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1 **Biotransformation of alkyl branched aromatic alkanoic naphthenic acids via two**
2 **pathways by a bacterial isolate with high 16S rRNA gene sequence similarity to**
3 ***Mycobacterium* spp.**
4
5 Running Title: Isolation and characterisation of an aromatic NA degrading
6 *Mycobacterium* spp.
7
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24 **Summary**

25 **Naphthenic acids (NAs) are complex mixtures of carboxylic acids found in**
26 **weathered crude oils and oil sands and are toxic, corrosive and persistent.**
27 **However, little is known about the microorganisms and mechanisms involved in**
28 **NA degradation. We isolated a sediment bacterium (designated IS2.3), with 99%**
29 **16S rRNA sequence identity to Mycobacterium, that degraded synthetic NAs (4′-*n*-**
30 **butylphenyl)-4-butanoic acid (*n*-BPBA) and (4′-*t*-butylphenyl)-4-butanoic acid (*t*-**
31 **BPBA). *n*-BPBA was readily oxidised with almost complete degradation (96% ±**
32 **0.4) compared to *t*-BPBA (73.7% ±4.26 degraded) by day 49. Cell counts increased**
33 **four-fold by day 14 but decreased five-fold by day 49 for both *n*- and *t*-BPBA.**
34 **At day 14, (4′-butylphenyl)ethanoic acid (BPEA) metabolites were**
35 **produced, with additional metabolites produced during *t*-BPBA degradation**
36 **identified by mass spectrometry of derivatives as (4'-carboxy-*t*-butylphenyl)-4-**
37 **butanoic acid and (4'-carboxy-*t*-butylphenyl)ethanoic acid; suggesting that IS2.3**
38 **used omega oxidation of *t*-BPEA to oxidise the *tert*-butyl side chain to produce**
39 **(4'-carboxy-*t*-butylphenyl)ethanoic acid, (primary route for biodegradation), but**
40 **that IS2.3 also produced this metabolite through initial omega oxidation of the**
41 ***tert*-butyl side chain of *t*-BPBA, followed by beta-oxidation of the alkanoic acid**
42 **side chain. In conclusion, a Mycobacterium-like isolate could degrade highly**
43 **branched aromatic NAs via two pathways and may be used as a model organism.**

44

45

46 **Introduction**

47 Naphthenic acids (NAs) are found principally in weathered crude oils and are
48 recalcitrant, corrosive and toxic (reviewed by Whitby, 2010). Concerns have been raised
49 about the potential deleterious effects of inputs of NAs to the environment (reviewed by
50 Headley and McMartin, 2004). NAs are a complex mixtures comprising predominantly
51 cycloaliphatic and straight chain and alkyl substituted acyclic carboxylic acids (Rowland
52 *et al*., 2011a-c). Although aromatic NAs make up a small percentage of some NA
53 mixtures (e.g. Rowland *et al*., 2011c,d), they may contribute disproportionately to the
54 overall toxicity and recalcitrance of NAs (Headley and McMartin, 2004; Johnson *et al*.,
55 2011). Despite their persistence and toxicity, little is known about the mechanisms
56 involved in aromatic NA degradation. A previous study reported a microbial consortium,
57 comprising predominantly *Burkholderia* spp., *Pseudomonas* spp. and *Sphingomonas*
58 spp. (identified by 16S rRNA analysis) which was capable of butylphenylbutanoic acid
59 (BPBA) degradation (Johnson *et al*., 2011). However, degradation of alkyl phenyl
60 alkanoic acids by a single pure culture has not yet been demonstrated and such studies
61 are needed if detailed mechanistic studies on NA degradation are to be undertaken to
62 increase the likelihood of achieving enhanced NA bioremediation.

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

70 Although *Mycobacterium* spp have not been shown to degrade aromatic NA
71 acids previously, they have been reported to degrade hydrocarbons such as branched
72 alkanes (*Mycobactorium fortuitum;* Cox *et al*., 1976) and polycyclic aromatic
73 hydrocarbons (PAH) (*Mycobacterium vanbaalenii* PYR-1; Stingley et al., 2004; Kim et
74 al. 2007; 2008). In this study, we aimed to isolate a microorganism from the
75 environment which could degrade BPBAs and thus potentially be used as a model
76 microorganism to study the pathways involved in aromatic NA degradation and to
77 facilitate NA bioremediation studies.
78

