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Multiple DMSP lyases in the γ-proteobacterium *Oceanimonas doudoroffii*

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Abstract The marine γ-proteobacterium *Oceanimonas doudoroffii* was shown to have at least three different enzymes, each of which can cleave dimethylsulfoniopropionate (DMSP), an abundant compatible solute made by different classes of marine phytoplankton. These various DMSP lyases have similarities, but also some differences to those that had been identified in other bacteria. This was demonstrated by cloning each of the corresponding genes and transferring them into other species of bacteria in which backgrounds they conferred the ability to catabolise DMSP, releasing dimethyl sulfide (DMS) as one of the products (Ddd^+ phenotype; DMSP-dependent DMS). One of these genes resembled *dddD,* which was in a cluster with other *ddd* genes variously involved in subsequent steps of DMSP catabolism, in DMSP import and in DMSP-dependent transcriptional regulation. The other two gene products both had sequence similarity to the previously identified DddP lyase. However, these two *Oceanimonas* DddP polypeptides were not particularly similar to each other and were in two different subbranches compared to those that had been studied in strains of the Roseobacter clade of bacteria. One of these *O. doudoroffii* enzymes, DddP1, most closely resembled gene products in a disparate group of microbes that included two bacteria, *Vibrio orientalis* and *Puniceispirillum marinum* and, more strikingly, some Ascomycete fungi that can catabolise DMSP. Previously, the only bacteria known to have multiple ways to catabolise DMSP were in the Roseobacter clade, which were also the only bacteria that had been shown to have functional DddP DMSP lyases. Thus *Oceanimonas doudoroffii* is unusual on more than one count and likely acquired its *dddD, dddP1* and *dddP2* genes by independent horizontal gene transfer events.

Keywords DddD · DddP · DMSP lyases · Gene regulation · Horizontal gene transfer · *Oceanimonas*

Introduction

In the 1990s, a number of laboratories described several individual species of microbes that catabolised dimethylsulfoniopropionate (DMSP) and conducted a series of biochemical and physiological experiments that revealed some key features of this important process (reviewed in Yoch 2002). Two very different general pathways were recognised; one of these

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involved an initial demethylation step, to form methylmercaptopropionate (MMPA), the other required cleavage of DMSP, releasing dimethyl sulfide (DMS) as one of the products. Although the catch-all term 'DMSP lyase' was (and still is) used to describe the enzymatic activity that cleaves DMSP in such a way as to liberate dimethyl sulfide, it was apparent early on that there must be several rather different types of polypeptide with this catalytic activity. This was because the lyases in different bacteria varied in their calculated sizes, their K_m and V_{max} values, their responses to potential inhibitors, and even such fundamental features as their proposed sub-cellular locations and the identities of the initially formed C3 catabolites (Yoch 2002).

The recent application of genetic methodology has begun to explain the basis of this heterogeneity at a molecular level. For example, we have described six different genes, namely *dddD* (Todd et al. 2007, 2010), *dddL* (Curson et al. 2008), *dddP* (Todd et al. 2009), *dddQ* (Todd et al. 2011a), *dddY* (Curson et al. 2011) and *dddW* (Todd et al. 2011b), which occur in a range of different bacteria and, in the case of *dddP*, in some fungi. All these genes encode enzymes that cleave DMSP, releasing DMS, yet their gene products are in different polypeptide families. In most cases (DddL, DddP, DddQ, DddY and DddW), the "other" C3 catabolite of the cleavage is acrylate, but the DddD enzyme generates 3-OH-propionate (3HP) as the first detectable C3 product. Furthermore, the different *ddd* genes are subject to different sorts of transcriptional regulation, mediated by a variety of transcriptional regulators, in response not only to the substrate DMSP, but in some cases to its catabolites acrylate or 3HP (Todd et al. 2010; Sullivan et al. 2011).

One of the earliest strains to be studied biochemically was a marine γ-Proteobacterium isolated off Hawaii, which was originally named *Pseudomonas doudoroffii* by Baumann et al. (1972), before its genus was reclassified as *Oceanomonas* (Brown et al. 2001) prior to a final correction to *Oceanimonas* (Anon 2001). This strain generated DMS from DMSP (Ledyard et al. 1993) and further studies in Yoch's laboratory indicated that it had a cytoplasmic DMSP lyase, some of whose features (e.g. pH optimum, response to inhibitors, and inducibility) were described (de Souza and Yoch 1995; Yoch et al. 1997). Indeed, a DMSP lyase from *O. doudoroffii* was purified and its N-terminal sequence was presented. Interestingly, this sequence was very similar to that of another DMSP lyase, made by the β-proteobacterium *Alcaligenes faecalis,* but biochemical evidence indicated that the *A. faecalis* enzyme was located at the bacterial cell surface, whereas it was cytoplasmic in *O. doudoroffii* (de Souza and Yoch 1995, 1996). We recently showed that the *Alcaligenes* DMSP lyase was encoded by the *dddY* gene, whose deduced gene product contained an N-terminal leader which, if cleaved by a signal peptidase, would yield a mature protein that would be targeted to the periplasm. Significantly, the sequence of the predicted N-terminus of this processed form of DddY corresponded to that which had been determined experimentally in *A. faecalis* (de Souza and Yoch 1996; Curson et al. 2011). Taken together, these observations raised the interesting possibility that *A. faecalis* and *O. doudoroffii* both contained versions of the DddY DMSP lyase but that these differed in their sub-cellular locations.

