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Dechloromonas agitata gen. nov., sp. nov. and *Dechlorosoma suillum* gen. nov., sp. nov., two novel environmentally dominant (per)chlorate-reducing bacteria and their phylogenetic position

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Previous studies on the ubiquity and diversity of microbial (per)chlorate reduction resulted in the isolation of 20 new strains of dissimilatory (per)chlorate-reducing bacteria. Phylogenetic analysis revealed that all of the isolates were members of the *Proteobacteria* with representatives in the α-, β- and γ-subclasses. The majority of the new isolates were located in the β-subclass and were closely related to each other and to the phototrophic *Rhodocyclus* species. Here an in-depth analysis of these organisms which form two distinct monophyletic groups within the *Rhodocyclus* assemblage is presented. Two new genera, *Dechloromonas* and *Dechlorosoma*, are proposed for these β-subclass lineages which represent the predominant (per)chlorate-reducing bacteria in the environment. The type species and strains for these new genera are *Dechloromonas agitata* strain CKB^T and *Dechlorosoma suillum* strain PS^T, respectively.

Keywords: *Dechloromonas*, *Dechlorosoma*, 16S rDNA, beta-*Proteobacteria*

INTRODUCTION

Recent concerns over the environmental contamination of ground waters and drinking waters with perchlorate has focused a significant amount of attention on the microbial metabolism of oxyanions of chlorine (Renner, 1998, 1999; Urbansky, 1998). Perchlorate contamination poses a significant health threat as preliminary toxicological studies have demonstrated that it has a direct effect on iodine uptake by the thyroid gland. In addition, at higher concentrations [6 mg (kg body wt)$^{-1}$ d^{-1}] perchlorate can result in fatal bone marrow disease. Prior to 1997, perchlorate was an unregulated compound and its presence in the environment has been primarily associated with the manufacture, handling and dismantling of munitions, in which it is used as a major component of rocket propellants and explosives (Urbansky, 1998). In 1997, with the development of highly sensitive analytical techniques (Wirt *et al.*, 1998), perchlorate contamination of drinking and recreational waters was identified throughout the US and particularly in the southwestern states of Utah, Nevada, Colorado and California. The California Environmental Protection Agency initiated a recommended maximum concentration limit (MCL) of 18 μg l^{-1} which, if exceeded, would require stoppage of water usage and remediation (Renner, 1998). Although the US Environmental Protection Agency recently increased this MCL to 32 μg l^{-1} (Renner, 1999), a value of 18 μg l^{-1} has been adhered to by several states throughout the US. In 1998, perchlorate was added to the US Environmental Protection Agency's drinking water candidate contaminant list and a regulatory decision regarding an MCL value for this compound is to be made by August 2001 following an extensive toxicological study and identification of potential remediation technologies. In the meantime, due to

Abbreviations: ClRB, (per)chlorate-reducing isolate(s); MCL, maximum concentration limit.

The GenBank accession numbers for the 16S rRNA sequences are listed in Methods.

down-sizing and regular maintenance of the munitions inventory, the US military is expected to produce another 165 million pounds (approx. 75000 tonnes) of perchlorate requiring treatment in the next 5 years (Wallace *et al.*, 1998).

Remediation efforts of perchlorate contamination have focused primarily on microbial processes because of the unique chemical stability and high solubility of perchlorate (Urbansky, 1998). Other physical/chemical technologies such as adsorption by activated charcoal, reverse osmosis or ion exchange have failed because of rapid saturation of active sites or the high cost, especially that associated with the processing of surface or groundwater contamination where excessively large volumes may require treatment. Although it has been recognized for more than 70 years that oxyanions of chlorine are suitable electron acceptors for microbial metabolism, this reductive process was originally identified with chlorate (Aslander, 1928) and was associated with nitrate-respiring organisms which simply used chlorate as a coincidental substrate for nitrate reductase (de Groot & Stouthamer, 1969; Hackenthal, 1965; Hackenthal *et al.*, 1964). Growth was not associated with this metabolism and chlorite was formed as a toxic end product of this metabolism (de Groot & Stouthamer, 1969; Hackenthal, 1965; Hackenthal *et al.*, 1964; Roldan *et al.*, 1994). Similar studies have not been done with perchlorate.

