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## Genotype versus phenotype in the circumscription of bacterial species: the case of *Pseudomonas stutzeri* and *Pseudomonas chloritidismutans*

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**Abstract** The phenotypic characteristic of strain AW-1<sup>T</sup> of *Pseudomonas chloritidismutans* that is most relevant from the taxonomic point of view appears to be the capacity of growth under anaerobic conditions using chlorate as electron acceptor. This property is not restricted to this species only within the genus *Pseudomonas*, since it is also present in strains of genomovars 1 or 5, and 3 of *Pseudomonas stutzeri*. *P. chloritidismutans* has been described as a non-denitrifying species, but the isolation of variants that are able to grow anaerobically in the presence of nitrate is possible after subcultivation under selective conditions. The subdivision of *P. stutzeri* into a number of species on the basis of these characteristics does not help to clarify the phylogenetic relationships among the members of an otherwise coherent group of strains, and the considerations presented in this communication support the reclassification of the new species name *P. chloritidismutans*, which in our opinion, should be considered as a Junior name of *P. stutzeri*. A multilocus sequence analysis, together with a phenotypic analysis of the anaerobic oxidative metabolism, gives new insights into the phylogeny and evolution of the species.

**Keywords** *P. stutzeri* · *P. chloritidismutans* · Denitrification · Multilocus sequence analysis (MLSA)

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### Introduction

*Pseudomonas stutzeri* is a remarkable species of the genus *Pseudomonas*. It has an exceptional range of physiological and biochemical capacities, and metabolizes a wide range of organic and mineral substrates. Members of the species mineralize organic substrates aerobically and anaerobically as denitrifiers. Some metabolize thiosulfate, obtaining energy chemolithotrophically (Sorokin et al. 1999). Other strains also fix dinitrogen (Vermeiren et al. 1999). The species is well-defined phenotypically by means of certain biochemical tests that distinguish *P. stutzeri* from other *Pseudomonas* species. However, intraspecifically, the species is biochemically very diverse (Palleroni 1984), which causes on occasion confusion as to its phenotypical identification. *P. stutzeri* can be isolated from many environmental and clinical specimens. The species is widespread, and can be found in many ecological niches (Bennasar et al. 1998a; Sikorski et al. 2002b), including extreme habitats such as hydrothermal vents (strain NF13; Ruby et al. 1981) or deep-sea sediments (strain MT-1; Tamegai et al. 1997).

Diversity within the species is not limited to physiological traits but also clearly reflected at the genetic level. At least 18 genomovars (gv.) have been distinguished within the species by DNA–DNA hybridization (García-Valdés et al. 2003; Sikorski et al. 2005). Genomovars are also clearly distinguished in most cases through phylogenetic analysis of the *rrn* operon (Guasp et al. 2000). For the moment, no consistent phenotypical traits in each genomovar justify the splitting of *P. stutzeri* into several independent species. Its extremely high genetic diversity, the highest so far described in a species, was demonstrated through MLEE studies (Rius et al. 2001; Sikorski et al. 1999) and through a multilocus gene study of representative strains of the species (Cladera et al. 2004). It was also suggested that *P. stutzeri* populations have a strong clonal structure, but the natural transformation ability

of many *P. stutzeri* strains (Sikorski et al. 2002a) explains the acquisition of new phenotypic traits not distributed in all members of the species or of a given genomovar.

Recently, the isolation and characterization of a chlorate-reducing bacterium, strain AW-1<sup>T</sup> (ATCC BAA-443<sup>T</sup>, DSM 13592<sup>T</sup>), was described by Wolterink et al. (2002). This strain was isolated from sludge of an anaerobic bioreactor treating chlorate- and bromate-polluted wastewater, as a bacterium able to grow anaerobically oxidizing organic matter, with chlorate as its electron acceptor. The morphological, physiological, biochemical and phylogenetic properties of strain AW-1<sup>T</sup> were compared with those of *P. stutzeri* strains DSM 5190<sup>T</sup> (gv.1) and DSM 50227, the reference strain of *P. stutzeri* gv. 3. Phylogenetic analysis of 16S rDNA clearly affiliated strain AW-1<sup>T</sup> to members of genomovar 3 (100% 16S rDNA sequence similarity to *P. stutzeri* DSM50227 and 98.6% sequence similarity to strain *P. stutzeri* DSM 5190<sup>T</sup>). Additionally, DNA–DNA similarity values of 80.5% with strain DSM 50227 demonstrated the closeness of the two strains. However, strain AW-1<sup>T</sup> was proposed as the type strain of a new species, *Pseudomonas chloritidismutans*, due to its ability to use chlorate as electron acceptor under anaerobic conditions and its inability to denitrify. This is the only trait that differentiates AW-1<sup>T</sup> from *P. stutzeri* DSM 50227, able to denitrify, but not to use chlorate. Strain AW-1<sup>T</sup> is the only representative of the species.

In order to clarify the taxonomic position of strain AW-1<sup>T</sup> further, and to clearly establish the status of this new species, several physiological, phenotypical and genetical studies were performed. The phylogenetic affiliation of strain AW-1<sup>T</sup> to genomovar 3 of *P. stutzeri* has been confirmed in a multilocus sequence analysis, and an adapted strain AW-1A able to grow under denitrifying conditions has been obtained.

