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1 **Isolation of isoprene degrading bacteria from soils, development of *isoA* gene**
2 **probes and identification of the active isoprene degrading soil community**
3 **using DNA-stable isotope probing**

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19 Isoprene, 2-methyl-1,3-butadiene, biodegradation, culture independent methods, molecular
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21 biosynthesis.

22

23 Originality-Significance Statement

24 Microorganisms with the ability to degrade the important climate-active gas isoprene are
25 abundant in the environment yet we know very little about them. Here we applied cultivation-
26 dependent and -independent methods (including the first example of the use of DNA-stable
27 isotope probing with this substrate) to greatly expand our knowledge of isoprene degraders,
28 which is a prerequisite to developing an understanding of isoprene biodegradation. We
29 provide genome sequence data on two new isolates, solid evidence for isoprene degradation
30 in genera not previously implicated and tools to investigate biodegradation of this important
31 atmospheric trace gas.

32

Abstract

Emissions of biogenic volatile organic compounds (bVOCs), are an important element in the global carbon cycle, accounting for a significant proportion of fixed carbon. They contribute directly and indirectly to global warming and climate change and have a major effect on atmospheric chemistry. Plants emit isoprene to the atmosphere in similar quantities to emissions of methane from all sources and each accounts for approximately one third of total VOCs. Although methanotrophs, capable of growth on methane, have been intensively studied, we know little of isoprene biodegradation. Here we report the isolation of two isoprene-degrading strains from the terrestrial environment and describe the design and testing of PCR primers targeting *isoA*, the gene encoding the active-site component of the conserved isoprene monooxygenase, which are capable of retrieving *isoA* sequences from isoprene-enriched environmental samples. Stable isotope probing experiments, using biosynthesized ^{13}C-labelled isoprene, identified the active isoprene-degrading bacteria in soil. This study identifies novel isoprene-degrading strains using both culture-dependent and, for the first time, culture-independent methods and provides the tools and foundations for continued investigation of the biogeography and molecular ecology of isoprene-degrading bacteria.

51 **Introduction**

52 Isoprene accounts for approximately one third of the total flux of volatile organic
53 compounds to the atmosphere, an amount that is similar to the methane flux
54 (Guenther et al., 2012; Kirschke et al., 2013). In the atmosphere, isoprene is rapidly
55 photochemically oxidized resulting in a short lifetime (of the order of hours) and
56 consequent low concentrations. Attack by hydroxyl or nitrate radicals or ozone leads
57 to a variety of products depending on temperature and pollutant (nitrogen oxides,
58 NO_x) levels (Atkinson and Arey, 2003). Overall, isoprene has a significant effect on
59 atmospheric chemistry and hence climate change, due both to the production of
60 greenhouse gases (principally ozone) and by reducing the hydroxyl radical-mediated
61 oxidizing capacity of the atmosphere, which increases the lifetime of methane
62 (Pacifico et al., 2009). In addition, isoprene oxidation-products form secondary
63 organic aerosols and cloud condensation nuclei, with implications for air quality and
64 climate (Fiore et al., 2012).

65 About 600 Tg y^{-1} isoprene is emitted to the atmosphere by terrestrial plants, although
66 not all plant species produce isoprene (Sharkey, 2013; Loreto and Fineschi, 2015).
67 Isoprene synthesis occurs in the chloroplast, via the enzyme isoprene synthase,
68 which converts dimethylallyl pyrophosphate to isoprene (Logan et al., 2000).
69 Isoprene protects plants against heat stress by reducing heat-induced cell-
70 membrane damage, enhances tolerance of reactive oxygen species and may affect
71 plant-insect interactions (Loivamäki et al., 2008; Sharkey et al., 2008; Vickers et al.,
72 2009; Sharkey, 2013). In the marine environment isoprene is released by
73 phytoplankton and macroalgae (Broadgate et al., 2004; Exton et al., 2015). Some
74 bacteria, including soil-dwelling species such as *Bacillus subtilis*, release isoprene,
75 as do some fungi (Kuzma et al., 1995; Julsing et al., 2007; Bäck et al., 2010),

76 although we lack a clear understanding of why these organisms produce isoprene. A
77 non-enzymatic reaction resulting in isoprene was reported in humans and other
78 animals, associated with the mevalonate pathway of cholesterol synthesis (Gelmont
79 et al., 1981).
80 Although atmospheric isoprene concentrations are low (<1 – 4 ppbv in one wide-
81 ranging study (Greenberg et al., 1999)), in the vicinity of isoprene sources (around or
82 below tree canopy level) concentrations are significantly higher. For example,
83 Wiedinmyer and colleagues measured ground level isoprene concentrations of 11
84 and 36 ppbv at sites in Texas and Missouri, respectively (Wiedinmyer et al., 2001;
85 Wiedinmyer et al., 2005). Soils can act as a biological sink for isoprene, at or below
86 these concentrations. In field chambers set up in temperate forest soils, isoprene
87 was rapidly depleted to below the 5 ppbv limit of detection (Cleveland and Yavitt,
88 1997; Cleveland and Yavitt, 1998). In continuous flow experiments conducted by
89 Gray et al. (2015), soils supplied with isoprene at concentrations of 2 – 200 ppbv
90 consumed isoprene at all concentrations, with a rate of 62 pmol g^{-1} h^{-1} at 20 ppbv.
91 These data demonstrate both the potential of soils to consume isoprene released
92 locally in soils and also to take up atmospheric isoprene, conclusions also reached in
93 mesocosm experiments by Pegoraro et al. (2005). Several bacterial strains,
94 tentatively assigned to the genera *Nocardia, Rhodococcus* (Actinobacteria) and
95 *Alcaligenes* (Betaproteobacteria), were isolated from isoprene enrichment cultures
96 and shown to grow on isoprene as sole growth substrate, (van Ginkel et al., 1987b;
97 van Ginkel et al., 1987a; Ewers et al., 1990; Cleveland and Yavitt, 1997) and, more
98 recently, *Pseudomonas, Alcaligenes* and *Klebsiella* isoprene-degrading strains were
99 isolated from rubber-contaminated soil (Srivastva et al., 2015). Strains were also
100 obtained from the marine environment, including representatives of Actinobacteria,

