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

Myriam El Khawand,1 Andrew T. Crombie,¹ Antonia Johnston,¹ Dmitrii V. Vavlline,² Joseph C. McAuliffe,² Jacob A. Latone,² Yuliya A. Primak,² Sang-Kyu Lee,² Gregg M. Whited,² Terry J. McGenity3 and J. Colin Murrell1

¹University of East Anglia, Norwich Research Park, Norwich, UK; ²DuPont Industrial Biosciences, 925 Page Mill Road, Palo Alto, CA 94304, USA; ³University of Essex, Wivenhoe Park, Colchester, UK.

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Corresponding author:
Andrew T. Crombie
School of Environmental Science, University of East Anglia, Norwich Research Park, Norwich NR4 7TJ, UK. Email: a.crombie@uea.ac.uk Telephone +44 (0)1603 592239

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

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

About 600 Tg y⁻¹ isoprene is emitted to the atmosphere by terrestrial plants, although not all plant species produce isoprene (Sharkey, 2013; Loreto and Fineschi, 2015). Isoprene synthesis occurs in the chloroplast, via the enzyme isoprene synthase, which converts dimethylallyl pyrophosphate to isoprene (Logan et al., 2000). Isoprene protects plants against heat stress by reducing heat-induced cell-membrane damage, enhances tolerance of reactive oxygen species and may affect plant-insect interactions (Loivamäki et al., 2008; Sharkey et al., 2008; Vickers et al., 2009; Sharkey, 2013). In the marine environment isoprene is released by phytoplankton and macroalgae (Broadgate et al., 2004; Exton et al., 2015). Some bacteria, including soil-dwelling species such as Bacillus subtilis, release isoprene, as do some fungi (Kuzma et al., 1995; Julsing et al., 2007; Bäck et al., 2010),
although we lack a clear understanding of why these organisms produce isoprene. A non-enzymatic reaction resulting in isoprene was reported in humans and other animals, associated with the mevalonate pathway of cholesterol synthesis (Gelmont et al., 1981).

Although atmospheric isoprene concentrations are low (<1 – 4 ppbv in one wide-ranging study (Greenberg et al., 1999)), in the vicinity of isoprene sources (around or below tree canopy level) concentrations are significantly higher. For example, Wiedinmyer and colleagues measured ground level isoprene concentrations of 11 and 36 ppbv at sites in Texas and Missouri, respectively (Wiedinmyer et al., 2001; Wiedinmyer et al., 2005). Soils can act as a biological sink for isoprene, at or below these concentrations. In field chambers set up in temperate forest soils, isoprene was rapidly depleted to below the 5 ppbv limit of detection (Cleveland and Yavitt, 1997; Cleveland and Yavitt, 1998). In continuous flow experiments conducted by Gray et al. (2015), soils supplied with isoprene at concentrations of 2 – 200 ppbv consumed isoprene at all concentrations, with a rate of 62 pmol g⁻¹ h⁻¹ at 20 ppbv. These data demonstrate both the potential of soils to consume isoprene released locally in soils and also to take up atmospheric isoprene, conclusions also reached in mesocosm experiments by Pegoraro et al. (2005). Several bacterial strains, tentatively assigned to the genera *Nocardia, Rhodococcus* (Actinobacteria) and *Alcaligenes* (Betaproteobacteria), were isolated from isoprene enrichment cultures and shown to grow on isoprene as sole growth substrate, (van Ginkel et al., 1987b; van Ginkel et al., 1987a; Ewers et al., 1990; Cleveland and Yavitt, 1997) and, more recently, *Pseudomonas, Alcaligenes* and *Klebsiella* isoprene-degrading strains were isolated from rubber-contaminated soil (Srivastva et al., 2015). Strains were also obtained from the marine environment, including representatives of Actinobacteria,
Bacteroidetes and Alpha- and Gammaproteobacteria (Acuña Alvarez et al., 2009).

