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J. A. Dijk · A. J. M. Stams · G. Schraa · H. Ballerstedt · J. A. M. de Bont · J. Gerritse

Anaerobic oxidation of 2-chloroethanol under denitrifying conditions by *Pseudomonas stutzeri* strain JJ

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Abstract A bacterium that uses 2-chloroethanol as sole energy and carbon source coupled to denitrification was isolated from 1,2-dichloroethane-contaminated soil. Its 16 S rDNA sequence showed 98% similarity with the type strain of *Pseudomonas stutzeri* (DSM 5190) and the isolate was tentatively identified as *Pseudomonas stutzeri* strain JJ. Strain JJ oxidized 2-chloroethanol completely to CO_2 with NO_3^- or O_2 as electron acceptor, with a preference for O_2 if supplied in combination. Optimum growth on 2-chloroethanol with nitrate occurred at 30 °C with a μ_{max} of 0.14 h^{-1} and a yield of 4.4 g protein per mol 2-chloroethanol metabolized. Under aerobic conditions, the μ_{max} was 0.31 h^{-1}. NO_2^- also served as electron acceptor, but reduction of $Fe(OH)_3$, MnO_2, SO_4^{2-}, fumarate or ClO_3^- was not observed. Another chlorinated compound used as sole energy and carbon source under aerobic and denitrifying conditions was chloroacetate. Various different bacterial strains, including some closely related *Pseudomonas stutzeri* strains, were tested for their ability to grow on 2-chloroethanol as sole energy and carbon source under aerobic and denitrifying conditions, respectively. Only three strains, *Pseudomonas stutzeri* strain LMD 76.42, *Pseudomonas putida* US2 and *Xanthobacter autotrophicus* GJ10, grew aerobically on 2-chloroethanol. This is the first report of oxidation of 2-chloroethanol under denitrifying conditions by a pure bacterial culture.

J. A. Dijk (✉) · A. J. M. Stams · G. Schraa
Laboratory of Microbiology, Wageningen University,
Hesselink van Suchtelenweg 4, 6703 CT Wageningen,
The Netherlands
e-mail: John.Dijk@wur.nl
Tel.: +31-317-483739
Fax: +31-317-483829

H. Ballerstedt · J. A. M. de Bont · J. Gerritse
TNO Environment, Energy and Process Innovation,
P.O. Box 342, 7300 AH Apeldoorn, The Netherlands

Introduction

2-Chloroethanol (CE) is used in industry mainly for the synthesis of insecticides and as a solvent. Very little is known about the emission and fate of CE in the environment due to the fact that CE is not included in routine analyses for soil pollution. CE is metabolized both in vivo and in vitro by mammalian alcohol dehydrogenase to 2-chloroacetaldehyde, which is considered to be mutagenic (McCann et al. 1975).

Several bacteria have been described that can degrade CE aerobically, including *Pseudomonas* sp. strain CE1, *Pseudomonas putida* US2, *Pseudomonas* sp. strain DCA1, *Xanthobacter autotrophicus* GJ10, *Pseudomonas* sp. strain GJ1, *Ancylobacter aquaticus* strains AD20 and AD25, and *Mycobacterium* sp. strain GP1 (Hage and Hartmans 1999; Janssen et al. 1985, 1984; Poelarends et al. 1999; Strotmann et al. 1990; Stucki et al. 1981; van den Wijngaard et al. 1992). Usually, degradation proceeds via the intermediates 2-chloroacetaldehyde and chloroacetate to glycolic acid. Recently, Poelarends et al. (1999) showed that *Mycobacterium* sp. strain GP1 degraded CE via ethylene oxide, without production of the 2-chloroacetaldehyde intermediate.

Although the aerobic CE-metabolizing *Pseudomonas* sp. strain GJ1 and *Xanthobacter autotrophicus* GJ10 are able to reduce NO_3^-, nothing is known about the degradation of CE under denitrifying conditions.

This report describes the isolation and characterization of *Pseudomonas stutzeri* strain JJ, the first microorganism known to grow anaerobically on CE with NO_3^- as electron acceptor. This is especially interesting, since many soils that are contaminated with chlorinated aliphatics are anoxic and NO_3^- is often present in the groundwater (Cox et al. 2000; Gerritse et al. 1999).

