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

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A novel alphaproteobacterium isolated from freshwater sediments, strain pMbN1, degrades 4-methylbenzoate to CO_2 under nitrate-reducing conditions. While strain pMbN1 utilizes several benzoate derivatives and other polar aromatic compounds, it cannot degrade *p*-xylene or other hydrocarbons. Based on 16S rRNA gene sequence analysis, strain pMbN1 is affiliated with the genus *Magnetospirillum*.

Aromatic compounds are structurally diverse and abundant in nature, ranging from alkylbenzenes and other crude oil components to lignin monomers and amino acids. Under anoxic conditions, which prevail in many habitats, most monoaromatic compounds are channeled via compound-specific reaction sequences into the central anaerobic benzoyl-coenzyme A (CoA) pathway for further degradation (5, 10). Among alkylbenzenes, *p*-xylene seems to be particularly difficult to degrade under anoxic conditions; for example, enrichment cultures of nitrate- (31) and sulfate-reducing bacteria (29, 44) with crude oil as the only source of organic carbon readily consumed *o*- and *m*-alkyltoluenes, but not the *p*-alkylated isomers. Attempts to isolate anaerobic bacteria growing with *p*-xylene as the sole source of carbon and energy have thus far not transcended the level of enrichment cultures (12,

TABLE 1 Anaerobically alkylbenzene- and alkyl benzoate-degrading proteobacteria[a]

Organism	Subgroup of *Proteobacteria*	Substrate for anaerobic growth		Cometabolic conversion of *p*-xylene to 4-methylbenzoate[d]	Reference(s)
		Alkylbenzene[b]	Benzoate/alkylbenzoate[c]		
Nitrate-reducing bacteria					
Thauera aromatica K172	*Beta-*	Toluene	Benzoate	+	2, 4
"*Aromatoleum aromaticum*" EbN1	*Beta-*	Toluene, ethylbenzene	Benzoate	ND	28
"*Aromatoleum*" sp. pCyN1	*Beta-*	Toluene, *p*-cymene, *p*-ethyltoluene	Benzoate, 4-isopropylbenzoate, 4-ethylbenzoate	ND	13
Magnetospirillum sp. pMbN1	*Alpha-*		Benzoate, 3-methylbenzoate, 4-methylbenzoate	ND	This study
Sulfate-reducing bacteria					
Desulfobacula toluolica strain Tol2	*Delta-*	Toluene	Benzoate	+	27, 30
Strain oXyS1	*Delta-*	Toluene, *o*-xylene	Benzoate, 2-methylbenzoate	ND	14
Strain mXyS1	*Delta-*	Toluene, *m*-xylene	Benzoate, 3-methylbenzoate	ND	14
Iron-reducing bacterium					
Geobacter metallireducens GS15	*Delta-*	Toluene	Benzoate	ND	20
Phototrophic bacterium					
Blastochloris sulfoviridis ToP1	*Alpha-*	Toluene	Benzoate	ND	48

[a] For a detailed overview of anaerobically alkylbenzene-degrading bacteria, see reference 43.
[b] None of the listed strains is capable of anaerobic degradation of *p*-xylene.
[c] Except for *Magnetospirillum* sp. pMbN1, none of the listed strains is capable of anaerobically degrading 4-methylbenzoate.
[d] Toluene-utilizing cells cometabolically convert *p*-xylene into 4-methylbenzoate as a dead-end product. +, positive for conversion; ND, not determined.