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

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

DNA stable isotope probing (DNA-SIP) is a cultivation-independent technique with the ability to identify active substrate-consuming organisms in environmental
samples (Radajewski et al., 2000; Dumont and Murrell, 2005). The method relies on incubation of samples with stable-isotope-labelled growth substrate. The incorporation of isotope (typically $^{13}$C or $^{15}$N) into biomass (including DNA), enables the identification of active microorganisms following separation of labelled and unlabelled DNA by isopycnic (density gradient) centrifugation.

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

Results and Discussion

Enrichment and isolation of two novel terrestrial isoprene-utilizing bacteria

Our initial aim was to isolate isoprene degraders from contrasting environments in order to provide sequence data for later cultivation-independent approaches. Since
isoprene consumption has previously been observed in soils and since isoprene is emitted from tree leaves, we used these as source material. Separate enrichments were set up, using either garden soil or Horse Chestnut (*Aesculus hippocastanum*) leaves, in minimal medium and incubated with isoprene. Both enrichments consumed isoprene and two isolates (designated SC4 and LB1), capable of growing on isoprene as sole source of carbon and energy, were obtained from soil and leaf samples, respectively. The nearly complete (1521 nucleotides) 16S rRNA gene sequences of both strains SC4 and LB1 were identical to strains of both *Rhodococcus opacus* and *R. wratislaviensis* (Fig. S1). Strains SC4 and LB1 also grew on acetate, succinate, glucose, fructose, propane and butane as sole source of carbon and energy (Table S1), in contrast to *Rhodococcus* sp. AD45, which does not grow on propane or butane (Crombie et al., 2015).

Genome sequencing

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

Identification of isoprene related genes in isolates

Our previous work (Crombie *et al.*, 2015) detected a cluster of 22 genes induced by isoprene, which appeared to be the complete inventory of isoprene-responsive
genes. In addition to isoABCDEF, encoding the monooxygenase, and isoGHIJ that encode a protein of unknown function, a dehydrogenase and two glutathione-S-transferases previously described (van Hylckama Vlieg et al., 2000), the cluster includes glutathione biosynthesis genes, predicted aldehyde dehydrogenases and a coenzyme-A disulfide reductase (Crombie et al., 2015). We therefore searched for homologous sequences in the genomes of strains SC4 and LB1, which are highly similar to each other in this region (over 99% nucleotide identity). Using the isoprene-responsive gene products previously identified in Rhodococcus sp. AD45 as query sequences in tBLASTn searches, we identified homologues of all of these 22 genes, with amino acid sequence identity ranging from 50–96% (Table S3). The most highly conserved were isoABCDEF encoding the multi-component IsoMO (81–96%), whereas a predicted protein of unknown function (SZ00_06083), highly induced by isoprene in Rhodococcus sp. AD45, shared 50% amino acid identity with sequences from these strains. We observed the same duplication of isoGHIJ (Fig. 2, 77 – 88% amino acid identity between copies) as is present in Rhodococcus sp. AD45. Between isoA and isoJ, both of the new strains contain a second copy (81% amino acid identity between copies), not present in Rhodococcus sp. AD45, of an aldehyde dehydrogenase (aldh1) which is located approximately 10,000 nucleotides (nt) upstream of the monooxygenase in Rhodococcus sp. AD45. A gene encoding a predicted coenzyme-A disulfide reductase is also present in two copies (69% amino acid identity between copies), although in strain LB1 (but not in strain SC4) one copy has a nucleotide insertion approximately 255 nt from the end, resulting in a frameshift mutation, suggesting this may not encode a functional protein. In comparison with Rhodococcus sp. AD45, five additional genes are present, in both strains, in the middle of the cluster. These are predicted to encode two hypothetical
proteins and an alpha/beta hydrolase domain-containing protein of unknown
function, an acetyl-CoA acetyltransferase, and a 3-hydroxyacyl-CoA dehydrogenase.
Interestingly, in strain LB1, another insertion in the acetyl-CoA acetyltransferase has
resulted in a frameshift mutation. None of these five genes are present in this region
of the *Rhodococcus* sp. AD45 genome, nor were remotely-located homologous
sequences induced by isoprene (Crombie et al., 2015), implying that they are not
essential for isoprene metabolism. In comparison with *Rhodococcus* sp. AD45,
strains LB1 and SC4 are more similar in this region of the genome, in terms of both
sequence identity and gene layout, with *R. opacus* PD630 (also included in Fig. 2).
Previously, using sequence data, we predicted, and confirmed, that *R. opacus*
PD630 could grow on isoprene (Crombie et al., 2015), although, beyond this, we
have no direct experimental data regarding isoprene-related gene function in this
strain.

