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Genome and proteome analysis of *Pseudomonas chloritidismutans* AW-1^T that grows on *n*-decane with chlorate or oxygen as electron acceptor

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Summary

Growth of *Pseudomonas chloritidismutans* AW-1^T on C7 to C12 *n*-alkanes with oxygen or chlorate as electron acceptor was studied by genome and proteome analysis. Whole genome shotgun sequencing resulted in a 5 Mbp assembled sequence with a G + C content of 62.5%. The automatic annotation identified 4767 protein-encoding genes and a putative function could be assigned to almost 80% of the predicted proteins. The distinct phylogenetic position of *P. chloritidismutans* AW-1^T within the *Pseudomonas stutzeri* cluster became clear by comparison of average nucleotide identity values of sequenced genomes. Analysis of the proteome of *P. chloritidismutans* AW-1^T showed the versatility of this bacterium to adapt to aerobic and anaerobic growth conditions with acetate or *n*-decane as substrates. All enzymes involved in the alkane oxidation pathway were identified. An alkane monooxygenase was detected in *n*-decane-grown cells, but not in acetate-grown cells. The enzyme was found when grown in the presence of oxygen or chlorate, indicating that under both conditions an oxygenase-mediated pathway is employed for alkane degradation. Proteomic and biochemical data also showed that both chlorate reductase and chlorite dismutase are constitutively present, but most abundant under chlorate-reducing conditions.

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Introduction

Pseudomonas chloritidismutans AW-1^T is a Gram-negative, facultative anaerobic bacterium isolated from chlorate and bromate-polluted wastewater (Wolterink *et al.*, 2002). Later studies showed that this bacterium is able to degrade C7 to C12 *n*-alkanes under anaerobic conditions with chlorate as electron acceptor (Mehboob *et al.*, 2009a). Generally, anaerobic biodegradation of *n*-alkanes is slow as reported for nitrate-reducing, iron-reducing, sulfate-reducing and methanogenic conditions (Rabus *et al.*, 1999; So and Young, 2001; Davidova *et al.*, 2006; Sherry *et al.*, 2013; Tan *et al.*, 2013). Information on biochemical mechanisms of anaerobic *n*-alkane degradation is limited. While aerobic bacteria employ oxygenases for initial attack, anaerobes initiate degradation of *n*-alkanes by either fumarate addition (Grundmann *et al.*, 2008), carboxylation (So *et al.*, 2003) or by yet unknown mechanisms (Aitken *et al.*, 2013; Embree *et al.*, 2014). Knowledge of alternative capacities for anaerobic degradation of hydrocarbons is important since oil spills in the environment may lead to problematic contamination of groundwater aquifers, which are often anoxic or may become anoxic due to oxygen consumption by aerobes.

Currently, *P. chloritidismutans* AW-1^T is the only known microorganism that can efficiently degrade medium-chain *n*-alkanes (C7-C12) in the absence of oxygen, presumably by generating molecular oxygen from chlorate (Mehboob *et al.*, 2009a). Recently, methane oxidation coupled to perchlorate reduction was shown as well (Luo *et al.*, 2015). Generation of molecular oxygen from a supplied electron acceptor such as chlorate represents an alternative for the fumarate addition or carboxylation mechanism in strict anaerobes (Mehboob *et al.*, 2010). Two enzymes are involved in chlorate reduction in *P. chloritidismutans* AW-1^T. Chlorate (ClO_3^-) is first reduced to chlorite (ClO_2^-) by chlorate reductase. Chlorite is then split into chloride (Cl^-) and oxygen (O_2) by chlorite dismutase, as found in other bacteria that reduce chlorate or perchlorate (Rikken *et al.*, 1996; Carlström *et al.*,

2013). Evidence for the occurrence of intracellular oxygen transfer from chlorite dismutase to the alkane oxygenase was previously obtained, but a gene encoding an alkane oxygenase could not be identified by using an array of specific primer sets (Mehboob *et al*., 2009a).

To get more insight into the *n*-alkane and chlorate metabolism, we sequenced the genome of *P. chloritidismutans* AW-1^T. Experimental validation of the predicted genes via proteomics was described to be among the best option for identifying the protein-coding genes (Ansong *et al*., 2008). Recent studies showed that the comparison of a complete six-frame translation of genome to a proteome data set can reveal many novel genes that are overlooked by automatic annotation (Baudet *et al*., 2010; Bitton *et al*., 2011). Enzymes involved in (per)chlorate reduction have been detected in the proteome of different bacteria (Bansal *et al*., 2009) and recently a proteomics approach was used to elucidate the metabolic pathways of the chlorate-reducing *Alicycliphilus denitrificans* BC (Oosterkamp *et al*., 2013a).

Here, we present the results of a combinatorial approach to get insight into the *n*-decane degradation pathway of *P. chloritidismutans* AW-1^T in the absence of oxygen, while generating molecular oxygen from chlorate. Results obtained from proteomic analyses of strain AW-1^T were compared with biochemical data of key enzymes. We also re-evaluated the phylogenetic relationship of *P. chloritidismutans* AW-1^T with *Pseudomonas stutzeri*. Previously, *P. chloritidismutans* AW-1^T was proposed as the type strain of a novel species (Wolterink *et al*., 2002). Subsequently, by sequence analysis of the 16S rRNA genes in combination with four housekeeping genes (Cladera *et al*., 2006) suggested that *P. chloritidismutans* AW-1^T and *P. stutzeri* are the same species. However, later studies confirmed the existence of different phylogenetic classes in the *P. stutzeri* group (Scotta *et al*., 2013). The comparative genomics approach using the available genomes of six *P. stutzeri* strains supported the assignment of strain AW-1^T to *P. chloritidismutans*.