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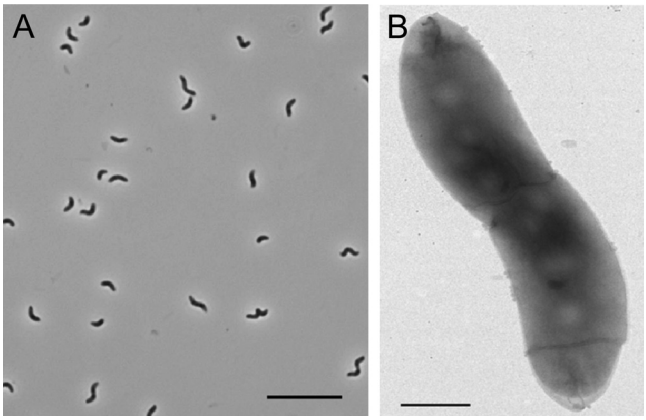

FIG 1 Micrographs of strain pMbN1. (A) Phase contrast micrograph. Bar, 10 μm. (B) Electron micrograph of negatively stained cell. Bar, 0.5 μm.

25, 32, 46). The recalcitrance of *p*-xylene is unlikely to result from difficulties with initial substrate activation, since early experiments with anaerobically toluene-degrading bacteria demonstrated *p*-xylene conversion to 4-methylbenzoate as a dead-end metabolite (4, 27) and analysis of *p*-xylene-consuming enrichment cultures revealed formation of (4-methylbenzyl)succinate as the initial metabolite (3, 25, 32). Thus, the subsequent degradation of 4-methylbenzoate appears to be the bottleneck in anaerobic *p*-xylene degradation, agreeing with the inability of presently known alkylbenzene-degrading (facultatively) anaerobic isolates to utilize 4-methylbenzoate (Table 1) (for an overview, see reference 42). Moreover, studies with wastewater-treating anaerobic bioreactors demonstrated the markedly longer time requirement for 4-methylbenzoate degradation than that for benzoate and phthalate isomers (16).

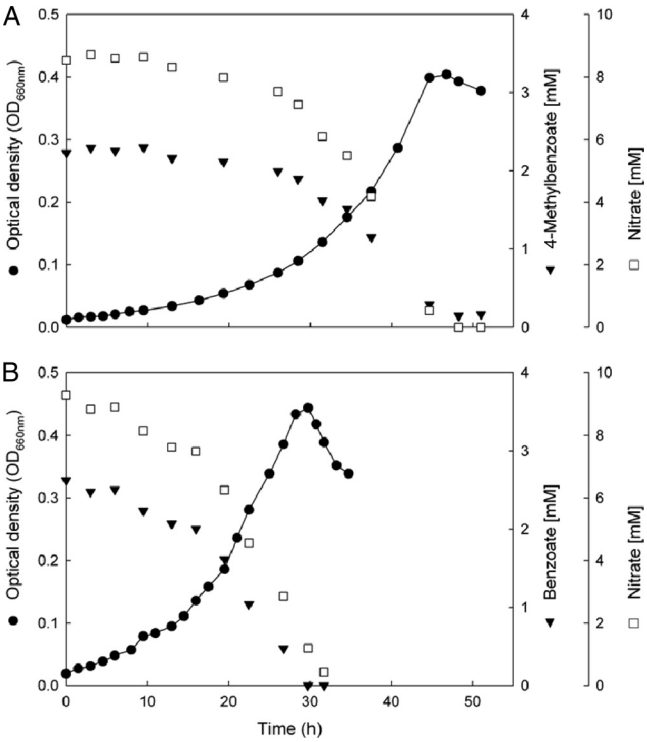

FIG 2 Anaerobic growth of strain pMbN1 with benzoates under nitrate-reducing conditions. (A) With 4-methylbenzoate. (B) With benzoate. Symbols are as indicated along the *y* axes. No intermediate formation of nitrite could be detected.