101 Bacteroidetes and Alpha- and Gammaproteobacteria (Acuña Alvarez et al., 2009).
102 None of these terrestrial isolates was extensively characterized and the best
103 documented isoprene degrader to date is *Rhodococcus* sp. AD45, a Gram-positive
104 actinobacterium isolated nearly 20 years ago from freshwater sediment by the group
105 of Dick Janssen (van Hylckama Vlieg *et al.,* 1998).

106 In *Rhodococcus* sp. AD45, isoprene is oxidized to epoxyisoprene (1,2-epoxy-2-
107 methyl-3-butene) by a four-component soluble diiron centre monooxygenase
108 (SDIMO) with homology to enzymes including the soluble methane monooxygenase
109 (sMMO) and alkene/aromatic monooxygenases (van Hylckama Vlieg et al., 2000;
110 Leahy et al., 2003). The epoxide is then conjugated with glutathione, catalyzed by
111 glutathione-*S*-transferase (GST) (IsoI) and oxidized in two steps by a dehydrogenase
112 (IsoH) resulting in 2-glutathionyl-2-methyl-butenoic acid (Fig. 1) (van Hylckama Vlieg
113 et al., 1998; van Hylckama Vlieg et al., 1999). Interestingly, conjugation with
114 glutathione in *Rhodococcus* sp. AD45 contrasts with other alkene utilizers, which
115 often overcome the toxicity of epoxides by forming coenzyme M conjugates or by
116 hydrolysis (Ensign, 2001; Kottegoda et al., 2015). The genes encoding the
117 monooxygenase (*isoABCDEF*) and two subsequent enzymes, together with two
118 additional genes of unknown function, were cloned and sequenced (van Hylckama
119 Vlieg et al., 2000). Recently, we sequenced the genome of *Rhodococcus* sp. AD45
120 and showed, by mutagenesis, that isoprene monooxygenase (IsoMO) was essential
121 for isoprene metabolism. Using RNAseq, a cluster of 22 genes was identified, all of
122 which were induced by isoprene or the immediate product of isoprene oxidation,
123 epoxyisoprene (Crombie *et al.,* 2015).

124 DNA stable isotope probing (DNA-SIP) is a cultivation-independent technique with
125 the ability to identify active substrate-consuming organisms in environmental

126 samples (Radajewski et al., 2000; Dumont and Murrell, 2005). The method relies on
127 incubation of samples with stable-isotope-labelled growth substrate. The
128 incorporation of isotope (typically ^{13}C or ^{15}N) into biomass (including DNA), enables
129 the identification of active microorganisms following separation of labelled and
130 unlabelled DNA by isopycnic (density gradient) centrifugation.

131 Despite its abundance and climatic importance, our knowledge of isoprene in the
132 environment is heavily skewed towards production in plants and atmospheric
133 oxidation, with only a few studies investigating isoprene bio-degradation. Isoprene is
134 an abundant plant secondary metabolite, also produced in soils, and would provide a
135 good source of carbon and energy for bacteria. Strains capable of growth on
136 isoprene have frequently been isolated from diverse environments, albeit generally
137 not characterized at the molecular level. Our hypothesis was that isoprene degraders
138 are widely distributed and may play an important role in the biogeochemistry of this
139 environmentally important trace gas. Our aim was to isolate and sequence isoprene
140 degraders, and identify putative isoprene metabolic genes. We aimed to develop
141 gene probes to target key diagnostic markers of isoprene degradation and to identify
142 the active isoprene-assimilating organisms in soil enrichments, using DNA-SIP.

143

144

145 Results and Discussion

146 *Enrichment and isolation of two novel terrestrial isoprene-utilizing bacteria*

147 Our initial aim was to isolate isoprene degraders from contrasting environments in
148 order to provide sequence data for later cultivation-independent approaches. Since

149 isoprene consumption has previously been observed in soils and since isoprene is
150 emitted from tree leaves, we used these as source material. Separate enrichments
151 were set up, using either garden soil or Horse Chestnut (*Aesculus hippocastanum*)
152 leaves, in minimal medium and incubated with isoprene. Both enrichments
153 consumed isoprene and two isolates (designated SC4 and LB1), capable of growing
154 on isoprene as sole source of carbon and energy, were obtained from soil and leaf
155 samples, respectively. The nearly complete (1521 nucleotides) 16S rRNA gene
156 sequences of both strains SC4 and LB1 were identical to strains of both
157 *Rhodococcus opacus and R. wratislaviensis* (Fig. S1). Strains SC4 and LB1 also
158 grew on acetate, succinate, glucose, fructose, propane and butane as sole source of
159 carbon and energy (Table S1), in contrast to *Rhodococcus* sp. AD45, which does not
160 grow on propane or butane (Crombie et al., 2015).

