The ATP-binding cassette transporter Cbc (choline/betaine/carnitine) recruits multiple substrate-binding proteins with strong specificity for distinct quaternary ammonium compounds

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Summary

We identified a choline, betaine and carnitine transporter, designated Cbc, from *Pseudomonas syringae* and *Pseudomonas aeruginosa* that is unusual among members of the ATP-binding cassette (ABC) transporter family in its use of multiple periplasmic substrate-binding proteins (SBPs) that are highly specific for their substrates. The SBP encoded by the *cbcXWV* operon, CbcX, binds choline with a high affinity (K_m, 2.6 μM) and, although it also binds betaine (K_m, 24.2 μM), CbcXWV-mediated betaine uptake did not occur in the presence of choline. The CbcX orthologue ChoX from *Sinorhizobium meliloti* was similar to CbcX in these binding properties. The core transporter CbcWV also interacts with the carnitine-specific SBP CaiX (K_m, 24 μM) and the betaine-specific SBP BetX (K_m, 0.6 μM). Unlike most ABC transporter loci, *caiX*, *betX* and *cbcXWV* are separated in the genome. CaiX-mediated carnitine uptake was reduced by CbcX and BetX only when they were bound by their individual ligands, providing the first *in vivo* evidence for a higher affinity for ligand-bound than ligand-free SBPs by an ABC transporter. These studies demonstrate not only that the Cbc transporter serves as a useful model for exploring ABC transporter component interactions, but also that the orphan SBP genes common to bacterial genomes can encode functional SBPs.

Introduction

Transporters in the ATP-binding cassette (ABC) family are vital among living organisms due to their role in translocating substrates ranging from ions, sugars and amino acids to high-molecular-weight proteins (Biemans-Oldehinkel *et al.*, 2006; Davidson *et al.*, 2008). The

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core transporter contains two transmembrane spanning domains (TMDs) that provide a substrate-specific pathway across the membrane, and two nucleotide-binding domains (NBDs, or ATPases) that power the translocation (Davidson *et al.*, 2008). Prokaryotic ABC importers generally employ a third subunit, a substrate-binding protein (SBP), which is either free in the periplasm in Gram-negative bacteria or anchored in the external surface of the membrane in Gram-positive bacteria. SBPs are the determinants of substrate specificity and are indispensable to the import function of prokaryotic ABC transporters.

All characterized ABC transporter-associated SBPs involved in import consist of two lobes, or domains, that are linked by a flexible hinge (van der Heide and Poolman, 2002). The relative movement of these lobes creates open and closed conformations and allows SBPs to capture their ligands through a mechanism termed a Venus flytrap that involves very rapid ligand binding (Miller *et al.*, 1983). The ligand-bound SBP is thought to transmit a signal via the TMD to the NBD on the other side of the membrane, probably through co-ordinated events including ATP binding and hydrolysis, to deliver the substrate into the cell (Orelle *et al.*, 2008).

Structural and kinetic analyses of the ABC transporters crystallized to date have demonstrated that a single, monomeric SBP typically associates with the core transporter such that each SBP lobe interacts with one of the two subunits of the TMD (Davidson *et al.*, 2008). These interaction sites, however, differ among transporters and range from simple salt bridges formed between amino acid residues of the SBP and the TMD (Braun and Herrmann, 2007; Rees *et al.*, 2009) to extensive interactions such as a large periplasmic loop on the TMD that keeps the SBP closely associated even in the absence of substrate; this loop likely communicates substrate availability (Daus *et al.*, 2009; Grote *et al.*, 2009). Under optimal conditions in a Gram-negative bacterium, periplasmic SBPs can reach concentrations as high as 1 mM, or 50-fold more than the core transporter concentration (Bohl *et al.*, 1995), thus potentially improving bacterial scavenging of substrates at low concentrations. Following rapid binding of the substrates by SBPs in the periplasm, the core transporter likely discriminates between the substrate-bound and substrate-free SBPs, preferentially allowing the bound SBPs to dock prior to substrate translocation.

Quaternary ammonium compounds (QACs), including glycine betaine, choline and carnitine, are found in many microbial environments where they can provide osmotic protection and nutritional benefit. Many transporters that take up QACs for osmoprotection are ABC transporters. For example, the *Escherichia coli* transporter ProU (ProVWX), which was characterized more than two decades ago, transports QACs to combat hyperosmotic stress (May *et al.*, 1986). Since then many osmoregulatory QAC transporters have been characterized (Wood *et al.*, 2001). In contrast, few QAC transporters are known that function primarily in uptake for nutrition. The partially characterized ChoXWV choline transporter from *Sinorhizobium meliloti*, and its orthologue GbcXWV from *Rhizobium leguminosarum* bv. *viciae* 3841, are the only known QAC transporters for catabolism (Dupont *et al.*, 2004; Fox *et al.*, 2008). While characterizing the osmoprotective QAC transporters in the phytopathogen *Pseudomonas syringae*, we identified the osmoregulatory QAC transporters OpuC (Chen and Beattie, 2007) and BetT (Chen and Beattie, 2008) but also found evidence for a transporter that functions in QAC uptake for catabolism.

The goal of this work was to characterize this catabolism-associated QAC transporter in *P. syringae* as well as in *Pseudomonas aeruginosa*. During our investigation, we identified the CbcXWV (<u>c</u>holine/<u>b</u>etaine/<u>c</u>arnitine) transporter and found that the Cbc core transporter is atypical among ABC transporters in its ability to interact with at least three highly substrate-specific SBPs. A few ABC transporters are known to employ multiple SBPs with broader specificity; these include ArgT-HisJQMP of *Salmonella typhimurium*, which uses the two amino acid-binding SBPs HisJ and ArgT (Higgins and Ames, 1981; Wolf *et al.*, 1996), and the oligopeptide transporter Opp of *Borrelia burgdorferi*, which uses as many as three chromosomally encoded SBPs (OppA1–3) and two plasmid-borne SBPs (OppA4 and OppA5) (Wang *et al.*, 2004). Transport systems that involve multiple SBPs with distinct substrate specificity provide an opportunity to better understand some unanswered questions about the mechanism of ABC transport, including the mechanism of recognition between the core transporter and its cognate SBPs. Here, our *in vivo* studies involving simultaneous expression of the Cbc-associated SBPs and the Cbc core transporter provide the first *in vivo* demonstration of a core transporter preferentially interacting with ligand-bound versus ligand-free SBPs.

Results

The Cbc transporter of *Pseudomonas* spp. is required for the uptake of QACs under low-osmolarity conditions

Previously, we described two QAC transporters in *P. syringae*: the ABC transporter OpuC, which transports glycine betaine, choline, carnitine and acetylcholine (Chen and Beattie, 2007), and the betaine/carnitine/choline family transporter BetT, which transports choline and acetylcholine (Chen and Beattie, 2008). Whereas OpuC and BetT are functional under hyperosmotic conditions, the loss of a third *P. syringae* locus, PSPTO_0464-PSPTO_0462, reduced choline uptake under low-osmolarity conditions (Chen and Beattie, 2007), suggesting a role in choline uptake for catabolism. We generated mutants of *P. syringae* pv. *tomato* strain DC3000 that lacked this locus, DC *cbc*, as well as the *opuC* and/or *betT* loci, strains GB1–GB4 (Table 1), and examined the uptake properties of these mutants. In the absence of choline and betaine as potential inducers during cell growth, DC3000 cells transported 1.8 and 0.6 nmol min^{-1} (mg protein)$^{-1}$ of choline and betaine, respectively, when each was provided at a final concentration of 10 μM (Fig. 1). After growth with 10 mM choline or betaine as inducers, the initial uptake rate for these compounds increased three- to fourfold. GB1, a double mutant lacking *opuC* and *betT*, similarly exhibited a substrate-induced increase in uptake, whereas mutants GB2, GB3 and DC *cbc*, which all lack PSPTO_0464-PSPTO_0462, did not. This indicates that the transporter encoded by this locus was responsible for the inducible uptake. The triple transporter mutant GB4 did not transport detectable amounts of choline or betaine (Fig. 1), nor did it grow on these as sole C sources (Fig. S1), demonstrating that these three loci encode the full complement of transporters for choline and betaine uptake in DC3000. We designated the transporter encoded by PSPTO_0464-PSPTO_0462 as the Cbc transporter because of its ability to transport choline and betaine as well as carnitine, described below, and designated the genes *cbcXWV* for the SBP, TMD and NBD respectively.

Orthologues of *cbcXWV* are present in all 14 strains of *Pseudomonas* spp. for which the complete genome sequences are available, as shown for three strains in Fig. 2. Although catabolism-associated QAC transporters have not been characterized in *Pseudomonas* species, Diab *et al.* (2006) found that the expression of the gene encoding the *P. aeruginosa* CbcX orthologue, PA5378, was highly induced in cells grown on betaine as a sole source of carbon. To test if the putative transporter encoded by PA5378-PA5376 in *P. aeruginosa* is functionally similar to the Cbc transporter, we evaluated growth on selected QACs of three mutants from the non-redundant transposon mutant library of *P. aeruginosa* strain PA14 (Liberati *et al.*, 2006). The PA5376::tn, PA5377::tn and PA5378::tn mutants all failed to grow on choline (Fig. 3A) and betaine (Fig. S2) as sole C sources in the low-osmoticum medium ½-21C. These transposon mutants also failed to grow on L-carnitine (Fig. S2), suggesting that carnitine is an additional substrate for this transporter.