We set out to identify the gene(s) in *O. doudoroffii* that encoded its DMSP lyase(s) and found that this bacterium had a more complex assembly of such enzymes than had been anticipated.

Materials and methods

Strains, plasmids and growth conditions

Oceanimonas doudoroffii strain DSM 7028 was obtained from the DSMZ culture collection, Braunschweig, Germany. *O. doudoroffii* and *E. coli* strains were routinely grown in LB complete medium (Sambrook et al. 1989) at 28°C and 37°C respectively and *Rhizobium leguminosarum* strain 3841 (Young et al. 2006) was grown in TY complete medium at 28°C (Beringer 1974). Antibiotics were used at the following concentrations: ampicillin (100 μg ml^{-1}), streptomycin (200 μg ml^{-1}), tetracycline (5 μg ml^{-1}). *O. doudoroffii* was also grown in M9 minimal medium (Sambrook et al. 1989) for DMS assays with 10 mM succinate as carbon source. For growth tests, 1 ml of an overnight LB culture of *Oceanimonas* was spun down and the pellet washed three times with M9 buffer. Washed *Oceanimonas* cells were then inoculated (1:100) into M9 without added carbon source or supplemented with DMSP (1 mM, 5 mM), acrylate (1 mM, 5 mM), 3HP (1 mM, 5 mM) or DMS (1 mM, 5 mM).

In vivo and in vitro genetic manipulations

Plasmids were transferred by triparental conjugation into a newly made rifampicin resistant derivative of *O. doudoroffii* (strain J495) using the helper plasmid pRK2013 (Figurski and Helinski 1979) and into *E. coli* strain 803 (Wood 1966) by transformation as described in Wexler et al. (2001). Recombinant plasmids based on the expression vector pET21a (Merck4Biosciences, Darmstadt, Germany) were made by PCR amplification using primers shown in Supplementary Table S1, and transformed into *E. coli* BL21 (Studier and Moffat 1986). A library of *Oceanimonas* genomic DNA was made in the wide-host range cosmid pLAFR3 (Staskawicz et al. 1987) essentially as in Curson et al. (2008), as follows. A culture of *O. doudoroffii* was grown to late exponential phase in LB medium. Genomic DNA was isolated using a Qiagen genomic DNA kit and partially digested for various times with *Eco*R1 and aliquots were examined following electrophoresis in agarose gels to determine the approximate sizes of the genomic fragments. A treatment of 10 min was found to generate fragments that were 20–30 kb in size, suitable for cloning into pLAFR3, which accepts inserts of ca. 25 kb. This sample of DNA was ligated to *Eco*R1-digested pLAFR3, prior to in vitro packaging into heads of bacteriophage lambda and transfection into *E. coli* strain 803. The transfected cells were plated and counted for colony-forming units on LB agar containing 5 μg ml^{-1} tetracycline, to which pLAFR3 confers resistance; this showed that the library comprised ca 5,000 primary transfectants. Cosmid DNA, isolated from a random sample of five such colonies, was examined by gel electrophoresis, following digestion with *Eco*R1. In all cases, the recombinant cosmids contained different regions of genomic DNA and the total sizes of the cloned DNA in each cosmid was in the range 20–30 kb.

In order to make transcriptional fusions to *dddD*, *dddP1* and *dddP2*, primers were designed to amplify the relevant regions (see Fig. 1 and Supplementary Table 1) from the *O. doudoroffii* genome, with primers containing restriction sites (*Eco*R1 and *Xba*1 for *dddD* and *dddP1*; *Xba*1 and *Pst*1 for *dddP2*) to facilitate cloning into the wide host range *lacZ* reporter plasmid pMP220 (Spaink et al. 1987).

