# Growth of *Pseudomonas chloritidismutans* AW-1$^T$ on n-alkanes with chlorate as electron acceptor

**Farrakh Mehboob, Howard Junca, Gosse Schraa, Alfons J.M. Stams[1]**

F. Mehboob, G. Schraa, A. J. M. Stams ( ✉ )
Laboratory of Microbiology, Wageningen University,
Dreijenplein 10, 6703 HB,
Wageningen, The Netherlands.
e-mail: fons.stams@wur.nl.
Tel.: (+31) 317 483101
Fax: +(31) 317 483829.

Howard Junca
AG Biodegradation, Helmholtz-Zentrum für Infektionsforschung,
Inhoffenstraße 7/ D-38124,
Braunschweig, Germany.

**Abstract**

Microbial (per)chlorate reduction is a unique process in which molecular oxygen is formed during the dismutation of chlorite. The oxygen thus formed may be used to degrade hydrocarbons by means of oxygenases under seemingly anoxic conditions. Up to now no bacterium has been described that grows on aliphatic hydrocarbons with chlorate. Here, we report that *Pseudomonas chloritidismutans* AW-$1^T$ grows on n-alkanes (ranging from C7 until C12) with chlorate as electron acceptor. Strain AW-$1^T$ also grows on the intermediates of the presumed n-alkane degradation pathway. The specific growth rates on n-decane and chlorate and n-decane and oxygen were 0.5 $\pm$ 0.1 and 0.4 $\pm$ 0.02 $day^{-1}$, respectively. The key enzymes chlorate reductase and chlorite dismutase were assayed and found to be present. The oxygen dependent alkane oxidation was demonstrated in whole cell suspensions. The strain degrades n-alkanes with oxygen and chlorate, but not with nitrate, thus suggesting that the strain employs oxygenase-dependent pathways for the breakdown of n-alkanes.

## Introduction

Petroleum, a complex mixture of aromatic and aliphatic hydrocarbons, is one of the most common environmental contaminants. On average, saturated and aromatic hydrocarbons together make 80 % of the oil constituents (Widdel and Rabus 2001). Since the saturated hydrocarbon fraction is the most abundant in crude oil, its biodegradation is quantitatively most important in oil bioremediation (Head et al. 2006). n-Alkanes are relatively stable due to lack of functional groups, presence of only sigma bonds, non-polar nature and low solubility in water.

Aerobic microbial degradation of n-alkanes is known since almost a century (Söhngen 1913), and the mechanisms of degradation, with the enzymes and genes involved, are rather well understood (Berthe-Corti and Fetzner 2002; Head et al. 2006; van Beilen and Funhoff 2007; Wentzel et al. 2007). During aerobic degradation, molecular oxygen acts as a co-substrate and as a terminal electron acceptor (Berthe-Corti and Fetzner 2002; Chayabutra and Ju 2000). Oxygenases incorporate molecular oxygen into the n-alkanes to form the corresponding alcohols, which are further degraded by beta oxidation (Wentzel et al. 2007). Since intermediates do not accumulate, the initial step of oxygen incorporation seems to be the rate limiting step. (Chayabutra and Ju 2000).

Insight into anaerobic degradation of n-alkanes is limited. The first step of anaerobic degradation of n-alkanes is thermodynamically difficult, and has been proposed to occur in the sulfate-reducing bacterium strain Hxd3 via carboxylation (So et al. 2003). Molecular evidence for a mechanism of n-alkane activation through fumarate addition was obtained recently (Callaghan et al. 2008; Grundmann et al. 2008). Anaerobic degradation of n-alkanes is slow compared to aerobic degradation

(Wentzel et al. 2007), and only a few denitrifying and sulfate-reducing bacteria have been isolated (Ehrenreich et al. 2000; So and Young 1999).

Microbial (per)chlorate reduction is a process that yields molecular oxygen, a property that has application possibilities in the bioremediation of polluted anoxic soils (Coates et al. 1998; Tan et al. 2006; Weelink et al. 2008). During chlorate reduction, chlorate ($ClO_3^-$) is reduced to chlorite ($ClO_2^-$) by the enzyme chlorate reductase. Chlorite is then split into $Cl^-$ and $O_2$ by chlorite dismutase (Rikken et al. 1996; Wolterink et al. 2002). The oxygen released during chlorate reduction might be used to degrade n-alkanes by oxygenases.

Here we report the finding that *Pseudomonas chloritidismutans AW-1*$^T$, a chlorate reducing bacterium, that was previously isolated in our laboratory with acetate as carbon and energy source is able to grow on n-decane with oxygen or chlorate as electron acceptor. This finding suggests that an additional function of chlorite dismutation is to generate molecular oxygen to perform oxygenase-dependent reactions to support growth on n-alkanes.

## Materials and Methods

### Inoculum, media, cultivation and counting

*Pseudomonas chloritidismutans* strain AW-1$^T$ (DSM 13592$^T$) was isolated in our laboratory (Wolterink et al. 2002) and was kindly provided by Servé Kengen. For experiments with nitrate, it was adapted to nitrate by repeated sub-culturing on acetate and nitrate, while gradually decreasing the oxygen concentration according to Cladera et al. (2006).

The medium for *P. chloritidismutans* strain AW-1$^T$ was based on the medium described by Dorn et al. (1974). The composition of the medium (in grams per liter of anaerobic demineralized water) was as follows: $Na_2HPO_4.2H_2O$, 3.48; $KH_2PO_4$ 1; resazurin, 0.005; $CaCl_2$, 0.009; Ammonium Iron (III) citrate, 0.01; $NH_4SO_4$, 1; $MgSO_4.7H_2O$, 0.04. Vitamins and trace elements were added as described by Holliger et al. (1993) supplemented with $Na_2SeO_3$, 0.06; $NaWO_4.2H_2O$ 0.0184. The pH of the medium was 7.3.