DC3000 cannot grow on carnitine (Chen and Beattie, 2007) and therefore is not a good strain for characterizing transporters involved in carnitine uptake for catabolism. *P. syringae* pv. *syringae* strain B728a, however, grew well on carnitine and exhibited significant uptake activity (Fig. 3B). We deleted the B728a loci that were homologous to the DC3000 *opuC*, *betT* and *cbc* loci, generating the triple mutant strain BT. BT failed to transport choline, betaine and carnitine (Fig. 3B). The deficiencies in choline and carnitine uptake by BT were complemented by introduction of the vector pME6041 expressing *cbcXWV* from DC3000, B728a or PAO1 (Fig. 3C). Similarly, the deficiencies in choline uptake by PA14 mutants with insertions in individual *cbc* loci were complemented by *cbcXWV* from either PAO1 or B728a (Fig. S3), with the *cbcXWV* from DC3000 not evaluated, and deficiencies in the growth of the mutants in choline were complemented by *cbcXWV* from either PAO1 (Fig. 3A) or DC3000 (data not shown).

CbcXWV is sufficient to transport choline and betaine but not carnitine

The Cbc transporters in B728a and DC3000 share 98–100% identity at the amino acid level, yet B728a transports carnitine and DC3000 does not (Fig. 3B). To investigate the basis for this difference, we examined the function of the Cbc transporter when expressed in *E. coli* strain MKH13. MKH13 cells transformed with the vector pME6041 were deficient in choline and betaine uptake (Table 2), as expected based on the absence of transporters for these compounds (Kempf and Bremer, 1995). MKH13 has a carnitine transporter CaiT, which has no measurable activity under these conditions. This was expected based on the fact that *caiT* gene expression requires carnitine and anaerobic conditions (Buchet *et al.*, 1998) and CaiT requires high internal concentrations of γ-butyrobetaine for uptake based on its L-carnitine/γ-butyrobetaine antiporter activity (Jung *et al.*, 2002). Interestingly, cells of MKH13 transformed with pME6041 expressing *cbcXWV* from PAO1, pCbc$_{PAO1}$, accumulated choline and betaine but little to no carnitine. Cells of MKH13 transformed with either pCbc$_{DC3000}$ or pCbc$_{B728a}$ accumulated little to no choline, betaine or carnitine (data not shown); this low activity was probably due to poor expression of *cbcXWV* in MKH13, despite their clear expression in *P. syringae* (Fig. 3C). When these genes were expressed in *E. coli* strain Acella under the control of the T7*lac* promoter, they significantly increased choline and betaine uptake, indicating successful heterologous expression, but again did not affect carnitine uptake (Table 2). This finding that the Cbc transporter encoded by *cbcXWV*

is sufficient for choline and betaine uptake but not for carnitine uptake suggests that Cbc-mediated transport of L-carnitine requires a component that is present in B728a and PA14 but is missing in DC3000 and the *E. coli* strains MKH13 and Acella.

CaiX, a carnitine-specific binding protein, is required for Cbc-mediated uptake of L-carnitine

In previous studies with *P. aeruginosa* PA14, we demonstrated that a non-polar deletion of a putative SBP gene near a carnitine catabolic locus eliminated growth on L-carnitine (Wargo and Hogan, 2009). Among the four strains of *P. syringae* with sequenced genomes (B728a, DC3000, T1 and 1448A), an orthologue of this gene, PA5388, is present in B728a, which grows on carnitine, but is absent in the other three, which do not (data not shown). A knockout mutant of B728a lacking the SBP orthologue Psyr_2916, designated B2916Ko, was dramatically reduced in carnitine but not choline or betaine uptake (Fig. 3B), whereas a B728a mutant lacking an additional putative SBP, Psyr_0028, which exhibits 41% identity at the amino acid level to Psyr_2916, was not altered in QAC uptake (Fig. 3B). Introduction of pME6041 expressing Psyr_2916 complemented the carnitine-uptake deficiency of B2916Ko (Fig. 4A) as well as of a PA14 mutant lacking PA5388 (Fig. 4B). Introduction of Psyr_2916, as well as Psyr_2916 and an adjacent putative transcriptional regulator Psyr_2915 (Wargo and Hogan, 2009), into DC3000 conferred carnitine uptake (Fig. 4C) but not catabolism (data not shown). This inability to grow on carnitine was consistent with the absence of orthologues to carnitine oxidation genes, which were recently identified in PA14 (Wargo and Hogan, 2009). These findings provide compelling evidence that Psyr_2916 and PA5388, hereafter referred to as CaiX, enable Cbc-mediated carnitine uptake in *P. syringae* and *P. aeruginosa* respectively.

The lack of competition between choline and betaine for uptake by Cbc suggests the involvement of an additional choline- or betaine-binding SBP in *P. syringae*

To better understand the substrate specificity of the Cbc transporter, we performed competition uptake assays with strains expressing *cbcXWV*, using strain derivatives that differ in the origin of the *cbcXWV* and *caiX* components. Strain BT, which expressed an endogenous *cai*X, exhibited similar inhibition profiles regardless of whether *cbcXWV* was expressed via pCbc$_{B728a}$ (homologous expression) or pCbc$_{DC3000}$ and pCbc$_{PAO1}$ (heterologous expression) (Table 3). As expected, the uptake of radiolabelled betaine by all of the strains was strongly inhibited by the presence of a 100-fold excess of unlabelled betaine, with similar self-inhibition occurring for choline and carnitine. CbcXWV can transport both choline and betaine; therefore, we predicted that the uptake of one substrate would be inhibited by the other substrate, especially when the latter was supplied at a 100-fold excess. Surprisingly, the uptake of radiolabelled betaine was not inhibited, or was only marginally inhibited, by choline nor was the uptake of radiolabelled choline inhibited by betaine in any of the strains (Table 3). This lack of competition was also observed when *cbcXWV* was expressed in DC3000 mutant GB4 via pCbc$_{B728a}$ and pCbc$_{PAO1}$, as well as in DC3000 mutant GB1 via the endogenous, single-copy *cbcXWV* (data not shown); *caiX* is absent in DC3000 and consequently had no influence. The mutual lack of competition between betaine and choline cannot be reconciled with CbcX being the sole SBP for these compounds. These results therefore suggest that the Cbc core transporter interacts with an

additional choline- or betaine-binding SBP and that the substrate-bound SBPs do not significantly compete for Cbc-mediated transport; we discuss our investigation of these predictions below.

BetX is a betaine-specific binding protein that interacts with the Cbc core transporter CbcWV

Plasmids that express a full-length *cbcXWV* locus were not useful for identifying additional choline- or betaine-binding SBPs because of overlap in the substrate specificities of these SBPs with CbcX (Table 2). To express *cbcWV* at a high level, but separately from the associated SBP-encoding *cbcX*, we first constructed the plasmid pN*cbcXWV*, which is a broad-host-range plasmid carrying $cbcXWV_{PAO1}$ under the control of the *nptII* promoter. When expressed in the QAC transporter-deficient strain BT, pN*cbcXWV* conferred betaine, choline and carnitine uptake (Fig. 5). Moreover, when expressed in *E. coli* MKH13, *pNcbcXWV* conferred sufficiently high expression to increase the rate of choline and betaine uptake 10-fold more than was conferred by $pCbc_{PAO1}$ (data not shown). A plasmid expressing only the core transporter, pN*cbcWV*, was constructed by deleting *cbcX* from pN*cb-cXWV*; it restored carnitine uptake to BT (Fig. 5), consistent with an association between the native CaiX SBP and the core transporter. The ability of pN*cbcWV* to restore betaine but not choline uptake to BT indicates that BT produces a Cbc-interacting SBP that specifically binds betaine.

We evaluated the genes Psyr_3758 and Psyr_0028 as candidates for a betaine-binding, Cbc-interacting SBP protein, and used *caiX* (Psyr_2916) as a control. Psyr_3758 and Psyr_0028 were annotated as putative SBPs in B728a (Feil *et al.*, 2005) and were predicted to interact with CbcW based on their high scores (> 0.85) as predicted functional partners of CbcW using the STRING database of known and predicted protein–protein interactions (Jensen *et al.*, 2009). Knockout mutants lacking these putative SBP genes, including mutants BT3758Ko, BT0028Ko and BT2916Ko, served as hosts for the SBP-expressing plasmids. As expected, the BT-derived mutants were all deficient in the uptake of betaine, carnitine and choline when expressing only the empty pME6041 vector with the *nptII* promoter, pN (Table 4). In the presence of the complete Cbc transporter encoded by pN*cbcXWV*, mutant BT2916Ko was deficient in carnitine uptake, consistent with the loss of CaiX; in contrast, the BT3758Ko and BT0028Ko mutants took up significant amounts of all of the substrates, consistent with the presence of chromosomally encoded CaiX and plasmid-borne CbcX (Table 4). In the presence of the Cbc core transporter encoded by pN*cbcWV*, mutant BT3758Ko, but not BT2916Ko and BT0028Ko, was deficient in betaine uptake, indicating that Psyr_3758 functions as a SBP for betaine; Psyr_3758 was thus designated BetX. The ability of BT3758Ko expressing pN*cbcXWV* to take up betaine is consistent with the presence of CbcX (PA5378) as a betaine-binding SBP; thus, the Cbc transporter appears to have redundant SBPs for betaine uptake. Uptake in these studies demonstrated expression of the endogenous *caiX* and *betX* genes in BT even in the absence of exogenous QACs as inducers. Although CbcX mediates approximately 99% of the choline uptake at low (10 μM) and high (1 mM) [^{14}C]choline concentrations, residual choline uptake was detectable when pN*cbcWV* was expressed in *P. syringae* but not in *E. coli* MKH13 (data not shown), suggesting that one or more additional SBPs can contribute to choline uptake in *P. syringae*.

