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ORIGINAL ARTICLE

DddY, a periplasmic dimethylsulfoniopropionate lyase found in taxonomically diverse species of Proteobacteria

Andrew RJ Curson, Matthew J Sullivan, Jonathan D Todd and Andrew WB Johnston
School of Biological Sciences, University of East Anglia, Norwich, UK

The abundant compatible solute dimethylsulfoniopropionate (DMSP) is made by many marine algae. Different marine bacteria catabolise DMSP by various mechanisms, some of which liberate the environmentally important gas dimethyl sulfide (DMS). We describe an enzyme, DddY, which cleaves DMSP into DMS plus acrylate and is located in the bacterial periplasm, unlike other DMSP lyases that catalyse this reaction. There are *dddY*-like genes in strains of *Alcaligenes, Arcobacter* and *Shewanella,* in the β-, ε- and γ-proteobacteria, respectively. In *Alcaligenes, dddY* is in a cluster of *ddd* and *acu* genes that resemble, but also have significant differences to, those in other bacteria that catabolise both DMSP and acrylate. Although production of DMS and transcription of *Alcaligenes dddY* are both apparently inducible by pre-growth of cells with DMSP, this substrate must be catabolised to form acrylate, the *bona fide* coinducer.
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Introduction

The volatile dimethyl sulfide (DMS) is the most important biogenic sulfurous compound that is transferred from the oceans to the atmosphere. When DMS or its photochemically oxidised products return to land via precipitation, this represents a major step in the transfer of sulfur from marine to terrestrial environments (Lovelock *et al.*, 1972; Kiene *et al.*, 2000). DMS has other important effects; it is a chemoattractant for marine animals, including seals, copepods and some seabirds (Steinke *et al.*, 2006; DeBose and Nevitt, 2008) and its oxidation products function as cloud condensation nuclei, affecting albedo (Vallina and Simó, 2007).

DMS is made by microbial catabolism of the compatible solute dimethylsulfoniopropionate (DMSP), an anti-stress molecule synthesised in large amounts ($\sim 10^9$ tons worldwide annually) by many phytoplankton, seaweeds and a few angiosperms, including the salt marsh Cordgrass *Spartina* (see Stefels *et al.*, 2007). There is remarkable biochemical and genetic diversity by which different bacteria catabolise DMSP. Globally, most DMSP is degraded via demethylation, this pathway being prevalent in abundant marine α-proteobacteria known as the Roseobacters and in the ubiquitous SAR11 lineage (Howard *et al.*, 2006). But this pathway liberates no DMS.

We recently identified four wholly different enzymatic gene products that act on DMSP, generating DMS. One of these (DddD) cleaves DMSP into DMS plus 3-hydroxypropionate (3HP) and three 'DMSP lyases' (DddL, DddP and DddQ) each generate DMS plus acrylate, although they are in wholly different polypeptide families. The *dddD* gene is mostly found in marine γ-proteobacteria, whereas *dddL, dddP* and *dddQ* usually occur in the Roseobacters (Curson *et al.*, 2008, 2010; Todd *et al.*, 2007, 2009, 2010, 2011). Some *ddd* genes are subject to horizontal gene transfer (HGT), even extending to interdomain transfer of *dddP* among bacteria and Ascomycete fungi (Todd *et al.*, 2007, 2009; Kirkwood *et al.*, 2010b).

The *dddP* and *dddQ* genes are more abundant than *dddD* and *dddL* in the published metagenomes of marine bacteria, most notably the Global Ocean Sampling data set (Rusch *et al.*, 2007), suggesting their widespread occurrence in the environment. These *ddd* genes, however, are rarer in these metagenomes than *dmdA,* which encodes the DMSP demethylase (Howard *et al.*, 2008). These *ddd* and *dmdA* genes also occur in bacteriophage metagenomes, pointing to phage as likely vehicles for HGT (Raina *et al.*, 2010).

Correspondence: AWB Johnston, School of Biological Sciences, University of East Anglia, Earlham Road, Norwich, Norfolk, NR4 7TK, UK.
E-mail: a.johnston@uea.ac.uk
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In this study, we describe the genes for another, unusual DMSP lyase called DddY. We identified it in the β-proteobacterium *Alcaligenes faecalis* M3A, which de Souza and Yoch (1995a) isolated from intertidal sediment containing the DMSP-producing *Spartina*. *A. faecalis* M3A grows well on DMSP and acrylate as carbon sources, converting the former to the latter (Ansede *et al.*, 1999), before further conversion of acrylate to 3HP. This DMSP lyase was purified and was located at the bacterial cell surface (de Souza and Yoch, 1995a, b; Yoch *et al.*, 1997), unlike other DMSP lyases, which are cytoplasmic (ARJ Curson, unpublished).

By identifying the *dddY* gene, we confirmed the novelty of this DMSP lyase in *A. faecalis* M3A and show that similar enzymes occur in other, unrelated bacteria.