In the last two decades, five organisms have been identified which can couple growth to the reduction of chlorine oxyanions (Malmqvist *et al.*, 1994; Rikken *et al.*, 1996; Romanenko *et al.*, 1976; Stepanyuk *et al.*, 1992; Wallace *et al.*, 1996). Only two of these organisms have been characterized both phenotypically and genotypically (Malmqvist *et al.*, 1994; Wallace *et al.*, 1996). Although not demonstrated in all cases, it has been assumed that these organisms can couple growth to the reduction of both chlorate and perchlorate. However, the recent isolation of an organism that can grow by the reduction of chlorate but not perchlorate has indicated that this assumption may be incorrect (J. D. Coates, unpublished data). Recent studies in our laboratory have indicated that the ubiquity of microbial (per)chlorate respiration is much more extensive than was previously assumed (Coates *et al.*, 1999b). Our studies resulted in the isolation and identification of more than 20 new dissimilatory (per)chlorate-reducing isolates (ClRB) from a broad diversity of environments, including both pristine and contaminated soils and sediments. The ClRB represented a broad phylogeny with members in the α-, β and γ-subclasses of the *Proteobacteria*; however, the majority were placed in the β-subclass. These organisms were closely related to each other and to the phototrophic *Rhodocyclus* species. Here we report on the identification of two novel groups of ClRB within the β-subclass of the *Proteobacteria*. The fact that members of these groups have been identified and isolated in nearly all environments screened in our laboratory suggest that members of these groups may represent the predominant (per)chlorate-reducing bacteria in the environment. In-depth descriptions of the phenotypic characteristics of these organisms have been published elsewhere (Bruce, 1999; Coates *et al.*, 1999b; Michaelidou *et al.*, 2000).

METHODS

Sources of soils and sediments. The organisms were isolated from samples collected from a broad diversity of environments as described previously (Coates *et al.*, 1999b), including pristine and contaminated soils, sediments and waste sludges. The isolates were obtained using a standard shake-tube technique (Bruce, 1999) with acetate as the electron donor and chlorate as the electron acceptor. All cultures were maintained in both active liquid stocks as well as anaerobic frozen stocks in 10% glycerol at -70 °C.

Medium and culturing conditions. Standard anaerobic culturing techniques were used throughout (Hungate, 1969). The medium was boiled under N_2/CO_2 (80:20) to remove dissolved O_2 and then dispensed into anaerobic pressure tubes or serum bottles under N_2/CO_2, capped with thick butyl rubber stoppers and sterilized by autoclaving. The basal medium was bicarbonate-buffered freshwater medium that had been used previously for culturing strain CKB^T (Bruce, 1999). Sodium salts of acetate and chlorate (10 mM each) were used as the electron donor and acceptor, respectively, which were added from sterile anoxic stocks.