*Development and validation of functional gene markers targeting isoA*

Using the sequence data from these isoprene-degrading strains, we designed
probes to detect isoprene-related genes in environmental samples. *isoA* codes for
the alpha-subunit of the hydroxylase of IsoMO and contains the diiron centre active
site and was shown to be essential for isoprene degradation in *Rhodococcus* sp.
AD45 (Crombie *et al.*, 2015). We therefore selected *isoA* as target for a functional
gene probe. To expand the diversity of *isoA* sequences, we also retrieved *isoA*
genes from draft sequences of a *Gordonia* strain and a *Mycobacterium* strain
previously isolated from surface sediment from the Colne estuary (Essex, UK),
identified as *Gordonia* i37 and *Mycobacterium* AT1 (Acuña Alvarez *et al.*, 2009;
The isoA sequences of strains SC4, LB1, i37 and AT1, together with those of *Rhodococcus* sp. AD45 (SZ00_06091) and *R. opacus* PD630 (Pd630_LPD03572), were aligned at the amino acid level, and conserved regions were used to design primers targeting isoA. To exclude other non-isoprene-degrading members of the SDIMO family, sequences of *mmoX* and *xamoA*, encoding the alpha subunits of sMMO from *Methylosinus trichosporium* OB3b (Cardy et al., 1991), and alkene monooxygenase from *Xanthobacter autotrophicus* Py2 (Zhou et al., 1996), respectively, were also included in the alignment. The isoA primers were validated by PCR amplification of template DNA from contrasting sources (Table S4): (i) genomic DNA from 15 isoprene-degrading isolates, (ii) DNA extracted from three isoprene-enriched soils and four isoprene enrichments of marine and estuarine water and sediment, and (iii) control DNA extracted from eight non-isoprene-degrading isolates able to grow on alkanes, alkenes or aromatic compounds. We obtained PCR products of the expected size using DNA extracted from all the isoprene-degrading isolates and enrichments, but not from any of the non-isoprene degraders. PCR products from enrichments were cloned and analysed by restriction fragment length polymorphism (RFLP) (Table S5). Representatives of each operational taxonomic unit (OTU) were sequenced, (all of which appeared to be isoA sequences) and aligned at the amino acid level with IsoA sequences obtained from the sequenced genomes. A phylogenetic tree of the isoA nucleotide sequences (1011 nt) was constructed from the alignment (Fig. 3). All the sequences, although from diverse phylogenetic groups including both Gram-positive and Gram-negative strains, were relatively similar (> 86% amino acid identity between sequences), but could be broadly separated into two groups in which the terrestrial sequences and those from the low-salinity environment of Hythe, on the
Colne estuary, were distinct from marine and other estuarine sequences similar to IsoA of *Gordonia* i37.

