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Biotransformation of alkyl branched aromatic alkanoic naphthenic acids via two pathways by a bacterial isolate with high 16S rRNA gene sequence similarity to *Mycobacterium* spp.

Running Title: Isolation and characterisation of an aromatic NA degrading *Mycobacterium* spp.

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Summary

Naphthenic acids (NAs) are complex mixtures of carboxylic acids found in weathered crude oils and oil sands and are toxic, corrosive and persistent. However, little is known about the microorganisms and mechanisms involved in NA degradation. We isolated a sediment bacterium (designated IS2.3), with 99% 16S rRNA sequence identity to Mycobacterium, that degraded synthetic NAs (4′-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA) and (4′-*t*-butylphenyl)-4-butanoic acid (*t*-BPBA). *n*-BPBA was readily oxidised with almost complete degradation (96% ± 0.4) compared to *t*-BPBA (73.7% ±4.26 degraded) by day 49. Cell counts increased four-fold by day 14 but decreased five-fold by day 49 for both *n*- and *t*-BPBA.

At day 14, (4′-butylphenyl)ethanoic acid (BPEA) metabolites were produced, with additional metabolites produced during *t*-BPBA degradation identified by mass spectrometry of derivatives as (4'-carboxy-*t*-butylphenyl)-4-butanoic acid and (4'-carboxy-*t*-butylphenyl)ethanoic acid; suggesting that IS2.3 used omega oxidation of *t*-BPEA to oxidise the *tert*-butyl side chain to produce (4'-carboxy-*t*-butylphenyl)ethanoic acid, (primary route for biodegradation), but that IS2.3 also produced this metabolite through initial omega oxidation of the *tert*-butyl side chain of *t*-BPBA, followed by beta-oxidation of the alkanoic acid side chain. In conclusion, a Mycobacterium-like isolate could degrade highly branched aromatic NAs via two pathways and may be used as a model organism.

Introduction

Naphthenic acids (NAs) are found principally in weathered crude oils and are recalcitrant, corrosive and toxic (reviewed by Whitby, 2010). Concerns have been raised about the potential deleterious effects of inputs of NAs to the environment (reviewed by Headley and McMartin, 2004). NAs are a complex mixtures comprising predominantly cycloaliphatic and straight chain and alkyl substituted acyclic carboxylic acids (Rowland *et al*., 2011a-c). Although aromatic NAs make up a small percentage of some NA mixtures (e.g. Rowland *et al*., 2011c,d), they may contribute disproportionately to the overall toxicity and recalcitrance of NAs (Headley and McMartin, 2004; Johnson *et al*., 2011). Despite their persistence and toxicity, little is known about the mechanisms involved in aromatic NA degradation. A previous study reported a microbial consortium, comprising predominantly *Burkholderia* spp., *Pseudomonas* spp. and *Sphingomonas* spp. (identified by 16S rRNA analysis) which was capable of butylphenylbutanoic acid (BPBA) degradation (Johnson *et al*., 2011). However, degradation of alkyl phenyl alkanoic acids by a single pure culture has not yet been demonstrated and such studies are needed if detailed mechanistic studies on NA degradation are to be undertaken to increase the likelihood of achieving enhanced NA bioremediation.

In contrast, a number of isolates can metabolise non-aromatic cyclohexane carboxylic acid (CHCA) either through beta-oxidation (e.g. *Pseudomonas putida* and *Alcaligenes faecalis*; reclassified as *Achromobacter denitrificans*; Blakley, 1974; 1978; Blakley and Papish 1982); or via a pathway similar to benzoate degradation (e.g. *Corynebacterium cyclohexanicum*) (Tokuyama and Kaneda, 1973), or via the aromatisation of the cyclohexane ring (e.g. *Arthrobacter* spp.; reclassified as *Arthrobacter globiformis*) (Blakley, 1974).

Although *Mycobacterium* spp have not been shown to degrade aromatic NA acids previously, they have been reported to degrade hydrocarbons such as branched alkanes (*Mycobactorium fortuitum;* Cox *et al*., 1976) and polycyclic aromatic hydrocarbons (PAH) (*Mycobacterium vanbaalenii* PYR-1; Stingley et al., 2004; Kim et al. 2007; 2008). In this study, we aimed to isolate a microorganism from the environment which could degrade BPBAs and thus potentially be used as a model microorganism to study the pathways involved in aromatic NA degradation and to facilitate NA bioremediation studies.

Results

Isolation and characterisation of an aromatic NA-degrading isolate

A single colony derived from a sediment sample was successfully isolated on washed MSM agar plates that contained *n*-BPBA as the sole carbon and energy source (but was unable to grow on control plates without *n*-BPBA). The isolate (designated IS2.3) possessed white, diffuse colonies and light microscopy observations showed that when IS2.3 was grown on MSM agar containing *n*-BPBA it was a non-motile, non-filamentous, short Gram-positive rod (Fig. 1A). However, when IS2.3 was grown in liquid MSM containing 1% (w/v) glucose, it developed a filamentous form (Fig. 1B), which could be easily disrupted following manual shaking of the flask (Fig. 1C).

The metabolic capability of isolate IS2.3 was investigated using BIOLOG plates and the data are presented in Table 1. IS2.3 grew on various monosaccharides (including α-D-glucose, D-galacturonic acid, D-mannose, D-xylose), disaccharides

(including D-trehalose, gentiobiose, maltose, sucrose and turanose) as well as polysaccharides such as dextrin. In addition, IS2.3 also grew well on acetic acid, glycerol and surfactants such as Tween40 and Tween80. No growth was observed in abiotic controls. By contrast IS2.3 was unable to grow on any of the PAHs, alkanes and alcohols tested and including ethanol, methanol, 1-methylnaphthalene, phenanthrene, pristane, tetradecane, phenylbutanoic acid and sodium benzoate.

Phylogenetic analysis of the 16S rRNA gene from isolate IS2.3.

The 16S rRNA gene from IS2.3 was sequenced, a Jukes Cantor DNA-distance and neighbour joining analysis was performed and the phylogenetic tree is presented in Fig. 2. IS2.3 showed the closest phylogenetic sequence similarity to *Mycobacterium aurum* (Tsukamura) ATC23070 (Tsukamura and Tsukamura, 1966) with a high bootstrap value (100%). The 16S rRNA gene sequence of IS2.3 also clustered with other *Mycobacterium* spp. including *M. fluoranthenivorans, M. neoaurum* and *M. frederiksbergense* but was more distantly related to *M. tuberculosis*, *M. bovis* and the PAH degrader *M. vanbaalenii* PYR-1. BLASTN analysis demonstrated that IS2.3 had 99% 16S rRNA gene sequence identity to several *Mycobacterium* spp. (Supplementary Table 1).