Results

Analysis of the P. chloritidismutans *AW-1^T draft genome*

Whole genome sequencing of *P. chloritidismutans* AW-1^T resulted in 5 Mbp assembled sequence data in 77 contigs, of which 56 contigs are larger than 1 kbp. Overall, the GC content of the sequenced genome was 62.5%. Evidence for the occurrence of plasmids was not obtained. The RAST annotation service (Aziz *et al*., 2008) identified 4767 protein-encoding genes and subsequently could assign putative functions to almost 80% of these. *Pseudomonas chloritidismutans* AW-1^T was proposed as the type strain of a novel species (Wolterink *et al*., 2002). Based on 16S rRNA gene sequence analysis, *P. chloritidismutans* AW-1^T is most closely related to *P. stutzeri*. The relationship between *P. chloritidismutans* AW-1^T and *P. stutzeri* has subsequently been re-evaluated in a multilocus phylogeny approach with a set of four 'housekeeping genes'. This suggested that they are the same species (Cladera *et al*., 2006). With the availability of the *P. chloritidismutans* AW-1^T draft genome, a calculation of the average nucleotide identity (ANI) between the *P. chloritidismutans* AW-1^T and the six available *P. stutzeri* genomes is possible. In general, a threshold ANI value of 95% for species circumscription is used (Goris *et al*., 2007; Richter and Rosselló-Móra, 2009). Pairwise ANI Blastn values of *P. chloritidismutans* AW-1^T and the available *P. stutzeri* strains, including A1501, ATCC 17588, DSM 4166, DSM 10701 and RCH2, showed average ANI values of $87.13 \pm 2.18\%$ and with *P. stutzeri* CCUG 29243 of 97%. In *P. chloritidismutans* AW-1^T, the complete Nar type operon for dissimilatory nitrate reduction, cellulose synthesis genes, type IV fimbrial biogenesis, chromate resistance and transport genes and CRISPR-associated family proteins are missing, which are present in *P. stutzeri* A1501. On the other hand, *P. stutzeri* A1501 and the other sequenced *P. stutzeri* strains lack the genes coding for the four subunits of chlorate reductase and chlorite dismutase. The alkane monooxygenase genes are also exclusively present in *P. chloritidismutans* AW-1^T. Physiological analysis confirmed that *P. stutzeri* A1501 is not able to grow on *n*-decane and cannot reduce chlorate or perchlorate.

Analysis of the genes coding for chlorate reduction of P. chloritidismutans *AW-1^T*

Pseudomonas chloritidismutans AW-1^T reduces chlorate to chlorite by chlorate reductase, and in a separate reaction, chlorite is converted to chloride by chlorite dismutase, while producing molecular oxygen. Both enzymes of strain AW-1^T have been characterized previously (Wolterink *et al*., 2003; Mehboob *et al*., 2009b). Since the chlorate reductase-encoding genes are relatively poorly characterized, the RAST service was not able to detect the genes encoding the four subunits of chlorate reductase directly. However, sequences that were misinterpreted by RAST to encode 'respiratory nitrate reductase alpha beta and gamma chain' were matching the N-terminal protein sequences of the alpha, beta and gamma subunits of chlorate reductase obtained from the previously purified chlorate reductase (Wolterink *et al*., 2003). The relative position of these N-terminal sequences, which were obtained from the mature alpha and gamma subunits, suggested the presence of a signal peptide for secretion of the alpha and gamma subunit, but not of the beta subunit. This was confirmed by SignalP3

(Bendtsen *et al*., 2004) and TatP analyses (Bendtsen *et al*., 2005) (Fig. S1). The alpha subunit pre-protein has a conserved twin-arginine motif located between the N-region and the hydrophobic region of the signal sequence and thus is most likely secreted by the twin-arginine translocation pathway (TAT) pathway. In contrast, the Sec secretion pathway is likely involved in secretion of the gamma subunit (Fig. S1). The beta subunit and delta subunit were predicted to have no signal peptide for secretion.

The arrangement of the *P. chloritidismutans* AW-1^T chlorate reductase encoding genes is the same as for *Ideonella dechloratans* and *A. denitrificans*, i.e. *clrABDC* (Thorell *et al*., 2003; Clark *et al*., 2013; Oosterkamp *et al*., 2013b). A transposase was found 6 kb downstream of the chlorate reductase genes, which points towards a lateral transfer of the *clrABDC* gene cluster. Like described for most genomic islands, the GC content (55%) of the 12 kb region from the *clrABDC* gene cluster up until the transposase gene is markedly different from the average GC content (62.8%) of the *P. chloritidismutans* AW-1^T genome. The chlorate reductase genes were located on a genomic island in an initial assembly of the genome of *P. chloritidismutans* based on 454-generated sequences. Similarly, in the final genome sequence, a contig (AOFQ01000041) contained proposed *clrA* and *clrB* sequences with a transposase downstream of *clrA*. These genes are located at the end of the contig, and sequences encoding ClrC and ClrD are located at the end of another contig (AOFQ01000018).

