Characterization of the Osmoprotectant Transporter OpuC from *Pseudomonas syringae* and Demonstration that Cystathionine-β-Synthase Domains Are Required for Its Osmoregulatory Function[∇][†]

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The plant pathogen *Pseudomonas syringae* may cope with osmotic stress on plants, in part, by importing osmoprotective compounds. In this study, we found that *P. syringae* pv. tomato strain DC3000 was distinct from most bacterial species in deriving greater osmoprotection from exogenous choline than from glycine betaine. This superior osmoprotection was correlated with a higher capacity for uptake of choline than for uptake of glycine betaine. Of four putative osmoregulatory ABC transporters in DC3000, one, designated OpuC, functioned as the primary or sole transporter for glycine betaine and as one of multiple transporters for choline under high osmolarity. Surprisingly, the homolog of the well-characterized ProU transporter from *Escherichia coli* and *Salmonella enterica* serovar Typhimurium did not function in osmoprotection. The *P. syringae* pv. tomato OpuC transporter was more closely related to the *Bacillus subtilis* and *Listeria monocytogenes* OpuC transporters than to known osmoprotectant transporters in gram-negative bacteria based on sequence similarity and genetic arrangement. The *P. syringae* pv. tomato OpuC transporter had a high affinity for glycine betaine, a low affinity for choline, and a broad substrate specificity that included acetylcholine, carnitine, and proline betaine. Tandem cystathionine-β-synthase (CBS) domains in the ATP-binding component of OpuC were required for transporter function. The presence of these CBS domains was correlated with osmoregulatory function among the putative transporters examined in DC3000 and was found to be predictive of functional osmoregulatory transporters in other pseudomonads. These results provide the first functional evaluation of an osmoprotectant transporter in a *Pseudomonas* species and demonstrate the usefulness of the CBS domains as predictors of osmoregulatory activity.

Prokaryotic ATP-binding cassette (ABC) transporters contrast with eukaryotic ABC transporters, which function mainly in export, by functioning mainly in the import of compounds that have bound to associated periplasmic proteins or lipoproteins. Genes encoding ABC transporters are the largest group of paralogous genes in bacterial genomes, particularly in bacteria associated with plants and soils (23, 43). For example, genomic sequences predict 119 transporters belonging to the ABC superfamily in the plant pathogen *Pseudomonas syringae* pv. tomato strain DC3000 and 200 in the plant symbiont *Sinorhizobium meliloti* strain 1021 but only 69 in *Escherichia coli* strain K-12 (43). The predominance of importers is illustrated by the prediction of 146 uptake systems but only 18 export systems in *S. meliloti* (43); this predominance may be due to their role in high-affinity acquisition of diverse nutrients (37).

Osmoregulatory ABC transporter systems contribute to bacterial adaptation to hyperosmolarity as well as to heat and chilling stresses (2, 4). The uptake of osmoprotectant compounds, i.e., compounds that can serve as or be converted to compatible solutes, can result in sufficient solute accumulation to provide osmotic homeostasis to cells. Since the ProU transporters of *E. coli* and *Salmonella enterica* were first identified as

osmoregulatory ABC transporters more than 2 decades ago (11, 21), osmoregulatory ABC transporters have been identified primarily in gram-positive bacteria. These include OpuA, OpuB, and OpuC from *Bacillus subtilis* (30), Gbu and OpuC from *Listeria monocytogenes* (3), and OpuA (BusA) from *Lactococcus lactis* (52). The *Erwinia chrysanthemi* OusB transporter is among the few identified in a gram-negative species and is similar to ProU in sequence, substrate range, expression, and activation profile (14). *S. meliloti* ABC transporters that function in the uptake of osmoprotectant compounds have also been identified (1, 8, 19, 28), but the primary or sole function of each appears to be for catabolism rather than osmoprotection. Greater functional knowledge of osmoregulatory ABC transporters in gram-negative bacteria would improve our ability to identify such transporters among multiple candidates in a given organism, as has been attempted in studies examining structure-function relations within these transporters (45).