DMS assays

To assay DMS production in *Oceanimonas*, an overnight culture was diluted (1:100) into M9 minimal medium containing 10 mM succinate, with or without 2 mM DMSP, acrylate or 3HP as potential inducers and incubated for 16 h at 28°C. Then, 1 ml culture was spun down and washed three times in M9 minimal medium lacking any carbon source or inducer and 285 μl of washed cells were added to 2 ml GC vials (Alltech Associates) in a final volume of 300 μl with the substrate DMSP (5 mM). Assay vials were incubated at 22°C before DMS production was quantified by gas chromatography with a flame photometric detector (Focus GC; Thermo Scientific) and a 30 m × 0.53 mm ID-BP1 5.0 μm capillary column (SGE Europe, Milton Keynes, UK). DMS concentrations were calculated by regression analysis based on an eight-point calibration with standard DMS solutions (1–300 μM DMS). To assay DMS production in *E. coli* strain BL21 expressing cloned *ddd* genes, an overnight culture was inoculated (1:100) into LB and grown to an OD_{600} of ~0.7 before induction with 0.2 mM isopropyl-*β*-D-1-thiogalactopyranoside (IPTG) for 16 h at 30°C then the cells were spun down, resuspended in 0.5 ml M9 minimal medium and sonicated (4 × 10 s). Following centrifugation to remove debris, lysates were assayed for DMS production as above. Rates of DMS production are expressed as pmol DMS μg $protein^{-1}$ min^{-1}, with protein concentrations estimated using a BIO-Rad protein assay. Screening the *Oceanimonas* library for DMS production in *Rhizobium leguminosarum* was done as above using individual *Rhizobium* transconjugants grown overnight in TY medium supplemented with 5 mM DMSP. DMS degradation by *Oceanimonas* was tested by diluting cells (1:100) from an overnight culture in complete medium to vials containing minimal medium plus 10 mM succinate and 0.1 mM DMS. The concentrations of DMS that remained in the headspace were measured after incubation at 22°C for 20 h.

Bioinformatics and *in silico* analysis

Searches for homologues and sequence analyses were done using NCBI BLAST and the DNAstar-Lasergene v6 package. Sequences were aligned using ClustalV. Sequencing of the cosmids that contained the *dddD,*

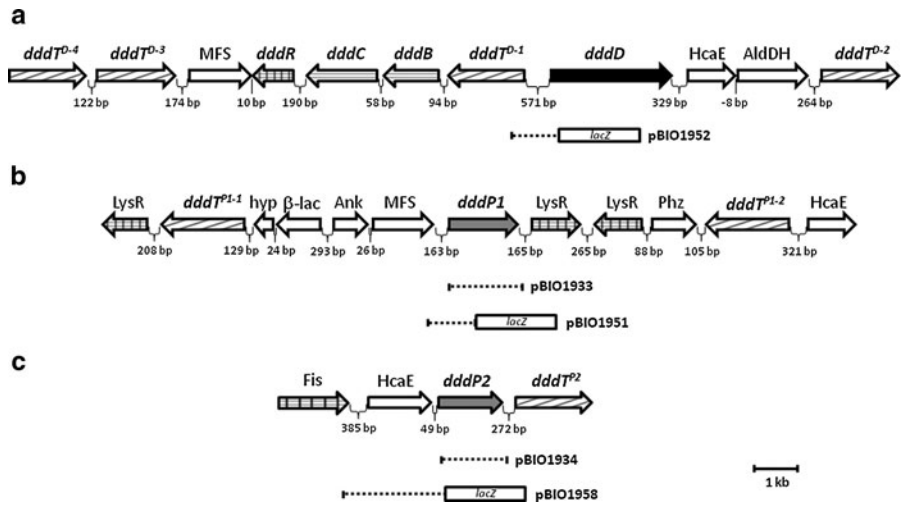

Fig. 1 Arrangement of genes near *dddD* (**a**), *dddP1* (**b**), and *dddP2* (**c**) in *Oceanimonas doudoroffii*. Genes marked with diagonal lines encode BCCT-type transporters, those with horizontal lines are predicted to be involved in downstream steps in DMSP catabolism and those that are hatched are regulatory. The numbers of base pairs in the intergenic spaces are marked and cloned regions in the recombinant plasmids pBIO1933, pBIO1934, pBIO1951, pBIO1952 and pBIO1958 are shown below the genes. *MFS* major facilitator superfamily transporter, *HcaE* oxygenase family polypeptide with C-terminal Rieske domain, *AldDH* aldehyde dehydrogenase, *hyp* hypothetical protein, *β-lac* β-lactamase, *Ank* ankyrin repeat protein, *Phz* phenazine biosynthesis protein. *Fis* and *LysR* are transcriptional regulators in the *Fis* and *LysR* super-families respectively

dddP1 and *dddP2* genes and the draft genome of *Oceanimonas* DSM 7028 were done at the Department of Biochemistry, University of Cambridge, UK. Sequencing of the genome was done using a Roche 454 Genome Sequencer FLX and produced 118,560 reads and 46.8 Mb of raw data, representing an average 10× coverage of the genome. The reads assembled to 193 contigs, with an average size of 27.6 kb, the largest contig being 155.9 kb and totalling 3,829,948 bp. Cosmid sequences were deposited at NCBI Genbank with accession numbers (JN541238, JN541239, JN541240) and the *Oceanimonas* partial genome sequence was deposited at IMG (http://img.jgi.doe.gov/).