A Blastp analysis (Altschul *et al*., 1997) shows that the molybdopterin containing alpha subunit of chlorate reductase (ClrA) of *P. chloritidismutans* AW-1^T is 86% identical to a hypothetical protein of *Halomonas jeotgali* and 45% identical with the ClrA of *A. denitrificans* BC and *I. dechloratans*. Similarly, the Fe-S containing beta subunit of chlorate reductase (ClrB) of *P. chloritidismutans* is 92% identical with the chemotaxis protein CheY of *H. jeotgali* and 60% identical to ClrB of *A. denitrificans* BC and *I. dechloratans*. The gamma subunit of chlorate reductase (ClrC) has 70% identity with gamma precursor of dimethylsulfide dehydrogenase of *Pseudomonas* sp. 12M76_air and 36% identity with the ClrC of *A. denitrificans* BC. The delta subunit of chlorate reductase (ClrD) has 62% identity with hypothetical protein of *H. jeotgali* and 30% with ClrD of *Dechloromonas aromatica* RCB. In perchlorate reductases, ClrD is not part of the mature enzyme, but is a cytoplasmic chaperone required for the assembly of the enzyme (Thorell *et al*., 2003; Bender *et al*., 2005).

The gene encoding chlorite dismutase (Cld) is not part of the genomic island that contains the chlorate reductase encoding genes. In the final genome sequence, the transposase is located in between the ClrA and Cld encoding genes. According to Blastp analysis, the Cld of *P. chloritidismutans* is 98% identical to the Cld of *Dechloromonas hortensis*, 93% identical to Cld of *D. aromatica* and 59% identical to Cld of *A. denitrificans* and *I. dechloratans*.

Shotgun proteomic analysis of P. chloritidismutans *AW-1^T under five different growth conditions*

Shotgun tandem mass spectrometry is an effective high-throughput approach for protein identification and quantification (Sadygov *et al*., 2004). For effective large-scale protein identification, a complete proteome is a prerequisite. Most contig ends of the draft genome of *P. chloritidismutans* AW-1^T consist of sequence repeats hampering further assembly with the present data. However, with 25 to 30× sequence coverage of the present 77 contigs, it is likely that the vast majority of protein-encoding genes was identified.

To study the differential expression of proteins involved in chlorate and nitrate reduction and in *n*-decane utilization, three anaerobic (decane + chlorate, acetate with + nitrate, acetate + chlorate) and two aerobic (decane + oxygen, acetate + oxygen) growth conditions were selected. For each condition approximately 20 000 to 25 000 MS/MS spectra were obtained from trypsin-digested protein mixtures of cell free extracts. Overall, out of 118 362 spectra obtained 45 518 peptide spectral matches were found over the five conditions with a false discovery rate (FDR) of < 5% and a peptide-spectrum match (PSM) efficiency of 38% (Table 1), which is a reasonable value that is more often found (Ivanov *et al*., 2014). Expression data were obtained from 2133 proteins. Differential protein expression was measured by spectral counting where the number of spectra matched to peptides from a protein is used as a proxy for relative protein abundance (Liu *et al*., 2004). To study differential protein expression, a subset of 1151 proteins was

Table 1. Number of MS spectra obtained, peptide spectrum matches (PSM) and proteins detected from trypsin digested extracts of *P. chloritidismutans* AW-1^T grown under five different conditions.

	Acetate + Oxygen	Acetate + Nitrate	Acetate + Chlorate	Decane + Oxygen	Decane + Chlorate	Total
Spectra	25 214	19 580	21 988	25 228	26 352	11 8362
Number of PSM	8737	7742	8627	9459	10 953	45 518
Number of proteins detected	1298	1071	1311	1273	1442	2133

selected which, over the five growth conditions applied, have accumulated at least five PSM.

Differential expression and enzyme activities of proteins involved in electron acceptor utilization

Compared with growth of strain AW-1^T with acetate and oxygen, the expression of 65 proteins was at least threefold increased during growth with acetate and chlorate. Similarly the expression of 49 proteins was increased at least threefold in strain AW-1^T cells grown with *n*-decane and chlorate in comparison with cells grown with *n*-decane and oxygen (Table S1). Expression of seven proteins was high in chlorate-grown cells irrespectively of the carbon source. Three of these proteins are directly involved in chlorate reduction, being chlorite dismutase and the alpha and gamma subunit of chlorate reductase. Enzyme activities of chlorate reductase and chlorite dismutase were also higher in chlorate-grown cells (Table 2). The other four proteins that were increased in expression in chlorate-grown cells relative to oxygen-grown cells were outer membrane (OM) protein OprC involved in Fe/haem transport (PseChl_2938), cytochrome c oxidase *cbb3*-type dihaem CcoP subunit (PseChl_3700), pterin-4-alpha-carbinolamine dehydratase (PseChl_4640) suggested to have a role in molybdopterin cofactor metabolism (Naponelli *et al*., 2008) and enoyl-CoA hydratase (PseChl_679).

