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ORIGINAL ARTICLE

Manganese uptake in marine bacteria; the novel MntX transporter is widespread in Roseobacters, Vibrios, Alteromonadales and the SAR11 and SAR116 clades

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We showed that two very different manganese transporters occur in various important genera of marine bacteria. The ABC transporter encoded by *sitABCD* of the model Roseobacter-clade bacterium *Ruegeria pomeroyi* DSS-3 is required for Mn^{2+} import and was repressed by the Mur (Manganese uptake regulator) transcriptional regulator in Mn-replete media. Most genome-sequenced Roseobacter strains contain SitABCD, which are in at least two sub-groups, judged by their amino-acid sequences. However, a few Roseobacters, for example, *Roseovarius nubinhibens,* lack *sitABCD*, but these contain another gene, *mntX*, which encodes a predicted inner membrane polypeptide and is preceded by *cis*-acting Mur-responsive MRS sequences. It was confirmed directly that *mntX* of *Roseovarius nubinhibens* encodes a manganese transporter that was required for growth in Mn-depleted media and that its expression was repressed by Mur in Mn-replete conditions. MntX homologues occur in the deduced proteomes of several bacterial species. Strikingly, all of these live in marine habitats, but are in distantly related taxonomic groups, in the γ- and α-proteobacteria. Notably, MntX was prevalent in nearly all strains of Vibrionales, including the important pathogen, *Vibrio cholerae.* It also occurs in a strain of the hugely abundant *Candidatus* Pelagibacter (SAR11), and in another populous marine bacterium, *Candidatus* Puniceispirillum marinum (SAR116). Consistent with this, MntX was abundant in marine bacterial metagenomes, with one sub-type occurring in an as-yet unknown bacterial clade.
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Introduction

Manganese (Mn) is an essential metal, being found in a wide range of enzymes, particularly those involving redox chemistry, as well as being a critical component of the photosystem II in plants, algae and cyanobacteria (Wieghardt, 1989). Although abundant in the Earth's crust, it is scarce in the oceans, reaching only low nanomolar concentrations in near-surface waters (Landing and Bruland, 1987; Middag *et al.,* 2011). Marine manganese can take the form of insoluble oxides (Mn^{3+} and Mn^{4+}), as well as the soluble, Mn^{2+} version, which can arise via photochemical reduction of Mn oxides (Sunda and Huntsman, 1994). Conversely, several bacteria contain enzymes that oxidise Mn(II) (Tebo *et al.,* 2005); such strains include some Roseobacters (see below) and other classes of marine bacteria.

Studies on manganese import in a range of different terrestrial bacteria have revealed two, widely used, but very different, specialised Mn^{2+} transporters, termed MntH and SitABCD, with some bacteria (for example, *Salmonella*), harbouring both systems (Boyer *et al.,* 2002). MntH is an inner membrane symporter that resembles the Nramp proteins of eukaryotes (Makui *et al.,* 2000). In contrast, SitABCD is an ABC-transporter type system, initially thought to be involved in iron uptake - hence its somewhat misleading acronym of *Salmonella* iron transporter (Zhou *et al.,* 1999). Recently, Hohle *et al.* (2011) described a novel ion channel polypeptide, MnoP, which facilitates Mn^{2+} transport across the outer membrane of *B. japonicum*, the microsymbiont of soybeans: this polypeptide is confined to a few close relatives of *Bradyrhizobium.*

Bacteria are known to use two different, dedicated, Mn-responsive transcriptional regulators, termed MntR and Mur (Manganese uptake

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regulator). The former controls manganese homoeostasis in *Escherichia coli,* regulating the expression of four genes, including the MntH transporter, by Mn-dependent binding to their operator sequences in Mn-replete cells (Yamamoto *et al.,* 2011). Bioinformatic analyses strongly indicated that a few α-proteobacterial species also contain MntR, repressing expression of their cognate MntH or SitABCD transporters in response to Mn availability (Rodionov *et al.,* 2006). However, the manganese regulons of α-proteobacterial species that have been examined directly, in rhizobia and the mammalian pathogen *Brucella,* are controlled by a very different transcriptional regulator, Mur. When complexed with Mn^{2+}, Mur represses transcription by binding to *cis*-acting 'Mur-responsive sequences' (MRSs), located at the Mn-repressed promoters of the *sitABCD* transporter operon and of promoters that precede other Mn-regulated genes (Chao *et al.,* 2004; Díaz-Mireles *et al.,* 2004, 2005; Platero *et al.,* 2007; Hohle *et al.*, 2011; Menscher *et al.*, 2012). The Mur protein resembles the iron responsive regulator Fur (ferric uptake regulator), which controls iron homoeostasis in many different bacteria (Lee and Helmann, 2007). In the α-proteobacteria, the Mur version of the Fur superfamily likely evolved from Fur, *sensu stricto,* but responds to a different, although similar, metal and regulates only a few transcriptional units. In turn, this may be because bacteria in this sub-phylum no longer use Fur for iron homoeostasis; instead, they employ evolutionarily distinct transcriptional regulators like Irr and, in some lineages, RirA (Rudolph *et al.,* 2006; Johnston *et al.,* 2007).

To date, there are no direct studies on the mechanisms of Mn acquisition or Mn-responsive gene regulation in any marine microbes. Among the most populous, widespread classes of marine bacteria in the near-surface waters of the World's oceans are the Roseobacters, a taxonomically coherent group of >25 genera within the Rhodobacterales Family of α-proteobacteria ('Roseobase' at http://www.roseobase.org/; Buchan *et al.,* 2005; Brinkhoff *et al.,* 2008). The acquisition and the responses of these bacteria to various nutrients, including trace metals, will therefore have important effects on the corresponding biogeochemical cycles in the oceans.

