The LysR-type PcaQ protein regulates expression of a protocatechuate-inducible ABC-type transport system in *Sinorhizobium meliloti*

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The LysR protein PcaQ regulates the expression of genes encoding products relevant to the degradation of the aromatic acid protocatechuate (3,4-dihydroxybenzoate), and we have previously defined a PcaQ DNA-binding site located upstream of the target *pcaDCHGB* operon in *Sinorhizobium meliloti*. In this work, we show that PcaQ also regulates the expression of the *S. meliloti* *smb20568-smb20787-smb20786-smb20785-smb20784* gene cluster, which is predicted to encode an ABC transport system. ABC transport systems have not been shown before to transport protocatechuate, and we have designated this gene cluster *pcaMNVWX*. The transcriptional start site of *pcaM* was mapped, and the predicted PcaQ DNA-binding site was located at –73 to –58 relative to this site. Results from electrophoretic mobility shift assays with purified PcaQ and from expression assays indicated that PcaQ activates expression of the transport system in the presence of protocatechuate. To investigate this transport system further, we generated a *pcaM* deletion mutant (predicted to encode the substrate-binding protein) and introduced a polar insertion mutation into *pcaN*, a gene that is predicted to encode a permease. These mutants grew poorly on protocatechuate, presumably because they fail to transport protocatechuate. Genome analyses revealed PcaQ-like DNA-binding sites encoded upstream of ABC transport systems in other members of the α -proteobacteria, and thus it appears likely that these systems are involved in the uptake of protocatechuate.

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INTRODUCTION

The β -ketoadipate pathway is a peripheral metabolic pathway through which a variety of lignin-derived and anthropogenic aromatic substrates can be degraded following their conversion to one of two common intermediates, either protocatechuate or catechol (Harwood & Parales, 1996). The Gram-negative legume microsymbiont *Sinorhizobium meliloti* encodes the protocatechuate branch of the β -ketoadipate pathway on the pSymB megaplasmid, and *S. meliloti* can utilize protocatechuate as a sole source of carbon (MacLean *et al.*, 2006). Protocatechuate catabolic genes are organized into two operons (*pcaDCHGB* and *pcaIJF*), whose expression is regulated by the transcriptional regulators PcaQ and PcaR, respectively (MacLean *et al.*, 2006, 2008). While systems involved in the active transport of aromatic acids such as protocatechuate have been described in many species, including *Pseudomonas putida* (Harwood *et al.*, 1994;

Nichols & Harwood, 1997), a protocatechuate transport system has yet to be identified in *S. meliloti* or any other member of the α -proteobacteria. Furthermore, *S. meliloti* appears to lack protein homologues of the PcaK major facilitator superfamily of aromatic acid transport proteins (Collier *et al.*, 1997; Williams & Shaw 1997; D'Argenio *et al.*, 1999; Ledger *et al.*, 2009).

PcaQ is a member of the LysR family of regulatory proteins, and requires the co-effectors β -carboxy-*cis*,*cis*-muconate and γ -carboxymuconolactone to induce expression of *pcaDCHGB* in *Agrobacterium tumefaciens* and *S. meliloti* (Parke, 1993, 1996; MacLean *et al.*, 2006, 2008). LysR-type transcriptional regulators participate in the regulation of a wide variety of physiological processes, including carbon and nitrogen metabolism, aromatic and amino acid degradation, virulence in plant and animal pathogens, quorum sensing, and the oxidative stress response (Maddocks & Oyston, 2008). As a general model, LysR proteins activate gene expression via interaction with a recognition (or repression) binding site that is positioned upstream of a target promoter and contains the primary binding determinant that is necessary for an interaction of

Abbreviations: α CTD-RNAP, RNA polymerase alpha subunit; Gm, gentamicin; Gm^r, Gm-resistant; Gm^s, Gm-sensitive; MFS, major facilitator superfamily; Nm, neomycin; Rif, rifampicin; RNAP, RNA polymerase; Sm, streptomycin; Sp, spectinomycin; Sp^r, Sp-resistant; sucrose^r, sucrose-resistant.

high affinity (Parsek *et al.*, 1992; Akakura & Winans, 2002). In the presence of a co-effector, the regulator interacts with a downstream activation binding site that is required for the induction of gene expression (Huang & Schell, 1991; Parsek *et al.*, 1992; Bundy *et al.*, 2002). To activate transcription, many LysR proteins have been demonstrated to require direct interaction with the C-terminal domain of the RNA polymerase (RNAP) alpha subunit (α CTD-RNAP) (Tao *et al.*, 1993; Chugani *et al.*, 1997; McFall *et al.*, 1998; Fritsch *et al.*, 2000; Park *et al.*, 2002). In 1992, Goethals and co-workers identified a LysR DNA-binding motif (TN₁₁A) present in the majority of binding sites (Goethals *et al.*, 1992); however, the *in silico* prediction of LysR regulatory binding sequences is compromised by the prevalence of this motif within AT-rich intergenic regions.

We have previously described a PcaQ DNA-binding motif that is located upstream of *pcaD* in *S. meliloti* and is conserved in related rhizobia such as *A. tumefaciens* (MacLean *et al.*, 2008). In this work, we identify putative PcaQ DNA-binding sites positioned upstream of genes in members of the classes α -, β - and γ -Proteobacteria. In 11 α -proteobacteria, PcaQ DNA-binding sites were located upstream of genes encoding ABC-type transport systems, and in the case of *S. meliloti*, we demonstrate that PcaQ regulates the expression of these ABC transport genes and that the gene encoding the solute-binding protein is required for growth on protocatechuate as carbon source.

METHODS

Bacterial strains and growth conditions. All plasmids and bacterial strains used throughout this study are described in Table 1. *Escherichia coli* strains were grown aerobically in Luria-Bertani (LB) broth at 37 °C. *S. meliloti* strains were grown aerobically at 30 °C in LB broth supplemented with 2.5 mM MgSO₄ and 2.5 mM CaCl₂ (LBmc). M9 minimal medium (Difco) salts were supplemented with 1.0 mM MgSO₄, 0.25 mM CaCl₂, 1 μ g D-biotin ml⁻¹ and 10 ng CoCl₂ ml⁻¹. Filter-sterilized 0.5 % (v/v) glycerol, 10 mM succinate or 5 mM protocatechuate (Sigma-Aldrich) was added as indicated to M9 minimal medium as a source of carbon. For *E. coli* strains, the following antibiotic concentrations were used (μ g ml⁻¹): chloramphenicol (Cm), 20; kanamycin (Kn), 10; gentamicin (Gm), 10. For *S. meliloti*, the concentrations used were: streptomycin (Sm), 200; spectinomycin (Sp), 200; neomycin (Nm), 200; gentamicin (Gm), 60; rifampicin (Rif), 20.