During anaerobic growth with acetate and nitrate versus aerobic growth with acetate, 96 proteins are at least threefold higher in expression (Table S1). When nitrate is added to anoxic cells of *P. stutzeri*, nitrate is reduced to nitrite (Körner and Zumft, 1989). In the subsequent denitrification pathway, NO is produced from nitrite by the enzyme Nir (nitrite reductase) which in *Pseudomonas* species is carried out by cd_1 Nir with haem as cofactor. The membrane-bound enzyme Nor (nitric oxide reductase) catalyses NO conversion into N_2O. Both enzyme systems are present in *P. chloritidismutans* AW-1^T and are strongly induced in acetate and nitrate-grown cells (Table 2).

In *P. stutzeri*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, the respiratory nitrate reductase (Nar) is used for nitrate reduction. Nar is encoded by the narGHJI operon and under the control of NarXL, a nitrate-responsive two-component system. The genes for the nitrate sensor, *narX*, and the response regulator, *narL*, are located immediately upstream of the *nar* operon in *P. stutzeri* (Härtig *et al*., 1999), *P. aeruginosa* (Schreiber *et al*., 2007) and *P. fluorescens* (Philippot *et al*., 2001). The *narGHJI* operon and upstream *narXL* nitrate sensory system are absent in the draft genome of *P. chloritidismutans* AW-1^T, but the *napDABC* operon is present and at least the NapA catalytic subunit is shown to

Table 2. Number of PSM detected and enzyme activity (in bold) of key enzymes in *P. chloritidismutans* AW-1^T grown under five different growth conditions.

	Protein (number)	Acetate + oxygen	Acetate + nitrate	Acetate + chlorate	Decane + oxygen	Decane + chlorate
Number of PSM	Chlorate reductase – alpha subunit (PseChl_4948)	15	47	95	48	158
	Chlorate reductase – beta subunit (PseChl_4949)	10	13	31	18	50
	Chlorate reductase – gamma subunit (PseChl_4951)	4	0	17	2	12
	Chlorate reductase – delta subunit (PseChl_4950)	1	2	11	2	4
Activity (U mg^{-1})	**Chlorate reductase**	**1.7 ± 0.1**	**10.6 ± 0.9**	**21.3 ± 0.1**	**0.47 ± 0.04**	**46.3 ± 3.2**
Number of PSM	Chlorite dismutase (PseChl_5209)	25	24	100	5	121
Activity (U mg^{-1})	**Chlorite dismutase (PseChl_5209)**	**0.15 ± 0.01**	**7.9 ± 1.5**	**6.8 ± 1.2**	**0.25 ± 0.0**	**7.4 ± 2.1**
Number of PSM	Nitrate reductase – catalytic subunit (PseChl_3101)	2	36	17	20	29
Activity (U mg^{-1})	**Nitrate reductase**	**bdl**	**0.73 ± 0.10**	**bdl**	**bdl**	**bdl**
Number of PSM	Cytochrome cd1 nitrite reductase (PseChl_4607)	2	195	6	1	0
Activity (U mg^{-1})	**Nitrite reductase**	**bdl**	**0.23 ± 0.42**	**0.0021 ± 0.00046**	**n.d**	**n.d**

PSM, Peptide Spectrum Match; n.d, not determined; bdl, below detection limit.

be expressed in nitrate-grown cells (Table 2). Since purified chlorate reductase from *P. chloritidismutans* AW-1^T cannot use nitrate as electron acceptor (Wolterink *et al.*, 2003), this periplasmic Nap nitrate reductase is most probably responsible for anaerobic nitrate reduction in acetate and nitrate-grown cells in *P. chloritidismutans* AW-1^T. Nitrate and nitrite reductase activities under these different growth conditions are also presented in Table 2.

Differential expression of proteins involved in n*-decane degradation*

A total of 97 proteins showed at least threefold more expression in *n*-decane and chlorate- versus acetate and chlorate-grown cells. Out of these proteins, at least 17 proteins were only detected in *n*-decane-grown cells (Table S1). When comparing oxygen-grown *n*-decane and oxygen-grown acetate cells, a total of 124 proteins were threefold more expressed when grown with *n*-decane, while 11 proteins were exclusively detected in *n*-decane-grown cells.

Alkane hydroxylation is the key step in *n*-alkane degradation. In *P. chloritidismutans* AW-1^T, alkane monooxygenase (PseChl_5262) is an integral membrane protein that is exclusively detected in cell-free extracts of *n*-decane-grown cells. Like all other members of this superfamily, there are eight conserved histidines within three His boxes and a HYG motif that is highly conserved in AlkB hydroxylases. These histidine residues are reported to be catalytically essential and proposed to be the ligands for the iron atoms (Fig. S2). We neither found any other alkylsuccinate synthase nor any other alkane oxygenase or cytochrome P450 genes in the genome or proteome that might be involved in alkane metabolism. Table 3 shows the number of PSMs found for alkane monooxygenase and other proteins and enzymes possibly involved in transport and further oxidation of *n*-decane. The terminal oxidation of *n*-alkanes by alkane monooxygenase generates primary fatty alcohols, which are further oxidized to aldehydes and fatty acids by alcohol and aldehyde dehydrogenases. Table 3 lists alcohol and aldehyde dehydrogenases that may be involved in *n*-decane degradation. Among the enzymes likely involved in β-oxidation, most of the enzymes listed in Table 3 were highly expressed during growth with *n*-decane. Based on proteome analysis, proteins PseChl_3353 to 3354 and PseChl_4798 to PseChl_4803 are putatively involved in the degradation of fatty acids (Table 3; Table S1).

