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Diversity of DMSP transport in marine bacteria, revealed by genetic analyses

Lei Sun · Andrew R. J. Curson · Jonathan D. Todd · Andrew W. B. Johnston

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Abstract The enzyme product of the *dddD* gene, found in several different marine bacteria, acts on dimethylsulfoniopropionate (DMSP), liberating dimethyl sulfide (DMS) and generating 3-OH-propionate as the initially detected C3 product. In many bacteria, *dddD* is near genes whose sequence suggests that they encode a DMSP transporter. These are of two very different types, in the BCCT (betaine-carnitine-choline transporter) family or resembling members of the ABC super-family that import betaines. Even within these two families, the amino acid sequences of these putative transporters are not particularly similar to each other. Genes for the predicted DMSP transporters of *Halomonas* and *Marinomonas* (both BCCT type) and of *Burkholderia ambifaria* AMMD (ABC-type) were each cloned and introduced into an *Escherichia coli* mutant (MKH13) that is defective in betaine uptake, and so fails to catabolise DMSP even when a cloned *dddD* gene was present, due to the failure of the substrate to be imported. DMSP-dependent DMS production (Ddd^+ phenotype) was restored by introducing any of these cloned transporters into MKH13 containing *dddD*. Other marine bacteria use a range of enzymes, called DddL, DddP, DddQ, DddW and DddY, to cleave DMSP, but the various *ddd* genes that encode them are usually unlinked to any that are predicted to encode betaine transporters. We identified one gene in *Sulfitobacter* sp. EE-36 and two in *Roseovarius nubinhibens* ISM, which, when cloned and introduced into *E. coli* MKH13, overcame its osmotic sensitivity when it was grown with DMSP or other exogenous betaines. These genes all encoded BCCT transporters, but were unlinked to any known genes involved in DMSP catabolism in these two strains of α-proteobacteria.

Keywords ABC transporter · BCCT transporter · *ddd* Genes · DMSP · *Halomonas* · *Marinomonas* · *Roseovarius* · *Sulfitobacter*

Lei Sun and Andrew R. J. Curson contributed equally to the work.

L. Sun · A. R. J. Curson · J. D. Todd · A. W. B. Johnston (✉)
School of Biological Sciences, University of East Anglia, Norwich NR4 7TJ, UK
e-mail: a.johnston@uea.ac.uk

Present Address:
L. Sun
Department of Biosciences, University of Kent, Canterbury CT2 7NJ, UK

Introduction

Direct uptake experiments reveal that many marine bacteria, including the cyanobacteria *Synechococcus* and *Prochlorococcus*, as well as many heterotrophs, can actively assimilate DMSP (Malmstrom et al. 2004; Vila-Costa et al. 2006). Indeed, the hugely abundant SAR11 clade of α-proteobacteria are only able to use

reduced forms of sulfur, such as DMSP, and cannot assimilate sulfate, whose high oxidation state deters its subsequent use as a source of biologically incorporated sulfur (Tripp et al. 2008). And, several eukaryotic marine plankton, including diatoms, also sequester this abundant molecule (Vila-Costa et al. 2006).

DMSP, once imported into microbial cells, is subject to various biotransformations. Some of the catabolic pathways involve enzymatic cleavage of DMSP, with the release of dimethyl sulfide (DMS) as one of the initial products, the colloquial term for such enzymes being "DMSP lyases". We have used genetics to identify several DMSP lyases in a range of different bacteria, and in a few eukaryotic fungi. With one of these, termed DddD, the "other" initial catabolite is 3-OH-propionate (Todd et al. 2007). In contrast, five other lyases, DddL, DddP, DddQ, DddW and DddY, each generate acrylate plus DMS, even though they are in different polypeptide families (Curson et al. 2008, 2011b; Todd et al. 2009, 2011a, b). Another bacterial enzyme, termed DmdA, demethylates DMSP to form methylmercaptopropionate, the first step of a demethylation pathway that is important in terms of global DMSP catabolism, but which liberates no DMS (Howard et al. 2006).

In addition to the *ddd* and *dmdA* genes that encode the "primary" catabolic enzymes that act on DMSP, some bacteria have other, closely linked *ddd* genes that encode polypeptides that are variously involved in downstream catabolic steps, gene regulation or, relevant to the work described here, the transport of DMSP (see Johnston et al. 2008).