The first Roseobacter whose genome was sequenced is *Ruegeria pomeroyi* DSS-3 (Moran *et al.,* 2004), which is something of a reference strain for genetic and genomic studies on this clade (Moran *et al.*, 2012; Curson *et al.,* 2011). This strain has a homologue of the Mur regulator and its genome includes an operon that likely encodes a SitABCD-type Mn^{2+} transporter, but it lacks MntH and has no homologue of the MntR transcriptional regulator (Rodionov *et al.,* 2006). Here, we describe genetic analyses of Mn^{2+} uptake and its regulation in *Ruegeria pomeroyi* DSS-3. Furthermore, comparative genomic bioinformatic analyses of other Roseobacters uncovered a novel Mn^{2+} transporter, which occurs in a range of important marine bacteria and is widespread in marine microbial metagenomes.

Materials and methods

Strains, media and growth conditions

Ruegeria pomeroyi wild-type DSS-3 (Moran *et al.*, 2004) was used as the source of genomic DNA for PCR amplifications. A Rif^R derivative, J470 (Todd *et al.*, 2011), was the recipient for plasmids in triparental conjugational matings, using the helper plasmid pRK2013 (Figurski and Helinski, 1979). Strain 803 (Wood, 1966) was the routine *E. coli* host.

Ruegeria pomeroyi was grown at 28 °C in complete half-strength YTSS medium (González *et al.*, 2003), or in minimal RSS medium before β-galactosidase assays (Rossen *et al.*, 1985). RSS minimal medium was based on the marine basal medium of Baumann and Baumann (1981), but the 'sea salts' was replaced with (l^{-1}): 17 g NaCl, 1.5 g $MgCl_2$, 0.75 g $CaCl_2$, 0.75 g KCl, 20 μM $FeCl_3$, plus the vitamin solution of González *et al.* (1997). Where appropriate, $MnCl_2$ was added, to 20 μM.

DNA manipulations and plasmid constructions

Routine manipulations of DNA were as in Wexler *et al.* (2001).

Construction of recombinant plasmids and mutants

The approach used to make insertions into *sitA* and *mur* involved the cloning of fragments internal to each of these genes into a suicide plasmid that was transferred by conjugation into *Ruegeria pomeroyi,* selecting for mutants in which the plasmid had integrated into the corresponding, genomic version of the gene (see Supplementary information, which also describes how the *lacZ* transcriptional fusions and other recombinant plasmids were made).

The sequences of the primers that were used for the various manipulations are shown in Supplementary Table S3.

Results

Ruegeria pomeroyi DSS-3 sitABCD is required for Mn^{2+} *import and is regulated by Mur*

Inspection of the *Ruegeria pomeroyi* DSS-3 genome had revealed two sets of genes with a likely role in Mn uptake and in regulating this process (Rodionov *et al.,* 2006). The consecutive genes *SPO3366, SPO3365, SPO3364* and *SPO3363* are predicted to encode the ABC-class Mn^{2+} transporter SitABCD (Figure 1a); *sitA* encodes the periplasmic binding protein, *sitB* the ATPase and *sitC* and *sitD* the two inner membrane permease polypeptides. The other relevant gene is *SPO2477,* a single-gene transcriptional unit whose product is 67% identical to Mur of