## Materials and methods

### Bacterial strains

*Pseudomonas chloritidismutans* AW-1<sup>T</sup> (Wolterink et al. 2002) was acquired from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany. Other strains used that represent the *P. stutzeri* genomovars discussed were described in a previous analysis (Cladera et al. 2004).

### Media and growth conditions

Luria Bertani (LB) medium (Miller 1972) was used for routine culture. For anaerobic growth, strains were inoculated into M9 mineral medium (Miller 1972) supplemented with 0.2% maltose as electron donor, and 0.1% KNO<sub>3</sub> or NaClO<sub>3</sub> as electron acceptor, in tubes

with an overlay of Vaseline, or in 30 ml serum vials with 20 ml mineral medium and an argon atmosphere. For the isolation of strain AW-1A, M9 was solidified with 1.5% agar and plates incubated on a GasPak container (BBL, Hampshire, England) with an anaerobic atmosphere generator Anaerocult A, and with Anaerostek (Merck, Darmstadt, Germany) to confirm anaerobic conditions. Growth temperature was always 30°C.

### Denitrification tests

The method of Alef (1995) was followed. Cells were grown in serum vials for 3 days under anaerobic conditions in mineral medium with 1% maltose, 0.1% KNO<sub>3</sub> and 0.1% Na<sub>2</sub>MoO<sub>4</sub>·2H<sub>2</sub>O. One millilitre (ml) of acetylene was introduced at the gas phase to inhibit nitrous oxide reductase activity, and the vials were incubated for a further 24 h. Two-hundred and fifty microliters of gas phase were injected in a Shimadzu GC8A gas chromatograph, equipped with a thermal conductivity detector and a molecular Sieve 5A column (2 m × 1/8) to detect nitrous oxide.

### Anaerobic growth with nitrate and chlorate of strains AW-1<sup>T</sup> and AW-1A

Serum vials with minimal medium were used for growth experiments. Control vials were prepared without electron acceptor. Anaerobiosis was achieved by flushing the medium with sterile argon. Nitrate in samples taken at different times was quantified by a flow injection analysis, QuickChem (Lachai Instruments). Chlorate was quantified by an ionic interchange chromatography apparatus (DIONEX) provided with IonPac AS9-HC column. Na<sub>2</sub>CO<sub>3</sub> (9.0 mM) was used as eluent with a flux of 1.0 ml/min. Growth was measured by counting viable cell numbers on LB plates incubated aerobically, and following absorbance at 600 nm with an Ultrospec III spectrophotometer (Pharmacia LKB).

### DNA extraction

Bacterial genomic DNA for PCR amplification was obtained by lysis with sodium dodecyl sulfate (SDS)-proteinase K and treatment with cetyltrimethyl ammonium bromide (CTAB), as described by Wilson (1987).

### PCR amplification and DNA sequencing

Primers, PCR and sequencing conditions for 16S rDNA, ITS1 and the *rpoD*, *gyrB*, *nosZ*, *catA* genes have been published (Cladera et al. 2004). Amplification primers for the chlorate dismutase gene, *clt*, were reported by Bender et al. (2004). *nirS* and *nirK* primers were designed

by Bennasar from the conserved regions retrieved from available data bases: nirU364 (5'-CCAAGCAGATCTA TTTCGA-3') and nirL1888 (5'-GTGTTGTASACGTT GAACTTRCCGG-3') for *nirS*, and CU-U808 (5'-GGS GCGCTTGCGCCG-3') and CU-L1648 (5'-GAACTT GCCGGTCGCCAGAC-3') for *nirK*. PCR conditions for *nirS* were: after denaturation at 94°C for 1 min, a total of 30 cycles were performed with template DNA using the following profile. Denaturation was performed at 94°C for 1 min, with primer annealing at 50°C for 1 min and primer extension at 72°C for 2 min. For amplification of *nirK*, the same conditions were applied, except that the annealing temperature was 55°C and extension was at 72°C for 1 min. The same primers were used for sequencing reactions.

16S rDNA, ITS1 and the *rpoD*, *gyrB*, *nosZ*, *catA* genes from strain AW-1<sup>T</sup> were aligned with those of 26 *P. stutzeri* strains from the different genomovars, two *P. balearica* strains and *P. aeruginosa* and *P. mendocina* type strains. The corresponding phylogenetic trees were constructed and a hypothetical multilocus tree was calculated to represent the combined molecular evolutionary relationships, as already described in Cladera et al. (2004).

Signature positions for genomovars in the 16S rDNA sequences previously published (Bennasar et al. 1996) were identified in the 16S rDNA sequence of the chlorate-reducing strains used in this study for their assignment to genomovar.

An ERIC-PCR protocol described by Bennasar et al. (1998b) was used in a test to confirm the identity of the adapted AW-1A strain as a variant of AW-1<sup>T</sup>. Sequences used are available in public databases (Cladera et al. 2004).

