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Pseudomonas chloritidismutans sp. nov., a non-denitrifying, chlorate-reducing bacterium

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A Gram-negative, facultatively anaerobic, rod-shaped, dissimilatory chlorate-reducing bacterium, strain AW-1^T, was isolated from biomass of an anaerobic chlorate-reducing bioreactor. Phylogenetic analysis of the 16S rDNA sequence showed 100% sequence similarity to *Pseudomonas stutzeri* DSM 50227 and 98·6% sequence similarity to the type strain of *P. stutzeri* (DSM 5190^T). The species *P. stutzeri* possesses a high degree of genotypic and phenotypic heterogeneity. Therefore, eight genomic groups, termed genomovars, have been proposed based upon ΔT_m values, which were used to evaluate the quality of the pairing within heteroduplexes formed by DNA–DNA hybridization. In this study, DNA–DNA hybridization between strain AW-1^T and *P. stutzeri* strains DSM 50227 and DSM 5190^T revealed respectively 80·5 and 56·5% similarity. DNA–DNA hybridization between *P. stutzeri* strains DSM 50227 and DSM 5190^T revealed 48·4% similarity. DNA–DNA hybridization indicated that strain AW-1^T is not related at the species level to the type strain of *P. stutzeri*. However, strain AW-1^T and *P. stutzeri* DSM 50227 are related at the species level. The physiological and biochemical properties of strain AW-1^T and the two *P. stutzeri* strains were compared. A common characteristic of *P. stutzeri* strains is the ability to denitrify. However, in growth experiments, strain AW-1^T could use only chlorate or oxygen as an electron acceptor and not nitrate, perchlorate or bromate. Strain AW-1^T is the first chlorate-reducing bacterium described that does not possess another oxyanion-reduction pathway. Cell extracts of strain AW-1^T showed chlorate and bromate reductase activities but not nitrate reductase activity. *P. stutzeri* strains DSM 50227 and DSM 5190^T could use nitrate or oxygen as an electron acceptor, but not chlorate. Chlorate reductase activity, in addition to nitrate reductase activity, was detected in cell extracts of both *P. stutzeri* strains. Chlorite dismutase activity was absent in extracts of both *P. stutzeri* strains but was present in extracts of strain AW-1^T. Based on the hybridization experiments and the physiological and biochemical data, it is proposed that strain AW-1^T be classified as a novel species of *Pseudomonas*, *Pseudomonas chloritidismutans* sp. nov. The type strain is strain AW-1^T (= DSM 13592^T = ATCC BAA-443^T).

Keywords: *Pseudomonas chloritidismutans* sp. nov., chlorate reduction, chlorite dismutation, denitrification

INTRODUCTION

Contamination of the environment with oxyanions, such as chlorate (ClO_3^-), is caused by human activities. Chlorate is used in a wide range of applications. It has been used as a herbicide in agriculture (Agaev *et al.*, 1986) and it is used for the on-site generation of the bleaching agent chlorine dioxide (ClO_2) in the paper and pulp industry (Germgard *et al.*, 1981). The compound may be toxic for certain organisms: chlorate added to drinking water can cause haemolytic

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The GenBank accession number for the 16S rDNA sequence of strain AW-1^T is AY017341.