79 **Results**

80 **Isolation and characterisation of an aromatic NA-degrading isolate**

81 A single colony derived from a sediment sample was successfully isolated on
82 washed MSM agar plates that contained *n*-BPBA as the sole carbon and energy source
83 (but was unable to grow on control plates without *n*-BPBA). The isolate (designated
84 IS2.3) possessed white, diffuse colonies and light microscopy observations showed that
85 when IS2.3 was grown on MSM agar containing *n*-BPBA it was a non-motile, non-
86 filamentous, short Gram-positive rod (Fig. 1A). However, when IS2.3 was grown in
87 liquid MSM containing 1% (w/v) glucose, it developed a filamentous form (Fig. 1B),
88 which could be easily disrupted following manual shaking of the flask (Fig. 1C).
89 The metabolic capability of isolate IS2.3 was investigated using BIOLOG plates
90 and the data are presented in Table 1. IS2.3 grew on various monosaccharides
91 (including α-D-glucose, D-galacturonic acid, D-mannose, D-xylose), disaccharides

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92 (including D-trehalose, gentiobiose, maltose, sucrose and turanose) as well as
93 polysaccharides such as dextrin. In addition, IS2.3 also grew well on acetic acid,
94 glycerol and surfactants such as Tween40 and Tween80. No growth was observed in
95 abiotic controls. By contrast IS2.3 was unable to grow on any of the PAHs, alkanes and
96 alcohols tested and including ethanol, methanol, 1-methylnaphthalene, phenanthrene,
97 pristane, tetradecane, phenylbutanoic acid and sodium benzoate.
98

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

100 The 16S rRNA gene from IS2.3 was sequenced, a Jukes Cantor DNA-distance
101 and neighbour joining analysis was performed and the phylogenetic tree is presented in
102 Fig. 2. IS2.3 showed the closest phylogenetic sequence similarity to *Mycobacterium*
103 *aurum* (Tsukamura) ATC23070 (Tsukamura and Tsukamura, 1966) with a high
104 bootstrap value (100%). The 16S rRNA gene sequence of IS2.3 also clustered with
105 other *Mycobacterium* spp. including *M. fluoranthenivorans, M. neoaurum* and *M.*
106 *frederiksbergense* but was more distantly related to *M. tuberculosis*, *M. bovis* and the
107 PAH degrader *M. vanbaalenii* PYR-1. BLASTN analysis demonstrated that IS2.3 had
108 99% 16S rRNA gene sequence identity to several *Mycobacterium* spp. (Supplementary
109 Table 1).
110

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

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

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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 1.6×10^5 cfu/ mL (at day 0) to 7.3
124 $\times 10^5$ cfu/ mL (at day 14) (p=0.05) for *n*-BPBA; and from 2.2×10^5 cfu/ mL (at day 0) to
125 7.6×10^5 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 1.5×10^5 cfu/ mL (at day 49) (p=0.044)
127 for *n*-BPBA and 2.0×10^5 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

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

161 394, absent). The base peak ion (*m/z* 277) is then explained by the favourable benzylic
162 cleavage with loss of a mass 117 moiety (Fig. 4C). Charge retention on the latter results
163 in the *m/z* 117 ion, also observed. The ion *m/z* 350 was assigned as due to losses of
164 44 (carbon dioxide) from the putative molecular ion of the bis-TMS ester. This was
165 observed previously in the mass spectra of the TMS esters of a number of synthetic
166 alkylphenylethanoic acids (Rowland *et al*., 2011d). Thus we assign the two new
167 metabolites as (4'-carboxy-*t*-butylphenyl)-4-butanoic acid and (4'-carboxy-*t*-
168 butylphenyl)ethanoic acid respectively, suggesting two divergent pathways for *tert*-
169 BPBA degradation (Fig. 5).
170

171 **Discussion**

172 We successfully isolated a sediment bacterium (designated IS2.3), related to
173 *Mycobacterium spp.* (99% 16S rRNA sequence identity) that degraded aromatic
174 alkanoic NAs (*n*-BPBA and *t*-BPBA) and identified two new metabolites as (4'-carboxy-*t*-
175 butylphenyl)-4-butanoic acid and (4'-carboxy-*t*-butylphenyl)ethanoic acid respectively,
176 suggesting two divergent pathways for *tert*-BPBA degradation.
177 Although degradation of aromatic alkanoic NAs has been demonstrated
178 previously using a mixed consortium (Johnson *et al*., 2011), this appears to be the first
179 report of a single isolate with the metabolic capability to transform aromatic alkanoic
180 NAs. During BPBA degradation both (4′-*n*-butylphenyl)ethanoic acid (*n*-BPEA) and (4′-
181 *tert*-butylphenyl)ethanoic acid (*t*-BPEA) metabolites were produced. Similar metabolites
182 have also been identified previously during degradation of both aromatic alkanoic NAs
183 (Johnson *et al*., 2011), and alicyclic alkanoic NAs (Smith *et al*., 2008; Rowland *et al*.,

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184 2011e) suggesting that BPBA degradation by IS2.3 proceeded via the same pathway as
185 was found previously. However, Johnson *et al*. (2011) demonstrated that *n*-BPEA was
186 completely metabolised after 49 days incubation using a mixed consortium, whereas
187 this did not occur with IS2.3. This suggests that either *n*-BPEA was not bioavailable to
188 IS2.3 or IS2.3 was incapable of producing the required extracellular enzymes for *n*-
189 BPEA metabolism.