*P. chloritidismutans* strain AW-1$^T$ was cultivated in 120-ml flasks containing 40 ml of medium at 30 $^oC$. The medium was made in anaerobic water and dispensed in the flasks under continuous flushing with nitrogen. The bottles were closed with viton stoppers (Maag Technik, Dübendorf, Switzerland) and aluminum crimp caps, and the head space was replaced by $N_2$ gas (140 kPa). All solutions that were added to the medium were made anaerobic and autoclaved at 121 $^oC$ for 20 minutes. The $CaCl_2$ was autoclaved separately to avoid precipitation and added aseptically to the already autoclaved salt solution. Vitamins and trace elements were filter sterilized. Chlorate and nitrate were supplied from an 0.4 M stock solution to get a final concentration of 10 mM. Pure oxygen was added from a sterilized gas stock. To prepare a stock solution of n-decane, a flask was made anaerobic by flushing with $N_2$ and then autoclaved. 10 ul (1.28 mM final concentration) of sterile 99 % pure n-decane (Merck) was added to the medium through a 0.2 micron membrane filter. For mass balance analyses 1 mM of n-decane was added from a 50 mM stock solution of n-decane in acetone. The inoculum size for cultivation was 10 % (v/v). Other n-alkanes tested were n-propane, n-butane, n-pentane, n-hexane, n-heptane, n-octane, n-nonane, n-undecane, n-dodecane, n-tetradecane and n-hexadecane. 1-decanol was added from an anaerobic filter sterilized solution, while sodium decanoate was added from an anaerobic, autoclaved 0.4 M stock solution.

Cell numbers were enumerated by phase contrast microscopy using a Bürker-Türk counting chamber at 1000X magnification.

Three aerobic alkane utilizing bacteria, *Alcanivorax borkumensis* SK2 (DSM 11573), *Acinetobacter* sp. strain (DSM 17874) and *Acinetobacter baylyi* (DSM 14961), and the non-alkane degrading *Pseudomonas putida* KT2440 (DSM 6125) were obtained from the DSMZ, Braunschweig, Germany.

**Preparation of cell free extracts**

Cell free extracts of strain AW-1$^T$, grown in anaerobic medium with n-decane as sole carbon and energy source and chlorate as electron acceptor, were prepared anaerobically as previously described by Wolterink et al. (2002). The only modification was the centrifugation of whole cells at 13000 rpm for 10 minutes at 4 °C. Cell free extracts were stored under a $N_2$ gas phase at 4 °C in 12 ml serum vials.

The protein content of the cell free extract fraction was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

**Enzyme activity measurements**

Chlorate reductase and chlorite dismutase activities were determined with cell free extracts. Chlorate reductase activity was determined spectrophotometrically as described by Kengen et al. (1999), by monitoring the oxidation of reduced methyl viologen 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 chlorate per minute.

Chlorite dismutase activity was determined by measuring oxygen production with a Clark-type oxygen electrode (Yellow Spring Instruments, Yellow Springs, Ohio, USA) as described by 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.

Alkane oxidation activity was determined by measuring the decrease in n-decane concentration in time by gas chromatography with whole cell suspensions. Cells were harvested by centrifugation at 13000 rpm for 10 min. at 4 °C. Cells were washed and suspended in the buffer and then starved for two days to decrease the endogenous activity. After starvation cells were suspended in a 15 mM phosphate buffer containing 2.5 mM n-decane added from a 50 mM stock solution in acetone. The reaction mixture was placed in a shaker set at 180 rpm at 30 °C. Two-ml samples were taken in duplicate periodically after 0, 5, 10, 15, and 30 minutes. The n-decane was extracted as described by Staijen et al. (2000). Cells treated for 10 minutes at 100 $^{o}$C were used as controls. One unit (U) of activity is defined as the amount of enzyme required to convert 1 µmol of n-decane per minute. Starved cells, resuspended in the anoxic buffer, flushed with nitrogen and reduced with 3 mM cystein were used as anaerobic control.

Alcohol dehydrogenase activity was determined spectrophotometrically in the reductive direction at 30 °C using a spectrophotometer (U-2010, Hitachi). The activities were assayed in 1 ml reaction mixtures containing 15 mM sodium phosphate buffer with 0.3 mM NADH, and 0.5 mM aldehyde. The decrease in $A_{340}$ was monitored to assess the activity. One unit of enzyme activity is defined as the amount enzyme required to oxidize one µmol of NADH. We also tried to determine the alcohol oxygenase activity by the above mentioned spectrophotometric method using NADH and air flushed reaction mixtures.

**Analytical techniques**

Chlorate, chloride, nitrate, nitrite were measured, as described by Scholten and Stams (1995) after separation on a Dionex column (Ionpac AS9-SC) (Breda, The

Netherlands), with a conductivity detector. Potassium fluoride (2 mM) was used as internal standard.

Oxygen was analyzed by gas chromatography with a GC-14B apparatus (Shimadzu, Kyoto, Japan) equipped with a packed column (Molsieve 13x 60/80 mesh, 2 m length, 2.4 mm internal diameter; Varian, Middelburg, The Netherlands) and a thermal conductivity detector. The oven temperature used was 100 °C and the injector and detector temperatures were 90 and 150 °C respectively. Argon was used as the carrier gas at a flow rate of 30 ml $min^{-1}$.

$CO_2$ was analyzed by gas chromatography on a Chrompack CP9001 gas chromatograph fitted with a TCD detector (Henstra and Stams 2004). pH was measured by a glass microelectrode connected to a pH meter (Radiometer, Copenhagen). Total amount of bicarbonate present inside the flask was calculated by using the Henderson-Hesselbach equation. At 30 °C, the α and pK' are 0.665 and 6.348, respectively (Breznak and Costilow 1994).

n-Decane was extracted from 40 ml culture with 20 ml of n-hexane by shaking for 3 hours, and then separating the two phases through a separating funnel. n-Decane was analyzed in the hexane phase after adding octane as internal standard. One microliter was injected with a CP9010 autosampler in a CP9001 gas chromatograph (Chrompack), equipped with FID detector and having a Chrompack Sil 5 CB capillary column (length, 25 m; diameter, 0.32 mm; df, 1.2 um) with nitrogen, 50 kPa inlet pressure, as carrier gas. The temperature of the injector, column and the detector was 250, 100 and 300 °C, respectively.