In Gram-negative bacteria, the lipopolysaccharide layer in the OM is an efficient barrier for hydrophobic molecules, such as *n*-alkanes, long-chain fatty acids and aromatic hydrocarbons destined for biodegradation (Nikaido, 2003). Transport of these molecules across the OM requires an OM transport protein of the OMPP1/FadL/TodX family. The *P. chloritidismutans* AW-1^T homologue of this OM transport protein, PseChl_3904, is strongly induced in *n*-decane-grown cells.

Discussion

Pseudomonas chloritidismutans *AW-1^T draft genome*

Pseudomonas chloritidismutans AW-1^T is a Gram-negative, facultative anaerobic, chlorate-reducing

Table 3. Differential expression of proteins possibly involved in *n*-decane degradation by *P. chloritidismutans* strain AW-1^T.

	Peptide spectrum matches under the indicated growth conditions					
Protein	Ace/Oxy	Ace/Nit	Ace/Chl	Dec/Oxy	Dec/Chl	Annotation
PseChl_5262	0	0	0	13	12	Alkane-1-monooxygenase (EC 1.14.15.3)
PseChl_5274	0	0	0	33	24	Choline dehydrogenase (EC 1.1.99.1)
PseChl_1086	2	3	2	6	4	Alcohol dehydrogenase (EC 1.1.1.1)
PseChl_2870	2	0	18	29	16	Quinoprotein alcohol dehydrogenase
PseChl_2941	1	10	6	13	8	Alcohol dehydrogenase (EC 1.1.1.1)
PseChl_3299	0	4	0	0	9	Alcohol dehydrogenase (EC 1.1.1.1)
PseChl_3821	0	1	3	2	5	Alcohol dehydrogenase (EC 1.1.1.1)
PseChl_4648	9	12	9	10	19	Alcohol dehydrogenase (EC 1.1.1.1)
PseChl_1330	1	4	8	10	12	Aldehyde dehydrogenase (EC 1.2.1.3)
PseChl_3577	2	24	7	28	38	Aldehyde dehydrogenase (EC 1.2.1.3)
PseChl_3833	7	2	4	4	10	Aldehyde dehydrogenase (EC 1.2.1.3)
PseChl_5170	0	0	0	13	13	Aldehyde dehydrogenase (EC 1.2.1.3)
PseChl_5028	4	3	7	51	42	Acyl-CoA dehydrogenase
PseChl_4798	0	0	0	26	27	Acyl-CoA dehydrogenase-like
PseChl_4800	0	0	0	7	37	AMP-dependent synthetase and ligase
PseChl_3353	10	18	15	103	135	Enoyl-CoA hydratase (EC 4.2.1.17)
PseChl_3626	0	0	0	2	5	3-hydroxyacyl-CoA dehydrogenase
PseChl_3354	4	5	6	30	25	3-ketoacyl-CoA thiolase (EC 2.3.1.16)
PseChl_4799	0	0	0	12	24	3-ketoacyl-CoA thiolase (EC 2.3.1.16)

Ace/Oxy, acetate/oxygen; Ace/Nit, acetate/nitrate; Ace/Chl, acetate/chlorate; Dec/Oxy, *n*-decane/oxygen; Dec/Chl, n-decane/chlorate.

bacterium originally isolated with acetate and chlorate (Wolterink *et al*., 2002). Strain AW-1^T is a versatile organism that can also degrade C7 to C12 *n*-alkanes in the presence of oxygen or under anaerobic conditions with chlorate as electron acceptor (Mehboob *et al*., 2009a). The ability to use chlorate as an electron acceptor is a characteristic that distinguishes *P. chloritidismutans* AW-1^T from other *Pseudomonas* species. Up to date 59 *Pseudomonas* genome sequences can be retrieved form the NCBI sequence repository (ftp://ftp.ncbi.nih.gov/genbank/genomes/Bacteria/) and on the average these genomes code for approximately 4889 proteins. The draft genome of *P. chloritidismutans* AW-1^T is no exception to that and harbours 4767 protein-encoding genes, which is more than the 3815 to 4300 protein encoding genes of the six closest related *P. stutzeri* strains. Comparison of the ANI of several *P. stutzeri* strains and *P. chloritidismutans* AW-1^T shows that *P. chloritidismutans* AW-1^T and *P. stutzeri* CCUG 29243 are distinct form five other *P. stutzeri* strains. Further full genome-based phylogeny is needed to clarify the phylogenetic position of *P. stutzeri* and *P. chloritidismutans* subgroups.