### Phylogenetic analysis

Partial 16S rDNA sequences were aligned with 16S rDNA reference sequences in the ARB package (<http://www.arb-home.de>). Ambiguously and incorrectly aligned positions were aligned manually on the basis of conserved primary sequence and secondary structure. Phylogenetic trees were inferred by neighbor-joining with the Jukes–Cantor model to calculate evolutionary distances. Short sequences were added to the tree by using the parsimony insertion tool in ARB, and the percentage of sequence similarity was determined.

Multilocus consensus trees for housekeeping genes and for the *clt* gene were constructed as described in Cladera et al. (2004).

### Nucleotide sequence accession numbers

EMBL accession numbers of *Pseudomonas* AW-1<sup>T</sup> are: for ITS1, AJ880096; *rpoD*, AJ880091; *gyrB*, AJ880092; *catA*, AJ880094; *nosZ*, AJ880093; *clt*, AJ880095; *nirS*, AJ 884572.

## Results

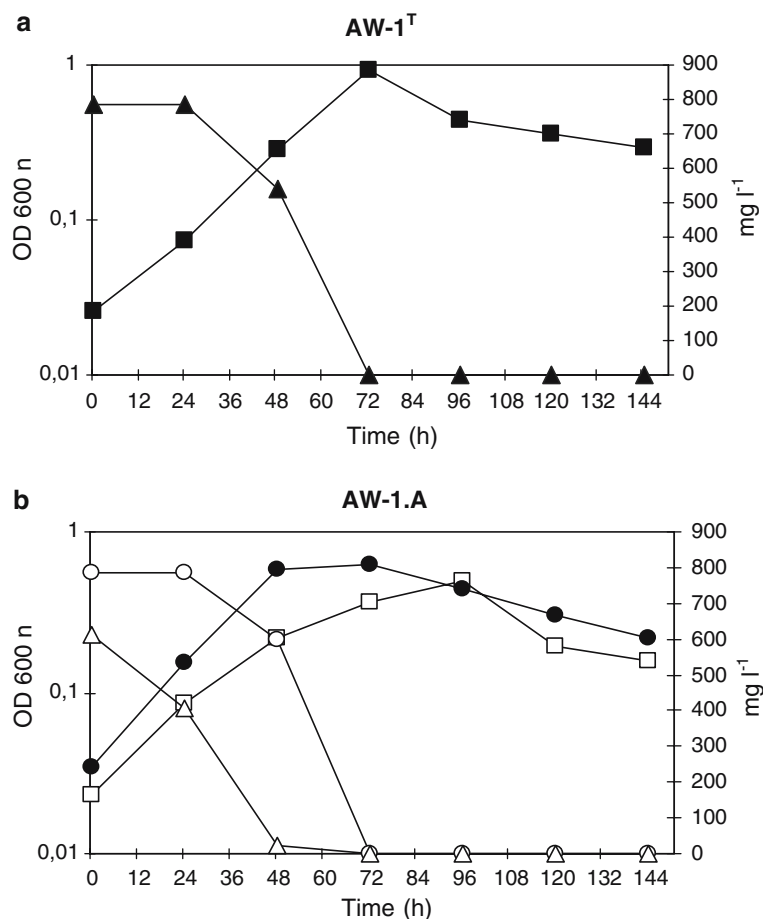
Selection of strain AW-1A, a variant of strain AW-1<sup>T</sup> able to grow under denitrification conditions

Strain AW-1<sup>T</sup> was cultured aerobically in minimal denitrification medium. Three subcultures of 100 µl in 5 ml medium were done every 48 h, to adapt the cells to the presence of nitrate. The same process was continued with two more subcultures, but adding 0.17% agar to the growth medium to prevent an excessive oxygen diffusion into the medium, and a subsequent series of 4 subcultures were done in the same medium with a 1 ml Vaseline layer at the top of the semisolid medium to obtain anaerobic conditions. Samples of the cultures thus obtained were inoculated on plates with the same medium and incubated for 7 days on a Gaspak container under anaerobic conditions. One of the well-developed colonies, AW-1A, was selected for further study. Strain AW-1A was confirmed as a variant of AW-1<sup>T</sup> by homogeneous colonial morphology after cells were plated on LB plates in each subculture, and by an ERIC-PCR test, which showed identical patterns for both strains but pattern different from other member of gv.3 (Fig. A given as supplementary material online). The same was true with the results obtained by sequencing the *clt* and *nirS* genes.

### Growth under anaerobic conditions with chlorate and nitrate as electron acceptors

Anaerobic growth was followed in serum vials with mineral medium, with chlorate or nitrate as electron acceptors and with sterile argon in the gas phase. Growth curves were determined and results are given in Fig. 1. Strain AW-1<sup>T</sup> grew anaerobically with chlorate ( $t_d = 25$  h,  $\mu = 0.04$  h<sup>-1</sup>; maximal growth after 72 h was  $5.95 \times 10^8$  cfu/ml, when chlorate was exhausted), but no growth was detected with nitrate. However, strain AW-1A not only grew anaerobically with chlorate ( $t_d = 29.4$ ,  $\mu = 0.034$  h<sup>-1</sup>,  $4.65 \times 10^7$  cfu/ml), but also showed anaerobic growth dependent on the presence of nitrate. Nitrate was consumed during the exponential growth phase, and was exhausted when cells reached the stationary phase ( $t_d = 17.86$ h,  $\mu = 0.056$  h<sup>-1</sup>,  $5.2 \times 10^8$  cfu/ml). Maximal growth of strain AW-1A was higher with nitrate and was reached after 48 h incubation time, when nitrate was depleted from the growth medium and growth rate was also lower. Nitrite was not detected in any sample. No growth of strain AW-1A was detected in a control experiment without electron acceptor. Denitrification capacity was also determined by the production of nitrous oxides, when nitrous oxide reductase activity was inhibited by acetylene (data not shown).