Degradation of *n*- and *tert*-BPBA by isolate IS2.3

Degradation of *n*-, and *t*-BPBA by IS2.3 was investigated and *n*-BPBA was more readily degraded than *t*-BPBA, demonstrating that BPBA degradation rates decreased as the degree of alkyl branching increased (Fig. 3) as observed previously with a mixed

115 microbial enrichment culture (Johnson *et al*. 2011). When IS2.3 was incubated with *n*-
116 BPBA as the sole carbon and energy source, almost complete degradation occurred by
117 day 49 with only 4.0% (±0.4) remaining (Fig. 3). By day 14, 30.1% (±10.9) of *n*-BPBA
118 was degraded but by day 35, 92.3% (±1.7) had been degraded (Fig. 3), which
119 corresponded to a rate of 1.96 µg day^{-1}. When IS2.3 was incubated with *t*-BPBA as the
120 sole carbon and energy source, by day 14, 33.0% (±12.6) of *t*-BPBA had been
121 degraded and by day 35 and 49, *t*-BPBA degradation had increased to 61.1% (±7.0)
122 and 73.7% (±4.26) respectively (Fig. 3) corresponding to a rate of 1.50 µg day^{-1}.
123 Cell counts significantly increased up to four-fold from 1.6 x 10^5 cfu/ mL (at day 0) to 7.3
124 x 10^5 cfu/ mL (at day 14) (p=0.05) for *n*-BPBA; and from 2.2 x 10^5 cfu/ mL (at day 0) to
125 7.6 x 10^5 cfu/ mL (at day 14) (p=0.007) for *tert*-BPBA compared to controls. After day 14
126 cell numbers significantly decreased five fold to 1.5 x 10^5 cfu/ mL (at day 49) (p=0.044)
127 for *n*-BPBA and 2.0 x 10^5 cfu/ mL (at day 49) (p=0.005) for *tert*-BPBA. NaOH controls
128 revealed no significant increase in cell numbers during the 49 day incubation (p=0.4).
129 During *n*-BPBA degradation, a metabolite was produced (by day 14) and based
130 on a comparison of the GC retention time and the mass spectrum of the unknown with
131 that of a synthetic acid (TMS ester; Rowland *et al*., 2011d), this metabolite was
132 identified as (4′-*n*-butylphenyl)ethanoic acid (*n*-BPEA), as previously reported for the
133 degradation of *n*-BPBA by a mixed microbial enrichment culture (Johnson *et al*., 2011).
134 As demonstrated with *n*-BPBA, degradation of *t*-BPBA proceeded with the
135 production of a metabolite at day 14, the TMS ester of which had a retention time and
136 mass spectrum which corresponded to that of synthetic (4′-*tert*-butylphenyl)ethanoic
137 acid (*t*-BPEA; TMS ester; Rowland *et al*., 2011d) a metabolite identified previously from

degradation of *t*-BPBA by a mixed culture (Johnson *et al*. 2011). In addition to *t*-BPEA, degradation of *tert*-BPBA by IS2.3 also produced two further metabolites, the first by day 35 and a second metabolite by day 42. The mass spectrum of the TMS-derivatised first–eluting metabolite was characterised by a base peak ion at *m/z* 249 and no obvious molecular ion (Fig. 4A). Ions at *m/z* 338 and 322 were however, tentatively assigned as due to losses of 28 (carbon monoxide) and 44 (carbon dioxide) from a putative molecular ion (*m/z* 366, absent) of the bis-TMS ester of (4'-carboxy-*t*-butylphenyl)ethanoic acid. The abundant ion (*m/z* 249) is explained by a very favourable double benzylic fragmentation. The *m/z* 73 (B^+) ion is typical of charge retention on the TMS groups, for which again there are now two possibilities, for this *bis*-derivatised diacid, increasing the abundance. To confirm these conjectures, a sample of the metabolites was also derivatised by refluxing with BF_3/methanol. This would be expected to produce the dimethyl esters of a diacid. As expected, GC-MS of these products revealed a component with a mass spectrum characterised by a clear molecular ion (*m/z* 250; Fig. 4B) and a base peak ion at *m/z* 191, attributed to a double benzylic fragmentation and loss of one or other of the methylcarboxy moieties (Fig. 4B) of (4'-carboxy-*t*-butylphenyl)ethanoic acid, dimethyl ester. (The base peak ion *m/z* 191 was also observed previously in the mass spectrum of the methyl ester of synthetic *t*-BPEA, due to a triple benzylic cleavage (Rowland *et al*., 2011d) and herein in the methyl ester of the corresponding *t*-BPEA metabolite (data not shown)). The mass spectrum of the TMS-derivatised second–eluting metabolite was characterised by a base peak ion at *m/z* 277 and no obvious molecular ion (Fig. 4C). We assign this metabolite tentatively to (4'-carboxy-*t*-butylphenyl)-4-butanoic acid (molecular ion *m/z*

394, absent). The base peak ion (*m/z* 277) is then explained by the favourable benzylic cleavage with loss of a mass 117 moiety (Fig. 4C). Charge retention on the latter results in the *m/z* 117 ion, also observed. The ion *m/z* 350 was assigned as due to losses of 44 (carbon dioxide) from the putative molecular ion of the bis-TMS ester. This was observed previously in the mass spectra of the TMS esters of a number of synthetic alkylphenylethanoic acids (Rowland *et al*., 2011d). Thus we assign the two new metabolites as (4'-carboxy-*t*-butylphenyl)-4-butanoic acid and (4'-carboxy-*t*-butylphenyl)ethanoic acid respectively, suggesting two divergent pathways for *tert*-BPBA degradation (Fig. 5).

Discussion

We successfully isolated a sediment bacterium (designated IS2.3), related to *Mycobacterium spp.* (99% 16S rRNA sequence identity) that degraded aromatic alkanoic NAs (*n*-BPBA and *t*-BPBA) and identified two new metabolites as (4'-carboxy-*t*-butylphenyl)-4-butanoic acid and (4'-carboxy-*t*-butylphenyl)ethanoic acid respectively, suggesting two divergent pathways for *tert*-BPBA degradation.

