Arch Microbiol (2000) 173 : 146–153
Digital Object Identifier (DOI) 10.1007/s002039900124

ORIGINAL PAPER

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Isolation and characterization of two new methanesulfonic acid-degrading bacterial isolates from a Portuguese soil sample

Received: 11 August 1999 / Accepted: 10 November 1999 / Published online: 8 December 1999

Abstract Two novel bacterial strains that can utilize methanesulfonic acid as a source of carbon and energy were isolated from a soil sample collected in northern Portugal. Morphological, physiological, biochemical and molecular biological characterization of the two isolates indicate that strain P1 is a pink-pigmented facultative methylotroph belonging to the genus *Methylobacterium*, while strain P2 is a restricted methylotroph belonging to the genus *Hyphomicrobium*. Both strains are strictly aerobic, degrade methanesulfonate, and release small quantities of sulfite into the medium. Growth on methanesulfonate induces a specific polypeptide profile in each strain. This, together with the positive hybridization to a DNA probe that carries the *msm* genes of *Methylosulfonomonas methylovora* strain M2, strongly endorses the contention that a methanesulfonic acid monooxygenase related to that found in the previously known methanesulfonate-utilizing bacteria is present in strains P1 and P2. The isolation of bacteria containing conserved *msm* genes from diverse environments and geographical locations supports the hypothesis that a common enzyme may be globally responsible for the oxidation of methanesulfonate by natural methylotrophic communities.

Key words Sulfur · Methanesulfonic acid · Methylotroph · α-Proteobacteria · Monooxygenase · *Hyphomicrobium* · *Methylobacterium*

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Introduction

Methanesulfonic acid (CH_3SO_3H) is a compound produced in the atmosphere by the natural processes of oxidation of dimethylsulfide brought about by the free radicals NO_x and OH (Andreae 1986). Dimethylsulfide originates from the decomposition of algal, cyanobacterial and marine-plant biomass in oceans and salt marshes (Steudler and Peterson 1984; Kwint and Kramer 1995). Many of these organisms accumulate dimethylsulfoniopropionate as an internal compatible solute in response to external osmotic pressure. This osmolyte is then released into sea water upon death of the cells and is degraded by bacteria and fungi to acrylate and dimethylsulfide (de Zwart and Kuenen 1992; Bacic and Yoch 1998). Dimethylsulfide itself is in part degraded in situ by methylotrophic bacteria (Kiene and Bates 1990), but, due to its volatile nature, some of it escapes to the gas phase. The largest contribution of organic sulfur from the oceans to the atmosphere is in the form of dimethylsulfide, amounting to 4×10^{10} kg year^{-1} (Kelly and Smith 1990). Estimates indicate that up to 50% of the atmospheric dimethylsulfide is converted to methanesulfonate (Andreae 1986; Mihalopoulos et al. 1992), which subsequently, due to its hygroscopic characteristics, takes part in the formation of cloud condensation nuclei, falling back to Earth within precipitation. Despite an estimated rate of deposition of ca. 10^{10} kg year^{-1}, methanesulfonate accumulation in nature has been recorded only in the perennial ices of Antarctica (Saigne and Legrand 1987) and Greenland (Whung et al. 1994). In no other environment has methanesulfonate ever been detected at appreciable levels. These data suggest that methanesulfonate is rapidly degraded by naturally occurring communities.

Methanesulfonate can be used as a source of sulfur, but not of carbon, by certain microorganisms (Uria-Nickelsen et al. 1993a, 1993b; Key et al. 1998; van der Ploeg et al. 1998; Kertesz et al. 1999; Reichenbecher and Murrell 1999). However, the C_1 moiety of the molecule can otherwise support the growth of methylotrophic microbes. Indeed, two bacterial strains that use methanesulfonate as

sole source of carbon and energy were isolated from soil and sea water, respectively. *Methylosulfonomonas methylovora* strain M2 was enriched from a soil sample collected in the Midlands, UK (Kelly and Baker 1990; Baker et al. 1991). *Marinosulfonomonas methylotropha* strain PSCH4 was obtained from sea water collected on the south coast of Great Britain, English Channel (Thompson et al. 1995). Both these isolates belong to the α-subgroup of the Proteobacteria (Holmes et al. 1997) and metabolize methanesulfonate through: (1) an oxygenation/cleavage reaction that produces sulfite and formaldehyde, and (2) subsequent steps of dehydrogenation that yield in succession formate and carbon dioxide (Kelly et al. 1994). Both these strains assimilate carbon in the form of formaldehyde through the serine pathway and possess an inducible, NADH-dependent multimeric monooxygenase (methanesulfonic acid monooxygenase) responsible for the first step in the catabolism of methanesulfonate (Kelly et al. 1994; Thompson et al. 1995; Higgins et al. 1996). The genes coding for methanesulfonic acid monooxygenase (*msmABCD*) were cloned from strain M2 and sequenced (Higgins et al. 1997; De Marco et al. 1999). The results showed that this enzyme complex is composed of a reductase and a ferredoxin that transport electrons from NADH to a terminal hydroxylase (large plus small subunit), characterized by a mononuclear, iron-containing reactive center.