**Detection of alkane oxygenase genes**

For the detection of putative genes encoding alkane oxygenase, the genomic DNA was extracted from cultures of strain AW-$1^T$ grown on n-decane and chlorate using a

FastDNA SPIN kit for Soil (Qbiogene). The extracted DNA was precipitated with isopropanol and vacuum dried. The primers developed by Whyte et al. (2002), Heiss-Blanquet et al. (2005), Kloos et al. (2006) and van Beilen et al. (2006) were used for the detection of the alkane oxygenases *alkB, alkM, almA, P450*. In addition, we developed some degenerated primers targeting *alkB, almA* and Acyl CoA dehydrogenase. PCR was carried out under the following conditions: final volume of 50 µl of 1X PCR Buffer (Promega) supplemented with $MgCl_2$, (2.5 mM), 200 µM dNTP´s, 0.5 µM of each primer and 0.3 U/µl Taq DNA Polymerase (Promega). The amplification conditions used were as follows: an initial denaturation step at 94 °C for 3 min, 35 times of a three steps cycle of 94 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 minute, a final elongation step of 72 °C for 8 min. Polymerization reactions were stopped by cooling the samples at 4 °C. In addition, a gradient PCR using a temperature gradient of 40-65 °C was done with the *alkB* and *Cyt P450* primers used above and also with the primers designed by Smits et al. (1999), Smits et al. (2002), Kohno et al. (2002), and the degenerate primers used by Kubota et al. (2005). Sequences of the primers used in this study are given in table 1. The positive controls of amplification were obtained in reactions with primers targeting *alkB*, and CYP153 genes using genomic DNA extracted from *Alcanivorax borkumensis* SK2, in reactions with primers targeting *almA*, with *Acinetobacter* sp. strain DSM 17874 genomic DNA and with *Acinetobacter baylyi* DSM 14961 in reactions targeting *alkM*.

**Nucleotide sequence accession numbers**

The DNA sequence of a putative acyl-CoA dehydrogenase gene of *P. chloritidismutans* AW-$1^T$ was deposited in the GenBank/EMBL/DDBJ under accession number FJ477383.

## Results

### n-Alkane degradation

*Pseudomonas chloritidismutans* AW-1$^T$ uses n-decane as a sole source of carbon and energy. Growth on n-decane and chlorate was indicated by the increase in optical density (Fig. 1a). An OD of 0.34 corresponds to a bacterial count of $1.31 \times 10^9$. Growth followed n-decane degradation as indicated by $CO_2$ formation, chlorate reduction and chloride production (Fig. 1a). No growth was observed in controls without inoculum or without n-decane or controls without chlorate (results not shown). The specific growth rate on n-decane and chlorate was 0.5 $\pm$ 0.1 per day (doubling time 1.4 $\pm$ 0.2 day). After 7 days, 87 % of the 1 mM of the added n-decane was oxidized. The oxidation of 1 mM of n-decane led to a reduction of 9.2 $\pm$ 0.7 mM of chlorate and yielded 7.7 $\pm$ 0.6 mM of bicarbonate and 8.3 $\pm$ 0.8 mM of chloride. The balance fits relatively well with the theoretical stoichiometry of complete oxidation of n-decane coupled to chlorate reduction:

$$C_{10}H_{22} + 10.33\ ClO_3^- \rightarrow 10\ HCO_3^- + 10.33\ Cl^- + H_2O + 10\ H^+ \qquad (1)$$

The bacterium also grows aerobically on n-decane (Fig. 1b). The specific growth rate on n-decane and molecular oxygen was 0.4 $\pm$ 0.02 per day (doubling time 1.7 $\pm$ 0.1). Growth and $CO_2$ production were not observed in the presence of n-decane and nitrate, using *P. chloritidismutans* adapted to growth on nitrate and acetate (results not shown).

### Other substrates utilized

Apart from n-decane other n-alkanes were also screened as possible substrates with chlorate as electron acceptor. Strain AW-1$^T$ grew with C7 to C12 n-alkanes, but not with smaller n-alkanes. It grew equally well on odd and even chain n-alkanes. Strain AW-1$^T$ grew equally well on C8 till C11, while growth on C7 and C12 was slower.

Strain AW-1$^T$ also grew on the possible intermediates of the aerobic n-alkane degradation pathway, namely, 1-decanol and decanoate. Table 2 shows the amount of bicarbonate formed with various substrates using different electron acceptors. With n-decane and 1-decanol as substrates, bicarbonate was formed with chlorate and oxygen, but not with nitrate as electron acceptor. With decanoate and nitrate, bicarbonate was also formed.

**Enzyme assays**

Extracts of cells grown on n-decane and chlorate and on acetate and chlorate showed chlorate reductase and chlorite dismutase activity (Table 3). The specific chlorite dismutase activity is dependent on the amount of cell extract and the chlorite concentration (Mehboob et al. 2009). The chlorite dismutase presented in Table 3 is the activity under optimal conditions.

Alkane oxidation activity could be measured with starved cells grown on n-decane and chlorate. A relatively small amount of activity was also observed with starved whole cells grown on acetate and chlorate (Table 3). No alkane oxidation was observed with the anoxic control. Cell free extract of alkane grown cells did not show alcohol oxygenase activity, but we found an activity of 0.06 U/mg of protein of $NAD^+$ dependent decanol dehydrogenase. This was quite surprising as during the growth experiment strain AW-1$^T$ was unable to grow with decanol and nitrate. Our strain is known to grow with ethanol and chlorate (Wolterink et al. 2002). We checked and found that strain AW-1$^T$ is able to grow with ethanol using oxygen, chlorate and nitrate as electron acceptors. Even ethanol and nitrate adapted cells could not grow on the decanol and nitrate. Cell free extract of decane grown culture showed 2 fold higher activity of 0.15 U/mg of protein with acetaldehyde.