DMSP is a sulfonium analogue of β-alanine betaine, an osmoprotectant used by some stress-tolerant plants in the Plumbaginaceae (Hanson et al. 1994), and it also resembles glycine betaine, which is an osmolyte for most terrestrial organisms. Sulfur is abundant in the oceans, but nitrogen is scarce, relative to their concentrations in terrestrial and freshwater environments. This perhaps explains why DMSP is widely used in marine algae, whereas the nitrogen-containing glycine betaine is the osmolyte of choice in terrestrial and freshwater environments. Despite the attraction of this adaptive explanation, there is little direct evidence that osmotic protection is the primary role of DMSP in those marine and littoral organisms that make the molecule. There are reports that some plankton respond to increasing salinity by raising their intracellular concentrations (Dickson and Hirst 1987; Karsten et al 1992), but in other organisms, increased salinity does not affect their intracellular levels of DMSP (see Otte et al. 2004).

Kiene et al. (1998) showed that assemblages of microbes in seawater imported ^{14}C-labeled glycine betaine and ^{35}S-labeled DMSP with similar kinetics and that the addition of unlabeled forms of one of these substrates inhibited the uptake of the labeled version of the other. This strongly suggests that these two molecules share one or more common transporters. There have been some direct demonstrations that DMSP is an effective osmoprotectant, but these were mostly done in artificial conditions, with organisms such as the enteric bacteria *Escherichia coli, Salmonella* and *Klebsiella*, which almost certainly never encounter DMSP in their own natural environments (Cosquer et al. 1999). Nevertheless, these studies confirmed that DMSP can be imported by the same transporters as those used for betaines and related molecules, and so have generated a body of knowledge on how DMSP is transported into bacteria.

Betaine import is mediated by at least two very different types of bacterial transporters, in the BCCT (betaine-choline-carnitine-transporter) and the ABC (ATP-binding cassette) families of proteins. The former of these (reviewed by Ziegler et al. 2010), comprise a homo-trimer of a polypeptide with twelve trans-membrane α-helices. Rather confusingly, genes for BCCT transporters have different nomenclatures in different bacteria; e.g. *opuD, betP* and *betT* encode the BCCT polypeptides of *Bacillus subtilis*, *Corynebacterium glutamicum*, and *E. coli* respectively.

The ABC transporters are one of the largest families of proteins on Earth. Their canonical components (in Gram negative bacteria) comprise a periplasmic substrate-binding protein, a transporter in the cytoplasmic membrane and an ATPase to provide the energy, although there are many variations on this theme in different bacteria (see Eitinger et al. 2011). ABC transporters involved in betaine import are exemplified by the ProU and OpuA systems of *E. coli* and *B. subtilis* respectively. Note that their nomenclature is not standard, so that (e.g.) "ProU" comprises three polypeptides — ProX is the periplasmic binding protein, ProW the cytoplasmic membrane transporter and ProV the ATPase (Gowrishankar 1989), and in *B. subtilis*, the corresponding functions are supplied respectively by the OpuAC, OpuAB and OpuAA polypeptides (Kempf et al. 1997).

Here, we describe the diversity of DMSP import into different bacteria. This includes *in silico* considerations of the products of predicted transporter genes in *ddd* gene clusters and the functional ratification of some of these. We also describe some unlinked genes that encode DMSP importers in two species of Roseobacters, an important marine alpha-proteobacterial clade, many of which catabolise DMSP.

Materials and methods

Bacterial strains, plasmids and general growth conditions

Strains of *E. coli* were grown routinely at 37°C in liquid or solid LB medium.

Genetic manipulations

Cloning, DNA isolation and routine genetic manipulations involving plasmid transfer by transformation or conjugation were as described in Wexler et al. (2001).

Assays

To measure DMS production by *E. coli*, cells from overnight LB cultures of the various strains were diluted into M9 minimal medium, adjusted to $OD_{600} = 0.2$, and placed in sealed 2 ml vials (12 × 32 mm, Alltech Associates). DMSP was added to a final concentration of 5 mM and, as necessary, vials were also supplemented with NaCl (final concentration 0.2 M). After incubating the vials for 24 h at 28°C, DMS was assayed essentially as in Todd et al. (2007).

Growth on hypersaline media was examined by growing overnight cultures of the *E. coli* strains in LB, then diluting these 10^{-4} and plating 20 µL on LB medium containing 0.6 M NaCl, with or without added osmolyte (0.1 mM).

In silico analysis

BLAST searches were done using NCBI BLAST and protein sequences were aligned using Megalign and Editseq in the DNASTAR-Lasergene v6 package, with alignments done using ClustalV.