**Fig. 1** **a** Growth curve of strain AW-1<sup>T</sup> (filled square) with chlorate as electron acceptor (closed triangle). **b** Growth curves of strain AW-1A with chlorate (open square) or nitrate as electron acceptor (closed circle). Chlorate (open circle). Nitrate (open triangle).



## Genomic analysis

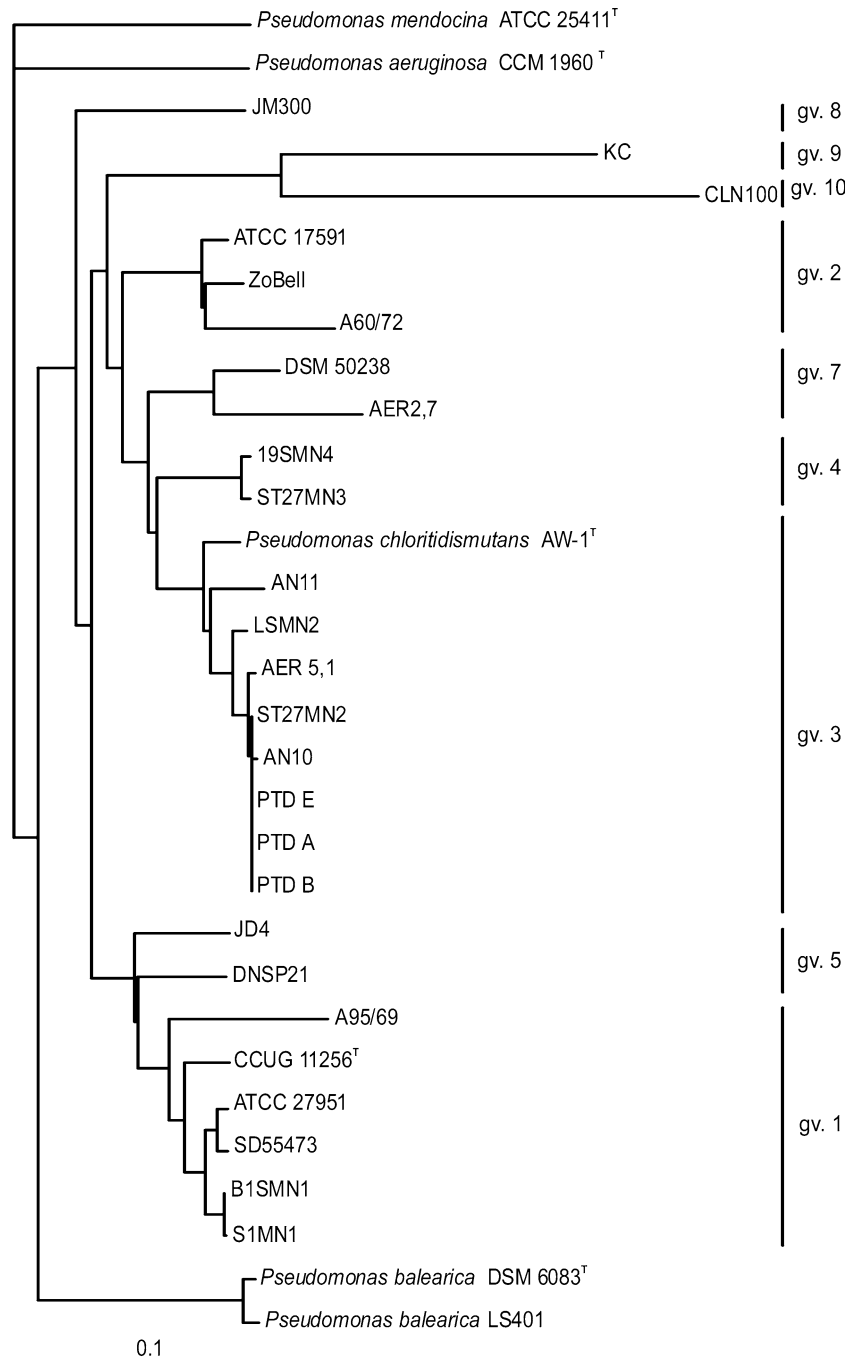
Several housekeeping genes previously studied in 26 *P. stutzeri* strains were also analyzed in strain AW-1<sup>T</sup>, and a consensus phylogenetic tree was constructed as described by Cladera et al. (2004). As expected, strain AW-1<sup>T</sup> clustered together with all members of genomovar 3 (Fig. 2). Essential genes for denitrification and chlorate reduction were detected in strain AW-1<sup>T</sup> and in the adapted strain AW-1A by PCR amplification and sequencing. These genes codify for nitrous oxide reductase (*nosZ*, amplicon of 453 nucleotides) and nitrate reductase (*nirS*, amplicon of 262 nucleotides). *nirS* sequences were identical in strains AW-1<sup>T</sup> and AW-1A, confirming that AW-1A is a variant of AW-1<sup>T</sup>. *nirK* could not be amplified.