16S rDNA sequencing and analysis. PCR and sequencing of the 16S rRNA genes was performed as described previously (Coates *et al.*, 1999b). Sequence entry and manipulation were performed with the MacVector 6.5 sequence analysis software program for the Macintosh (Oxford Molecular Group). Sequences of selected 16S rRNAs were downloaded from the Ribosomal Database Project (Maidak *et al.*, 2000) and GenBank (Benson *et al.*, 1998) into the computer program SeqApp (Gilbert, 1993). 16S rDNA sequences of ClRB were manually added to the alignment using secondary structure information for proper alignment (alignment available on request). Complete 16S rDNA sequences were generated for 14 ClRB strains. For the remaining six ClRB strains, partial 16S rDNA sequences were determined. Only those regions sequenced in all of the organisms (815 nt) were used in the subsequent phylogenetic analyses (included *Escherichia coli* positions 434–767, 807–1182 and 1266–1362). Distance, parsimony and maximum-likelihood analysis of the aligned sequences was performed on a Power Macintosh G3 using PAUP* 4.0 (Swofford, 1999). Bootstrap analysis was conducted on 100 replications using a heuristic search strategy to assess the confidence level of various clades. GenBank accession numbers for sequences represented in Fig. 1 are as follows: *Treponema pallidum* (M88726), *Magnetospirillum gryphiswaldense* (Y10109), isolate WD (AF170352), *Azospirillum brasilense* (Z29617), isolate TTI (AF170353), *Comamomas testosteroni* (M11224), *Ideonella dechloratans* (X72724), isolate FL2 (AF288771), isolate FL8 (AF288772), isolate FL9 (AF288773), strain CKB^T (AF047462), isolate CL (AF170354), isolate NM (AF170355), isolate CL24+ (AF288774), isolate CL24 (AF288775), *Ferribacterium limneticum* (Y17060), isolate MissR (AF170357), isolate CCO (AF288776), isolate SIUL (AF170356), *Rhodocyclus tenuis* (D16209), *Rhodocyclus purpureus* (M34132), *Azoarcus evansii* (X77679), '*Azoarcus denitrificans*' (L33689), *Thauera*

selenatis (X68491), *Azoarcus indigens* (L15531), *Duganella zoogloeoides* (previously *Zoogloea ramigera*; X74913), strain PS^T (AF170348), isolate SDGM (AF170349), isolate Iso1 (AF170350), isolate Iso2 (AF170351), Gill symbiont of *Thyasira flexuosa* (L01575), isolate NSS (AF170359), *Pseudomonas stutzeri* (U26415), isolate PK (AF170358), isolate CFPBD (AF288777), *Wolinella succinogenes* ATCC 29543 (M26636) and *Helicobacter pylori* (M88157).

G+C analysis. Analysis of the G+C content of the chromosomal DNA was performed by the Identification Service of the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (DSMZ) using the HPLC method and conditions as described by Mesbah *et al.* (1989) and Tamaoka & Komagata (1984). Calibration of the method was performed with non-methylated λ DNA (Sigma) (Mesbah *et al.*, 1989).

RESULTS AND DISCUSSION

Phylogenetic analysis

Although ClRB representatives can be found throughout the *Proteobacteria* (Coates *et al.*, 1999b; Michaelidou *et al.*, 2000; Wallace *et al.*, 1996), the current work demonstrates that the majority (15 strains) of the ClRB isolated to date for which 16S rDNA sequence data are available (22 strains) are closely related to each other and are classified within the *Rhodocyclus* assemblage in the β-subclass of the *Proteobacteria* (Fig. 1). Analyses of the 16S rDNA sequences indicated that these (per)chlorate-reducing isolates form two distinct monophyletic groups within the β-subclass (Fig. 1). One of these groups, represented by our previously published ClRB strain CKB^T (Bruce, 1999), contains 11 strains and is relatively diverse with 16S rDNA distances among this group ranging from 0 to 3·9 %. The other group is represented by strain PS^T (Coates *et al.*, 1999b; Michaelidou *et al.*, 2000) and contains four isolates that are more phylogenetically compact than the members of the CKB group with distances only ranging from 0 to 0·12 %. Representative ClRB from the CKB group are most closely related to the Fe(III)-reducer *Ferribacterium limneticum* (Cummings *et al.*, 1999), while PS group members are most closely related to the phototroph *Rhodocyclus tenuis*.