Materials and methods

Strains, plasmids and microbiology

Strains and plasmids are shown in Supplementary Table S1. *E. coli*, *Alcaligenes faecalis* M3A, *Shewanella oneidensis* MR-1 (Venkateswaren *et al.*, 1999), *S. putrefaciens* CN-32 (Fredrickson *et al.*, 1998) and *Pseudomonas putida* 1290 (Leveau and Lindow, 2005) were grown routinely on LB medium, *Shewanella halifaxensis* DSM17350 (Zhao *et al.*, 2006) on Difco marine agar 2216 and *Arcobacter nitrofigilis* DSM7299 on Columbia blood agar (Oxoid, Basingstoke, UK) supplemented with 5% horse blood or *Arcobacter* marine agar (McClung *et al.*, 1983). Minimal media were M9 (Sambrook *et al.*, 1989) for *E. coli* and *P. putida*, Alcaligenes basal medium (ABM) for *A. faecalis* (de Souza and Yoch, 1995a) and *Shewanella* minimal medium (SMM) for *Shewanella* sp. (Supplementary Materials). *E. coli* was grown at 37 °C, *Alcaligenes* and *Arcobacter* at 30 °C and *Shewanella* at 22 °C. For *Alcaligenes* and *Pseudomonas*, succinate and glucose (10 mM) were used, respectively, as carbon sources in minimal media or, where appropriate, DMSP, acrylate or 3HP, each at 5 mM.

In vivo *and* in vitro *genetic manipulations*

Plasmids were transferred by conjugation in triparental crosses (Figurski and Helinski, 1979) into *Alcaligenes* and *Pseudomonas* or by transformation into *E. coli* (Wexler *et al.*, 2001). Genomic mutations in *A. faecalis acu* and *ddd* genes were made by cloning fragments internal to each gene into pBIO1879 (Todd *et al.*, 2010), a derivative of suicide plasmid pK19*mob* (Schäfer *et al.*, 1994) into which a spectinomycin-resistance cassette had been cloned, to allow counter-selections. These plasmids were mobilised from *E. coli* into the *A. faecalis* Rif^R derivative J481 selecting for transfer of Spc^R (200 μg ml^{-1}). *Alcaligenes* transconjugants were checked for resistance to kanamycin (20 μg ml^{-1}), then by PCR, using primers external to the expected integration site. Promoter fusions in the wide host-range reporter plasmid pMP220 (Spaink *et al.*, 1987) and *dddY* clones in the expression vector pET21a (Novagen, Darmstadt, Germany) were made by PCR amplification using primers shown in Supplementary Table S2. All clones were sequenced by Genome Enterprise Ltd (Norwich, UK). The genomic library of *A. faecalis* M3A was made as in Curson *et al.* (2008). This involved partial *Eco*RI digestion of *A. faecalis* M3A genomic DNA, ligated to the wide host-range cosmid pLAFR3 (Staskawicz *et al.*, 1987), also cut with *Eco*RI, before packaging into Gigapack III XL (Stratagene, Stockport, UK) and transfecting *E. coli* strain 803. This produced a library of ~10 000 primary transfectants. Cosmid pBIO1895 was sequenced at the Department of Biochemistry, University of Cambridge.

Enzyme assays

E. coli BL21 containing cloned *dddY* was grown to OD_{600} of 0.5 at 37 °C in LB broth containing 200 μM IPTG to induce *dddY* expression. Cultures were spun at 13 200 rpm and cells were resuspended in 1 ml M9 minimal medium. Then 300 μl was placed in a sealed 2 ml vial (Alltech Associates, Carnforth, UK), DMSP (5 mM, pH 6.5) was added and vials were incubated at 22 °C. DMS in the headspace was assayed by gas chromatography after 10 min (Todd *et al.*, 2009). To measure DMS production in other bacteria, strains were grown overnight in minimal medium, with, or without 2 mM DMSP, acrylate or 3HP, cells were washed, resuspended in minimal medium and assayed as above. Fractionation of *E. coli* expressing DddY used the PeriPreps Periplasting Kit (Epicentre Biotechnologies, Madison, WI, USA), 10 μl of each fraction being diluted in 50 mM Tris-HCl, pH8 and assayed as above. Rates are expressed as pmol DMS μg $protein^{-1}$ min^{-1}. Protein concentrations were estimated by Bradford assay (Bio-Rad, Hemel Hempstead, UK).

Conversion of [1-^{13}C]DMSP to labelled acrylate was assayed by nuclear magnetic resonance as follows. *E. coli* strain BL21 with or without cloned *dddY* was grown overnight in complete medium and cultures were adjusted to equivalent OD_{600} values. Cells were pelleted, and then resuspended in M9 made with D_2O (> 99.9%), with 10 mM glycerol as C source, 10 mM [1-^{13}C]DMSP and 0.2 mM IPTG. Cells were incubated overnight at 28 °C, lysed with perchloric acid (final concentration 5% v/v) and incubated on ice for 10 min. Cell debris was spun down and nuclear magnetic resonance was done as in Todd *et al.* (2010).

To assay β-galactosidase, *Alcaligenes* strains containing pMP220 clones were inoculated into ABM with or without inducers (2 mM), grown overnight and assayed as in Rossen *et al.* (1985).

Bioinformatics

NCBI BLAST was used to search for homologues and metagenomes were searched using CAMERA

in the 'GOS: All ORF Peptides (P)' database (version 1.3.2.32). Alignments used Megalign in the DNAStar software package and Genedoc. Signal peptide predictions were from SignalP v3.0 (http://www.cbs.dtu.dk/services/SignalP/). Sequence of the *Alcaligenes faecalis* M3A *ddd/acu* region is deposited at Genbank, accession number HQ226120.