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

206 It is known that hydrocarbon biodegradation is greatly inhibited by terminal
207 branching (Schaeffer *et al*., 1979) and previous studies have shown that hydrocarbons
208 with terminal dimethyl branches are relatively resistant to microbial oxidation (Hammond
209 and Alexander, 1972). However, IS2.3 may be efficient in utilising *tert*-branched side
210 chains. Furthermore, *Mycobacterium* spp have been shown to degrade the highly
211 branched squalane (2,4-,6,10,15,19,23-hexamethyltetracosane) via oxidation of the
212 terminal carbon as the initial step (Berekaa and Steinbushel, 2000), and IS2.3 has 99%
213 16S rRNA gene sequence identity to *Mycobacterium* spp. *M. tuberculosis* possesses a
214 ω-hydroxylase enzyme for this step (Johnston *et al*., 2009). Interestingly, Pirnik *et al*.
215 (1974) observed that "ω-oxidation of long chain acids with ω-1 methyl branching seems
216 slow enough to permit at least one cycle of beta oxidation before a dicarboxylic acid is
217 fully established". We observed a similar phenomenon.

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

228 It has been previously shown that Mycobacteria require build up of carbon dioxide (up to
229 8% v/v) in the headspace before exponential growth occurs (Ratledge, 1982). In the
230 present study, it was also found that static cultures of IS2.3 decreased incubation times
231 required for growth (data not shown). Furthermore, members of Mycobacteria such as
232 *Mycobacterium aurum* Tsukamura (Tsukamura and Tsukamura, 1966) have been
233 shown to grow both at 28°C and 37°C (Tsukamura, 1966). In contrast, although IS2.3
234 grew at both 20°C and 30°C; the isolate was unable to grow at 37°C (data not shown).
235 Furthermore, degradation rates of *n*-BPBA by IS2.3 at 30°C were not significantly
236 different from those at 20°C ($p = 0.513$) (data not shown).

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

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

256 **Experimental Procedures**

257 **Culture isolation and light microscopy**

258 (4′-*n*-butylphenyl)-4-butanoic acid (*n*-BPBA) and (4′-*t*-butylphenyl)-4-butanoic
259 acid (*t*-BPBA) were synthesised as described previously (Smith et al., 2008). Sediment
260 samples (top 3 cm) were obtained from Avonmouth (51:31:28N, 2:41:04W). Cation/
261 anion analysis was performed using Dionex ICS-3000 and data were as follows: acetate
262 (160.7 μM), bromide (313.3 μM), calcium (489.9 μM), chlorate (170.5 μM), chloride
263 (179.4 mM), fluoride (225.7 μM), lithium (1.86 μM), magnesium (2435. 1 μM), nitrate
264 (78.54 μM), phosphate (1,68 μM), potassium (613.1 μM) sodium (24.5 mM), strontium
265 (8.40 μM) and sulphate (7949 μM). Total organic carbon was 30.34 ± 0.37 mg g^{-1} as
266 determined using a Shimadzu TOC-VCHS analyser. Sediment samples were pre-
267 enriched in 25 mL of minimal salts medium (MSM) containing 1% (v/v) heavy crude oil
268 (Tia Juana Pesado) as the sole carbon source as previously described (Johnson *et al*.,
269 2011).

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

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

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

281 Degradation experiments were set up by inoculating IS2.3 (2% v/v) into 25 mL
282 MSM containing *n*- or *t*-BPBA as described previously (Johnson *et al*., 2011). Viability
283 checks of killed controls were performed by streaking onto R2A agar (Fluka) and
284 incubating at 20°C for two days prior to inoculation. Destructive sampling was carried
285 out at days 0, 14, 35 and 49 and BPBAs extracted using ethyl acetate (HPLC, Fisher)
286 as described previously (Smith *et al*., 2008; Johnson *et al*., 2011). Aromatic acid
287 extracts were analysed by on a Thermo Finnigan Trace gas chromatograph Ultra
288 coupled with a Thermo Finnigan Trace DSQ mass spectrometer and Thermo AS3000
289 autosampler. Samples were injected with a 1 µL splitless injection (injector temperature
290 250°C) onto a 15 m x 250 µm x 0.25 µm Rtx®-1MS column with integrated guard
291 (Restek) using helium as the carrier gas at a constant flow of 1 mL min^{-1}. Oven
292 temperatures were programmed as above with an initial increase of 40°C to 300°C at
293 10°C min^{-1} and a final hold at 300°C for 10 min. The transfer line was again held at
294 230°C onto a source for the mass spectrometer of 230°C which was in full scan mode
295 (scan range 50-550 Da). Data were analysed and integrated with Xcalibur ver. 1.4 SR1.