Prokaryotic ABC transporters typically consist of a periplasmic (or lipoprotein) substrate-binding protein, two integral membrane proteins (permeases), and two peripheral membrane proteins that bind and hydrolyze ATP. Recently, tandem cystathionine-β-synthase (CBS) domains in the ATPase component of the OpuA transporter of *L. lactis*, designated OpuAC, were shown to function in osmosensing by enabling OpuA activation by threshold levels of ionic osmolytes (7, 35). Based on this function, we propose that tandem CBS domains may be a predictive feature of functional osmoregulatory ABC transporters.

In this study, we examined the osmoregulatory ABC trans-

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TABLE 1. Plasmids and strains used in this study

Strain or plasmid	Description/relevant genotype	Reference or source
Escherichia coli		
DH5α	Host for cloning	Invitrogen
MG1655	Host for mutagenesis	15
MKH13	Δ*putPA101* Δ*proP2* Δ*proU*::*spc-608* Δ*betTIBA*; Sp^r	31
Pseudomonas syringae pv. tomato		
DC3000	Wild type; Rf^r	41
0462::pKO	DC3000 with PSPTO_0462::pKnockout-Ω; Rf^r Sp^r	This work
3060::pKO	DC3000 with PSPTO_3060::pKnockout-Ω; Rf^r Sp^r	This work
4575::pKO	DC3000 with PSPTO_4575::pKnockout-Ω; Rf^r Sp^r	This work
5273::pKO	DC3000 with PSPTO_5273::pKnockout-Ω; Rf^r Sp^r	This work
DC3000Δ*opuCA*	DC3000 with ΔPSPTO_4575; Rf^r	This work
Plasmids		
pKnockout-Ω	Suicide vector for rapid gene inactivation in *P. syringae*; Sm^r Sp^r	55
pKD13	Template for *kan* cassette flanked by FLP recombination target sites; Ap^r Km^r	15
pKD46	Encodes λ Red recombinase; *repA101ts*; Ap^r	15
pFlp2	Encodes Flp recombinase, suicide vector in *P. syringae*; Ap^r	26
pRK2013	RP4 transfer functions for mobilization; Km^r	17
pRK2073	pRK2013 with Tn7 in the Km^r gene; Sm^r Sp^r	6
pKO4575-8	pKnockout-Ω containing 9,018-bp PCR-amplified fragment with PSPTO_4575–PSPTO_4578	This work
pME6041	*E. coli*-*Pseudomonas* shuttle and cloning vector; *oriV*$_{pVS1}$ *oriV*$_{p15A}$ *oriT*; Km^r	24
pME*opuC*	pME6041 containing a 9.6-kb genomic fragment with PSPTO_4575–PSPTO_4578	This work

porters in a member of the genus *Pseudomonas*. *Pseudomonas* species have been extensively studied due to their medical, agricultural, and environmental importance. For example, *Pseudomonas aeruginosa* and *P. syringae* serve as major models for elucidating the molecular mechanisms of animal and plant pathogenesis, whereas *Pseudomonas putida* serves as a model for bioremediation studies, *Pseudomonas fluorescens* as a model for biological control of phytopathogens, and *Pseudomonas stutzeri* as a model for understanding denitrification, natural transformation, and pollutant degradation processes. These various ecological functions are each influenced by the abilities of the bacteria to survive and maintain metabolic activity in their natural habitats despite often fluctuating and stressful environmental conditions, including high salinity and low water content. Here, we provide the first functional characterization of an osmoregulatory ABC transporter in a pseudomonad by using *P. syringae*, a widespread epiphyte that must cope with fluctuating osmotic conditions on and in aerial plant leaves (5, 25, 56), and show that tandem CBS domains are an effective predictor of function among putative osmoregulatory ABC transporters, at least in *Pseudomonas* species.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* strains were grown at 37°C in LB medium (39) or M63 medium (46) supplemented with vitamin B$_1$ (0.0005%). *E. coli* strains harboring the temperature-sensitive plasmid pKD46 were grown at 30°C. *P. syringae* strains were grown at 28°C in King's B medium (32), minimal medium MinA (39), or the low-osmoticum medium 1/2-21C (5, 22), which contained, per liter, 0.5 g of NH$_4$Cl, 1.745 g of Na$_2$HPO$_4$ · 7H$_2$O, 1.395 g of KH$_2$PO$_4$, 3 g of glucose, and 20 ml of Huntner's mineral solution (48) and was modified by the addition of 0.6 g of succinate to promote bacterial growth. In the absence of supplemental NaCl, 1/2-21C medium had an osmolality of 95 mosmol/kg H$_2$O; the addition of NaCl to 0.22 M increased the osmolality to 490 mosmol/kg. Osmolality was determined using an osmometer (Osmomette A; Precision Systems Inc., Sudbury, MA). Antibiotics were used at the following