Although degradation of aromatic alkanoic NAs has been demonstrated previously using a mixed consortium (Johnson *et al*., 2011), this appears to be the first report of a single isolate with the metabolic capability to transform aromatic alkanoic NAs. During BPBA degradation both (4′-*n*-butylphenyl)ethanoic acid (*n*-BPEA) and (4′-*tert*-butylphenyl)ethanoic acid (*t*-BPEA) metabolites were produced. Similar metabolites have also been identified previously during degradation of both aromatic alkanoic NAs (Johnson *et al*., 2011), and alicyclic alkanoic NAs (Smith *et al*., 2008; Rowland *et al*.,

2011e) suggesting that BPBA degradation by IS2.3 proceeded via the same pathway as was found previously. However, Johnson *et al*. (2011) demonstrated that *n*-BPEA was completely metabolised after 49 days incubation using a mixed consortium, whereas this did not occur with IS2.3. This suggests that either *n*-BPEA was not bioavailable to IS2.3 or IS2.3 was incapable of producing the required extracellular enzymes for *n*-BPEA metabolism.

In addition to the BPEA metabolites, degradation of *t*-BPBA by IS2.3 also produced (4'-carboxy-*t*-butylphenyl)-4-butanoic acid and (4'-carboxy-*t*-butylphenyl)ethanoic acid metabolites that have not been identified previously. This suggests that IS2.3 is capable of omega oxidation of the *tert*-butyl side chain of *t*-BPEA to produce (4'-carboxy-*t*-butylphenyl)ethanoic acid which is the major route for degradation, but additionally, IS2.3 is also capable of producing the final (4'-carboxy-*t*-butylphenyl)ethanoic acid metabolite through initial omega oxidation of the *tert*-butyl side chain of 4'-*t*-BPBA, side chain, followed by beta-oxidation of the (4'-carboxy-*t*-butylphenyl)-4-butanoic acid intermediate to the final (4'-carboxy-*t*-butylphenyl)ethanoic acid through a minor pathway. Although a mixed culture oxidized *n*-BPBA to a diacid metabolite, this is the first report an individual microorganism capable of oxidising the *tert* branched alkyl side chain of a NA. Production of identifiable diacids by such mechanisms may help to explain the detection of so-called O_4 (viz: diacid) species in NAs by electrospray ionisation mass spectrometry (e.g. Headley *et al*., 2011) and the postulation of diacids in oil sands process water NAs from nuclear magnetic resonance spectroscopy data (Frank *et al*., 2009).

It is known that hydrocarbon biodegradation is greatly inhibited by terminal branching (Schaeffer *et al*., 1979) and previous studies have shown that hydrocarbons with terminal dimethyl branches are relatively resistant to microbial oxidation (Hammond and Alexander, 1972). However, IS2.3 may be efficient in utilising *tert*-branched side chains. Furthermore, *Mycobacterium* spp have been shown to degrade the highly branched squalane (2,4-,6,10,15,19,23-hexamethyltetracosane) via oxidation of the terminal carbon as the initial step (Berekaa and Steinbushel, 2000), and IS2.3 has 99% 16S rRNA gene sequence identity to *Mycobacterium* spp. *M. tuberculosis* possesses a ω-hydroxylase enzyme for this step (Johnston *et al*., 2009). Interestingly, Pirnik *et al*. (1974) observed that "ω-oxidation of long chain acids with ω-1 methyl branching seems slow enough to permit at least one cycle of beta oxidation before a dicarboxylic acid is fully established". We observed a similar phenomenon.

Mycobacteria have also been shown to metabolise a range of other branched acyclic isoprenoid alkanes as well as to beta-oxidise *n*- and methyl substituted alkanes (Cox *et al*., 1976) and to dioxygenate PAHs, with the complete pyrene degradation pathway elucidated in *Mycobacterium vanbaalenii* PYR-1 (Kim *et al*., 2007), which shares 96% 16S rRNA sequence similarity to IS2.3 (over 1,452 bp). However, the terminal carbons in branched alkanes such as squalane are only *iso*-branched (dimethyl) and therefore not as highly branched as the side chain in *t*-BPEA (trimethyl). Moreover, in the present study, IS2.3 was not able to catabolise the aromatic ring of *n*- or *t*-BPBA, which may have possibly been due to sub-optimal incubation times or experimental conditions for complete mineralisation.

It has been previously shown that Mycobacteria require build up of carbon dioxide (up to 8% v/v) in the headspace before exponential growth occurs (Ratledge, 1982). In the present study, it was also found that static cultures of IS2.3 decreased incubation times required for growth (data not shown). Furthermore, members of Mycobacteria such as *Mycobacterium aurum* Tsukamura (Tsukamura and Tsukamura, 1966) have been shown to grow both at 28°C and 37°C (Tsukamura, 1966). In contrast, although IS2.3 grew at both 20°C and 30°C; the isolate was unable to grow at 37°C (data not shown). Furthermore, degradation rates of *n*-BPBA by IS2.3 at 30°C were not significantly different from those at 20°C ($p = 0.513$) (data not shown).

Mycobacteria are considered generalists, utilising a wide range of substrates including glycerol and amino acids (Hartmans *et al*., 2006). By contrast, our findings suggest that IS2.3 is more a specialist as it grew on the various mono-, di- and polysaccharides, glycerol, amino acids, surfactants and organic acids such as acetic acid. It is perhaps no surprise that IS2.3 was able to utilise acetate, as acetyl-CoA would be the by-product of beta-oxidation of BPBAs. Despite this, IS2.3 was unable to grow on any of the PAHs, alkanes and alcohols tested. It is possible that IS2.3 can catabolise hydrocarbons with no observable increase in growth. Therefore, despite both a high 16S rRNA gene homology and some similarities in metabolic traits, between IS2.3 and members of the Mycobacteria, there are some physiological differences between IS2.3 and Mycobacteria. Further analysis is therefore required to unequivocally identify the taxonomic assignment of IS2.3. In conclusion, an environmental microorganism designated IS2.3 was successfully isolated with high 16S rRNA gene sequence identity to *Mycobacterium* spp. which could degrade both *n*-BPBA and the

more branched *t*-BPBA within weeks. Additional metabolites produced during *t*-BPBA degradation indicated that IS2.3 catabolised *t*-BPBA via two divergent pathways which to our knowledge have not been observed previously by an individual microorganism. Therefore, IS2.3 may make an appropriate model organism with which to study the pathways involved in aromatic NA biodegradation.