We compared the behaviour of the Cbc transporter and its interacting SBPs with the behaviour of the highly similar Cho transporter from *S. meliloti*, which mediates choline uptake (Dupont *et al.*, 2004). The full-length *choXWV* locus has not yet been cloned and characterized. In *P. syringae* BT strain derivatives, which lacked all endogenous QAC transporters, ChoXWV was able to transport betaine and carnitine as well as choline (Table 4). Although betaine transport did not require BetX, carnitine transport required CaiX (Table 4), suggesting that the ChoWV core transporter interacted with CaiX from the phylogenetically distinct species *P. syringae*. CbcXWV and ChoXWV differed in their capacity for choline transport, with choline uptake by CbcXWV increasing from 16 to 18 nmol (mg protein)$^{-1}$ to 562–648 nmol (mg protein)$^{-1}$ when the choline concentration was increased from 10 μM to 1 mM, but not increasing by ChoXWV when the choline concentration was similarly increased (Table 4). Similar results were observed when both of them were expressed heterologously in *E. coli* MKH13 (data not shown). These data demonstrate an intrinsic difference between CbcXWV and ChoXWV in their uptake capacity.

The SBPs interacting with the Cbc core transporter are highly substrate specific and confer distinct uptake affinities and uptake capacities upon the Cbc transporter

To better understand the substrate specificities among the SBPs CbcX, BetX and CaiX, we expressed the Cbc core transporter and its associated SBPs in *E. coli* strain MKH13, which lacks orthologues of *cbcX*, *betX* and *caiX*, and evaluated the subsequent uptake activities. The absence of Cbc-interacting SBPs in MKH13 was confirmed using MKH13 (pN*cbcWV*) (Table 5). The *caiX* and *betX* genes from PAO1, B728a and DC3000 were introduced into the pBR322-based vector pTOK2T, which enables moderate expression of the cloned genes by an undescribed promoter. As expected, MKH13 cells coexpressing *cbcWV* (on pN*cbcWV*) and *caiX* (on a pTOK2T derivative) were able to take up carnitine but not choline and betaine, demonstrating that CaiX was sufficient for, and specific to, carnitine uptake (Table 5). Similarly, BetX was sufficient for, and specific to, betaine uptake. Consistent with earlier observations, CbcX conferred uptake for choline and betaine but not carnitine. ChoX was also sufficient for the transport of choline and betaine, in contrast to an earlier report that excluded betaine as a substrate (Dupont *et al.*, 2004), but ChoWV, like CbcWV, depended on CaiX for carnitine uptake (Table 4). Cbc dependence on CaiX for carnitine uptake was also illustrated by the expansion of the substrate range when *cbcX* and *caiX* were coexpressed as compared with when *cbcX* was expressed (Table 5).

We examined the uptake kinetics of the Cbc transporter system when it utilized each of the distinct SBPs. Using MKH13 cells and thus an *in vivo* assay, we found that CbcX and ChoX each conferred a similarly high affinity for choline, with K_m values of 1.8–2.6 μM (Table 6). CbcX conferred an approximately 10-fold lower affinity for betaine than choline, and BetX exhibited a very high affinity for betaine, consistent with its role as a high-affinity betaine-binding protein. The CbcX-mediated choline and betaine transport activities were assayed using replicate samples of the same pool of cells to ensure similar concentrations of the transporter components, and thus the higher V_{max} for choline than betaine [34.6 versus 11.6 nmol min^{-1} (mg protein)$^{-1}$] suggested that CbcX-choline induced greater uptake activity in the core transporter than CbcX-betaine (Table 6). The *E. coli* MKH13 cells expressing BetX

and the core transporter exhibited the lowest V_{max} [4.8 nmol min^{-1} (mg protein)$^{-1}$] among the choline and betaine transport activities of the Cbc transporter. Although the relative expression of BetX and CbcX was not determined, thus preventing a direct comparison of the V_{max} values for BetX- and CbcX-mediated betaine uptake, we observed a lower betaine uptake capacity for BetX than CbcX even when comparing constructs with BetX expressed on a higher-copy plasmid than CbcX (data not shown). This suggests that BetX has a lower uptake capacity for betaine than CbcX, despite the fact that BetX bound betaine with a higher affinity than CbcX did. These relative kinetics of CbcX- versus BetX-mediated betaine uptake are consistent with a model predicting that a moderately lower binding affinity results in a faster release of substrate from the ligand-bound SBP into the core transporter, resulting in a higher uptake rate (Picon *et al.*, 2000). The ChoXWV transporter exhibited the lowest V_{max} among the transporter configurations shown in Table 6, suggesting that the interaction between ChoX-choline and ChoWV is less efficient than that between CbcX-choline or CbcX-betaine and CbcWV. A low capacity for choline uptake by ChoXWV was also indicated by the much lower level of choline uptake observed when *choXWV* was expressed on the expression vector pET15b in *E. coli* Acella cells (data not shown) as compared with the higher level of choline uptake by *cbcXWVDC3000* and *cbcXWV_{B728a}* when similarly expressed (Table 2). A low capacity for choline uptake by ChoXWV is also consistent with that observed when ChoXWV was expressed in *P. syringae* (Table 4) or *P. aeruginosa* (data not shown). We were unable to accurately assess the kinetics of CaiX-mediated carnitine uptake using MKH13; however, we did so using strain PA5378::tn(pN*cbcWV*) and observed a relatively low affinity and low uptake capacity of CbcWV-CaiX for carnitine. The apparent K_m (1.8 ± 0.36 μM) for choline uptake by ChoXWV in MKH13 cells was in excellent agreement with the reported K_D (2.3 μM) for choline binding to purified ChoX, as evaluated using a fluorescence-based equilibrium binding assay (Oswald *et al.*, 2008), and to the reported K_D (2.7 μM) from a binding assay with radiolabelled choline (Dupont *et al.*, 2004). This similarity between K_D and K_m suggests that the rate-limiting step is the donation of the ligand by the ligand-bound SBP after docking onto the membrane complexes (or core transporters) (Lanfermeijer *et al.*, 1999).

Competition among the SBPs occurs both for binding to the substrates and for docking of ligand-bound SBPs to the core transporter

To better understand the substrate specificity of the individual SBPs as they interacted with the core CbcWV transporter, we examined the ability of selected substrates to inhibit the uptake of a radiolabelled substrate when supplied in a 10- or 100-fold excess over the radiolabelled substrate. As expected, BetX-mediated [^{14}C]betaine uptake was inhibited by unlabelled betaine (94% reduction in uptake) (Table 7); it was also moderately inhibited (56%) by the betaine analogue dimethylglycine (DM) but not by choline, carnitine or the betaine analogue monomethylglycine (MM). CaiX-mediated [^{14}C]carnitine uptake was inhibited by unlabelled carnitine (91%) but not by other compounds (Table 7). These results are consistent with BetX and CaiX being betaine- and carnitine-specific SBPs respectively. CbcX-mediated [^{14}C]betaine uptake was strongly inhibited by unlabelled choline (99%), even more than by unlabelled betaine (87%), consistent with our kinetic data that CbcX binds choline with a much higher affinity than betaine. Interestingly, CbcX-mediated

[^{14}C]choline uptake was not inhibited by unlabelled betaine, even at a 100-fold excess. This finding was surprising in light of data indicating that Cbc can transport both betaine and choline (Tables 5 and 7). Together these data suggest that choline strongly competes against betaine for binding to CbcX; alternatively, CbcX-choline strongly competes against CbcX-betaine for docking to the core Cbc transporter. Unlike CbcX, ChoX-mediated choline uptake was inhibited by acetylcholine (Table 7), consistent with a previous report that ChoX binds acetylcholine (Dupont *et al.*, 2004). ChoXWV- but not CbcXWV-mediated [^{14}C]choline uptake was moderately inhibited (41%) by carnitine, reflecting another intrinsic difference between these transporters.

To gain insight into the interactions among these SBPs and the core transporter, we coexpressed multiple SBPs and evaluated uptake of target substrates. When we coexpressed *caiX* and *cbcX* in the presence of *cbcWV*, CaiX-mediated [^{14}C]carnitine uptake was moderately inhibited by choline (66%) and betaine (57%) (Table 7), which are the ligands for CbcX. Choline and betaine do not compete with carnitine for binding to CaiX, as indicated by the lack of competition by choline or betaine in the absence of CbcX (Table 7) and by the lack of choline or betaine uptake when CaiX was the sole SBP (Table 5); therefore, the reduction in [^{14}C]carnitine uptake likely resulted from reduced CaiX-carnitine docking to the core transporter. This suggests that CbcX-choline and CbcX-betaine have a higher affinity for docking, as supported by the fact that carnitine, even at a 100-fold excess, did not reduce CbcX-mediated choline or betaine uptake in the presence of CaiX (Table 7). Furthermore, the lack of inhibition of CaiX-mediated carnitine uptake by ligand-free CbcX, as was observed in a control with only water rather than choline or betaine (Table 7), indicates that ligand-free CbcX did not compete with ligand-bound CaiX for docking. This result is consistent with the Cbc core transporter having a higher affinity for a ligand-bound than a ligand-free SBP.