anaemia in rats (Condie, 1986). In plants and micro-organisms, chlorate may compete with nitrate in the nitrate reductase system (van Wijk & Hutchinson, 1995). However, chlorate-reducing bacteria are able to remove this oxyanion by chlorate respiration (Rikken *et al*., 1996). This process seems to be promising in comparison with conventional chemical and physical treatment technologies (Wallace *et al*., 1998; Malmqvist & Welander, 1992). Strain GR-1 was the first bacterium reported to reduce chlorate completely to chloride (Rikken *et al*., 1996). In the first step, chlorate is reduced to chlorite (ClO_2^-) by a chlorate reductase. In the second step, chlorite is disproportionated to chloride and oxygen by a chlorite dismutase. These enzymes have been purified and characterized (van Ginkel *et al*., 1996; Kengen *et al*., 1999). Energy for biosynthesis can be conserved during reduction of chlorate and oxygen. Dissimilatory chlorate reduction has also been studied in *Dechloromonas agitata* CKB^T (Bruce *et al*., 1999; Achenbach *et al*., 2001), *Ideonella dechloratans* (Malmqvist *et al*., 1994) and *Wolinella succinogenes* HAP-1 (Wallace *et al*., 1996). These organisms (except strain CKB^T) have been reviewed by Logan (1998). All chlorate-reducers were characterized as facultative anaerobes, except *W. succinogenes* HAP-1, which is a microaerophile (Wallace *et al*., 1998). The latter strain is the only one that uses H_2 as an electron donor. Besides chlorate, other electron acceptors can be used by chlorate-reducing strains. The use of nitrate is of special interest, because many nitrate reductases are able to reduce chlorate (Hochstein & Tomlinson, 1988). Several denitrifying bacteria reduce chlorate but, in general, this reduction is not coupled to growth (De Groot & Stouthamer, 1969; Oltmann *et al*., 1976). Moreover, these organisms lack chlorite dismutase, which is required for the removal of the toxic chlorite. Recently, it was shown that dissimilatory chlorate-reducing bacteria have a specific chlorate reductase and a specific nitrate reductase (Rikken *et al*., 1996). It was shown that perchlorate-grown cells of strain GR-1 were unable to reduce nitrate or nitrite, indicating that specific (per)chlorate reductases are involved. *D. agitata* CKB^T is a dissimilatory chlorate-reducer incapable of coupling growth to the reduction of nitrate (Bruce *et al*., 1999). These findings suggest that nitrate- and chlorate-reduction pathways are not necessarily related. This paper describes the isolation and characterization of a chlorate-reducing bacterium, strain AW-1^T. This strain was enriched from sludge of an anaerobic bioreactor treating a chlorate- and bromate-polluted wastewater. The physiological, biochemical and phylogenetic properties of strain AW-1^T were compared with those of *Pseudomonas stutzeri* strains DSM 5190^T and DSM 50227.

METHODS

Media and growth conditions. Enrichment and cultivation of strain AW-1^T was performed in strictly anaerobic medium containing the following (l^{-1}): KH_2PO_4, 0·41 g; Na_2HPO_4, 0·53 g; $NaClO_3$, 1·06 g; $CaCl_2$, 0·11 g; $MgCl_2$, 0·1 g; NH_4HCO_3, 0·44 g; $NaHCO_3$, 3·73 g; Na_2S . $9H_2O$, 0·5 g; and 1 ml resazurin solution (0·5 g l^{-1}), as well as acid and alkaline trace elements (each 1 ml l^{-1}) and vitamins (1 ml l^{-1}). The medium was boiled and afterwards cooled under N_2 to remove dissolved oxygen. Resazurin was present as an indicator of the redox potential. When oxygen was produced by dismutation of chlorite, the medium turned pink. When all of the chlorate and oxygen was consumed, the pink colour disappeared. The acid trace-element solution contained the following (l^{-1}): $FeCl_2$. $4H_2O$, 1·485 g; H_3BO_3, 0·062 g; $ZnCl_2$, 0·068 g; $CuCl_2$, 0·013 g; $MnCl_2$. $4H_2O$, 0·098 g; $CoCl_2$. $6H_2O$, 0·119 g; $NiCl_2$. $6H_2O$, 0·119 g; and 4·1 ml HCl (37 %). The alkaline trace-element solution contained the following (l^{-1}): Na_2SeO_3 . $5H_2O$, 0·026 g; Na_2WO_4 . $2H_2O$, 0·033 g; Na_2MoO_4 . $2H_2O$, 0·024 g; and NaOH, 0·40 g. The composition of the vitamin solution was as described by Wolin *et al*. (1963). Bacteria were cultured at 30 °C in 120 ml serum vials with 40 ml medium and a gas phase of 1·5 atm. N_2. The pH values were calculated using the Henderson–Hasselbach equation. At 30 °C, the α and pK′ are respectively 0·665 and 6·348 (Breznak & Costilow, 1994). The percentage of CO_2 in the headspace was changed, whereas the bicarbonate concentration was kept constant. In this way, pH values of 9 (0 % CO_2, v/v), 8·5 (1·2 % CO_2, v/v), 8 (3·9 % CO_2, v/v), 7·3 (20 % CO_2, v/v) and 6·6 (100 % CO_2, v/v) were obtained. Bottles were sealed with butyl rubber stoppers and aluminium caps. All solutions were sterilized by autoclaving at 121 °C for 30 min, except for the vitamin solution, which was filter-sterilized before addition. The batch cultures were incubated on an orbital shaker (100 r.p.m.) in the dark. Substrates were added from 0·8 M stock solutions to give a final concentration of 10 or 20 mM. Occasionally, oxygen was added to the gas phase; the concentrations were expressed as mmol dissolved oxygen l^{-1} medium. Synthetic medium, nutrient broth and strictly anaerobic media were used to test the denitrification and chlorate-reduction capacities of the *P. stutzeri* strains and strain AW-1^T. Synthetic medium was prepared as described by Matsubara *et al*. (1982) with modifications as described by Coyle *et al*. (1985). This synthetic medium contained the following (l^{-1}): L-asparagine hydrate, 2·0 g; trisodium citrate dihydrate, 7·0 g; KH_2PO_4, 2·0 g; $CaCl_2$. $2H_2O$, 0·2 g; $MgSO_4$. $7H_2O$, 4·0; $FeCl_3$. $6H_2O$, 0·02 g and NaCl; 20 g. The pH was adjusted to 6·6 with 5 M NaOH. Forty ml medium was added to 120 ml serum bottles. N_2 was used as the gas phase. Nutrient broth medium contained the following (l^{-1}): peptone, 5·0 g; nutrient broth, 3·0 g. The pH was adjusted to pH 7·0. For determination of the optimum pH, a pH range of 5–10 was used. The medium was boiled and afterwards cooled under N_2 to remove dissolved oxygen. Forty ml medium was added to 120 ml serum bottles. N_2 was used as the gas phase. Nutrient agar plates (pH 7·0) were prepared in the same way, except that 15 g agar l^{-1} was added.