Chlorite dismutase is an enzyme that disproportionates chlorite to chloride and oxygen, and is considered indicative of dechlorination capacity. Primers for chlorite dismutase (*clt*) were used to amplify a 409-nucleotide fragment of the gene in strains AW-1<sup>T</sup> and AW-1A. The *clt* gene is present in both strains, and their sequences are identical and very similar to *clt* in other chlorate-reducing strains (Bender et al. 2004): *Pseudomonas* strain PK (with one different aminoacid, S153 instead of T) and *Dechloromarinus chlorophilus* (three

different aminoacids N103, S159, A206, instead of H, T and V, respectively). Twenty-six strains of all *P. stutzeri* genomovars used in a previous study (Cladera et al. 2004) were tested by PCR amplification for the presence of the *clt* gene, and all results were negative. A *clt* phylogenetic tree was constructed with all the sequences available in the databases (Fig. 3). *clt* genes of strains AW-1<sup>T</sup> and AW-1A are identical and located in the same branch as *clt* of strains *Pseudomonas* PK and *D. chlorophilus*. As already indicated by Bender et al. (2004), the topology of the tree clearly shows that *clt* phylogeny does not correlate with 16S rDNA phylogeny of the chlorate- or perchlorate-reducing strains.

The percentage values of G+C, the relative use of synonymous codons (RSCU) and the codon bias index (CBI, the measure of the deviation from the equal use of synonymous codons) of the chlorite dismutase gene were determined and compared with those previously calculated for other genes of *P. stutzeri* strains (Cladera et al. 2004). The G+C content of the entire genomic DNA of *P. stutzeri* gv.3 strains so far described ranges from 62.4 to 63.7%. *P. chloritidismutans* AW-1<sup>T</sup> has a G+C content of 63.90% (Wolterink et al. 2002). G+C content in the housekeeping genes analyzed in strain AW-1<sup>T</sup> ranged from 58.7% for *nosZ* to 65.4% for *catA*, in the same range as the corresponding genes in the five

**Fig. 2** Multilocus consensus tree showing the molecular evolutionary relationships of the genes *rpoD*, *gyrB*, *NosZ*, *catA*, 16S rDNA, and ITS1 between *P. chloritidismutans* AW-1<sup>T</sup>, *P. aeruginosa*, *P. mendocina*, *P. balearica*, and 26 representative strains of nine genomovars described for *P. stutzeri*. The bar indicates sequence divergence



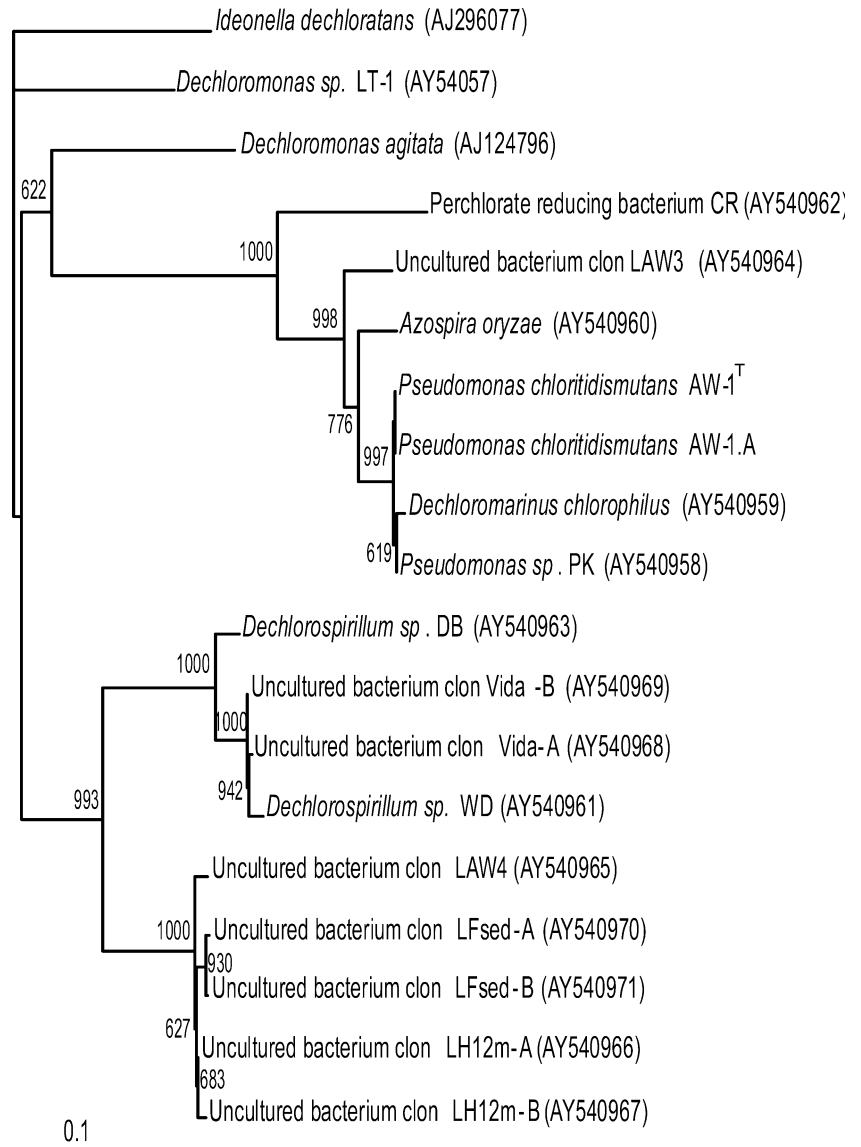
members of gv.3 studied (Table 1). In contrast, the *clt* gene has a clearly lower GC content of 50.8%, this is 13% below the value calculated for the whole genome of strain AW-1<sup>T</sup>. This value is similar to those calculated for other *clt* sequences from beta- and gammaproteobacteria retrieved from data bases (mean value of 50.0%). G+C % values of *clt* of alphaproteobacteria are higher, with a mean value of 59.5%.