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

Since all four of our sequenced terrestrial isolates were rhodococci and all isoA sequences retrieved were relatively similar, we used cultivation-independent methods to test whether a greater diversity of isoprene degraders existed in soils in a DNA-stable isotope experiment (DNA-SIP) using $^{13}$C-labelled isoprene, biosynthesized as described in Experimental Procedures. Soil microcosms (in triplicate for labelled substrate and duplicate for unlabelled-substrate controls) were incubated with 0.5% (v/v) isoprene, without any other amendments. Isoprene was consumed without an appreciable lag phase, and incubations were terminated after consumption of 10 μmol substrate g$^{-1}$ soil (15 days). DNA was extracted from $^{13}$C- and control $^{12}$C-isoprene incubations and separated into heavy and light fractions and used for 454 pyrosequencing of 16S rRNA genes. The unenriched soil at timepoint zero displayed a typically diverse community, comprising 50% Proteobacteria, with Planctomycetes, Actinobacteria, Bacteroidetes, Chloroflexi and Firmicutes contributing an additional 40% (Fig. 4). The major effect of the incubations was to greatly increase the relative abundance of Actinobacteria, while having a relatively minor effect on the remaining phyla (Fig. 4). Multivariate analysis (Fig. S2) shows that the unlabelled bacterial community of the $^{13}$C-isoprene incubations (i.e. light DNA fraction) was extremely similar to that of the timepoint zero community, suggesting that the change in community profile during the incubations was due to an enrichment of isoprene degraders. As expected, the community represented by the light fraction of the $^{12}$C-isoprene incubations, derived
from both isoprene-consumers and non-consumers, was extremely similar to the
total (unfractionated) DNA from $^{13}$C-incubations. The heavy fraction of $^{12}$C-isoprene
incubations, which contained only a very small fraction of the total DNA of $^{12}$C-
incubations, was less similar. This DNA results mainly from incomplete separation,
with minor effects due to factors such as GC content of DNA. In contrast to all of
these, the active isoprene degraders represented by the heavy fractions of the $^{13}$C
incubations formed a distinct community (Fig. S2) and displayed a dramatically
altered taxonomic profile (Fig. 5), becoming dominated by *Rhodococcus* sequences,
which comprised 88 ± 5% (mean ± s.d.) of the heavy fraction of $^{13}$C incubations.
These sequences were predominantly those of *Rhodococcus wratislaviensis*, *R.
koreensis* and *R. globerulus* (Fig. 5) and were 147- to 161-fold more abundant in the
heavy fractions compared to light fractions of $^{13}$C incubations, but without any
corresponding enrichment in the heavy fraction of $^{12}$C-incubations (Fig. S3). Also
enriched in the heavy fractions of $^{13}$C-incubations were members of the
Betaproteobacteria, *Comamonas* spp. and *Variovorax* spp., which together
comprised 6.5 ± 1.2% of the $^{13}$C heavy fractions (but were not detected in the $^{13}$C
light fractions), and were also enriched during the incubations, increasing from 0.3%
to 0.7% of the total community. These data clearly demonstrate that *Rhodococcus*
and, to a lesser extent, *Comamonas* and *Variovorax* spp. had assimilated carbon,
directly or indirectly, from labelled isoprene. Searches of the publicly available
databases did not identify high-similarity putative isoprene-related genes in the
family Comamonadaceae (which encompasses both *Comamonas* and *Variovorax*)
(see Experimental Procedures). Comparison with the PCR-based *isoA* analysis,
which generated similar sequences from diverse phylogenetic groups, suggests that
the isoprene-degrading members of the Comamonadaceae implicated in the SIP
experiment are not represented in the NCBI databases, or that they use genes or possibly pathways dissimilar to those of the characterized strains.