The chlorate reduction pathway of strain AW-1^T

Pseudomonas chloritidismutans AW-1^T has the ability to produce molecular oxygen by the chlorate reduction pathway. None of the other sequenced *Pseudomonas* genomes and plasmids encodes genes of the two key enzymes involved in chlorate reduction, chlorate reductase and chlorite dismutase, encoded by the *clrABDC* and *cld* genes respectively. Thus, this physiological trait differentiates *P. chloritidismutans* AW-1^T from other sequenced *Pseudomonas* species. An alignment of the protein sequences of the alpha, beta and gamma subunits of chlorate reductase of *P. chloritidismutans* with the N-terminal end of the alpha, beta and gamma subunits of the purified mature enzyme not only confirmed the identity of the Clr encoding genes, but also suggests that two secretion pathways are used in parallel to export the ClrABC enzyme to the periplasmic space. ClrB most likely first forms a complex with ClrA since there is no evidence for a signal peptide for ClrB. Like in *I. dechloratans* the ClrD protein may function as a cytoplasmic chaperone required for formation of this complex (Thorell *et al*., 2003; Bender *et al*., 2005). Subsequently, the TAT pathway is likely used to export the ClrAB complex, while the ClrC subunit uses the Sec pathway.

Table 2 shows that the enzymes directly involved in chlorate reduction, i.e. chlorate reductase and chlorite dismutase are constitutive. An increased abundance of all the subunits of chlorate reductase and of chlorite dismutase, and a concomitant increase in activity of both enzymes was observed during growth with chlorate. These results are in agreement with previous findings. Although the chlorate reductase of *P. chloritidismutans* AW-1^T appears to be oxygen sensitive (Wolterink *et al*., 2003), the bacterium can simultaneously reduce chlorate and oxygen when oxygen is added to a chlorate-reducing culture (Wolterink *et al*., 2002). Similarly, the chlorate reductase of *Pseudomonas* sp. PDA is reported to be constitutive (Steinberg *et al*., 2005).

Some other proteins essential for efficient chlorate reduction also have an increased abundance. Since chlorite dismutase is a haem-containing protein, an increase in the formation of haem is expected. Evidence for that is an increased abundance of the oxygen-dependent coproporphyrinogen III oxidase (PseChl_5183) involved in haem formation. We also found evidence for expression of PseChl_4604. Protein sequence PseChl_4604 is a mature soluble 10.9 kDa periplasmic cytochrome preceded by a predicted N-terminal signal peptide. This 8.6 kDa cytochrome shares the motif KLVGPxxKDVAAK with a soluble 9 kDa periplasmic cytochrome *c* involved in periplasmic chlorate reduction in *I. dechloratans* (Bäcklund *et al*., 2009; Bäcklund and Nilsson, 2011).

Nitrate reductase of strain AW-1^T

Pseudomonas chloritidismutans AW-1^T is most closely related to *P. stutzeri*. The six finished *P. stutzeri* genomes all encode the well-studied Nar operon involved in dissimilatory nitrate reduction. *Pseudomonas chloritidismutans* AW-1^T, which has previously been described as a non-denitrifying species, lacks this operon completely. Nevertheless, Cladera and colleagues (2006) and we have shown that variants able to grow anaerobically with nitrate are readily obtained, suggesting that the Nap periplasmic nitrate reductase can be used as a substitute under anaerobic conditions. Notably, the Nar nitrate reductase can also reduce chlorate (Bell *et al*., 1990). Due to the toxic effects of chlorite accumulation in the cytoplasm, a bacterial capability to generate molecular oxygen from chlorate may be not compatible with the presence of a cytoplasm-oriented, chlorate-reducing Nar nitrate reductase. The Nap nitrate reductase, in contrast, is not cytoplasm oriented and does not reduce chlorate (Oosterkamp *et al*., 2011).

Enzymes of strain AW-1^T involved in n-alkane degradation

Pseudomonas chloritidismutans AW-1^T is known to degrade *n*-alkanes via an oxygenase mediated pathway (Mehboob *et al*., 2009a). From Table 3, it is clear that an alkane monooxygenase (PseChl_5262) is exclusively present when the bacterium is grown with *n*-decane. The sequence of alkane monooxygenase of strain AW-1^T is

79% identical with a fatty acid desaturase of *P. aeruginosa* and 70% identical with the alkane monooxygenase of the oil-degrading bacterium *Oleispira antarctica* (Kube *et al*., 2013).

Rubredoxin (Rd) is a redox-active protein. One of its functions is storing and transferring electrons to alkane monooxygenase from Rd reductase. In *P. chloritidismutans* AW-1^T, Rd and Rd reductase are fused into a single open reading frame (PseChl_651). We were unable to find an alcohol oxygenase in the proteome as we postulated before (Mehboob *et al*., 2009a). Instead, we found a number of different alcohol dehydrogenases some of which are mainly present during growth with *n*-decane.

Apart from these proteins, which are directly involved in alkane degradation some other proteins putatively involved in alkane degradation are also abundant. PseChl_4162 is a porin of the oprD superfamily and strongly induced upon growth with *n*-decane, but under aerobic conditions only. PseChl_3904 appears to be a long chain fatty acid OM transporter and is strongly induced in *n*-decane-grown cells both under aerobic and chlorate-reducing conditions.

Taking into account the localization of chlorate reductase, differential expression of subunits of chlorate reductase, chlorite dismutase, ubiquinol-cytochrome-*c*-reductase, cytochrome *cbb3* oxidase, rubredoxin, rubredoxin reductase and presence and expression of an 8.6 kDa cytochrome in the genome, we propose that *n*-decane degradation with chlorate as electron acceptor proceeds in *P. chloritidismutans* AW-1^T as presented in Fig. 1.