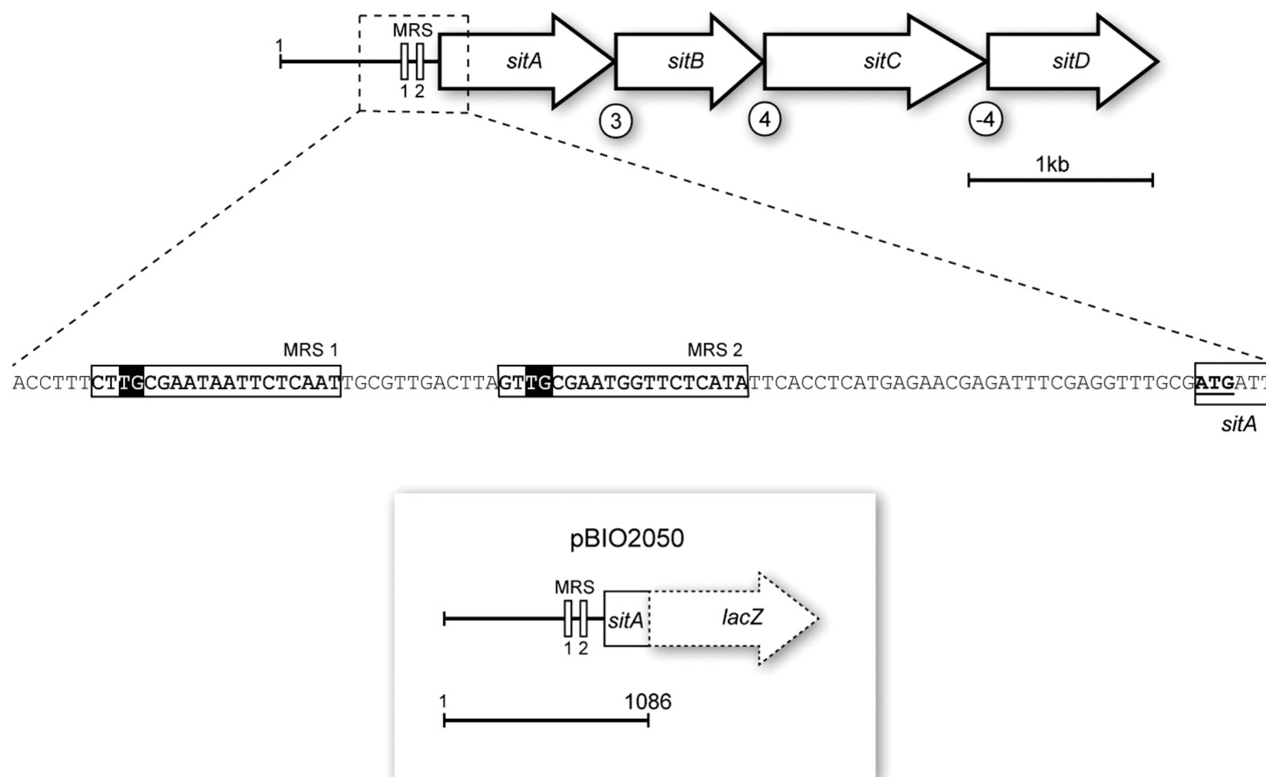

Figure 1 Maps of *sitABCD* genes of *Ruegeria pomeroyi* and their genetically modified derivatives. The open arrows in the top section show the dimensions of the individual genes in the *sitABCD* operon, the corresponding gene tags being *SPO3366, SPO3365, SPO3364* and *SPO3363*, respectively. Numbers of base pairs in intergenic spaces are shown, '-4' denoting overlap between *sitC* and *sitD.* The approximate locations of the two MRS regulatory sequences are indicated. The middle section shows the sequence upstream of the ATG start of *sitA*, the two MRS motifs being boxed. The conserved 'TG' base pairs in MRS1 and in MRS2 that were changed by site-directed mutagenesis are highlighted. The dimensions of the 1086-bp fragment used to construct the *sitA-lacZ* transcriptional fusion plasmid pBIO2050 are shown.

Rhizobium leguminosarum, which represses expression of *sitABCD* in that species. We confirmed that *Ruegeria pomeroyi sitABCD* and *mur* are indeed involved in Mn uptake as follows.

First, an insertional mutation was made into *sitA* (Supplementary information) and growth of the resultant mutant, strain J529, was compared with the wild type in minimal media that were replete or depleted for manganese. The two strains grew at similar rates in media supplemented with 20 μM $MnCl_2$, but in the absence of added manganese, the *sitA* mutant was severely reduced in its growth rate (Figure 2). Thus *Ruegeria pomeroyi* SitABCD is its major, and likely its only dedicated Mn transporter, with the slight growth of the mutant that is eventually seen in the Mn-depleted medium, perhaps being due to nonspecific import of the metal by other transporters.

To assess if *sitABCD* expression was affected by Mn^{2+} availability, we made a transcriptional fusion plasmid, pBIO2050, in which the *lacZ* reporter of the wide host-range promoter-probe vector pBIO1878 was controlled by the *sitA* promoter (Figure 1). This fusion plasmid was mobilised into wild-type *Ruegeria pomeroyi* by conjugation, and a purified transconjugant was grown in Mn-replete and Mn-depleted minimal media, before assaying β-galactosidase, encoded by the *lacZ* reporter. There was markedly greater (19-fold) expression in the $-$Mn than in $+$Mn medium (Table 1).

This differential expression was shown to be mediated by the Mur repressor by repeating this assay, but this time, the fusion plasmid pBIO2050 was transferred into a Mur$^-$ mutant (J528), which had an insertion in the *mur* gene, *SPO2477.* In this background, the *sitA–lacZ* fusion expressed β-galactosidase at high, constitutive levels in both $-$Mn and $+$Mn media (Table 1).

Just as in the region upstream of *sitABCD* of *Rhizobium leguminosarum* (Díaz-Mireles *et al.,* 2004), there are two motifs, centred 75 and 45 bp upstream of the ATG translational start of *Ruegeria pomeroyi sitA,* both of which resemble a regulatory 'MRS-box' (Rodionov *et al.*, 2006); these were termed MRS1 and MRS2, respectively (Figure 1). These *cis*-acting sequences bind to Mur and are required for Mur-dependent repression (Rodionov *et al.,* 2006). To investigate their roles in *Ruegeria,* we made site-directed mutations in each of them individually, and also in both MRSs, substituting their highly conserved GT residues with two A residues. We cloned the resultant mutant fragments