Experimental Procedures

Culture isolation and light microscopy

(4′-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA) and (4′-*t*-butylphenyl)-4-butanoic acid (*t*-BPBA) were synthesised as described previously (Smith et al., 2008). Sediment samples (top 3 cm) were obtained from Avonmouth (51:31:28N, 2:41:04W). Cation/ anion analysis was performed using Dionex ICS-3000 and data were as follows: acetate (160.7 µM), bromide (313.3 µM), calcium (489.9 µM), chlorate (170.5 µM), chloride (179.4 mM), fluoride (225.7 µM), lithium (1.86 µM), magnesium (2435. 1 µM), nitrate (78.54 µM), phosphate (1,68 µM), potassium (613.1 µM) sodium (24.5 mM), strontium (8.40 µM) and sulphate (7949 µM). Total organic carbon was 30.34 ± 0.37 mg g^{-1} as determined using a Shimadzu TOC-VCHS analyser. Sediment samples were pre-enriched in 25 mL of minimal salts medium (MSM) containing 1% (v/v) heavy crude oil (Tia Juana Pesado) as the sole carbon source as previously described (Johnson *et al*., 2011).

Pre-enrichment cultures were established by inoculating sediment 1% (w/v) onto washed MSM agar plates containing either *n*- or *t*-BPBA (final concentration of 2 mg L^{-1} in 0.1M NaOH (Fisher)). Plates were incubated statically at 20°C in the dark. Colonies

were selected and re-streaking onto fresh MSM agar containing the same concentration of either *n*- or *tert*-BPBA, as previously described. Colonies obtained were checked by re-streaked onto MSM agar containing no BPBAs and any isolates that grew were discarded. One colony was obtained that also failed to grow on control plates and was stored in 80% (v/v) glycerol at -80°C. Light microscopy observations utilised an Olympus BX41 microscope fitted with a digital camera and imaging system (Colorview II).

Degradation of *n*- and tert-BPBA by IS2.3

Degradation experiments were set up by inoculating IS2.3 (2% v/v) into 25 mL MSM containing *n*- or *t*-BPBA as described previously (Johnson *et al*., 2011). Viability checks of killed controls were performed by streaking onto R2A agar (Fluka) and incubating at 20°C for two days prior to inoculation. Destructive sampling was carried out at days 0, 14, 35 and 49 and BPBAs extracted using ethyl acetate (HPLC, Fisher) as described previously (Smith *et al*., 2008; Johnson *et al*., 2011). Aromatic acid extracts were analysed by on a Thermo Finnigan Trace gas chromatograph Ultra coupled with a Thermo Finnigan Trace DSQ mass spectrometer and Thermo AS3000 autosampler. Samples were injected with a 1 µL splitless injection (injector temperature 250°C) onto a 15 m x 250 µm x 0.25 µm Rtx®-1MS column with integrated guard (Restek) using helium as the carrier gas at a constant flow of 1 mL min^{-1}. Oven temperatures were programmed as above with an initial increase of 40°C to 300°C at 10°C min^{-1} and a final hold at 300°C for 10 min. The transfer line was again held at 230°C onto a source for the mass spectrometer of 230°C which was in full scan mode (scan range 50-550 Da). Data were analysed and integrated with Xcalibur ver. 1.4 SR1.

Cell Counts

During BPBA degradation cell counts were performed by dilution plating onto R2A Agar plates and incubating at 30°C for 48 h. Cell counts of NaOH controls were also performed.

Carbon substrate utilisation

Isolate IS2.3 was characterised using a BIOLOG *G2P MicroPlate*™ plate. Triplicate BIOLOG plates were set up, by inoculating 150 µL of IS2.3 into each well, and were incubated at 30°C for 24 h. The OD_{590} at 23.5°C of the three plates were measured on a VERSAmax tunable microplate reader (Molecular Devices) and analysed using SOFT Max Pro (version 3.1.1) software, against the substrate blank well. Isolate (IS2.3) was also inoculated (2% v/v) into MSM (10 mL) containing one of the following individual carbon substrates: cholesterol (10% w/v), phenylalanine (10% w/v), glycerol (10% v/v), ethanol (10% v/v), methanol (10% v/v), sodium benzoate (10% w/v), phenyl butyric acid (10% w/v), sodium acetate (5% w/v), naphthalene (1% w/v), fluoranthene (0.5% w/v), phenanthrene (0.5% w/v), 1-methylnaphthalene (10% v/v), tetradecane (10% v/v) and pristane (10% v/v). The inoculated cultures were incubated in the dark at 20°C for seven days and growth assessed by monitoring turbidity visually.

16S rRNA Gene Sequence Analysis

Colony PCR was performed on IS2.3 using a Gene Amp® PCR system 9700 Thermocycler (Applied Biosystems) in 50 µL PCR reactions containing: 1x buffer (Qiagen), 0.2 mM dNTPs (Fermentas), 0.4 µM each primers (pA/pH') (Edwards *et al*.,

1989), 2.5 U *Taq* DNA Polymerase (Qiagen). PCR cycling conditions were as follows: 95°C for 5 mins followed by 28 cycles of 94°C for 30 s, 57°C for 30 s and 72°C for 1.5 mins; then 72°C for 10 mins. PCR Products were purified using a QIAquick® PCR purification kit (Qiagen) according to the manufacturer's instructions, sequenced bidirectionally using the primers pA, pC and pC', pF' and pG' (Edwards *et al*., 1989) by GATC Biotech (Konstanz, Germany). Sequences were checked for ambiguous bases; the 16S rRNA gene consensus sequence was assembled to a total length of 1452 bp and submitted to Genbank under the accession number HQ224877.

Phylogenetic analysis of 16S rRNA Sequence from IS2.3

The 16S rRNA sequence recovered from IS2.3, together with selected sequences from the Genbank database were aligned using the RDP INFERNAL alignment tool (Nawrocki and Eddy, 2007). Phylogenetic analysis was performed using PHYLIP 3.4 with Jukes-Cantor distance and neighbor-joining methods (Jukes and Cantor, 1969; Saitou and Nei, 1987). Bootstrap analysis was based on 100 replicates using SEQBOOT (PHYLIP 3.4). Tree construction was performed using Treeview (WIN32) version 1.5.2 (Page, 1996).