Computational analyses. A consensus PcaQ DNA-binding site was generated using conserved sequences upstream of *pcaD* in *A. tumefaciens* C58 (Atu4542), *Mesorhizobium loti* MAFF303099 (mlr7206), *Rhizobium etli* CFN42 (RHE_PE00059), *R. leguminosarum* bv. *viciae* 3841 (pRL110089), *S. meliloti* Rm1021 (SMb20579) and *Sinorhizobium medicae* WSM419 (Smed_4206). The fully sequenced genomes and plasmids of all α -proteobacteria [217 genome and plasmid sequences, available as of December 2008; National Center for Biotechnology Information (NCBI) Genome Database], and selected members of the γ -proteobacteria [*Pseudomonas* (11 sequences)] and β -proteobacteria [*Burkholderia* and *Ralstonia* (85 sequences)], were scanned on both the positive and negative strands for the consensus PcaQ DNA-binding motif (5'-ATAAAY-(N)_x-RRTTAW-3', where x=3, 4 or 5), while allowing for one mismatch within the regulatory site. The output data were screened manually,

and putative binding sites positioned upstream of genes encoding aromatic acid catabolic enzymes and/or LysR-type regulators were extracted for further analysis. The sequence logo was generated by WebLogo (Crooks *et al.*, 2004) using as input all PcaQ DNA-binding sites identified upstream of target genes in members of the α - and γ -proteobacteria, as shown in Fig. 1 and listed in Table 2. Predicted binding sites in β -proteobacteria were excluded from the analysis due to a 1 bp deletion within the motif.

Construction of *S. meliloti* transporter mutants. We targeted the gene encoding a periplasmic solute-binding protein (*smb20568*) for disruption via introduction of an in-frame deletion. Primers (5'-TCGTCTAGAACGTGACGATGGTTCTGG-3' and 5'-TCTAAGCTTCGATCGTCATCAGCACCTG-3') were used to amplify a 1965 bp fragment, which was cloned into pUCP30T via *Xba*I and *Hind*III, yielding pTH1948. A *Pst*I digestion was performed upon pTH1948 to generate a 687 bp deletion within *smb20568* (in-frame deletion of 229 aa), to create plasmid pTH1949. The *S. meliloti* insert in pTH1949 (with the 687 bp deletion) was then subcloned into the suicide vector pJQ200 (Quandt & Hynes, 1993) in the following manner. pTH1949 was digested with *Xba*I and *Hind*III to liberate the *S. meliloti* insert DNA from the pUCP30T backbone. Klenow fragment of DNA polymerase I (New England Biolabs) was added to digested DNA to yield blunt-ended DNA fragments, which were resolved and purified from the agarose gel and cloned into the suicide vector pJQ200 uc-1 via *Sma*I to create pTH1959. This plasmid was transferred into wild-type *S. meliloti* strain RmP110, and single crossover recombinants were selected by plating on LB agar supplemented with Gm. A single purified Gm-resistant (Gm^r) transconjugant was grown overnight in LBmc in the absence of antibiotic selection. Aliquots of the overnight culture were plated on LB agar in the presence of 5 % sucrose to select for cells in which the integrated plasmid had recombined out of the genome. Sucrose-resistant (sucrose^r) colonies were patched on Gm to confirm the loss of the pJQ200 derivative plasmid. Ten sucrose^r and Gm-sensitive (Gm^s) strains were screened via whole-cell PCR amplification of *smb20568* for the presence of a 687 bp deletion; of these, five strains yielded products consistent with the incorporation of a deletion within this gene. Sequencing reactions were performed upon genomic DNA to confirm the genotype of each strain using primers complementary to a sequence external to the region cloned into pJQ200. One of the strains was selected for use in this study and was designated RmP1710.

A second transporter mutant strain was constructed through the insertion of an antibiotic cassette into *smb20787*. An Sm/Sp-resistance cassette was cloned into a *Sac*II site within *smb20787* as follows. Primers (5'-ATGCGGCCGCTGCATCGTTGGTTTGG-3' and 5'-ATGCGGCCGCAATAGCCGGTGACG-3') were used to PCR-amplify a sequence spanning a *Sac*II site located 187 bp downstream of the predicted translational start site of *smb20787*. The 1149 bp amplified product was cloned into plasmid pTH1883 via a *Not*I restriction site, yielding pTH1908. pTH1883 is a derivative of pVO155 (Oke & Long, 1999) in which the reporter gene *gusA* has been deleted; this plasmid was selected because it is unable to replicate in *S. meliloti* and thus may be used as a means of recombining the antibiotic cassette into the *S. meliloti* genome. The Sm/Sp-resistance cassette from pH45Q (Prentki & Krisch, 1984) was PCR-amplified and cloned into pTH1908 following digestion with *Sac*II to create pTH1917. This plasmid was transferred by conjugation into the Rif-resistant *S. meliloti* strain Rm5000; recombinants were selected by plating on LB agar supplemented with Rif and Sp. Transconjugants were patched on LB agar plus Nm to screen for loss of pTH1917.

Southern hybridization was performed to confirm integration of the Ω cassette within *smb20787* as follows. Briefly, primers (5'-CGAGATCGAGCGAGAGTACCAGC-3' and 5'-GGAGAACGTGACGATGGTTCTGG-3') were used to amplify a probe corresponding to *smb20787*. *Sal*I digests were performed upon genomic DNA

Table 1. Strains and plasmids used in this study

Abbreviations: Amp^r, ampicillin resistant; Kn^r, kanamycin resistant; Sm^r, streptomycin resistant; Tc^r, tetracycline resistant.