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

Conclusions

The isoprene concentrations used here are considerably in excess of those normally encountered in the environment and the aim was not to replicate environmental conditions, but rather to expand the diversity of known isoprene degraders. Draft genome sequences showed that isoprene monooxygenase was present in all our isoprene-degrading isolates, which enabled the development of *isoA* primers to effectively target this enzyme with high specificity. While we cannot exclude the possibility that some sequences may be missed, or that other isoprene-degrading
enzymes or pathways exist, these primers constitute an effective tool to identify isoprene-related gene sequences in environmental samples. DNA-SIP, to our knowledge the first time this technique has been used to identify isoprene assimilators, showed that the major isoprene utilisers in the microcosms were *Rhodococcus* strains and that members of the *Comamonadaceae* were also active in isoprene degradation. The fact that we did not obtain isolates from this family suggests that they may be resistant to cultivation under our laboratory conditions, emphasizing the importance of cultivation-independent techniques. This study confirms that soils readily consume isoprene and harbor a diverse community of isoprene degraders. Investigation of their diversity, abundance and mechanisms of isoprene degradation is essential to assess the environmental relevance of the global biological isoprene sink and the extent to which biodegradation moderates the effect on the atmosphere of this abundant and climate-active trace gas. This study provides the tools and foundations to further investigate these topics. Future experiments should search for novel genes and pathways involved in isoprene degradation, perhaps using SIP coupled with metagenomics. The isoprene-degrading community in the phyllosphere is also worthy of investigation, since our isolation of a strain from this environment, to our knowledge the first published example, suggests that isoprene degraders may be present or abundant on leaves, close to the major source of isoprene to the atmosphere.

**Experimental procedures**

*Cultivation of bacterial strains*

The terrestrial isoprene-degrading strains were routinely grown in minimal medium (CBS medium) which contained (per 1 litre): 0.1 g MgSO$_4$.7H$_2$O, 0.8 g NH$_4$Cl, 1.5 g
KH$_2$PO$_4$, 6.3 g Na$_2$HPO$_4$, and 10 ml of trace element solution (Tuovinen and Kelly, 1973) (pH 7.0). Marine isoprene degrading-bacteria were grown in mineral salts medium prepared according to Schaefer et al., (2002), except containing 20 g l$^{-1}$ NaCl and supplemented with Na$_3$VO$_4$ and Na$_2$SeO$_3$ (5 ng L$^{-1}$ each). Cultures were set up in serum vials (120 ml) sealed with grey butyl rubber seals or in Quickfit flasks (250 ml or 2 L) fitted with SubaSeal stoppers (Sigma-Aldrich) and isoprene was added (as gas) to a final concentration of 0.6% - 1% (v/v) by injection through the septum. The cultures were incubated at 30 °C, shaking at 150 rpm.

For growth tests on other gaseous substrates, 25 ml of CBS medium in serum vials (120 ml) was inoculated with isoprene-grown culture (5% inoculum) and incubated with 10% (v/v) substrate. Cultures with succinate, glucose, fructose or acetate (10 mM) as growth substrates were prepared in universal bottles (20 ml) containing 5 ml CBS medium inoculated with 5% of isoprene-grown culture. Isoprene (catalogue no. I19551) was obtained from Sigma Aldrich.

Isolation of isoprene-degrading strains

Isoprene enrichment cultures were set up using garden soil from Leamington Spa (UK) or leaves of a Horse Chestnut tree from the campus of the University of Warwick (Coventry, UK). Isoprene (0.6 % v/v) was added to 50 ml CBS minimal medium in flasks (250 ml) and inoculated with either 0.3 g soil or one leaf, cut into small pieces. The optical density of enrichment cultures was followed spectrophotometrically at 540 nm and isoprene uptake was monitored with a gas chromatograph fitted with a flame ionization detector (GC-FID) as described previously (Crombie et al., 2015). Enrichment cultures were streaked on CBS agar
plates and incubated at 30 °C in a desiccator with isoprene vapour (approximately 5% v/v). Colonies were subcultured until pure, confirmed by phase contrast microscopy (Zeiss Axioskop, UK). Marine strains for primer design and validation were isolated as described previously (Acuña Alvarez et al., 2009; Johnston, 2014).