The other known methanesulfonate-degrading isolate, *Ma. methylotropha* strain PSCH4, shows some physiological differences to strain M2, namely, in the range of multicarbon molecules it can utilize and in its requirement for higher concentrations of salt in the growth medium. However, despite the overall differences between the two strains and the fact that they appear only distantly related within the α-Proteobacteria, *Me. methylovora* and *Ma. methylotropha* show remarkable similarities in the way in which they metabolize methanesulfonate. Furthermore, unpublished data (Baxter et al., in preparation) show that the *msm* genes in the two bacteria share high degrees of identity.

We started this work with the aim of investigating whether methanesulfonate-utilizing microorganisms were present in a geographical location far from the one of the original isolations of *Me. methylovora* and *Ma. methylotropha* (Great Britain). Our goal included increasing knowledge of the ecophysiology of this class of bacteria and of the possible conservation of the methanesulfonic acid monooxygenase enzyme. We describe here the isolation of two novel methanesulfonate-utilizing bacteria from a soil sample collected in northern Portugal.

Materials and methods

Enrichments and growth of isolates

Enrichment and routine growth of isolates were carried out using the adapted minimal medium MinE, as described in Kelly et al. (1994), with the addition of 10 or 20 mM sodium methanesulfonate for enrichment and growth, respectively. For prolonged growth of pure cultures on methanesulfonate, a formula with stronger buffering power, MinE4, containing four times the concentration of potassium phosphate buffer (45 mM, pH 7.0) as in the original MinE, was used. Only when stated, the media were supplemented with 5,5'-dithiobis(2-nitrobenzoic acid), or Ellman's reagent (Johnston et al. 1975), at a final concentration of 1 mg ml^{-1}. Unless otherwise noted, the carbon sources tested were used at 10 mM.

Microscopy

Standard phase-contrast and epifluorescence microscopy were performed with a Nikon Labophot microscope. The Kit Gram of Sanofi Diagnostic Pasteur, France was used for Gram staining. Poly-β-hydroxy alcanoate granules were detected by a modified version of the staining procedure described in Ostle and Holt (1982): exponentially growing cells were harvested by centrifugation in a microfuge tube; 100 µl of a 1% (w/v) solution of Nile Blue A (Fluka) was added to the tube and used to resuspend the cell pellet; the tube was incubated at 55 °C for 10 min, cells were harvested by centrifugation, resuspended in 100 µl of 8% (by vol.) acetic acid, centrifuged again and resuspended in 100 µl of sterile distilled water. The cells were then re-centrifuged, resuspended in the residual liquid, spread onto a microscope glass slide and heat-fixed. Slides were kept in the dark until analyzed. Samples were visualized in UV light (excitation filter 450–490 nm; dichroic mirror 510 nm; barrier filter 520 nm). A JEOL CX100 microscope was used for electron microscopy. Aliquots from pure cultures were fixed in 2.5% (by vol) glutaraldehyde, spun onto formvar/carbon 400-mesh, 3-mm-diameter grids and contrasted with uranyl acetate 2% (w/v).

Growth studies

Growth rates and yields were measured using 50- or 100-ml cultures grown in 250 or 500 ml vigorously-shaking Erlenmeyer flasks. Growth rates were monitored by recording the optical density of cultures at 540 nm in a Shimadzu UV-1201 spectrophotometer. Yields were estimated by vacuum filtering measured volumes of the cultures on pre-weighed 0.45-µm pore filters which were subsequently dried at 70 °C until a stable weight was reached. The spectra of resistance to antibiotics of the two strains were determined using the antibiotic disk sensitivity assay by Difco Laboratories according to the manufacturer's instructions. Ampicillin, chloramphenicol, colistin, erythromycin, kanamycin, nalidixic acid, neomycin, penicillin, streptomycin, sulfonamides, tetracycline and trimethoprim were tested. The possibility of growth in anaerobiosis was evaluated by the following procedure: MinE sloppy agar (0.5% w/v) medium and the appropriate carbon source were autoclaved in transparent glass tubes and cooled to ca. 40 °C. Inocula from liquid cultures were added to the tubes and the contents completely mixed. The tubes were then cooled for the agar to solidify and incubated at 28 °C in the dark. To assess the ability of either strain to utilize nitrate as an electron acceptor in anoxic conditions, $NaNO_3$ (10 mM) was added as described in the Results section.