296 **Cell Counts**

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

300

301 **Carbon substrate utilisation**

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

314

315 **16S rRNA Gene Sequence Analysis**

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

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319 1989), 2.5 U *Taq* DNA Polymerase (Qiagen). PCR cycling conditions were as follows:
320 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
321 mins; then 72°C for 10 mins. PCR Products were purified using a QIAquick® PCR
322 purification kit (Qiagen) according to the manufacturer's instructions, sequenced
323 bidirectionally using the primers pA, pC and pC', pF' and pG' (Edwards *et al*., 1989) by
324 GATC Biotech (Konstanz, Germany). Sequences were checked for ambiguous bases;
325 the 16S rRNA gene consensus sequence was assembled to a total length of 1452 bp
326 and submitted to Genbank under the accession number HQ224877.
327

328 **Phylogenetic analysis of 16S rRNA Sequence from IS2.3**

329 The 16S rRNA sequence recovered from IS2.3, together with selected
330 sequences from the Genbank database were aligned using the RDP INFERNAL
331 alignment tool (Nawrocki and Eddy, 2007). Phylogenetic analysis was performed using
332 PHYLIP 3.4 with Jukes-Cantor distance and neighbor-joining methods (Jukes and
333 Cantor, 1969; Saitou and Nei, 1987). Bootstrap analysis was based on 100 replicates
334 using SEQBOOT (PHYLIP 3.4). Tree construction was performed using Treeview
335 (WIN32) version 1.5.2 (Page, 1996).
336

337 **Statistical Analysis**

338 Statistical analysis was carried out using SPSS v18.0 with ANOVA.
339

340

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458 **Figure Legends**

459 **Figure 1.** Gram stains of environmental isolate IS2.3 visualised using a light microscope
460 (A); in filamentous form before (B) and after (C) shaking.
461

462 **Figure 2**. Phylogenetic analysis of the 16S rRNA gene sequence from IS2.3. Included
463 are 16S rRNA gene sequences from type strains obtained from Genbank. Sequence
464 analysis was based on 1361 bp using Jukes-Cantor DNA distance and neighbour-
465 joining methods. *E. coli* was used as an outgroup. Bootstrap values represent
466 percentages from 100 replicates of the data and percentages >80% are shown. The
467 scale bar indicates 0.1 substitutions per nucleotide base.
468

469 **Figure 3.** Degradation of *n*- and *tert*-BPBA by IS2.3. Calculated as a percentage of
470 either *n*- or *tert*-BPBA remaining compared to killed controls. Error bars represent
471 standard deviation of the mean (*n*=3). *n*-BPBA (■), and *tert*-BPBA (♦)
472

473 **Figure 4.** Mass spectra of metabolites produced during degradation of *t*-BPBA. (A)
474 Mass spectrum of trimethylsilylated ester assigned to (4'-carboxy-*t*-butylphenyl)ethanoic
475 acid (*bis*-TMS ester). (B) Mass spectrum of dimethyl ester assigned to (4'-carboxy-*t*-
476 butylphenyl)ethanoic acid (C) Mass spectrum of trimethylsilylated ester assigned to (4'-
477 carboxy-*t*-butylphenyl)-4-butanoic acid (*bis*-TMS ester).
478
479 **Figure 5.** Postulated biotransformation of 4'-BPBA by IS 2.3

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481 **Table Legends**

482 **Table 1.** Growth of IS2.3 on various organic substrates. Growth was determined by

483 measuring mean absorbance at A_{595} (n=3).

484

485 **Supplementary Table 1**. BLASTN analysis of 16S rRNA gene sequence from IS2.3

486 compared to representative members of the Mycobacterium. 16S rRNA sequences from

487 type strains were obtained from Genbank.