TABLE 2 Anaerobic and aerobic growth tests of denitrifying strain pMbN1 with different aromatic and nonaromatic compounds[a]

Compound tested (concn)[b]	Growth[c]	
	Anaerobic	Aerobic
Aromatic compounds		
4-Methylbenzoate (1, 2.5)	+	+
3-Methylbenzoate (1, 2)	+	−
Benzoate (1, 4)	+	+
2-Aminobenzoate (1, 2)	+	−
4-Hydroxybenzoate (1, 4)	+	−
Phenylacetate (1, 4)	+	−
Cinnamate (2)	+	+
Hydrocinnamate (2)	+	+
p-Coumarate (2)	+	+
Phenol (0.5, 2)	+	−
p-Cresol (0.5, 2)	+	−
Benzyl alcohol (0.5, 2)	+	−
Benzaldehyde (0.5, 2)	+	−
Nonaromatic compounds		
Acetate (1, 5)	+	+
Propionate (1, 4)	+	+
Butyrate (1, 4)	+	+
Isobutyrate (1, 4)	−	+
Adipate (1, 5)	+	+
Lactate (5, 10)	+	+
Malate (1, 5)	+	+
Pyruvate (1, 5)	+	+
Succinate (1, 5)	+	+
Ethanol (1, 5)	+	+
Complex media		
Yeast extract (0.5% [wt/vol])	+	+
Peptone (0.5% [wt/vol])	−	−
Casamino acids (0.5, 2% [wt/vol])	−	−

[a] Further compounds tested but not utilized by strain pMbN1 under nitrate-reducing or oxic conditions are as follows (concentrations given in percent volume/volume refer to dilutions of poorly water soluble compounds in heptamethylnonane as inert carrier phase): toluene (2%), 4-ethyltoluene (2%), ethylbenzene (2%), propylbenzene (2%), *m*-xylene (2%), *p*-xylene (1%), 2-methylbenzoate (1, 2 mM), 4-ethylbenzoate (1, 2 mM), 4-propylbenzoate (0.5, 2 mM), 4-isopropylbenzoate (1, 2 mM), 4-butylbenzoate (0.5, 2 mM), 4-*tert*-butylbenzoate (0.5, 2 mM), terephthalate (0.5, 2 mM), resorcinol (1 mM), *o*-cresol (0.5, 2 mM), *m*-cresol (0.5, 2 mM), 1-phenylpropanol (0.5, 2 mM), (*R*)-1-phenylethanol (0.5, 2 mM), (*S*)-1-phenylethanol (0.5, 2 mM), acetophenone (1%), propiophenone (1%), *p*-cymene (2, 5%), α-phellandrene (1, 2%), α-terpinene (1, 2%), limonene (1, 2%), cyclohexane carboxylate (0.5, 2 mM), cyclohexanol (1, 2%), cyclohexane-1,2-diol (1, 5 mM), *n*-hexane (2%), glucose (1, 5 mM), fructose (1, 5 mM), all 20 amino acids (0.5, 2 mM), ascorbate (4 mM), formate (10, 20 mM), acetone (0.5, 2 mM).

[b] Each compound was tested at the concentrations shown in parentheses and given in mM unless otherwise indicated.

[c] +, optical density at 660 nm of >0.1; −, no growth.

In the present study, enrichment of bacteria degrading 4-methylbenzoate under nitrate-reducing conditions was attempted in defined mineral medium (28) at 28°C in 250-ml bottles containing 200 ml of mineral medium, 15 ml of mud mixture (collected from ditches and the Weser River in Bremen, Germany), 1.5 mM 4-methylbenzoate, and 5 mM $NaNO_3$. Nitrate consumption was monitored by high-pressure liquid chromatog-

TABLE 3 Quantification of 4-methylbenzoate (substrate) and nitrate consumption and of produced biomass by strain pMbN1[a]

Experiment	Amt of substrate added (mmol)	Amt of substrate disappeared[b] (mmol)	Amt of nitrate disappeared (mmol)	Amt of cell dry mass formed[c] (mg)	Amt of substrate dissimilated[d] (mmol)	Amt of electrons from substrate dissimilated[e] (mmol)	Amt of electrons consumed by nitrate reduction[f] (mmol)
Cells with limiting amt of substrate	0.34 (0.05)	0.34 (0.13)	1.4 (0.4)	21 (3)	0.25 (0.06)	9.0 (2.0)	7.0 (1.8)
Cells with excess amt of substrate	0.89 (0.07)	0.83 (0.17)	3.4 (0.2)	56 (6)	0.57 (0.05)	20.7 (2.0)	17.1 (1.2)
Cells without substrate (control)	0.00[g]	0[g]	0.0[g]	0.0[g]			0.0[g]
Sterile medium without cells (control)	0.83[g]	0[g]	0.0[g]				0.0[g]

