SUPPLEMENTARY MATERIAL

Anaerobic Degradation of 4-Methylbenzoate by a New Denitrifying Bacterium, Strain pMbN1

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Material and Methods

Figure S1

MATERIALS AND METHODS

Source of bacteria. Enrichments with 4-methylbenzoate under nitrate-reducing conditions were established using a homogenized mixture of mud samples collected from ditches and the river Weser in Bremen, Germany.

Media, isolation and cultivation. The denitrifying strain pMbN1 was cultivated in ascorbate-reduced (4 mM), bicarbonate-buffered defined mineral media under nitrate-reducing conditions (5 mM) as described previously (6). Establishment and sub-culturing of enrichments was carried out in ascorbate-free medium. Isolation of 4-methylbenzoate-degrading denitrifiers from sediment-free enrichment cultures was achieved via agar dilution series (9). Purity checks with various organic substances (incl. yeast extract) always yielded the same morphotype. Maintenance was conducted as previously described (6). Compounds tested for supporting anaerobic or aerobic growth of strain pMbN1 are indicated in Table 2. Anaerobic and aerobic growth experiments, determination of temperature and pH ranges, as well as testing for growth on agar plates were carried out in triplicates.

Anaerobic cultivation of strain pMbN1 was carried out in volumes of 15 ml in glass tubes (20 ml) sealed with butyl rubber septa and screw caps under N_2/CO_2 (90:10, v/v). Cultures were routinely incubated at 28°C. Cultures used for analysing intermediates of nitrate reduction had an Ar headspace and contained a phosphate buffered medium (11).

For aerobic cultivation of strain pMbN1, the same medium was used as described before, with the exception that no ascorbate and no $NaNO_3$ were added. $NaHCO_3$ was added as follows. Glass tubes (20 ml) for cultivation contained 4 ml medium and were sealed with butyl rubber septa and screw caps maintaining the oxic atmosphere. A volume of 120 µl $NaHCO_3$ (84 g/l) and 1.6 ml CO_2 were injected with CO_2-flushed syringes. To assure sufficient supply with oxygen, aerobic cultures were shaken (50 rpm) in horizontal position during incubation.

The temperature range of anaerobic growth of strain pMbN1 with 4-methylbenzoate was determined in a temperature gradient block essentially as described previously (5). The block consists of 31 evenly distributed triplicates of incubation wells. The linear temperature gradient ranged from 2.0–50.1°C. Cultivation was performed in volumes of 10 ml in glass tubes (15 ml) sealed with butyl rubber septa and screw caps under N_2/CO_2 (90:10, v/v).

The pH range of anaerobic growth of strain pMbN1 with 4-methylbenzoate was determined in medium adjusted to different final pH values. Cultivation was performed as described before in volumes of 15 ml in glass tubes (20 ml). The pH values of the media were adjusted with sterile NaOH (1 M) and HCl (1 M), respectively. The values ranged from pH 5 to pH 9 in steps of pH 0.5. Prior to inoculation the media were incubated at 28°C for at least 24 hours. Cultures were inoculated (5%, v/v) with cells grown at pH 7.4. The growth was determined by measuring the optical density at 660 nm in a spectrophotometer equipped with a tube holder (UV-1202, Shimadzu, Kyoto, Japan).

Growth of strain pMbN1 on solid medium was tested as described previously (10), using rich medium (LB, BHI) as well as a defined medium containing a mixture of organic substrates (4 mM benzoate, 5 mM pyruvate and 5 mM acetate) and incubating under oxic as well as nitrate-reducing conditions. Anaerobic incubations were carried out at 28°C in jars (Ochs, Bovenden-Lenglern, Germany) under an N_2-atmosphere.

Magnetotactic response was microscopically tested with cells of strain pMbN1 grown under microoxic conditions (approximately 2 – 7 µM O_2 at 28°C) as described before (8).

Electron microscopy. For negative staining, 30 µl of a freshly grown culture were washed with Tris-HCl buffer (100 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5). Cells were allowed to adsorb for 5 min onto Formvar- and carbon-coated cooper grids (3.05 mm/ 400 mesh; Plano GmbH, Wetzlar, Germany). The grids were stained for 4–5 min in 2% (*w/v*) uranyl acetate and washed in 2 drops of distilled water. Images were taken as digitized pictures with a Zeiss EM 902A electron microscope (Jena, Germany) operated as described before (7). Cellular dimensions of strain pMbN1 were determined from electron microscopic images using the program measureIT 5.0 (Olympus, Münster, Germany).

Determination of growth balance. The degradation balance of strain pMbN1 for 4-methylbenzoate was determined in flat glass bottles (500 ml total volume). The medium was prepared as described above. A volume of 380 ml of sterile medium was added to the bottles, supplemented with 4-methylbenzoate (from sterile stock solution) and anoxically sealed with butyl rubber stoppers and screw caps under N_2/CO_2 (90/10, v/v) atmosphere. After pre-incubation for 24 hours at 28°C, the bottles were inoculated each with 20 ml (5%, v/v) of a freshly grown 4-methylbenzoate-adapted culture by means of N_2-flushed sterile syringes. Triplicate growth cultures and duplicate controls were incubated at 28°C. Samples for optical density (OD) measurements at 660 nm and chemical analysis were taken with N_2-flushed syringes.