The RSCU values of the housekeeping genes sequenced from strain AW-1<sup>T</sup> are similar to those of *P. stutzeri* gv.3 strains, and of other genomovars of *P. stutzeri* (data retrieved from our own results and

coinciding with those available in databases: 82,284 codons of *P. stutzeri* strains, published by Nakamura et al. 2000). When these data are analyzed gene by gene, *rpoD*, *gyrB*, *nosZ* and *catA* have the same pattern of codon usage in *P. stutzeri* and *P. chloritidismutans*. However, RSCU values for 7 of 17 amino acids (G, E, V, K, Y, H and P) in *clt* gene in AW-1<sup>T</sup> are distinct from the other essential genes analyzed and closer to the *clt* gene from a distant species, such as *D. chlorophilus*.

CBI values calculated for AW-1<sup>T</sup> genes are 0.475 for *gyrB*, 0.639 for *rpoD*, 0.685 for *catA* and 0.753 for *nosZ*. These values are consistent with values calculated for

**Fig. 3** Neighbor-joining tree based on the *cld* gene sequence data resulting from a distance analysis performed with the Jukes–Cantor correction from strain AW-1<sup>T</sup>, AW-1A and gamma, beta and alphaproteobacteria strains available on the data bases. Bootstrap values of more than 600 (from 1,000 replicates) are indicated at the nodes. The *bar* indicates sequence divergence



housekeeping genes of *P. stutzeri*, and are clearly different from the CBI value of the *cld* gene, which is 0.271, consistent with *cld* for the gammaproteobacteria so far

analyzed (*Pseudomonas* PK is 0.277; *D. chlorophilus* is 0.280) and lower than CBI *cld* values for beta- (0.332 to 0.57) and alphaproteobacteria (0.638 to 0.683).

**Table 1** G+C% mean values of *rpoD*, *gyrB*, *nosZ* and *catA* genes in each *P. stutzeri* genomovar and of *cld* gene in chlorate-reducing related strains available in data bases

gv. (strains)	GC %					Mean	Range	<i>cld</i>
	<i>catA</i>	<i>gyrB</i>	<i>rpoD</i>	<i>nosZ</i>				
gv.1 (6)	63.67	60.68	63.18	60.45	61.62	63.4–65.0	-	
gv.2 (3)	63.67	55.83	59.64	58.28	59.36	60.9–62.3	-	
gv.3 (5)	65.3	58.15	63.15	58.14	60.39	62.4–63.7	-	
gv.4 (2)	64.74	58.3	61.94	60.37	60.51	62.0–62.2	-	
gv.5 (2)	64.42	61.07	63.73	59.05	62.07	63.4	-	
gv.7 (2)	65.38	59	62.32	59.16	60.61	64.9	-	
gv.8 (1)	66.35	59.95	63.35	59.82	62.36	-	-	
gv.9 (1)	-	58.1	60	58.9	59	-	-	
gv.10 (1)	-	58	60.3	58.9	58.55	-	-	
Mean value	64.62	58.83	62.41	59.13	60.49	-	-	
<i>Pseudomonas</i> AW-1 <sup>T</sup>	65.4	57.7	63.3	58.7	61.27	63.9	50.82	
<i>Pseudomonas</i> PK	-	-	-	-	-	-	50.82	

Number of strains analysed in each genomovar is indicated in brackets

16S rDNA sequences show 100% identity between AW-1<sup>T</sup> and the reference strain of *P. stutzeri* gv.3, DSM50227 (Wolterink et al. 2002). Other chlorate-reducing strains in the *P. stutzeri* group and their 16S rDNA sequences have been previously described: PK (Coates et al. 1999), CFPBD (Achenbach et al. 2001), PDA and PDB Logan et al. 2001). 16s rDNA sequences of strains PK and CFPBD are 99.65% and 99.62% identical with *P. stutzeri* gv.3 reference strain, and strains PDA and PDB are 100% identical with *P. stutzeri* gv.1 and gv.5 reference strains (CCUG11256 and DNSP21). The signature positions in the 16S rDNA sequences of these strains are consistent with their assignment to genomovars 3, and 1 or 5, respectively. A *P. stutzeri* phylogenetic tree, based on the 16S rDNA sequence and including these chlorate-reducing *Pseudomonas* strains, is shown in Fig. 4.

