine grained magnetite characteristic of magnetotactic bacteria when grown microaerophilically on iron-based medium. In addition, none of the

Magnetospirillum species tested could grow by dissimilatory (per)chlorate reduction or could dismutate chlorite into chloride and O_2.

Strain WD and strain PS were both isolated from swine waste lagoons and represent the first described examples of (per)chlorate-reducing bacteria that were isolated from an environment not known to be contaminated with oxyanions of chlorine. Even though the two strains differ morphologically, their physiologies are very similar (temperature, pH, salinity optima) and optimum growth for both organisms is observed on short chain fatty acids and dicarboxylic acids while respiring on perchlorate, chlorate, nitrate or oxygen. In contrast to most other known (per)chlorate-reducing isolates and similarly to its closest relatives, strain WD is a microaerophile. Of the previously described (per)chlorate-reducing bacteria, only *Wolinella succinogenes* strain HAP-1 is known to be microaerophilic.[40] However, in contrast to strain WD, *W. succinogenes* is a member of the epsilon subclass of the Proteobacteria and incompletely oxidizes organic compounds while growing with oxyanions of chlorine.[19] *W. succinogenes* can also couple the oxidation of H_2, an important endproduct of microbial fermentation processes, to the reduction of (per)chlorate which is unique among the described (per)chlorate-reducing bacteria.

Growth rates on either perchlorate or chlorate for both strains PS and WD are comparable over a broad range of (per)chlorate concentrations. Strains WD and PS grow best at a concentration of 10 mM of either electron acceptor respectively which is similar to previous observations made for *D. agitatus* strain CKB.[22] Growth rates of both strains PS and WD are not significantly decreased even at concentrations of 40 mM chlorate/perchlorate. Strain WD is remarkably tolerant to perchlorate and is capable of growth in concentrations as high as 80 mM or approximately 7000 ppm. Similarly to strain GR-1[41] and *D. agitatus* strain CKB,[22,24,26,27] both strains PS and WD demonstrate the ability to dismutate chlorite into O_2 and chloride in washed whole-cell suspensions. Although this metabolism was until recently assumed to be unique to the (per)chlorate-reducing organism strain GR-1, studies in our laboratory have demonstrated that this metabolism is also found in more than twenty other perchlorate-reducing isolates obtained from a broad diversity of environments and suggest that this is probably the central enzyme in the reductive pathway for chlorate and perchlorate used (per)chlorate-reducing microorganisms.[22,27]

The distinct phenotypic and genotypic differences of strains WD and PS from each other, from their closest relatives, and from the previously described (per)chlorate-reducing bacteria indicate that these organisms represent two new genera of perchlorate-reducers in the Proteobacteria. The names *Dechlorisoma suillus* and *Dechlorospirillum anomalous* are proposed for strains PS and WD, respectively.

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REFERENCES

1. Urbansky, E.T. "Perchlorate chemistry: implications for analysis and remediation." *Bioremediation Journal,* 1998, *2,* 81–95.

2. Germgard, U.; Teder, A. Tormund, D. "Chlorate formation during chlorine dioxide bleaching of softwood kraft pulp." *Paperi ja Puu,* 1981, *3,* 127–133.

3. Rosemarin, A.; Lehtinen, K. Notini, M. "Effects of treated and untreated softwood pulp mill effluents on Baltic sea algae and invertebrates in model ecosystems." *Nordweigen Pulp and Paper Research Journal,* 1990, *2,* 83–87.

4. Agaev, R.; Danilov, V.; Khachaturov, V.; Kasymov, B. Tishabaev, B. "The toxicity to warm-blooded animals and fish of new defoliants based on sodium and magnesium chlorates." *Uzb. Biol. Zh.,* 1986, *1,* 40–43.

5. Urbanski, T**.** *Composite propellants*., Pergamon Press: Oxford, 1988; pp. 602–620.•

6. Siddiqui, M. "Chlorine-ozone interactions: formation of chlorate." *Water Research,* 1996, *30,* 2160–2170.

7. Cosson, H. Ernst, W.R. "Photodecomposition of chlorine dioxide and sodium chlorite in aqueous solution by irradiation with ultraviolet light." *Industrial Engineering Chemistry Research,* 1994, *33,* 1468–1475.