## Detection of alkane oxygenase genes

Various primers at different annealing temperatures were used to detect the following alkane oxygenase genes: *alkB, alkM, almA,* and cytochrome P450 subfamily CYP153. Though we got the expected results in positive controls, with the available specific primers sets we were not able to detect any kind of known alkane oxygenase genes in our strains. We were able to amplify a sequence which was 51 % and 57 % similar to two acyl-CoA dehydrogenases involved in the degradation of n-alkane in *Acinetobacter* strain M-1 (Tani et al. 2002).

## Discussion

*Pseudomonas chloritidismutans* AW-1$^T$ is a gram-negative, facultative anaerobic, chlorate reducing bacterium, which has been isolated on acetate and chlorate in our laboratory (Wolterink et al. 2002). We tested its ability to grow on n-alkanes and found that strain AW-1$^T$ grows on n-alkanes with oxygen and chlorate as electron acceptor. Many Pseudomonades have the ability to grow aerobically on n-alkanes (Söhngen 1913; Wentzel et al. 2007), but strain AW-1$^T$ is the only known bacterium that grows on n-alkanes by supplying molecular oxygen formed by chlorite dismutation. The doubling time with n-decane and chlorate is 1.4 $\pm$ 0.2 days. Except for strain HxN1, which has a doubling time of 11 hours (Ehrenreich et al. 2000), all other anaerobic n-alkane degraders grow slower, e.g. strains Hxd3 and Pnd3 have doubling times of 9 days (Aeckersberg et al. 1998) and strain AK-01 has a doubling time of 3 days (So and Young, 1999). In contrast, doubling times of aerobic alkane degrading bacteria are approximately 1 hour for *P. aeruginosa* (Ertola et al. 1965) and 5 hours for *Rhodococcus* species (Bredholt et al. 1997).

Strain AW-1$^T$ grows on n-decane with chlorate and oxygen, but not with nitrate, suggesting the involvement of oxygenases. Oxygen is incorporated in n-decane through an oxygenase to form decanol. When chlorate is used as electron acceptor oxygen is formed by dismutation of chlorite. This is supported by the similar specific growth rates on n-decane with oxygen (0.5 $\pm$ 0.1 day$^{-1}$) or chlorate (0.4 $\pm$ 0.02 day$^{-1}$) as electron acceptor. Strain AW-1$^T$ also grows on possible aerobic intermediates, like 1-decanol and decanoate with oxygen and chlorate as electron acceptor. It was unable to utilize 1-decanol when nitrate was used as electron acceptor. This also suggests that in the conversion of decanol, an oxygenase as found by Buhler et al. (2000) and Katopodis et al. (1984) is involved. Growth on decanoate with nitrate suggests that no oxygenases are required for decanoate degradation. Growth of strain AW-1$^T$ with oxygen or chlorate was observed to be the fastest on decanoate followed by decanol and then n-decane.

We faced a problem in detecting alkane oxygenase activity with cell free extracts. This has also been observed by others (Katopodis et al. 1984; Tani et al. 2001) and was attributed to the poor solubility of the substrate (Smits et al. 2002; Tani et al. 2001), the instable nature of the alkane oxygenase complex (Katopodis et al. 1984; McKenna and Coon 1970; Ruettinger et al. 1974) and the involvement of unknown factors (Tani et al. 2001), like some unique electron transfer proteins (van Beilen et al. 2006). However, alkane oxidation activity could be demonstrated with whole cells grown on n-decane and chlorate. Almost 3.5 fold more activity was observed with the cells grown on decane and chlorate as compared with the cells grown on acetate and chlorate showing the induction of alkane oxygenase when grown on n-decane**.**

We were unable to detect an alcohol oxygenase in decane grown cell free extracts. However we found an alcohol dehydrogenase that has a more than 2 fold

higher activity for acetaldehyde than for decanal. Since the strain AW-1$^T$ grows with ethanol with oxygen, chlorate) and nitrate, but is unable to grow with decanol and nitrate,,it is unlikely that this alcohol dehydrogenase is involved in long chain alcohol oxidation. Instead the detected alcohol dehydrogenase only seems to be involved in growth with short chain alcohols.

We were unable to amplify an alkane oxygenase gene from our strain with new and known primers designed to detect different classes of alkane oxygenases. However, we amplified a sequence, similar to an acyl CoA dehydrogenase from strain AW-1$^T$. This acyl CoA dehydrogenase enzyme has been reported to be involved in n-alkane degradation in *Acinetobacter* strain M-1 (Tani et al. 2002). The same group proposed that a dioxygenase is involved in the initial oxidation (Finnerty pathway) of n-alkanes (Maeng et al. 1996). However, we have observed that the N-terminal sequence of this dioxygenase and the above mentioned acyl-CoA dehydrogenase are similar. This sequence seems highly conserved in all *Pseudomonas* genomes. Therefore, we also did a growth test on n-alkanes as carbon and energy source with *Pseudomonas* sp. KT2440, for which the genome sequence is available. The genome contains acyl-CoA dehydrogenase genes, but evidence for the presence of a conventional alkane hydroxylase system is lacking. No obvious aerobic growth of this strain on n-alkanes was found. Hence, we assume that the acyl-CoA dehydrogenase is not involved in the first step of activation of n-alkane, as reported by Maeng et al. (1996), but is important in a later reaction step of n-alkane degradation.

A reason why we were not able to detect alkane oxygenase genes could be that the alkane oxygenases have a very high sequence diversity (i.e. the protein sequence similarity between reported *alkB* types can be as low as 35%), especially among the *Pseudomonas* group. The *Pseudomonas* alkane oxygenases are as

distantly related to each other as to the alkane oxygenases from phylogenetically unrelated bacteria (Smits et al. 2002). This may have led to similar false negative results as reported by others (Chandler and Brockman 1996; Heiss-Blanquet et al. 2005; van Beilen et al. 2006). It may also be that a novel type of alkane oxygenase is involved in this process, of which the genes are not known yet and which may be specific for n-alkane degradation at low oxygen concentrations, as is evidently the case for growth under chlorate-reducing conditions. The extent of diversity of alkane oxygenases became apparent in recent research by Kuhn et al. (2009). They found that only one out of the 76 clones of the putative *alk* genes had a significant sequence similarity with previously known *alk* genes.