Results

The Alcaligenes faecalis *gene cluster involved in DMSP catabolism and DMS production*

To identify genes involved in DMSP catabolism, a genomic library of *A. faecalis* M3A was made in the wide host-range cosmid pLAFR3 (Materials and Methods section) and transferred *en masse* to the heterologous host *Pseudomonas putida* strain J450. This was the chosen recipient because it has many RNA polymerase sigma factors (Potvin *et al.*, 2008), so, *a priori,* should be better at expressing heterologous genes. Transconjugants that grew slowly on DMSP as sole carbon source appeared after ~7-days incubation, at a frequency of $\sim 10^{-3}$ per transconjugant. Two such colonies were purified; these also grew slowly on acrylate as sole carbon source and produced DMS on media containing DMSP (Ddd^+ phenotype). Plasmids were isolated from these *P. putida* transconjugants and transformed into *E. coli* strain 803 (Wood, 1966). When conjugated back to *P. putida*, both plasmids conferred slow growth on DMSP and on acrylate and a Ddd^+ phenotype to this host. Restriction digests showed that they contained overlapping cloned *Alcaligenes* genomic DNA. One of these, pBIO1895, was studied further.

Sequencing the ~30 kb cloned DNA in pBIO1895 revealed eight contiguous genes (Figure 1), five of which had previously been implicated in the ability of other bacteria to grow on DMSP (*ddd* genes) or acrylate (*acu*) as sole carbon sources (Todd *et al.*, 2010). Of particular interest, one gene, which we call *dddY,* encodes a polypeptide that corresponds to the DMSP lyase that was purified from *A. faecalis* M3A, and whose *N*-terminal sequence had been identified (de Souza and Yoch, 1995a, b, 1996). This allowed a comparison of the directly sequenced polypeptide with that of the *dddY* gene product, translated *in silico*—see below.

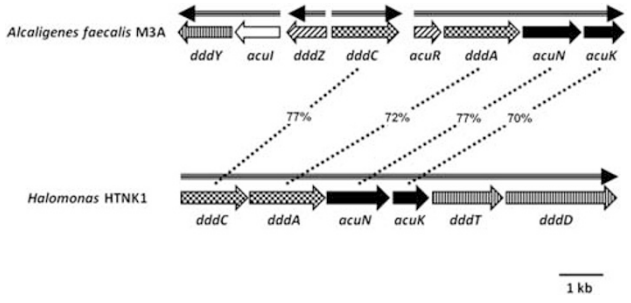

Figure 1 Comparison of the arrangements of *acu* and *ddd* genes in *Alcaligenes faecalis* M3A and *Halomonas* HTNK1. Locations, names and direction of transcription of the *acu* and *ddd* genes in *Halomonas* HTNK1 (Todd *et al.*, 2010) and *Alcaligenes faecalis* M3A are shown. Genes specifically involved in DMSP catabolism are shown with vertical lines, those for acrylate breakdown are black and those with a role with both substrates are chequered. Those with diagonal lines are regulatory. Striped arrows above the genes indicate their transcriptional organisation. The dotted lines show the equivalent genes in the two strains, along with the percentage identity of their gene products.

DddY is a periplasmic DMSP lyase

The deduced *dddY* product has a predicted M_r of 45.5 kDa. It has no overall similarity to any polypeptide with known function, so was hitherto a 'Domain Of Unknown Function'. However, it was strongly predicted (probability = 1.0 on SignalP 3.0) to be a periplasmic protein, with a 21 amino-acid leader (MQKRMLGGMVAGALACFQVQA) that would be cleaved from the holo-polypeptide by SecP protease (Figure 2). Importantly, this generates a processed polypeptide, the *N*-terminal sequence of which (AQFQCQDDVKPAAISAEE) corresponds very closely to the *A. faecalis* M3A DMSP lyase purified and sequenced by de Souza and Yoch (1996), although the deduced *dddY* gene product has a cysteine (underlined above) rather than the histidine that they found (Figure 2). This indicates that DddY is in the periplasm, as confirmed below.

To examine its function, we first made an insertional mutation into the genomic *A. faecalis dddY* gene. This completely abolished the resultant mutant's (J482) ability to make DMS from DMSP (Figure 3). Interestingly, though, it still grew normally on DMSP as sole carbon source, pointing to the presence of another, unknown, DMSP catabolic pathway in *A. faecalis*, the identification of which is currently under study.

We also amplified *dddY* from *A. faecalis* M3A genomic DNA and cloned the product into the expression vector pET21a. *E. coli* strain BL21 (Studier and Moffatt, 1986) harbouring the resultant plasmid (pBIO1912) efficiently generated DMS from DMSP (Table 1). Using [1-^{13}C]DMSP as substrate with *E. coli* containing pBIO1912, we confirmed by nuclear magnetic resonance that DddY was a DMSP lyase, as the labelled DMSP substrate had been converted to acrylate, in agreement with the earlier findings of Ansede *et al.* (1999) on the catabolic fate of DMSP in *A. faecalis* M3A itself.

E. coli containing cloned *dddY* was also used to show directly that DddY was located in the periplasm. *E. coli* cells expressing DddY were separated into periplasmic and spheroplastic fractions and these were assayed separately for DMSP-dependent DMS production. Consistent with the predicted location of DddY, the activity was ~11-fold greater in the periplasmic fraction (Table 1).