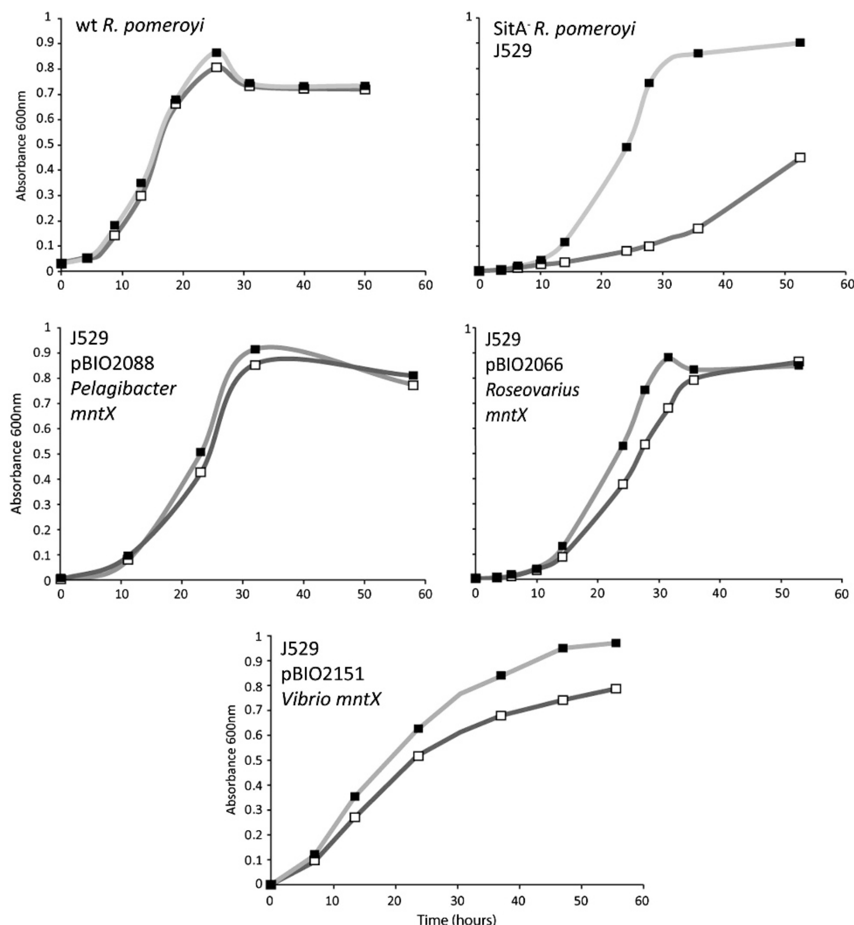

Figure 2 Effects of *sitA* and of the *mntX* genes of *Roseovarius nubinhibens*, *Ca.* Pelagibacter ubique and *Vibrio cholerae* on growth of *Ruegeria pomeroyi* in Mn-depleted minimal media. Cultures of *Ruegeria pomeroyi* wild type, or the SitA- mutant J529 or J529 corrected with cloned *mntX* of *Roseovarius nubinhibens* (pBIO2066), *Ca.* Pelagibacter ubique (pBIO2088) or *V. cholerae* (pBIO2150) were diluted into RSS minimal medium either lacking any added $MnCl_2$ (white squares), or supplemented with 10 μM $MnCl_2$ (black squares). Cultures were grown, with shaking, at 28 °C, and growth was measured by absorbance at 600 nm.

Table 1 The MRS motif and the *mur* genotype affect manganese-responsive expression of a *Ruegeria pomeroyi sitA-lacZ* transcriptional fusion.

	WT (sitA–lacZ)	*Mur⁻ (sitA–lacZ)*	*μMRS1 (sitA–lacZ)*	*μMRS2 (sitA–lacZ)*	*μMRS1 + μMRS2 (sitA–lacZ)*
Plus Mn	42 ± 1	1736 ± 178	294 ± 14	249 ± 5	1612 ± 41
Minus Mn	791 ± 30	1817 ± 204	628 ± 19	927 ± 23	1235 ± 98

The wild type *sitA–lacZ* fusion plasmid pBIO2050, or derivatives (pBIO2100, pBIO2101 and pBIO2102, respectively) containing mutations in the MRS1 (μMRS1) or MRS2 (μMRS1) or in both (μMRS1 + μMRS2) regulatory sequences were introduced into wild-type and Mur- mutant *Ruegeria pomeroyi* DSS-3 recipients. Transconjugants were grown in media that either did or did not have added $MnCl_2$ before assaying β-galactosidase in triplicate. Activities are shown in Miller units, with s.e.

into the reporter plasmid pBIO1878. These mutated plasmids were each introduced by conjugation into wild-type *Ruegeria pomeroyi* and assayed for β-galactosidase activity. Compared with the wild-type fusion, the mutation in the upstream MRS1 caused a ~seven-fold increase in β-galactosidase activity in Mn-replete conditions, but was little changed under Mn-limitation. Similarly, the mutation in MRS2 caused a six-fold higher expression of β-galactosidase than the wild type in Mn-replete conditions (Table 1). The fusion plasmid with mutations in both MRS1 and MRS2 was deregulated even further, under both Mn replete (38-fold) and Mn-depleted (1.5-fold) conditions. Thus, both MRS1 and MRS2 contribute to the Mn-responsive, Mur-dependent repression of the *sitABCD* operon.

Diversity of the mur, sitABCD and other genes involved in Mn uptake and sensing in the Roseobacters

Inspection of the genomes of 37 other Roseobacters showed that none of them contained the MntR

Table 2 Distribution of sub-types of the *sitA, mntX, mntH* and *mur* genes in genome-sequenced Roseobacter strains

Species \ Gene Name	*sitABCD*	*mntX*	*mntH*	*mur*
Citreicella SE45	[hatched]			[grid]
Citreicella sp. *357*			[black]	[grid]
Dinoroseobacter shibae DFL 12	[hatched]			[grey]
Jannaschia sp. CCS1	[grey]			[grey]
Loktanella vestfoldensis SKA53	[vertical stripes]			[grey]
Maritimibacter alkaliphilus HTCC2654	[grey]			[grey]
Oceanibulbus indolifex HEL45	[grey]			[grey]
Oceanicola batsensis HTCC2597	[grey]			[grey]
Oceanicola granulosus HTCC2516		[black]		[grey]
Octadecabacter antarcticus 307	[hatched]			[dotted] / [grey]
Octadecabacter arcticus 238	[hatched]			[grey]
Pelagibaca bermudensis HTCC2601	[hatched]			[dotted]
Phaeobacter gallaeciensis 2.10	[grey]			[grey]
Phaeobacter gallaeciensis BS107	[grey]			[grey]
Phaeobacter sp. Y4I	[hatched]			[grey]
Rhodobacterales bacterium HTCC2083	[grey]			[grey]
Rhodobacterales bacterium HTCC2150	[hatched]			[grey]
Rhodobacterales bacterium HTCC2255		[black]		[grey]
Rhodobacterales bacterium KLH11	[grey]			[grey]
Roseobacter denitrificans OCh 114	[grey]			[grey]
Roseobacter litoralis Och 149	[grey]			[grey]
Roseobacter sp. AzwK-3b	[grey]			[grey]
Roseobacter sp. CCS2	[hatched]			[grey]
Roseobacter sp. GAI101	[hatched]			[dotted]
Roseobacter sp. MED193	[grey]			[grey]
Roseobacter sp. SK209-2-6	[grey]			[grey]
Roseovarius nubinhibens ISM		[black]		[grey]
Roseovarius sp. 217	[grey]			[grey]
Roseovarius sp. TM1035	[grey]			[grey]
Ruegeria lacuscaerulensis ITI-1157	[grey]			[grey]
Ruegeria pomeroyi DSS-3	[grey]			[grey]
Ruegeria sp. R11	[grey]			[grey]
Ruegeria sp. TM1040	[grey]			[grey]
Ruegeria sp. Trich CH4B	[grey]		[black]	[grey]
Sagittula stellata E-37	[grey]			[grey]
Sulfitobacter NAS-14.1	[hatched]			[dotted]
Sulfitobacter sp. EE-36	[hatched]			[dotted]
Thalassiobium R2A62	[hatched]			[grey]