concentrations (μg ml^{-1}): ampicillin, 100; kanamycin, 50 for *P. syringae* or 20 for *E. coli*; rifampin, 100; and spectinomycin, 60.

Construction of knockout and deletion mutants of *P. syringae* pv. tomato strain DC3000 in putative osmoprotectant transporters. The suicide vector pKnockout-Ω (55) was used to generate insertional mutations in the following DC3000 genes: PSPTO_0462, PSPTO_3060, PSPTO_4575, and PSPTO_5273. An internal fragment of each gene was amplified using the primers listed in Table S1 in the supplemental material and was subsequently cloned into XcmI-digested pKnockout-Ω. Insert orientation was determined by PCR using the primer Carol1 and the forward or reverse primer for each target gene (see Table S1 in the supplemental material). Single recombinants were selected after introducing each pKnockout-Ω construct into DC3000 via a triparental mating with pRK2013 (17), and insertions were confirmed by PCR. The resulting mutants were designated 0462::pKO, 3060::pKO, 4575::pKO, and 5273::pKO.

To generate a PSPTO_4575 deletion mutant, the PSPTO_4575–PSPTO_4578 locus, including 1,561 bp upstream of PSPTO_4578 and 4,034 bp downstream of PSPTO_4575, was amplified using AccuPrime Hi Fi polymerase (Invitrogen, Carlsbad, CA) and the primer set 4575L1/4575L2 (see Table S1 in the supplemental material). The 9-kb product was cloned into XcmI-digested pKnockout-Ω, forming pKO4575-8, which was then introduced into *E. coli* strain MG1655(pKD46) (Table 1) by electroporation. PCR amplification of pKD13 by use of the primer set 4575H1/4575H2 (see Table S1 in the supplemental material) resulted in a PCR product that contained a *kan* cassette surrounded by FLP recombination target (FRT) sites and by 36-bp regions that share sequence similarity with the termini of PSPTO_4575. This chimeric fragment was introduced into MG1655(pKD46, pKO4575-8) by electroporation, resulting in the replacement of PSPTO_4575 through lambda Red recombinase-mediated recombination (15). This plasmid containing the marked deletion was mobilized into DC3000 via triparental matings with pRK2073 (6). Deletion mutants were identified as Rf^r Km^r Sp^s colonies and were confirmed by PCR. An unmarked deletion mutant was constructed by introducing pFlp2 (26).

To identify a genomic clone containing PSPTO_4575–PSPTO_4578, EcoRV-digested genomic DNA of DC3000 was ligated into pME6041 (24). This genomic library was introduced into the glycine betaine/choline transporter-deficient *E. coli* strain MKH13 (31) by electroporation, and a clone containing PSPTO_4575–PSPTO_4578 was identified based on restoration of growth on M63 medium amended with 0.6 M of NaCl and 1 mM of glycine betaine. The resulting strain was designated MKH13(pME*opuC*).