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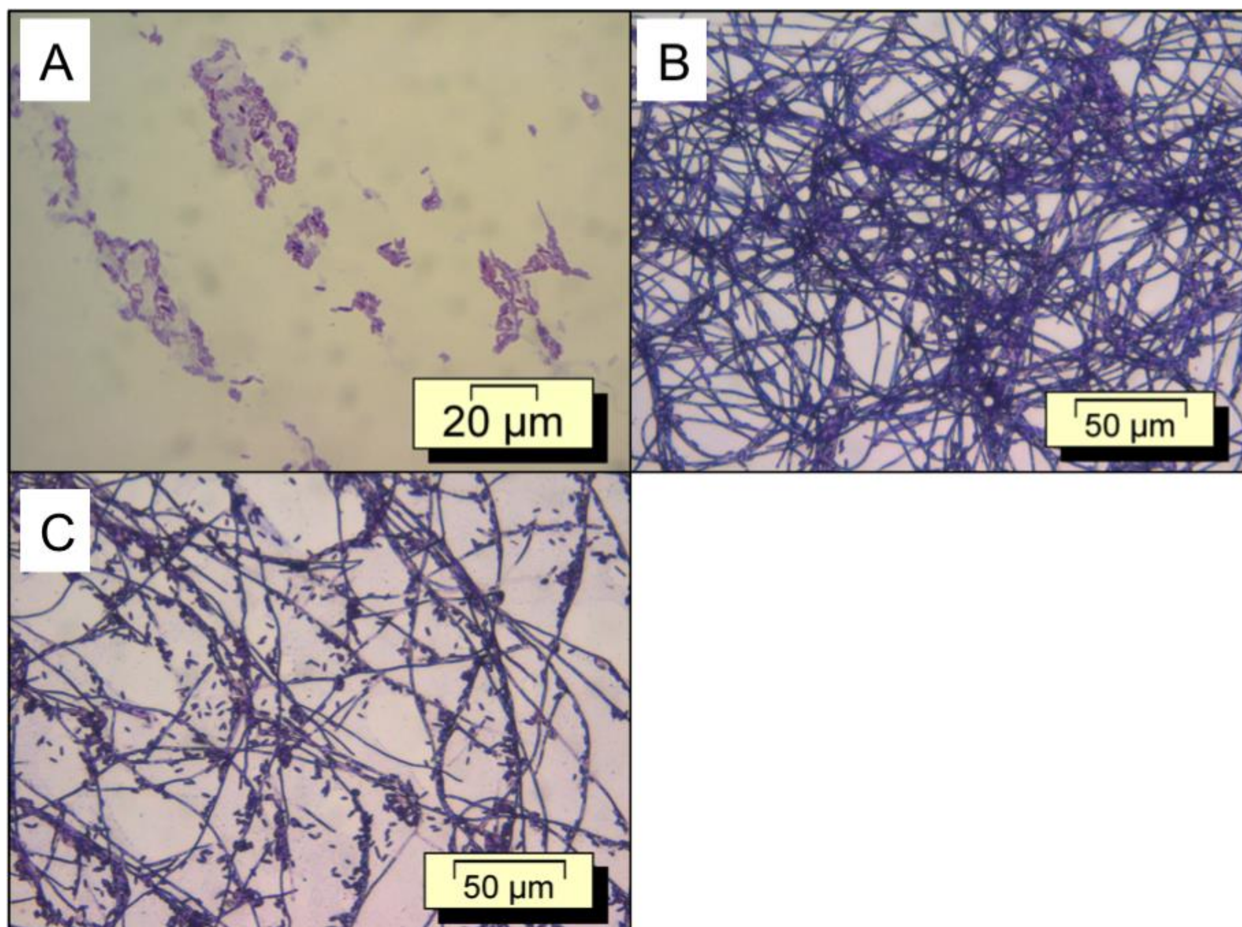

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493 **Figure 1**

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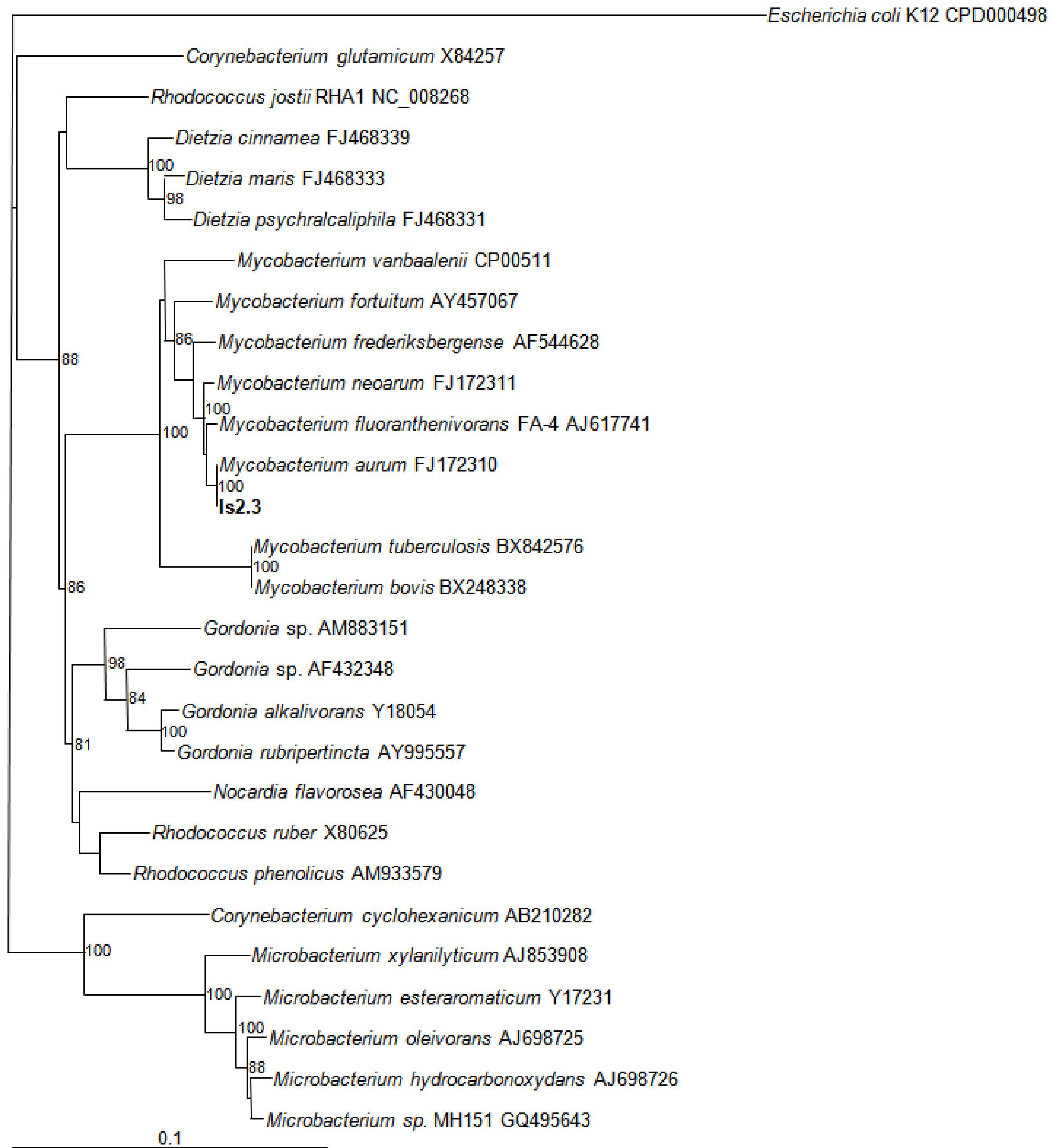