Materials and methods

Organisms

Pseudomonas stutzeri strain LMD 76.42 (Palleroni et al. 1970) and *Pseudomonas stutzeri* strain LMD 26.48 (Sijderius 1946) were purchased from The Netherlands Culture Collection of Bacteria (NCCB, Utrecht, The Netherlands). *Pseudomonas mendocina* (DSM 50017, type strain) (Skerman et al. 1980), *Pseudomonas stutzeri* (DSM 5190, type strain) (Skerman et al. 1980), (Middelhoven and Bakker 1982), *Xanthobacter autotrophicus* GJ10 (Janssen et al. 1984), *Pseudomonas stutzeri* strain KC (Criddle et al. 1990), *Pseudomonas putida* US2 (Strotmann et al. 1990) and *Alcaligenes xylosoxidans* subsp. *denitrificans* (DSM 6505) (Blake and Hegeman 1987) were obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany). *Pseudomonas* sp. strain P51 (van der Meer et al. 1987), *Pseudomonas putida* strain C 3024, *Pseudomonas stutzeri* strain 346B (DSM 6538) (Baggi et al. 1987) and *Pseudomonas aeruginosa* (ATCC 17933) (Çetin et al. 1965) were obtained from the culture collection of the Laboratory of Microbiology of Wageningen University. The new isolate, *Pseudomonas stutzeri* strain JJ, has been deposited in the DSM as strain DSM 15012.

Media and cultivation techniques

Batches were prepared under a N_2 atmosphere in 120-ml serum bottles that were crimp-sealed with butyl-rubber stoppers and contained 50 ml medium. Standard medium consisted of the following components: Na_2HPO_4/KH_2PO_4 buffer (20 mM, pH 7), 0.7 g $(NH_4)_2SO_4$/l, 0.1 g $MgSO_4 \cdot 7H_2O$/l , 0.05 g $CaCl_2 \cdot 2H_2O$/l , 1 ml trace elements/l (Gerritse et al. 1992) and 1 ml vitamin solution/l (Heijthuisen and Hansen 1986). Vitamins were filter sterilized (0.2-µm) and added to the media after autoclaving. Phosphate buffer was also added separately after autoclaving. Unless mentioned otherwise, CE (5 mM) was added as electron donor and $NaNO_3$ (10 mM) as electron acceptor from separately sterilized stock solutions. Batches were transferred weekly to fresh media to maintain actively growing cultures. Batch enrichments were initially incubated at 20 °C, but after isolation, strain JJ was incubated at 30 °C unless mentioned otherwise. Agar plates consisted of the above mentioned components plus 15 g Bacto-agar/l (Difco Laboratories, Detroit, Mich., USA). The plates were incubated in a nitrogen-flushed anaerobic jar or in air.

Enrichment, isolation and purity control

Strain JJ originates from an anaerobic enrichment culture growing on 1,2-dichloroethane as sole energy and carbon source in the presence of NO_3^- (Gerritse et al. 1999). One ml of the enrichment culture was transferred to standard medium, containing 1 mM CE and 5 mM $NaNO_3$. Upon growth, samples were plated on anoxic agar plates. Colonies were isolated from the plates and transferred to liquid medium. This process was repeated several times until a pure culture was obtained.

Purity was tested by phase-contrast microscopy and by aerobic growth on both solid and liquid standard media with glucose (5 mM) and yeast extract (5 g/l). Oxidase, catalase and Gram-type tests were done at The Netherlands Culture Collection of Bacteria (Utrecht, The Netherlands).

Identification

Physiological tests

The API 20NE and BIOLOG GN analyses were carried out at The Netherlands Culture Collection of Bacteria (Utrecht, The Netherlands).