Results

We showed that *O. doudoroffii* DSM 7028 grew well on M9 minimal medium in which DMSP (5 mM) was the sole carbon source, with single colonies appearing on plates after 72 h. However, it did not grow at the expense of either 5 mM or 1 mM acrylate or 3-OH-propionate (3HP), the C3 catabolites that are known to be formed following cleavage of DMSP by DddD (for 3HP) or by DddL, DddP, DddQ, DddW or DddY, all of which generate acrylate (see above), and which support the growth of some other bacteria that catabolise DMSP (Todd et al. 2010; Curson et al. 2011). Given that *Oceanimonas* can grow on DMSP, but not its C3 catabolites, we tested whether it could catabolise DMS, the other product of DMSP breakdown. *Oceanimonas* did not grow on 5 mM or 1 mM DMS as sole carbon source and did not degrade DMS when grown in minimal medium containing 10 mM succinate and 0.1 mM DMS.

When grown with DMSP in minimal media that also contained 10 mM succinate, the cells of *O. doudoroffii* emitted DMS (Ddd^+ phenotype). In order to test for induction of DMS production, *O. doudoroffii* was pregrown with or without DMSP, acrylate or 3HP as potential inducers. DMS production was increased, ~4-fold (0.215 pmol ug^{-1} min^{-1} compared to 0.046 pmol ug^{-1} min^{-1} without inducer), when the cells had been pregrown in the presence of DMSP. No induction of activity was seen with either acrylate or 3HP, two DMSP catabolites that are co-inducers in other bacteria (Yoch 2002; Todd et al. 2010; Curson et al. 2011; Sullivan et al. 2011).

These preliminary observations tally with earlier ones on this strain made by de Souza and Yoch (1995) and by Yoch et al. (1997). The levels of DMS

production described here for *O. doudoroffii* are similar to those reported for *Pseudomonas* J465 and *Psychrobacter* J466, but were lower than those for *Halomonas* sp. HTNK1, all of which also contain the *dddD* gene (Todd et al. 2010; Curson et al. 2010).

Cloning the *ddd* genes from *O. doudoroffii*

To identify the gene(s) responsible for the Ddd^+ phenotype of *O. doudoroffii,* we used a functional genetic approach, as has been done previously to clone the *ddd* genes from other bacteria (e.g. Todd et al. 2007). We made a genomic library of *O. doudoroffii* DNA, cloned in pLAFR3, a cosmid vector that accepts inserts of ~25 kb and which can be transferred by conjugation to a wide range of proteobacteria. Following transfection of *E. coli* strain 803 with the ligation mix, ~5,000 primary transfectants were obtained. These were pooled and used *en masse* as a donor culture in a conjugational, triparental cross in which *Rhizobium leguminosarum* strain 3841 (Young et al. 2006) was used as the recipient. The reason for choosing *R. leguminosarum* is that it more effectively expresses heterologous genes, probably because of its relatively large number of RNA polymerase sigma factors (Young et al. 2006). Individual transconjugant colonies were assayed for their ability to make DMS when grown with DMSP; of ~400 that were tested, three had a Ddd^+ phenotype. The cosmids were isolated from these three Ddd^+ transconjugants, transformed into *E. coli*, then mobilised back to *Rhizobium* by conjugation. In all cases, the newly constructed *Rhizobium* transconjugants had a Ddd^+ phenotype, confirming that the cosmids contained functional *ddd* genes.

Restriction digests of these three cosmids showed that they contained different regions of cloned *O. doudoroffii* genomic DNA, since they did not share any fragments of the same size. To identify the relevant *ddd* genes, we sequenced the cloned DNA in each cosmid and found that each of them contained a gene that encoded a product that resembled a previously identified Ddd enzyme. Thus, one cosmid (pBIO1932) contained a gene whose product is ~70% identical to the DddD enzymes that cleave DMSP into DMS plus 3HP. Functional *dddD* genes occur in a range of other γ-Proteobacteria, including strains of *Marinomonas, Halomonas* and *Pseudomonas,* and in a few strains of *Rhizobium* and *Burkholderia* (α- and β-Proteobacteria respectively), which may have acquired *dddD* by horizontal gene transfer (HGT) (Todd et al. 2007; Raina et al. 2010).