Although we did not evaluate whether BetX-betaine similarly reduces CaiX-mediated carnitine uptake in MKH13, we observed that CaiX-mediated carnitine uptake was reduced more by betaine than by choline in each of the three BT strain derivatives examined (Table 3). Given that betaine uptake involves BetX and CbcX whereas choline uptake involves only CbcX, this suggests that BetX-betaine reduced CaiX-mediated carnitine uptake. We also observed higher inhibition (75–88%) of carnitine uptake when *betX*, *caiX* and *cbcX* were simultaneously expressed, with *betX* and *caiX* chromosomally encoded (Table 3), than when only *caiX* and *cbcX* were expressed (57–66%) (Table 7), despite similar self-inhibition by unlabelled carnitine (94% versus 97%). This result also suggests that BetX-betaine reduces CaiX-mediated carnitine uptake. Together, this result suggests that BetX-betaine competes for docking with CaiX-carnitine.

Discussion

The Cbc transporter described here represents the first catabolism-associated QAC transporter to be characterized in *Pseudomonas* spp., despite the fact that catabolic uptake of QACs was reported in *P. aeruginosa* more than four decades ago (Kleber and Aurich, 1967). In addition to the core transporter comprised of a transmembrane protein (CbcW) and an ATPase (CbcV), the Cbc transporter system interacts with at least three SBPs: CbcX, which

binds choline with a high affinity and betaine with a much lower affinity; BetX, which binds betaine with a very high affinity; and CaiX, which binds carnitine with a moderate affinity and represents the first carnitine-specific SBP to be reported. These three SBPs are encoded by genes located in separate regions of the genome. The complexity of this Cbc transporter, compounded by the redundancy of osmoregulatory QAC transporters [e.g. OpuC, BetT and BetT-like transporters such as PA5291 in *P. aeruginosa* (Chen and Beattie, 2007; 2008)], may have hindered previous attempts to identify such transporters (Aurich and Kleber, 1970; Salvano *et al.*, 1989; Diab *et al.*, 2006; Galvao *et al.*, 2006). To minimize any compounding effects, we expressed Cbc transporter configurations in *E. coli* mutant MKH13, in which all QAC transporters and SBPs have been deleted or remained inactive under the test conditions (Kempf and Bremer, 1995). In addition, enzymes that are responsible for the conversion of choline into betaine were also deleted in MKH13. We characterized the components of the Cbc transporter in three different *Pseudomonas* spp. strains, as shown in Fig. 6.

Among characterized transporters, the *S. meliloti* ChoXWV transporter (Dupont *et al.*, 2004) shares the greatest sequence similarity with CbcXWV as well as shares several functional similarities. ChoXWV was initially reported to transport only choline (Dupont *et al.*, 2004); however, a subsequent report showed that the SBP ChoX binds betaine, albeit with a lower affinity than choline (Oswald *et al.*, 2008). Here, we provide direct evidence that ChoXWV transports betaine as well as choline and, moreover, that the core transporter ChoWV is similar to CbcWV in interacting with CaiX to transport carnitine. The complete genomes of strains of Rhizobiaceae do not contain genes encoding CaiX orthologues. The SBPs CbcX and ChoX share a high structural similarity, including the amino acid residues identified in ChoX as required for substrate binding (Fig. S5), and they bind choline and betaine with comparable affinity (Oswald *et al.*, 2008). Although CbcX mediates betaine uptake, betaine surprisingly did not inhibit CbcX-mediated choline uptake even when it was present at a 100-fold excess over choline. A similar lack of inhibition was reported for the transporter ChoXWV (Dupont *et al.*, 2004) and its orthologue GbcXWV (Fox *et al.*, 2008). This lack of competition by one substrate against another substrate has not been observed in osmoregulatory QAC transporters, such as *E. coli* ProU (Cosquer *et al.*, 1999a) and *P. syringae* OpuC (Chen and Beattie, 2007), and probably contributed to the initial conclusion that betaine was not a substrate for the Cho transporter. Such a lack of competition in uptake can be explained by better adaptation of ChoX, and potentially CbcX, to bind choline than betaine (Dupont *et al.*, 2004); for example, perhaps choline binding induces a conformational change that excludes betaine binding. Interestingly, among the CbcXWV$_{DC3000}$ and ChoXWV transporter components, the SBPs exhibited the highest sequence similarity, contrary to those of other ABC transporters in which the NBDs exhibit the highest and the SBP the lowest similarity (Davidson *et al.*, 2008). Based on the fact that *P. syringae* and *S. meliloti* have evolved to live in close association with plants, this unusual conservation profile may reflect a selection pressure for choline utilization in plants, as suggested by previous studies (de Rudder *et al.*, 2000; Chen and Beattie, 2008). Differences between the Cbc and Cho transporter include that the capacity for choline uptake is much higher by Cbc than Cho, that acetylcholine and carnitine inhibit choline uptake by Cho but not by Cbc, and that the encoding loci differ in their genomic context. The *cbcXWV* operon is located proximal to the AraC family transcriptional regulator GbdR (Fig. 2), which is

required for betaine catabolism (Wargo *et al.*, 2008), whereas the *choXWV* operon is not located near an obvious transcriptional regulator.

Sequence alignments among the QAC-specific SBPs characterized here reveal high conservation in many amino acid residues that are involved in substrate binding by ChoX (Horn *et al.*, 2006; Oswald *et al.*, 2008). This includes the three Trp residues Trp-43, Trp-90 and Trp-205 of ChoX (Fig. S5) that are critical to the 'cation–π' interaction characteristic of the binding of QAC ligands to their cognate SBPs. This cation–π interaction results from the trimethylammonium head moiety, which promotes delocalization of the positive charge over the three methyl groups and forms a bulky cation that interacts with three to five aromatic residues in the ligand-binding box of a SBP. This type of interaction contrasts with the H bonding that typically mediates ligand binding to SBPs. The differential recognition by the Cbc-associated SBPs of QACs that share a strong structural similarity demonstrates that these SBPs have exceptional specificity. Although unusual among the characterized SBPs of ABC transporters, perhaps the best example of such exquisite specificity is that of oxyanion-binding proteins, which have evolved to discriminate among highly similar ions such as phosphate and sulphate; they do this based on the protonation of phosphate at physiological pH (Quiocho, 1996). The specificity of the Cbc-associated SBPs for QAC binding must be based on recognition of the carboxyl versus hydroxyl group distinguishing betaine from choline (Smits *et al.*, 2008), and in the shorter versus longer acyl chain distinguishing betaine from carnitine. Future crystallization and binding affinity studies may provide insight into the molecular basis for SBP recognition of distinct QACs.

The Cbc transporter system offers many advantages as a model system for investigating the mechanisms underlying uptake by prokaryotic ABC transporters. The distinct substrate specificity of certain pairs of SBPs, such as CaiX and CbcX, provides an opportunity to use *in vivo* studies to examine SBP competition for docking to the core transporter. *In vivo* studies avoid some of the limitations inherent to purified-component studies, including altered behaviour due to purification and reconstitution processes (Rees *et al.*, 2009) and due to the absence of a full transporter when individual components are examined (Orelle *et al.*, 2008). For example, our studies demonstrated that the Cbc core transporter preferentially supported docking of a ligand-bound SBP, CbcX-choline or CbcX-betaine, over the ligand-free SBP CbcX based on the greater inhibition of CaiX-carnitine docking and transport by the ligand-bound SBPs. Although we predict that a core transporter would preferentially permit docking of a ligand-bound SBP over a ligand-free SBP, surprisingly, such preference has not been unambiguously demonstrated. On the contrary, studies using detergent-solubilized proteins or permeabilized proteoliposomes have commonly found that ligand-bound and ligand-free SBPs compete equally well for docking (Bohl *et al.*, 1995; Ames *et al.*, 1996). Another advantage of the Cbc transporter as a model system is that the QAC substrates are generally available or easily produced in radiolabelled form. They also have low molecular masses, which allow them to enter and rapidly diffuse in the periplasm; this avoids entry and diffusion as rate-limiting steps during transport. Moreover, bacterial strains that do not synthesize the QAC substrates, such as MKH13, can be easily obtained; this allows *in vivo* studies to be performed with the Cbc transporter without interference from endogenous substrates. This contrasts with most or all of the other ABC transporters that use multiple SBPs, most of which transport amino acids and oligopeptides.

The finding that BetX-betaine did not detectably compete with CbcX-choline for docking is intriguing and suggests the need for a better understanding of docking. It is possible that docking and substrate delivery are exceedingly fast and thus significant competition for docking is avoided. It is also possible that the Cbc core transporter was present in a similar or greater concentration than the SBPs and thus the number of docking sites was not limited. This latter possibility, however, seems unlikely for several reasons. First, uptake was not inhibited by the alternate substrate when CbcX and BetX were expressed in a variety of constructs that probably varied in the relative production of the core transporter and the SBPs; this included expression from the DC3000 genome as well as expression of homologous and heterologous plasmid-borne *cbcWV* in DC3000 and B728a. Second, the location of *cbcX* as the first gene in the *cbcXWV* operon suggests equal or greater transcript levels of *cbcX* versus the downstream core transporter genes, although the stability of the transcripts may vary. And third, SBP synthesis is generally subject to less feedback inhibition than the core transporter components because of SBP localization in the periplasm, which generally contributes to the much greater abundance of SBPs than their core transporter components (Bohl *et al.*, 1995). If we assume that the docking sites were in fact limited, then another possible explanation for the two-way lack of competition of ligand-bound CbcX and BetX is the presence of cooperative interactions, such as heterodimerization of the two SBPs. Although such heterodimerization has not been reported, the possibility of homodimerization has been elegantly demonstrated in another QAC transporter, the osmoregulatory OpuA transporter of *Lactococcus lactis* (Biemans-Oldehinkel and Poolman, 2003). This dimerization resulted from the association of two TMDs, each of which was fused to a SBP. Kinetic studies performed with purified transporters in proteoliposomes have suggested cooperative interactions between SBPs that stimulated either the docking of the SBPs on the core transporter or the translocation of the substrate.