Strain or plasmid	Relevant characteristics	Source or reference
<i>S. meliloti</i> strains		
RmP110	Rm1021 with wild-type <i>pstC</i> ; Sm ^r	Yuan <i>et al.</i> (2006)
RmP1676	RmP110 <i>pcaQ</i> :: Ω ; Sm ^r , Sp ^r	MacLean <i>et al.</i> (2008)
RmP1710	RmP110 Δ <i>pcaM</i> , 687 bp in-frame deletion created via pTH1959; Sm ^r , Gm ^s , sucrose ^r	This study
RmP1712	RmP110 <i>pcaN</i> :: Ω ; Sm ^r , Sp ^r	This study
RmP1811	RmP110 <i>pcaQ</i> :: Ω <i>pcaM</i> :: <i>gfp</i> ⁺ / <i>lacZ</i> ; Sm ^r , Sp ^r , Gm ^r	This study
Plasmids		
pFL1131	2619 bp fragment extending from within <i>smb20571</i> to <i>pcaM</i> (1 581 256–1 583 875 nt; pSymB) in pTH1522; Gm ^r	Cowie <i>et al.</i> (2006)
pHP45 Ω	pBR322 derivative with Ω element; Amp ^r , Sp ^r , Sm ^r	Prentki & Krisch (1984)
pJQ200 uc1	Suicide vector with <i>sacB</i> to select for plasmid excision; Gm ^r	Quandt & Hynes (1993)
pOT1	Broad-host-range <i>gfpuv</i> transcription reporter; Gm ^r	Allaway <i>et al.</i> (2001)
pUCP30T	Cloning vector; Gm ^r	GenBank accession no. U33752
pTH1227	Broad-host-range derivative of pFus1 with <i>P</i> _{tac} promoter inserted upstream of <i>gusA</i> ; Tc ^r	J. Cheng and T. M. Finan, unpublished results
pTH1705	Derivative of pTH1522; transcription fusion reporter plasmid; Gm ^r	Cowie <i>et al.</i> (2006)
pTH1883	pVO155 derivative lacking <i>gusA</i> , suicide plasmid in <i>S. meliloti</i> ; Amp ^r , Kn ^r	Laboratory collection
pTH1908	1149 bp PCR-amplified fragment spanning <i>pcaN</i> into pTH1883 via <i>NotI</i> ; Amp ^r , Kn ^r	This study
pTH1917	Ω Sm/Sp ^r into pTH1908 via <i>SacI</i> ; Amp ^r , Kn ^r , Sm ^r , Sp ^r	This study
pTH1948	1965 bp PCR-amplified fragment spanning <i>pcaM</i> into pUCP30T via <i>XbaI</i> / <i>HindIII</i> ; Gm ^r	This study
pTH1949	pTH1948 with a 687 bp deletion via <i>PstI</i> ; Gm ^r	This study
pTH1959	<i>XbaI</i> / <i>HindIII</i> insert from pTH1949 into pJQ200 via <i>SmaI</i> ; Gm ^r	This study
pTH1960	1139 bp PCR-amplified fragment spanning <i>pcaI</i> and <i>pcaR</i> intergenic region into pTH1705 via <i>BglII</i> / <i>KpnI</i> ; Gm ^r	This study
pTH2410	242 bp PCR product encompassing <i>pcaM</i> – <i>smb20569</i> intergenic region into pUCP30T via <i>XbaI</i> ; Gm ^r	This study
pTH2414	246 bp PCR-amplified insert from pTH2410 into pOT1 (<i>pcaM</i> :: <i>gfpuv</i>); Gm ^r	This study
pTH2424	A(–62)G site-directed mutation into pOT1; Gm ^r	This study
pTH2425	A(–62)C site-directed mutation into pOT1; Gm ^r	This study
pTH2426	C(–69)A site-directed mutation into pOT1; Gm ^r	This study
pTH2427	A(–59)G site-directed mutation into pOT1; Gm ^r	This study
pTH2428	T(–61)G site-directed mutation into pOT1; Gm ^r	This study
pTH2432	A(–73)G site-directed mutation into pOT1; Gm ^r	This study
pTH2433	A(–71)G site-directed mutation into pOT1; Gm ^r	This study
pTH2454	PCR-amplified <i>pcaQ</i> – <i>his</i> into pTH1227 via <i>BglII</i> / <i>EcoRI</i> ; Tc ^r	This study

isolated from two Sp-resistant (Sp^r) and Nm-sensitive (Nm^s) colonies and wild-type strain RmP110. In both putative *smb20787*:: Ω mutants, an ~2 kb shift (compared with wild-type) in one of the three hybridized *SalI* fragments was noted, consistent with the incorporation of the 2.1 kb cassette into the *SacI* site and excision of pTH1917. The *smb20787*:: Ω allele was transferred from one of the Rm5000 mutants into RmP110 through selection of Sp^r transductants. This strain (RmP110 *smb20787*:: Ω) was designated RmP1712.

Construction of reporter plasmids and plasmid pTH2454. The *smb20568*–*smb20569* intergenic region of *S. meliloti* was PCR-amplified (primers 5'-ATCTAGATACAGGCAGGAGCTGCTTCG-3' and 5'-ATTCTGCAGCAGAATGATCCTTCTCATATTCCT-3') and cloned into the broad-host-range plasmid pOT1 (Allaway *et al.*, 2001) via *XbaI* and *PstI* to create a transcriptional fusion with the promoterless reporter gene *gfp* (*smb20568*::*gfp*, i.e. *pcaM*::*gfp*). Plasmids were mobilized into the recipient *S. meliloti* wild-type strain

Fig. 1. Sequence logo representing PcaQ DNA-binding sites identified in the α - and γ -proteobacteria. The sequence logo was created using WebLogo (Crooks *et al.*, 2004).

Table 2. Description of putative PcaQ-binding motifs in proteobacteria

List of predicted PcaQ DNA-binding sites identified in the α -, β - and γ -proteobacteria. Gene annotations are as assigned by the relevant genome-sequencing projects. Nucleotides in bold type are substitutions or deletions that likely reduce the level of gene expression under inducing conditions.