Results

BCCT-type transport genes linked to *dddD*

The first DMSP lyase to be identified at a genetic level was DddD, which has a characteristic architecture of two tandemly arranged Class III CoA transferase domains, separated by a short inter-domain linker (Todd et al. 2007). In several, though not all, bacteria that contain *dddD,* this gene is in a cluster with several other *ddd* genes involved in DMSP catabolism, including one or more that encode predicted DMSP importers. Thus, in the marine γ-proteobacterium *Marinomonas* sp. MWYL1, *dddD* is a one-gene operon, transcribed divergently from *dddTBCR,* in which *dddT* encodes a predicted BCCT-type betaine transporter, *dddB* and *dddC* encode downstream catabolic enzymes and the *dddR* gene product is a transcriptional regulator (Fig. 1). Genes that are predicted to encode BCCT-type transporters occur near *dddD* of some other γ-proteobacteria including strains of *Halomonas*, *Psychrobacter* and *Pseudomonas,* and also in some more distantly related α-proteobacteria, such as *Sagittula stellata* E37 and *Pseudovibrio* sp. JE062, which are both in the Rhodobacterales (Fig. 1).

One strain, the γ-proteobacterium *Oceanimonas doudoroffii* DSM7028, has no less than four genes that likely encode BCCT-type transporters near its *dddD* gene (Curson et al. 2011a), with one of these, termed $dddT^{D1}$, being transcribed divergently from *dddD.* Furthermore, *O. doudoroffii* has three additional BCCT-type transporters, encoded by genes located near *dddP1* and *dddP2,* which both encode DMSP lyases that are very different from DddD (Curson et al. 2011a).

Interestingly, when the sequences of these DddT-like polypeptides were compared, they did not cluster in a DMSP-specific subset of the BCCT family, and do not reflect the taxonomic distances among the corresponding species. For example DddT of *Marinomonas* sp. MWYL1 is very similar (>70% identical) not only to corresponding polypeptides in other close relatives (e.g. *Marinobacter* sp. ELB 17), but it also closely resembles (68% identical) the product of the *dddT* gene of the α-proteobacterium *Pseudovibrio* sp. JE062. Conversely, DddT of *Halomonas* sp. HTNK1, is quite distinct from that of *Marinomonas* (only 30%