Enzyme assay and electrophoresis of protein

Cell-free extracts were obtained by disruption in a Heat Systems Ultrasonics Sonicator W-375 with 15 cycles of 30 s at 12-µm amplitude and 60% duty cycle intercalated with 15 cycles of 30 s off duty. The sonication buffer contained sodium phosphate buffer (50 mM, pH 7.8), NaCl (300 mM), phenylmethanesulfonyl fluoride (1 mM), EDTA (1 mM), lysozyme (1 mg ml^{-1}). The lysates were centrifuged at 90,000×*g* to eliminate particulate matter and the extracts were stored at –20 °C until used. The protein content of the cell-free extracts was measured by the Coomassie Protein Assay Reagent (Pierce) according to the manufacturer's instructions. Hydroxypyruvate reductase activity was measured as described in Krema and Lidstrom (1990). Cell-free extracts were analyzed by SDS-PAGE in 12.5% acrylamide gels according to standard procedures (Laemmli 1970).

DNA methods and PCR conditions

Genomic DNA was extracted by the lysozyme/Sarkosyl lysis method, followed by CsCl gradient purification, as described in Oakley and Murrell (1991).

The methods described in Sambrook et al. (1989) were used for DNA restriction, electrophoresis, Southern blotting, nick-translation of the probe and hybridization. The probe, containing the *msm* genes from *Me. methylovora* strain M2, was obtained by *Not*I digestion of a subclone of plasmid pDM5 (De Marco et al. 1999). The guanosine+cytosine content (mol% G+C) of the genomic DNAs was determined by the HPLC technique described in Mesbah et al. (1989) at the BCCM/LMG Culture Collection Laboratories, University of Gent, Belgium.

The 16S rRNA genes of the two strains were amplified by PCR using the primer set f27 and r519 (Lane 1991) under standard PCR conditions (30 cycles of 1 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C) with AmpliTaq DNA polymerase (Perkin Elmer). The amplified fragments were cloned into the pGEM T-Easy vector (Promega) and sequenced by Alta Bioscience, University of Birmingham, UK (Taq DyeDeoxy Terminator Cycle Sequencing and Model 373A gel apparatus, Applied Biosystems) using the universal –20 forward and reverse primers of *lacZ'*. The 16S rRNA gene sequences were analyzed with the Genetics Computer Group (GCG) Wisconsin Package (version 8.0.1-Unix, September 1994) and the programs SEQBOOT (100 iterations), DNADIST (Kimura 2-parameter), NEIGHBOR, DNAPARS and CONSENSE of the PHYLIP package (Felsenstein 1995). 16S rRNA sequences were obtained from the National Center for Biotechnology Information taxonomy database (http://www.ncbi.nlm.nih.gov/Taxonomy). An alignment of 44 sequences by 318 nucleotides was used.

Materials

Except where otherwise stated, all chemicals were of analytical grade and obtained from Aldrich or Sigma. Ethanesulfonic acid was purchased from Fluka (>98% purity). Methane was used as a 50:48:2 air:CH_4:CO_2 mixture.

Results

Isolation of methanesulfonic-acid-degrading strains

Two soil samples were used as the starting material for enrichment. The first sample was field soil with a low organic matter and high clay content. The second sample was collected in an orchard. This soil was much darker than the first and had a higher content of organic matter.

Around 20 g of each soil was deposited in closed jars with ca. 200 ml head-space. These sub-samples were kept in the jars for 3 months at room temperature (~20 °C) and moistened fortnightly by sprinklings with sterile methanesulfonate (10 mM). After 3 months incubation, ca. 1 g was collected from each sub-sample and used to inoculate two flasks containing 100 ml of minimal medium MinE and 10 mM methanesulfonate. These flasks were maintained with shaking at 28 °C until the pH of the medium fell to values of 5.5–6.0. At this point, aliquots of the decanted suspension were used to start sub-cultures. When growth was clearly detectable by eye as turbidity, dilutions to extinction were performed and the lowest dilutions exhibiting growth were analyzed for purity by microscopic inspection. Streaking to obtain single colonies on solid medium was also carried out in order to obtain pure strains.

We were not able to isolate any microorganism capable of growth on methanesulfonate from the first soil sample. The second, organic-rich soil yielded two different bacterial strains, both capable of utilizing methanesulfonate as sole carbon and energy source.