161

162 *Genome sequencing*

163 To identify isoprene-related gene sequences, we sequenced the genomes of strains
164 SC4 and LB1. Their genomes, 10.6 and 10.7 Mbp, are considerably larger than that
165 of *Rhodococcus* sp. AD45 (6.9 Mbp) and closer to that of *R. jostii* RHA1 (9.7 Mbp)
166 (McLeod et al., 2006; Crombie et al., 2015), whereas the GC contents (66.7 and
167 66.6% respectively) are typical of the genus (Table S2).

168

169 *Identification of isoprene related genes in isolates*

170 Our previous work (Crombie *et al.,* 2015) detected a cluster of 22 genes induced by
171 isoprene, which appeared to be the complete inventory of isoprene-responsive

172 genes. In addition to *isoABCDEF,* encoding the monooxygenase, and *isoGHIJ* that
173 encode a protein of unknown function, a dehydrogenase and two glutathione-*S*-
174 transferases previously described (van Hylckama Vlieg et al., 2000), the cluster
175 includes glutathione biosynthesis genes, predicted aldehyde dehydrogenases and a
176 coenzyme-A disulfide reductase (Crombie et al., 2015). We therefore searched for
177 homologous sequences in the genomes of strains SC4 and LB1, which are highly
178 similar to each other in this region (over 99% nucleotide identity). Using the
179 isoprene-responsive gene products previously identified in *Rhodococcus* sp. AD45
180 as query sequences in tBLASTn searches, we identified homologues of all of these
181 22 genes, with amino acid sequence identity ranging from 50–96% (Table S3). The
182 most highly conserved were *isoABCDEF* encoding the multi-component IsoMO (81–
183 96%), whereas a predicted protein of unknown function (SZ00_06083), highly
184 induced by isoprene in *Rhodococcus* sp. AD45, shared 50% amino acid identity with
185 sequences from these strains. We observed the same duplication of *isoGHIJ* (Fig. 2,
186 77 – 88% amino acid identity between copies) as is present in *Rhodococcus* sp.
187 AD45. Between *isoA* and *isoJ*, both of the new strains contain a second copy (81%
188 amino acid identity between copies), not present in *Rhodococcus* sp. AD45, of an
189 aldehyde dehydrogenase (*aldh1*) which is located approximately 10,000 nucleotides
190 (nt) upstream of the monooxygenase in *Rhodococcus* sp. AD45. A gene encoding a
191 predicted coenzyme-A disulfide reductase is also present in two copies (69% amino
192 acid identity between copies), although in strain LB1 (but not in strain SC4) one copy
193 has a nucleotide insertion approximately 255 nt from the end, resulting in a
194 frameshift mutation, suggesting this may not encode a functional protein. In
195 comparison with *Rhodococcus* sp. AD45, five additional genes are present, in both
196 strains, in the middle of the cluster. These are predicted to encode two hypothetical

197 proteins and an alpha/beta hydrolase domain-containing protein of unknown
198 function, an acetyl-CoA acetyltransferase, and a 3-hydroxyacyl-CoA dehydrogenase.
199 Interestingly, in strain LB1, another insertion in the acetyl-CoA acetyltransferase has
200 resulted in a frameshift mutation. None of these five genes are present in this region
201 of the *Rhodococcus* sp. AD45 genome, nor were remotely-located homologous
202 sequences induced by isoprene (Crombie et al., 2015), implying that they are not
203 essential for isoprene metabolism. In comparison with *Rhodococcus* sp. AD45,
204 strains LB1 and SC4 are more similar in this region of the genome, in terms of both
205 sequence identity and gene layout, with *R. opacus* PD630 (also included in Fig. 2).
206 Previously, using sequence data, we predicted, and confirmed, that *R. opacus*
207 PD630 could grow on isoprene (Crombie et al., 2015), although, beyond this, we
208 have no direct experimental data regarding isoprene-related gene function in this
209 strain.

210

211 *Development and validation of functional gene markers targeting isoA*

212 Using the sequence data from these isoprene-degrading strains, we designed
213 probes to detect isoprene-related genes in environmental samples. *isoA* codes for
214 the alpha-subunit of the hydroxylase of IsoMO and contains the diiron centre active
215 site and was shown to be essential for isoprene degradation in *Rhodococcus* sp.
216 AD45 (Crombie *et al.,* 2015). We therefore selected *isoA* as target for a functional
217 gene probe. To expand the diversity of *isoA* sequences, we also retrieved *isoA*
218 genes from draft sequences of a *Gordonia* strain and a *Mycobacterium* strain
219 previously isolated from surface sediment from the Colne estuary (Essex, UK),
220 identified as *Gordonia* i37 and *Mycobacterium* AT1 (Acuña Alvarez et al., 2009;