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503 **Figure 2.**

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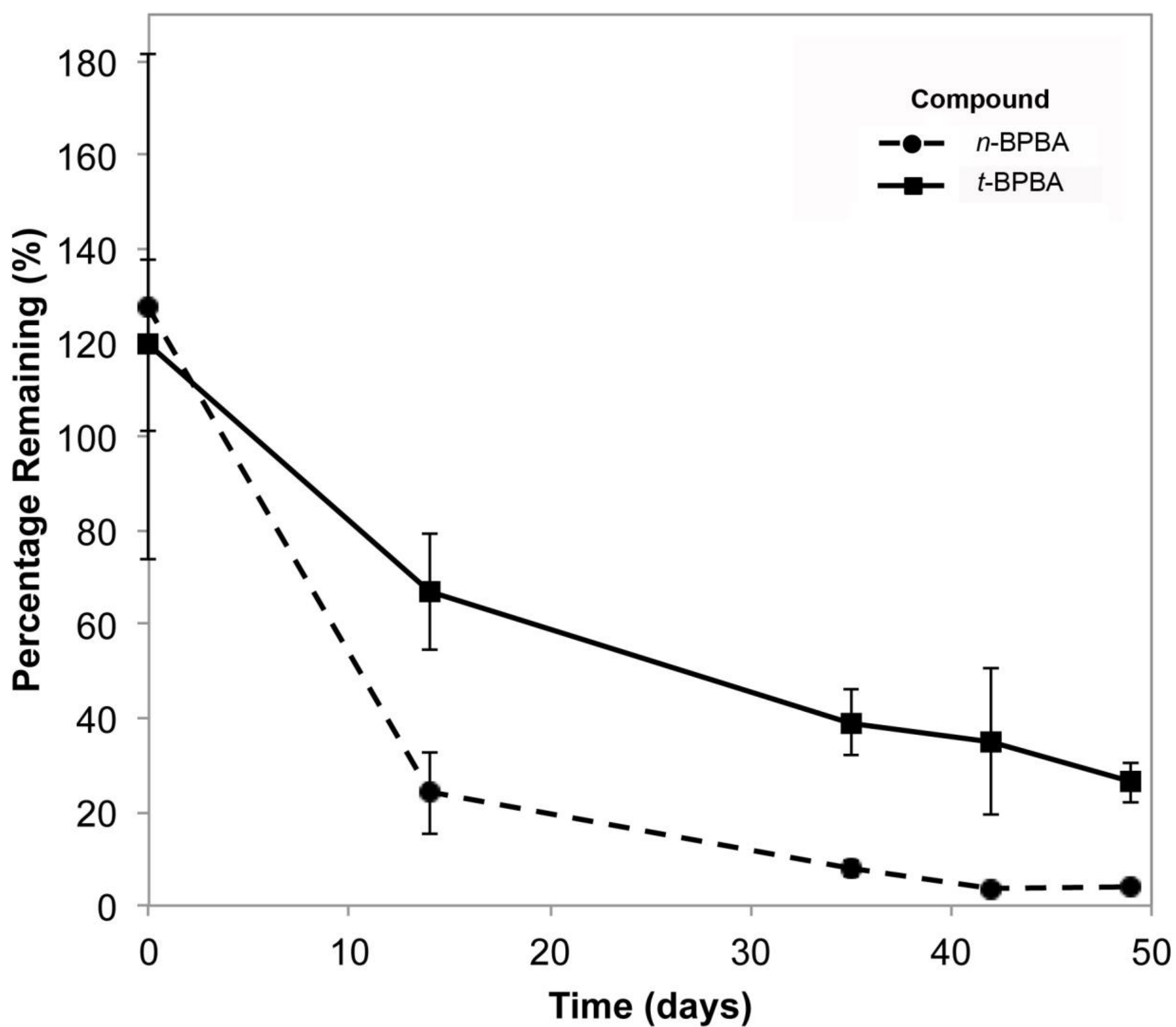