Osmoprotection assay. Bacterial growth in 1/2-21C or MinA medium amended with 0.3 M of NaCl and 1 mM of choline, glycine betaine, L-glutamate,

acetylcholine, carnitine, DL-pipecolate, trehalose, taurine, sucrose, or L-proline was monitored spectrophotometrically to evaluate the osmoprotection conferred by each compound. Late-log-phase cells were inoculated to a density of 10^7 cells/ml, and growth was monitored either in test tubes based on optical density at 600 nm (OD_{600}) or in microtiter plates based on measurements at both 630 nm and 450 nm to compensate for the optical interference of water condensation within the wells. The dual-wavelength measurements were converted into OD_{600} values by determining the OD_{600} and OD_{630}/OD_{450} values for the same cultures and performing regression analysis to obtain the following relationship: $y = 0.0344 + 0.477x + 6.621x^2$, where x was the OD_{630}/OD_{450} measurement and y was the OD_{600} measurement. All cultures were incubated at 28°C with shaking.

Transport assays. [methyl-^{14}C]choline and [methyl-^{14}C]proline (specific activity of 55 mCi/mmol) were obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [methyl-^{14}C]glycine betaine was prepared by the oxidation of [methyl-^{14}C]choline as described by Ko et al. (33). Proline betaine was prepared from proline as described by Musich and Rapoport (42) but was not purified beyond the filtration step. Cells were prepared by growing them in 1/2-21C or MinA medium to mid-log phase (OD_{600} of 0.3 to 0.5) and suspending washed cells in the same medium to an OD_{600} of 0.1 to 0.2 for glycine betaine uptake or to an OD_{600} of 1 for all other studies. Following introduction of NaCl, cells were incubated at 28°C for 2 to 3 h with shaking to allow the induction and expression of transporters.

The initial uptake rates were measured after adding a radiolabeled substrate to 0.5 ml of cells, shaking it for 2 min for glycine betaine uptake or 5 min for choline and proline uptake, and terminating uptake by centrifugation at 13,000 × g. The supernatant was immediately removed from each pellet, the cells were washed with 1 ml of medium that had the same osmolarity as the incubation medium, and the cells were suspended in 1 ml of ScintiVerse BD (Fisher Scientific, Fair Lawn, NJ). The radiolabel in the cells was determined using a liquid scintillation counter (Tri-Carb liquid scintillation analyzer, model 2100TR; Packard Instrument Co., Meriden, CT). Each sample was counted four times, and the average value was used as the reading.

For kinetic studies, the radiolabeled substrates were used at final concentrations of 1 to 100 μM for glycine betaine and choline (specific activity ranged from 6 to 600 μCi/mmol) and 1 to 2,000 μM for choline (specific activity ranged from to 0.6 to 1,199 μCi/mmol). For competition experiments, unlabeled compounds were used at final concentrations of 100 μM and 1 mM, whereas [methyl-^{14}C]glycine betaine and [methyl-^{14}C]choline were used at a final concentration of 10 μM (with specific activity of 0.55 mCi/mmol). The protein content of cell suspensions was determined using the Bio-Rad Bradford assay (Bio-Rad, Hercules, CA) following incubation of a subsample of cells in 1 M of NaOH at 95°C for 5 min. The data from the kinetic experiments were fit with the Michaelis-Menten equation, and the apparent affinity constant (K_m) and maximal rate of uptake (V_{max}) were determined.

Construction and analysis of PSPTO_4575 deletion derivatives. C-terminal deletions of the PSPTO_4575 protein were constructed by amplifying PSPTO_4575 by PCR using one upstream primer, 5′-CTGGCCATCATCGCC GACCTG-3′, with each of eight downstream primers with sequence similarity to distinct endpoints within the PSPTO_4575 gene. The resulting fragments were cloned into the EcoRV site of pME6041 and were introduced into the PSPTO_4575 deletion mutant by electroporation. The abilities of the cloned genes to complement the PSPTO_4575 deletion were evaluated based on growth on MinA medium containing 0.5 M NaCl and 2 mM glycine betaine and choline.