In-fills with the same shading patterns represent polypeptides that closely resemble each other. In the case of the SitA, B, C and D polypeptides, all four components of this transport system showed a coherent relatedness pattern; thus all four polypeptides of (e.g.) *Sulfitobacter* were most similar to those in strains (e.g. *Octadecabacter*) with the same in-fill pattern. Empty boxes represent absence of the corresponding polypeptide. Note that *O. antarcticus* 307 has two Mur sequences, one resembling that in most of the Roseobacters (including *Ruegeria pomeroyi*) and one that is similar to those in *Sulfitobacter, Pelagibaca* and *Roseobacter* GAI101.

transcriptional regulator, but that all of them contained at least one gene whose product was within the Mur branch of the Fur super-family. However, we discerned different sub-types of these Mur polypeptides, judged by their sequences in different strains and species (Table 2; Supplementary Figure S1). In most Roseobacter strains, the Mur polypeptide closely resembles (>70% identical) that of *Ruegeria pomeroyi* DSS-3 (above). However, those of *Sulfitobacter, Citreicella, Pelagibaca*, and one strain (GAI101) of *Roseobacter* are only ~40% identical to that of *Ruegeria pomeroyi*, with those of *Sulfitobacter, Pelagibaca bermudensis* and *Roseobacter* GAI101 being very similar to each other. *Citreicella* Mur is very similar (65% identical) to that of *Sinorhizobium meliloti,* a ratified manganese-responsive regulator in that species (Platero *et al.,* 2007). Finally, one Roseobacter strain, *Octadecabacter antarcticus* 307, had two Mur polypeptides; one resembled that in *Ruegeria pomeroyi* and the other is of the *Sulfitobacter/Roseobacter* GA101 type. These different sub-types of Mur are seen in a relatedness tree, which also includes Mur polypeptides from other bacteria (Supplementary Figure S1A, B, and C).

The ABC-type transporter SitABCD is also widespread in the Roseobacters, with all but four strains (see below) containing the corresponding four genes, arranged contiguously (Table 2). Closer examination of the sequences of SitA, SitB, SitC and SitD showed that in most strains, these closely resemble (~70% identical) those in *Ruegeria pomeroyi* DSS-3 itself (Table 2). Several other Roseobacters contained a somewhat distinct version of the SitABCD polypeptides, which were very similar in the different strains (~77% identity) but less so (40%–50% identity) to those of the *Ruegeria pomeroyi* group (Table 2). This second sub-class of Roseobacter SitABCD polypeptides closely resembled (~75% identical) those of various strains of *Rhizobium, Sinorhizobium* and *Agrobacterium,* and other bacteria, whose function as manganese transporters has been ratified (Diaz-Mireles *et al.,* 2004; Davies and Walker, 2007). Finally, the SitABCD polypeptides of *Loktanella vestfoldensis* SKA53 are somewhat divergent from those homologues in all the other Roseobacters and more closely resembles deduced SitA-like polypeptides in two other marine bacteria, *Halomonas elongata* and *Pelagibacterium halotolerans*.

It was noted that all those strains with a form of the Mur polypeptide that did not closely resemble that of *Ruegeria pomeroyi* (see above) also had SitABCD polypeptides that were somewhat divergent from those in that species (Table 2). However, some strains with the 'non–*Ruegeria pomeroyi*' version of SitABCD (for example, *Dinoroseobacter shibae*) did have the 'conventional' form of Mur that resembles that of *Ruegeria pomeroyi* (Table 2).

Although the SitA polypeptides may be of different sub-classes, all the corresponding *sitA* genes are preceded by sequences that strongly resemble MRS motifs, indicating that they are regulated in a similar fashion, via the Mur transcriptional regulator (Supplementary Figure S2).

Two Roseobacter strains, *Ruegeria* sp. TrichCH4B and *Citreicella* sp. 357, contain a gene (*SCH4B_0376* and *C357_18417*, respectively) whose product resembles MntH, a very different Mn^{2+} transporter, being ~50% (*E* value $1e^{-120}$) identical to *E. coli* MntH. These *mntH* genes were both preceded by convincing MRSs, strongly suggesting that they, too, are regulated by Mur, in response to Mn^{2+} availability (Supplementary Figure S2). *Ruegeria* sp. TrichCH4B contains both MntH *and* SitABCD, but in *Citreicella sp.* 357, MntH is its *only* recognisable manganese transporter (Table 2).

MntX of Roseovarius nubinhibens is a Mur-regulated Mn^{2+} transporter

As mentioned above, four genome-sequenced Roseobacter strains lack any SitABCD homologues. One of these, *Citreicella* sp 357, contains MntH, but *Roseovarius nubinhibens* ISM, *Oceanicola granulosus* HTCC2516 and *Rhodobacterales* strain HTCC2255 lack any previously defined Mn^{2+} transporter. However, these strains, and *only* these three strains, each contain a gene, termed *mntX,* with two significant features; it is preceded by an MRS motif (Supplementary Figure S2; Rodionov *et al.,* 2006), and likely encodes an inner membrane integral protein, with eight or nine predicted trans-membrane helices as shown by SPLIT 4.0 (Juretic *et al.,* 2002), PREDTMBB (Bagos *et al.,* 2004), pSORTB (Yu *et al.,* 2010) and iMembrane (Kelm *et al.,* 2009) analyses. These MntX polypeptides are ~400 amino acids in length (for example, 387 for *Roseovarius nubinhibens ISM_02005* gene product) and are categorised as ‘function unknown’, with their C-terminal 300 amino acids corresponding to a domain of unknown function (DUF2899 (pfam11449)) and with a less well-conserved *N*-terminal region. Thus, *mntX* likely encodes a novel manganese transporter that substitutes for SitABCD and/or MntH and is regulated by Mur, in response to Mn availability. (This MntX has no similarity to a hydrophobic Mn exporter, also termed MntX, described in *Neisseria meningitides* by Veyrier *et al.* (2011)).