Description of strain P1

Strain P1 formed pink, smooth, circular, convex mucoid colonies of 0.5- to 3-mm diameter on agar-containing medium. The pink pigmentation, which was quite intense after growth on C_1 compounds, became more orange when strain P1 was grown on acetate or ethanol. Exposure to UV light revealed no fluorescence. When observed by phase-contrast microscopy, strain P1 grown on a C_1 substrate appeared as large motile rods showing no visible external or internal morphological features. When grown on acetate or ethanol, the cells were full of transparent spherical vesicles, possibly caused by accumulation of poly-β-hydroxy alcanoates. The fact that acetate-grown cells of strain P1,

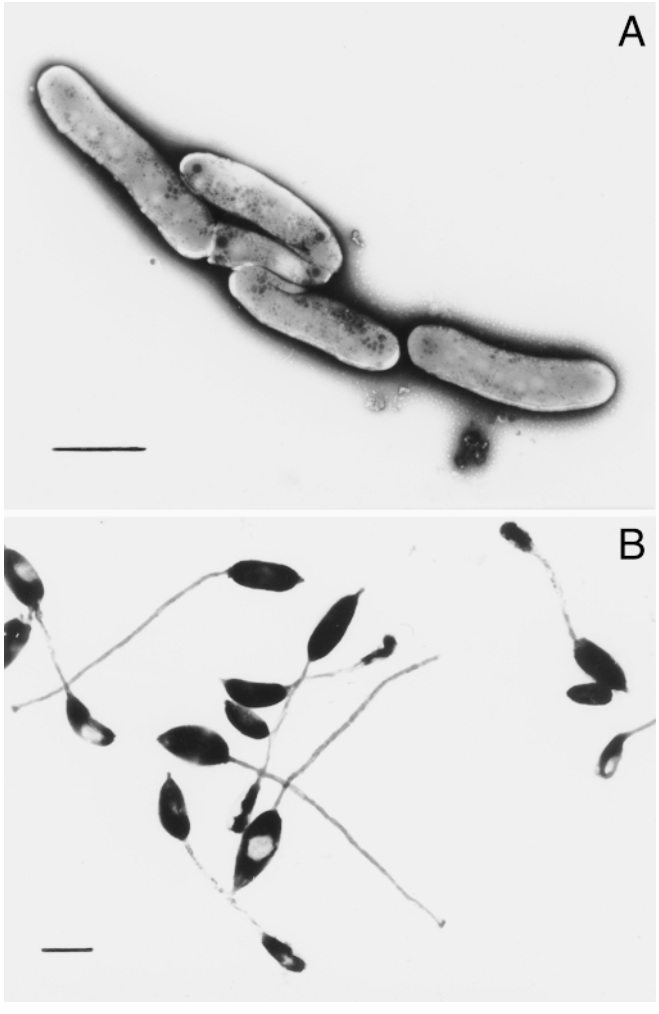

Fig. 1 Electron micrograph of cells of **A** strain P1 and **B** strain P2. *Bar*=1 μm

when stained with Nile Blue A, were replete with orange, light-emitting fluorescent bodies supports this hypothesis. Cells of strain P1 sampled during exponential growth on methylamine showed fewer fluorescent vesicles per cell (1–2). In stationary phase, only a small proportion of the cells contained fluorescent bodies.

Strain P1 stained negative with the Gram method. Transmission electron microscopy (Fig. 1A) confirmed that strain P1 cells were simple rods (2–2.6 μm in length, ca. 0.5 μm across) with no appreciable morphological features. The characteristics of strain P1 are summarized in Table 1.

Strain P1 tested negative for oxidase and weakly positive for catalase and grew on a variety of C_1 compounds as well as many non-methylotrophic substrates (Table 2). Cysteate, malate, lactate and glycine had an inhibitory effect. The presence of any of these compounds at concentrations of 10 mM suppressed not only growth in the presence of methylamine or methanesulfonate (both at 20 mM), but also the usual, residual background growth of strain P1 visible on MinE-agar plates even with no added carbon source.

The growth spectrum of strain P1 indicates that it is a facultative methylotroph. When tested in an agar-column experiment, strain P1 grew on methanesulfonate or methanol only at the air/agar interface, regardless of the presence of 10 mM $NaNO_3$, indicating that it is a strict aerobe. Strain P1 was capable of growth at 15–32 °C, but did not grow at 100 °C or 37 °C. There were no significant differences in its growth rate between 23 and 32 °C. The optimum pH for the growth (on methanol) of strain P1 was 6.0. At this slightly acidic pH, a shorter lag phase and relatively rapid growth (t_d=5.9 h) were observed and the strain showed no sign of flocculation at the end of growth, whereas this phenomenon was evident at pH 7 and 8.