Micro-organisms. *P. stutzeri* strains DSM 50227 and DSM 5190^T were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Strain AW-1^T was isolated in this study.

Enrichment cultures and isolation of strain AW-1^T. Approximately 2 g sludge, taken from an anaerobic bioreactor treating chlorate- and bromate-polluted wastewater, was added to strictly anaerobic medium. Chlorate (10 mM) and acetate (10 mM) were used as electron acceptor and electron donor, respectively. N_2 was used as the gas phase. Enrichment cultures were checked for chlorate reduction and growth by visual and microscopic inspection. After a second

subculture, a pure culture of strain AW-1^T was obtained by repeated application of the roll-tube dilution method, as described by Hungate (1969), using chlorate and acetate as substrates.

Determination of growth parameters. All growth parameters of strain AW-1^T were checked in strictly anaerobic medium pre-reduced with sulfide. The growth rate was determined by measuring the increase in OD_{430}. Doubling times were determined by linear regression of a exponentially plotted growth curve. Cell counts were obtained by plating dilution series on nutrient agar plates. The following electron donors (10 mM) were tested with ClO_3^- (10 mM) as electron acceptor: acetate, propionate, glucose, maltose, mannitol, malate, lactate, arabinose, hydrogen, glycine, glycerol, formate, gluconate, ethanol, starch, citrate and succinate. The following electron acceptors (10 mM) were tested with acetate (10 mM) as electron donor: perchlorate, chlorate, chlorite, nitrate, nitrite, bromate, sulfate, selenate and oxygen. All anions were supplied as sodium salts. The temperature optimum was determined in medium at pH 8·5, in the range 10–42 °C. The pH optimum was determined in medium at 30 °C using a pH range of 6·6–9. For comparison with the type strain of *P. stutzeri* (DSM 5190^T), the pH optimum was also determined in nutrient broth medium in a pH range of 5–10. The optimum salt concentration was determined at 30 °C and pH 8·5 using a NaCl range of 1–40 g l^{-1}. The effect of oxygen on chlorate reduction was investigated. Oxygen was either added together with chlorate at the start of the incubation or was added to a culture in which chlorate reduction was already going on.

Cellular characterization. Micrographs were taken using transmission electron microscopy. The Gram-stain type was determined using Gram staining and electron microscopy. Protocols described previously by Plugge *et al.* (2000) were used.