Betaine and choline are known components of plant and animal tissues, with the former most often associated with osmoprotection and the latter present as a precursor for betaine as well as the major membrane lipid phosphatidylcholine. In contrast, carnitine is prevalent in animal tissues but rarely reported in plant tissues; thus, carnitine transport and metabolism has been characterized in the animal pathogen *P. aeruginosa* (Kleber and Aurich, 1966; 1967; Wargo and Hogan, 2009) but not in *P. syringae* or other phytopathogens. In fact, of the four *P. syringae* strains for which the genome is fully sequenced, only B728a can grow on carnitine and has the predicted carnitine catabolism genes. Among 30 *P. syringae* strains collected from at least 13 distinct host plants, however, 50% were capable of growth on carnitine (C. Chen and G.A. Beattie, unpubl. data). Almost all of the strains grew on betaine, choline and acetylcholine, although, surprisingly, carnitine typically supported growth that was the fastest and to the highest cell densities. Thus, for carnitine-catabolizing strains, adaptation of the Cbc transporter to interact with a carnitine-binding SBP could have provided a significant ecological fitness benefit. For phytopathogens, this benefit may be realized on germinating seedlings (Hirano *et al.*, 1997), where carnitine may be present due to the active oxidation of fatty acids (Bourdin *et al.*, 2007).

We can only speculate on the ecological advantages that led to a transporter that recruits multiple substrate-specific SBPs rather than a single SBP with broad substrate specificity.

These substrate-specific SBPs appear to be conserved across *Pseudomonas* spp. based on that all of the *Pseudomonas* spp. strains sequenced to date have orthologues of CbcX, BetX and CaiX, with the exception that the *P. syringae* strains DC3000, T1 and 1448A lack CaiX orthologues. *Burkholderia* spp. such as *B. amifaria* and *B. vietnamiensis* have up to seven SBPs, and Rhizobiaceae members have up to three SBPs, that are predicted to interact with Cbc-like transporters. These bacterial species are commonly found as living free in the soil or in association with plants or animals. The chemical complexity of these habitats suggests the possibility of a diversity of QAC compounds, some of which could be toxic to the bacteria (Anthoni *et al.*, 1991). Such toxicity of QAC analogues has been demonstrated in studies with *S. meliloti* (Cosquer *et al.*, 1999b). Thus, one possible ecological advantage for evolving SBPs that are highly discriminating among QACs is to allow them to avoid binding toxic QAC derivatives. Moreover, the finding that toxic betaine analogues inhibited the growth of bacteria that were capable of catabolizing QACs but not of those using QACs solely as osmoprotectants (Cosquer *et al.*, 1999b) suggests that it would be more advantageous to evolve highly substrate-specific transporters for catabolic uptake than for osmoprotective uptake. Thus, assuming that these proteobacterial genera encounter multiple QACs, including toxic QAC analogues, the presence of QAC transporters such as OpuC with broad specificity may provide osmoprotection, but the presence of a transporter such as Cbc with multiple, highly substrate-specific SBPs may maximize catabolic potential while minimizing damage due to the import of toxic compounds. Interestingly, the predicted result of increased tolerance to toxic QAC compounds may be reflected in the observation that, among microorganisms, *Pseudomonas* spp. are particularly tolerant to biocidal QACs that are widely used as sanitizing agents (Langsrud *et al.*, 2003).

Experimental procedures

Bacterial strains and growth conditions

The bacterial strains and plasmids used are listed in Table 1. *P. syringae* strains were maintained in King's B medium (King *et al.*, 1954) at 28°C, whereas *E. coli* strains and *P. aeruginosa* strains were maintained in LB at 37°C. To evaluate growth on selected QACs, cells were grown in the low-osmoticum medium ½-21C (Halverson and Firestone, 2000; Chen and Beattie, 2007). Unless otherwise indicated, *Pseudomonas* spp. strains were grown in ½-21C containing 20 mM pyruvate, a non-repressive carbon source (Sage and Vasil, 1997), for the transport assays. *E. coli* strains were grown in M63 medium (Silhavy *et al.*, 1984) containing glucose (0.2%) and vitamin B1 (0.0005%) for the transport assays. When necessary, antibiotics were used at the following concentrations (µg ml^{-1}): ampicillin (Amp), 100; gentamicin (Gm), 60; kanamycin (Km), 500 for *P. aeruginosa*, 50 for *P. syringae* and 20 for *E. coli*; rifampin (Rf), 100; and tetracycline (Tet), 20.

Construction of plasmids

The integration vectors pKnockout-Ω (Windgassen *et al.*, 2000), pTOK2T and pT*sacB* were used for the generation of *P. syringae* mutants. pTOK2T was constructed by modifying the vector pTOK2, which is a pBR322 derivative containing the highly effective *mob* region from RSF1010 (Kitten and Willis, 1996), to allow blue-white screening. To repair the dysfunctional *lacZα* gene, a 60 bp DNA fragment from pKnockout-Ω was inserted into

pTOK2 and a blue colony was identified; the sequence of the modified region is shown in Fig. S6. PCR products were introduced into the SmaI site of pTOK2T to generate the pT-based plasmids (Table 1). The counter-selectable vector pT*sacB* was constructed by inserting a 3.5 kb XmnI–EcoRV fragment containing the *sacB* gene from pFlp2 (Hoang *et al.*, 1998) into the XmnI site of pTOK2T.

The Phusion high-fidelity DNA polymerase (New England Biolabs, Ipswich, MA) was used to amplify all DNA fragments. The expression plasmids pET-*cbc*$_{B728a}$ and pET-*cbc*$_{DC3000}$ were constructed by introducing NdeI- and BamHI-restricted PCR products into pET15b (Novagen). The plasmids pCbc$_{B728a}$, pCbc$_{DC3000}$ and pCbc$_{PAO1}$ were constructed by introducing the *cbc* loci from B728a, DC3000 and PAO1, respectively, which were amplified by PCR using the primers shown in Table S1, into the EcoRV site of pME6041 (Heeb *et al.*, 2000). The plasmids pME2916 and pME2915-16 were similarly constructed (Table 1). The high-level expression vector pN was constructed by introducing a 733 bp DNA fragment containing the *nptII* promoter (Axtell and Beattie, 2002) (Fig. S7) into the EcoRV site of pME6041 such that it regenerated the EcoRV site. Plasmid pN*cbcXWV* was constructed by introducing a DNA fragment containing *cbcXWV*$_{PAO1}$ into the EcoRV site of pN. Primers facing outward from *cbcX* (PA5378) in pN*cbcXWV*, PAproXF and PAproXR (Table S1), were treated with T4 polynucleotide kinase (Promega, Madison, WI) and were used with pN*cbcXWV* to create pN*cbcWV*; the PCR product was digested with DpnI to remove residual plasmid DNA before incubation with T4 DNA ligase (Promega, Madison, WI). Successful construction was verified by PCR and partial sequencing.

Generation of mutants

The generation of DC3000 strains GB1, GB2, GB3 and GB4 has been described previously (Chen and Beattie, 2008). The *cbcXWV* locus was deleted in DC3000 by using splice-overlap-extension (SOE) PCR to amplify 1–2 kb of the regions immediately flanking the locus and replacing the locus with a *kan* cassette surrounded by FRT sites, as we have done previously (Chen and Beattie, 2008). The amplified fragment was cloned into pKnockout-Ω, which was transformed into DC3000. A screen for double recombinants followed by eviction of the *kan* cassette by introducing pFlp2 (Hoang *et al.*, 1998) resulted in the generation of DC Δ*cbc* (Table 1).

Knockout mutants of B728a were generated by amplifying the internal region of the target genes and cloning the fragment into the SmaI site of pTOK2T (Table 1). The resulting plasmids were conjugated into B728a and mutants were selected as single recombinants. Attempts to generate deletion mutants of B728a using pKnockout-Ω derivatives were not successful. The *betT* gene was deleted in B728a by using SOE-PCR and a *kan* cassette surrounded by FRT sites, similar to the strategy used for the DC3000 mutants, but with the integration vector pTOK2T and plasmid mobilization via tri-parental mating, as described previously (Chen and Beattie, 2007). The *opuC* and *cbcXWV* loci were deleted by performing SOE-PCR to generate an unmarked deletion of the target loci between the flanking regions, cloning it into pT*sacB*, mobilizing it into B728a, and selecting initially for single recombinants, with a subsequent screen for a second recombination event following growth in King's B broth containing 10% sucrose for 1 day and plating on King's B agar

plates containing 10% sucrose. The triple deletion mutant BT was obtained by generating an *opuC cbcXWV* double mutant, followed by introduction of the marked *betT* deletion and subsequent eviction of the *kan* cassette. The quadruple mutants BT0028Ko, BT2916Ko and BT3758Ko were obtained by conjugating the integration vectors pT0028Ko, pT2916Ko and pT3758Ko, respectively, into the BT strain.

The PA14 transposon mutants were obtained from the non-redundant PA14 transposon mutant library. The transposon insertion sites were verified using the two-round arbitrary PCR protocol described by Liberati *et al.* (2006).