Based on all the physiological features, enzyme measurements and the amplification of an acyl-coA dehydrogenase gene, we propose a hypothetical n-alkanes (C7-C12) degradation pathway as depicted for n-decane in Fig. 2. We suggest that oxygen formed in the dismutation of chlorite is used to convert n-decane to decanol and decanol to decanal by means of oxygenases. Decanal is further oxidized to decanoate, which upon activation is degraded by β-oxidation

This is the first report of the degradation of aliphatic hydrocarbons with chlorate, both as electron acceptor and as source of oxygen needed for the oxygenase activity. The degradation of aromatic hydrocarbons with chlorate has recently been described (Tan et al. 2006; Weelink et al. 2008). This study adds to the possibility to apply microorganisms with oxygenase-dependent pathways for the bioremediation of anoxic soils polluted with compounds that are difficult to degrade in the absence of molecular oxygen.

**Acknowledgements**

We thank the Dutch Center for Soil Quality Management and Knowledge Transfer (SKB) The Netherlands, the Wageningen Institute for Environment and Climate Research (WIMEK) The Netherlands, European Commission BIOTOOL project (GOCE 003998) and the Higher Education Commission of Pakistan (HEC) for providing funds for the present study. We thank Willem van Berkel and Hauke Smidt for expert advice; Wim Roelofsen and Silke Kahl for excellent technical assistance.

## References

Aeckersberg F, Rainey FA, Widdel F (1998) Growth, natural relationships, cell fatty acids and metabolic adaptation of sulfate-reducing bacteria utilizing long-chain alkanes under anoxic conditions. Arch Microbiol 170:361-369.

Berthe-Corti L, Fetzner S (2002) Bacterial metabolism of n-alkanes and ammonia under oxic, suboxic and anoxic conditions. Acta Biotechnol 22: 299-336.

Bradford MM (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem 72:248-54.

Bredholt H, Josefsen K, Vatland A, Bruheim P, Eimhjellen K (1998) Emulsification of crude oil by an alkane-oxidizing *Rhodococcus* species isolated from seawater. Can J Microbiol 44:330-340.

Breznak JA, Costilow RN (1994) Physiological factors in growth. In: Gerhardt P, Murray RGE, Woods WH, Krieg NR (eds) Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington, D.C. pp 139-141.

Buhler B, Schmid A, Hauer B, Witholt B (2000) Xylene monooxygenase catalyzes the multistep oxygenation of toluene and pseudocumene to corresponding alcohols, aldehydes, and acids in *Escherichia coli* JM101. J Biol Chem 275:10085-10092.

Callaghan AV, Wawrik B, Chadhain SMN, Young LY, Zylstra GB (2008) Anaerobic alkane-degrading strain AK-01 contains two alkylsuccinate synthase genes. Biochem and Biophysic Res Commun 366:142–148.

Chandler DP, Brockman FJ (1996) Estimating biodegradative gene numbers at a JP-5 contaminated site using PCR. Appl Biochem Biotechnol 57/58:971–982.

Chayabutra C, Ju LK (2000) Degradation of n-hexadecane and its metabolites by *Pseudomonas aeruginosa* under microaerobic and anaerobic denitrifying conditions. Appl Environ Microbiol 66:493-498.

Cladera AM, García-Valdés E, Lalucat J (2006) Genotype versus phenotype in the circumscription of bacterial species: the case of *Pseudomonas stutzeri* and *P. chloritidismutans*. Arch. Microbiol. 184:353–361.

Coates JD, Bruce RA, Haddock JD (1998) Anoxic bioremediation of hydrocarbons. Nature 396:730.

Dorn E, Hellwig M, Reineke W, Knackmuss H-J (1974) Isolation and characterization of a 3-chlorobenzoate degrading pseudomonad. Arch Microbiol 99:61-70.

Ehrenreich P, Behrends A, Harder J, Widdel F (2000) Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. Arch Microbiol 173:58–64.

Ertola RJ, Lilly D, Webb FC (1965) Production of cell tissue from hydrocarbons by a microbiological process. Biotechnol Bioeng 7:309-319.

Grundmann O, Behrends A, Rabus R, Amann J, Halder T, Heider J, Widdel F (2008) Genes encoding the candidate enzyme for anaerobic activation of *n*-alkanes in the denitrifying bacterium, strain HxN1. Environ Microbiol 10:376-385.

Head IM, Jones DM, Roling WFM (2006) Marine micro-organisms make a meal of oil. Nature Rev Microbiol 4:173-182.

Heiss-Blanquet S, Benoit Y, Maréchaux C, Monot F (2005) Assessing the role of alkane hydroxylase genotypes in environmental samples by competitive PCR. J. Appl. Microbiol 99:1392–1403.

Henstra AM, Stams AJM (2004) Novel physiological features of *Carboxydothermus hydrogenoformans* and *Thermoterrabacterium ferrireducens.* Appl Env Microbiol 70:7236-7240.

Holliger C, Schraa G, Stams AJM, Zehnder AJB (1993) A highly purified enrichment culture couples the reductive dechlorination of tetrachloroethene to growth. Appl Env Microbiol 59:2991-2997.

Katopodis AG, Wimalasena K, Lee J, May SW (1984) Mechanistic studies on non-heme iron monooxygenase catalysis: epoxidation, aldehyde formation, and demethylation by the v-hydroxylation system of *Pseudomonas oleovorans*. J Am Chem Soc 106:7928–7935.

Kengen SWM, Rikken GB, Hagen WR, van Ginkel CG, Stams AJM (1999) Purification and characterization of (per)chlorate reductase from the chlorate-respiring strain GR-1. J Bacteriol 181:6706-6711.