Species	Class*	Nucleotide sequence	Gene annotation	Locus
<i>Azorhizobium caulinodans</i> ORS 571	α	ATAA CCCTCAGG TTAT	<i>pcaDHGB</i>	AZC_1444
<i>A. tumefaciens</i> C58	α	ATAA TCCACAGG TTAT	<i>pcaDCHGB</i>	Atu4542
<i>Brucella abortus</i> S19	α	ATAA CCTGTGAG TTAT	<i>pcaDCHG</i>	BAbS19_II05600
<i>B. abortus</i> S19	α	ATAA CTGTTCGG ATAT	ABC-type transporter	BAbS19_II05550
<i>Brucella canis</i> ATCC 23365	α	ATAA CCTGTGAG TTAT	<i>pcaDCGB</i>	BCAN_B0644
<i>B. canis</i> ATCC 23365	α	ATAA CTGTTCGG ATAT	ABC-type transporter	BCAN_B0648
<i>Brucella melitensis</i> 16M	α	ATAA CCTGTGAG TTAT	<i>pcaDCHGB</i>	BMEII0638
<i>B. melitensis</i> 16M	α	ATAA CTGTTCGG ATAT	ABC-type transporter	BMEII0633
<i>Brucella ovis</i> ATCC 25840	α	ATAA CCTGTGAG TTAT	<i>pcaCG</i>	BOV_A0606
<i>B. ovis</i> ATCC 25840	α	ATAA CTGTTCGG ATAT	ABC-type transporter	BOV_A0610
<i>Brucella suis</i> 1330	α	ATAA CCTGTGAG TTAT	<i>pcaDCHGB</i>	BRA0643
<i>B. suis</i> 1330	α	ATAA CTGTTCGG ATAT	ABC-type transporter	BRA0648
<i>M. loti</i> MAFF303099	α	ATAA CCCCCGAG TTAA	<i>pcaDCHGBpobA</i>	mlr7206
<i>M. loti</i> MAFF303099	α	CTAA CCCCAGGG TTAA	ABC-type transporter	mlr7204
<i>Ochrobactrum anthropi</i> ATCC 49188	α	ATAA CCCTGTAG TTAT	<i>pcaDCHGB</i>	Oant_3725
<i>R. etli</i> CIAT 652	α	ATAA CTCCTAAG TTAT	<i>pcaDCHGB</i>	RHECIAT_PA0000063
<i>R. etli</i> CIAT 652	α	ATAA CCAACAGA TCAT	ABC-type transporter	RHECIAT_CH0003683
<i>R. etli</i> CFN42	α	ATAA CCTCGGAG TTAT	<i>pcaDCHGB</i>	RHE_PE00059
<i>R. etli</i> CFN42	α	ATAA CCGGCAA TCAT	ABC-type transporter	RHE_CH03445
<i>R. leguminosarum</i> bv. <i>viciae</i> 3841	α	ATAA TTGTACAG TTAT	ABC-type transporter	RL3906
<i>R. leguminosarum</i> bv. <i>viciae</i> 3841	α	ATAA CTCCACGG TTAT	<i>pcaDCHGB</i>	pRL110089
<i>R. leguminosarum</i> bv. <i>trifolii</i> WSM2304	α	ATAA CTCCCAGG TTAT	<i>pcaDCHGB</i>	Rleg2_5939
<i>S. meliloti</i> Rm1021	α	ATAA CCGGGGGA TTAT	ABC-type transporter	<i>smb20568</i>
<i>S. meliloti</i> Rm1021	α	ATAA CCTCCTGG TTAA	<i>pcaDCHGB</i>	<i>smb20579</i>
<i>S. medicae</i> WSM419	α	ATAA CCGGGGAA TTAT	ABC-type transporter	Smed_4217
<i>S. medicae</i> WSM419	α	ATAA CTCCCTGG TTAA	<i>pcaDCHGB</i>	Smed_4206
<i>Burkholderia mallei</i> NCTC 10229	β	ATAA CGGTAG TTAT	<i>pcaHG</i>	BMA10229_0245
<i>Burkholderia multivorans</i> ATCC 17616	β	ATAA CCACCCG TTAT	<i>pcaHG</i>	BMULJ_04089
<i>Burkholderia pseudomallei</i> 1106a	β	ATAA CAGTTAG TTAT	<i>pcaHG</i>	BURPS1106A_A1760
<i>Burkholderia phymatus</i> STM815	β	ATAA CGTAACG TTAT	<i>pcaHG</i>	Bphy_3889
<i>Burkholderia vietnamiensis</i> G4	β	ATAA CTCCCGG TTAT	<i>pcaHG</i>	Bcep1808_4740
<i>Ralstonia solanacearum</i> MolK2	β	ATAA CATCCGG TTAT	<i>pcaHG</i>	RSMK00708
<i>Pseudomonas aeruginosa</i> PA7	γ	ATAA CCAAACGG TTAT	<i>pcaHG</i>	PSPA7_0231
<i>P. aeruginosa</i> PAO1	γ	ATAA CCAAATGG TTAT	<i>pcaHG</i>	PA0153
<i>P. aeruginosa</i> UCBPP-PA14	γ	ATAA CCAAATGG TTAT	<i>pcaHG</i>	PA14_01900
<i>Pseudomonas fluorescens</i> Pf-5	γ	ATAA CCATTTGG TTAT	<i>pcaHG</i>	PFL_5396
<i>P. putida</i> W619	γ	ATAA CCATTTGG TTAA	<i>pcaHG</i>	PputW619_0780
<i>Pseudomonas stutzeri</i> A1501	γ	ATAA CCCCTGGG TTAT	<i>pcaHG</i>	PST_1249
<i>Pseudomonas syringae</i> pv. <i>phaseolicola</i> 1448A	γ	ATAA CCATTTGG TTAT	<i>pcaHGKBC</i>	PSPPH_2096
<i>P. syringae</i> pv. <i>tomato</i> str. DC3000	γ	ATAA CCAATTGG TTAT	<i>pcaHGKBC</i>	PSPTO_2338

*Proteobacterial class.

RmP110 and PcaQ-minus strain RmP1676 via triparental conjugation with MT616 (pRK600) (Finan *et al.*, 1986).

Plasmid pTH1979 contains *pcaQ* cloned into the expression vector pET-21a (Novagen) to create a translational fusion with a hexahistidine tag at the N terminus of the *S. meliloti* protein (MacLean *et al.*, 2008). This plasmid was used as template DNA for the PCR amplification of *pcaQ-his* using the primers 5'-GTGAGATCTAAGAAGGAGATATACATATGATCGAC-3' and 5'-

AGAATTCGTTAGCAGCCGGATCTCAGTG-3' into the broad-host-range replicating plasmid pTH1227 via *Bgl*II and *Eco*RI to create plasmid pTH2454, in which *pcaQ-his* is expressed from the P_{tac} promoter. After sequencing (to confirm the absence of mutations within the coding sequence), the plasmid was transferred via conjugation into *S. meliloti* strain RmP1811 (RmP110 *pcaQ*:: Ω *smb20568*::*gfp*+/*lacZ*), in which expression of *pcaQ* has been disrupted through the integration of an Sp/Sm antibiotic-resistance cassette. RmP1811 also contains a transcriptional fusion (*smb20568*::*gfp*+/*lacZ*) created through the

integration of plasmid pFL1131 (Cowie *et al.*, 2006), which allows expression of *smb20568* to be monitored via β -galactosidase assays.

Site-directed mutagenesis. To modify the PcaQ DNA-binding site located upstream of *smb20568*, plasmid pTH2410 was used as a template for mutagenesis reactions and was constructed as follows. Primers (5'-ATCTAGATACAGGCAGGAGCTGCTTCG-3' and 5'-ATCTAGAGCCAGAATGATCCTTCTCATATTCCT-3') were used to PCR-amplify the *smb20568*–*smb20569* intergenic region, which was cloned into pUCP30T (GenBank accession no. U33752) via *Xba*I. Site-directed mutagenesis reactions were performed using Platinum *Pfx* DNA polymerase (Invitrogen) according to the supplier's protocol. After amplification, 20 U *Dpn*I (New England Biolabs) was added directly to the reaction mixture, which was incubated at 37 °C for 2 h. Plasmid DNA was purified using QIAquick spin columns (Qiagen) and transformed into chemically competent *E. coli* DH5 α . Transformants were selected via Gm resistance, and plasmids were sequenced to confirm the presence of site-directed mutations by Mobix Lab (McMaster University, Hamilton, ON, Canada). For the purpose of constructing *gfp* reporter fusions, the pUCP30T derivative plasmids (each carrying a site-directed mutation) were used as template DNA for the PCR amplification of the *smb20568*–*smb20569* intergenic region using the same primers as those described for the amplification of the wild-type regulatory region.