Phylogenetic identification of strain AW-1^T. The 16S rDNA sequence and the G+C content of the DNA of strain AW-1^T were determined by the DSMZ. The 16S rRNA gene sequence was determined by direct sequencing of PCR-amplified 16S rDNA as described by Rainey *et al.* (1996). Approximately 95% of the 16S rDNA sequence was analysed. The G+C content of the DNA was determined by HPLC as described by Mesbah *et al.* (1989). The 16S rDNA sequence was analysed with ARB software (Ludwig & Strunk, 1996). The 16S rRNA gene similarity values were calculated by a pairwise comparison of sequences within the alignment. A neighbour-joining tree was constructed based on evolutionary distance values (Saitou & Nei, 1987). These tools were implemented in the ARB software. Sequences of chlorate-reducing bacteria and the denitrifying strains of *P. stutzeri* were introduced into the ARB software package. These sequences were derived from the National Center for Biotechnology Information database (Bethesda, MD, USA) or the ARB software package. DNA–DNA hybridization was also done at the DSMZ. DNA from strain AW-1^T (= DSM 13592^T) and *P. stutzeri* strains DSM 50227 and DSM 5190^T was isolated by chromatography on hydroxyapatite by using the procedure of Cashion *et al.* (1977). DNA–DNA hybridization was carried out as described by De Ley *et al.* (1970), with the modification described by Huß *et al.* (1983) and Escara & Hutton (1980). Renaturation rates were computed with the TRANSFER.BAS program (Jahnke, 1992).

Preparation of cell extracts. All procedures were performed anaerobically. Strain AW-1^T was grown in strictly anaerobic medium with chlorate as electron acceptor. The *P. stutzeri* strains were grown in synthetic medium with nitrate as electron acceptor. Whole cells were harvested from 200 ml cultures by centrifugation at 9000 r.p.m. for 9 min at 10 °C. The cell pellet was washed once with 15 mM potassium phosphate/sodium phosphate buffer (1·55 g K_2HPO_4 l^{-1}; 0·85 g NaH_2PO_4 . H_2O l^{-1}), pH 7·2. Cells were resuspended in the same buffer (2 ml). To prepare cell extracts, 1 ml cell suspension was sonicated for 30 s and cooled on ice for 30 s; this cycle was repeated five times. The cell-debris fraction was removed by centrifugation at 13000 r.p.m. for 1 min at room temperature. Cell extracts were stored under a N_2 gas phase at 4 °C.

Enzyme activity measurements. Chlorate reductase and chlorite dismutase activities were determined in cell extracts of strain AW-1^T and *P. stutzeri* strains DSM 50227 and DSM 5190^T. Chlorate reductase and nitrate reductase activities were determined spectrophotometrically as described previously by Kengen *et al.* (1999) by monitoring the oxidation of reduced methyl viologen at 578 nm and 30 °C. The following electron acceptors (supplied as sodium salts) were tested: perchlorate, chlorate, chlorite, bromate, nitrate, selenate, sulfate, iodite and iodate. In this assay, 10 µl of a 0·25 M stock solution and 10 µl cell-extract fraction were added to the reaction mixture. Chlorite dismutase activity was determined by measuring oxygen production with a Clark-type oxygen electrode (Yellow Spring Instruments). The reaction vessel was kept at 30 °C. The reaction mixture contained 2·8 ml 15 mM potassium phosphate/sodium phosphate buffer (pH 7·2) and 10 µl cell extract or 10 µl whole cells. The reaction was started by injecting a concentrated solution of sodium chlorite into the vessel to give a concentration of 0·2 mM. The reaction was run for 5 min, but only the initial, linear part was used to calculate the rate. One unit (U) of activity is defined as the amount of enzyme required to convert 1 µmol chlorite or chlorate min^{-1}. The protein content of the cell extract fraction was determined according to the method of Bradford (1976), with BSA as the standard.

Other analyses. Electron acceptors were analysed by HPLC as described previously by Scholten & Stams (1995). Acetate, hydrogen and oxygen levels were measured by GC as described by Stams *et al.* (1993).

RESULTS AND DISCUSSION

Enrichment and isolation of strain AW-1^T

A sludge sample was transferred to a 120 ml serum bottle with strictly anaerobic medium containing chlorate as electron acceptor and acetate as electron donor. In subcultures, within 3 days, growth was observed coupled to the disappearance of chlorate and acetate and the formation of chloride. After a second subculture, a pure culture was finally obtained by repeated application of the roll-tube dilution method.