8. Stanbury, J.B. Wyngaarden, J.B. "Effect of perchlorate on the human thyroid gland." *Metabolism,* 1952, *1,* 533–539.

9. Calabrese, E.J.; Moore, G. Brown, R. "Effects of environmental oxidant stressors on individuals with a G-6-PD deficiency with particular reference to an animal model." *Environmental Health Perspectives,* 1979, *29,* 49–55.

10. Heffernan, W.P.; Guion, C. Bull, R.J. "Oxidative damage to erythrocyte induced by sodium chlorite in vivo." *Journal of Environmental Pathology and Toxicology,* 1979, *2,* 1487–1499.

11. Singelmann, E.; Wetzel, E.; Adler, G. Steffen, C. "Erythrocyte membrane alterations as the basis of chlorate toxicity." *Toxicology,* 1984, *30,* 135–147.

12. van Wijk, D.J. Hutchinson, T.H. "The ecotoxicity of chlorate to aquatic organisms: a critical review." *Ecotoxicology and Environmental Safety,* 1995, *32,* 244–253.

13. van Wijk, D.J.; Croon, S.G.M. Garttener-Arends, I.C.M. "Toxicity of chlorate and chlorite to selected species of algae, bacteria, and fungi." *Ecotoxicology and Environmental Safety,* 1998, *40,* 206–211.

14. Rosemarin, A.; Mattsson, J.; Lehtinen, K.; Notini, M. Nylen, E. "Effects of pulp mill chlorate (ClO_3^-) on *Fucus vesiculosus* - a summary of projects." *Ophelia,* 1986, *4,* 219–224.

15. de Groot, G.N. Stouthamer, A.H. "Regulation of reductase formation in *Proteus mirabilis*. I. Formation of reductases and enzymes of the formic hydrogenlyase complex in the wild type and in chlorate resistant mutants." *Archives of Microbiology,* 1969, *66,* 220–233.

16. Roldan, M.D.; Reyes, F.; Moreno-Vivian, C. Castillo, F. "Chlorate and nitrate reduction in phototrophic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*." *Current Microbiology,* 1994, *29,* 241–245.

17. Korenkov, V.; Romanenko, V.; Kuznetsov, S. Voronov, J. "Process for purification of industrial waste waters from perchlorates and chlorates." *US Patent,* 1976, *3,943,055,*

18. Malmqvist, A.; Welander, T.; Moore, E.; Ternstrom, A.; Molin, G. Stenstrom, I.-M. "*Ideonella dechloratans* gen. nov., sp. nov., a new bacterium capable of growing anaerobically with chlorate as an electron acceptor." *Systematic and Applied Microbiology,* 1994, *17,* 58–64.

19. Wallace, W.; Ward, T.; Breen, A. Attaway, H. "Identification of an anaerobic bacterium which reduces perchlorate and chlorate as *Wolinella succinogenes*." *Journal of Industrial Microbiology,* 1996, *16,* 68–72.

20. Stepanyuk, V.; Smirnova, G.; Klyushnikova, T.; Kanyuk, N.; Panchenko, L.; Nogina, T. Prima, V. "New species of the *Acinetobacter* genus *Acinetobacter thermotoleranticus* sp. nov." *Mikrobiologiya,* 1992, *61,* 347–356.

21. Rikken, G.; Kroon, A. van Ginkel, C. "Transformation of (per)chlorate into chloride by a newly isolated bacterium: reduction and dismutation." *Applied Microbiology and Biotechnology,* 1996, *45,* 420–426.

22. Bruce, R.A.; Achenbach, L.A. Coates, J.D. "Reduction of (per)chlorate by a novel organism isolated from a paper mill waste." *Environmental Microbiology,* 1999, *1,* 319–331.

23. Bruce, R.A. "The microbiology and bioremediative potential of (per)chlorate-reducing bacteria. " Master's Thesis, Southern Illinois University, 1999

24. Coates, J.D.; Bruce, R.A.; Patrick, J.A. Achenbach, L.A. "Hydrocarbon bioremediative potential of (per)chlorate-reducing bacteria." *Bioremediation Journal,* 1999, *3,* (in press).