221 Johnston, 2014). The *isoA* sequences of strains SC4, LB1, i37 and AT1, together
222 with those of *Rhodococcus* sp. AD45 (SZ00_06091) and *R. opacus* PD630
223 (Pd630_LPD03572), were aligned at the amino acid level, and conserved regions
224 were used to design primers targeting *isoA*. To exclude other non-isoprene-
225 degrading members of the SDIMO family, sequences of *mmoX* and *xamoA*,
226 encoding the alpha subunits of sMMO from *Methylosinus trichosporium* OB3b (Cardy
227 et al., 1991), and alkene monooxygenase from *Xanthobacter autotrophicus* Py2
228 (Zhou et al., 1996), respectively, were also included in the alignment.
229 The *isoA* primers were validated by PCR amplification of template DNA from
230 contrasting sources (Table S4): (i) genomic DNA from 15 isoprene-degrading
231 isolates, (ii) DNA extracted from three isoprene-enriched soils and four isoprene
232 enrichments of marine and estuarine water and sediment, and (iii) control DNA
233 extracted from eight non-isoprene-degrading isolates able to grow on alkanes,
234 alkenes or aromatic compounds. We obtained PCR products of the expected size
235 using DNA extracted from all the isoprene-degrading isolates and enrichments, but
236 not from any of the non-isoprene degraders. PCR products from enrichments were
237 cloned and analysed by restriction fragment length polymorphism (RFLP) (Table S5).
238 Representatives of each operational taxonomic unit (OTU) were sequenced, (all of
239 which appeared to be *isoA* sequences) and aligned at the amino acid level with IsoA
240 sequences obtained from the sequenced genomes. A phylogenetic tree of the *isoA*
241 nucleotide sequences (1011 nt) was constructed from the alignment (Fig. 3). All the
242 sequences, although from diverse phylogenetic groups including both Gram-positive
243 and Gram-negative strains, were relatively similar (> 86% amino acid identity
244 between sequences), but could be broadly separated into two groups in which the
245 terrestrial sequences and those from the low-salinity environment of Hythe, on the

246 Colne estuary, were distinct from marine and other estuarine sequences similar to
247 IsoA of *Gordonia* i37.
248

249 *Active isoprene-assimilating bacteria identified by DNA-stable isotope probing (SIP)*

250 Since all four of our sequenced terrestrial isolates were rhodococci and all *isoA*
251 sequences retrieved were relatively similar, we used cultivation-independent
252 methods to test whether a greater diversity of isoprene degraders existed in soils in a
253 DNA-stable isotope experiment (DNA-SIP) using ^{13}C-labelled isoprene,
254 biosynthesized as described in Experimental Procedures. Soil microcosms (in
255 triplicate for labelled substrate and duplicate for unlabelled-substrate controls) were
256 incubated with 0.5% (v/v) isoprene, without any other amendments. Isoprene was
257 consumed without an appreciable lag phase, and incubations were terminated after
258 consumption of 10 μmol substrate g^{-1} soil (15 days). DNA was extracted from ^{13}C-
259 and control ^{12}C-isoprene incubations and separated into heavy and light fractions
260 and used for 454 pyrosequencing of 16S rRNA genes. The unenriched soil at
261 timepoint zero displayed a typically diverse community, comprising 50%
262 Proteobacteria, with Planctomycetes, Actinobacteria, Bacteroidetes, Chloroflexi and
263 Firmicutes contributing an additional 40% (Fig. 4). The major effect of the
264 incubations was to greatly increase the relative abundance of Actinobacteria, while
265 having a relatively minor effect on the remaining phyla (Fig. 4). Multivariate analysis
266 (Fig. S2) shows that the unlabelled bacterial community of the ^{13}C-isoprene
267 incubations (i.e. light DNA fraction) was extremely similar to that of the timepoint
268 zero community, suggesting that the change in community profile during the
269 incubations was due to an enrichment of isoprene degraders. As expected, the
270 community represented by the light fraction of the ^{12}C-isoprene incubations, derived

271 from both isoprene-consumers and non-consumers, was extremely similar to the
272 total (unfractionated) DNA from ^{13}C-incubations. The heavy fraction of ^{12}C-isoprene
273 incubations, which contained only a very small fraction of the total DNA of ^{12}C-
274 incubations, was less similar. This DNA results mainly from incomplete separation,
275 with minor effects due to factors such as GC content of DNA. In contrast to all of
276 these, the active isoprene degraders represented by the heavy fractions of the ^{13}C
277 incubations formed a distinct community (Fig. S2) and displayed a dramatically
278 altered taxonomic profile (Fig. 5), becoming dominated by *Rhodococcus* sequences,
279 which comprised 88 ± 5% (mean ± s.d.) of the heavy fraction of ^{13}C incubations.
280 These sequences were predominantly those of *Rhodococcus wratislaviensis, R.*
281 *koreensis* and *R. globerulus* (Fig. 5) and were 147- to 161-fold more abundant in the
282 heavy fractions compared to light fractions of ^{13}C incubations, but without any
283 corresponding enrichment in the heavy fraction of ^{12}C-incubations (Fig. S3). Also
284 enriched in the heavy fractions of ^{13}C-incubations were members of the
285 Betaproteobacteria, *Comamonas* spp. and *Variovorax* spp., which together
286 comprised 6.5 ± 1.2% of the ^{13}C heavy fractions (but were not detected in the ^{13}C
287 light fractions), and were also enriched during the incubations, increasing from 0.3%
288 to 0.7% of the total community. These data clearly demonstrate that *Rhodococcus*
289 and, to a lesser extent, *Comamonas* and *Variovorax* spp. had assimilated carbon,
290 directly or indirectly, from labelled isoprene. Searches of the publicly available
291 databases did not identify high-similarity putative isoprene-related genes in the
292 family Comamonadaceae (which encompasses both *Comamonas* and *Variovorax*)
293 (see Experimental Procedures). Comparison with the PCR-based *isoA* analysis,
294 which generated similar sequences from diverse phylogenetic groups, suggests that
295 the isoprene-degrading members of the Comamonadaceae implicated in the SIP