Morphological and physiological characterization of strain AW-1^T

The strain obtained was a Gram-negative, facultatively anaerobic, motile, rod-shaped organism (Fig. 1). The size of the cells was 0·5–2 µm, as measured by

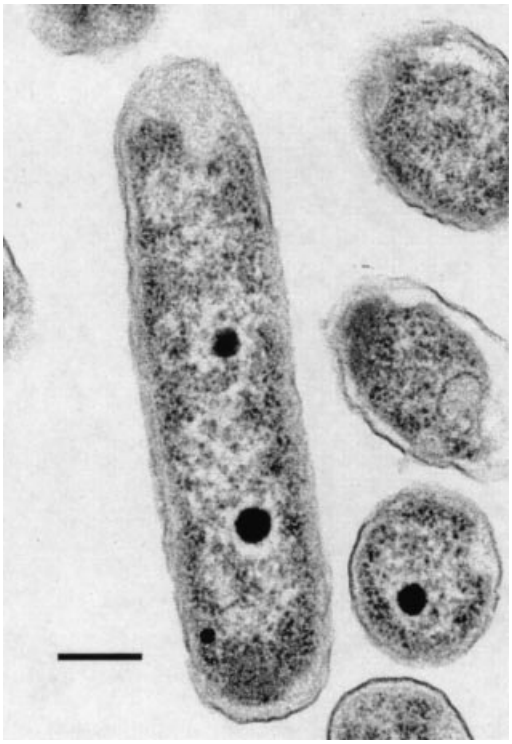

Fig. 1. Electron micrograph showing strain AW-1^T. Electron-dense structures are polyphosphate particles. Bar, 0·25 μm.

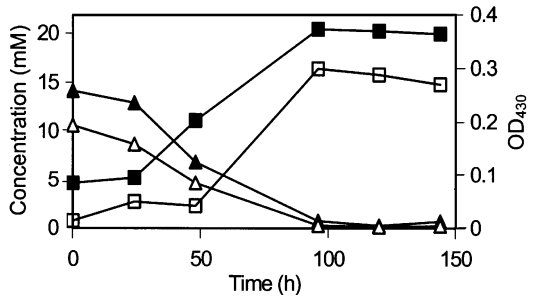

Fig. 2. Growth curve of strain AW-1^T with acetate (△) as electron donor and chlorate (▲) as electron acceptor in a batch culture. ■, Chloride; □, OD_{430}.

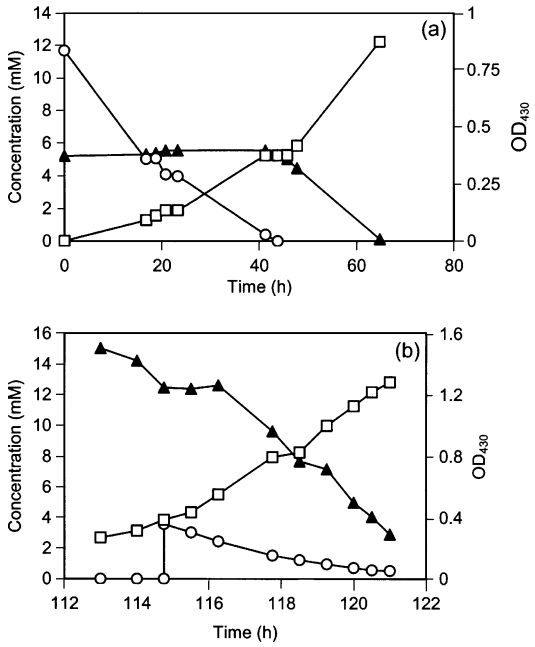

Fig. 3. Competition experiments in strictly anaerobic medium with chlorate (▲). Oxygen (○) was added at the start of the experiment (a) or at $t = 115$ h (b). □, OD_{430}.