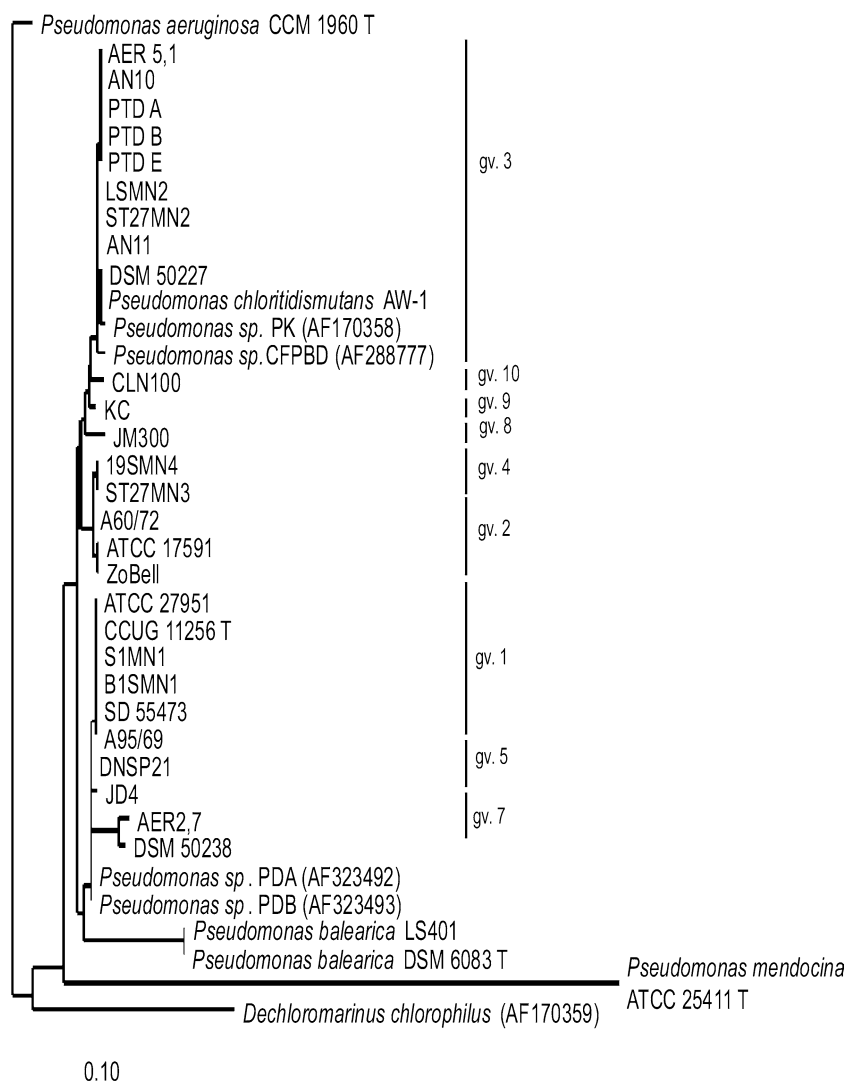
## Discussion

In this paper, we show the presence of denitrification pathway genes in strain AW-1<sup>T</sup> and that an adapted

spontaneous variant, able to express these genes and to grow anaerobically with nitrate as denitrifier, can be obtained with simple subculturing techniques. Cases of loss and recovery of activity and functionality in *Pseudomonas* strains have been previously published. Three strains of *P. aureofaciens* reported inability to denitrify gained this ability after “adaptation” in nitrate medium (Palleroni and Doudoroff 1972). *P. stutzeri* strain ZoBell of genomovar 2 was described as non-motile, but it recovered its flagellation after three passages onto soft agar plates (Rosselló-Mora et al. 1993). Although the molecular mechanisms controlling these adaptations were not described, it is clear that the strain AW-1<sup>T</sup> possesses the basic phenotypic traits that are characteristic of *P. stutzeri*.