DNA isolation, amplification, sequence determination, and analysis of 16S rDNA

Genomic DNA was isolated from whole cells by a combined method of SDS-treatment and bead beating in the presence of phenol, followed by subsequent phenol-chloroform-isoamylalcohol (25:24:1) extraction (Duarte et al. 1998; Ralebitso et al. 2001). The final DNA extracts were further purified, using the Wizard DNA Clean-Up System (Promega, Leiden, The Netherlands). PCR amplification of the 16S rRNA gene was done using 1 µl DNA extract as template, bacteria-specific primers FD1 and RP2 (Weisburg et al. 1991) (*Escherichia coli* numbering 8–27, and 1512–1492 (Brosius et al. 1978), AmpliTaq LD DNA-polymerase (Perkin Elmer Life Sciences, Zaventem, Belgium) and a thermal cycler temperature program as follows: denaturation at 95 °C for 5 min; touch down PCR step: 10 cycles of 94 °C for 30 s, 65°C–55°C for 30 s, 72 °C for 1 min; 27 similar cycles at 55 °C annealing temperature and with a 20-s extension of elongation phase per cycle. PCR product was purified via agarose gel separation stained with 1× Nuclistain (National Diagnostics, Manville, USA) and subsequent DNA extraction using a DNA extraction kit (MBI Fermentas, St. Leon-Rot, Germany). The purified 16S rDNA was ligated into a pGEM-T Easy Vector (Promega) and transformed into E.coli XL 1-Blue-MRF′ cells (Stratagene, La Jolla, Calif., USA). The transformed cells were subjected to blue-white color screening, randomly picked white colonies were grown overnight in selective LB-medium as described elsewhere (Sambrook et al. 1989), and recombinant plasmids were subsequently isolated using QIAprep Spin Miniprep kit (Qiagen, Westburg b.v., Leusden, The Netherlands). Cloned inserts were reamplified using pGEM-T-Easy Vector binding primers PG1f and PG2r (Hantke 2000), and subsequently digested with restriction enzymes *Msp*I and *Rsa*I (Promega, Leiden, The Netherlands) overnight at 37 °C. The restriction patterns were monitored to confirm uniformity of the inserts using 4 % agarose gels in 1× TBE-buffer (Sambrook et al. 1989). Bidirectional sequence was determined by MWG Biotech AG (Ebersberg, Germany) using universal SP6 and T7 primers and primers 984f (Heuer et al. 1999) and 907r (Muyzer et al. 1995). The determined sequence was compared with 16S rDNA sequences obtained from the EMBL data library using the Fasta3 program (Pearson 1990).

Characterization of the CE-degrading strain JJ

To determine the optimum growth temperature, batch cultures of strain JJ with 5 mM CE and 10 mM NO_3^- were incubated at 4, 12, 20, 25, 30, 35 and 45 °C, respectively. Liquid samples (1, 5 ml) were taken at different time points and the optical density at 660 nm (OD_{660}) was determined. Specific growth rates were obtained by plotting the natural logarithm of the OD_{660} against time. The determination of the optimum pH was done in the same way. Batch cultures of strain JJ were incubated at pH 6, 6.5, 7, 7.5, 8, 8.5, 9 and 9.5.

Electron donor utilization

Initially, utilization of chlorinated electron donor was screened in 10-ml vials containing 5 ml standard medium and crimp-sealed with butyl-rubber stoppers. Chlorinated electron donors were added from autoclaved stock solutions to a final concentration of 1 mM. Media were incubated anaerobically with 5 mM NO_3^-, or under an air atmosphere. After 8 weeks of incubation, the Cl^- concentration was determined. Batches with a chloride production >0.2 mM were transferred to standard media in 120-ml serum bottles with 5 mM electron donor and incubated anaerobically with 10 mM NO_3^-, or under air, respectively. After 3 weeks, growth was determined by visual detection of increased turbidity.

Growth on non-chlorinated electron donors was tested in 120-ml serum bottles with 50 ml standard medium, containing 10 mM electron donor with 10 mM NO_3^-, or under air, respectively. After

3 weeks, growth was determined by visual detection of increased turbidity.

Electron acceptor utilization

Electron acceptors were added according to the theoretical amounts of reduction equivalents needed for the complete oxidation of 5 mM CE: $NaNO_3$ (10 mM), $NaNO_2$ (17 mM), MnO_2 (25 mM), $Fe(OH)_3$ (50 mM), Na_2SO_4 (6 mM), $NaClO_3$ (8 mM), O_2 (air atmosphere; 12 mmol O_2/l medium) and fumarate (25 mM). Amorphous $Fe(OH)_3$ was prepared by neutralizing a 0.4 M $FeCl_3$ solution with 1 M NaOH until a pH of 7 was achieved (Lovley and Phillips 1986). MnO_2 was prepared by mixing equal amounts of 0.4 M MnO_4 and 0.6 M $MnCl_2$ and adjusting the pH to 10 with 1 M NaOH (Lovley and Phillips 1988; Murray 1974). After preparation, the $Fe(OH)_3$ and MnO_2 suspensions were washed four times with milli-Q water by centrifugation. All stock solutions were flushed with N_2 before use. After 4 weeks of incubation, the CE concentration of the batches with the respective electron acceptors was determined. When more than 2 mM of CE had been consumed, the electron acceptor was considered to be utilized.