We confirmed these predictions for the *mntX*-containing strain, *Roseovarius nubinhibens* ISM. First, a 1574-bp fragment that included its *mntX* gene (*ISM_02005*) plus upstream regulatory sequences was amplified from genomic DNA and cloned into the wide host-range vector pOT2 (Allaway *et al.,* 2001), forming pBIO2066. When mobilised into the *Ruegeria pomeroyi* $SitA^-$ mutant J529, this plasmid fully restored the ability to grow on Mn-depleted medium (Figure 2).

To study the regulation of *mntX* of *Roseovarius nubinhibens* ISM, we made a *lacZ* transcriptional fusion. This comprised a 569-bp fragment that spanned its predicted promoter, plus its predicted MRS *cis*-acting regulatory sequence, and extending into the first 180 bp of the *mntX* structural gene, cloned into the promoter-probe plasmid pBIO1628, forming pBIO2067. This plasmid was mobilised by conjugation into wild-type *Roseovarius nubinhibens* and into wild-type *Ruegeria pomeroyi.* For both species, the β-galactosidase activities were 16-fold greater when transconjugants were grown in Mn-depleted than in Mn-replete medium, with, respectively, 1607 and 94 Miller units. To show that this regulation was mediated by Mur, the pBIO2067 fusion was mobilised into the *Ruegeria pomeroyi* Mur^- mutant J528. In this background, the fusion was expressed at high level in both +Mn and −Mn media (1861 and 1889 Miller units, respectively). Thus, Mur of one Roseobacter strain can regulate *mntX* of a different species, consistent with their similar MRS box sequences (Supplementary Figure S2).

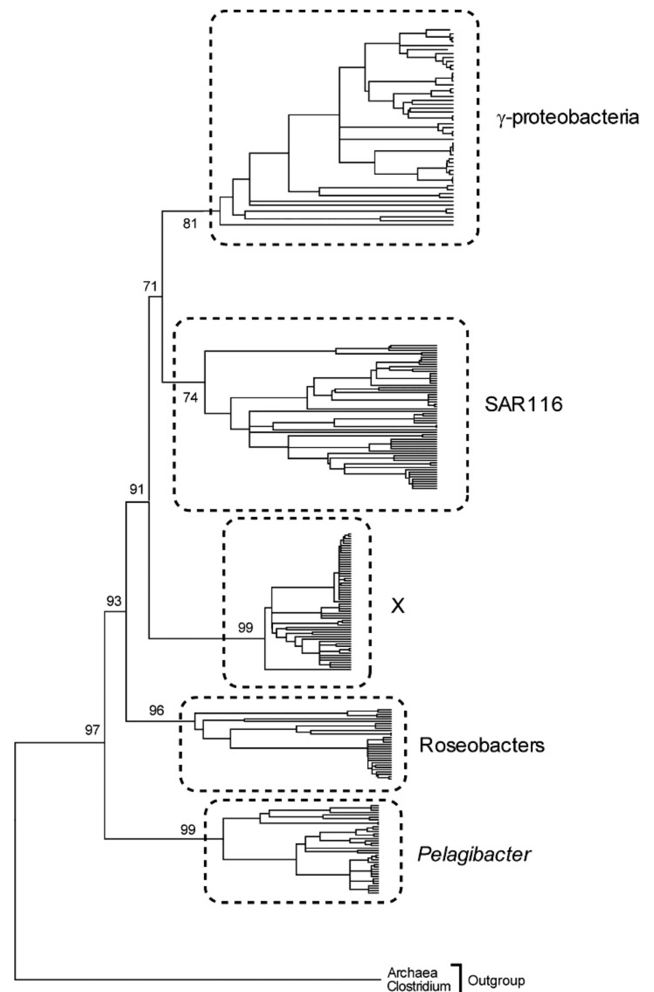

Figure 3 Neighbour-joining tree of MntX polypeptides in known bacteria and in the GOS marine metagenome. Using Mega5, a tree of the MntX homologues in the bacteria described in the text and in the GOS marine metagenome was constructed, bootstrap values being presented. These homologues were of five sub-classes as indicated, each corresponding to those found in the bacterial clade that is shown. Group ‘X’ contains no sequences from any known bacterium. The number of GOS sequences found in each of the five groups are indicated. Accession numbers of the individual GOS sequences are in Supplementary Table S2 and blow-up versions of the five branches are shown in Supplementary Figure S3, A–E.

MntX in other, important bacterial clades

There are many significant MntX homologues, in addition to those in the three Roseobacter strains, above. These homologues were restricted to a few genera in the Orders Vibrionales, Altermonadales and Oceanospirillales of γ-proteobacteria and in two α-proteobacterial clades, namely SAR11 and SAR116. Significantly, all these taxa are sea-dwelling. These MntX polypeptides fell into four subclasses, each being associated with one of the above bacterial lineages (Figure 3), as described below.

Vibrionales: Among this Order of marine bacteria, there are genome-sequenced strains in *Aliivibrio* (*A. salmonicida*), *Photobacterium* and *Vibrio* itself. The single *Aliivibrio* and three *Photobacterium* strains contain MntX homologues as do all but four of the 99 sequenced strains of *Vibrio* itself. The exceptions were strains of *Vibrio campbellii*, *Vibrio caribbenthicus*, *Vibrio nigripulchritudo* and *Vibrio sinaloensis*, but these contained at least one other known Mn^{2+} transporter, either SitABCD alone (*V. caribbenthicus, V. sinaloensis, V. nigripulchritudo*) or both SitABCD plus MntH (*V. campbellii* DS40M4). Furthermore, the *sitABCD* operon of *V. caribbenthicus* and *V. sinaloensis* abuts a *mur*-like gene whose product resembled those of *Citreicella* and *Sulfitobacter* (Supplementary Figure S1). Finally, some *Vibrio* and *Photobacterium* strains contain MntH and MntX, but not SitABCD (Supplementary Table S1). (The available *Vibrio* genomes of all strains with no known Mn transporters were in draft form and were not included).