Dry mass of cells was determined when cultures reached the stationary growth phase. Samples (100 ml) were collected with sterile N_2-flushed syringes and washed with Tris-HCl buffer (100 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5). The cell pellets were then dried at 80°C to constant weight. Two cultures without growth substrate inoculated with 4-methylbenzoate-adapted cells served as blank for the determination of biomass formed.

Chemical analysis. 4-Methylbenzoate, nitrate and nitrite in the culture supernatant were quantified using a high performance liquid chromatography system (Sykam, Fürstenfeldbruck, Germany) as described before (6). Compound separation was achieved by means of an anion exchange column (IBJ A3, High Speed NO_x, 4 × 60 mm; Sykam GmbH), using 20 mM NaCl in 45 % ethanol as eluent. For compound detection an S 3200 UV-detector (Sykam) set at 220 nm was used. The retention times for 4-methylbenzoate, nitrate and nitrite were 4.1 min, 2.5 min and 1.8 min, respectively. The detection limits for all three compounds were at 25 µM.

Ammonium was measured using the indophenol method essentially as described by Marr (1988). Samples were diluted 1:100 in distilled H_2O. A volume of 100 µl solution A (0.3 M phenol and 0.1 mM sodium nitroprusside) and 100 µl solution B (0.5 mM NaOH and 0.26 % NaClO) were added to 1 ml of diluted sample or standard. The reaction was incubated at room temperature for 1 h in the dark and absorption was measured at 635 nm.

N_2 and N_2O were analyzed by gas chromatography essentially as described recently (11). The gas chromatograph (GC-8A; Shimadzu, Duisburg, Germany) was equipped with a thermal conductivity detector and a CP PoraPLOT Q column (3 mm × 2 mm; Agilent, Waldbronn, Germany). The column temperature was set at 40°C and argon was used as carrier gas (15.0 ml min^{-1}). A gas-tight syringe equipped with a gas valve was used to withdraw samples from culture headspaces and a sample volume of 1 ml was injected per analysis into the gas chromatograph.

Determination of G+C-content. The G+C-content was inferred from a merged draft genome sequence of strain pMbN1 (5 Mbp; 251 contigs; M. Kube and R. Reinhard, personal communication).

Nucleotide sequence, accession number and phylogenetic analysis. The 16S rRNA gene sequence of strain pMbN1 was detected in the genomic shotgun database with BLASTN (1) and then manually inspected. The start and stop positions of this gene were determined with RNAmmer (2). The sequence is deposited under EMBL accession number FQ790395. Alignment of 16S rRNA gene sequences and phylogenetic tree construction was performed using version "silva 100" of the SILVA database (4) and the ARB software package (3).

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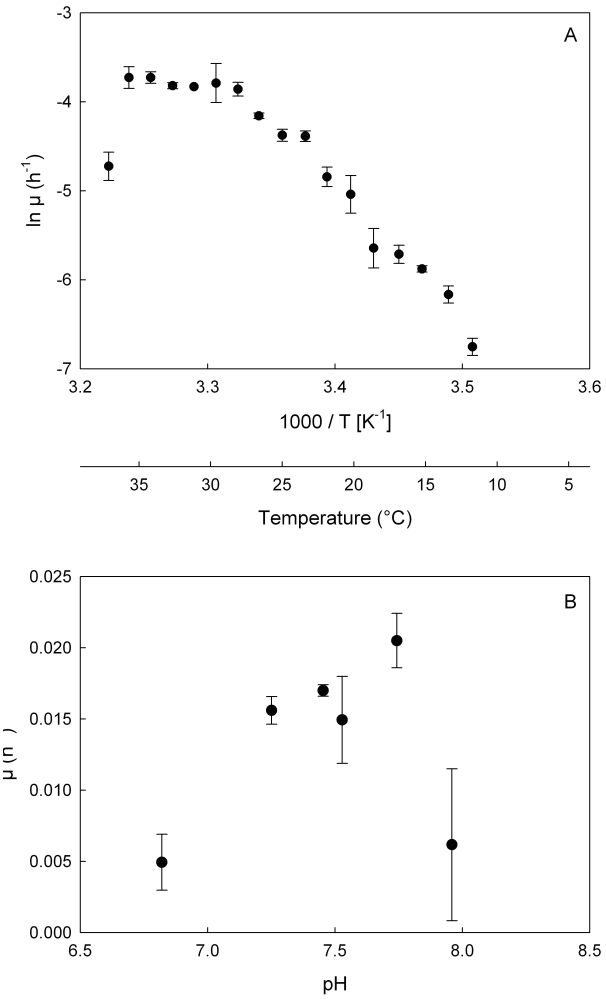

FIG. S1. Temperature and pH range for growth of strain pMbN1 with 4-methylbenzoate under nitrate-reducing conditions. (A) Arrhenius plot of temperature range determined from incubation experiments in a temperature gradient block. (B) pH dependent anaerobic growth rates.