Kloos K, Munch JC, Schloter M (2006) A new method for the detection of alkane-monooxygenase homologous genes (alkB) in soils based on PCR-hybridization. J Microbiol Meth 66:486–496.

Kohno T, Sugimoto Y, Sei K, Mori K (2002) Design of PCR primers and gene probes for general detection of alkane-degrading bacteria. Microbe and Environ 17:114-121.

Kubota M, Nodate M, Hirose YM, Uchiyama T (2005) Isolation and functional analysis of cytochrome P450 CYP153A genes from various environments. Biosci Biotechnol Biochem 69:2421–2430.

Kuhn E, Bellicanta GS, Pellizari VH (2009) New *alk* genes detected in antarctic marine sediments. *Env Microbiol* 11:669-673.

Maeng JH, Sakai Y, Tani Y, Kato N (1996) Isolation and characterization of a novel oxygenase that catalyzes the first step of *n*-alkane oxidation in *Acinetobacter* sp. strain M-1. J Bacteriol 178:3695-3700.

McKenna EJ, Coon MJ (1970) Enzymatic ω-oxidation. IV. Purification and properties of the ω-hydroxylase of *Pseudomonas oleovorans*. J Biol Chem 245:3882–3889.

Mehboob F, Wolterink AFWM, Vermeulen AJ, Jiang B, Hagedoorn P-L, Stams AJM, Kengen SWM (2009) Purification and characterization of a chlorite dismutase from *Pseudomonas chloritidismutans*. FEMS Microbiol Lett 293:115-121.

Rikken GB, Kroon AGM, van Ginkel CG (1996) Transformation of (per)chlorate into chloride by a newly isolated bacterium: reduction and dismutation. Appl Microbiol Biotechnol 45:420-426.

Ruettinger RT, Olson ST, Boyer RF, Coon MJ (1974) Identification of the ω-hydroxylase of *Pseudomonas oleovorans* as a nonheme iron protein requiring phospholipid for catalytic activity. Biochem Biophys Res Commun 57:1011–1017.

Scholten JCM, Stams AJM (1995) The effect of sulfate and nitrate on methane formation in a fresh water sediment. Antonie van Leeuwenhoek 68:309-315.

Smits TH M, Röthlisberger M, Witholt B, van Beilen JB (1999) Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. Environ Microbiol 1:307-318.

Smits TH M, Balada SB, Witholt B, van Beilen JB (2002) Functional analysis of alkane hydroxylases from gram-negative and gram-positive bacteria. J Bacteriol 184:1733-1742.

So CM, Young LY (1999) Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. Appl Environ Microbiol 65:2969–2976.

So CM, Phelps CD, Young LY (2003) Anaerobic transformation of alkanes to fattty acids by a sulfate-reducing bacterium, strain Hxd3. Appl Environ Microbiol 69:3892-3900.

Söhngen NL (1913) Benzin, Petroleum, Paraffinöl und Paraffin als Kohlenstoff- und Energiequelle für Mikroben. Zentralbl Bakteriol Parasitenkd Infektionskr Hyg. Abt. 2, 37:595–609 (in German).

Staijen IE, van Beillen JB, Witholt B (2000) Expression, stability and performance of the three-component alkane mono-oxygenase of *Pseudomonas oleovorans* in *Escherichia coli*. Eur J Biochem 267:1957-1965.

Tan NCG, van Doesburg W, Langenhoff AAM, Stams AJM (2006) Benzene degradation coupled with chlorate reduction in a soil column study. Biodegradation 17:11-17.

Tani A, Ishige T, Sakai Y, Kato N (2001) Gene structures and regulation of the alkane hydroxylase complex in *Acinetobacter* sp. strain M-1. J Bacteriol 183:1819–1823.

Tani A, Ishige T, Sakai Y, Kato N (2002) Two acyl-coA dehydrogenase of *Acinetobacter* sp. strain M-1 that uses very long chain n-alkanes. J Biosci Bioeng 94:326-329.

van Beilen JB, Funhoff EG, van Loon A, Just A, Kaysser L, Bouza M, Holtackers R, Rothlisberger M, Li Z, Witholt B (2006) Cytochrome P450 alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. Appl Environ Microbiol 72:59-65.

van Beilen JB, Funhoff EG (2007) Alkane hydroxylase involved in microbial alkane degradation. oxidation by *Pseudomonas oleovorans.* Appl Microbiol Biotechnol 74:13-21.

Widdel F, Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol 12:259-276.

Weelink SAB, Tan NCG, ten Broeke H, van den Kieboom C, Van Doesburg W, Langenhoff AAM, Gerritse J, Junca H, Stams AJM (2008) Isolation and

characterization of *Alicycliphilus denitrificans* strain BC that grows on benzene with chlorate as electron acceptor. Appl Env Microbiol 74:6672-6681.

Wentzel A, Ellingsen TE , Kotlar HK, Zotchev SB, Throne-Holst M (2007) Bacterial metabolism of long-chain n-alkanes. Appl Microbiol Biotechnol 76:1209-1221.

Whyte LG, Schultz A, van Beilen JB, Luz AP, Pellizari V, Labbe D, Greer CW (2002) Prevalence of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon-contaminated and pristine soils. FEMS Microbiol Ecol 41:141–150.

Widdel F, Rabus R (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. Curr Opin Biotechnol 12:259-276.

Wolterink AFWM, Jonker AB, Kengen SW, Stams AJM (2002) *Pseudomonas chloritidismutans* sp. nov., a non-denitrifying, chlorate-reducing bacterium. Int J Syst Evol Microbiol 52:2183-2190.