Based on the unique phenotypic (Bruce, 1999; Coates *et al.*, 1999b; Michaelidou *et al.*, 2000) and genotypic characteristics of the organisms in this assemblage of ClRB, we propose that the two groups of ClRB within the β-subclass of the *Proteobacteria* represent two new genera within the *Rhodocyclus* assemblage. The names *Dechloromonas* gen. nov. and *Dechlorosoma* gen. nov. are proposed for the CKB and PS group members, respectively. *Dechloromonas agitata* sp. nov. strain CKB^T (Bruce, 1999) is the type strain for the *Dechloromonas* genus and *Dechlorosoma suillum* sp. nov. strain PS^T (Michaelidou *et al.*, 2000) is the type strain for the *Dechlorosoma* genus. Unlike other members of the *Rhodocyclus* assemblage, these organisms are capable of dissimilatory (per)chlorate reduction and chlorite dismutation (Bruce, 1999; Coates *et al.*, 1999b; Michaelidou *et al.*, 2000). In addition, 16S rDNA sequences of the species of the two groups within this assemblage of ClRB shared no more than 94·1 % similarity. For comparison, *Rhodocyclus tenuis* shares 94·7 % 16S rDNA sequence similarity to the most similar *Dechloromonas* species (strain CKB^T) and 94·0 % similarity to the most similar *Dechlorosoma* species (strain Iso2). The G+C content for these two groups of ClRB is also distinct with *Dechloromonas agitata* CKB^T at 63·5$\pm$0·3 mol % and *Dechlorosoma suillum* PS^T at 65·8$\pm$0·2 mol % (mean$\pm$SD, $n = 3$). *Rhodocyclus tenuis* has a G+C content of 64·8 mol % (Trüper & Imhoff, 1992).

All of the (per)chlorate-reducing *Dechloromonas* and *Dechlorosoma* species are heterotrophic facultative anaerobic respirers (Bruce, 1999; Coates *et al.*, 1999b; Michaelidou *et al.*, 2000). Other common features include the ability to couple growth to the complete oxidation of acetate with chlorate or perchlorate as sole electron acceptor and the ability to dismutate chlorite into chloride and O_2 (Coates *et al.*, 1999b). Some of the isolates can alternatively use nitrate as an electron acceptor (Coates *et al.*, 1999b) and nitrate is completely reduced to N_2. Chlorite dismutation is a central step in the enzymic reductive pathway of dissimilatory (per)chlorate reduction that is common to all tested dissimilatory (per)chlorate-reducing bacteria (Coates *et al.*, 1999b). (Per)chlorate is completely reduced to innocuous chloride.

Ideonella dechloratans, the only other previously described (per)chlorate-reducer which has been shown to be a member of the β-subclass of the *Proteobacteria*, was not closely related to either group and showed a maximum similarity of 90·1 % to *Dechloromonas agitata* strain CKB^T. Although previous studies have stated that the ClRB strain GR-1 is also a member of the β-subclass of the *Proteobacteria* (Rikken *et al.*, 1996), this has never been demonstrated and the 16S rRNA sequence data are not available for comparative analysis.

We had previously shown that *Rhodocyclus tenuis* is incapable of reducing (per)chlorate (Bruce, 1999) and that washed whole-cell suspensions of this species were incapable of dismutating chlorite into chloride and O_2. None of the other close relatives to *Dechloromonas* and *Dechlorosoma* that were tested could grow and reduce (per)chlorate or dismutate chlorite in washed whole-cell suspensions (data not shown; Coates *et al.*, 1999b; Michaelidou *et al.*, 2000).

Residing within the *Dechloromonas* group is a previously characterized Fe(III)-reducer, *Ferribacterium limneticum* (Cummings *et al.*, 1999), that shares 97·3 % 16S rDNA sequence similarity to its closest relative, the ClRB strain MissR. Interestingly, *Ferribacterium limneticum* is a strict anaerobic respirer unable to reduce (per)chlorate (data not shown). None of the ClRB isolated in this study were able to reduce ferric iron (Coates *et al.*, 1999b). In addition, similarly to *Rhodocyclus tenuis* and in contrast to all tested