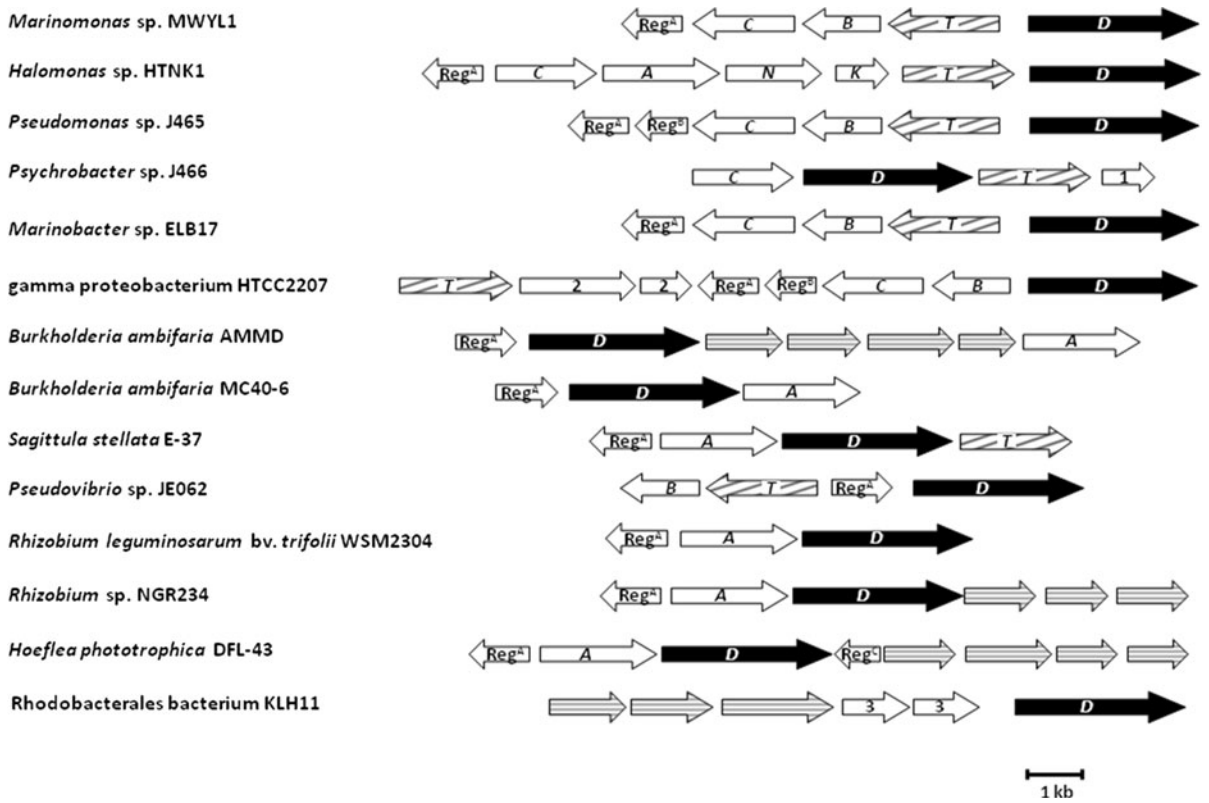

Fig. 1 Gene maps showing transporter genes linked to *dddD* in different bacteria. Selected bacterial species are listed and genes, drawn to scale, are portrayed as individual arrows. The *dddD* genes are filled in with black. BCCT-type transporter genes have diagonal stripes and those that encode ABC systems are shown with horizontal stripes. Other linked genes are unfilled. Those whose function is known (or inferred) to be involved in DMSP catabolism are tagged with different letters that correspond to the particular *ddd* gene. Those whose function is not known to be associated with DMSP have numbers, listed below, and different types of transcriptional regulators are also marked. *A* DddA—glucose-methanol-choline oxidoreductase, *B* DddB—iron-containing alcohol dehydrogenase, *C* DddC—methylmalonate semialdehyde dehydrogenase, *N* AcuN—Class III acyl-CoA transferase, *K* AcuK—enoyl-CoA dehydratase, Reg^A LysR-type regulator, Reg^B IclR-type regulator, Reg^C GntR-type regulator, *1* Enoyl-CoA hydratase, *2* Hypothetical protein, *3* Agmatinase, The bacterial strains, with the gene accession numbers of their *dddD* genes shown in brackets are: *Marinomonas* sp. MWYL1 (YP_001342872.1); *Halomonas* sp. HTNK1 (ACV84065.1); *Pseudomonas* sp. J465 (ACY01992.1); *Psychrobacter* sp. J466 (ACY02894.1); *Marinobacter* sp. ELB17 (ZP_01735760.1); *Burkholderia ambifaria* AMMD (YP_776185.1); *Burkholderia ambifaria* MC40-6 (YP_001811485.1); *Sagittula stellata* E-37 (ZP_01746034.1); *Pseudovibrio* sp. JE062 (ZP_05082536.1); *Rhizobium leguminosarum* bv. *trifolii* WSM2304 (YP_002278602.1); *Rhizobium* sp. NGR234 (YP_002822700.1); *Hoeflea phototrophica* DFL-43 (ZP_02165273.1); Rhodobacterales bacterium KLH11 (ZP_05125228.1)

identical), even though both these strains are γ-proteobacteria.

ABC-type transport genes linked to *dddD*

An even more striking indication of the diversity of potential DMSP transporter genes is that in some other bacteria, namely *Burkholderia ambifaria* AMMD (β-proteobacterium), *Rhizobium* sp. NGR234, *Rhodobacterales* bacterium KLH11 and *Hoeflea phototrophica* DFL-43 (all α-proteobacteria), *dddD* is closely linked to genes that encode the components of ABC transporters (Fig. 1). However, as with the BCCT-type transporters (above), these ABC polypeptides do not form a coherent, closely linked subset of the ABC super-family. For example, the ABC transporters in *Rhodobacterales* bacterium KLH11 and *Rhizobium* sp. NGR234 are both annotated as being in the proline/glycine betaine transport sub-group of the ABC transporters (COG2113), represented by OpuAC (see Igarashi and Kashiwagi 2010), yet their periplasmic binding proteins are only 23% identical to each other.

Similarly, the corresponding polypeptides in *B. ambifaria* and *H. phototrophica* are both predicted to be in a different sub-family (COG0687), of spermidine/putrescine binding transport systems, yet, here too, these predicted DMSP transporters are not closely related to each other (9% identical).

Direct confirmation of the function of predicted DMSP transporters

Todd et al. (2010) showed directly that *dddT* of *Halomonas* sp. HTNK1 encoded a DMSP transporter by introducing the cloned gene into a mutant *E. coli* strain (MKH13) that was defective in betaine uptake because of multiple mutations in *putPA, proP* and *proU,* which respectively encode a Na^+/proline symporter, an integral membrane betaine transporter and an ABC transporter system for betaine uptake (Haardt et al. 1995). The *Halomonas dddT* restored the ability of the mutant to import [1-^{14}C]DMSP and it also complemented the hyper-sensitivity of MKH13 to NaCl in the medium, when exogenous osmolytes such as DMSP and glycine betaine were added to the medium (Todd et al. 2010).