Growth on CE

Batch cultures of strain JJ were grown in 1-l screw-cap flasks with butyl-rubber stoppers, containing 500 ml medium with 5 mM CE and 10 mM NO_3^-. The flasks were inoculated with 1–10 ml of an exponentially growing batch culture and incubated at 30 °C in a shaking waterbath (90 rpm). The optical densities and the CE, NO_3^-, NO_2^-, NH_4^+ and Cl^- concentrations were monitored over time.

Experiments with NO_3^- and O_2 present at the same time were carried out in 500-ml screw-cap flasks with butyl-rubber stoppers. The flasks contained 250 ml medium with 10 mM CE, 1 mM O_2 and 2 mM NO_3^-. The inoculum was pre-grown on CE and a combination of NO_3^- and O_2. The optical densities and the CE, NO_3^- and O_2 concentrations were monitored over time.

Comparison of the newly isolated strain JJ with other bacterial strains

Aerobic growth on CE of different bacterial strains was tested in standard medium containing 2.5 mM CE. After 3 weeks, the OD_{660} was determined. For anaerobic growth experiments with CE, batch cultures were pre-grown on medium containing: 27 g sodium succinate/l , 1 g KNO_3/l, 1 g K_2HPO_4/l, 1 g $(NH_4)_2SO_4$/l, 0.1 g$MgSO_4$/l, 0.05 g $CaCl_2$ /l, and 1 ml trace elements/l (see Media and cultivation techniques) to induce denitrification. Subsequently, the batch cultures were transferred to anoxic standard medium (2% inoculum) containing 2.5 mM CE and 5 mM NO_3^-. After 3 weeks, growth was determined by visual detection of increased turbidity.

Analytical methods

OD_{660} was determined in a Perkin-Elmer 55 A UV-VIS spectrophotometer. Cl^-, NO_3^- and NO_2^- were determined by suppressor-mediated ion chromatography (Dionex, Breda, The Netherlands) and conductivity detection. Eluent consisted of 1.8 mM Na_2CO_3 and 1.7 mM $NaHCO_3$ at a flow rate of 1 ml/min. The chromatograph was equipped with an IonPac AS9-SC column (Dionex, Breda, The Netherlands). NH_4^+ was determined colorimetrically according to the Berthelot reaction (Richterich 1965). CE was measured by extracting 2-ml samples with 1 ml diethylether (containing 0.1 mM 1-butanol as internal standard) and injecting 2 μl into a Hewlett Packard HP 6890 gas chromatograph, equipped with a CPWax 52 CB column (length, 30 m; diameter, 0.32 mm; Chrompack, Middelburg, The Netherlands), using an FID detector (300 °C). The oven temperature was 80 °C isothermal and the detection limit was 50 μM. CE concentrations are presented as nominal concentrations in the batch cultures, assuming that all CE was present in the liquid phase. O_2 and N_2 were measured on a Chrompack CP9000 gas chromatograph equipped with a CP Molsieve 5Å column (length, 30 m; diameter, 0.53 mm) and a TCD detector (150 °C). The oven temperature was kept at 50 °C. Protein was measured with the Lowry reagent with bovine serum albumin as a standard (Lowry et al. 1951).

Chemicals

All chemicals were of analytic grade with a purity of more than 97% and obtained from commercial sources.

Nucleotide sequence accession numbers

The nucleotide sequence was deposited in the GenBank database under accession number AF411219.

Results

Enrichment and isolation of strain JJ

A 1,2-dichloroethane (1,2-DCA)-degrading, denitrifying enrichment culture was previously obtained from 1,2-DCA-contaminated soil (Gerritse et al. 1999). Upon subculturing, the enrichment culture lost the ability to degrade 1,2-DCA. However, after supplying this culture with CE, a presumed intermediate of 1,2-DCA degradation, a well growing enrichment culture on CE (5 mM) and NO_3^- (10 mM) was obtained (Dijk et al. 2000). From this enrichment strain, JJ was isolated.