Altermonadales MntX homologues occur in strains of *Colwellia, Alteromonas*, *Pseudoalteromonas*, all of which are Altermonadales γ-proteobacteria. Here too, there is diversity within a genus; thus, *Alteromonas macleodii* ATCC 27126 contains *mntX* but strain 'Deep ecotype' does not (Supplementary Table S1).

The *Oceanospirillales* also include some strains with *mntX* (*Marinomonas mediterranea MMB-1* and *M. posidonica* IVIA-Po-181), but others in the same genus (MWYL1) lack it (Supplementary Table S1).

Two other important marine α-proteobacteria contain *mntX* genes. These two clades, SAR11 (*Ca.* Pelagibacter ubique) and SAR116 (*Ca.* Puniceispirillum marinum) were first detected by culture-independent analysis of bacterial DNA sequences from the Sargasso Sea (Rappe *et al.,* 2002; Stingl *et al.,* 2007), but have now been grown in pure culture and some have been genome-sequenced.

The SAR11 bacteria are the most abundant on Earth (Morris *et al.,* 2002). Of four sequenced strains, one, *Ca.* Pelagibacter sp. IMCC9063, encodes a homologue of MntX (*SAR11G3_00277*), which is 39% identical to that of *Roseovarius nubinhibens*. Interestingly, this *mntX*-like gene is adjacent to a divergently transcribed gene (*SAR11G3_00278*) whose product is predicted to be in the MntR family, being 50% identical to the *E. coli* version of this manganese-responsive transcriptional repressor. Strain IMCC9063 is in Pelagibacter subgroup 3, rather distantly related to other genome-sequenced strains (HTCC7211, HTCC1062 and HTCC1002) (Oh *et al.,* 2011), all of which lack MntX. Indeed, these three strains lack genes for any known Mn^{2+} transporters.

SAR116 clade bacteria are also widespread throughout the oceans, although not as abundant as the SAR11 group (Morris *et al.,* 2012). The genome-sequenced SAR116 strains, *Ca.* Puniceispirillum marinum IMCC1322 (Oh *et al.,* 2010) and sp. HIMB100 both contain genes, (SAR116_1166 and *HIMB100_00004540*, respectively) whose products are 46% identical to MntX of *Roseovarius nubinhibens*.

MntX of Ca. Pelagibacter *sp. IMCC9063 and Vibrio cholerae O1 biovar El Tor str. N16961 are Mn^{2+} transporters*

We tested directly if two of these predicted *mntX* genes encoded functional Mn^{2+} transporters, again by trying to correct the growth phenotype of the $SitA^-$ mutant of *Ruegeria pomeroyi.* We chose *Ca.* Pelagibacter sp. IMCC9063 and *V. cholerae,* because these are especially important bacteria, although for very different reasons.

For *Ca.* Pelagibacter sp. IMCC9063, we acquired a 1345-bp fragment containing intact *mntX* (*SAR11G3_00277*), whose codon usage was optimised (using OPTIMIZER (Puigbò *et al.,* 2007)) for expression in the Roseobacters (whose GC content is ~60%, compared with ~30% in Pelagibacter). This *mntX*-like gene was sub-cloned into the wide host-range vector pRK415 (Keen *et al.,* 1988) and the resulting plasmid (pBIO2088) was conjugated into the *Ruegeria pomeroyi* DSS-3 $SitA^-$ mutant J529. It fully corrected the mutant's growth defect in Mn-deficient medium (Figure 2).

Similarly, we created a plasmid, pBIO2150, which contained the *mntX* gene (*VC1688*) of *V. cholerae* O1 biovar eltor str. N16961 cloned in the wide host-range vector pRK415. When conjugated into the *Ruegeria pomeroyi* $SitA^-$ mutant J529, the *V. cholera mntX* gene in this plasmid also fully complemented the mutant's growth defect in Mn-deficient medium (Figure 2).

Distribution of MntX polypeptides in marine metagenomes

Given that MntX homologues were found in diverse marine bacterial clades, it was of interest to examine their distribution in bacterial metagenomic data sets, most notably those obtained in the Global Ocean Sampling (GOS) expeditions (Rusch *et al.,* 2007; Yooseph *et al.,* 2007). When MntX of *Roseovarius nubinhibens* was used in BLASTP-based interrogations of these sequence reads, in CAMERA (Sun *et al.,* 2010), we found many homologues (469 with *E* values $<9.4\,e^{-20}$), only ~10-fold fewer than when RecA of a number of different bacterial taxa were individually used as the probes. These MntX-like polypeptides occurred in nearly all the sampling sites, in the Atlantic, Pacific and Indian Oceans, further emphasising their abundance in marine bacteria (Supplementary Table S2).

Of these 469 sequence reads, 183 had >230 bp (corresponding to >59% of the total MntX polypeptide sequence) of alignment to *Roseovarius nubinhibens mntX* and these were included in the neighbour-joining tree of MntX polypeptides (Figure 3; Supplementary Figure S3 A–E). These show that the GOS sequences fall into five different branches of the

MntX family, four of which mapped onto those represented by the MntX polypeptides of known bacterial strains (see above). Of these four groups, the most populous (59 sequences) was the one that closely resembled the SAR116-type, followed by the Pelagibacter version (35 sequences), then the Roseobacters (27 sequences) and, most infrequently, the γ-proteobacterial group, with two GOS representatives. Significantly, 59 closely related sequences (Clade X in Figure 3) were in a cluster that did not include any sequences from known organisms, so may originate from an as-yet unknown marine bacterial clade. It was striking that the DNA in the GOS reads that included these Clade X-like *mntX* genes all had very low (~30%) GC contents, as do the genomes of the SAR11 clade. Although some of these reads included a flanking gene, none of these encoded a taxonomically diagnostic product, so the origin of these *mntX* genes is not known.