transmission electron microscopy. Colonies in anaerobic agar were circular and pale orange. Colonies on nutrient broth plates were wrinkled, coherent and had a pale brown or sometimes reddish brown colour. This is the same morphology as described for *P. stutzeri* by Stolp & Gadkari (1981). Reduction of 14·2 mM chlorate resulted in the oxidation of 10·6 mM acetate and the formation of 15·6 mM chloride (Fig. 2), giving a stoichiometry of 1·0 : 0·75 : 1·1. This is close to that expected theoretically when chlorate is reduced to chloride with acetate, as is described by Rikken *et al.* (1996): $ClO_3^- + 0{\cdot}75\ CH_3COO^- \rightarrow 1{\cdot}5\ HCO_3^- + H^+ + Cl^-$. During the exponential growth phase, chlorite, the intermediate compound in chlorate reduction, was not detected. With acetate as electron donor, growth of strain AW-1^T could be observed only when ClO_3^- or O_2 was used as the electron acceptor. When ClO_4^-, ClO_2^-, NO_3^-, NO_2^-, BrO_3^-, SO_4^{2-} or SeO_4^{2-} were tested, no growth could be detected. Compared with strain GR-1 [ClO_3^-, ClO_4^-, O_2, NO_3^-, Mn(IV); Rikken *et al.*, 1996] and *D. agitata* CKB^T (ClO_3^-, ClO_4^-, O_2; Bruce *et al.*, 1999), the electron-acceptor range of strain AW-1^T is rather limited. This indicates that a specific chlorate reductase is involved that cannot reduce perchlorate or nitrate. Strain AW-1^T differs in this aspect from other chlorate-reducing bacteria. Apparently, strain AW-1^T does not possess another oxyanion-reduction pathway. However, a recently isolated bacterium also showed only chlorate reduction (Achenbach *et al.*, 2001). In strictly anaerobic medium, strain AW-1^T grew with doubling times of 1·5 and 1·2 h using chlorate and oxygen as the respective electron acceptors. In a competition experiment, the effect of oxygen on chlorate reduction was analysed. When oxygen and chlorate were both present at the beginning of the experiment, chlorate reduction started only when the oxygen had been consumed (Fig. 3a). When oxygen was added to a chlorate-reducing culture, oxygen and chlorate were consumed simultaneously (Fig. 3b). Similar results were obtained by Rikken *et al.* (1996) with washed cells of strain GR-1. These results suggest that the expression of chlorate reductase is inhibited by the presence of oxygen. However, when chlorate reduction proceeds, addition of oxygen does not inhibit the chlorate-reduction process itself. With

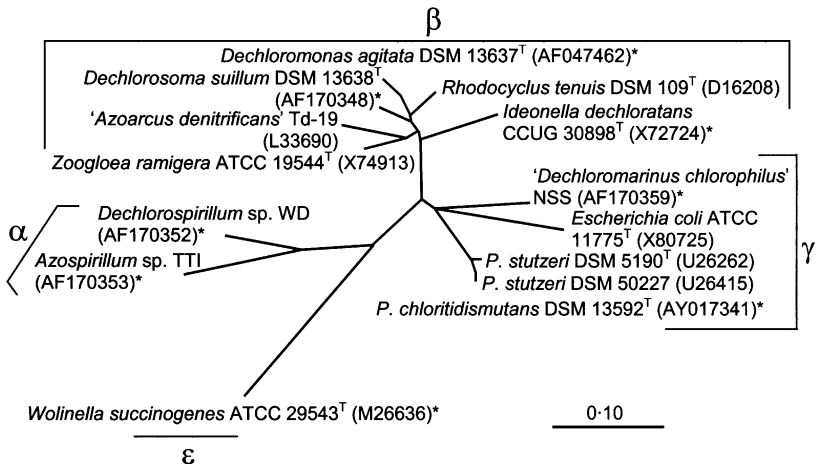

Fig. 4. Neighbour-joining tree based on 16S rDNA sequences, showing the phylogenetic position of strain AW-1^T among the subclasses of the *Proteobacteria*. Chlorate-reducing bacteria are indicated by asterisks. Bar, 0·1 evolutionary distance.

chlorate as electron acceptor, growth of strain AW-1^T was observed with acetate, propionate, glucose, maltose, gluconate, mannitol, glycerol, ethanol, starch or citrate as electron donors and carbon sources. No growth was observed with malate, succinate, lactate, glycine, arabinose or H_2 ($+CO_2$). In the absence of chlorate or oxygen, glucose and maltose were not fermented. The pH range in strictly anaerobic medium was defined by using the buffer capacity of bicarbonate/CO_2; a pH range of 6·6 (100 % CO_2) to 9·0 (100 % N_2) was obtained. In this medium, growth occurred within a pH range of 8·0–9·0, with an optimum at pH 8·5. In nutrient broth medium, growth occurred within a pH range of 7·0–9·0, with an optimum at pH 7·5. For *P. stutzeri* DSM 5190^T, a pH optimum of 7·0 was described, although growth was possible up to pH 9 (Van Niel & Allen, 1952). Because the strictly anaerobic medium was used for isolation, the temperature and salinity range were determined in this medium. Strain AW-1^T could grow in a concentration range of 1–40 g NaCl l^{-1}. The highest growth rates were obtained between 20 and 40 g NaCl l^{-1}. However, at these high NaCl concentrations, cells lysed more rapidly in the stationary phase. The optimal temperature was 30 °C, the lower and upper limits being 10 and 37 °C.