To confirm experimentally that some of the other predicted transporters can import DMSP, we again took advantage of *E. coli* MKH13, but used a different phenotype to measure the correction of this mutant. We reasoned that MKH13 would not catabolise DMSP even if a gene that encoded a DMSP lyase was introduced into it, since the DMSP substrate would not be imported. This was ratified as follows. The *dddD* gene of *Rhizobium* sp. NGR234 (Trinick 1973) was cloned into the plasmid vector pUC18 (Messing et al. 1983) to form pBIO1609. This plasmid was transformed into *E. coli* MKH13 and also into its wild type parent, MC4100 (Casadaban 1976) and the resulting transformants were assayed for DMS production when grown on media with DMSP. The cloned *dddD* gene in pBIO1609 did confer a Ddd^+ phenotype to the wild type *E. coli* but not to MKH13, for which DMS production was only at background (buffer alone) levels (Fig. 2).

As a prelude to examining if the BCCT-type transporters of *Halomonas* sp. HTNK1 and *Marinomonas* sp. MWYL1 and the PotABCD ABC-type transporter of *Burkholderia ambifaria* AMMD could overcome the defect in the *E. coli* mutant MHK13, the corresponding genes were amplified from the corresponding genomic DNA, then cloned into the wide host-range plasmid pRK415 (Keen et al. 1988) such that the cloned genes would be transcribed from a vector promoter. Each of these plasmids was mobilised in tri-parental conjugational crosses (Figurski and Helinski 1979) into *E. coli* MKH13 containing pBIO1609 and the three sets of transconjugants were assayed for their Ddd^+ phenotype. All were found to make DMS from DMSP, with the levels of activity conferred by the two *dddT* genes from *Halomonas* and *Marinomonas* in the MKH13 background being significantly greater than that by the cloned *potABCD* genes of *B. ambifaria* AMMD (Fig. 2). The effectiveness of BCCT transporters of several different bacteria is enhanced when cells are grown in hypersaline conditions, due to a conformational change of the proteins in response to the ionic strength (see Ziegler et al. 2010). Consistent with this, when 0.2 M NaCl was present in the medium of MC4100/pBIO1609 prior to assaying its Ddd phenotype, the levels of DMS were significantly greater than when it was grown in the regular M9 medium, which contains only 0.04 M NaCl (Fig. 2). A similar NaCl-dependent enhancement in DMS production was also seen when each of the cloned *dddT* genes (but not the *potABCD* genes) were introduced into the mutant MKH13 containing pBIO1609 (Fig. 2).

We also examined the effects of these cloned transporters on salt tolerance of MKH13. As expected, they all allowed this mutant to grow on medium that contained 0.1 mM DMSP as the osmolyte, plus 0.6 M NaCl, a salt concentration that is tolerated by the wild type strain MC4100 but not by the mutant MKH13 itself. We examined the protective effects of two other potential osmolytes, glycine betaine and choline, both at 0.1 mM; these also overcame the growth defect on NaCl in the MKH13 derivatives that harboured *dddT* of *Marinomonas* or *Halomonas*, or *potABCD* of *Burkholderia.* Thus, although their genetic locations suggest that the primary roles of these genes are for DMSP uptake, they nevertheless encode transporters that import other betaine substrates, in keeping with earlier findings on the limited levels of substrate specificity seen in other BCCT polypeptides (Ziegler et al. 2010).

Lack of DMSP transport genes near *dddL, dddP, dddQ, dddW* and *dddY*

In addition to DddD, the DddL, DddP, DddQ, DddW and DddY DMSP lyases also act on DMSP, but these