The other two cosmids, pBIO1930 and pBIO1931, each contained genes, termed *dddP1* and *dddP2* respectively, whose products were 50% identical to each other and had sequence similarity to DddP, a DMSP lyase in the M24 metallopeptidase family (Todd et al. 2009; Kirkwood et al. 2010a). Previously, *dddP* had only been found in some strains of Roseobacters, an abundant group of marine α-Proteobacteria, and, more surprisingly, in some Ascomycete fungi (Kirkwood et al. 2010b; Todd et al. 2009).

Bioinformatics-based description of the *ddd* and nearby genes in *Oceanimonas*

As shown in Fig. 1, the *dddD*-like gene of *O. doudoroffii* is transcribed divergently from a predicted four-gene operon whose promoter-proximal gene, $dddT^{D\text{-}1}$, encodes a BCCT-type transporter that is likely involved in the uptake of DMSP (see Sun et al. 2011). Downstream of $dddT^{D\text{-}1}$ are *dddB* and *dddC,* which respectively encode an Fe-containing dehydrogenase and an enzyme related to methylmalonate-semialdehyde dehydrogenase, with the promoter-distal gene, *dddR,* encoding a polypeptide in the LysR super-family of transcriptional regulators (Fig. 1a). This arrangement is the same as in the γ-Proteobacteria *Marinomonas* sp. MWYL1 and *Pseudomonas* sp. J465, which also grow well on DMSP (Todd et al. 2007; Curson et al. 2010) and the products of all the genes in the *O. doudoroffii dddD* and *dddTBCR* operons are very similar (at least 70% identical) to their corresponding homologues in both these strains. However, one novel feature of this region in *O. doudoroffii* was the presence of three further genes ($dddT^{D\text{-}2}$, $dddT^{D\text{-}3}$ and $dddT^{D\text{-}4}$) that are predicted to encode BCCT-type transporters (Fig. 1a) that likely import DMSP, though this has not yet been ratified experimentally (Sun et al. 2011). None of the other genes in the *dddD* cluster (Fig. 1a) has any known link with DMSP catabolism in other bacteria.

Turning to the two *O. doudoroffii dddP*-like genes, the outcomes of BLAST-based comparisons of their products with those in the databases were more unusual. As mentioned above, the *dddP* gene had only been described in Roseobacter strains among the

Bacteria, but it also occurs in the eukaryotic fungal pathogens *Aspergillus oryzae*, *A. sydowii* and *Fusarium culmorum* (Todd et al. 2009; Kirkwood et al. 2010b). Interestingly, the *O. doudoroffii* DddP1 polypeptide was more similar (>67% identical, *E* value $<1e^{-176}$) to these fungal sequences than to the previously identified Roseobacter types (~55% identical). Furthermore, two recently available bacterial genome sequences, of the marine γ-Proteobacterium *Vibrio orientalis* CIP 102891 (Yang et al. 1983; http://www.ebi.ac.uk/ena/data/view/Project:40487) and *Puniceispirillum marinum* IMCC1322, in the SAR116 clade of α-proteobacterium (Oh et al. 2010) have deduced polypeptides (gene tags VIA_000771 and SAR116_1427 for *V. orientalis* and *P. marinum* respectively) that cluster with these fungal DddPs and with DddP1 of *O. doudoroffii.* Indeed, DddP of *V. orientalis* is more similar (~80% identical) to *O. doudoroffii* DddP1 than to any other polypeptide in current databases.

The *O. doudoroffii dddP1* gene likely forms a single gene transcriptional unit, lying 5′ of a predicted regulatory gene in the LysR family (see Fig. 1b), whose product has no particular similarity to any known transcriptional regulator of any other *ddd* genes. The only other nearby genes of note encode two transporters of the BCCT type, one of which is 5′ of *dddP1* and separated by four intervening genes and the other 3′ of *dddP1* and separated by three intervening genes (see Sun et al. 2011).

The *dddP2* gene and its deduced product differ from that of *dddP1* in a number of ways. Although a member of the M24 family of peptidases (*E* value 7.8 e^{-42}), DddP2 is rather distantly related to those ratified versions of the enzyme that have DMSP lyase activity in other bacteria, in the Roseobacter clade (43% identity to *Roseovarius nubinhibens* DddP). Further, DddP2 is not particularly similar to the polypeptides represented by DddP1, to which it is ~50% identical. Instead, DddP2 is closest in sequence to polypeptides in the deduced proteomes of strains of some unrelated bacteria that have no known links to DMSP catabolism, including those in the genera *Burkholderia* and *Rhizobium/Sinorhizobium* (β- and α-Proteobacteria, respectively).