Table 1 Summary of the main characteristics of strains P1 and P2. Unit of activity (U) is 1 nmol min^{-1}. *Ery* Erythromycin, *Kan* kanamycin, *Neo* neomycin, *Pen* penicillin, *Str* streptomycin, *Tet* tetracycline

Strain	P1	P2
Cell morphology	Large rods	Small ovoids with hyphae
Cell size (μm)	2.5×0.5	1.5×0.6
Gram reaction	Negative	Negative
DNA G+C content	67.5 mol%	59.4 mol%
Hydroxypyruvate reductase V_{max} [U (mg protein)$^{-1}$]	675	1122
Catalase	Weak	+
Oxidase	–	–
Sulfite release	+	+
Antibiotic sensitivity	Kan, Neo, Str, Tet	Ery, Kan, Neo, Pen, Str, Tet
Optimum growth temperature (°C)	23–32	28–32
Optimum growth pH	6	8
Molar yield on methanesulfonate (g mol^{-1})	6–9	3.2–3.4
Molar yield on methylamine (g mol^{-1})	13–14	6.7–8.4

Table 2 Growth profiles of strains P1 and P2. Substrates that did not support the growth of either strains: dimethylsulfide, methylsulfate, formamide, propanesulfonic acid, butanesulfonic acid, benzenesulfonic acid, taurine (2-amino ethanesulfonic acid), isethionate (2-hydroxy ethanesulfonic acid), succinate, citrate, benzoate, glucose, mannose, mannitol

Substrate	Strain	
	P1	P2
Methanesulfonate	+	+
Monomethylamine	+	+
Dimethylamine	–	+
Trimethylamine	–	+
Methanol	+	+
Formaldehyde (2 mM)	+	–
Formate	+	+
Methane	–	–
Ethanesulfonate	+	–
Ethanol	+	–
Glycerol	+	–
Pyruvate	+	–
Acetate	+	–
Aspartate	+	–
Maltose	+	–
Yeast extract	+	–

The presence of NaCl did not produce a marked inhibitory effect on the growth of strain P1. When tested on methylamine plus NaCl (0.5% w/v), strain P1 grew after a slightly longer lag phase than the salt-free control and at a slightly lower rate (t_d=11.4 h vs 10.4 h), but reached a comparable maximum OD_{540}. The presence of 1% (w/v) NaCl produced a longer lag phase, but did not prevent the culture from growing up to a final optical density comparable to that of the NaCl-free control, although growth occurred at a lower rate (t_d=13.4 h).

When grown on methanesulfonate, the pH of the culture dropped rapidly to stabilize around 5.5–5.0 at the end of growth. It was demonstrated (data not shown) that, using MinE medium and in the presence of an initial concentration of methanesulfonate of 20 mM, the factor limiting growth was the resulting low pH, since adjustment to pH 7.0 during early stationary phase restarted growth temporarily. Therefore, for routine growth of strain P1, a minimal medium (MinE4) with higher buffering power (phosphate buffer concentration four times higher than in MinE) was subsequently used. This resulted in higher growth yields. In MinE4, methanesulfonate 20 mM became the factor limiting growth.

With the addition of Ellman's reagent, a yellow halo appeared around the colonies when strain P1 was grown on methanesulfonate, whereas it was not visible when methanol or methylamine was used as carbon source. This demonstrated the production and release of sulfite during growth on methanesulfonate. Strain P1 transformed methanesulfonate into cell mass with an efficiency of 6–9 g mol^{-1}. Methylamine gave molar yields between 13 and 14 g mol^{-1}.

The presence of high hydroxypyruvate reductase activity (a key enzyme of the serine pathway for the assimilation of carbon) in cell-free extracts was clearly evident (Table 1) after growth on methanesulfonate, while it was not detected in cell-free extracts of acetate-grown strain P1. SDS-PAGE analysis was performed with cell-free ex-