β -Galactosidase and GFP assays. A transcriptional fusion between *S. meliloti* *pcaI* and the reporter gene *lacZ* was created as follows. The intergenic region between *pcaI* and *pcaR* was amplified using primers (5'-ATCAGATCTGAGTTCGTCGACGATCTCC-3' and 5'-TAGGTACCCAGTTGATCGACATCACC-3'). The amplified product was cloned into the reporter plasmid pTH1705 (Cowie *et al.*, 2006) via *Bgl*II and *Kpn*I to create *pcaI::gfp*+/lacZ. The resulting plasmid (pTH1960) is unable to replicate in *S. meliloti*; however, it carries an ~1 kb region that is homologous to the *S. meliloti* *pcaI*–*R* intergenic region. pTH1960 was introduced into RmP110, RmP1710 and RmP1712 through triparental conjugation with the *E. coli* helper strain MT616 (pRK600) (Finan *et al.*, 1986). Transconjugants in which pTH1960 had recombined into the pSymB megaplasmid were isolated by demanding growth on LB agar plus Sm and Gm.

β -Galactosidase enzyme assays were performed as previously described (MacLean *et al.*, 2006) upon *S. meliloti* strains subcultured into M9 minimal medium with glycerol and/or protocatechuate as carbon sources. *Gfp* fluorescence was assayed as previously described (MacLean, *et al.*, 2008). Relative fluorescence was determined by dividing the emission output of each sample by its respective OD₆₀₀.

Primer extension and electrophoretic mobility shift assays. Total RNA was isolated from an *S. meliloti* RmP110 culture grown aerobically at 30 °C in LBmc $\pm$ 5 mM protocatechuate to OD₆₀₀ 0.8. RNA extraction was performed using a hot phenol method, as previously described (MacLellan *et al.*, 2005). Approximately 60 μ g total RNA was used in each extension reaction, as previously described (MacLellan *et al.*, 2005). Two primers were used to yield extension products (5'-CCAGAATGATCCTTCTCATATTCCTCC-3' and 5'-CGACGACTCCGACCTTGATCGTATCC-3'). Sequenase Version 2.0 DNA Sequencing kits (USB) were used for sequencing reactions, which were performed upon plasmid pFL1131. The same primers were used in both sequencing and primer extension reactions.

A 246 bp probe spanning the *smb20568* (*pcaM*) and *smb20569* intergenic region (–149 to +87; *smb20568* transcriptional start site) was PCR-amplified using primers (5'-ATCTAGATACAGGCAGGAGCTGCTTCG-3' and 5'-ATTCTGCAGCAGAATGATCCTTCTCATATTCCT-3'). PCR products were purified using a polyacrylamide gel before labelling reactions were performed using [γ -³²P]ATP (Perkin Elmer) and T4 polynucleotide kinase. Assays were performed

using purified PcaQ-His, as previously described (MacLean *et al.*, 2008).

RESULTS

Prediction of putative PcaQ-binding sites in proteobacteria

A consensus PcaQ DNA-binding motif was generated using the experimentally characterized binding site upstream of *pcaD* in *S. meliloti* (MacLean *et al.*, 2008) and conserved sites upstream of *pcaD* in related rhizobia. Using the consensus sequence (5'-ATAAAY-(N)_x-RRTTAW-3', where $x=3, 4$ or 5), we scanned the genomes of 313 α -, β - (*Burkholderia* and *Ralstonia*) and γ -proteobacteria (*Pseudomonas*) for potential regulatory sites. The majority of genomes yielded between zero and three hits for putative PcaQ DNA-binding sites. The quality of putative PcaQ DNA-binding sites was assessed based upon the position of a mismatch (if present) within the predicted regulatory sequence, as compared with the consensus sequence, and the proximity of the predicted site to likely target genes (i.e. upstream of a gene annotated to encode a product involved in aromatic acid catabolism or a LysR-type protein). Using these criteria, we identified candidate PcaQ DNA-binding sites within the genomes of species from all three groups of proteobacteria (Fig. 1, Table 2). In the α -proteobacteria, conserved binding sites were located upstream of *pcaD* (encoding β -ketoadipate enol-lactone hydrolase), consistent with previous reports documenting the regulation of this gene by PcaQ in *S. meliloti* and *A. tumefaciens* (Parke, 1993; MacLean *et al.*, 2006). In β - and γ -proteobacteria, PcaQ DNA-binding sites were positioned upstream of the operon *pcaHG*, encoding the β and α subunits of protocatechuate 3,4-dioxygenase, which mediates the first step in the metabolism of protocatechuate, a ring cleavage of the aromatic acid through the incorporation of oxygen (Stanier & Ingraham, 1954). Furthermore, we identified putative PcaQ DNA-binding sites located upstream of genes encoding ABC-type transport systems in 10 members of the α -proteobacteria, including *S. meliloti*. Expression of one of these ABC-type transport systems (encoded by *smb20568*–*smb20784* in *S. meliloti*) has previously been shown to be upregulated by growth with protocatechuate (Mauchline *et al.*, 2006). As we show herein that the *smb20568*–*smb20784* gene cluster is required for growth with protocatechuate, we designate these genes *pcaMNVWX*. This transport gene cluster is located approximately 8 kb downstream of the *pcaDCHGB* operon on the pSymB megaplasmid.

Identification of the *pcaM* transcriptional start site

We have determined that the intergenic region between *pcaM* and *pcaN* does not contain a promoter (MacLean, 2008), and the non-coding regions located between the remaining transport genes (ranging from 3 to 19 nt between each gene) are unlikely to include a promoter. Thus it is

probable that expression of the entire transport gene cluster is dependent upon a promoter located upstream of *pcaM* (*smb20568*). A primer extension analysis was performed to identify the *pcaM* transcription start site as a means of further elucidating the regulation of this gene cluster.

Total RNA was isolated from wild-type *S. meliloti* strain RmP110 grown in the presence and absence of protocatechuate, and extension reactions were performed using two different primers. With both primers, at least two extension products were obtained using RNA isolated from *S. meliloti* grown with and without protocatechuate (Fig. 2a). The larger of the two extension products corresponds to a transcriptional start site located 29 nt upstream of the predicted *pcaM* translational start codon. A greater product yield for this transcript was obtained using RNA isolated from cells grown with protocatechuate; this is consistent with an upregulation of *pcaM* expression in the presence of protocatechuate. The smaller extension product corresponds to a transcriptional start site located within the predicted coding sequence of *pcaM* and is likely due to the premature termination of the extended product. The predicted PcaQ DNA-binding site was located -73 to -58 upstream of the ATG translational start codon of *pcaM* (Fig. 2b), and we note that this relative position is comparable with that of the experimentally characterized binding site -72 to -57 upstream of *pcaD* (MacLean *et al.*, 2008).