Based on the size of its upstream intergenic region, it is likely that *dddP2* of *O. doudoroffii* is co-transcribed with a gene whose product is in the HcaE family (COG4638). Members of this family are dioxygenases, with a catalytic C-terminal domain linked to an N-terminal region that resembles the family of Rieske proteins, which are involved in transferring the electrons necessary for the oxygenase reactions. There is no known role for such a protein in DMSP catabolism but we noted that Rieske family polypeptides are also encoded by a gene found downstream of *dddD* in *Oceanimonas* and by a gene divergently transcribed from the BCCT transporter 3′ of *Oceanimonas dddP1* (see Fig. 1). The *dddP2* of *O. doudoroffii* lies upstream of a gene that encodes a BCCT-type transporter, which is therefore a candidate for being involved in DMSP import, although, in this case, the intergenic spacing suggests that this gene would not be co-transcribed with *dddP2* (Fig. 1c; see Sun et al. 2011).

Ratification of the function of the *Oceanimonas doudoroffii dddP* genes

Given that both DddP1 and DddP2 of *Oceanimonas* were rather different from those DddP polypeptides that we had studied previously, we set out to confirm that they had functional DMSP lyase activity as follows. The *dddP1* and *dddP2* genes were individually cloned into the expression vector pET21a (in pBIO1933 and pBIO1934 respectively), following their amplification from genomic DNA, using primers that corresponded to sequences immediately 5′ and 3′ of these genes (see Fig. 1 and Supplementary Table 1). The resultant recombinant plasmids were each transformed into *E. coli* strain BL21 and the cell-free extracts were assayed for their Ddd phenotypes. In both cases, the transformants generated DMS when they were grown with DMSP, those with the cloned *dddP2* being considerably higher than those with *dddP1* (values of 151 and 12.4 pmol μg protein^{-1} min^{-1}, respectively).

Regulation of *Oceanimonas dddD*, *dddP1* and *dddP2* genes

It was shown previously (de Souza and Yoch 1995), and confirmed here (see above), that DMS production in *Oceanimonas* is induced by DMSP. We therefore examined if this was associated with enhanced expression of one or more of the *ddd* genes that we had identified. To do this, we made a series of transcriptional fusions in the wide host-range promoter-probe

plasmid pMP220 (see "Materials and methods"), which contains a reporter *lacZ* gene that lacks its own promoter. Fragments that spanned the promoter regions of *dddD, dddP1* and *dddP2* (see Fig. 1) were amplified from genomic DNA and cloned into pMP220 and the resultant plasmids were individually mobilised into *O. doudoroffi* strain J495 (rif^R derivative) by conjugation. The transconjugants were then assayed for β-galactosidase activity (encoded by *lacZ*) after pre-growth in the presence or absence of 2 mM DMSP, acrylate or 3HP. It was found that expression of the *dddD-lacZ* transcriptional fusion (pBIO1952) was dramatically increased, ca. 50-fold by pre-growth in DMSP, with values of 119 and 5779 Miller units following growth in the absence and presence of DMSP respectively. No induction of this *dddD-lacZ* fusion was obtained following growth in acrylate or 3HP, consistent with the failure of these compounds to induce DMS production. In contrast to the *dddD* fusion, the *dddP1-lacZ* fusion (pBIO1951) was expressed constitutively, with low-level expression irrespective of the presence or absence of any of the potential inducers, and the *dddP2-lacZ* fusion (pBIO1958) was expressed at even lower levels, which were barely detectable under all conditions tested.

Thus the enhanced Ddd^+ phenotype seen when cells of *Oceanimonas* were grown in DMSP is most likely due to the increase in expression of its *dddD* gene.

Failure to find a *dddY*-like gene in the genome of *Oceanimonas doudoroffii*

As mentioned above, de Souza and Yoch (1996) purified a DMSP lyase from *O. doudoroffii*. This enzyme had a similar size (48 kDa) to those of the deduced DddP1 and DddP2 polypeptides (50 and 48 kDa respectively). However, the experimentally determined N-terminal sequence (AQFQSQDDV KPASIDAWSGK), which resembles that of the processed DddY polypeptide of *Alcaligenes* (de Souza and Yoch 1996; Curson et al. 2011) does not match that of either the *dddP1* or *dddP2* gene products. The DddD polypeptide is much larger, with a deduced Mr of 92 kDa; not surprisingly, the deduced N-terminal sequence of the *O. doudoroffii* DddD does not correspond to that found by de Souza and Yoch (1996).