The presence of oxygenases and the absence of anaerobic hydrocarbon degradative enzyme systems in the *D. aromatica* RCB (Salinero *et al*., 2009), *A. denitrificans* BC (Oosterkamp *et al*., 2013b) and *P. chloritidismutans* AW-1^T (present study) suggests that these hydrocarbon-degrading bacteria employ oxygenase-mediated pathways while generating oxygen via chlorite dismutation.

Concluding remarks

This is the first report of a proteomic analysis of a bacterium able to degrade *n*-alkanes in the absence of external oxygen, generating oxygen via chlorite dismutation. This study provides evidence that the previously postulated oxygenase-mediated pathway is employed by *P. chloritidismutans* when grown with *n*-alkanes and chlorate (Mehboob *et al*., 2009a). It is further shown that there are separate pathways for the reduction of chlorate and nitrate and that chlorate reductase and chlorite dismutase activity is increased when the bacterium is grown with chlorate and decreased when grown with oxygen.

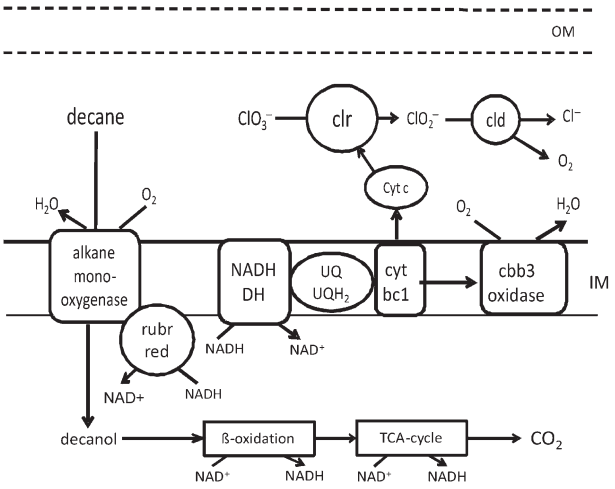

Fig. 1. Proposed model of anaerobic *n*-decane degradation. After reduction of chlorate by chlorate reductase the resulting chlorite is decomposed into chloride ions and molecular oxygen. Under anaerobic conditions, the produced oxygen can be used by alkane-1-monooxygenase to oxidize decane to decanol. OM, outer membrane; IM, Inner membrane; clr, chlorate reductase; cld, chlorite dismutase; Cyt C, 8.6 kDa periplasmic cytochrome; cbb3 oxidase, cbb3-type cytochrome c oxidase; UQ/UQH2, ubiquinone/ubihydroxyquinone; rubr/red, rubredoxin and rubredoxin reductase, which in *P. chloritidismutans* AW-1^T are fused into a single protein.

Experimental procedures

Strains, media and cultivation

Pseudomonas chloritidismutans strain AW-1^T (DSM 13592^T) was isolated in our laboratory (Wolterink *et al*., 2002). For experiments with nitrate, it was adapted to nitrate as described (Mehboob *et al*., 2009a). *Pseudomonas stutzeri* strain A1501 (GenBank Accession No. CP000304) was obtained from the Collection of Institute Pasteur (CIP, Paris, France).

Pseudomonas chloritidismutans strain AW-1^T was grown in medium as described previously (Mehboob *et al*., 2009a) and cultivated at 30°C in 1 l flasks containing 600 ml of medium. For anaerobic growth experiments, the medium was prepared with anoxic water and dispensed in the flasks under continuous flushing with nitrogen. The bottles were closed with butyl rubber stoppers and aluminum caps, and the headspace was replaced with N_2 gas (140 kPa). All solutions that were added to the medium were anoxic and autoclaved at 121°C for 20 min. Sodium salts of chlorate and nitrate were supplied from a 0.4 M stock solution to a final concentration of 10 mM.

Preparation of cell-free extracts

All the samples were taken during the late logarithmic phase and cell free extracts of strain AW-1^T were prepared under anaerobic conditions as previously described (Mehboob *et al*., 2009a). Cell free extracts were stored in 12 ml serum vials at −80°C for proteomic analysis and under anaerobic conditions at –20°C for biochemical analysis.

The protein content of the cell free extract fraction was determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions with bovine serum albumin as standard.

Enzyme activity measurements

Cell-free extract was used to assay chlorate reductase, nitrate reductase and nitrite reductase activities spectrophotometrically in triplicates as described previously (Kengen *et al.*, 1999). The substrate-dependent oxidation of reduced methyl viologen was monitored at 578 nm and 30°C. One unit (U) of enzyme activity is defined as the amount of enzyme required to convert 1 μMol of substrate per minute.

Chlorite dismutase activity was determined by measuring chlorite-dependent oxygen production with a Clark-type oxygen electrode (Yellow Spring Instruments, Yellow Springs, OH, USA) as described previously (Wolterink *et al.*, 2002). One unit (U) of activity is defined as the amount of enzyme required to convert 1 μMol of chlorite per minute.