296 experiment are not represented in the NCBI databases, or that they use genes or
297 possibly pathways dissimilar to those of the characterized strains.

298 Previous studies of terrestrial environments have isolated *Alcaligenes, Klebsiella* and
299 *Pseudomonas* isoprene-degrading strains in addition to the Actinobacteria *Nocardia*
300 and *Rhodococcus*, although some of these identifications were not based on
301 molecular data and most strains were not extensively characterized (van Ginkel et
302 al., 1987b; van Ginkel et al., 1987a; Ewers et al., 1990; Cleveland and Yavitt, 1997;
303 van Hylckama Vlieg et al., 1998; Srivastva et al., 2015). In our SIP incubations, 16S
304 rRNA gene sequences of *Alcaligenaceae* spp. were slightly enriched during the
305 incubations, increasing from 0.3% to 0.6% of the total community, but were
306 concentrated (14:1) in the non-labelled light DNA fractions. *Pseudomonas* spp.,
307 present at 0.9% of the initial community, decreased to 0.1% following isoprene
308 incubations and were also not labelled, whereas *Klebsiella* were not detected at any
309 point. These data indicate that these taxa had not assimilated carbon from isoprene
310 under our experimental conditions.

311

312 *Conclusions*

313 The isoprene concentrations used here are considerably in excess of those normally
314 encountered in the environment and the aim was not to replicate environmental
315 conditions, but rather to expand the diversity of known isoprene degraders. Draft
316 genome sequences showed that isoprene monooxygenase was present in all our
317 isoprene-degrading isolates, which enabled the development of *isoA* primers to
318 effectively target this enzyme with high specificity. While we cannot exclude the
319 possibility that some sequences may be missed, or that other isoprene-degrading

320 enzymes or pathways exist, these primers constitute an effective tool to identify
321 isoprene-related gene sequences in environmental samples. DNA-SIP, to our
322 knowledge the first time this technique has been used to identify isoprene
323 assimilators, showed that the major isoprene utilisers in the microcosms were
324 *Rhodococcus* strains and that members of the *Comamonadaceae* were also active
325 in isoprene degradation. The fact that we did not obtain isolates from this family
326 suggests that they may be resistant to cultivation under our laboratory conditions,
327 emphasizing the importance of cultivation-independent techniques. This study
328 confirms that soils readily consume isoprene and harbor a diverse community of
329 isoprene degraders. Investigation of their diversity, abundance and mechanisms of
330 isoprene degradation is essential to assess the environmental relevance of the
331 global biological isoprene sink and the extent to which biodegradation moderates the
332 effect on the atmosphere of this abundant and climate-active trace gas. This study
333 provides the tools and foundations to further investigate these topics. Future
334 experiments should search for novel genes and pathways involved in isoprene
335 degradation, perhaps using SIP coupled with metagenomics. The isoprene-
336 degrading community in the phyllosphere is also worthy of investigation, since our
337 isolation of a strain from this environment, to our knowledge the first published
338 example, suggests that isoprene degraders may be present or abundant on leaves,
339 close to the major source of isoprene to the atmosphere.

340 **Experimental procedures**

341 *Cultivation of bacterial strains*

342 The terrestrial isoprene-degrading strains were routinely grown in minimal medium
343 (CBS medium) which contained (per 1 litre): 0.1 g $MgSO_4.7H_2O$, 0.8 g NH_4Cl, 1.5 g

344 KH_2PO_4, 6.3 g Na_2HPO_4, and 10 ml of trace element solution (Tuovinen and Kelly,
345 1973) (pH 7.0). Marine isoprene degrading-bacteria were grown in mineral salts
346 medium prepared according to Schaefer et al., (2002), except containing 20 g l^{-1}
347 NaCl and supplemented with Na_3VO_4 and Na_2SeO_3 (5 ng L^{-1} each). Cultures were
348 set up in serum vials (120 ml) sealed with grey butyl rubber seals or in Quickfit flasks
349 (250 ml or 2 L) fitted with SubaSeal stoppers (Sigma-Aldrich) and isoprene was
350 added (as gas) to a final concentration of 0.6% - 1% (v/v) by injection through the
351 septum. The cultures were incubated at 30 °C, shaking at 150 rpm.
352 For growth tests on other gaseous substrates, 25 ml of CBS medium in serum vials
353 (120 ml) was inoculated with isoprene-grown culture (5% inoculum) and incubated
354 with 10% (v/v) substrate. Cultures with succinate, glucose, fructose or acetate (10
355 mM) as growth substrates were prepared in universal bottles (20 ml) containing 5 ml
356 CBS medium inoculated with 5% of isoprene-grown culture. Isoprene (catalogue no.
357 I19551) was obtained from Sigma Aldrich.
358