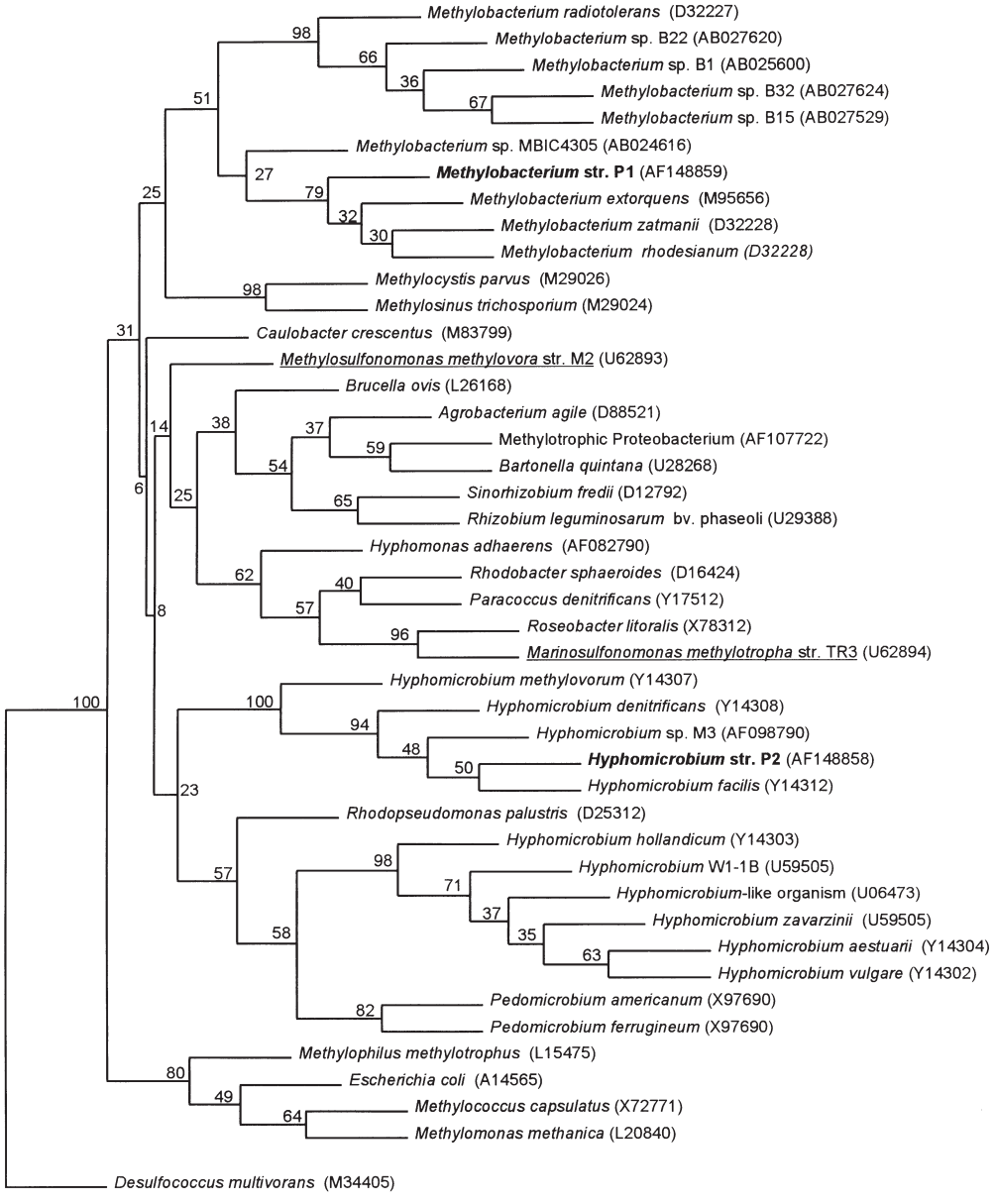

Fig. 2 Phylogenetic tree obtained by the parsimony method with the 16S rRNA sequences of strains P1 and P2 (*boldface*), the other known methanesulfonate degraders (*underlined*) and a sample of other Proteobacteria (methylotrophs and non-methylotrophs) of the α, β, γ and δ subgroups. The accession number for each sequence is reported in *parentheses*. Bootstrap values at the nodes are for 100 iterations

tracts of strain P1 grown on methanesulfonate or methanol. Several specific polypeptides (of ca. 16 19, 33, 42 and 65 kDa) were induced during growth on methanesulfonate, suggesting that an inducible, multimeric enzymatic system is responsible for the oxidation of methanesulfonate. A polypeptide of ca. 67 kDa, which is probably the large subunit of methanol dehydrogenase, was evident during growth on methanesulfonate and methanol.

The molar G+C content of DNA from strain P1 was 67.5 mol%. The 5' portion of the 16S rRNA gene of this strain was amplified and sequenced. The application of parsimony and neighbor-joining methods to infer a phylogenetic tree using this sequence (Accession No. AF148859) confirmed (bootstrap values 79 and 88%, respectively) that the 16S rRNA sequence of strain P1 clusters among those of Methylobacteria (Fig. 2).

*Sal*I- and *Xho*I-cut DNA from strain P1 was used in a hybridization experiment in which the *msm* (methanesulfonic acid monooxygenase) gene cluster from *Me. methylovora* strain M2 (De Marco et al. 1999) served as a probe. Two *Sal*I bands (2.4 and 3 kb) and three *Xho*I bands (1.5, 1.8 and 2.0 kb) hybridized to the probe (Fig. 3). This revealed the presence in strain P1 of genes homologous to *msmABC* and *D* of strain M2 and strongly suggests that strain P1 oxidizes methanesulfonate through the action of a monooxygenase similar to the one present in *Me. methylovora*.