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Al. faecalis M3A                                          MQKRMLGGMVAGALACFQVQAAQFQcQDDVKPAAISAEEQ
Ar. nitrofigilis DSM 7299 (Arnit_0113)     MGHVKILKDNCIKWGSMILVTFVLMTNAKAGDLPTFLDKKDILNANFKCLG--TTTKYSKEEQ
Ar. nitrofigilis DSM 7299 (Arnit_2767)        MFILK----KLGVILILTNLLYG--AGLEIPNYLDKKDILNAEFKCLS--EPVKYNKKDQ
Shewanella baltica OS155 (Sbal_0256)             MK------KTIVALSLLSANGAMAADLPNFLDQSDIDNAQFKCLG--EPIKYSKADQ
S. frigidimarina NCIMB 400 (Sfri_3895)        MDDMK------YNTLLVMMLLSSSAMASEIPSFLDKNDVDNAEFTCLG--AESQYDKNDQ
S. halifaxensis HAW-EB4 (Shal_0333)              MK------YTMLLGSLLLSNGLVAAELPDFLDKKDINDAQFQCLG--ESPNYNAQDQ
S. pealeana ATCC 700345 (Spea_3937)              MK------YQILLGCLLASNGLAAAELPDFLDANDINNAQFQCLG--ESPKYSGEDQ
S. piezotolerans WP3 (swp_0222)                  MK------TKMLLGALLLSNGAFAAGIPDFLDAKEVKSAEFVCLG--EQPSYSAKDK
S. putrefaciens CN-32 (Sputcn32_3534)            MK------KTIIALSLLAVNGAMAADLPDFLDQSEIDNAQFKCLG--EPVTYSKTDQ
S. woodyi ATCC 51908 (Swoo_0277)                 MK------YMVLFSGLLFSNVLVASELPDFLDMNDIENAKFQCLG--ESPQYSAQDQ
S. sp. MR-4 (Shewmr4_2151)                       MK------KAIIALSLLSINAAMAANLPDYLDETDIYNARFKCLG--EPVKHSKADQ
S. sp. MR-7 (Shewmr7_2228)                       MK------KAIIALSLLSINAAMAANLPDYLDETDIYNARFKCLG--EPVKHSKADQ
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Figure 2 *N*-terminal regions of the DddY proteins in *Alcaligenes, Arcobacter* and *Shewanella*. The deduced *N*-terminal DddY holopolypeptides of strains of *Alcaligenes, Shewanella* and *Arcobacter* are shown. The underlined region in the *N*-terminal region of *Alcaligenes faecalis* M3A DddY corresponds to that which was directly sequenced, with a cysteine (lower case 'c') being found here, instead of the histidine residue reported by de Souza and Yoch (1996). The 'white on black' residues are the *N*-terminal amino acids of the processed polypeptides, as predicted by SignalP v3.0. Residues with grey backgrounds are those that may correspond in the leader sequences of the two DddY polypeptides of *Arcobacter nitrofigilis*. 'Al.' and 'Ar.' refer to *Alcaligenes* and *Arcobacter* strains, respectively, and gene numbers are shown in brackets.

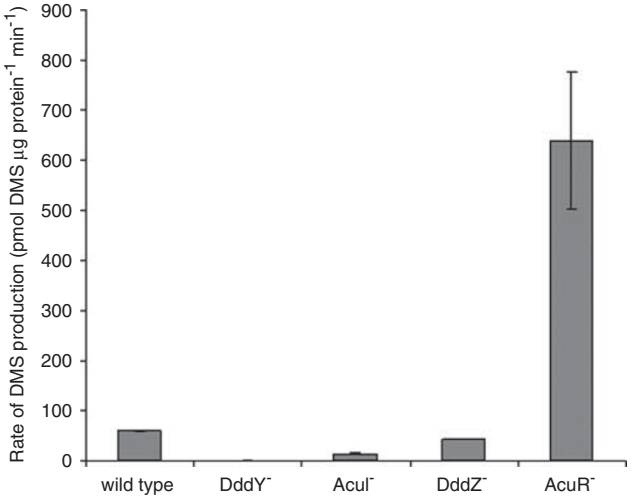

Figure 3 Effects of mutations in *acu* and *ddd* genes on DMSP-dependent DMS production in *Alcaligenes faecalis*. Wild-type *Alcaligenes faecalis* and mutant derivatives with insertions in *dddY, acuI, dddZ* or *acuR,* as indicated, were assayed for DMSP-dependent DMS production. Error bars are shown.

DddY homologues in other bacterial genomes and in metagenomes

DddY is not present in the deduced proteomes of any other *Alcaligenes* species whose genome sequences are available, and it has no close homologues in the current (August 2010) NCBI data base. However, it is ~35% identical over its entire length to a polypeptide made by some, but not all, species of the γ-proteobacterium *Shewanella,* namely *S. putrefaciens* CN-32, *S. woodyi* ATCC 51908, *S. baltica* OS155, *S. sp.* MR-7 and MR-4, *S. piezotolerans* WP3, *S. pealeana* ATCC 700345, *S. frigidimarina* NCIMB 400 and *S. halifaxensis* HAW-EB4*S*. It also has a similar level of identity to two polypeptides, encoded by two unlinked genes (Arnit_2767 and Arnit_0113, see http://genome.jgi-psf.org/arcni/arcni.home.html) in the ε-proteobacterium *Arcobacter nitrofigilis* DSM7299 (Pati *et al*., 2010). The sequences of these polypeptides in the different *Shewanella* species and in *A. nitrofigilis* closely resemble each other (~75% identical in amino acid sequence). Although all the DddY-like polypeptides in *Shewanella* and one of those (gp Arnit_0113) of *Arcobacter* are strongly predicted to be periplasmic, their *N*-terminal leaders are more diverse than the rest of the polypeptides (Figure 2). The sub-cellular location of the other DMSP lyase (gp Arnit_2767) in *Arcobacter* is uncertain; SignalP 3.0 had a low probability value for its being in the periplasm, although there is some sequence conservation in the *N*-terminal regions of both DddY polypeptides of this species (Figure 2).