Phylogenetic, physiological and biochemical comparison of strain AW-1^T and *P. stutzeri* strains

Sequencing of 16S rDNA showed that strain AW-1^T belongs to the *γ-Proteobacteria*. The 16S rDNA sequence of strain AW-1^T showed 100 % similarity to that of *P. stutzeri* DSM 50227 and 98·6 % similarity to that of *P. stutzeri* DSM 5190^T. The 16S rDNA similarity between strain AW-1^T and *P. stutzeri* strain PK was 99·4 %. The latter strain was isolated by Coates *et al.* (1999) and is also a chlorate-reducing bacterium. The phylogenetic position of strain AW-1^T is shown in Fig. 4. The figure shows that chlorate-reducing bacteria are present in all subclasses of the *Proteobacteria*, making it a phylogenetically diverse group of micro-organisms. The G+C content of strain AW-1^T is 63·9 mol %. The G+C contents of *P. stutzeri* strains DSM 50227 and DSM 5190^T are respectively 63·7±0·2 and 65·0±1·0 mol % (Rosselló *et al.*, 1991). DNA–DNA hybridization using strain AW-1^T and *P. stutzeri* DSM 50227 or *P. stutzeri* DSM 5190^T respectively showed 80·5 and 56·5 % similarity. DNA–DNA hybridization between *P. stutzeri* strains DSM 50227 and DSM 5190^T showed 48·4 % similarity. It is agreed that, when two strains are compared and the similarity of 16S rDNA sequences is greater than 97 %, the strains might be members of the same species (Stackebrandt & Goebel, 1994). Because the 16S rRNA molecule is small and contains little information, priority is given to DNA–DNA hybridization. To differentiate two species, the DNA–DNA similarity should be less than 60–70 % (Stackebrandt & Goebel, 1994). According to these criteria, strain AW-1^T, and also *P. stutzeri* DSM 50227, are not related to *P. stutzeri* DSM 5190^T at the species level. Instead, strain AW-1^T and *P. stutzeri* DSM 50227 are related at the species level. Many studies have demonstrated that the species *P. stutzeri* has a high degree of phenotypic and genotypic heterogeneity (Stanier *et al.*, 1966; Palleroni *et al.*, 1970; Rosselló *et al.*, 1991). DNA–DNA hybridization (based on the calculation of ΔT_m values, which were used to evaluate the quality of the pairing within heteroduplexes formed by DNA–DNA hybridization) together with G+C-content studies showed the existence of eight genomic groups, called genomovars (Rosselló *et al.*, 1991; Rosselló-Mora *et al.*, 1996). This subdivision was confirmed by 16S rDNA sequencing (Bennasar *et al.*, 1996), lipid analysis, protein analysis (Rosselló-Mora *et al.*, 1994), macrorestriction fragment analysis of genomic DNA (Ginard *et al.*, 1997) and internally transcribed 16S–23S rRNA gene spacer regions (Guasp *et al.*, 2000). Based on differences in 16S rRNA sequences together with differential phenotypic traits, genomovar 6 was renamed *Pseudomonas balearica* (Bennasar *et al.*, 1996). Because DNA–DNA hybridizations in this study showed that *P. stutzeri* DSM 5190^T was not related at the species