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

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

**Genome sequencing, annotation and mining**

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

The genome of *Gordonia* i37 was sequenced at Oregon State University (USA) using a Roche 454 pyrosequencing platform. The genomes of *Mycobacterium* AT1, *Rhodococcus* SC4 and *Rhodococcus* LB1 were sequenced using Illumina GAIIx at the University of Warwick Genomics Facility (Coventry, UK). Reads were assembled into contigs using CLC Genomics Workbench for de novo assembly (CLC bio,
Aarhus, Denmark). The genome sequences were uploaded to RAST (Rapid Annotation using Subsystem Technology) for annotation. Local nucleotide databases were constructed using NCBI BLAST in BioEdit. The database was mined using tBLASTn with the amino acid sequences of genes shown to be important in isoprene metabolism in *Rhodococcus* sp. AD45 as query sequences. These Whole Genome Shotgun projects have been deposited at DDBJ/EMBL/GenBank under the accession numbers LTCZ00000000 (SC4) and LSBM00000000 (LB1). Versions described in this paper are LTCZ01000000 and LSBM01000000. Cloned *isoA* sequences and *isoA* gene sequences of *Mycobacterium* AT1 and *Gordonia* i37 have been deposited under accession numbers KU870702 – KU870744 and KU870745 and KU870746, respectively.

**isoA Primer design and PCR**

Deduced amino acid sequences of *isoA* and other related enzymes were aligned using ClustalW in Mega6 (Tamura et al., 2013). The alignment was visually inspected for conserved regions specific to isoprene degraders, which were not conserved among homologous sequences from related SDIMO enzymes. Primers for *isoA* amplification, containing a maximum of three degenerate bases, were designed based on these regions (5′-TGCATGGTGARCAYATG-3′ and 5′-GRTCYTGYTCGAAGCACCACTT-3′), yielding a predicted amplicon of 1015 bp (*Rhodococcus* sp. AD45 template). These primers were used with a touchdown PCR protocol: an initial step at 94 ºC for 3 min was followed by 19 cycles of denaturation at 94 ºC for 30 seconds, annealing at variable temperatures for 45 seconds, and extension at 72 ºC for 60 seconds. The annealing temperature, initially 72 ºC, was
decreased by 1 °C per cycle until 54 °C and maintained at this temperature for a further 25 cycles, followed by a final extension at 72 °C for 5 min.

Clone libraries

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

Search for iso genes in the Comamonadaceae

Tblastn (Altschul et al., 1990) was used to search the NCBI nr and genome databases and 153 whole genome shotgun projects deposited in NCBI, for isoprene-degrading gene sequences among the Comamonadaceae. Using IsoA from Rhodococcus sp. AD45 as query, genes with 46 – 48% amino acid identity were identified in the genomes of Hydrogenophaga sp. T4, Comamonas badia DSM 17552 and Variovorax paradoxus ZNC0006. Genes encoding the other subunits of the monooxygenase were identified in the Hydrogenophaga and Comamonas genomes, but no identifiable additional isoprene metabolic genes were found nearby, and no hits to IsoI were found in any of the genomes in any location (evalue 0.0001).
The isoA homologues were more similar to characterized toluene monooxygenase genes, such as touA from *Pseudomonas stutzeri* OX1 (67 – 73% amino acid identity) (Bertoni et al., 1998), suggesting that isoprene may not be their natural substrate.