[a] Incubation experiments were carried out in anoxic flat glass bottles with a culture volume of 400 ml containing 3.4 mmol nitrate. Numbers in parentheses represent the standard deviation calculated from triplicate experiments.
[b] Difference between substrate added and substrate recovered at the end of incubation in the culture supernatant.
[c] The amount of cell dry mass added with the inoculum has been subtracted.
[d] Differences between substrate dissimilated and substrate assimilated. The assimilated amount of 4-methylbenzoate was calculated according to the following equation: $17\ C_8H_7O_2^- + 8\ HCO_3^- + 25\ H^+ + 50\ H_2O \rightarrow 36\ C_4H_7O_3$. Thus, 1 mg of cell dry mass requires 0.00458 mmol 4-methylbenzoate.
[e] Thirty-six moles of electrons are derived from 1 mol of 4-methylbenzoate if oxidized to CO_2.
[f] Electrons consumed = 5 × (nitrate added − nitrate remaining). Nitrite has not been observed in detectable quantities during growth.
[g] No detectable deviations in duplicate experiments.

raphy (HPLC), and nitrate was several times replenished upon depletion. The initial enrichment culture grew within 14 days. The sediment-free subcultures obtained after four transfers displayed a doubling time of 10 h, resembling those of earlier reported enrichments with 4-methylbenzoate and nitrate (13). After several passages, the sediment-free enrichment cultures were dominated by spirillum-shaped cells. Isolation of 4-methylbenzoate-degrading nitrate-reducing bacteria was then attempted with anoxic agar-dilution series (41). Whitish colonies were retrieved by means of finely-drawn Pasteur pipettes and transferred to liquid media. The best-growing strain was chosen for further analysis and designated pMbN1. For further cultivation of strain pMbN1 under nitrate-reducing conditions, the reductant ascorbate (4 mM) was routinely added to the mineral medium. All of the following growth experiments were conducted in triplicate. Additional information on materials and methods is provided in the supplemental material.

The isolate strain pMbN1 had a spirillum-like shape (Fig. 1) with dimensions of 1.8 to 3.6 μm by 0.6 to 0.8 μm. Cells stained as Gram-negative and were motile. However, they did not display a magnetotactic response during microaerobic growth (33; D. Schüler, personal communication). Strain pMbN1 did not grow on solid media (rich or mineral) under oxic or nitrate-reducing conditions; anaerobic incubation of plates was carried out at 28°C in jars under an N_2 atmosphere as recently described (45).

The temperature range of anaerobic growth of strain pMbN1 with 4-methylbenzoate was tested in a temperature gradient block. The observed temperature range of growth was 11.9 to 37.2°C, with an optimum range of 26.2 to 35.7°C (determined by means of maximal growth rates [μ_{max}]; see the Arrhenius plot in Fig. S1A in the supplemental material). All subsequent cultures were incubated at 28°C. The pH range of anaerobic growth with 4-methylbenzoate was pH 6.8 to pH 8.0, with an optimum around pH 7.3 to pH 7.7 (determined using μ_{max}; see Fig. S1B in the supplemental material).

Approximate doubling times of strain pMbN1 during anaerobic growth with 4-methylbenzoate and benzoate were 9.5 h (specific μ_{max}, 0.10 h^{-1}) and 6.1 h (specific μ_{max}, 0.16 h^{-1}), respectively (Fig. 2).