359 *Isolation of isoprene-degrading strains*

360 Isoprene enrichment cultures were set up using garden soil from Leamington Spa
361 (UK) or leaves of a Horse Chestnut tree from the campus of the University of
362 Warwick (Coventry, UK). Isoprene (0.6 % v/v) was added to 50 ml CBS minimal
363 medium in flasks (250 ml) and inoculated with either 0.3 g soil or one leaf, cut into
364 small pieces. The optical density of enrichment cultures was followed
365 spectrophotometrically at 540 nm and isoprene uptake was monitored with a gas
366 chromatograph fitted with a flame ionization detector (GC-FID) as described
367 previously (Crombie et al., 2015). Enrichment cultures were streaked on CBS agar

368 plates and incubated at 30 °C in a desiccator with isoprene vapour (approximately
369 5% v/v). Colonies were subcultured until pure, confirmed by phase contrast
370 microscopy (Zeiss Axioscop, UK). Marine strains for primer design and validation
371 were isolated as described previously (Acuña Alvarez et al., 2009; Johnston, 2014).
372

373 *DNA extraction, amplification and sequencing of 16S rRNA genes*

374 DNA was extracted from isolates and enrichment cultures using the FastDNA Spin
375 Kit for Soil (MP Biomedicals), following the manufacturer's instructions. For
376 identification of strains, 16S rRNA genes were amplified using 27f / 1492R primers
377 (Lane, 1991). Amplicons were purified, cloned into pGEMT-easy vector (Promega),
378 and sequenced with M13 primers (Invitrogen).

379 *Genome sequencing, annotation and mining*

380 High molecular-mass genomic DNA was extracted from 500 ml mid-late exponential
381 cultures of isoprene-degrading strains following the Marmur extraction method
382 (Marmur, 1961) except that the sodium dodecyl sulfate (SDS) concentration was
383 increased to 2% (w/v) and the incubation period at 55 °C in
384 sucrose/ethylenediaminetetraacetic acid/Tris (SET) buffer and SDS extended to 5
385 hours to achieve better cell lysis.

386 The genome of *Gordonia* i37 was sequenced at Oregon State University (USA)
387 using a Roche 454 pyrosequencing platform. The genomes of *Mycobacterium* AT1,
388 *Rhodococcus* SC4 and *Rhodococcus* LB1 were sequenced using Illumina GAIIx at
389 the University of Warwick Genomics Facility (Coventry, UK). Reads were assembled
390 into contigs using CLC Genomics Workbench for de novo assembly (CLC bio,

391 Aarhus, Denmark). The genome sequences were uploaded to RAST (Rapid
392 Annotation using Subsystem Technology) for annotation. Local nucleotide databases
393 were constructed using NCBI BLAST in BioEdit. The database was mined using
394 tBLASTn with the amino acid sequences of genes shown to be important in isoprene
395 metabolism in *Rhodococcus* sp. AD45 as query sequences. These Whole Genome
396 Shotgun projects have been deposited at DDBJ/EMBL/GenBank under the
397 accession numbers LTCZ00000000 (SC4) and LSBM00000000 (LB1). Versions
398 described in this paper are LTCZ01000000 and LSBM01000000. Cloned *isoA*
399 sequences and *isoA* gene sequences of *Mycobacterium* AT1 and *Gordonia* i37 have
400 been deposited under accession numbers KU870702 – KU870744 and KU870745
401 and KU870746, respectively.
402

403 isoA *Primer design and PCR*

404 Deduced amino acid sequences of *isoA* and other related enzymes were aligned
405 using ClustalW in Mega6 (Tamura et al., 2013). The alignment was visually
406 inspected for conserved regions specific to isoprene degraders, which were not
407 conserved among homologous sequences from related SDIMO enzymes. Primers
408 for *isoA* amplification, containing a maximum of three degenerate bases, were
409 designed based on these regions (5´-TGCATGGTCGARCAYATG-3´ and 5´-
410 GRTCYTGYTCGAAGCACCACTT-3´), yielding a predicted amplicon of 1015 bp
411 (*Rhodococcus* sp. AD45 template). These primers were used with a touchdown PCR
412 protocol: an initial step at 94 °C for 3 min was followed by 19 cycles of denaturation
413 at 94 °C for 30 seconds, annealing at variable temperatures for 45 seconds, and
414 extension at 72 °C for 60 seconds. The annealing temperature, initially 72 °C, was

415 decreased by 1 °C per cycle until 54 °C and maintained at this temperature for a
416 further 25 cycles, followed by a final extension at 72 °C for 5 min.

417

418 *Clone libraries*

419 Clone libraries were constructed from *isoA* amplicons using DNA extracted from
420 isoprene enrichments of soil, sediment or water. The purified *isoA* amplicons were
421 cloned into pGEMT Easy vector (Promega) prior to transformation into *E. coli* TOP10
422 cells (Invitrogen) following the manufacturers' instructions. Clones were screened by
423 PCR using M13 primers and restriction fragment length polymorphism (RFLP) using
424 *Eco*RI and *Msp*I restriction enzymes (Fermentas). Clones yielding identical
425 restriction fragment profiles on agarose gels were designated operational taxonomic
426 units (OTUs) and representatives of each OTU were sequenced using M13 primers.