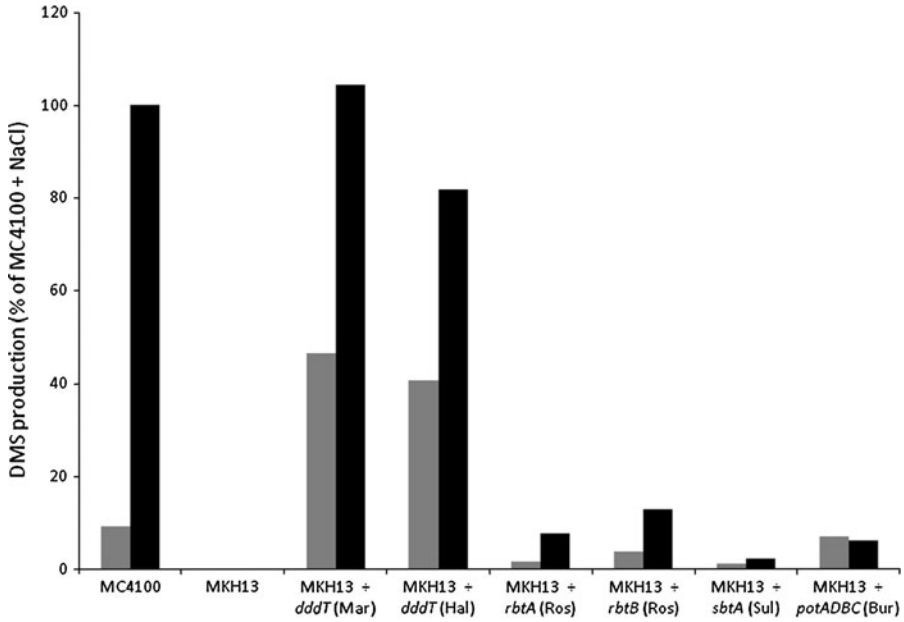

Fig. 2 Effects of cloned BCCT-type and ABC-type transporters on the Ddd phenotype of the permease mutant *E. coli* MKH13 containing the cloned *dddD* gene. The strains used were derivatives of *E. coli* wild type (MC4100) or the betaine uptake mutant (MKH13) that also contain the *dddD* gene of *Rhizobium* NGR234, cloned in plasmid pBIO1609. In some cases, the strains also contained plasmids into which transporter genes identified in this study had been cloned. Levels of DMS production are given as a %age, relative to the 100% value of wild type MC4100/pBIO1609, pregrown in +NaCl medium. The bacterial sources of the cloned transporter genes are as follows: Mar, *Marinomonas* sp. MWYL1; Hal, *Halomonas* sp. HTNK1; Ros: *Roseovarius nubinhibens* ISM; Sul, *Sulfitobacter* sp. EE-36; Bur: *Burkholderia ambifaria* AMMD. Assays were done in triplicate on each strain following growth in M9 minimal medium (*grey bars*) and in M9 with NaCl added to a final concentration of 0.2 M (*black bars*)

five enzymes generate DMS plus acrylate, even though they are in different polypeptide families (Curson et al. 2008, 2011b; Todd et al. 2011a, b). The DddL, DddP, DddW, and DddQ enzymes occur mostly in the Roseobacter clade of α-proteobacteria, although there is evidence of horizontal gene transfer (HGT) of *dddP* to other bacteria and even to some Ascomycete fungi (Kirkwood et al. 2010; Todd et al. 2009). Unlike *dddD,* most examples of the *dddL, dddP*, *dddW* and *dddQ* genes in different bacteria have no nearby genes that encode potential DMSP transporters. An exception is the γ-proteobacterium *Oceanimonas doudoroffii,* which contains versions of both *dddD* and *dddP* (de Souza and Yoch 1995; Curson et al. 2011a). As mentioned above, its *dddD* gene has four nearby genes that encode BCCT transporters, and three BCCT transporters are encoded by genes close to its two *dddP* genes, with two of these ($dddT^{P1-1}$ and $dddT^{P1-2}$) being in the vicinity of *dddP1* and one of them ($dddT^{P2}$) lying immediately downstream of *dddP2* (Curson et al. 2011a).

Finally, the DddY DMSP lyase, found in a strain of *Alcaligenes* (β-proteobacteria), is unique (so far) in being in the bacterial periplasm and not the cytoplasm, the sub-cellular location of the other DMSP lyases (see Yoch 2002; Curson et al. 2011b). Although there are nearby *ddd* catabolic and regulatory genes, none of these encodes a transporter, consistent with the fact that the periplasmic DddY has no need for an active DMSP import system (Curson et al. 2011b).

Identification of DMSP transporters in *Sulfitobacter* sp. EE-36 and *Roseovarius nubinhibens* ISM

The Roseobacter strains *Sulfitobacter* sp EE-36 (González et al. 1996) and *Roseovarius nubinhibens* ISM (González et al. 1999) catabolise DMSP. Both have at least one DMSP lyase, DddL in the case of *Sulfitobacter* sp. EE-36 (Curson et al. 2008), whereas *R. nubinhibens* ISM has no less than three lyases, encoded by *dddP* plus two versions of *dddQ* (Todd

et al. 2009, 2011a). Furthermore, *R. nubinhibens* ISM also contains the DmdA DMSP demethylase (Howard et al. 2006).

We set out to identify DMSP transport genes from both these strains and again used the betaine transport defective strain *E. coli* MKH13, this time exploiting its salt sensitivity in the presence of exogenously supplied betaine, relative to that of the wild type MC4100. This approach has been successfully applied to isolate betaine transport genes in other organisms, e.g. *betP* of *Corynebacterium glutamicum* (Peter et al. 1996) and the *ota* genes that encode an ABC betaine transporter in the archaeon *Methanosarcina mazei* (Schmidt et al. 2007). In general terms, it involves the construction of a genomic library from the bacterium of interest, followed by its introduction *en masse* into MKH13, screening for derivatives whose salt sensitivity is corrected on media containing a betaine osmolyte, then analysing the heterologous, cloned DNA.