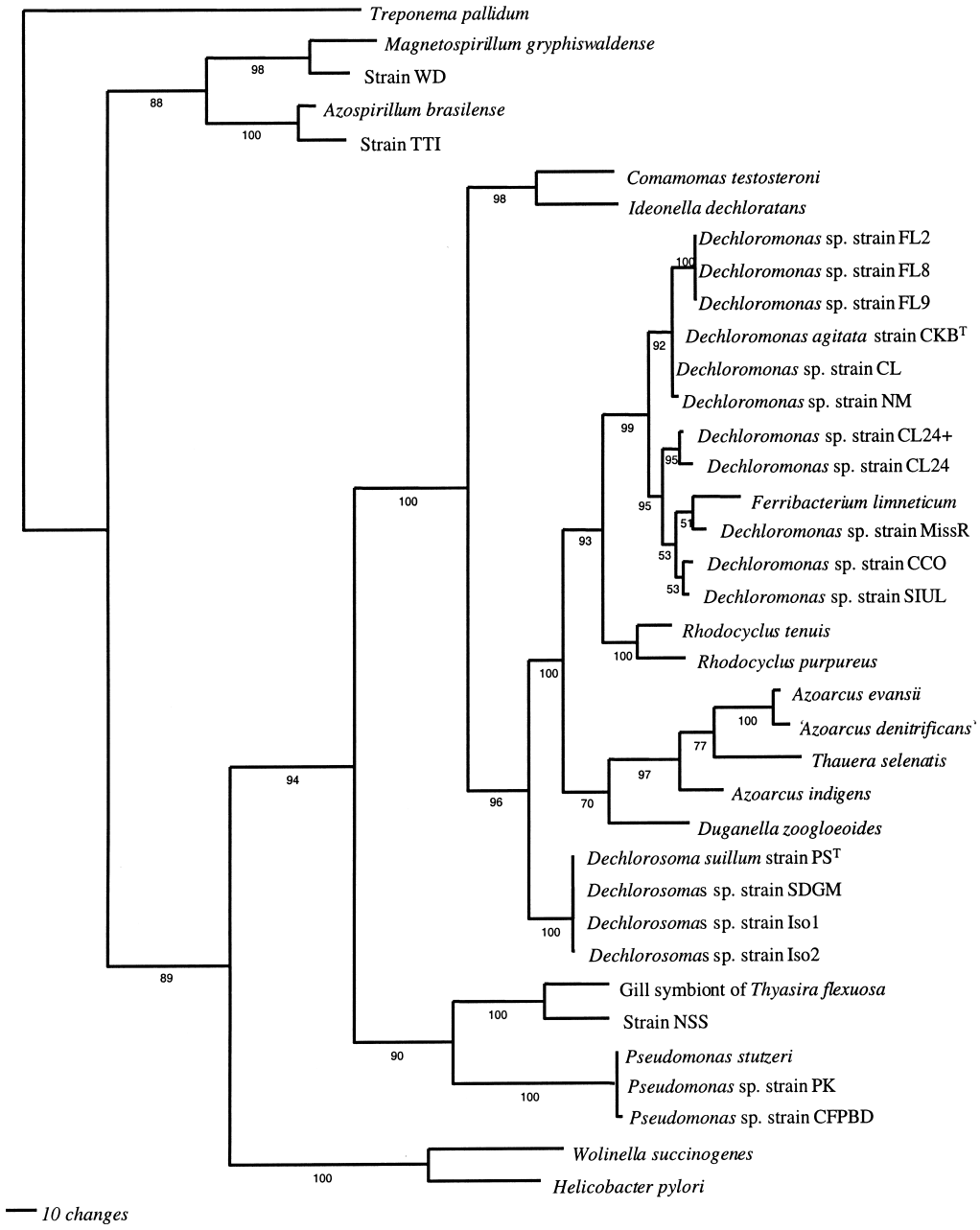

Fig. 1. Phylogenetic tree of newly isolated (per)chlorate-reducing bacteria and their closest relatives using a heuristic search with parsimony analysis. Tree length, 1205; C.I. minus uninformative sites, 0·502; R.I., 0·742. Bootstrap support values from 100 replications are indicated for each node. This figure represents one of two most parsimonious trees with the only difference being the topology between *Dechloromonas* sp. strains MissR, CCO and SIUL, and *Ferribacterium limneticum*.