Transport assays

[*Methyl*-^{14}C]choline and L-carnitine (specific activity of 55 mCi mmol^{-1}) were obtained from American Radiolabelled Chemicals (St. Louis, MO). [*Methyl*-^{14}C]glycine betaine was prepared by the oxidation of [*methyl*-^{14}C]choline (Chen and Beattie, 2007). Unless indicated otherwise, *P. syringae* and *P. aeruginosa* cells were grown in ½-21C medium to an optical density at 600 nm (OD$_{600}$) of 0.3–0.5 (mid-log phase) and *E. coli* cells were grown in M63 medium to an OD$_{600}$ of 0.6–0.7 (mid-log phase). For bacterial cells grown in the presence of QACs, cells were washed 3× with ½-21C medium to remove residual QACs. Bacterial cells grown in the absence of QACs were generally washed twice with ½-21C medium (*Pseudomonas* spp.) or M63 medium (*E. coli*), and re-suspended with the same medium to an OD$_{600}$ of 1 for evaluation of QAC uptake. Total uptake, initial uptake rates, inhibition of uptake and uptake kinetics were obtained as previously described (Chen and Beattie, 2007; Chen and Beattie, 2008).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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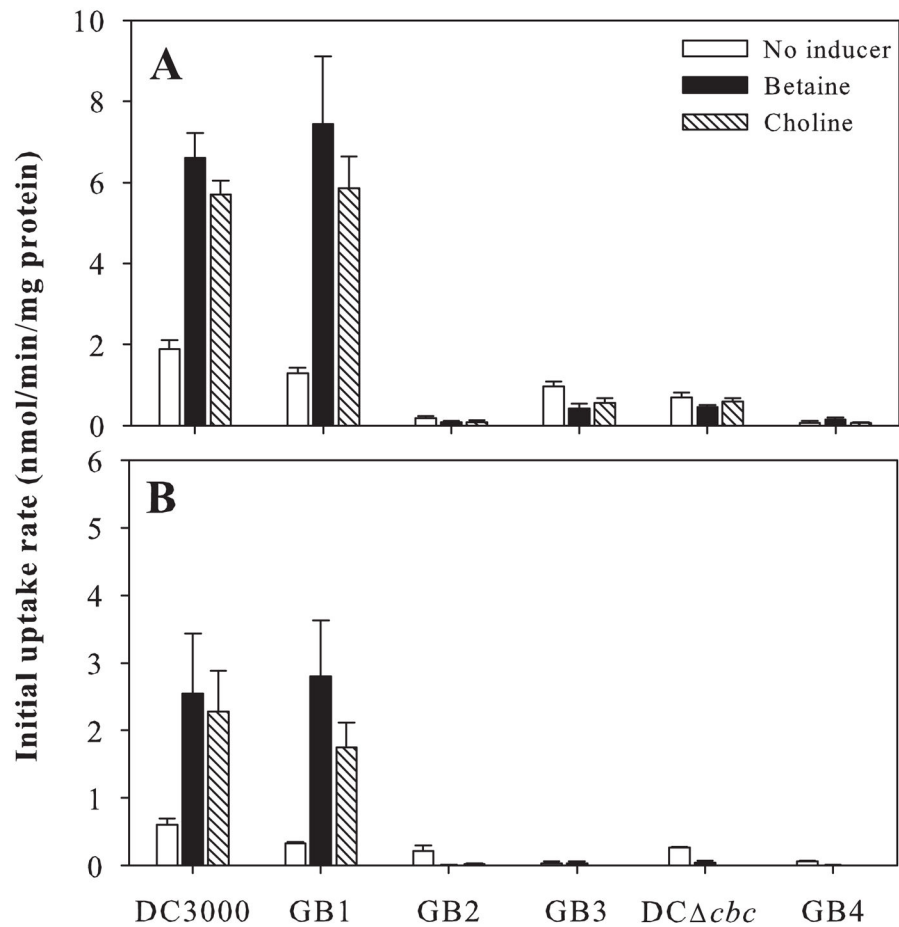
Fig. 1.
Choline (A) and betaine (B) uptake by *P. syringae* pv. *tomato* DC3000 and mutants lacking one or more loci encoding the CbcXWV, OpuC and BetT transporters. The wild-type DC3000 and the strains GB1–GB4 and DC *cbc* (Table 1) were grown in the absence of choline or betaine, designated no inducer, or in the presence of betaine or choline (final concentration 10 mM), allowing these compounds to serve as inducers. As a catabolic product of choline, betaine may also serve as an inducer in the choline-amended cells. Uptake was measured using 10 μM [^{14}C]choline (A) or [^{14}C]betaine (B). Values are mean uptake rates + standard error of the mean (SE) (n = 3).
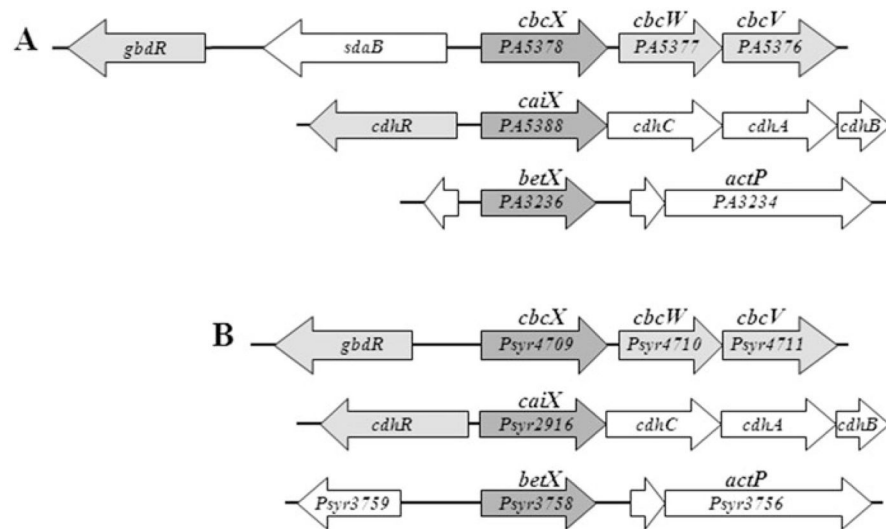
Fig. 2.
Genomic arrangement of the *cbcXWV* loci and related loci in (A) *P. aeruginosa* PA14 and PAO1 and (B) *P. syringae* strain B728a. The following genes are shown: *gbdR* [transcriptional regulator influencing betaine catabolism (Wargo *et al.*, 2008)], *sdaB* (L-serine dehydratase), *cbcXWV* (ABC transporter), *cdhR* [transcriptional regulator influencing carnitine catabolism (Wargo and Hogan, 2009)], *caiX* (carnitine-specific binding protein), *cdhCAB* [carnitine catabolic proteins (Wargo and Hogan, 2009)], *betX* (betaine-specific binding protein) and *actP* (acetate permease).
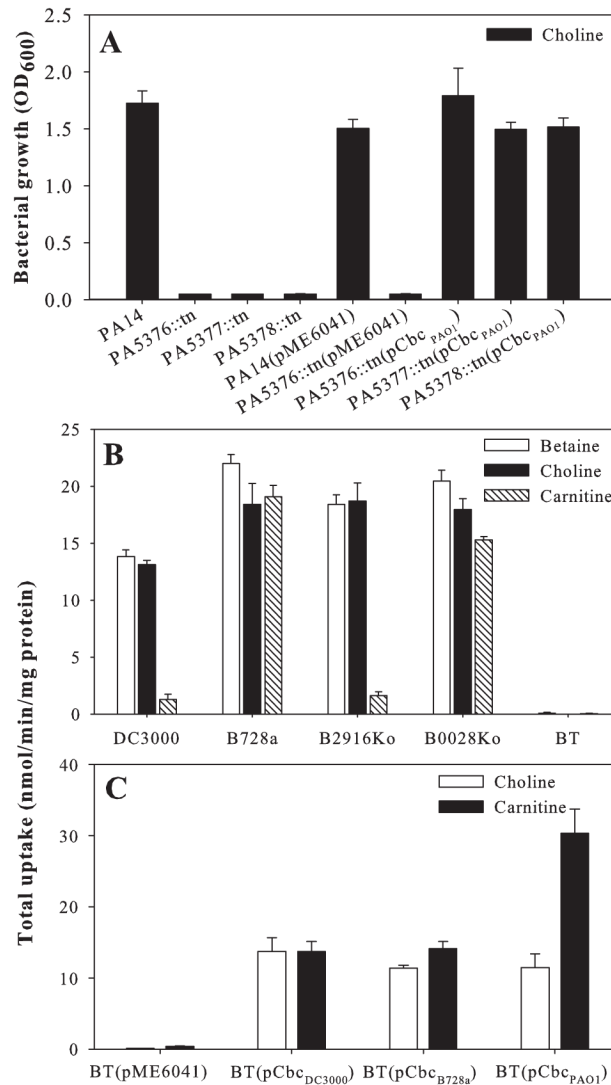
Fig. 3.

Complementation of *P. aeruginosa* and *P. syringae* transporter-deficient mutants by plasmids expressing *cbcXWV* from various strains.

A. Growth of PA14, PA14 containing insertions in individual *cbc* genes, and the PA14 mutants containing pME6041 or pME6041 expressing *cbcXWV* from PAO1.

B. Uptake of radiolabelled betaine, choline or carnitine (1 mM) by DC3000, B728a, B728a with insertions in the putative SBP loci Psyr_2916 (B2916Ko) and Psyr_0028 (B0028Ko), and B728a lacking *opuC*, *betT* and *cbc* (BT).

C. Uptake of radiolabelled choline and carnitine (1 mM) by strain BT containing the vector pME6041 or pME6041 expressing *cbcXWV* from DC3000, B728a or PAO1.