No *Shewanella* or *Arcobacter* species were previously known to make DMS—indeed, no DMSP catabolism had been described in any of the ε-proteobacteria subphylum. Therefore, we examined the Ddd phenotypes of *S. putrefaciens* CN-32, *S. halifaxensis* HAW-EB4*S* and *Arcobacter nitrofigilis* DSM7299 and found that all three strains produced DMS from DMSP. Under these conditions, these two *Shewanella* species produced less DMS than *Alcaligenes faecalis,* but *Arcobacter nitrofigilis* made more than either. In contrast, *S. oneidensis* MR-1, a *Shewanella* species that lacks a *dddY*-like gene, made no DMS (Table 1).

To show that *dddY* of *Shewanella* is involved in DMS production, the *dddY* (gene tag Sputcn32_3534; http://genome.jgi-psf.org/shepu/shepu.home.html) of *S. putrefaciens* CN-32 was cloned into pET21a to form pBIO1913 and an *E. coli* transformant containing this plasmid had a Ddd$^+$ phenotype (Table 1).

Table 1 DMSP-dependent DMS production by different bacteria that contain or lack the *dddY* gene

Strain[a]	*Genotype/Description*	*DMS production*[b]
Alcaligenes faecalis J481	Wild type	60.1 (2.2)
Shewanella oneidensis MR-1	Wild type (no *dddY*)	0.05 (0.002)
S. putrefaciens CN-32	Wild type	1.7 (0.02)
S. halifaxensis HAW-EB4S	Wild type	0.59 (0.2)
Arcobacter nitrofigilis DSM 7299	Wild type	114.2 (13.7)
E. coli BL21:pET21a	Vector only control	0.05 (0.02)
E. coli BL21:pBIO1912	Cloned *dddY* of *A. faecalis*	559.7 (116)
E. coli BL21:pBIO1912	Cloned *dddY* of *A. faecalis*, periplasmic fraction	966.2 (318.5)
E. coli BL21:pBIO1912	Cloned *dddY* of *A. faecalis*, spheroplast fraction	89.9 (0.1)
E. coli BL21:pBIO1913	Cloned *dddY* of *S. putrefaciens*	12.4 (2.19)

Abbreviations: DMS, dimethyl sulfide; DMSP, dimethylsulfoniopropionate.
[a]Cells of wild-type *Alcaligenes*, *Shewanella* and *Arcobacter* strains, and *E. coli* strain BL21 with or without cloned *dddY* genes, were assayed for DMS production after growth in minimal medium.
[b]Rates of DMS production are expressed in pmol DMS μg protein^{-1} min^{-1} (average of two independent experiments) with standard errors shown in brackets.

We also searched for DddY polypeptides in metagenomic databases. Only one significant homologue was found, in sewage treatment bacteria (ctg6139; http://www.ncbi.nlm.nih.gov/pubmed/16998472); its polypeptide product was 35% identical to DddY of *Shewanella* over the 180 *C*-terminal amino acids that were available in this individual sequence read. Notably, there were no DddY homologues in the Global Ocean Sampling metagenome (Rusch *et al.*, 2007), which is well represented with homologues of the other DMS-emitting enzymes (DddD, DddL, DddP and DddQ) and the DMSP demethylase DmdA (Howard *et al.*, 2008; Raina *et al.*, 2010; Todd *et al.*, 2011).

Comparison of the ddd/acu gene clusters of A. faecalis *M3A and* Halomonas *HTNK1*

Near *dddY* of A. *faecalis* M3A, we noted that there are several *ddd* and *acu* genes, which resemble those involved in DMSP and acrylate catabolism in other bacteria that grow on DMSP and which have a Ddd$^+$ phenotype. For example, the γ-proteobacterium *Halomonas* HTNK1 catabolises acrylate and DMSP using pathways that are initially independent, but which converge on a shared catabolite, 3HP (Todd *et al.*, 2010; Figure 4). DMSP is converted to 3HP via an unusual Class III acyl-CoA transferase encoded by *dddD*, whereas two other enzymes, a dehydratase (*acuK* gene product) and a conventional CoA transferase (encoded by *acuN*) function together to generate 3HP from acrylate. The 3HP is converted to malonate semi-aldehyde via an alcohol dehydrogenase encoded by *dddC* and then, via the aldehyde dehydrogenase *dddA* gene product, to acetyl CoA, then into central metabolism.

The *ddd/acu* gene cluster of *A. faecalis* M3A also contains *acuN, acuK, dddA* and *dddC* genes, all of which products are at least 70% identical to the corresponding *Halomonas* enzymes, although the relative