Enzymatic reaction of chlorate to chlorite by nitrate reductase occurs as a competitive reaction between nitrate and chlorate in certain denitrifying bacteria. However, chlorate reduction by most denitrifiers is not an energy-yielding process, and very few denitrifying bacteria employ perchlorate or chlorate as a terminal electron acceptor (Logan et al. 2001). Bacteria capable

**Fig. 4** Phylogenetic affiliation of the chlorate-reducing gammaproteobacteria and *P. stutzeri* representative strains based on the 16S rDNA sequences. Bar indicates sequence divergence



of chlorate reduction are, however, widely distributed in the environment, soil water and waste-water. Phenotypically, the ability to reduce chlorate in strain AW-1<sup>T</sup> is the only distinctive characteristic that differentiates *P. chloritidismutans* from its closest phenotypically and genomically related strains. This property is not exclusive to strain AW-1<sup>T</sup>, because other strains of *P. stutzeri* belonging to gv.1 or gv.5 (PDA and PDB) and strains PK and CFPBD of gv.3 were described, earlier than *P. chloritidismutans*, as able to grow with chlorate (Logan, 2001). Allocation of these strains to their respective genomovars was based on the phylogenetic analysis of the 16S rDNA sequence and on the presence of the signature nucleotides of genomovars 1 or 5 and gv.3.

In this paper, we demonstrate that chlorate reductase activity is present in more than one genomovar, but not in all their members. The lack of congruence between the phylogenetic analysis of the *cld* gene and the 16S rDNA of chlorate-reducing bacteria, and the similarly conserved molecular structures of the purified chlorite dismutases have led to the suggestion that the metabolic capacity to grow by means of the dissimilation of perchlorate or chlorate is a result of lateral gene transfer in the environment, not only between gammaproteobacteria such as *P. stutzeri* and *D. chlorophilus*, but also between gamma- and betaproteobacteria (Achenbach et al. 2001; Bender et al. 2004; Coates et al. 1999).

Our results from *cld* gene analysis in strain AW-1<sup>T</sup> (phylogenetic tree, GC content, RSCU, CBI), *cld*'s presence in other strains of a different genomovar of *P. stutzeri* and its absence in all strains in our collection that have been tested, confirm the results of Bender et al. (2004) that it may have been acquired through lateral gene transfer, given the fact that this phenomenon is not uncommon in *P. stutzeri*, as shown by its capability for natural transformation (Sikorski et al. 2002a). We conclude that strain AW-1<sup>T</sup> is a *P. stutzeri* strain with the additional characteristic of growing anaerobically with chlorate as electron acceptor. Similar conclusions were obtained in the study of naphthalene degradation by *P. stutzeri* strains. This is a metabolic characteristic present in certain members of 5 different genomovars of the species, and these genes are probably transferred laterally (Cladera et al. 2004). A transposase gene located downstream of *cld* gene has been described (Coates et al. 2004) and Bosch et al. (2000) also found transposases related to the naphthalene catabolic genes in *P. stutzeri*.

'Genomovar' is an intraspecific subdivision devised to harbor a genomically coherent group of strains that cannot be distinguished phenotypically from other genomovars (Rosselló et al. 1991; Ursing et al. 1995). Each of them may be regarded as a provisional intraspecific status susceptible to be reclassified as new species once a discriminative, and stable phenotypic property can be provided. In this regard, *P. stutzeri* strain DSM50227 is the reference strain of gv.3 (Rosselló et al. 1991), and we demonstrate in this communication that strain AW-1<sup>T</sup> belongs by both genomic and physi-

ological traits to gv.3. Should gv.3 be reclassified as a new species distinct from *P. stutzeri*, then DSM50227 will become the type strain of the species, and AW-1<sup>T</sup> should be regarded as a member of that taxon. The proposal of *P. chloritidismutans* as a new species was based on the presence of a characteristic chlorate dissimilatory pathway and on the absence of a dissimilatory nitrate reduction respiration, despite the high DNA–DNA similarities shown with gv.3 reference strain (Wolterink et al. 2002). The demonstration that indeed AW-1<sup>T</sup> harbors and expresses the denitrification capability, a common trait of *P. stutzeri* strains, together with the suspicions of a horizontal transfer of the chlorate dissimilatory pathway, indicate some of the pitfalls of monothetic approaches in bacterial taxonomy (Rosselló-Mora and Amann 2001). As shown in other cases (Rosselló-Mora et al. 1993), here both traits had given undue prominence, a fact that hampered the inclusion of AW-1<sup>T</sup> within its deserved taxon. The predominance of a polythetic approach to prokaryotic taxonomy since the mid of last century had been essential for the establishment of a rather stable taxonomic classification system (Rossello-Mora and Amann 2001). Since then, it is clear that the taxonomic coherence of a species should be shown by allowing phenotypic traits to be covariant, and not immutable as in the monothetic taxonomies.

Finally, it is quite comforting to see that the genomic and phylogenetic coherence of a species is supported in this case by the nitrate dissimilatory phenotype. This trait is common within the species. It is actually a good candidate to belong to that group of genes that may constitute the genetic backbone of this species (Lan and Reeves 2000; Cladera et al. 2004). If the DNA–DNA similarity results, alone or a multigenic sequencing approach, are accepted for species delineation, then *P. stutzeri* should be split into 17 more different species, one for each described genomovar. However, this situation would not help, in our view, to clarify the taxonomic position of a phylogenetically and phenotypically coherent group of strains such as *P. stutzeri*. We do not see yet clear reasons to split *P. stutzeri* in 17 new species just because clear, stable, phenotypic properties discriminating *P. stutzeri* genomovars have not been found. Therefore, we propose to consider "*P. chloritidismutans*" a junior name of *P. stutzeri*.

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