In an attempt to identify the *Oceanimonas* gene whose product includes this proposed N-terminal sequence, we obtained a near-complete genomic sequence of this strain, comprising a total of 3,829,948 bp, in 193 contigs. All of the predicted single-copy bacterial genes that we searched for, namely *recA,* 16S rRNA*, rpoB, rpoD, rpoS, rpoZ, gyrA, ftsZ, dnaK, infB, atpD, groEL, sodA,* as well as the regions represented by the cosmids described above, were present in the available sequence, indicating that it contained the great majority of the *O. doudoroffii* genome. Based on analysis of the read numbers and sequencing coverage of the genome, it was predicted to have covered 98.5% of the genome.

However, a search of this newly acquired genome yielded no sequences that matched the N-terminal sequence (see above) of the DMSP lyase described by de Souza and Yoch (1996). It is not clear if this was because it was encoded by a gene in a region that had not been sequenced or if, perhaps, the gene had been lost by spontaneous deletion in the intervening years since they analysed this strain.

We also noted that the partial *O. doudoroffii* genome did not contain any genes whose products resembled the other known DMSP lyases namely DddL, DddW or DddQ, nor was there any sign of a polypeptide sequence that corresponded to the DmdA DMSP demethylase (Howard et al. 2006).

Discussion

The work presented here complements and extends earlier physiological and biochemical studies on the ability of the marine bacterium *Oceanimonas doudoroffii* to catabolise DMSP (de Souza and Yoch 1995; Yoch et al. 1997). This strain contains at least three enzymes with DMSP cleavage activity, representing two very different families, the DddD CoA-transferases and the DddP M24 peptidases. The presence of multiple mechanisms of DMSP catabolism has been found in other bacteria, but, to our knowledge, these have been confined to the Roseobacter clade. Thus, it has been known for some time that some individual Roseobacter strains can both demethylate DMSP and can cleave it via "DMSP lyase" activities that liberate DMS (González et al. 1999, 2003). Recent genetic and genomic analyses have shown that most strains of Roseobacter whose genomes have been sequenced

contain *dmdA,* which encodes DMSP demethylase (Howard et al. 2006; Newton et al. 2010), plus at least one DMSP lyase. Indeed, some strains contain multiple DMSP lyases, with their own particular portfolios, encoded by different *ddd* genes. For example, *Ruegeria pomeroyi* DSS-3 has *dddQ*, *dddP* and *dddW,* whereas *Roseovarius nubinhibens* has two versions of *dddQ* plus *dddP* (Todd et al. 2009, 2011a, b).

This multiplicity of DMSP catabolic systems in the Roseobacters is in keeping with the importance of DMSP in the lifestyles of these bacteria (Newton et al. 2010). By the same token, our finding of multiple *ddd* genes in a γ-Proteobacterium implies that DMSP may be a key substrate for *O. doudoroffii,* as further illustrated by the *dddT*-like genes near *dddD, dddP1* and *dddP2,* with no less than four such predicted BCCT transporter genes being seen in the vicinity of *dddD.* It will be of interest to gauge the relative contributions of these different transporters in the natural environment (see Sun et al. 2011). To date, there are no reports of DMSP catabolism in other *Oceanimonas* strains and no other genome sequences of this genus or the closely related *Oceanisphaera* (Ivanova et al. 2004) are available, so there are no bioinformatic data on the prevalence of *ddd* and *dmdA* genes in this genus.

Given that *O. doudoroffii* contains both DddP and DddD, it was surprising that neither acrylate nor 3HP, respectively the initial catabolites of these enzymes, acted as carbon sources, unlike the situation in some other DMSP-catabolising bacteria (e.g. Todd et al. 2010). However, a strain of *Pseudomonas* that contains *dddD* and grows well on DMSP also fails to use 3HP as a carbon source (Curson et al. 2010). One possible explanation for this is that such bacteria lack effective acrylate and/or 3HP transporters. Some bacteria can grow at the expense of DMS as sole carbon source (Schäfer et al. 2010), so one other possibility is that *O. doudoroffii* might catabolise some of the DMS formed by DMSP cleavage. However, we found no evidence that this strain could grow when provided with DMS as sole carbon source. Indeed, when O. *doudoroffii* was grown in medium that contained both DMS plus a conventional carbon source (succinate), there was no detectable removal of the exogenous DMS.

DMS production in *O. doudoroffii* had been shown previously to be induced by DMSP (de Souza and Yoch 1995) and we obtained strong evidence that this is due to the markedly increased levels of transcription of its *dddD* gene when the cells were pregrown in DMSP. It has been noted that the expression of *dddD* in several other bacteria is also highly induced by the DMSP substrate (Todd et al. 2007, 2010). In contrast, transcription of the *Oceanimonas dddP1* gene was at low levels and was unaffected by any of the potential co-inducers tested here. More strikingly, *dddP2* was not expressed at detectable levels in any of our conditions. Although the *dddP*-like genes in some bacteria of the Roseobacter clade are induced by DMSP (Todd et al. 2009), the factor of increase is modest (2–4-fold). It remains to be seen if either or both of these *dddP*-like genes in *Oceanimonas* are expressed in response to some unknown environmental signal that has so far eluded us in laboratory conditions.