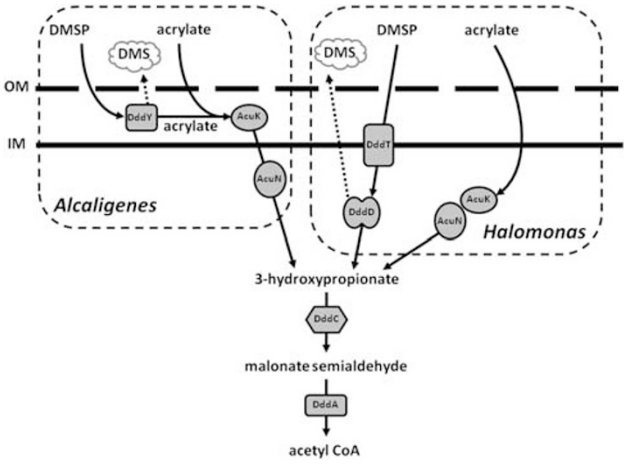

Figure 4 Pathways of DMSP and acrylate catabolism in *Halomonas* HTNK1 and *Alcaligenes faecalis* M3A. Likely pathways for DMSP and acrylate catabolism in *Halomonas* HTNK1 and *Alcaligenes faecalis* M3A are shown separated by dashed boxes (see Todd *et al.*, 2010). In *Alcaligenes,* the DddY and AcuK polypeptides are in the periplasmic space between the inner and outer membrane (IM and OM, respectively), whereas DddD and AcuK of *Halomonas* are cytoplasmic. *Halomonas* also has the DddT BCCT-type transporter in the inner membrane—no such transporter is found with *Alcaligenes*.

locations and transcriptional organisation of these genes differ in the two strains (Figure 1; see below). One other, significant difference concerns AcuK, which is involved in the initial step in acrylate catabolism (Todd *et al.*, 2010). AcuK of *Alcaligenes,* but not of *Halomonas,* has an 18 amino-acid leader that is strongly predicted to be removed by SecP protease.

The most notable difference between the *Alcaligenes* and *Halomonas* clusters is that the former uses the periplasmic DddY, whereas *Halomonas* has a totally different, cytoplasmic, enzyme DddD, which also releases DMS from DMSP (Todd *et al.*, 2007, 2010). Consistent with this,

upstream of *Halomonas dddD* is a gene, *dddT*, which encodes a BCCT-type transporter that imports DMSP into the cytoplasm. In contrast, DddY requires no transporter and there is no nearby transporter gene.

Three other genes are embedded in the *Alcaligenes* cluster. Two of these, *acuR* and *dddZ,* encode polypeptides in the TetR and LysR superfamilies of transcriptional regulators, respectively (see below). The third of these 'extra' genes is *acuI,* which encodes a member of the highly conserved zinc-containing super-family of alcohol dehydrogenases (PF08240). There are hundreds of close homologues (>72% identical) of *A. faecalis* AcuI in many, taxonomically diverse bacteria. AcuI-like polypeptides are encoded by genes in *ddd* clusters of other, known Ddd^+ bacteria including *Halomonas* and *Rhodobacter,* but the products of these *acuI*-like genes do not form a particular outgroup compared with the mass of other PF08240 family members and their exact functions are unknown.

Effects of mutations in the ddd/acu gene cluster of Alcaligenes faecalis *M3A*

The phenotype of the $DddY^-$ mutant of *Alcaligenes faecalis* pointed to the existence of another, unknown, pathway for DMSP catabolism in this strain (see above). Further evidence for this came from the effects of mutations in the other structural genes *acuI* (strain J483), *acuN* (J488), *acuK* (J489), *dddA* (J487), *dddC* (J485) and the two regulatory genes, *acuR* (J486) and *dddZ* (J484). Mutations in two of these genes affected DMSP-dependent DMS production. The $AcuI^-$ mutant (J483) produced less DMS than wild type (by ~four-fold), most likely due to polar effects on *dddY* expression, which is in the same transcriptional unit (see below). In contrast, the $AcuR^-$ mutant J486 produced ~11-fold more DMS than wild type, consistent with AcuR being a negatively functioning regulator, as predicted from its sequence, and confirmed below.

All the mutants grew as well as the wild type on 5 mM DMSP, consistent with the presence of another, unknown pathway for DMSP catabolism in *A. faecalis*. On acrylate, the $AcuI^-$ and $DddY^-$ mutants grew normally, but the $DddZ^-$ (J484), $DddC^-$(J485), $AcuR^-$(J486), $DddA^-$(J487), $AcuN^-$(J488) and $AcuK^-$(J489) mutants all failed to grow on this substrate. The $AcuR^-$ mutant phenotype is likely due to polarity on the downstream *dddA*, *acuN* and *acuK* genes. The $DddZ^-$ mutant may be defective on acrylate because this LysR-type regulator is required for expression of genes involved in acrylate catabolism—this will be presented elsewhere.

Using the above experimental and bioinformatic information, we constructed a likely pathway for the catabolism of DMSP and acrylate in *A. faecalis* (Figure 4). This resembles that which we had elucidated for *Halomonas*, the key differences being (i) the subcellular locations of the initial enzymatic biotransformations of DMSP and of acrylate and (ii) that in *Halomonas*, the 3HP intermediate is formed independently from DMSP and from acrylate, but in *Alcaligenes,* the reactions are sequential, with DMSP being converted to acrylate, then to 3HP.

Regulation of acu *and* ddd *genes of* Alcaligenes faecalis *M3A*

DMS production in *A. faecalis* M3A was shown to be inducible, not only by the substrate DMSP, but also, more surprisingly, by the catabolites acrylate and 3HP (Yoch, 2002). We confirmed this, showing that pre-growth of *A. faecalis* in 2 mM DMSP, acrylate or 3HP led to increased DMS production by factors of 33-, 27- and 15-fold respectively compared with the controls. We set out to establish whether this induction was due to enhanced expression of *dddY*.