level to strain AW-1^T and *P. stutzeri* DSM 50227, physiological and biochemical characterization of these strains was done to allow further description of strain AW-1^T. *P. stutzeri* is known to be a vigorous denitrifier that is capable of growth on nitrate, nitrite and nitrous oxide (Van Niel & Allen, 1952; Palleroni *et al.*, 1970; Bergan, 1981; Zumft, 1997). Growth experiments were done in a strictly anaerobic medium, a synthetic medium and a nutrient broth medium. In all three media, strain AW-1^T was able to couple chlorate reduction to growth, whereas the *P. stutzeri* strains were unable to do this. Conversely, nitrate reduction occurred only in synthetic and nutrient broth media inoculated with *P. stutzeri* strains, but not with strain AW-1^T. Apparently, strain AW-1^T is a chlorate-reducing bacterium that does not possess another oxyanion-reduction pathway. These results were confirmed by the data derived from the enzyme assays. Strain AW-1^T showed only chlorate and bromate reductase activities. The specific activities were respectively 9·0 and 8·6 U mg^{-1} for chlorate and bromate. Although the *P. stutzeri* strains could not couple growth to chlorate reduction, the enzyme assays revealed chlorate reductase and nitrate reductase activities. The specific reductase activities for strain *P. stutzeri* DSM 50227 were respectively 0·9 and 1·6 U mg^{-1} for nitrate and chlorate. Strain *P. stutzeri* DSM 5190^T showed specific reductase activities of 4·3 and 3·4 U mg^{-1} for nitrate and chlorate, respectively. These data suggest that *P. stutzeri* contains a nitrate reductase with chlorate-reducing activity. Apparently, the bacterium cannot gain energy from chlorate reduction. This confirms findings from other denitrifying bacteria (De Groot & Stouthamer, 1969; Oltmann *et al.*, 1976). *P. stutzeri* cannot grow on chlorate, probably because the chlorite dismutase is absent; no activity of this enzyme was found in *P. stutzeri* strains DSM 50227 and DSM 5190^T. Chlorate is reduced but the product, chlorite, is not dismutated into chloride and oxygen. Chlorite may be toxic for these bacteria. Extracts of strain AW-1^T did show chlorite dismutase activity, with a specific activity of 134 U (mg protein)$^{-1}$. Strain AW-1^T and *P. stutzeri* strains DSM 50227 and DSM 5190^T contain an enzyme to reduce chlorate, but the presence of chlorite dismutase differs for strain AW-1^T and *P. stutzeri* strains DSM 50227 and DSM 5190^T. This enzyme seems to be essential for growth with chlorate as electron donor.

Strain AW-1^T showed 100 % 16S rDNA similarity and 80·5 % DNA–DNA hybridization to *P. stutzeri* DSM 50227. However, comparison with *P. stutzeri* DSM 5190^T showed that strain AW-1^T is not related to the type strain at the species level. Also, physiological and biochemical comparisons of strain AW-1^T with *P. stutzeri* strains DSM 50227 and DSM 5190^T showed that these strains can be differentiated with respect to the capacity for dissimilatory chlorate reduction, chlorite dismutation and denitrification. Therefore, the name *Pseudomonas chloritidismutans* sp. nov. is proposed for strain AW-1^T.

Description of *Pseudomonas chloritidismutans* sp. nov.

Pseudomonas chloritidismutans (chlo.ri.ti.dis.mu′tans. N.L. n. *chloris* chlorite; L. part. pres. *dismutans* splitting; N.L. part. pres. *chloritidismutans* chlorite-splitting).

Cells are Gram-negative, facultatively anaerobic, motile rods. Cells are 0·5–2 μm in size. Colonies growing in anaerobic agar are circular and pale orange. Colonies on nutrient broth plates are wrinkled, coherent and have a pale brown or sometimes reddish brown colour. In strictly anaerobic medium prereduced with sulfide, the growth characteristics as follows: growth range, 10–37 °C, optimum 30 °C; pH range for growth, pH 8·0–9·0, optimum pH 8·5. In nutrient broth medium, growth is in the pH range 7·0–9·0, with an optimum at pH 7·5. Doubling times are respectively 1·5 and 1·2 h for chlorate and oxygen. Growth occurs in media containing up to 40 g NaCl l^{-1}. At high NaCl concentrations, cells in stationary phase may lyse. Growth occurs on the following: acetate, propionate, glucose, maltose, gluconate, mannitol, ethanol, starch, glycerol and citrate. There is no growth on malate, succinate, lactate, glycine, arabinose or H_2. Glucose and maltose are not fermented. Chlorate and oxygen are used as electron acceptors. No growth with perchlorate, chlorite, nitrate, nitrite, bromate, sulfate or selenate. Cell extracts contain chlorate reductase and bromate reductase activities. Chlorite is converted to chloride and oxygen by a chlorite dismutase. The type strain belongs to the *γ-Proteobacteria*. The 16S rDNA sequence is 98·6 % similar to that of *P. stutzeri* DSM 5190^T. DNA–DNA hybridization between strain AW-1^T and *P. stutzeri* DSM 5190^T showed 56·5 % similarity. The G+C content is 63·9 mol %. The type strain, strain AW-1^T (= DSM 13592^T = ATCC BAA-443^T), was obtained from biomass of an anaerobic bioreactor treating chlorate- and bromate-polluted wastewater.

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