Expression of an ABC-type transport system is regulated by PcaQ

To determine whether PcaQ is required for the protocatechuate-inducible expression of the *S. meliloti* transport

system, the regulatory region upstream of a gene (*pcaM*) encoding the periplasmic solute-binding protein was cloned into the broad-host-range reporter plasmid pOT1, to generate a transcriptional fusion with the reporter gene *gfp*. Expression of *pcaM::gfp* (pTH2414) was induced greater than sixfold in wild-type cells cultured with protocatechuate, confirming that expression of this gene cluster is responsive to growth with the aromatic acid. In contrast, expression of the transporter gene was not induced in RmP1676 (RmP110 *pcaQ::Ω*), indicating that the LysR-type regulator PcaQ is required for the upregulation of *pcaM* expression (Table 3). As a control, we included *pcaD::gfp* (pTH2276), and observed that *pcaD* expression was not upregulated in RmP1676, consistent with the results of previous studies (MacLean *et al.*, 2006, 2008). Furthermore, we observed that expression of *pcaM* in a *pcaR* mutant (RmP110 *pcaR::Ω*) background was comparable with that observed in the wild-type strain (MacLean, 2008). Thus, PcaR is not required for the regulation of *pcaM* expression.

In separate experiments, we confirmed that supplying *pcaQ* *in trans* is sufficient to allow protocatechuate-dependent *pcaM* expression in a *pcaQ* mutant (Table 4). Thus, cultures of *S. meliloti* RmP1811 (*pcaQ::Ω pcaM::gfp*+/lacZ) containing the empty expression plasmid pTH1227 showed no induction of β -galactosidase activity upon addition of protocatechuate. In contrast, assays of cultures of *S. meliloti* RmP1811 containing plasmid pTH2454 expressing PcaQ with a hexahistidine tag showed a sixfold induction of β -galactosidase activity when grown in the presence of protocatechuate. These results indicate that the His-tagged PcaQ has the ability to regulate *pcaM*

Fig. 2. Regulation of an ABC-type transport system encoded by *pcaMNVWX*. (a) Primer extension reactions were performed using mRNA isolated from *S. meliloti* wild-type strain RmP110 grown in the absence (lane 1) and presence (lane 2) of protocatechuate. Arrowheads (right) indicate the extension products obtained using two primers (results shown for one primer only), and an arrowhead (left) identifies the corresponding nucleotide. (b) Comparison of *S. meliloti* *pcaD* and *pcaM* regulatory regions (this work; MacLean *et al.*, 2008). The -35 hexanucleotide region for each gene is underlined and the PcaQ DNA-binding sites are shaded in grey. Invariant nucleotides are indicated by asterisks. (c) Electrophoretic mobility shift assay for PcaQ binding to the intergenic region upstream of *pcaM*. A 246 bp radiolabelled probe (extending from -149 to $+87$; *pcaM*) was incubated in the presence of increasing concentrations of purified PcaQ. Lanes 1–8 contained 0, 0.2, 0.5, 1.2, 2.5, 4.9, 12.4 and 24.7 nM PcaQ.

Table 3. Expression of *pcaM::gfp* in *S. meliloti*

Strain genotype	Gene fusion	GFP fluorescence* (SD)	
		Uninduced	Induced
RmP110			
(pTH2414)	<i>pcaM</i>	2443 (69)	15 173 (2663)
(pTH2276)	<i>pcaD</i>	1047 (38)	16 115 (572)
(pOT1)	None	353 (20)	145 (108)
RmP110			
<i>pcaQ::Ω</i>			
(pTH2414)	<i>pcaM</i>	2204 (198)	1227 (55)
(pTH2276)	<i>pcaD</i>	422 (99)	208 (102)
(pOT1)	None	298 (54)	512 (112)

*Shown are means of assays performed in triplicate using *S. meliloti* strains subcultured into M9 minimal medium with 0.5% (v/v) glycerol in the absence (uninduced) and presence (induced) of 5 mM protocatechuate. Data are representative of three independent experiments. SD, Standard deviation of assays performed in triplicate.

expression *in vivo*, and that the presence of the N-terminal tag does not interfere with PcaQ regulatory activity.

We performed electrophoretic mobility shift assays using purified PcaQ-His to directly examine whether PcaQ binds to the regulatory region upstream of *pcaM*. PcaQ was shown to bind labelled probe (extending from -149 to +87, with respect to the transcriptional start site of *pcaM*) in the absence of a co-inducing metabolite (Fig. 2c), consistent with previous reports describing the binding of this and other LysR proteins to DNA independently of a co-effector (Fisher *et al.*, 1988; Schell & Poser, 1989; Rothmel *et al.*, 1991; Wang *et al.*, 1992; MacLean *et al.*, 2008). In contrast, PcaQ did not shift a probe consisting of the *S. meliloti* *pcaI*-*pcaR* intergenic sequence, even when up to 50 ng purified protein was included in a parallel assay (data not shown). As PcaQ does not regulate the expression of *pcaI*, we included

Table 4. Complementation of an *S. meliloti* *pcaQ* mutant by PcaQ-His

Strain	β -Galactosidase activity* (SD)	
	Uninduced	Induced
RmP1811 (pTH1227)	117 (9)	116 (25)
RmP1811 (pTH2454)	115 (12)	696 (14)

*Shown in Miller units are means of assays performed in triplicate using *S. meliloti* strains subcultured into M9 minimal medium with 0.5% (v/v) glycerol in the absence (uninduced) and presence (induced) of 5 mM protocatechuate. Data are representative of three independent experiments. SD, Standard deviation of assays performed in triplicate.

this probe as a control to demonstrate that the interaction between PcaQ and sequence upstream of *pcaM* is specific. Our assays therefore indicate that PcaQ is directly involved in regulating expression of the transporter gene through an interaction with a binding site located within the *pcaM*-*smb20569* intergenic region.

Mutagenesis of the predicted PcaQ DNA-binding site reveals a functional role

We wished to determine whether the PcaQ DNA-binding site predicted by our *in silico* analyses was essential to the regulation of *pcaM* expression in response to growth with protocatechuate. We targeted six conserved positions within the predicted PcaQ DNA-binding site for mutagenesis, and determined whether each mutation affected the regulation of *pcaM* expression (Fig. 3). The introduction of mutations within five of the six nucleotides targeted for mutagenesis (as underlined: 5'-ATAACCGGGGATTAT-3') abolished the protocatechuate-inducible expression that was observed in the wild-type regulatory sequence (Fig. 3, pTH2414). In four of these mutations (corresponding to plasmids pTH2427, pTH2428, pTH2432 and pTH2433), the nucleotide substitution decreased the fold-induction observed in protocatechuate-cultured cells, and it is likely that these mutations interfered with the ability of PcaQ to bind upstream of *pcaM*, based upon an analysis of the DNA-binding site associated with *pcaD* (MacLean *et al.*, 2008). Substitution of an adenine with a cytosine at a position that invariably encodes a purine in all predicted DNA-binding motifs (5'-ATAACCGGGGATTAT-3') did not exert a strong effect upon the regulated expression of *pcaM* (Fig. 3, pTH2425); however, substitution with a guanine at the same position almost doubled the level of induced *pcaM* expression (pTH2424).