Genome sequencing and annotation

The genome *P. chloritidismutans* strain AW-1^T was sequenced to 20× depth of coverage using both Roche 454 pyrosequencing with GS FLX Titanium chemistry and Illumina HiSeq. An initial draft genome sequence based on the 454 pyrosequencing data was assembled using the Newbler assembler (454 Life Sciences) into 112 contigs. This genome sequence was improved by combining the 454 reads with Illumina HiSeq reads. These sequence reads were assembled into contigs using the Ray assembler (version 2.1.0) which resulted in a final genome sequence consisting of 5.0 Mbp assembled sequence data in 77 contigs (Boisvert *et al.*, 2010). A 25 to 30× sequence coverage of the contigs was obtained. The genome sequences were automatically annotated using the RAST tool and in the NCBI prokaryotic genome annotation pipeline (Angiuoli *et al.*, 2008; Aziz *et al.*, 2008). ANI Blastn values were calculated with JSpecies (Richter and Rosselló-Móra, 2009). Signal peptides were predicted using the SignalP 3.0 and TatP software (Bendtsen *et al.*, 2004; 2005). The genomic sequences of *P. chloritidismutans* strain AW-1^T have been deposited in GenBank under the Accession No. NZ_AOFQ00000000.

Sample preparation for tandem-mass spectrometry

A comparative analysis of the proteome of cells grown under the five different conditions (acetate with oxygen, nitrate or chlorate and decane with oxygen or chlorate) was made. Equal amounts of each sample (250 μg protein) were separated on 12% SDS polyacrylamide gels, and gels were stained according to the manufacturer's protocol using Colloidal Coomassie brilliant blue staining (Invitrogen, Carlsbad, CA, USA). Each of the gel lanes was cut into five slices, and slices were cut into small pieces of approximately 1 mm^3. After washing twice with ultra-pure water, gel samples were treated with 50 mM dithiothreitol (DTT) in 50 mM NH_4HCO_3 (pH 8.0) for 1 h at 60°C. DTT solution was decanted and samples were alkylated with 100 mM iodoacetamide in NH_4HCO_3 (pH 8.0) for 1 h at room temperature in the dark with occasional mixing. The iodoacetamide solution was decanted and samples were washed with NH_4HCO_3 (pH 8.0). Gel pieces were rehydrated in 10 ng μl^{-1} trypsin (sequencing grade modified trypsin; Promega, Madison, WI, USA) and digested overnight at 37°C. To maximize peptide extraction, the solution from trypsin digest was transferred to new tubes, and gel pieces were subjected to two rounds of 1 min sonication, the first round with 5% trifluoroacetic acid (TFA) and the second round with 15% acetonitrile and 1% TFA. After each of these two rounds, solutions were removed and added to the original trypsin digests. Samples were analysed with an nLC (Proxeon EASY) connected to a LTQ-Orbitrap XL (Thermo electron) (Lu *et al.*, 2011).

Mass spectrometry database searching

The resulting spectra from the MS analysis were submitted to a local implementation in the Open Mass Spectrometry Search Algorithm (OMSSA) search engine (Geer *et al.*, 2004). MS/MS spectra were searched against a protein database derived from a six-frame translation of *P. chloritidismutans* strain AW-1^T and a decoy reverse database constructed from the reverse of all the six frame translation output.

All OMSSA searches used the following parameters: a precursor ion tolerance of 0.2 Da, fragment ion tolerance of 0.3 Da, a missed cleavage allowance of up to and including 2, fixed carbamide methylation, variable oxidation of methionine and deamination of glutamine and asparagine.

The expect value threshold was determined iteratively from the FDR and was set at 0.01. With this setting an FDR of $< 5\%$ is expected.

The FDR was calculated as follows: PSM were ranked by their E-value for each identified spectrum with a threshold E-value < 0.01 and the top hit identified peptide sequence was selected. Top hit spectral matches to peptides in the reversed database were used for FDR calculation as described (Elias and Gygi, 2007). Unambiguously assigned peptides were used to identify expressed proteins. Spectral counting was subsequently used for comparative quantification (Liu *et al.*, 2004). A G-test ($P < 0.025$) was used to identify if the changes in expression between different growth conditions are significant.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Identification of chlorate reductase encoding genes in *P. chloritidismutans* AW-1^T. Alignment of the predicted ClrA, ClrB and ClrC protein sequences with N-terminal sequences of the alpha, beta and gamma subunits of the purified enzyme (Accession No. P83448-50). Underlined: Putative signal peptide predicted by TatP (ClrA) and SignalP3 (ClrC). The conserved twin-arginine motif [regular expression: RR.(FGAVML)(LITMVF)] located between the N region and the hydrophobic region of the signal sequence is indicated in bold.

Fig. S2. Alignment of alkane-1-monooxygenases. AlkB hydroxylases have eight conserved and catalytically essential histidines (*) within three His boxes (Shaded). P_chlor, *Pseudomonas chloritidismutans* AW-1^T alkane-1-monooxygenase (PseChl_5262); *P_mendo*, *P. mendocina* ymp (YP001188029); A_ADP1, *Acinetobacter* ADP-1 AlkM (AJ002316); P_GPO1, *P. putida* GPo1 Alk B (AJ245436); P_aerug, *P. aeruginosa* Alk B1 (NP251264).

Table S1. Abundances of proteins of P. chloritidismutans strain AW-1T grown under 5 different growth conditions.