Identification of strain JJ

Strain JJ was identified by using the API 20NE, the ID 32 GN and BIOLOG GN systems and by sequencing the 16S rDNA. The API 20NE, ID 32 GN and BIOLOG GN tests identified strain JJ as *Pseudomonas stutzeri* (99.5% probability). The 16S rDNA sequence was compared with the BLAST database. Strain JJ showed 98% similarity with the type strain of *Pseudomonas stutzeri* (DSM 5190; U26262) (Bennasar et al. 1996). Therefore, the isolate was tentatively designated as *Pseudomonas stutzeri* strain JJ. The general characteristics of strain JJ are presented in Table 1.

Electron donor and acceptor utilization

No difference in the use of electron donor substrates was observed between aerobic and denitrifying conditions. Under both conditions, strain JJ grew on ethanol, propanol, butanol, acetate, pyruvate, glucose, chloroethanol and chloroacetate. The following substrates were not utilized by strain JJ: methanol, lactate, 1,1-dichloroethane, 1,2-dichloroethane, 1,1,1-trichloroethane, 1-

Table 1 Characteristics of *Pseudomonas stutzeri* strain JJ

Gram stain	–
Cell morphology	Rod-shaped
Cell size	1–2.5×0.6–1.3 μm
Colony morphology	Two types; wrinkled and smooth
Oxidase	+
Catalase	+
Motility	+
Spore formation	–
pH optimum	±7.5
Temp. optimum	30 °C
Growth at 4 °C	–
Growth at 40 °C	+
Growth substrates	Glycogen, glucose, maltose, 3-methylglucose, α-methyl-D-glucoside, β-methyl-D-glucoside, α-methyl-D-mannoside, palatinose, stachyose, D-trehalose, turanose, D-sorbitol, xylitol, dextrin, α-cyclodextrin, inulin, proline, L-alanine, lactamide, acetate, α-hydroxybutyrate, β-hydroxybutyrate, caprate, citrate, gluconate, L-glutamate, itaconate, α-ketoglutarate, α-ketovalerate, D-lactate-methylester, D-malate, L-malate, propionate, pyruvate and methylpyruvate
No growth	Amygdalin, L-arabinose, D-arabitol, arbutin, cellobiose, D-fructrose, D-galactose, gentiobiose, mannose, D-melibiose, rhamnose, D-ribose, salicin, sedoheptulosan, D-sucrose, D-tagatose, D-xylose, inositol, mannitol, mannan, γ-hydroxybutyrate, γ-hydroxyphenylacetate, L-lactate, mono-methylsuccinate, succinate, *N*-acetyl L-glutamate, D-galacturonate, alaninamide, D-alanine, L-alanine, L-alanyl-glycine, L-asparagine, glycyl-L-glutamate, L-pyroglutamate, L-serine, putrescine, 2,3-butanediol, glycerol, adenosine, 2′-deoxy adenosinosine, thymidine, uridine, histidine, γ-cyclodextrin, adenosine-5′-monophosphate, uridine-5′-monophosphate, frutose-6-phosphate, glucose-1-phosphate, glucose-6-phosphate, D-L-g-glycerol phosphate, 2-ketogluconate, *N*-acetyl glucosamine, adipate, suberate, 3-hydroxybenzoate, 4-hydroxybenzoate and phenyl-acetate

chloropropane, 2-chloropropane, 1,2-dichloropropane, 1,3-dichloropropane, 1,3-dichloro-2-propanol, 2-chloropropanol, vinyl chloride, 1,1-dichloroethene, *cis*-1,2-dichloroethene, *trans*-1,2-dichloroethene, trichloroethene and tetrachloroethene.

During growth on CE, strain JJ reduced NO_3^-, NO_2^- and O_2. Not utilized as electron acceptor were SO_4^{2-}, $Fe(OH)_3$, MnO_2, ClO_3^- and fumarate.

Growth on CE

Growth on CE with NO_3^- started after a lag phase of about 12 hours and proceeded according to a maximum specific growth rate (μ_{max}) of 0.14 h^{-1} (Fig. 1A). After 45 days, 4.8 mM of Cl^- was produced and 4.4 mM CE had been consumed (Fig. 1B). Concurrently, 12.2 mM of NO_3^- was reduced, mainly to NO_2^- (6.2 mM) and presumable to N_2 (not measured), since NH_4^+ was not produced. The total amount of protein formed was 19.4 mg/l, corresponding to a yield of 4.4 g protein per mol CE metabolized. When grown on CE and NO_3^-, strain JJ displayed a temperature optimum at 30 °C. No growth was observed at 4 °C or at 45 °C.