Substitution of a cytosine at a position that is highly conserved amongst the PcaQ regulatory sites (5'-ATAACCGGGGATTAT-3'; pTH2426) elicited a level of *pcaM::gfp* expression that was greater than twofold that observed in uninduced cells carrying the wild-type regulatory sequence. Furthermore, expression of *pcaM::gfp* was comparable in cells grown in the presence and absence of protocatechuate when this substitution was made, and in *S. meliloti* strains with (RmP110) and without (RmP1676) PcaQ, indicating that the elevated expression of *pcaM* was independent of PcaQ-mediated regulation. This constitutive expression might be attributed to the generation of an UP element, an AT-rich sequence upstream of a promoter that interacts with α CTD-RNAP (Ross *et al.*, 1993). However, the position of the mutation (-69) was farther upstream than might be expected to influence expression (typically -60 to -40), and the altered regulatory sequence did not exhibit a strong similarity to the consensus UP element (Estrem *et al.*, 1999). In any case, the transversion of C→A within the context of the AT-rich PcaQ-binding site appeared to strengthen interactions with the RNAP in a manner that was independent of PcaQ activation.

Fig. 3. Analysis of *pcaM::gfp* expression in *S. meliloti* wild-type and *pcaQ::Ω* strains. *S. meliloti* strains carrying plasmids as indicated were subcultured into M9 minimal medium with 0.5% (v/v) glycerol with or without 5 mM protocatechuate, and incubated at 30 °C for 4–6 h. Point mutations were introduced into a putative PcaQ DNA-binding site as shown, and assays were performed to assess the effect of mutagenesis upon the regulation of *pcaM* expression. Reported is the mean expression as determined from three independent experiments; in each experiment, assays were performed in triplicate. Error bars, SD observed between experiments. White bars, *S. meliloti* RmP110 (wild-type), uninduced; light-grey bars, *S. meliloti* RmP110, induced; dark-grey bars, *S. meliloti* RmP1676 (RmP110 *pcaQ::Ω*), uninduced; black bars, *S. meliloti* RmP1676, induced.

An ABC-type transport system is required for growth with protocatechuate in *S. meliloti*

As a means of assessing whether the *pcaMNVWX* (*smb20568–smb20784*) cluster is involved in the uptake of protocatechuate, we constructed two mutant strains. *S. meliloti* strain RmP1710 (RmP110 Δ *pcaM*) carries an in-frame deletion of the gene encoding the periplasmic solute-binding protein (*pcaM*) of the transporter, whereas RmP1712 (RmP110 *pcaN::Ω*) contains an antibiotic-resistance cassette within a permease gene (*pcaN*) (Fig. 4a). The *S. meliloti* mutants exhibited growth kinetics comparable with those of the wild-type strain (RmP110) when subcultured into minimal medium supplemented with succinate (Fig. 4b) or glycerol (data not shown). However, both transporter mutant strains grew poorly when cultured with protocatechuate as a sole carbon source, and did not grow as well as the wild-type strain in the presence of protocatechuate and succinate (Fig. 4c, d). The growth phenotype exhibited by RmP1710 and RmP1712 in the presence of protocatechuate was complemented by the cosmid pTH178 (MacLean, 2008), which

carries the ABC-type transport system genes *pcaMNVWX* and the *pcaDCHGB* operon (MacLean *et al.*, 2006). Strain RmG879 (Rm1021 *pcaG::Tn5*) is unable to metabolize protocatechuate (MacLean *et al.*, 2006), and was included as a control in the growth assays. As expected, this strain grew well in the presence of succinate; however, it was unable to utilize protocatechuate as a sole source of carbon.

The data obtained from the growth curves (Fig. 4b–d) suggested that while the transporter mutants RmP1710 and RmP1712 grew poorly when offered protocatechuate as a sole carbon source, sufficient protocatechuate may enter the cell to permit some metabolism of the compound, which is best reflected by the growth of the transporter mutants (as compared with the *pcaG* mutant) cultured in minimal medium with protocatechuate and succinate (Fig. 4c). We monitored the expression of *pcaI::lacZ* in wild-type and transporter mutant strains of *S. meliloti*, and determined that expression was indeed induced by growth with protocatechuate in all three strains (Fig. 4e). Induction of *pcaI* expression requires the pathway metabolite β -ketoadipate, thus confirming passive diffusion or uptake via

Fig. 4. (a) Schematic depiction of the *S. meliloti* protocatechuate transport and catabolic genes on the pSymB megaplasmid. *pcaMNVWX* (*smb20568*–*smb20784*) (shaded grey) encode a protocatechuate-inducible ABC-type transport system required for growth with protocatechuate (this work). (b–e) Growth of *S. meliloti* strains subcultured into M9 minimal medium with (b) 10 mM succinate, (c) 10 mM succinate and 5 mM protocatechuate or (d) 5 mM protocatechuate as sole carbon sources. Inoculum was from washed LBmc-grown cells and cultures were incubated with shaking at 30 °C for 57.5 h; growth of each strain was assayed in triplicate cultures and the mean OD₆₀₀ is shown; error bars, SD. (e) Expression of *pcaI::lacZ* was assayed in wild-type (RmP110) and transporter mutant backgrounds (RmP1710 and RmP1712) in minimal medium with carbon sources as indicated; Pca, protocatechuate.

alternative transporter(s) of protocatechuate across the membrane (and subsequent metabolism) in the absence of the inducible transport system. Nonetheless, uptake in the *pcaM* and *pcaN* mutant strains is apparently not sufficient to support the growth of the transporter mutants in the absence of an additional carbon source.