Statistical Analysis

Statistical analysis was carried out using SPSS v18.0 with ANOVA.

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Figure Legends

Figure 1. Gram stains of environmental isolate IS2.3 visualised using a light microscope (A); in filamentous form before (B) and after (C) shaking.

Figure 2. Phylogenetic analysis of the 16S rRNA gene sequence from IS2.3. Included are 16S rRNA gene sequences from type strains obtained from Genbank. Sequence analysis was based on 1361 bp using Jukes-Cantor DNA distance and neighbour-joining methods. *E. coli* was used as an outgroup. Bootstrap values represent percentages from 100 replicates of the data and percentages >80% are shown. The scale bar indicates 0.1 substitutions per nucleotide base.

Figure 3. Degradation of *n*- and *tert*-BPBA by IS2.3. Calculated as a percentage of either *n*- or *tert*-BPBA remaining compared to killed controls. Error bars represent standard deviation of the mean (*n*=3). *n*-BPBA (■), and *tert*-BPBA (♦)

Figure 4. Mass spectra of metabolites produced during degradation of *t*-BPBA. (A) Mass spectrum of trimethylsilylated ester assigned to (4'-carboxy-*t*-butylphenyl)ethanoic acid (*bis*-TMS ester). (B) Mass spectrum of dimethyl ester assigned to (4'-carboxy-*t*-butylphenyl)ethanoic acid (C) Mass spectrum of trimethylsilylated ester assigned to (4'-carboxy-*t*-butylphenyl)-4-butanoic acid (*bis*-TMS ester).

Figure 5. Postulated biotransformation of 4'-BPBA by IS 2.3

Table Legends

Table 1. Growth of IS2.3 on various organic substrates. Growth was determined by measuring mean absorbance at A_{595} (n=3).

Supplementary Table 1. BLASTN analysis of 16S rRNA gene sequence from IS2.3 compared to representative members of the Mycobacterium. 16S rRNA sequences from type strains were obtained from Genbank.

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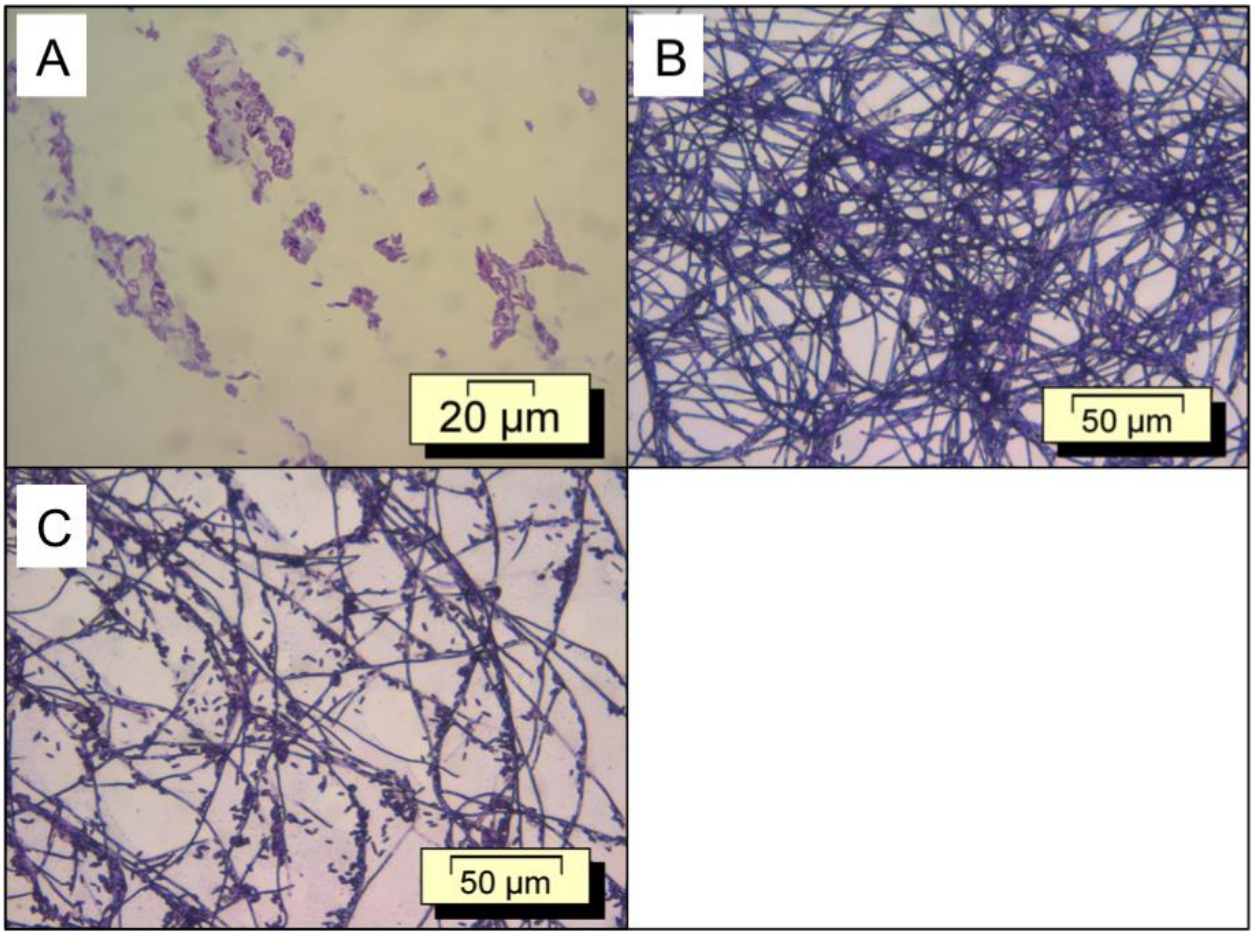