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

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

**DNA stable isotope probing (SIP) experiments**

For SIP incubations, 5 g soil (pH 7.4), collected from the upper 5 cm (after removal of vegetation and leaf litter), in the vicinity of Willow (*Salix fragilis*) trees on the
University of East Anglia campus, was incubated in serum vials (120 ml volume) with
0.5% (v/v) isoprene in triplicate (labelled substrate) or duplicate (unlabelled
substrate). Vials were incubated at room temperature (22 °C) in the dark. Headspace
isoprene concentration was monitored by GC-FID. When substrate was depleted,
additional isoprene was injected through the septum to the original concentration.
Incubations were terminated and DNA extracted from the soil when 10 µmol g⁻¹ had
been consumed. Control incubations with autoclaved soil were also conducted. DNA
extracted from samples was separated into heavy (¹³C-labelled) and light
(unlabelled) fractions as previously described (Neufeld et al., 2007). Briefly, 1-2 µg
DNA was added to caesium chloride solution (final density of 1.725 g ml⁻¹) and
subjected to density gradient ultracentrifugation (177,000 x g, 40 h, 20 °C, Beckman
Vti 65.2 rotor). The contents of each tube was separated into 12 – 15 fractions and
the density of each fraction measured by refractometry (AR200 digital refractometer,
Reichert Inc., Buffalo, USA). DNA from each fraction was precipitated, resuspended
in nuclease-free water and characterized by 16S rRNA gene analysis using
denaturing gradient gel electrophoresis (DGGE). DGGE showed unique profiles in
heavy, compared to light, fractions of ¹³C-incubations while the ¹²C incubations
exhibited similar profiles between fractions, indicating that heavy fractions from ¹³C
incubations contained the DNA of isoprene degraders (data not shown). Based on
fraction density and DGGE profiles, fractions 7 and 11 were identified as containing
labelled and unlabelled DNA, respectively, designated “heavy” and “light” and used
for downstream analysis. DNA was obtained from six different treatments: extracted
from timepoint zero (one sample); extracted following ¹³C-isoprene incubation but
prior to fractionation (unfractionated); and after separation into heavy and light
fractions for both ¹³C- and ¹²C-isoprene incubations. For ¹³C heavy fractions triplicate
samples were analysed individually, but for the other treatments the DNA was pooled prior to analysis.

**Denaturing gradient gel electrophoresis (DGGE)**

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

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

Labelled (heavy) and unlabelled (light) DNA from SIP incubations was characterized by sequencing of 16S rRNA gene amplicons generated by PCR using the primers 27Fmod (5´-AGRGTTTGATCMTGGCTCAG-3´) and 519Rmodbio (5´-GTNTTACNGCGGCKGCTG-3´) using a Roche 454 FLX titanium instrument at MR DNA (Molecular Research LP), Shallowater, USA. Sequence data were processed at MR DNA using a published pipeline (Dowd et al., 2008; Capone et al., 2011). Briefly, the Q25 reads were stripped of barcodes and primers. Short sequences (<200 bp), sequences with ambiguous base calls and those with >6 bp homopolymer runs were removed. Remaining sequences were denoised using a custom pipeline, OTUs clustered at 97% sequence identity, chimeric sequences were removed using Uchime (Edgar et al., 2011) and taxonomy was assigned using BLASTn against the
RDPII/NCBI database (v 11.1) (Cole et al., 2014). An average of 3,320 sequences per sample were used for analysis.

Statistical Analysis

The Bray-Curtis distance measure was applied to log-transformed relative abundance and used to generate non-metric multidimensional scaling (NMDS) plots using Primer 6 (Primer-E, Plymouth, UK).

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**Figure Legends**

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

**Figure 2.** The isoprene metabolic gene cluster from *Rhodococcus* sp. AD45 (Crombie et al., 2015), together with similar regions from *R. opacus* PD630 and strains SC4 and LB1. The contigs containing the genes are identified by horizontal lines and numbers below. The monooxygenase genes are shown in red, and other colours indicate genes of the corresponding predicted function between strains. Locus tags and gene names are indicated with angled text. Locus tag prefixes: *R.*
sp. AD45, SZ00_; R. opacus PD630, Pd630_LP; R. sp. SC4, AXA44_; R. sp. LB1, AZG88_.

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

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

**Figure 5.** Bacterial communities derived from labelled isoprene DNA-SIP enrichments and fractionation. The bar chart shows 16S rRNA gene-based composition of the unenriched soil community (timepoint zero), the isoprene-enriched total community (unfractionated) and labelled (heavy) and unlabelled (light)
components separated by isopycnic centrifugation and fractionation. The symbols on
the x-axis correspond with those shown in Fig. S2. The isoprene-assimilating
community is represented by the heavy fractions of the three replicate $^{13}$C
incubations (solid red diamonds). T-0, timepoint zero; U-F, unfractionated; H, heavy
fraction; L, light fraction. The inset (b) shows the species composition of the
rhodococci in the heavy fractions (mean of three replicates)