**Table 1: Different primers sets used in this study targeting conserved regions of *alkB*, *almA*, *Cytochrome P450 Subfamily CYP153*, and *Acyl CoA dehydrogenase* genes.**

| Primer Name | Sequence (5' ⟶ 3') | Reference |
|---|---|---|
| | ***alkB*** | |
| TS2Sf<br>TS2Smodf<br>TS2Smod2f | AAYAGAGCTCAYGARYTRGGTCAYAAG<br>AAYAGAGCTCAYGARITIGGICAYAAR<br>AAYAGAGCTCAYGARITITCICAYAAR | Smits et al., 1999 |
| DEG1RE<br>DEG1RE2 | GTRAGICTRGTRGTRCGCTTAAGGTG<br>GTRTCRCTRGTRGTRCGCTTAAGGTG | |
| alkMUp<br>alkMDn | CGGGGTAAGCATGAATAGCT<br>CGTACAGCTACTTGGTGGAC | Tani et al., 2001 |
| Alk-1F<br>Alk -1R | CATAATAAAGGGCATCACCGT<br>*GATTTCATTCTCGAAACTCCAAAC* | Kohno et al., 2002 |
| Alk-3F<br>Alk-3R | *CCGTAGTGCTCGACGTAGTT*<br>*CAGGCGTTCTTCGGGTTGCGCTGCTCGA* | |
| AlkBpaFwd<br>AlkBpaRv2 | AACTGGAATTCACGATGTTTGA<br>CTGCCCGAAGCTTGAGCTAT | Smits et al., 2002 |
| AlkBpaBfw<br>AlkBpaBrv | GGAGAATTCTCAGACAATCT<br>GAGGCGAATCTAGAAAAAACTG | |
| B5 –Eco<br>B3-Hind | GGAGAATTCCAAATGCTTGAG<br>TTTGTGAAAGCTTTCAACGCC | |
| Pp alkB-F<br>Pp alkB-R | TGGCCGGCTACTCCGATGATCGGAATCTGG<br>CGCGTGGTGATCCGAGTGCCGCTGAAGGTG | Whyte et al., 2002 |
| Rhose<br>Rhoas | ACGGSCAYTTCTACRTCG<br>CCGTARTGYTCGAGRTAG | Heiss-Blanquit et al., 2005 |
| Pseuse1<br>Pseuas1 | GARCATAATAARGGBCATC<br>AGCARWCCGTARTGYTCA | |
| Pseuse2<br>Pseuas2 | AYGTSCGYGGCCACCATGT<br>CGACGTAGTTGAYGAYYTCC | |
| Acinse<br>Acinas | ACWCCTGAAGATCCRGCWTC<br>TRTTCCATCTAGCTCWGGC | |
| alkB-1f<br>alkB-1r | AAYACNGCNCAYGARCTNGGNCAYAA<br>GCRTGRTGRTCNGARTGNCGYTG | Kloos et al., 2006 |
| alkBF<br>alkBR | GSNCAYGARYTSRKBCAYAA<br>GCRTGRTGRTCNSWRTGNCGYTG | This study |
| | ***Cytochrome P450 Subfamily CYP153*** | |
| CF<br>CR | ATGTTYATHGCNATGGAYCCNC<br>NARNCKRTTNCCCATRCANCKRTG | Kubota et al., 2005 |
| P450fw1<br>P450rv3 | GTSGGCGGCAACGACACSAC<br>GCASCGGTGGATGCCGAAGCCRAA | Van Beilen et al., 2006 |
| | ***almA*** | |
| almA-F1<br>almA-R1<br>almA-R2 | CCBGGBATYCGBTCNGAYTCNGAYATGT<br>GGHGADCGYTGYARCATVGTNACGTNACBATGYTRCARCG HTCDCC<br>CANAVVCGYTSRTCCCANGGVTTRTATAYAABCCNTGGGAY SARCGBBTNTG | This study |
| | ***Acyl-Co A dehydrogenase*** | |
| Acyl-F1<br>Acyl-R1<br>Acyl-R2 | GGYTCNATYGARCABAARATGGG<br>CCCCAYTCRCGRATRWARCCRTGVCCRCCRAA<br>TGRAYRCCRTTRGTRCCTTCRTARAT | This study |

**Table 2: Formation of bicarbonate (in mM) by strain AW-1$^T$ during growth on different substrates.** Values for decane are after 9 days, while for decanol and decanoate the samples were analyzed after 7 days

| | Electron acceptor | | |
|---|---|---|---|
| **Substrate** | **$O_2$** | **$ClO_3^-$** | **$NO_3^-$** |
| **n-Decane** | **10.6 ± 1.1** | **7.9 ± 0.3** | **0.7 ± 0.2** |
| **1-Decanol** | **9.8 ±1.3** | **9.8 ± 0.3** | **0.2 ± 0.1** |
| **n-Decanoate** | **8.3 ± 0.4** | **8.6 ± 0.4** | **8.0 ± 0.9** |

**Table 3: Activities of chlorate reductase, chlorite dismutase and alkane oxygenase of strain AW-1$^T$ grown on acetate and chlorate or on n-decane and chlorate.** (*The alkane oxidation activity was measured with whole cells and is average of the activities at four different time points i.e. after 5, 10, 15 & 30 min.)

| | **Specific enzyme activity (U/mg of protein)** | |
|---|---|---|
| | **Acetate + $ClO_3^-$** | **n-Decane + $ClO_3^-$** |
| **Chlorate reductase** | **11.4 ± 0.3** | **26.6 ± 1** |
| **Chlorite dismutase** | **7.7 ± 1.4** | **2.8 ± 0.7** |
| **Alkane oxygenase*** | **26 ± 9** | **93 ± 31** |

## Figure Legends

**Figure 1: Growth of strain AW-1$^T$ (a) with decane and chlorate and (b) with decane and oxygen.** Values are means of three replicates. The bars represent standard deviation. Dotted line with open circles (Θ) represents the OD at 600nm. The continuous lines represent ♦ chlorate utilized; ✕ $O_2$ utilization; ■ chloride produced and ▲ bicarbonate formed.

**Fig. 2: Hypothetical pathway of degradation of n-decane coupled to chlorate reduction.** Oxygen released from chlorite dismutation is used by a presumed oxygenase to incorporate in the n-alkane molecule to form an alcohol and later on an aldehyde. Further degradation may occur in the absence of oxygen.