427

428 *Search for iso* genes in the *Comamonadaceae*

429 Tblastn (Altschul et al., 1990) was used to search the NCBI nr and genome
430 databases and 153 whole genome shotgun projects deposited in NCBI, for isoprene-
431 degrading gene sequences among the Comamonadaceae. Using IsoA from
432 *Rhodococcus* sp. AD45 as query, genes with 46 – 48% amino acid identity were
433 identified in the genomes of *Hydrogenophaga* sp. T4, *Comamonas badia* DSM
434 17552 and *Variovorax paradoxus* ZNC0006. Genes encoding the other subunits of
435 the monooxygenase were identified in the *Hydrogenophaga* and *Comamonas*
436 genomes, but no identifiable additional isoprene metabolic genes were found nearby,
437 and no hits to IsoI were found in any of the genomes in any location (evalue 0.0001).

438 The *isoA* homologues were more similar to characterized toluene monooxygenase
439 genes, such as *touA* from *Pseudomonas stutzeri* OX1 (67 – 73% amino acid identity)
440 (Bertoni et al., 1998), suggesting that isoprene may not be their natural substrate.

441 *Biosynthesis of [1,2,3,4,5-^{13}C]-2-methyl-1,3-butadiene ([U-^{13}C]-isoprene) from D-[U-*
442 *^{13}C]-glucose*

443 Uniformly labelled ^{13}C-isoprene was prepared utilizing an isoprene-producing
444 engineered variant of *E. coli* BL21, expressing a functional mevalonate pathway and
445 an isoprene synthase, as biocatalyst. Specifically, the genes encoding thiolase,
446 mevalonate synthase, and 3-hydroxy-3-methyl-glutaryl- (HMG-) CoA reductase
447 (*mvaE, mvaS*) were derived from *Enterococcus faecalis*, the gene encoding
448 mevalonate kinase was derived from *Methanosarcina mazei*, and the genes
449 encoding phosphomevalonate kinase, mevalonate pyrophosphate decarboxylase
450 and isopentyl-PP isomerase were from *Saccharomyces cerevisiae*. This recombinant
451 pathway serves to channel carbon from glucose to the isoprene precursor
452 dimethylallyl pyrophosphate (DMAPP). The final intracellular enzymatic step to
453 convert DMAPP to isoprene was catalyzed by recombinant isoprene synthase from
454 *Populus alba*. This strain was designated *E. coli* CMP1082 and has been described
455 in detail elsewhere (McAuliffe et al., 2015). Details of fermentation and isoprene
456 recovery and its ^{1}H NMR spectrum are included in Supporting Information.

457

458 *DNA stable isotope probing (SIP) experiments*

459 For SIP incubations, 5 g soil (pH 7.4), collected from the upper 5 cm (after removal
460 of vegetation and leaf litter), in the vicinity of Willow (*Salix fragilis*) trees on the

461 University of East Anglia campus, was incubated in serum vials (120 ml volume) with
462 0.5% (v/v) isoprene in triplicate (labelled substrate) or duplicate (unlabelled
463 substrate). Vials were incubated at room temperature (22 °C) in the dark. Headspace
464 isoprene concentration was monitored by GC-FID. When substrate was depleted,
465 additional isoprene was injected through the septum to the original concentration.
466 Incubations were terminated and DNA extracted from the soil when 10 μmol g^{-1} had
467 been consumed. Control incubations with autoclaved soil were also conducted. DNA
468 extracted from samples was separated into heavy (^{13}C-labelled) and light
469 (unlabelled) fractions as previously described (Neufeld et al., 2007). Briefly, 1-2 μg
470 DNA was added to caesium chloride solution (final density of 1.725 g ml^{-1}) and
471 subjected to density gradient ultracentrifugation (177,000 x *g*, 40 h, 20 °C, Beckman
472 Vti 65.2 rotor). The contents of each tube was separated into 12 – 15 fractions and
473 the density of each fraction measured by refractometry (AR200 digital refractometer,
474 Reichert Inc., Buffalo, USA). DNA from each fraction was precipitated, resuspended
475 in nuclease-free water and characterized by 16S rRNA gene analysis using
476 denaturing gradient gel electrophoresis (DGGE). DGGE showed unique profiles in
477 heavy, compared to light, fractions of ^{13}C-incubations while the ^{12}C incubations
478 exhibited similar profiles between fractions, indicating that heavy fractions from ^{13}C
479 incubations contained the DNA of isoprene degraders (data not shown). Based on
480 fraction density and DGGE profiles, fractions 7 and 11 were identified as containing
481 labelled and unlabelled DNA, respectively, designated “heavy” and “light” and used
482 for downstream analysis. DNA was obtained from six different treatments: extracted
483 from timepoint zero (one sample); extracted following ^{13}C-isoprene incubation but
484 prior to fractionation (unfractionated); and after separation into heavy and light
485 fractions for both ^{13}C- and ^{12}C-isoprene incubations. For ^{13}C heavy fractions triplicate

486 samples were analysed individually, but for the other treatments the DNA was
487 pooled prior to analysis.