(per)chlorate-reducing bacteria, washed whole-cell suspensions of *Ferribacterium limneticum* did not dismutate chlorite into chloride and O_2. This is in contrast to all known ClRB, including the isolates described in this study, which are facultative anaerobes capable of coupling growth to the dissimilation of either perchlorate or chlorate (Coates *et al.*, 1999b). The distinct physiological differences between *Ferribacterium limneticum* and the closely related ClRB imply that *Ferribacterium limneticum* is in fact a member of a separate genus.

Although the ClRB *Wolinella succinogenes* strain HAP-1 was originally thought to be a strict anaerobe, a recent study indicated that it is, in fact, a microaerophile (Wallace *et al.*, 1998). This is similar to one of our isolates, '*Dechlorospirillium anomolous*' strain WD, a member of the α-subclass of the *Proteobacteria*

closely related to a magnetotactic *Magnetospirillum* sp. (Michaelidou *et al*., 2000) which also grows preferentially under microaerophilic conditions.

Significance

To date, very little is known about micro-organisms capable of dissimilatory (per)chlorate reduction and until recently only three organisms had been described both phenotypically and genotypically which are capable of this metabolism (Bruce, 1999; Malmqvist *et al*., 1994; Wallace *et al*., 1996). Here and in previous work we describe several new (per)chlorate-reducing organisms that were isolated from a broad diversity of environments (Coates *et al*., 1999b; Michaelidou *et al*., 2000). Sequence analyses of the 16S rDNAs from the isolates indicated that all were members of three (α, β and γ) of the five subclasses of the *Proteobacteria* (Coates *et al*., 1999b). A previously described (per)chlorate-reducing bacterium, *Wolinella succinogenes* strain HAP-1 (Wallace *et al*., 1996), was shown to be a member of the ε-subclass. These results demonstrate that the phylogenetic diversity of dissimilatory (per)chlorate-reducing bacteria is far greater than was previously considered. Most proteobacterial subclasses contain only a few ClRB; however, the majority of the ClRB isolated to date reside in the β-subclass. The fact that the majority of the known (per)chlorate-reducing isolates are either *Dechloromonas* or *Dechlorosoma* species suggests that these groups are likely to represent the predominant (per)chlorate-reducing bacteria in the environment. This is further supported by the fact that, in almost every environment screened in our studies, members of these two groups were either isolated or their presence was identified by molecular analyses (Bruce, 1999; Coates *et al*., 1999a, b; Michaelidou *et al*., 2000). Although the Fe(III)-reducing organism, *Ferribacterium limneticum*, is an anomaly amongst this assemblage of organisms, its environmental significance is still unknown as there is only a single representative of this genus to date (Cummings *et al*., 1999).

In addition to the treatment of chlorate and perchlorate contamination, previous studies in our lab have demonstrated that the unique metabolic capabilities of (per)chlorate-reducing bacteria can alternatively be used for the treatment of other contaminants, including heavy metals, radionuclides (J. G. Lack, S. Chaudhuri & J. D. Coates, unpublished data) and hydrocarbons (Coates *et al*., 1998, 1999a). The only known natural source of (per)chlorate is from mineral deposits in Chile (Ericksen, 1983; Schilt, 1979) and the presence of (per)chlorate in the environment is the result of anthropogenic contamination over the last 100 years. It is thus surprising that such a phylogenetically diverse set of organisms should have evolved the ability to grow by dissimilatory (per)-chlorate reduction in such a short time frame and suggests that the metabolic capability to grow by the dissimilation of (per)chlorate is the result of a horizontal gene transfer event in the environment. This hypothesis is further supported by the fact that some ClRB are almost identical phenotypically and genotypically to organisms not capable of (per)chlorate reduction. For example, the γ-*Proteobacteria* strain PK is 99·8 % similar to *Pseudomonas stutzeri* based on 16S rDNA sequence analysis and phenotypically shares many characteristics of a pseudomonad; yet, strain PK is able to grow by dissimilatory (per)chlorate reduction while *Pseudomonas stutzeri* cannot (Coates *et al*., 1999b). The specific genetic mechanisms that confer (per)chlorate reduction and the true role of ClRB in the environment have yet to be identified.