So, to clone genes that encode DMSP transporter(s) in *Sulfitobacter* sp. EE-36 and *Roseovarius nubinhibens* ISM, we used previously constructed gene libraries of these two strains, in which sub-genomic fragments, ~25 kb in size, had been cloned into the wide host-range cosmid pLAFR3 (Curson et al. 2008; Todd et al. 2009). In separate, tri-parental conjugational crosses (Figurski and Helinski 1979), the cosmids of the two libraries were introduced *en masse* into *E. coli* MKH13, selecting transconjugants that were resistant to tetracycline, specified by pLAFR3. When these colonies were then replica-plated onto medium that contained 0.1 mM DMSP plus 0.6 M NaCl, ~0.1% of the transconjugants grew in these hypersaline conditions. We confirmed that these salt-tolerant derivatives contained cloned DMSP transporter genes from *Sulfitobacter* and from *Roseovarius* as follows.

Cosmid DNA was isolated from six transconjugants for each set of crosses and these were examined by restriction digestions. With the *Sulfitobacter* clones, all the cosmids appeared to contain overlapping cloned DNA and one of these, termed pBIO1768, was chosen for further study. However, when the DNA in the libraries was from *Roseovarius,* the restriction digest patterns were of two types, suggesting that two different transporter genes had been cloned; one representative cosmid of each type, called pBIO1758 and pBIO1762, were studied further.

To locate the cloned DNA relative to the genome sequence of the two strains, the termini of the three cosmids were sequenced, using primers to either side of the cloning site in pLAFR3. By such means, the genes in the intervening cloned DNA were deduced by examining the corresponding genomic regions in *Sulfitobacter* (see http://www.roseobase.org/roseo/ee36.html) and *Roseovarius* (see http://www.roseobase.org/roseo/nubinhibens.html). In all three cosmids, the cloned DNA contained genes that were strongly predicted to encode BCCT-type transporters. Thus, the *Sulfitobacter* sp. EE-36 cosmid pBIO1768 contained the gene EE36_10469, which we term *sbtA,* and the *R. nubinhibens* ISM cosmids pBIO1758 and pBIO1762 contained, respectively, ISM_12165 (*rbtA*) and ISM_11110 (*rbtB*). Judged by their genomic locations and orientations, *sbtA, rbtA* and *rbtB* are all predicted to comprise one-gene transcriptional units, with no sign of any nearby genes known to be involved in DMSP catabolism. To confirm their functions, the individual *sbtA, rbtA* and *rbtB* genes were amplified from the corresponding cosmids and cloned into wide host-range, conjugational plasmid vectors (pRK415 in the case of *rbtB* and pOT2 (Allaway et al. 2001) for *sbtA* and *rbtA*). The resultant plasmids were each introduced by conjugation into MKH13; in all cases, the transconjugants grew on hypersaline (0.6 M NaCl) medium in the presence of the osmolytes DMSP, glycine betaine or choline.

We also examined if *sbtA, rbtA* or *rbtB* could confer a Ddd^+ phenotype to strain MKH13 containing *dddD,* cloned in pBIO1609 (see above), by conjugating the plasmids containing each of these genes into this *E. coli* strain. Each set of transconjugants made DMS at levels that were significantly above background and were further enhanced, approximately twofold, when the *E. coli* cells were in M9 medium to which extra NaCl had been added. However, the levels of DMS production were considerably less than those conferred by cloned *dddT* of *Halomonas* or *Marinomonas* (Fig. 2).

The sequences of the SbtA, RbtA and RbtB polypeptides were not particularly closely related to each other or to the BCCT-type transporters encoded by genes close to *dddD* (see above). During the course of these comparisons we also noted that the RbtA polypeptide had an extra 60 amino acids (from positions ~300−360) which were absent from most other BCCT-type polypeptides, such as the DddTs of

Halomonas and *Marinomonas,* but which occur in several BCCT-type transporters from several other species of the Roseobacter clade. The significance of this is not known, though it is clear from the functional assays with RbtA that this form of BCCT-type polypeptide does function as a betaine importer.

Discussion

Recent genetic studies of bacterial catabolism of DMSP have revealed remarkable diversity in this process, at several levels. Thus, the identification of the genes whose products cleave DMSP, releasing DMS as one of the products, has shown that these enzymes are of at least six different types. Furthermore, the *dddD, dddP* and *dddY* genes occur, sporadically, in an unexpectedly wide range of microbes, including some fungi, most likely through a number of independent HGT events.