When both O_2 and NO_3^- were simultaneously present in a batch culture, strain JJ had a preference for O_2. The μ_{max} during the aerobic growth phase on CE was 0.31 h^{-1}. When the oxygen concentration dropped to below approximately 500 μM, strain JJ started to reduce NO_3^- to NO_2^-. NO_2^- was subsequently reduced to most likely N_2 gas, since no NH_4^+ formation was detected.

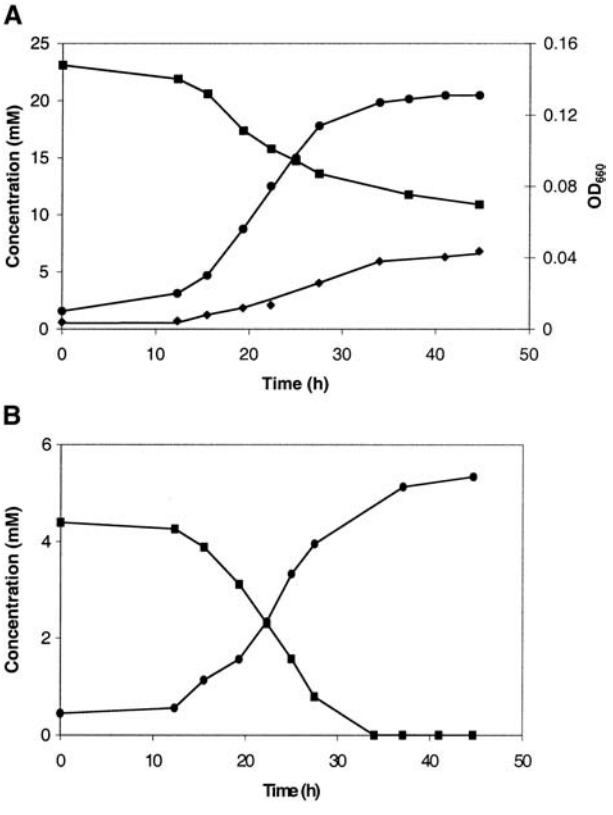

Fig. 1A, B Degradation of 2-chloroethanol (5 mM) with nitrate (10 mM) as electron acceptor by *Pseudomonas stutzeri* strain JJ. **A** Increase in optical density at 660 nm (●), the consumption of NO_3^- (■) and the formation of nitrite (◆). **B** Degradation of 2-chloroethanol (■) and the release of chloride (●)

Table 2 The ability of different *Pseudomonas* strains, *Xanthobacter autotrophicus* GJ10 and *Alcaligenes xylosoxidans* subsp. *denitrificans* to grow on 2-chloroethanol under denitrifying or oxic conditions

Species	Growth conditions		
	Succinate+NO_3^-	Chloroethanol+NO_3^-	Chloroethanol+O_2
Strain JJ	+	+	+
Pseudomonas stutzeri type strain	+	−	−
Pseudomonas stutzeri strain LMD 76.42	+	−	+
Pseudomonas stutzeri strain LMD 26.48	+	−	−
Pseudomonas stutzeri strain KC	+	−	−
Pseudomonas stutzeri strain 346B	+	−	−
Pseudomonas mendocina type strain	+	−	−
Pseudomonas sp. strain P51	−	−	−
Pseudomonas putida strain C 3024	−	−	−
Xanthobacter autotrophicus GJ10	+	−	+
Pseudomonas aeruginosa	+	−	−
Pseudomonas putida US2	+	−	+
Alcaligenes xylosoxidans subsp. *denitrificans*	+	−	−

Comparison of newly isolated strain JJ with other bacterial strains

Eleven bacterial strains were tested for their ability to grow on CE under both aerobic and denitrifying conditions (Table 2). *Pseudomonas* sp. strain P51 and *Pseudomonas putida* strain P42 could not grow on succinate with NO_3^- as electron acceptor. *Pseudomonas stutzeri* strain LMD 76.42, *Pseudomonas putida* US2 and *Xanthobacter autotrophicus* GJ10 were able to oxidize CE under aerobic conditions. The other tested strains were able to denitrify, but did not oxidize CE. Only strain JJ was able to oxidize CE under both aerobic and denitrifying conditions.

Discussion

This report describes the isolation and characterization of a bacterium that is able to grow on CE under aerobic and denitrifying conditions. The isolated strain was designated as *Pseudomonas stutzeri* strain JJ and is the first pure bacterial culture described that is capable of anaerobic oxidation of CE with NO_3^- as electron acceptor.