In addition to 4-methylbenzoate, a wide range of other benzoate derivatives, phenylpropanoids, and aliphatic carboxylates were anaerobically utilized by strain pMbN1. All compounds tested for supporting aerobic or anaerobic growth are indicated together with the applied concentrations in Table 2. Remarkably, strain pMbN1 could not grow anaerobically with other 4-alkylbenzoates, *p*-xylene, or other hydrocarbons, indicating a specialization for benzoate derivatives and simple aliphatic carboxylates. Although strain pMbN1 is facultatively aerobic, it could degrade only a few aromatic compounds under oxic conditions. The nutritional specialization of strain pMbN1 is further underpinned by its inability to utilize amino acids or carbohydrates (Table 2) and by the lack of lithoautotrophic growth with H_2 (10% [vol/vol] in gas headspace) as the electron donor and nitrate (10 mM), chlorate (5 and 10 mM), or perchlorate (5 and 10 mM) as the electron acceptor.

During growth with 4-methylbenzoate under nitrate (NO_3^-)-reducing conditions, strain pMbN1 did not intermediately excrete nitrite (NO_2^-), as do other aromatic compound-degrading denitrifiers, such as "*Aromatoleum aromaticum*" EbN1 (28). At the end of nitrate-limited growth (10 mM NO_3^- consumed), no accumulation of ammonium (NH_4^+) was observed, but formation of 1.3 mM dinitrogen monoxide (N_2O) and 2.4 mM dinitrogen (N_2) was detected. This suggests that strain pMbN1 reduces nitrate via denitrification. NO_3^- and NO_2^- were quantified using an HPLC system equipped with an anion exchange column and an UV detector as described previously (28). NH_4^+ was spectrophotometrically measured using the indophenol method (23). Formation of N_2O and N_2 was determined by gas chromatography as recently described (47).

The biochemical challenges imposed by the *para*-methyl group of 4-methylbenzoate on the dearomatization of this compound as well as on the subsequent reaction sequence (ring cleavage and β-oxidation) raised a question about the capacity of strain pMbN1 to anaerobically oxidize 4-methylbenzoate completely to CO_2. Thus, the degradation of this compound coupled to denitrification was balanced (Table 3) using cultures (400 ml) of strain pMbN1 provided with limiting (0.34 mmol) or excess (0.89 mmol) amounts of 4-methylbenzoate relative to the added