**Fig. 1a :**

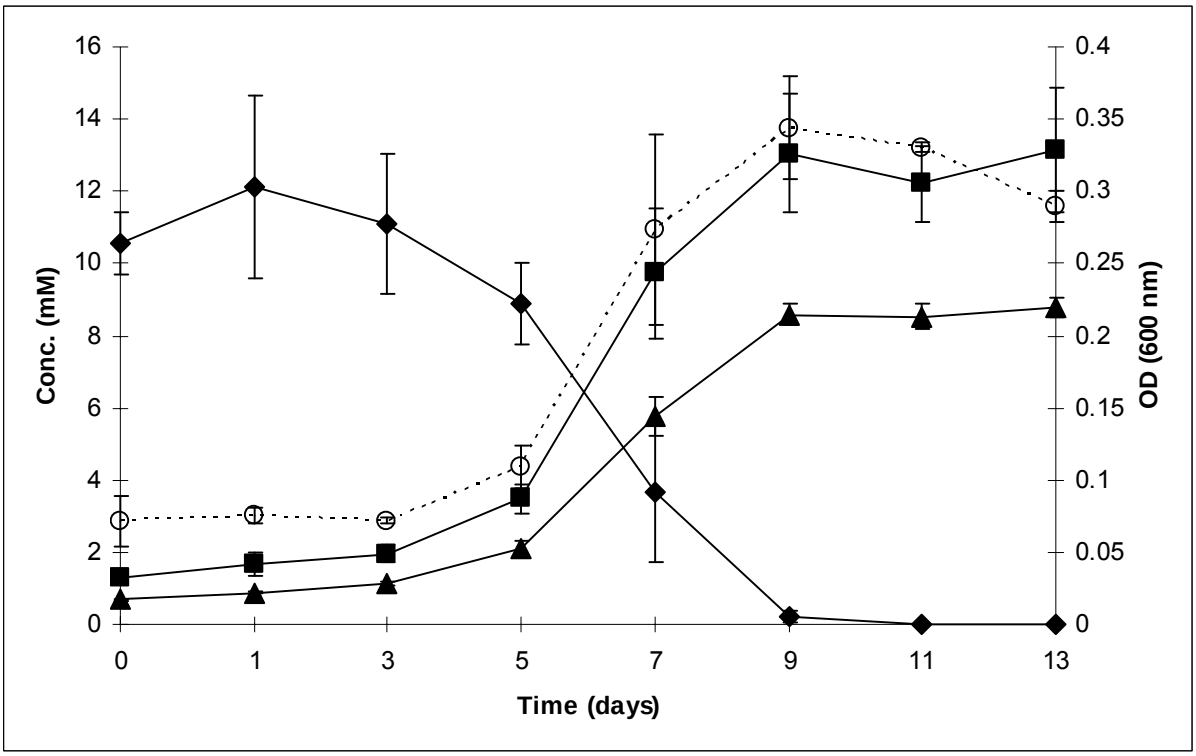
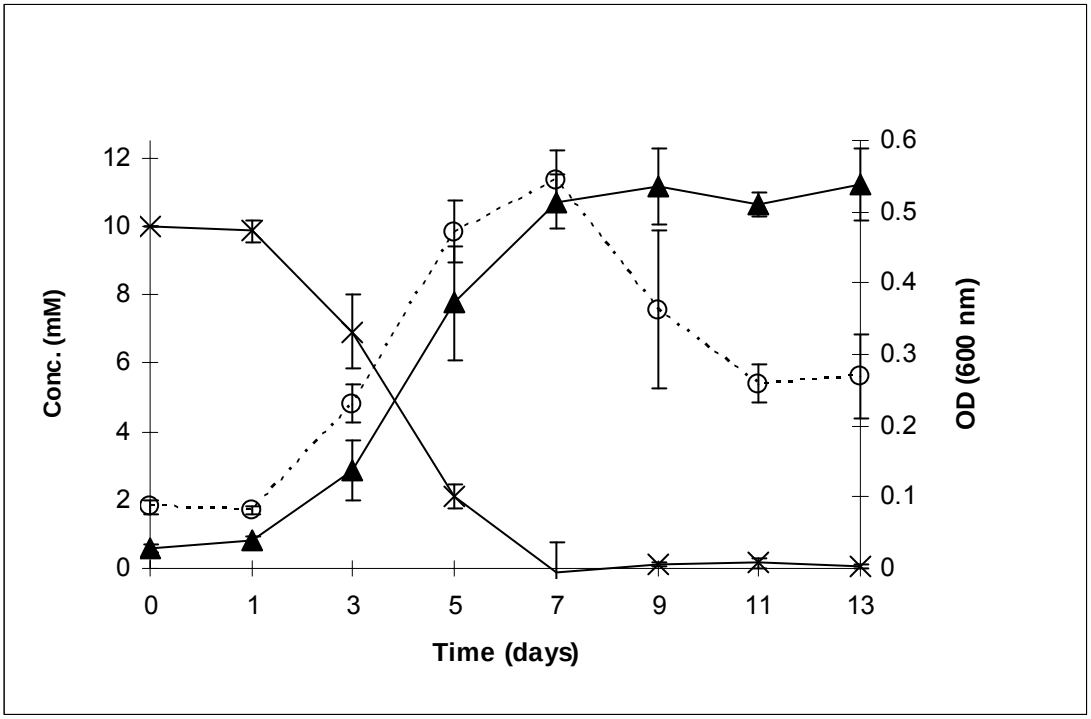

Conc. (mM)
OD (600 nm)
Time (days)
0
1
3
5
7
9
11
13
0
2
4
6
8
10
12
14
16
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4

**Fig.1b :**


Conc. (mM)
OD (600 nm)
Time (days)
0
1
3
5
7
9
11
13
0
2
4
6
8
10
12
0
0.1
0.2
0.3
0.4
0.5
0.6