488

489 *Denaturing gradient gel electrophoresis (DGGE)*

490 Bacterial 16S rRNA genes were amplified using primers 341F-GC (Muyzer et al.,
491 1993) and 907R (Muyzer et al., 1998). The PCR products were run on an 8% (w/v)
492 polyacrylamide gel with a 30% - 70% linear denaturant gradient. Electrophoresis was
493 carried out for 16 h, at 80 V, 60 °C using the DCode^{TM} Universal Mutation Detection
494 System (Bio-Rad), stained with SYBR® Gold Nucleic Acid Gel Stain (Invitrogen) and
495 imaged using a BioRad GelDoc system.

496

497 *Bacterial 16S rRNA gene analysis by 454 pyrosequencing*

498 Labelled (heavy) and unlabelled (light) DNA from SIP incubations was characterized
499 by sequencing of 16S rRNA gene amplicons generated by PCR using the primers
500 27Fmod (5′-AGRGTTTGATCMTGGCTCAG-3′) and 519Rmodbio (5′-
501 GTNTTACNGCGGCKGCTG-3′) using a Roche 454 FLX titanium instrument at MR
502 DNA (Molecular Research LP), Shallowater, USA. Sequence data were processed at
503 MR DNA using a published pipeline (Dowd et al., 2008; Capone et al., 2011). Briefly,
504 the Q25 reads were stripped of barcodes and primers. Short sequences (<200 bp),
505 sequences with ambiguous base calls and those with >6 bp homopolymer runs were
506 removed. Remaining sequences were denoised using a custom pipeline, OTUs
507 clustered at 97% sequence identity, chimeric sequences were removed using
508 Uchime (Edgar et al., 2011) and taxonomy was assigned using BLASTn against the

509 RDPII/NCBI database (v 11.1) (Cole et al., 2014). An average of 3,320 sequences
510 per sample were used for analysis.
511
512 Statistical Analysis
513 The Bray-Curtis distance measure was applied to log-transformed relative
514 abundance and used to generate non-metric multidimensional scaling (NMDS) plots
515 using Primer 6 (Primer-E, Plymouth, UK).
516
517
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685

686 **Figure Legends**

687 **Figure 1**. Isoprene metabolism in *Rhodococcus* sp. AD45. Enzymes: IsoABCDEF,
688 isoprene monooxygenase; IsoI, glutathione-S-transferase; IsoH, dehydrogenase.
689 HGMB, 1-hydroxy-2-glutathionyl-2-methyl-3-butene; GMB, 2-glutathionyl-2-methyl-3-
690 butenal; GMBA, 2-glutathionyl-2-methyl-3-butenoic acid; SG, glutathione; GSH,
691 reduced glutathione.

692

693 **Figure 2**. The isoprene metabolic gene cluster from *Rhodococcus* sp. AD45
694 (Crombie et al., 2015), together with similar regions from *R. opacus* PD630 and
695 strains SC4 and LB1. The contigs containing the genes are identified by horizontal
696 lines and numbers below. The monooxygenase genes are shown in red, and other
697 colours indicate genes of the corresponding predicted function between strains.
698 Locus tags and gene names are indicated with angled text. Locus tag prefixes: *R.*

699 sp. AD45, SZ00_; *R*. opacus PD630, Pd630_LPD; *R*. sp. SC4, AXA44_; *R*. sp. LB1,
700 AZG88_.

701

702

703 **Figure 3**. Phylogenetic tree of *isoA* genes from isolates and environmental clones,
704 constructed using the Maximum Likelihood method in MEGA6 (Tamura et al., 2013).
705 All positions containing gaps and missing data were eliminated and there were 1011
706 nucleotide positions in the final dataset. The scale bar shows nucleotide substitutions
707 per site. Bootstrap values (500 replications) greater than 50% are shown at the
708 nodes. Isoprene-degrading isolates are shown in bold. Cloned *isoA* sequences
709 (Tables S4 and S5) are labelled with the sample site followed by clone identification
710 number. WCO_L4, Western Channel Observatory station L4; FW, freshwater.

711

712

713 **Figure 4**. Soil bacterial community (based on 16S rRNA gene amplicons) of labelled
714 isoprene DNA-SIP microcosms. The pie charts show (left) the unenriched soil
715 community from timepoint zero and (right) the ^{13}C- isoprene-enriched total
716 community (prior to isopycnic centrifugation and DNA fractionation). For the right
717 hand chart, DNA from triplicate enrichments was pooled prior to analysis.

718

719

720 **Figure 5**. Bacterial communities derived from labelled isoprene DNA-SIP
721 enrichments and fractionation. The bar chart shows 16S rRNA gene-based
722 composition of the unenriched soil community (timepoint zero), the isoprene-
723 enriched total community (unfractionated) and labelled (heavy) and unlabelled (light)

724 components separated by isopycnic centrifugation and fractionation. The symbols on
725 the x-axis correspond with those shown in Fig. S2. The isoprene-assimilating
726 community is represented by the heavy fractions of the three replicate ^{13}C
727 incubations (solid red diamonds). T-0, timepoint zero; U-F, unfractionated; H, heavy
728 fraction; L, light fraction. The inset (b) shows the species composition of the
729 rhodococci in the heavy fractions (mean of three replicates)

730