Based on the present findings, the reaction equation for growth of strain JJ on CE plus NO_3^- is: CE+$2HNO_3 \rightarrow 2CO_2+HCl^-+N_2+3H_2O$ and CE+$5HNO_3 \rightarrow 2CO_2+HCl^-+5HNO_2+2H_2O$. When NO_3^- was supplied in excess (23.1 mM NO_3^-+4.4 mM CE), nitrite accumulated (up to 6.2 mM) in batches of strain JJ. It is known that nitrate can have an inhibitory effect on nitrite reduction by *Pseudomonas stutzeri* (Kodoma et al. 1969; Körner and Zumft 1989). Also, the nitrate concentration in the pre-culture is important, since nitrate starvation inactivates the existing capacity of NO_2^- reduction (Xu and Enfors 1996).

Various other bacterial strains that were tested, including some closely related *Pseudomonas stutzeri* strains, did not grow on CE with NO_3^-, indicating that the ability to grow on CE with NO_3^- is an exceptional feature of *Pseudomonas stutzeri* strain JJ. Possibly, strain JJ expresses a 2-chloroethanol dehydrogenase or a hydrolytic 2-chloroethanol dehalogenase, enabling it to grow on CE under denitrifying conditions. However, it is remarkable that *Xanthobacter autotrophicus* GJ10 cannot grow on CE with NO_3− This strain is able to denitrify, to grow on CE aerobically, and it contains both dehydrogenases and dehalogenases, which do not require oxygen (Janssen et al. 1985, 1984). More insight into the regulation of the CE degradation pathway under denitrifying conditions may clarify this observation.

The ability of strain JJ to grow on CE and chloroacetate under denitrifying conditions may be important for survival in its natural habitat. The aquifer from which strain JJ was obtained was anoxic and contained small amounts of NO_3^- (<10 μM), chlorinated aliphatics, including 1,2-DCA (up to 3.5 mM), vinyl chloride and various chlorinated methanes and propanes (Gerritse et al. 1999). The ability to use low-chlorinated aliphatics as substrates under denitrifying conditions may add significantly to the competitive strength of strain JJ in the environment. In addition, if O_2 and NO_3^- are both present in the field, strain JJ can easily switch from aerobic growth to denitrification, when O_2 becomes limited, and vice versa.

It remains unclear which chlorinated compound(s) served as growth substrate for strain JJ in the field. CE and chloroacetate are degradation products from 1,2-DCA. Unfortunately, CE and chloroacetate are not covered in the standard analyses of the pollutants at this location. Another possibility would be that strain JJ could grow on 1,2-DCA in a mixed culture, since the enrichment from which strain JJ originates was able to degrade 1,2-DCA under denitrifying conditions (Gerritse et al. 1999). Finally, strain JJ may have lost the ability to degrade 1,2-DCA during isolation and cultivation on CE in the laboratory. The transfer and expression of a 1,2-DCA dehalogenase, e.g. from *Xanthobacter autotrophicus* GJ10, might enable strain JJ to grow on 1,2-DCA (Janssen et al. 1985).

The observation that denitrifying bacteria can use chlorinated aliphatics may have important implications for the natural attenuation and bioremediation of contaminated aquifers. The presence of NO_3^- in the groundwater may enable biodegradation of chlorinated aliphatics that entered the soil directly or have been formed in situ via partial dechlorination. Anaerobic oxidation pathways are of particular importance when chlorinated contaminated plumes spread from reduced (e.g. methanogenic, sulfate-reducing) to more oxidized (e.g. iron-reducing and denitrifying) redox zones. These groundwater situations frequently occur at polluted sites in Western Europe.

Nitrate-coupled bioremediation has several advantages because nitrate reducers grow rapidly, NO_3^- dissolves well in water and it does not form precipitates in anoxic groundwater, which frequently clog infiltration wells during aerobic bioremediation.

Oxidation of vinyl chloride and 1,2-DCA has also been demonstrated under denitrifying conditions (Cox et al. 2000; Dijk et al. 2000; Gerritse et al. 1999). Since no microorganisms that are able to oxidize vinyl chloride or 1,2-DCA with NO_3^- have been described thus far, the isolation of such bacteria would provide useful information for natural attenuation and bioremediation.

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