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506 **Figure 3**

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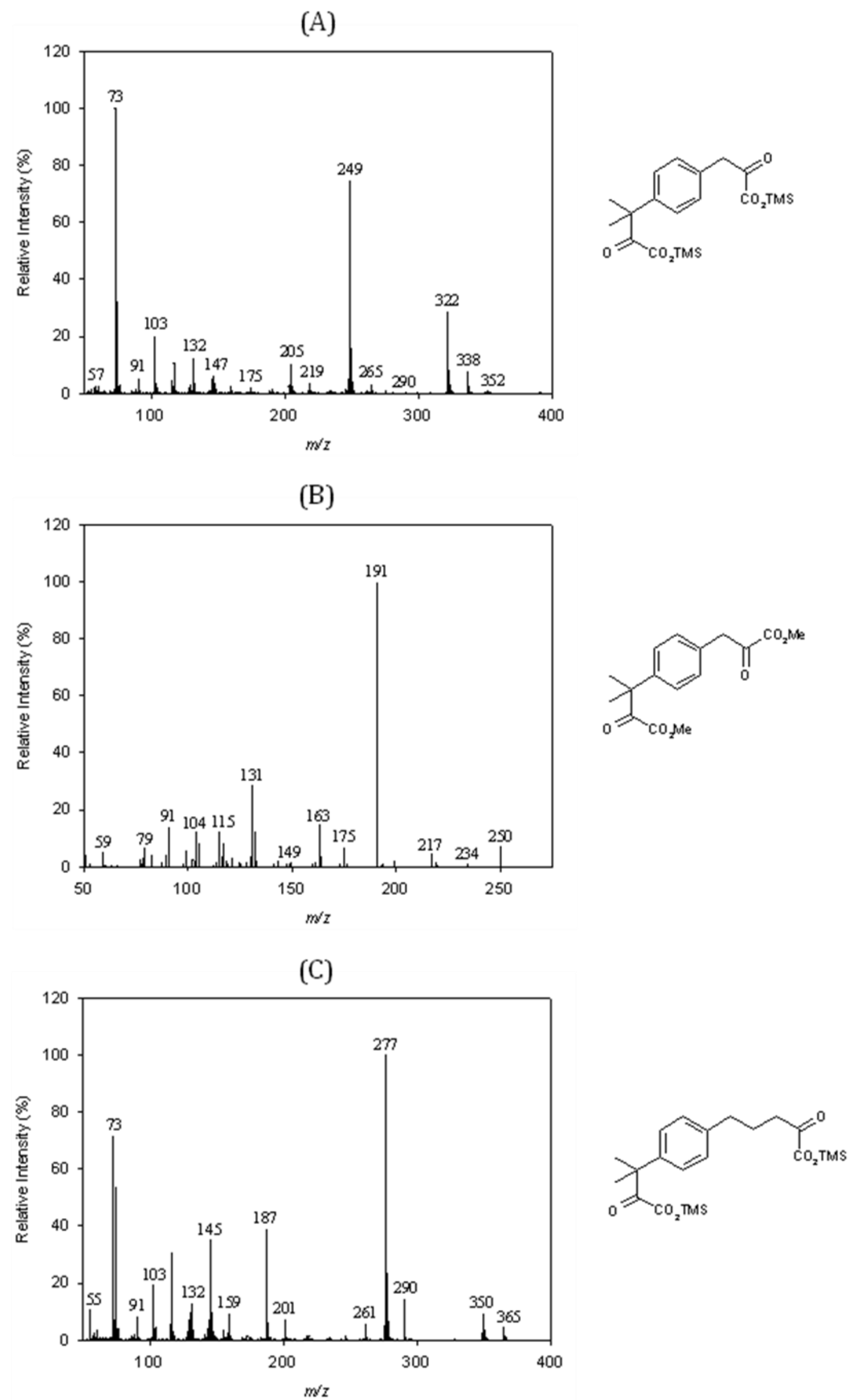