25. Coates, J.D.; Bruce, R.A.; Patrick, J. Achenbach, L.A. "Stimulation of benzene degradation in anaerobic sediments by microbial chlorite dismutation." *Proceedings of the 5th International Petroleum Environmental Conference,* 1999.

26. Coates, J.D.; Bruce, R.A. Haddock, J.D. "Anoxic bioremediation of hydrocarbons." *Nature,* 1998, *396,* 730.

27. Coates, J.D.; Michaelidou, U.; Bruce, R.A.; O'Connor, S.M.; Crespi, J.N. Achenbach, L.A. "The ubiquity and diversity of dissimilatory (per)chlorate-reducing bacteria." *Applied and Environmental. Microbiology.,* 1999, *65*, 5234–5241.

28. Achenbach, L.A.; Bruce, R.A.; Michaelidou, U. Coates, J.D. "Phylogenetic analysis of dissimilatory (per)chlorate-reducing bacteria and development of molecular probes." *Applied and Environmental. Microbiology,* 1999, (in press)

29. Hackenthal, E.; Mannheim, W.; Hackenthal, R. Becher, R. "Die reduktion von perchlorat durch bakterien. I. untersuchungen an intakten zellen." *Biochemical Pharmacology,* 1964, *13,* 195–206.

30. Hackenthal, E. "Die reduktion von perchlorat durch bacterien - II. Die identitat der nitratreduktase und des perchlorat reduzierenden enzyms aus *B. cereus*." *Biochemical Pharmacology,* 1965, *14,* 1313–1324.

31. Stouthamer, A. "Nitrate reduction in *Aerobacter aerogenes*. I. Isolation properties of mutant strains blocked in nitrate assimilation and resistant against chlorate." *Archives of Microbiology.,* 1967, *56,* 68–75.

32. Coates, J.D.; Michaelidou, U.; O'Connor, S.M.; Bruce, R.A. Achenbach, L.A. "The diverse microbiology of (per)chlorate reduction." *218th National Meeting of the American Chemical Society,* 2000, (in press).

33. Tayeh, M.A. Madigan, M.T. "Malate dehydrogenase in phototrophic purple bacteria: purification, molecular weight, and quaternary structure." *Journal of Bacteriology,* 1987, *169,* 4196–4202.

34. Hungate, R.E. "A roll tube method for cultivation of strict anaerobes." *Methods in Microbiology.,* 1969, *3B,* 117–132.

35. Nickrent, D.L. "From field to film: rapid sequencing methods for field-collected plant species." *BioTechniques,* 1994, *16,* 470–475.

36. Maidak, B.L.; Olsen, G.J.; Larsen, N.; Overbeek, R.; McCaughey, M.J. Woese, C.R. "The RDP (Ribosomal Database Project)." *Nucleic Acids Research,* 1997, *25,* 109–111.

37. Gilbert, D.G. "SeqApp. version 1.9a157" 1993, Biocomputing Office, Biology Dept., Indiana University, Bloomington, IN.

38. Swofford, D.L. "PAUP*: Phylogenetic Analysis Using Parsimony (and other methods)." 1999, Version 4.0. Sinauer Associates, Sunderland, MA. Smithsonian Institution, Washington, DC.

39. Romanenko, V.I.; Korenkov, V.N. Kuznetsov, S.I. "Bacterial decomposition of ammonium perchlorate." *Mikrobiologiya,* 1976, *45,* 204–209.

40. Wallace, W.; Beshear, S.; Williams, D.; Hospadar, S. Owens, M. "Perchlorate reduction by a mixed culture in an up-flow anaerobic fixed bed reactor." *Journal of Industrial Microbiology & Biotechnology,* 1998, *20,* 126–131.

41. van Ginkel, C.; Rikken, G.; Kroon, A. Kengen, S. "Purification and characterization of chlorite dismutase: a novel oxygen-generating enzyme." *Archives of Microbiology,* 1996, *166,* 321–326.