The sequence and the local genomic geography of the *O. doudoroffii dddD* gene was conventional in the sense that *dddD* was clustered with other genes (*dddT, dddB, dddC* and *dddR*) that were in the same, or very similar, relative positions in other γ-proteobacteria such as *Marinomonas* and *Pseudomonas* (Todd et al. 2007; Curson et al. 2010), and the sequences of the corresponding products of all these genes were very similar in these different strains.

A different situation pertains to the two *Oceanimonas dddP* genes. The DddP1 polypeptide was very similar to the gene products in a miscellany of other microbes, ranging from two unrelated bacteria, *Vibrio orientalis* and *Puniceispirillum marinum* through to some eukaryotic fungi. The finding of members of this out-branch of the DddP polypeptides in such a disparate group of organisms is strong evidence that these were acquired by repeated instances of HGT. This conclusion is bolstered by the fact that of >20 strains of *Vibrio* that have been sequenced, *V. orientalis* CIP 102891 is the only one that contains *dddP*. It is not clear why this subset of the *dddP* gene should, apparently, be more prone to HGT to other bacteria than the forms that are represented by the DddP enzymes in the Roseobacters.

In its primary sequence, DddP2 differs not only from DddP1, but also from the DddP polypeptides in the Roseobacter clade (Todd et al. 2009). Nevertheless it can be a functional enzyme, as shown by the high level of DMSP-dependent production seen in *E. coli* containing *dddP2*. Indeed, its activity when expressed in the heterologous host *E. coli* was significantly

higher than that with the cloned *dddP1*. The behaviour of *dddP2* has some similarities to that of another gene, *dddL,* which encodes a wholly different type of DMSP lyase, in the Roseobacter species *Dinoroseobacter shibae* DFL 12. This gene, when cloned and expressed in *E. coli,* confers the ability to make DMS from DMSP even though *D. shibae* DFL 12 itself does not make DMS (Dickschat et al. 2010) and the *dddL* gene is not expressed at detectable levels (JD Todd, unpublished observations).

There have been several reports on the frequencies of the different *ddd* and *dmd* genes in the massive metagenomic databases, most importantly those in the Global Ocean Sampling (GOS) data set (Rusch et al. 2007). These have shown that the *dmdA* demethylase is the most abundant, and that *dddP* and *dddQ* are widespread, compared to other *ddd* genes such as *dddL* and *dddD* (Todd et al. 2009; Newton et al. 2010). The census-taking of the DddP-type of DMSP lyase in the GOS was based on the numbers of close homologues of the originally described version of this enzyme, identified in the Roseobacters. Performing the same sort of survey with the newly identified *Oceanimonas* DddP1 and DddP2 polypeptides shows that these, too, have close homologues (*E* values $<e^{-86}$) in the GOS, and that they are both relatively abundant, with census numbers that are each approximately a fifth of the number of metagenomic homologues that closely resembled the original DddP enzyme, as exemplified by the versions found in the Roseobacters and described by Todd et al. (2009).

De Souza and Yoch (1996) predicted that *O. doudoroffii* has a DMSP lyase that resembles the recently characterized periplasmic DddY of *Alcaligenes faecalis* (Curson et al. 2011), but that it has an important difference, in that it was proposed to be cytoplasmic in *Oceanimonas.* However, despite sequencing an estimated 98.5% of the *O. doudoroffii* genome, we did not find any match for the sequence of the published N-terminal region of this enzyme. Since the DMSP lyases of *A. faecalis* and of *O. doudoroffii* are immunologically cross-reactive (de Souza and Yoch, 1996), we plan to use antibody against over-expressed DddY of the former species to examine the production of the corresponding protein in *Oceanimonas* and will attempt to identify the corresponding gene, in case it is in a region which, by chance, was not represented in our partial genome sequence of this strain.

In strains of different Roseobacters that have multiple ways of catabolising DMSP, the relative importance of the different pathways was shown to depend on environmental factors such as the concentration of the DMSP substrate (González et al. 2003). Having now found a multiplicity of DMSP catabolic pathways in a different type of bacterium, it will be of interest to determine which of the DddP- and DddD-mediated routes are most important for *Oceanimonas* and to establish if and how the relative contributions of these enzymes are affected by factors in the natural environments of this bacterium.

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