Cells were grown with 20 mM choline (A) or with 10 mM choline and 10 mM betaine (B and C) as sole C sources and optical density at 600 nm (OD_{600}) was recorded after 24 h (with an initial OD_{600} of 0.05). Values represent the mean + SE ($n = 3$).
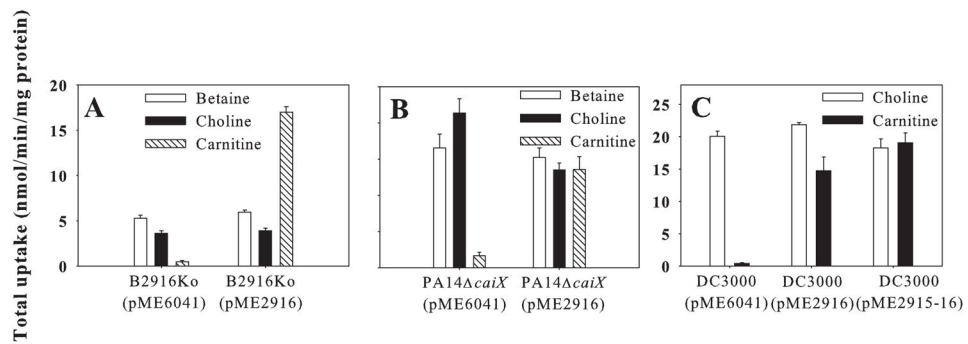
Fig. 4.
Complementation of uptake activity in *P. syringae* and *P. aeruginosa* strain derivatives by plasmids expressing Psyr_2916 (*caiX*) from *P. syringae* B728a. Uptake of radiolabelled betaine, choline and carnitine (1 mM) by derivatives of strain (A) B2916Ko, in which Psyr_2916 was insertionally inactivated, (B) PA14 *caiX*, in which PA5388 was deleted, and (C) DC3000. Strain derivatives included those expressing only pME6041 or pME6041 containing *caiX_{B728a}* (pME2916) or *caiX_{B728a}* and the adjacent putative transcriptional regulator *cdhR* (pME2915-16) (Wargo and Hogan, 2009). Values are mean + SE (*n* = 3).
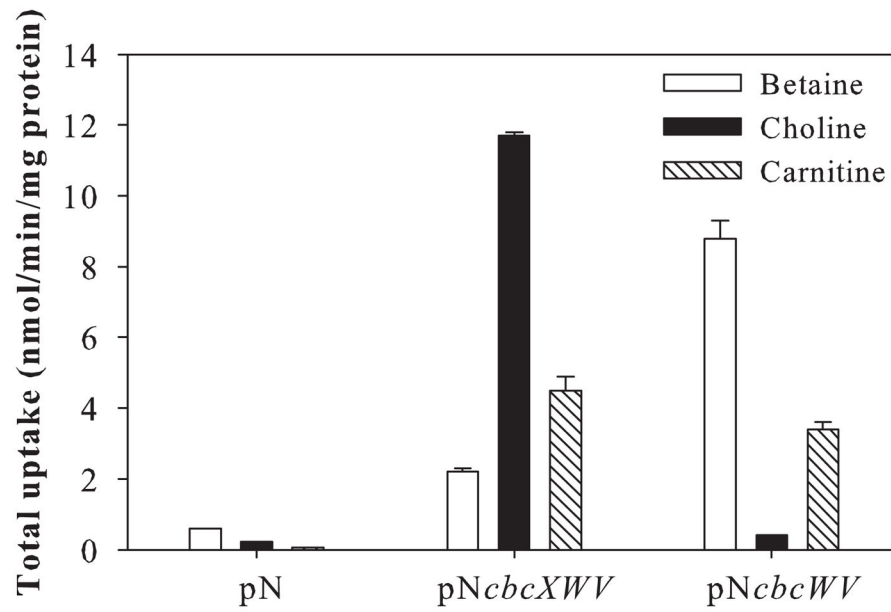
Fig. 5.
Complementation of betaine, choline and carnitine uptake in the QAC transporter-deficient *P. syringae* strain BT by vectors expressing *cbcWV* and *cbcXWV*. Cells were grown in King's B medium, washed with water, and re-suspended in ½-21C medium for the transport assay using radiolabelled substrates at a concentration of 10 μM. Values are mean + SE ($n = 3$).

Fig. 6.
Model of the interacting components comprising the Cbc transporter of *P. aeruginosa* and *P. syringae.* (A) Diagrammatic model and (B) list of Cbc transporter components. This study has provided functional evidence for all of the components shown.

Table 1

Strains and plasmids used in this study.

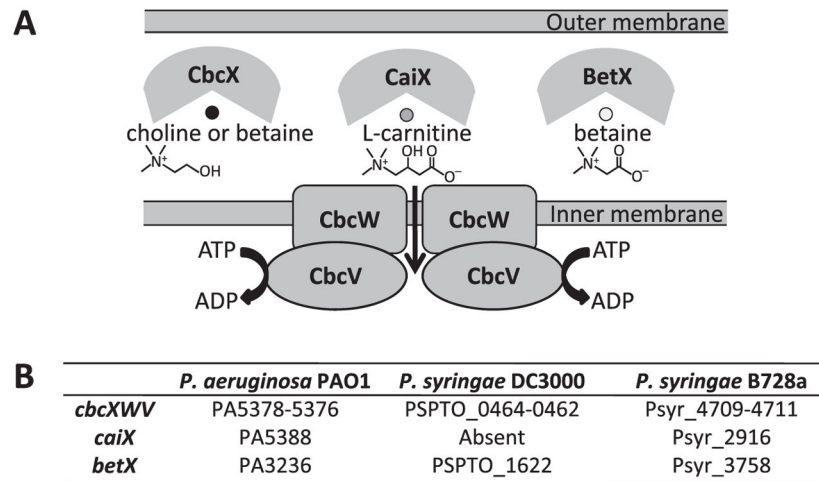| Strain or plasmid	Description/relevant genotype	Reference or source
Strains		
Escherichia coli		
Acella	Host supporting pET15b-based expression	EdgeBio
MKH13	*putPA proP2 betTIBA proU::spc*; Sp^r	Kempf and Bremer (1995)
Pseudomonas syringae pv. *tomato*		
DC3000	Wild type; Rf^R	Moore *et al.* (1989)
DC *cbc*	DC3000 *cbcXWV*; Rf^r	This work
GB1	DC3000 *betT opuCA*; Rf^r	Chen and Beattie (2008)
GB2	DC3000 *betT cbcXWV*; Rf^r	Chen and Beattie (2008)
GB3	DC3000 *opuCA cbcXWV*; Rf^r	Chen and Beattie (2008)
GB4	DC3000 *betT opuCA cbcXWV*; Rf^r	Chen and Beattie (2008)
Pseudomonas syringae pv. *syringae*		
B728a	Wild type; Rf^R	Loper and Lindow (1987)
B0028Ko	B728a Psyr_0028::Tet; Rf^r	This work
B2916Ko	B728a *caiX*::Tet; Rf^r	This work
BT	B728a *betT opuC cbcXWV*; Rf^r	This work
BT0028Ko	B728a *betT opuC cbcXWV* Psyr_0028::Tet	This work
BT2916Ko	B728a *betT opuC cbcXWV caiX*::Tet	This work
BT3758Ko	B728a *betT opuC cbcXWV betX*::Tet	This work
Pseudomonas aeruginosa		
PA14	Wild type	Rahme *et al.* (1995)
PA14 *caiX*	PA14 PA5388	Wargo and Hogan (2009)
PA5376:tn	PA14 with transposon insertion in PA5376; Gm^r	Liberati *et al.* (2006)
PA5377:tn	PA14 with transposon insertion in PA5377; Gm^r	Liberati *et al.* (2006)
PA5378:tn	PA14 with transposon insertion in PA5378; Gm^r	Liberati *et al.* (2006)
Plasmids		
pTOK2	Suicide vector; Tet^r	Kitten and Willis (1996)
pTOK2T	pTOK2 with restored *lacZ* activity; Tet^r	This work
pT*sacB*	pTOK2T with *sacB*; Tet^r	This work
pT-PA5388	pTOK2T with *caiX* from *P. aeruginosa* PAO1	This work
pT-B2916	pTOK2T with *caiX* from B728a	This work
pT-PA3236	pTOK2T with *betX* from *P. aeruginosa* PAO1	This work
pT-B3758	pTOK2T with *betX* from B728a	This work
pT-D3758	pTOK2T with *betX* from DC3000	This work
pME6041	Broad-host-range vector; Km^r	Heeb *et al.* (2000)
pCbc_PAO1	pME6041 with *cbcXWV* from *P. aeruginosa* PAO1	This work
pCbc_B728a	pME6041 with *cbcXWV* from B728a	This work
pCbc_DC3000	pME6041 with *cbcXWV* from DC3000	This work

Strain or plasmid	Description/relevant genotype	Reference or source
pME2916	pME6041with *caiX* from B728a	This work
pME2915-16	pME6041with *caiX* and *cdhR* from B728a	This work
pET15b	Expression vector	Novagen
pET-*cbc*$_{B728a}$	pET15b with [His]$_6$-*cbcXWV*$_{B728a}$	This work
pET-*cbc*$_{DC3000}$	pET15b with [His]$_6$-*cbcXWV*$_{DC3000}$	This work
pN	pME6041 with *nptII* promoter	This work
pN*cbcWV*	pN with *cbcWV* from *P. aeruginosa* PAO1	This work
pN*cbcXWV*	pN with *cbcXWV* from *P. aeruginosa* PAO1	This work
pN*choXWV*	pN with *choXWV* from *S. meliloti* 1021	This work

Table 2

Uptake activities of *E. coli* strains expressing *cbcXWV* from various *Pseudomonas* spp.