The *A. faecalis dddY* gene is predicted to be co-transcribed with the upstream *acuI* gene, with only a small gap (68 bps) between them. A fragment spanning the proposed promoter region of this operon, upstream of *acuI,* was cloned into the wide host-range *lacZ* promoter-probe plasmid pMP220. The resulting *acuI-lacZ* plasmid, pBIO1905, was mobilized into *A. faecalis* M3A and the transconjugants were assayed for β-galactosidase activity following growth of the cells in minimal medium and also in media supplemented with DMSP, acrylate or 3HP, each at 2 mM. As shown in Figure 5, β-galactosidase activity was significantly enhanced (~six-fold above background) when the cells were pre-grown with acrylate or with DMSP, but only slightly (<two-fold) with 3HP. In contrast, a fragment that spanned the *acuI-dddY* intergenic region, cloned into pMP220 to form pBIO1904, did not

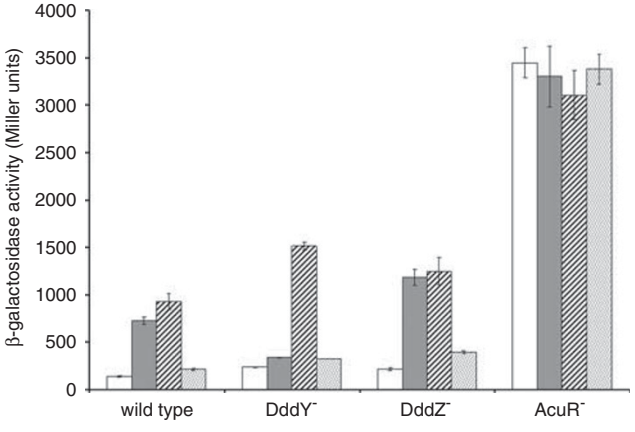

Figure 5 Effects of different co-inducers and mutations in regulatory genes on expression of *Alcaligenes faecalis acuI-dddY* operon. Wild-type *Alcaligenes faecalis* and mutant derivatives with insertions in *dddY, acuR* or *dddZ* as indicated, and containing the *acuI-lacZ* transcriptional fusion plasmid pBIO1905, were grown in minimal media with no inducer (no fill), or with DMSP (grey), acrylate (diagonal stripes) or 3HP (stippled), each at 2 mM. Cells were assayed for β-galactosidase activity as described in the text. Errors bars are shown.

confer any β-galactosidase activity under any growth conditions, consistent with *acuI* and *dddY* being co-transcribed.

In an attempt to identify the gene(s) responsible for regulating transcription of the *acuI-dddY* operon, pBIO1905 was mobilised into the AcuR$^-$ and DddZ$^-$ mutant strains (J486 and J484, respectively). Compared with wild type, the *acuR* mutation caused markedly increased, constitutive expression of the *acuI-lacZ* fusion, even in the absence of any co-inducer, consistent with the hyper-production of DMS in the AcuR$^-$ mutant (see above). In contrast, the *dddZ* mutation did not affect expression of the fusion under any of the growth conditions. The fusion plasmid was also mobilized into the DddY$^-$ mutant strain, J482. In this background, DMSP no longer functioned as a co-inducer, although acrylate retained its co-inducing ability. This suggests that DMSP itself is not a co-inducer, but must first be converted to the likely *bona fide* co-inducer acrylate by the DddY lyase.

Discussion

More than 10 years ago, Yoch's laboratory demonstrated three features of DMSP catabolism in *Alcaligenes faecalis* M3A: (i) DMSP cleavage occurred near the bacterial cell surface, not in the cytoplasm (ii) acrylate was the immediate catabolite of DMSP, followed by its conversion to 3HP and (iii) DMSP-dependent DMS production was induced by the DMSP substrate, and by some of its catabolites. The work described here provides a genetic explanation for these biochemically based observations.

From its sequence, and ratified by direct measurement, DddY is indeed a periplasmic enzyme. Apart from its *N*-terminal leader, it does not resemble any enzyme with known function and has no recognisable functional motifs. Therefore, DddY must use novel mechanisms to cleave DMSP into acrylate plus DMS, compared with the other lyases that effect the same biotransformation, namely DddP, DddL and DddQ (Curson *et al.*, 2008; Kirkwood *et al.*, 2010a; Todd *et al.*, 2009, 2011).

The pathway of DMSP catabolism in *Alcaligenes* resembles that of *Halomonas,* but also has some differences, stemming from the different compartments of the primary enzymes, DddD and DddY, which generate DMS from DMSP in the cytoplasm and periplasm, respectively. These different subcellular locations are consistent with (a) the presence of a dedicated DMSP transporter gene, *dddT,* in *Halomonas,* but not in *Alcaligenes* and (b) the deduced periplasmic location of the *Alcaligenes* AcuK acrylate dehydratase, whereas AcuK of *Halomonas* is cytoplasmic. AcuK is homologous to *E. coli* CaiD, a hydratase that functions in concert with the CoA transferase, CaiB, to hydrate crotonobetaine to L-carnitine, the CaiD-mediated hydration being followed by transfer of CoA from carnitine via CaiB (Elssner *et al.*, 2001). In *Alcaligenes,* the second step in the conversion of acrylate to 3HP is predicted to be mediated by AcuN, which is homologous to CaiB, and lacks a leader, so is likely to be cytoplasmic. These bioinformatically based predictions tally with earlier biochemical studies showing that acrylate was converted to 3HP at the *Alcaligenes* cell surface (de Souza and Yoch, 1995a, b). Genomic mutations in the *ddd* and *acu* genes in *A. faecalis* caused no detectable effects on growth on DMSP as a carbon source. Thus, *A. faecalis* must have other ways of degrading this substrate. Other bacteria can catabolise DMSP in more than one way—for example, *Ruegeria pomeroyi* demethylates DMSP via the DmdA demethylase (Howard *et al.*, 2006) and has at least two DMSP lyases, DddP and DddQ (Todd *et al.*, 2009, 2011). We have access to a draft genome sequence of *A. faecalis* M3A and noted that it lacks *dmdA* and has no known other *ddd* genes, so this additional inferred DMSP catabolic pathway must involve some as yet unidentified gene products. In contrast to the lack of effects with DMSP as carbon source, *A. faecalis* AcuN$^-$, AcuK$^-$, DddA$^-$ and DddC$^-$ mutants were defective for growth on acrylate, consistent with the pathway shown in Figure 4.