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509 **Figure 4**

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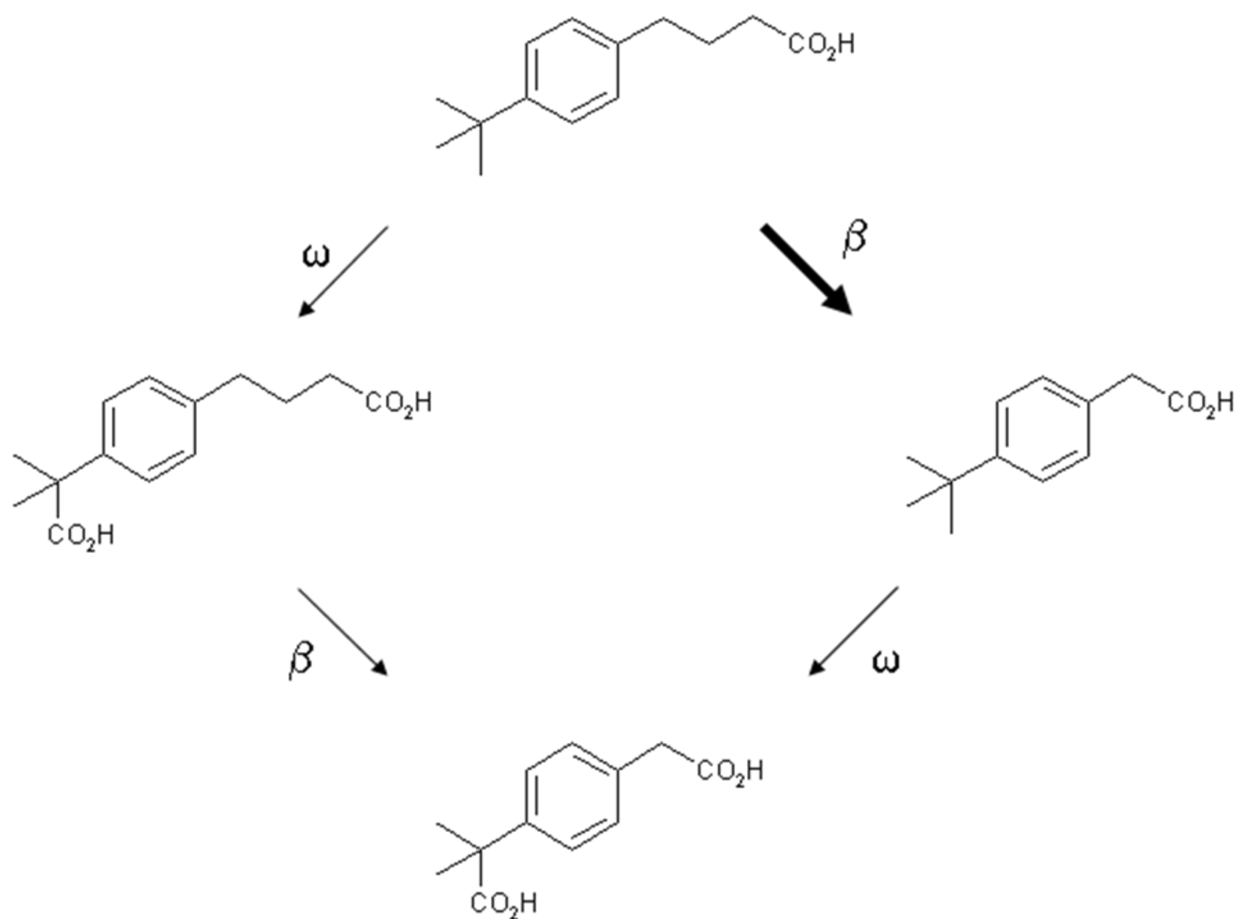

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513 **Figure 5**
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519 **Table 1**

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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Compound	Response	Line
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	++	527
L-Malic acid	-	
Lactamide	-	528
N-Acetyl-L-glutamic acid	++	
p-Hydroxyphenylacetic acid	++	529
Propionic acid	++	
Pyruvatic acid methyl ester	-	530
Pyruvic acid	+	
Succinamic acid	-	531
Succinic acid	-	
Succinic acid mono-methyl ester	++	532
		533
Polyols		
D-Arabitol	++	534
D-L-α-Glycerol phosphate	++	
D-Mannitol	++	535
D-Sorbitol	-	
Glycerol	**+++**	536
m-Inositol	-	
Xylitol	++	537
		538
Surfactants		
Tween40	**+++**	539
Tween80	**+++**	
		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

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545 **Supplementary Table 1**

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547

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

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