Figure 1

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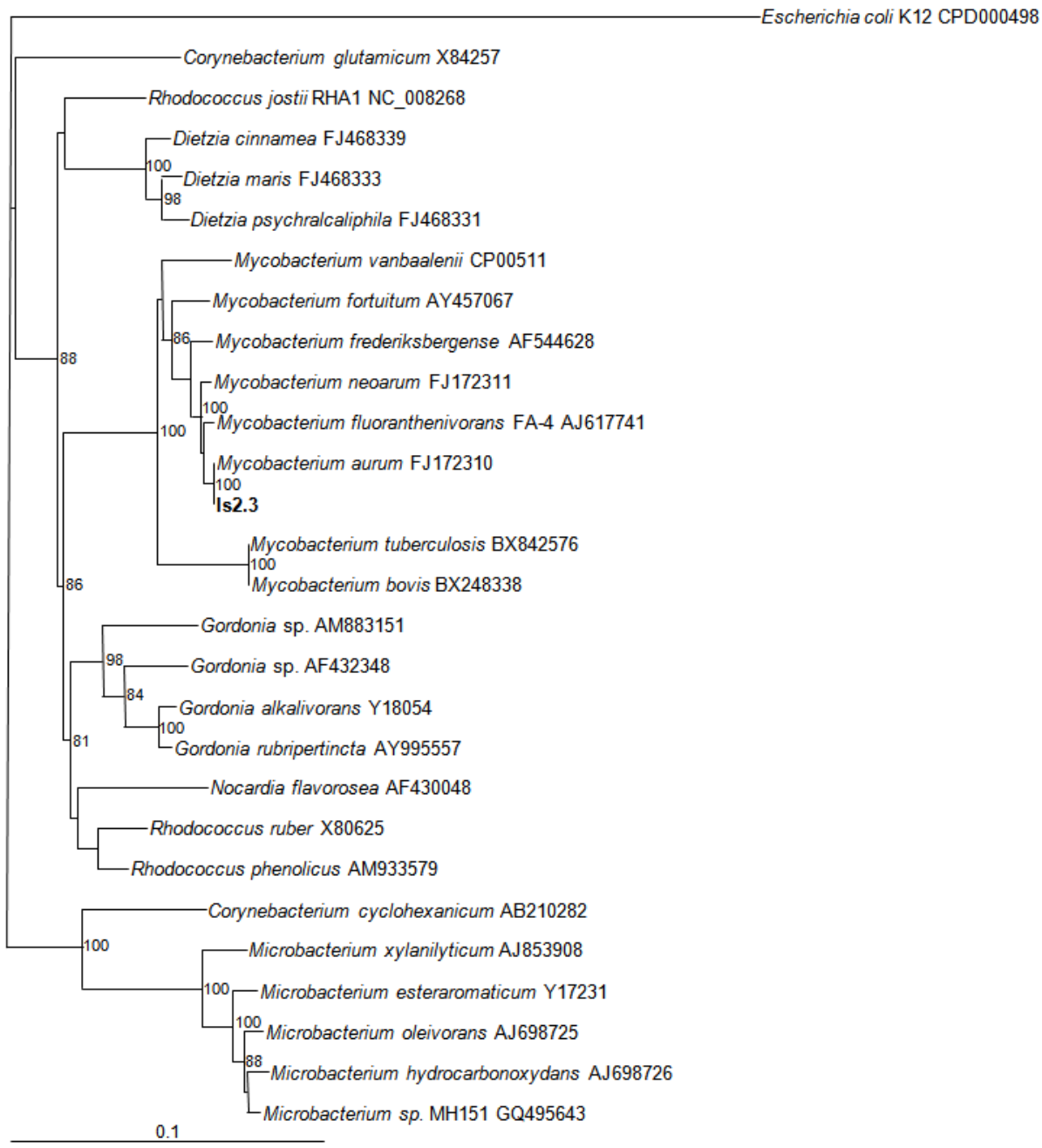

Figure 2.

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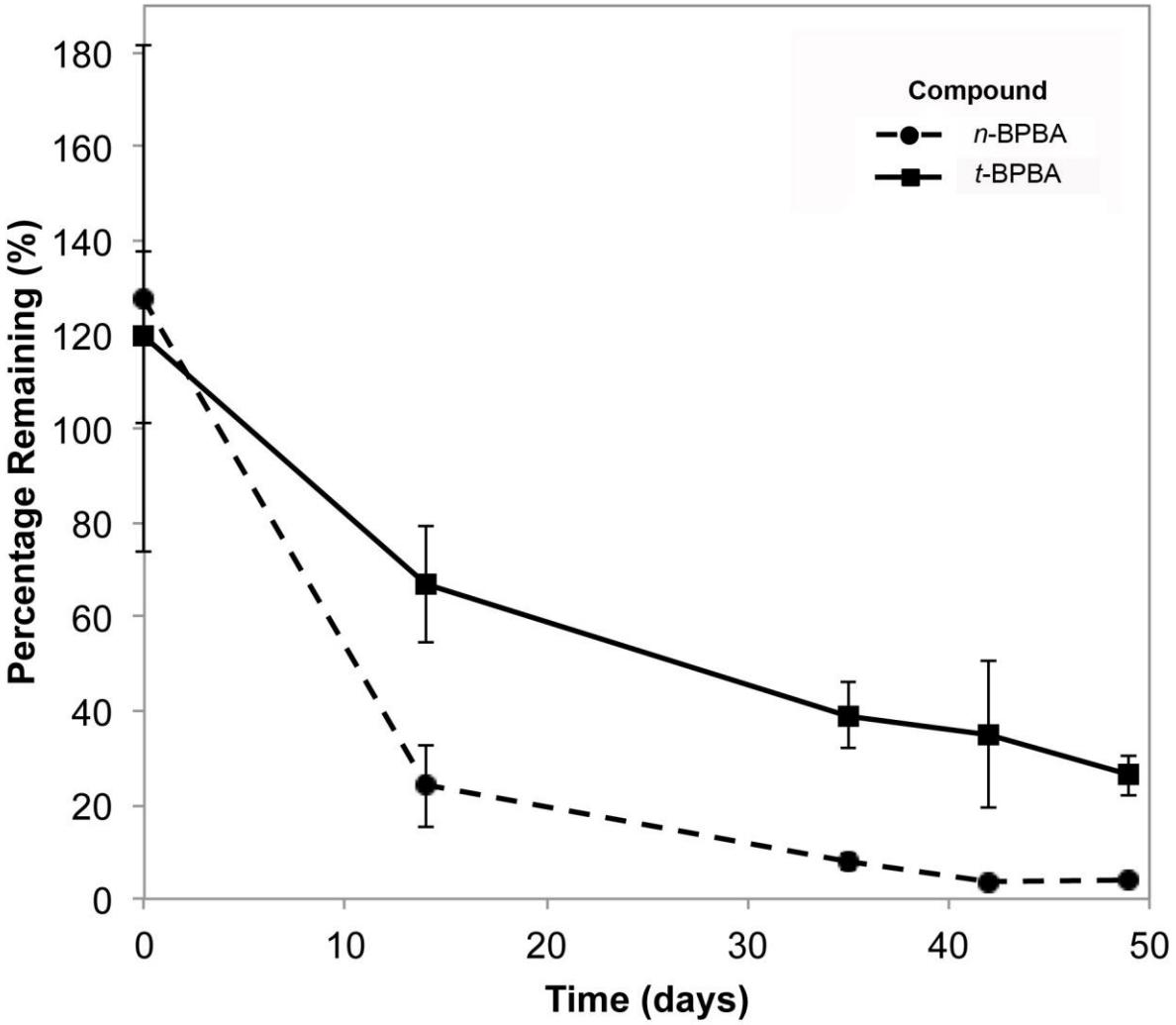