The regulation of the *ddd/acu* cluster in *A. faecalis* may be complex, as it contains two predicted regulatory genes, *acuR* and *dddZ,* the former being a ratified regulator of *acuI-dddY*. Being in the TetR family, AcuR is expected to be a repressor (Ramos *et al.*, 2005). Consistent with this, the AcuR$^-$ mutant strain overproduces DMS, and has high-level, constitutive expression of the *acuI-dddY* operon. The repression is relieved by DMSP, but this requires its conversion to acrylate, or some further catabolite, which is the *bona fide* co-inducer. For reasons that are unknown, induction by DMSP catabolites is a feature of genes involved in DMSP catabolism, not only in *A. faecalis,* but also in other bacteria.

We have not found a role for *dddZ,* as the *acuI-dddY* operon was expressed normally in the DddZ$^-$ mutant, as were the *dddC, dddZ* and *acuR-dddA-acuNK* transcriptional units (unpublished). However, a DddZ$^-$ mutant fails to grow on acrylate, suggesting a role in the expression of some unknown acrylate catabolic genes. Most LysR family members function positively in the presence of a cognate co-inducer to activate expression of their target transcriptional units (Maddocks and Oyston, 2008). Maybe, DddZ requires some co-inducer compound that remains to be identified in order to exert its regulatory properties.

Like other *ddd* genes, *dddY* seems to have spread by HGT among distantly related lineages, as close homologues occur in species of *Alcaligenes, Shewanella* and *Arcobacter* (β-, γ- and ε-proteobacteria, respectively). Although these are only distantly related to each other, members of these three genera are all associated with microaerobic

environments. Thus, *Shewanella sp.* have flexible respiratory abilities and are mostly marine, occurring in sediments and other anoxic conditions, although some strains come from aerobic seawater (Hau and Gralnick, 2007; Fredrickson *et al.*, 2008). The two *Shewanella* species examined here, *S. putrefaciens* CN-32 and *S. halifaxensis* HAW-EB4*S*, are from anaerobic subsurface shale sandstone and marine sediment, respectively. Interestingly, *Arcobacter nitrofigilis* DSM7299 is a diazotroph isolated from sediment around *Spartina* roots, the same type of environment as for *A. faecalis*. *Arcobacter* is closely related to *Campylobacter* (*A. nitrofigilis* was originally *Campylobacter nitrofigilis*) and contains pathogens such as *A. butzleri*, but this genus is also environmentally important, being abundant in hydrothermal vents (Moussard *et al.*, 2006). Further evidence for HGT came from the observation that a DMSP lyase in the marine Chlorophyte *Ulva curvata* cross-reacted with antibody raised against the *A. faecalis* DMSP lyase (de Souza *et al.*, 1996). However, the algal polypeptide (78 kDa) was larger than the 48 kDa bacterial lyase, and it will be of interest to compare these two enzymes at a molecular level to see if this represents another case of inter-Domain HGT, as was found with the DddP DMSP lyase (Todd *et al.*, 2009; Kirkwood *et al.*, 2010b).

We noted that the *dddY* of *Shewanella* species and one of those in *A.nitrofigilis (*Arnit_2767) are adjacent to genes, the products of which are membrane-bound cytochromes, raising the question that in these bacteria, DddY may have a role in anaerobic respiration, rather than in food supply, as occurs in *A. faecalis*. In this connection, *Desulfovibrio acrylicus,* uses exogenous acrylate as an electron acceptor for anaerobic respiration. It also has a DMSP lyase with some features that resemble DddY (van der Maarel *et al.*, 1996a, b); this enzyme could supply acrylate when these bacteria have access to DMSP.

The association of *dddY* with bacteria that favour microaerobic environments may also account for the lack of DddY homologues in the published metagenomes of marine bacteria, as these represent the aerobic upper reaches of the oceans. Marine sediments harbour vast numbers of live bacteria, as much as 30% of the world's total (Schippers *et al.*, 2005). The DddY DMSP lyase, though rare in the upper oceans, may be abundant in such environments, particularly where DMSP is available, as occurs in saltmarshes where *Spartina* can form the predominant flora. Future surveys of the microbial metagenomes of such ecosystems may therefore be instructive in this regard.

Perhaps the most striking feature to emerge from recent genetic work on bacterial DMS production is the remarkable diversity in the genes and hence the enzymes and pathways that are involved in this process in different bacteria. DddY was the first DMSP lyase to be characterised, in 1995, and our analysis of the corresponding gene expands the novelty of this enzyme. Future work on DddY therefore, can range from ecological examinations of its importance in anoxic habitats to molecular enzymology, aimed at elucidating the catalytic mechanisms that this hitherto 'Domain of Unknown Function' uses to cleave its environmentally important substrate.

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