Discussion

Despite the importance of metal acquisition by marine bacteria, there have been few genetic experimental studies on this subject, the work on the effects of iron on the transcriptome and proteome of *Ca.* Pelagibacter ubique by Smith *et al.* (2010) being a recent exception.

The starting point for our study, on the mechanism and regulation of manganese uptake in a model Roseobacter strain, *Ruegeria pomeroyi* DSS-3, generated no real surprises. As in other α-proteobacteria, notably the rhizobia, this Roseobacter used the ABC-type transporter SitABCD, whose expression was regulated by the Mur transcriptional regulator, which binds to regulatory, MRS, *cis*-acting sequences, to repress transcription under Mn-replete conditions. This likely applies to most of the other Roseobacters, on the basis of their possession of all these components of the manganese homoeostatic machinery. It was clear, though, that both the SitABCD transporters and the Mur regulators were in different sub-classes in different strains and that, mostly, there was an association between the versions of each of these sets of polypeptides in individual strains; thus, if a given strain had a SitABCD transporter that closely resembled that of (for example) *Ruegeria pomeroyi*, then so did its Mur regulator, in most cases (see Table 2).

More interesting were those few Roseobacter strains that lacked the SitABCD transporter, leading us to investigate the novel MntX polypeptide. This was first invoked in the Roseobacters as a possible manganese transporter on the basis of circumstantial bioinformatic evidence, as (a) it occurred only in those few Roseobacter strains that lacked SitABCD, (b) the *mntX* gene is preceded by a regulatory MRS motif and (c) the MntX polypeptide is predicted to be membrane-bound. We now have direct genetic evidence that the MntX of *Roseovarius nubinhibens* ISM is functional and is regulated in response to Mn availability via the Mur transcriptional regulator. Within the Roseobacter clade, it appears that nearly all strains either contain the SitABCD or the MntX system but not both; a few have the very different, third class of Mn^{2+} transporter MntH instead of (in *Citreicella* sp. 357) or, as well as (*Ruegeria* sp. Trich CH4B) one of the other types. In such stains with multiple systems of Mn^{2+} transport, it will be of interest to determine their relative importance, perhaps under different environmental conditions.

It is now clear that MntX is more widespread in the proteobacteria, in the Oceanospirillales, Altermonadales and Vibrionales in the γ-subphylum and in the SAR11, SAR116 and Roseobacter clades of α-proteobacteria. It is striking that despite their taxonomic diversity, all these Orders are largely or exclusively marine in their habitats. Notably, though, even within a single genus (for example, *Marinomonas* or *Ca.* Pelagibacter) some species/strains harbour *mntX* and others do not. Although this type of distribution may point to the acquisition of *mntX* by horizontal gene transfer, the fact that the MntX's within any given clade (for example, Vibrionales) closely resemble each other compared with those that are in other taxa, suggests that the horizontally acquired gene was stably maintained throughout the evolution of that Order.

Given that cholera is still a major killer, with 3–5 million cases and around 120 000 deaths annually worldwide, the finding of the MntX transporter in many species and genera of the Vibrionales, and in *V. cholerae* in particular, may prompt some future work on this system in these important pathogens. A microarray study has shown that the expression of the *mntX* gene, *VC1688*, in *V. cholerae* 0395 was repressed slightly (2.9-fold) in Fe-replete compared with Fe-depleted medium (Mey *et al.,* 2005). However, to our knowledge, there have been no studies on the effects of manganese on its transcription, or, indeed on the role of manganese acquisition on the virulence and/or environmental survival of *V. cholerae.* It will therefore be of interest to examine the effects of mutating this gene on these phenotypes.

MntX occurs in several bacteria (SAR11, SAR116 and Roseobacters) that are abundant in the oceans, so it was not surprising to see large number of homologues in marine metagenomes. MntX homologues in the GOS metagenome are slightly less abundant than those of the SitABCD type and 3.7-fold more common than the MntH homologues in that database. Interestingly, 59 out of 183 MntX polypeptides formed a tightly related cluster that did not include members of any known bacterial species. This cluster was characterised by a very low GC content, as also occurs in the SAR11 genomes. However, we do not know if this cluster represents a novel, unknown clade of bacteria that coincidentally has a high AT:GC ratio or if there are SAR11 strains that contain this form of MntX but whose

genome sequences are not currently available. We saw no convincing homologues of MntX in any other metagenomic database–from soils, or gut, for example–stressing the association of the MntX polypeptide and marine environments.

Our observations on the distribution of MntX in the various marine bacteria have other implications. Perhaps most significant is that some strains lack any of the known manganese transporters MntH, MntX and SitABCD. Several of these may therefore possess another, as yet unidentified system or else have evolved a biochemistry in which Mn is required in low amounts, if at all. Given that three of the sequenced strains of the important SAR11 *Ca.* Pelagibacter ubique are of this type, this topic warrants further investigation.

The fact that *mntX* seems to be confined to marine bacteria prompts some questions; is it a more effective transporter of the very low concentrations of manganese that prevail in the oceans, and/or does it operate more effectively under saline conditions, as occurs for other systems, such as the betaine/carnitine/choline transporter (see Ziegler *et al.,* 2010)?

Manganese is very scarce in the open oceans, so one might expect the manganese transporters to be expressed constitutively under most natural circumstances. The very fact that the Mn-responsive Mur, plus its *cis*-acting MRS targets have been retained suggests that a significant proportion of the population of cells must spend sufficient time in naturally occurring Mn-replete conditions to justify the retention over evolutionary time of a negatively-acting regulator that can respond to such nutritional largesse for this metal. Such conditions might apply, for example, if the cells are transiently in the vicinity of a dense plankton bloom or if present in sediments, as recently demonstrated for Roseobacter strains (Lenk *et al.*, 2012). Indeed, this consideration may apply more broadly, encompassing analogous regulatory systems that sense other essential metals, such as molybdenum, nickel or iron, which are also rare in the seas.

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