It is not only these "primary" Ddd enzymes that exhibit such diversity. As described here, this is also a feature of the "ancillary" polypeptides involved in DMSP transport, encoded by nearby *ddd* genes. To date, such linked transporter genes are nearly always found only in those strains in which the *dddD* gene is responsible for the bacterial Ddd^+ phenotype. Mostly, *dddD* is found in γ-proteobacteria strains that grow well on DMSP as sole carbon source and it occurs in clusters with other *ddd* genes for transcriptional regulators and enzymes that catalyse further steps in the DMSP catabolic pathways, in addition to the transport gene(s). This investment of what appear to be sets of integrated, multi-gene cassettes may reflect the importance of DMSP as a food source for the bacteria that use the DddD catabolic system. And, the finding of no less than four different genes that encode BCCT-type transporters near *dddD* of *Oceanimonas doudoroffii* may emphasise the advantage of having efficient DMSP uptake machinery in this species.

Despite their close linkage to *dddD*, not only are these transporters of wholly different types (ABC or BCCT) but even within these two sets of polypeptide super-families, they do not form coherent sub-groups. It is possible, therefore, that these genes were acquired in a series of independent HGT events with, presumably, different bacteria acting as the source donors of the horizontally acquired genes.

In contrast to *dddD,* other primary genes that encode DMSP lyases, namely *dddL, dddP*, *dddQ, dddW, dddY,* rarely, if at all, have linked DMSP transport genes. In the unusual case of the γ-proteobacterium *O. doudoroffii,* both of its *dddP*-like genes have nearby genes that encode BCCT-type transporters. In the special case of *dddY,* the lack of a nearby transporter gene is easily explained, since the DddY DMSP lyase is in the periplasm, so no inner membrane transporter is needed. However, the DddL, DddP, DddQ, and DddW lyases are cytoplasmic, so this explanation cannot apply. These four enzymes are, for the most part, found in the Roseobacters, most of which also contain *dmdA*, which encodes the DMSP demethylase that catalyses the initial step in the demethylation pathway and which is important for DMSP assimilation in these bacteria. We noted that downstream of, and likely co-transcribed with, the *dmdA* genes of several Roseobacter strains (e.g. *Oceanibulbus indolifex* HEL-45 and *Ruegeria* sp. R11) there was a gene whose product was in the BCCT family of betaine transporters. However, there were no genes that were predicted to encode DMSP transporters near *dmdA* of other Roseobacters, including *Ruegeria pomeroyi* DSS-3 and *Roseovarius nubinhibens* ISM.

The work described here shows that at least two Roseobacters, *Roseovarius nubinhibens* ISM and *Sulfitobacter* sp. EE-36, contain genes that encode BCCT-type transporters and that these can import DMSP, in addition to other betaines such as glycine betaine and choline. Although these "housekeeping" betaine transporters may be sufficient for the uptake of the DMSP substrate in these bacteria, we did find that SbtA, RbtA and RbtB were not as effective as the *Halomonas* or *Marinomonas* DddT transporters in the admittedly artificial situation in which their behaviour in the heterologous background, *E. coli,* was examined. Given the importance of DMSP as a nutrient for the Roseobacters (Newton et al. 2010), it is perhaps surprising that several strains of this clade do not appear to have dedicated, or "back-up" DMSP transporters encoded by genes close to those involved in DMSP catabolism. However, Moran et al. (2004) noted that the *Ruegeria pomeroyi* genome had no less than five sets of genes that might encode betaine transporters. RbtA and RbtB represent the only BCCT type transporters in the deduced *Roseovarius nubinhibens* ISM proteome, but *Sulfitobacter* sp. EE-36 has

three predicted BCCT transporters, only one of which, SbtA, was examined here. Furthermore, both these species have predicted ProU-type ABC transporters, one in the case of *Sulfitobacter* and three different ones in *R. nubinhibens*. So, the Roseobacters may use a multiplicity of ways to import DMSP. Indeed, it cannot be excluded that some wholly uncharacterised form of DMSP import system applies to these bacteria.

In connection with this, it is also noteworthy that in microarray experiments in cells of *Ruegeria pomeroyi* DSS-3, grown in the presence and absence of DMSP, there was no marked differential expression of any genes that encode potential BCCT transporters but there was enhanced transcription of some other transporters, of unknown function, following pre-growth in DMSP (Bürgmann et al. 2007; M. Kirkwood, personal communication).

It is apparent, therefore, that the diversity of the DMSP transporters mirrors that which has emerged from recent studies on other aspects of the catabolism of substrate. It will be of interest to learn which, if any, of the systems described above is of particular importance in natural environments and/or if there are some other, as yet undiscovered mechanisms.

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