DISCUSSION

The LysR-type protein PcaQ participates in the regulation of *pcaD* expression in *S. meliloti* and *A. tumefaciens* (Parke, 1993, 1996; MacLean *et al.*, 2006, 2008), and we here report that expression of a gene encoding a component of an

ABC-type transport system (*pcaM*) is likewise regulated by PcaQ in *S. meliloti*. Transport systems involved in the uptake of aromatic acids primarily belong to the major facilitator superfamily (MFS) of transporter proteins (Harwood *et al.*, 1994; Collier *et al.*, 1997; Williams & Shaw, 1997; Leveau *et al.*, 1998; D'Argenio *et al.*, 1999; Chaudhry *et al.*, 2007) and have been described predominantly in the γ -proteobacteria. Within the α -proteobacteria, protocatechuate (and *p*-hydroxybenzoate) uptake has been described in *R. leguminosarum*, yet the genetic identity of the transport system has never been established (Wong *et al.*, 1991). We show that the *smb20568–smb20784* transport genes are required for growth with protocatechuate (Fig. 4), that expression of this system is protocatechuate-inducible (Table 3), and that it is subject to regulation by the β -ketoadipate regulatory protein PcaQ (Figs 2 and 3). We conclude that the system encoded by *smb20568–smb20784* is a protocatechuate transport system, and propose that these genes be designated *pcaMNVWX* (Fig. 4a).

The organization of aromatic acid catabolic, regulatory and transport genes within supraoperons is well-documented (Harwood & Parales, 1996), and we have furthermore observed that transport systems are often encoded near genes relevant to the catabolism of a substrate. We have identified PcaQ DNA-binding sites upstream of ABC-type transport systems in *S. medicae*, *R. etli*, *R. leguminosarum*, *M. loti* and *Brucella* species (Fig. 1, Table 2), and (as with *S. meliloti*) we note that these systems are located in close proximity to aromatic acid catabolic genes. ABC-type transport genes are also located near to the *pcaQCHG* gene cluster in the marine bacteria *Roseobacter* sp. MD193 (MED193_18204 to MED193_18229) and *Phaeobacter gallaeciensis* (RG210_05172 to RG210_05147), but appear to be absent in *Roseobacter* sp. GAI101, *Ruegeria pomeroyi* DSS-3, and related species (Buchan *et al.*, 2004). Nonetheless, it seems likely that as a general rule, ABC-type transport systems mediate the uptake of protocatechuate and related aromatic acids in α -proteobacteria, which represents a departure from the MFS-dominated systems characterized to date.

An interesting result from the *in silico* prediction of PcaQ-binding sites was the identification of such motifs upstream of *pcaHG* in *Pseudomonas*, *Ralstonia* and *Burkholderia* (Fig. 1, Table 2). The genomes of these genera were selected for inclusion in our analyses based upon an annotation of the LysR-type protein PcaQ in the genome of at least one species from each group. While the presence of a PcaQ homologue in *Pseudomonas* has been noted (Overhage *et al.*, 1999; Jiménez *et al.*, 2002), there is currently no experimental evidence supporting the regulation of *pca* genes by PcaQ in these bacteria. In *Acinetobacter baylyi*, *pcaHG* form part of a large operon (*pcaIJFBDKCHG*) that is regulated by the IclR-type regulatory protein PcaU (Gerischer *et al.*, 1998). In contrast, expression of *pcaDCHGB* is modulated by PcaQ in *S. meliloti* and *A. tumefaciens* (Parke, 1993; MacLean *et al.*, 2006), and we

now offer *in silico* evidence that PcaQ may also regulate the expression of these genes in certain members of the β - and γ -proteobacteria. We note that the binding sites identified in the β -proteobacteria atypically contain a 7 nt spacer between the conserved left- and right-hand arms of the binding site; all other predicted sites encode 8 nt. Further study is required to confirm that PcaQ does regulate *pcaHG* expression in these species, and to determine what effect (if any) such a deletion might have upon the regulation of gene expression in *Ralstonia* and *Burkholderia*.

We have previously described the effect of introducing point mutations within a PcaQ DNA-binding site upstream of *pcaD* in *S. meliloti* (MacLean *et al.*, 2008), and the identification of a second putative binding site upstream of *pcaM* presented an opportunity to expand upon our earlier analyses. Each of the six positions targeted for mutagenesis in this study (underlined) appears to be relevant to the regulation of *pcaM* expression (5'-ATAACCGGGGATTAT-3'), as replacement of any of these conserved nucleotides resulted in an altered expression of the transporter gene (Fig. 3). In addition, we note a general correlation between the degree of nucleotide conservation at a particular position and the severity of the regulatory phenotype upon mutagenesis. For example, the greatest effect upon the regulated expression of both *pcaD* and *pcaM* was observed upon mutagenesis of A(–59)G, a position that is invariant in all PcaQ-binding sites predicted in this study (Fig. 1, Table 2).

Interestingly, the substitution of A(–62)G (5'-ATAACCGGGGATTAT-3'; pTH2424) doubled the level of *pcaM* expression (>20-fold induction) in response to the aromatic acid. It is worth emphasizing that this particular A(–62)G mutation yields a dyad motif that more closely matches the majority of predicted DNA-binding sites [5'-ATAACY-(N)₄-RGTTAW-3']. The *pcaM* sequence is one of only four predicted PcaQ DNA-binding sites to encode an adenine (as opposed to a guanine) at this position (Table 2), and each of these four sites is associated with an ABC-type transport system which presumably mediates the transport of protocatechuate (in *R. etli*, *S. meliloti* and *S. medicae*). In total, of the 11 predicted PcaQ DNA-binding sites positioned upstream of transport genes, 10 contain nucleotide substitutions or deletions that likely reduce the level of gene expression under inducing conditions (Table 2, bold type), based upon mutagenesis of comparable positions in sites upstream of *pcaD* (MacLean *et al.*, 2008) or *pcaM* (this study). In comparison, we note that no such mutations are present within binding sites associated with the catabolic genes (*pcaDCHGB* and *pcaHG*). The functional significance of encoding transport systems with a decreased responsiveness to protocatechuate (in terms of gene expression) imparts an interesting physiological consequence. Protocatechuate (and particularly the metabolite β -carboxy-*cis,cis*-muconate) is toxic if allowed to accumulate intracellularly (Parke *et al.*, 2000), and a highly expressed aromatic acid transport system may be

maladaptive in an environment rich in protocatechuate and related compounds, particularly if the transport system has a high affinity for its substrate. For example, it has been proposed that a mutational hotspot in the protocatechuate uptake system *vanK* of *Acinetobacter baylyi* may enhance the adaptability of this species in a soil environment by providing a subset of the population with a decreased ability to transport protocatechuate and thus a greater resistance to protocatechuate exposure (D'Argenio *et al.*, 1999; Parke *et al.*, 2000). It is interesting to speculate that the preferential accumulation of mutations within PcaQ DNA-binding sites upstream of transport genes may reflect an adaptation to limit the active import of protocatechuate into the cell by limiting the expression of a relevant transport system. Alternatively, it is possible that the absence of such mutations in sites associated with *pca* catabolic genes may be due to stronger selective pressure acting against systems in which the expression of these (catabolic) genes is compromised. Indeed, these alternative scenarios are not mutually exclusive.

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