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506 **Figure 3**

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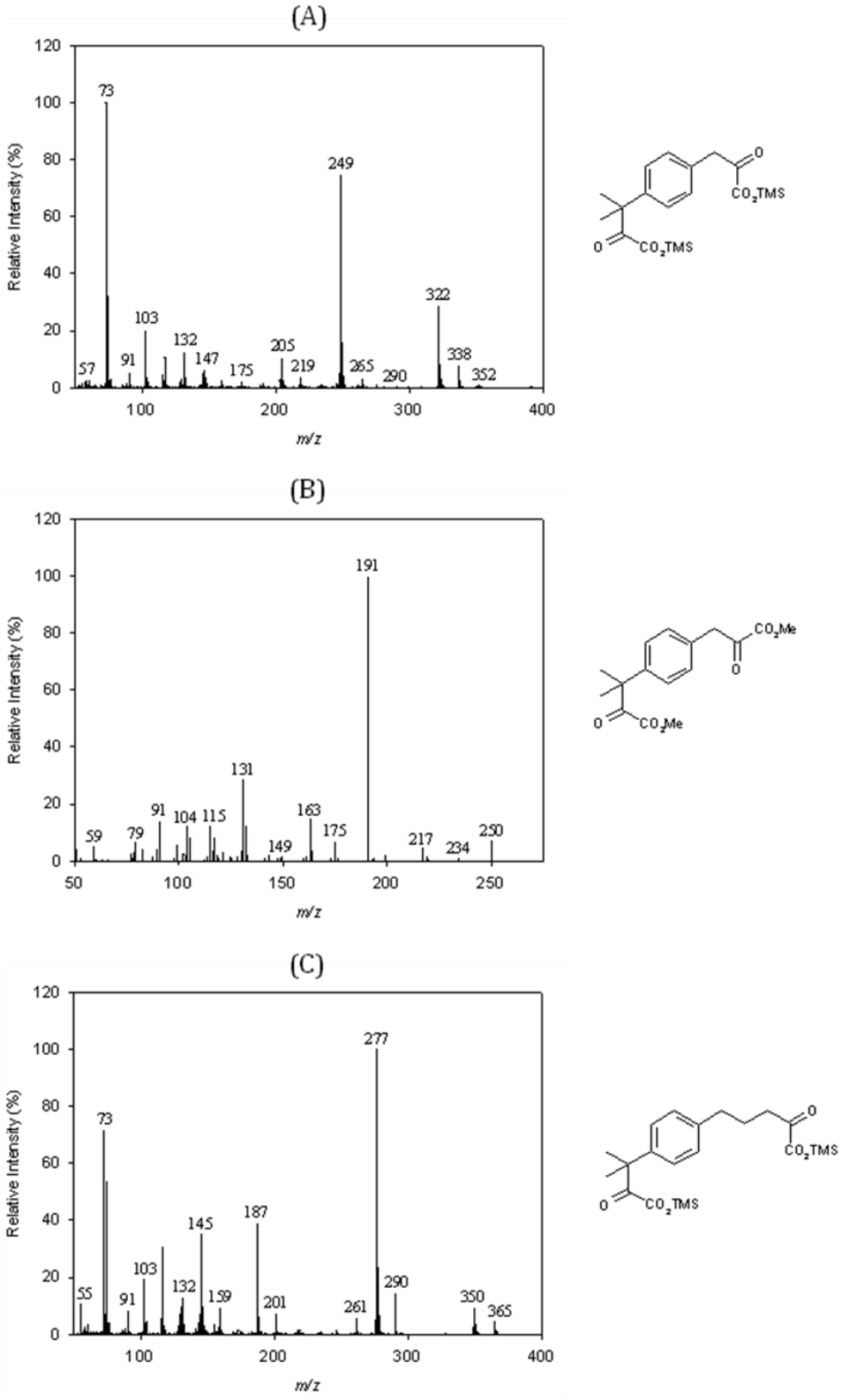