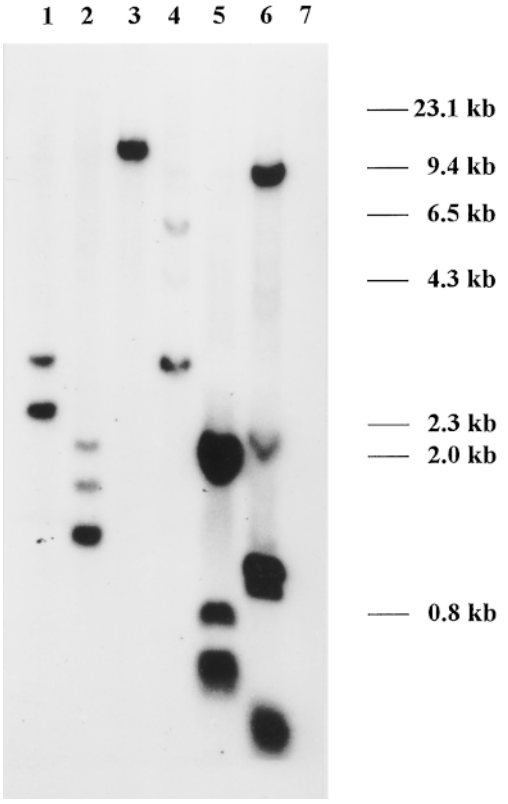

Fig. 3 Southern blot obtained using the *msm* genes *msmABCD* from *Methylosulfonomonas methylovora* strain M2 as a probe and genomic DNA as target. *Lane 1* Strain P1/*Sal*I, *lane 2* strain P1/*Xho*I, *lane 3* strain P2/*Sal*I, *lane 4* strain P2/*Xho*I; *lane 5* strain M2/*Sal*I, *lane 6* strain M2/*Xho*I, *lane 7* λ/*Hin*dIII markers

Description of strain P2

Strain P2 formed small colonies (diameter 0.1–0.5 mm) on methanesulfonate agar and larger colonies (diameter 1–2 mm) on methylamine agar plates. Colonies were smooth and white in reflected light, whereas in transmitted light they appeared yellow-brownish and contained granules. Colonies were circular, convex, with entire edges, shiny and mucoid, and part of the colony grew below the surface of the agar. They showed no pigmentation under both white and UV light.

Strain P2 was a small, motile gram-negative rod (1.3–1.8×0.5–0.8 μm) with polar hyphae. Transmission electron microscopy of this strain confirmed its *Hyphomicrobium*-like cell morphology, with hyphae apparent as rigid tubules (Fig. 1B). The presence of a minor hypha opposite the major hypha could be observed in most cells. A few cells appeared as non-terminal branching buds along a hypha. In several cells a less electron-dense central area was visible by electron microscopy. Staining with Nile Blue A demonstrated that it corresponded to a large single granule of poly-β-hydroxy alkanoates.

Strain P2 tested negative for oxidase and positive for catalase and grew using a variety of one-carbon compounds (Table 2). This strain was tested on the same range of substrates as strain P1 and was incapable of growing on any of the multi-carbon compounds. Cysteate, malate, lactate and glycine showed the same inhibitory effect on strain P2 as recorded for strain P1.

Strain P2 appears to be a restricted methylotroph. In the agar-column experiment, it showed the ability to grow utilizing methanesulfonate and methanol only at the air/agar interface, regardless of the presence of $NaNO_3$ (10 mM). Strain P2 grew at 18–32 °C. Growth was completely inhibited at 15 °C and 37 °C. Optimum growth (t_d=12 h) at pH 7 was obtained at 28–30 °C. The pH optimum for strain P2 was 8 (t_d=11.5 h). Strain P2 was more sensitive than strain P1 to the presence of salt in the medium, with strong inhibition of growth at 0.5% (w/v) NaCl. The drop in pH produced by growth on methanesulfonate observed in strain P1 was present also with strain P2; therefore, MinE4 was used when methanesulfonate was the carbon source.

Strain P2, when growing on methanesulfonate, released sulfite. Molar growth yields for strain P2 were 3.2–3.4 g mol^{-1} for methanesulfonate and 6.7 to 8.4 g mol^{-1} for methylamine, considerably lower than the yields observed for strain P1 and *Me. methylovora* strain M2 (Kelly et al. 1994). High hydroxypyruvate reductase activities were found in cell-free extracts of methanesulfonate-grown cultures (Table 1). We were not able to test for hydroxypyruvate reductase activity in a negative control for this organism, since strain P2 does not grow on any of the non-C_1 compounds we tried. Cell-free extracts of strain P2 grown on methanesulfonate or methanol were examined by SDS-PAGE and revealed the presence of a few polypeptides (of ca. 21, 28, 33, 42 and 43 kDa) apparently induced by methanesulfonate. As found for strain P1, methanol dehydrogenase appeared to be induced in strain P2 during growth on either substrate.