Strain[b]	Plasmid	Total uptake [nmol (mg protein)$^{-1}$]^a		
		Choline	Betaine	Carnitine
MKH13	pME6041	0.2 (0.1)	0.5 (0.5)	0.5 (0.3)
MKH13	pCbc$_{PAO1}$	12.1 (1.1)	5.8 (1.1)	0.8 (0.4)
Acella[c]	pET15b	0	8.2	0
Acella	pET-*cbc*$_{DC3000}$	15.0 (2.4)	17.0 (1.8)	0.59 (0.82)
Acella[c]	pET-*cbc*$_{B728a}$	19.2	16.2	0

^{a}Uptake was evaluated using radiolabelled choline, betaine or carnitine (1 mM). Unless otherwise indicated, average values (SE) are shown ($n = 10$).

^{b}The Acella cells were grown with 1 mM IPTG for promoter induction.

^{c}Uptake values for these constructs represent a single colony; these values were verified in a separate experiment.

Table 3

Abilities of various compounds to competitively inhibit the uptake of radiolabelled choline, betaine or carnitine by the QAC transporter-deficient BT strain expressing *cbcXWV* from B728a, DC3000 and PAO1.

Competitor	[14C] substrate	% inhibition of uptake of [14C]substrate[a]		
		BT(pCbc$_{B728a}$)	BT(pCbc$_{DC3000}$)	BT(pCbc$_{PAO1}$)
Water	Betaine	0	0	0
Betaine	Betaine	93	89	83
Choline	Betaine	3	−6	−6
Carnitine	Betaine	5	−2	2
Water	Choline	0	0	0
Betaine	Choline	19	12	9
Choline	Choline	93	89	84
Carnitine	Choline	5	−6	1
Water	Carnitine	0	0	0
Betaine	Carnitine	83	88	84
Choline	Carnitine	75	81	77
Carnitine	Carnitine	93	94	94

[a]Cells were grown with 10 mM choline and 10 mM betaine and uptake was evaluated using radiolabelled substrates (5 μM) in the presence of unlabelled competitors (0.5 mM) for 10 min. The steady-state total uptake in water, and thus in the absence of competitors, was: [14C]betaine uptake, 1.8, 1.8 and 1.6 nmol (mg protein)$^{-1}$; [14C]choline, 5.8, 5.4 and 5.0 nmol (mg protein)$^{-1}$; [14C]carnitine, 9.5, 5.0 and 15.2 nmol (mg protein)$^{-1}$, for BT(pCbc$_{DC3000}$), BT(pCbc$_{B728a}$), BT(pCbc$_{PAO1}$) respectively. Values are the mean % inhibition ($n = 4$), and the results are representative of two independent experiments. The standard deviation did not exceed 5%.

Table 4

Uptake activities of strains expressing the core Cbc or Cho transporters in the presence and absence of the putative chromosomally encoded SBPs Psyr_2916, Psyr_3758 and Psyr_0028 and the plasmid-encoded SBPs CbcX and ChoX.

Plasmid	Host[b]	Total uptake [nmol (mg protein)$^{-1}$][a]			
		Betaine (10 µM)	Carnitine (10 µM)	Choline (10 µM)	Choline (1 mM)
pN	BT2916Ko	0.0	0.1	0.0	0
pN	BT3758Ko	0.0	0.0	0.0	0
pN	BT0028Ko	0.0	0.0	0.0	0
pN*cbcXWV*	BT2916Ko	16.0	0.5	16.2	644
pN*cbcXWV*	BT3758Ko	14.3 (0.24)	13.0 (0.3)	16.4 (0.18)	648 (27.5)
pN*cbcXWV*	BT0028Ko	16.1 (0.41)	7.0 (0.4)	18.2 (0.36)	562 (6.8)
pN*cbcWV*	BT2916Ko	17.2	0.3	0.15	8.6
pN*cbcWV*	BT3758Ko	0.1 (0.01)	15.1 (0.16)	0.15 (0.05)	7.1 (0.6)
pN*cbcWV*	BT0028Ko	17.2 (0.2)	16.3 (0.91)	0.11 (0.05)	8.9 (1.4)
pN*choXWV*[c]	BT2916Ko	3.0	0.1	6.5	6.1
pN*choXW*	BT3758Ko	3.0 (0.64)	1.8 (0.4)	8.6 (1.28)	7.4 (0.8)
pN*choXW*	BT0028Ko	2.8 (0.3)	1.2 (0.1)	7.8 (0.07)	8.5 (1.4)

[a]Cells were re-suspended in ½–21C containing 0.3% glucose for the uptake measurements. Values represent a single replication for the BT2916Ko derivatives and the mean (SE) for the BT3758Ko ($n = 3$) and B0028Ko ($n = 2$) derivatives.

[b]The host strains were derived from strain BT, and thus lacked OpuC, BetT and Cbc due to deletions, as well as lacked Psyr_2916/CaiX (BT2916Ko), Psyr_3758/BetX (BT3758Ko) and Psyr_0028 (B70028Ko) due to knockout insertions (Table 1).

[c]The plasmid pN*choXWV* expressed the *S. melliloti* ChoXWV transporter.

Table 5

Uptake activities of the *E. coli* QAC transporter-deficient mutant MKH13 coexpressing components of the *P. aeruginosa* Cbc transporter or *S. meliloti* Cho transporter (Plasmid 1) and putative periplasmic substrate-binding proteins (SBPs) (Plasmid 2).

| Plasmid 1 | Plasmid 2 | SBP(s) present | Total uptake [nmol (mg protein)$^{-1}$]^a | | |
			Choline	Betaine	Carnitine
pN	None	None	0	0	0
pN*cbcWV*	None	None	0	0	0
pN*cbcWV*	pTOK2T	None	0	0	0
pN*cbcWV*	pT-PA5388	CaiX$_{PAO1}$	0	0	3.8
pN*cbcWV*	pT-B2916	CaiX$_{B728a}$	0	0	3.7
pN*cbcWV*	pT-PA3236	BetX$_{PAO1}$	0	21.6	0
pN*cbcWV*	pT-B3758	BetX$_{B728a}$	0	11.9	0
pN*cbcWV*	pT-D3758	BetX$_{DC3000}$	0	6.1	0
pN*cbcXWV*	None	CbcX$_{PAO1}$	44	12	0
pN*choXWV*	None	ChoX	4.7	2.7	0
pN*cbcXWV*	pT-PA5388	CbcX/CaiX$_{PAO1}$	26.7	19.4	2.9
pN*cbcXWV*	pT-B2916	CbcX/CaiX$_{B728a}$	22.5	13.8	0.3

^{a}Uptake was evaluated using radiolabelled substrates (10 µM). Values represent a single replication, with multiple measurements for the SBPs cloned from distinct strains indicating variability among the measurements for each SBP tested.

Table 6

Summary of the uptake kinetics of the Cbc and Cho transporters.

| Host | Plasmid 1 | Plasmid 2 | SBP present | [^{14}C] Substrate | Uptake kinetics[a] | |
					K_m	V_{max}
MKH13	pN$cbcXWV$	None	CbcX	Choline	2.6 (0.66)	34.6 (2.2)
MKH13	pN$cbcXWV$	None	CbcX	Betaine	24.2 (2.4)	11.6 (0.6)
MKH13	pN$cbcWV$	pTPA3236	BetX	Betaine	0.6 (0.16)	4.8 (0.18)
MKH13	pN$choXWV$	None	ChoX	Choline	1.8 (0.36)	1.2 (0.05)
PA5378::tn	pN$cbcWV$	None	CaiX$_{PAO1}$	Carnitine	24.2 (8.3)	3.8 (0.33)

[a]The data were fit with the Michaelis–Menten equation (Fig. S4). The units for the apparent affinity constant (K_m) are μM and for the maximal rate of uptake (V_{max}) are nmol min^{-1} (mg protein)$^{-1}$. Values shown are mean (SE) ($n = 4$).

Table 7

Competitive uptake of radiolabelled choline, betaine and carnitine in the *E. coli* transporter-deficient mutant MKH13 expressing selected transporter components.[a]

Plasmid 1	Plasmid 2	SBP(s)	[14C] Substrate	% inhibition of uptake of substrate with the following unlabelled competitors[a]							
				W	MM	DM	Bet	Cho	Car	AC	PC
pN*cbcWV*	pT-PA3236	BetX	Betaine	0	−13	56	94	−8	3	NT	NT
pN*cbcWV*	pT-PA5388	CaiX	Car	0	0	2	5	5	91	NT	NT
pN*cbcXWV*	pT-PA5388	CaiX/CbcX	Car	0	−1	7	57	66	97	NT	NT
pN*cbcXWV*	None	CbcX	Betaine	0	−3	−5	87	99	3	NT	NT
pN*cbcXWV*	pT-PA5388	CbcX/CaiX	Betaine	0	3	−4	88	99	10	NT	NT
pN*cbcXWV*	None	CbcX	Choline	0	2	4	2	92	6	9	1
pN*cbcXWV*	pT-PA5388	CbcX/CaiX	Choline	0	2	−8	−1	96	−2	NT	NT
pN*choXWV*	None	ChoX	Choline	0	−4	−6	3	98	41	88	5

[a]Uptake was evaluated using using radiolabelled substrates (10 µM) in the presence of unlabelled competitors (1 mM). The steady-state total uptake in absence of competitors (Water) were, by row, 8.6, 14, 11, 9.8, 11.8, 17.7, 29.5 and 16.7 nmol (mg protein)$^{-1}$. W, water; MM, monomethylglycine; DM, dimethylglycine; Bet, glycine betaine; Cho, choline; Car, carnitine; AC, acetylcholine; PC, phosphorylcholine; NT, not tested. Values are the mean percentage inhibition ($n = 3$–4), and the results are representative of two independent experiments.