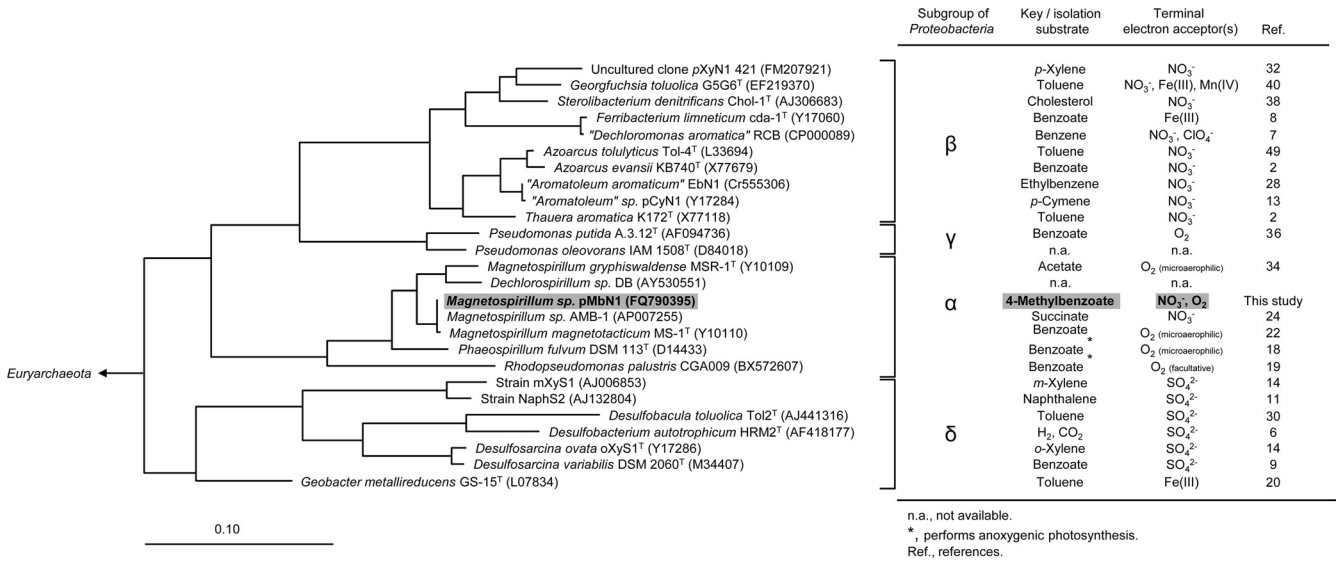

FIG 3 Phylogenetic tree of 16S rRNA gene sequence from strain pMbN1. The cluster shown represents selected members of the *Proteobacteria*. Scale bar, 10% sequence divergence.

amount of electron acceptor (3.4 mmol nitrate). Determination of the growth balance was based on quantifying consumption of 4-methylbenzoate and nitrate (by HPLC as described above), as well as on formation of biomass when cultures reached the stationary growth phase. Determination of the dry mass of cells involved a washing step with Tris buffer (100 mM Tris-HCl, 5 mM $MgCl_2$, pH 7.5) and drying at 80°C to constant weight. The addition of limiting amounts of 4-methylbenzoate resulted in its complete depletion, while about half of the provided nitrate was recovered. In the experiment with excess amounts of 4-methylbenzoate, 7.2% (±0.6%) of the organic substrate was recovered, while the added nitrate was completely consumed. The average molar growth yield of strain pMbN1 determined from these two organic substrate conditions was 65 g of dry mass per mol 4-methylbenzoate. The amount of electrons formed from dissimilation of 4-methylbenzoate was close to the amount of electrons consumed by nitrate reduction, in agreement with the following stoichiometric equation for complete substrate oxidation:

$$C_8H_7O_2^- + 7.2\ NO_3^- + 0.4\ H_2O + 0.2\ H^+ \rightarrow 8\ HCO_3^- + 3.6\ N_2$$

$$\Delta G^{\circ\prime} = -3{,}583\ \text{kJ/mol 4-methylbenzoate}$$

Calculation of free energy ($\Delta G^{\circ\prime}$) is based on standard values (37, 39). The dissimilated amount of 4-methylbenzoate was about 20% higher than required for nitrate reduction, as has previously also been observed for the anaerobic oxidation of ethylbenzene and *p*-cymene in the denitrifying *Aromatoleum* strains EbN1 and pCyN1, respectively (13, 28), assumed to result from partial conversion of the substrate to unknown organic compounds.

The G+C content of 65.9 mol% was inferred from a merged draft genome sequence of strain pMbN1 (5 Mbp, 251 contigs; M. Kube and R. Reinhardt, personal communication). The 16S rRNA gene sequence was retrieved and finished from the genomic shotgun database using the BLASTN (1) and RNAmmer (17) programs. Phylogenetic analysis using the Silva database (26) and the ARB software package (21) revealed affiliation of strain pMbN1 with the genus *Magnetospirillum* of the *Alphaproteobacteria* (34) (Fig. 3). Thus, strain pMbN1 is phylogenetically distinct from the *Aromatoleum*/*Azoarcus*/*Thauera* cluster within the *Betaproteobacteria*, which comprises the majority of currently known aromatic compound-degrading denitrifiers (15, 43). Some members of the genus *Magnetospirillum* were previously shown to degrade various aromatic compounds under nitrate-reducing conditions; growth tests with the isomers of methylbenzoate were not reported (35). Among members of the related genus *Phaeospirillum*, utilization of aromatic compounds seems to be less well studied (18).

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