DNA from strain P2 cells had a Ge+Ce content of 59.4 mol%. Information on the 16S rRNA of this strain was obtained by PCR amplification of the relevant gene, and cloning and automated DNA sequencing of the first third of the gene. Analysis of the sequence (accession number AF148858) with the programs available in the PHYLIP package firmly placed strain P2 within the Hyphomicrobia (bootstrap values 100% with either parsimony and neighbor-joining methods; Fig. 2). *Sal*I- and *Xho*I-cut strain P2 genomic DNA was also probed, in the same hybridization experiment as strain P1, with the *msm* genes (De Marco et al. 1999) from *Me. methylovora*. One *Sal*I band (around 12 kb in size) and two major *Xho*I bands (sizes ca. 3 and 6 kb) hybridized strongly to the probe (Fig. 3), indicating that strain P2, like strain P1 and *Ma. methylotropha* strain PSCH4 (Baxter et al., in preparation), harbors genes homologous to those encoding the methanesulfonate monooxygenase in strain M2. Strain P2 very likely utilizes a related enzyme complex for the oxidation of methanesulfonate.

Discussion

The aim of this work was to obtain new strains of methanesulfonate-oxidizing bacteria from a new geographical

location in order to assess the biodiversity of these type of organisms and to demonstrate their ubiquity in the environment. Two bacterial strains capable of growth on methanesulfonate as sole carbon and energy source were isolated from one of two soil enrichments. The first strain, P1, is a facultative methylotroph that can be classified as a *Methylobacterium*. The second strain, P2, is a restricted methylotrophic bacterium with several characteristics that suggest its classification as a *Hyphomicrobium* species. Both strains are mesophilic gram-negative bacteria that probably oxidize methanesulfonate to CO_2 and H_2SO_4 in a manner similar to that proposed for *Me. methylovora* strain M2 (Kelly et al. 1994). Strain P1 is more efficient than strain P2 in the conversion of methanesulfonate into biomass (Table 1) with yields closer to those obtained by *Me. methylovora* strain M2.

Like strain M2 and *Ma. methylotropha* strain PSCH4, both strains P1 and P2 release traces of sulfite into the medium when growing on methanesulfonate. This supports the contention that a monooxygenase is responsible for the oxidation of methanesulfonate to formaldehyde and sulfite. Some microorganisms, such as *Paracoccus denitrificans* GB17 (Wodara et al. 1997), *Beggiatoa* strain MS-81-1c (Hagen and Nelson 1997), and *Comamonas* strain P53 (Reichenbecher and Murrell 1999), are known to oxidize sulfite to sulfate (ΔG_0=–258 kJ mol^{-1}; Kelly 1999). We think it possible that our methanesulfonate-degrading isolates could obtain energy from this oxidation. If this is the case, these bacteria would be defined as lithoheterotrophs.

Both strains P1 and P2 possess a hydroxypyruvate reductase activity (in the case of strain P1 we showed its inducible character), which is an enzyme diagnostic for the serine pathway. This suggests that both strains assimilate C_1 carbon at the level of formaldehyde using the serine pathway, as do *Me. methylovora* strain M2, *Ma. methylotropha* strain PSCH4 and the other known strains of *Hyphomicrobium* and *Methylobacterium*. SDS-PAGE and Southern blot hybridization profiles suggest the presence in both strains of an inducible, multimeric methanesulfonic acid monooxygenase related to that found in *Me. methylovora* strain M2.

Both methylamine and methanesulfonate-grown cells of either strain show the presence in the soluble fraction of a ca. 67 kDa peptide, which is very likely the large subunit of methanol dehydrogenase. This phenotype is apparently in contrast with the fact that the catabolism of neither methylamine nor methanesulfonate should produce methanol at any stage. The induction of methanol dehydrogenase in similar conditions has been reported for the other methanesulfonate-degrading bacteria (Thompson et al. 1995; Higgins et al. 1996) and for other methylotrophs (Stouthamer 1992), and it has been suggested that this enzyme has a role in the detoxification of high levels of formaldehyde.

It appears that all the known methanesulfonate degraders, despite their varied origins and phylogenetic positions, share a common enzyme complex, namely, methanesulfonic acid monooxygenase. Indeed, we have demonstrated the presence in the two new strains of genes homologous to the *msm* genes from *Me. methylovora* strain M2. The subsequent cloning and sequencing of *msm* genes from these two new genera of methanesulfonate-utilizing bacteria will allow the design of PCR primers that can be used to directly determine the distribution of these organisms in a wide variety of environments.

Acknowledgements We thank Manuela Pinto for her kind assistance with the electron microscopy. P.D.M. was supported by a postdoctoral grant from Fundação Ciência e Tecnologia (BPD/9977/96).

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