Description of *Dechloromonas* gen. nov.

Dechloromonas [De.chlo.ro.mo′nas. L. pref. *de* from; Gr. adj. *chloros* green (chlorine); Gr. fem. n. *monas* unit, monad; N.L. fem. n. *Dechloromonas* a dechlorinating monad].

Rod-shaped, Gram-negative cells, $0{\cdot}5 \times 2$ μm, non-spore-forming, non-fermenting, facultative anaerobe. Cells are motile by a single polar flagellum and occur singly or in chains of two to three cells. A strictly respiring, complete oxidizer that oxidizes acetate with O_2, ClO_3^-, ClO_4^- or NO_3^- as alternative electron acceptors. Perchlorate and chlorate are completely reduced to chloride. Cells contain *c*-type cytochrome(s). Type species is *Dechloromonas agitata*.

Description of *Dechloromonas agitata* sp. nov.

Dechloromonas agitata (a.gi.ta′ta. L. fem. part. adj. *agitata* agitated, highly active).

Cells can grow with O_2, ClO_3^- or ClO_4^- as alternative electron acceptors. Organics used as alternative electron donors include propionate, butyrate, lactate, succinate, yeast extract, fumarate and malate. The reduced form of the humic substances analogue 2,6-anthrahydroquinone disulfonate, Fe(II) or sulfide can also serve as alternative electron donors coupled to the reduction of chlorate. Fe(II) is oxidized to insoluble amorphous Fe(III) oxide while sulfide is oxidized to elemental sulfur. Cells contain *c*-type cytochrome(s). Optimum growth is observed at 35 °C, pH 7·5 and 1 % NaCl with acetate (10 mM) as electron donor and chlorate (10 mM) as electron acceptor. G + C content is 63·5 mol %. Type strain, CKB^T, has been deposited in the American Type Culture Collection under ATCC 700666^T and in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under DSM 13637^T.

Description of *Dechlorosoma* gen. nov.

Dechlorosoma [De.chlo.ro.so′ma. L. pref. *de* from; Gr. adj. *chloros* green (chlorine); Gr. neut. n. *soma* body; N.L. neut. n. *Dechlorosoma* dechlorinating body].

Cells are Gram-negative, facultative anaerobic, non-spore-forming, non-fermentative rods, $1{\cdot}0 \times 0{\cdot}3$ μm. Cells are motile by a single polar flagellum and occur singly or in chains. Simple organic fatty acids are used as electron donors with O_2, ClO_3^-, ClO_4^- or NO_3^- as alternative electron acceptors. Organic electron donors are completely oxidized and perchlorate or chlorate are completely reduced to chloride. Cells contain *c*-type cytochrome(s). Type species is *Dechlorosoma suillum*.

Description of *Dechlorosoma suillum* sp. nov.

Dechloromonas suillum [su.il′lum; L. neut. adj. *suillum* pertaining to swine (Michaelidou *et al.*, 2000)].

Cells use acetate, propionate, butyrate, Casamino acids, lactate and ethanol as alternative electron donors with O_2, (per)chlorate or nitrate as electron acceptor. Nitrate is reduced to N_2 gas. Optimum growth is observed at 37 °C and pH 6·5 in freshwater medium (0% NaCl). G+C content is 65·8 mol%. Type strain, PS^T, has been deposited in the American Type Culture Collection under ATCC BAA-33^T and in the Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH under DSM 13638^T.

Habitat

Dechloromonas agitata was obtained from (per)chlorate-reducing enrichments from samples collected from a pulp and paper plant waste pulp sludge in Pennsylvania. *Dechlorosoma suillum* was obtained from (per)chlorate-reducing enrichments from samples collected from a primary treatment lagoon of swine waste at the Agricultural Research Facility at Southern Illinois University, Carbondale campus. Acetate (10 mM) was used as the electron donor in the enrichment and isolation cultures for both organisms.

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