Figure 4

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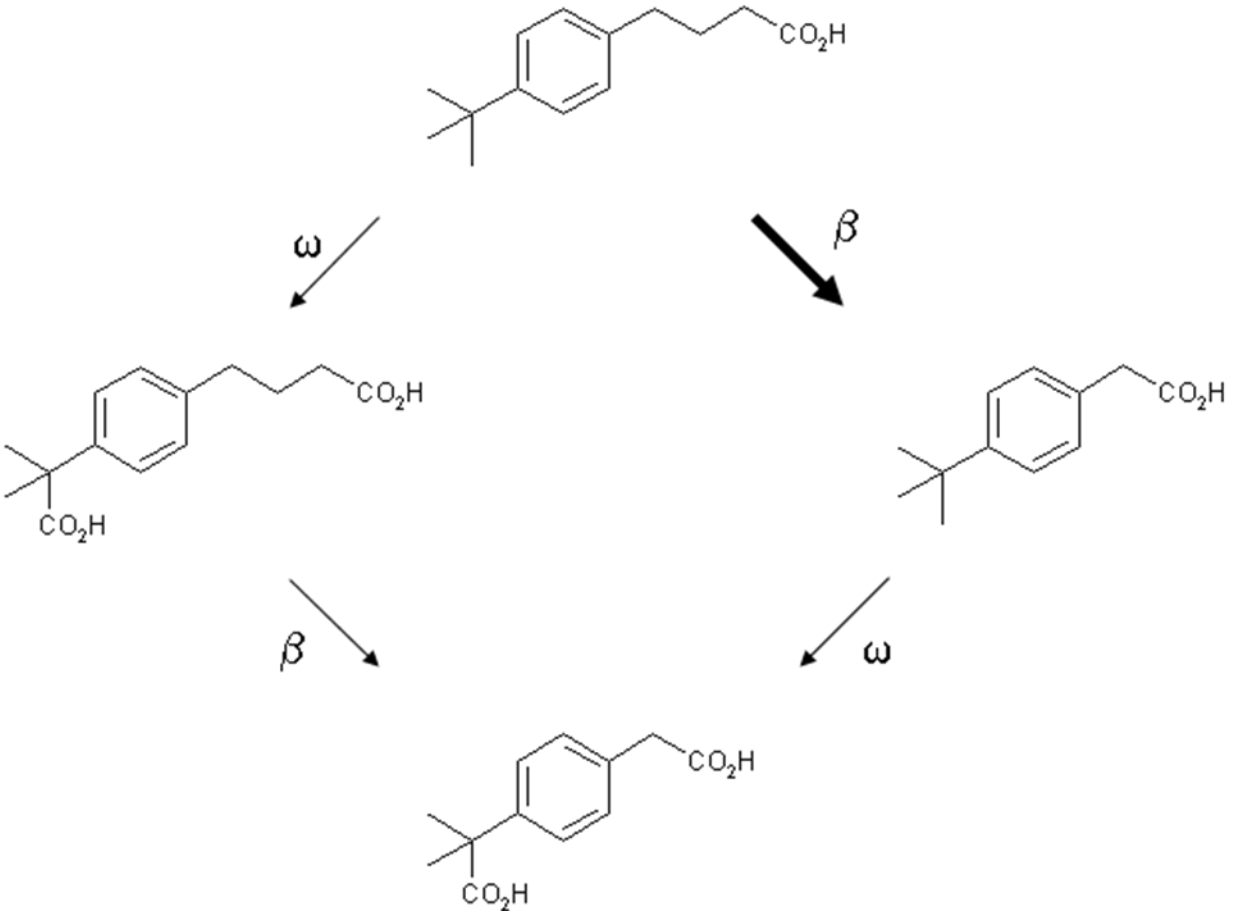

Figure 5

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519 **Table 1**

Substrate	Relative growth[†]
Alcohol	
2,3-butanediol	**+++**
Amine	
Putrescine	+
Amino Acids	
D-Alanine	+
Glycyl-L-glutamic acid	+
L-Alanine	+
L-Alanyl-glycine	++
L-Asparagine	++
L-Glutamic acid	++
L-Pyroglutamic acid	++
L-Serine	+
Oligopeptides	
L-Alaninamide	**+++**
Glycosides	
α-Methyl-D-galactoside	-
α-Methyl-D-glucoside	++
Amygladin	+
Arbutin	+
β-Methyl-D-galactoside	-
β-Methyl-D-glucoside	++
β-Methyl-D-mannoside	-
Salicilin	-
Monosaccharides	
3-Methylglucose	+
α-D-Glucose	**+++**
α-D-Glucose-1-phosphate	-
D-Fructose	+
D-Fructose-6-phosphate	+
D-Galactose	+
D-Galacturonic acid	**+++**
D-Glucose-6-phosphate	-
D-Mannose	**+++**
D-Psicose	+

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D-Ribose	+
D-Tagatose	+
D-Xylose	**+++**
L-Arabinose	+
L-Fucose	++
L-Rhamnose	+
N-Acetyl-D-glucosamine	++
N-Acetyl-D-mannosamine	++
Sedoheptulosan	-
Disaccharides	
α-D-Lactose	++
D-Cellobiose	++
D-Melibiose	+
D-Trehalose	**+++**
Gentiobiose	**+++**
Lactulose	+
Maltose	**+++**
Palatinose	+
Sucrose	**+++**
Turanose	**+++**
Trisaccharides	
D-Melezitose	++
D-Raffinose	+
Maltotriose	++
Tetrasaccharide	
Stachyose	-
Polysaccharides	
α-Cyclodextrin	-
β-Cyclodextrin	+
Dextrin	**+++**
Glycogen	++
Inulin	-
Mannan	++
Nucleosides	
2-Deoxyadenosine	-
Adenosine	++
Adenosine-5-monophosphate	-
Inosine	+
Thymidine	++
Thymidine-5-monophosphate	+

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Uridine	++	520
Uridine-5-monophosphate	+	
		521
Organic Acids		
α-Hydroxybutyric acid	++	522
α-Ketoglutaric acid	++	
α-Ketovaleric acid	++	523
Acetic acid	**+++**	
β-Hydroxybutyric acid	++	524
D-Gluconic acid	++	
D-Lactic acid methyl ester	++	525
D-Malic acid	-	
γ-Hydroxybutyric acid	++	526
L-Lactic acid	++	
L-Malic acid	-	527
Lactamide	-	
N-Acetyl-L-glutamic acid	++	528
p-Hydroxyphenylacetic acid	++	
Propionic acid	++	529
Pyruvatic acid methyl ester	-	
Pyruvic acid	+	530
Succinamic acid	-	
Succinic acid	-	531
Succinic acid mono-methyl ester	++	
		532
Polyols		533
D-Arabitol	++	
D-L-α-Glycerol phosphate	++	534
D-Mannitol	++	
D-Sorbitol	-	535
Glycerol	**+++**	
m-Inositol	-	536
Xylitol	++	
		537
Surfactants		538
Tween40	**+++**	
Tween80	**+++**	539
		540

† Relative response as measured by A_{590} compared to controls. (-) A_{590} 0.000-0.005; (+) A_{590} 0.005-0.009; (++) A_{590} 0.010-0.099; (+++) A_{590} 0.100-1.300. Entries in bold are statistically significant ($p<0.05$). 541 542

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Supplementary Table 1

Closest match	Percentage 16S rRNA gene sequence similarity (%)
Mycobacterium aurum sp. FJ172310	99
Mycobacterium neoaurum sp. FJ172311	99
Mycobacterium fluoranthenivorans FA-4 AJ617741	99
Mycobacterium frederiksbergense AF544628	99
Mycobacterium fortuitum AY457067	98
Mycobacterium vanbaalenii PYR-1 CP000511	96
Mycobacterium tuberculosis H37Rv BX849576	94
Mycobacterium bovis subsp. *bovis* BX248338	94