Identification of osmoregulatory ABC transporter genes from other pseudomonads. Genomic libraries of *P. syringae* pv. syringae B728a (34) and *P. aeruginosa* PAO1 (50) were constructed by ligating EcoRV- or PvuII-restricted genomic DNA into the EcoRV site of pME6041. The PSPTO_0462 and PSPTO_5269 genes were deleted in the PSPTO_4575 deletion mutant, described above, by using the lambda Red recombinase-mediated recombination system and the primers shown in Table S1 in the supplemental material, resulting in the construction of a DC3000 triple deletion mutant. The genomic libraries were transferred into this triple mutant by electroporation, and the transformants were plated on MinA media containing 0.5 M NaCl and either glycine betaine or choline (2 mM). The cloned fragments that permitted growth of the DC3000 triple mutant in the presence of either betaine or choline were sequenced.

CBS domain identification and homology search. Amino acid sequences of the ATP-binding component of characterized and putative osmoregulatory ABC transporters were obtained from the GenBank database (http://www.ncbi.nlm .nih.gov). Alignments of these amino acid sequences were performed using ClustalW (http://www.ebi.ac.uk/clustalw/). The CBS domains were identified and evaluated using the Pfam HMM database (http://pfam.janelia.org/hmmsearch .shtml) and Motif Search (http://motif.genome.jp/).
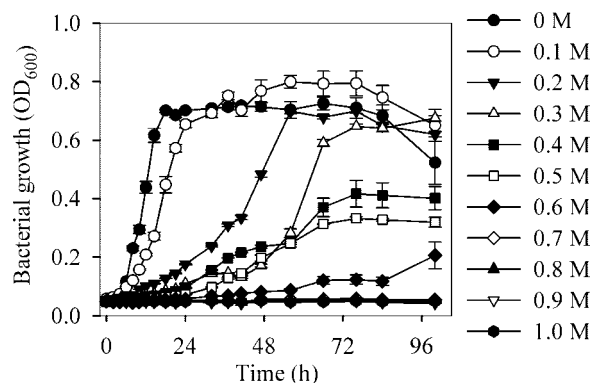

FIG. 1. Osmotolerance of *P. syringae* pv. tomato DC3000. Cells were grown in 1/2-21C medium amended with NaCl at the indicated concentrations. The initial concentrations were approximately 10^7 CFU/ml. Values shown are means ± standard errors of the mean (SEM) ($n = 4$).

RESULTS

Choline provides better osmoprotection than glycine betaine to DC3000. The growth of *P. syringae* pv. tomato strain DC3000 in the low-osmolarity medium 1/2-21C was significantly delayed by NaCl concentrations as low as 0.1 M (Fig. 1), with an even more dramatic delay caused by 0.2 M. In the presence of NaCl concentrations of 0.4 M or higher, the final growth yield of DC3000 was reduced by at least 50% compared to that of the 0 M control. No growth was observed in the presence of NaCl concentrations of 0.7 M or higher, even after 7 days of incubation (data not shown).

Glycine betaine and choline each provided strong osmoprotection (Fig. 2A). Surprisingly, choline provided consistently better protection than glycine betaine when provided at 1 mM (Fig. 2A). This contrasts with previous reports of other bacterial species for which glycine betaine provides better osmoprotection than choline (9, 33, 40). This superior osmoprotection by choline occurred at choline concentrations of ≥50 μM when examined after 8 h of growth (data not shown) and at concentrations of ≥100 μM when examined after 12 h (Fig. 2B). Acetylcholine provided good osmoprotection. Glutamate provided intermediate protection and growth to a lower final density than choline and glycine betaine, whereas carnitine supported delayed growth (Fig. 2A). No osmoprotection was observed in the presence of proline, ectoine, DL-pipecolate, mannitol, maltose, succinate, sucrose, trehalose, or taurine (data not shown). No significant bacterial growth occurred at NaCl concentrations of 0.7 M or higher, regardless of the presence of osmoprotectants or incubation time (data not shown).

The abilities of the potential osmoprotectants to serve as sole C sources were determined to better understand their use in osmoprotection. When provided at a final concentration of 10 mM, choline, glutamate, proline, and sucrose supported strong growth of DC3000, whereas DL-pipecolate, acetylcholine, and glycine betaine supported some growth (Fig. 2C). Glycine betaine supported significantly more growth when it was provided at a concentration of 20 mM (data not shown). Carnitine was unique among the osmo-protection-active compounds in its inability to serve as a
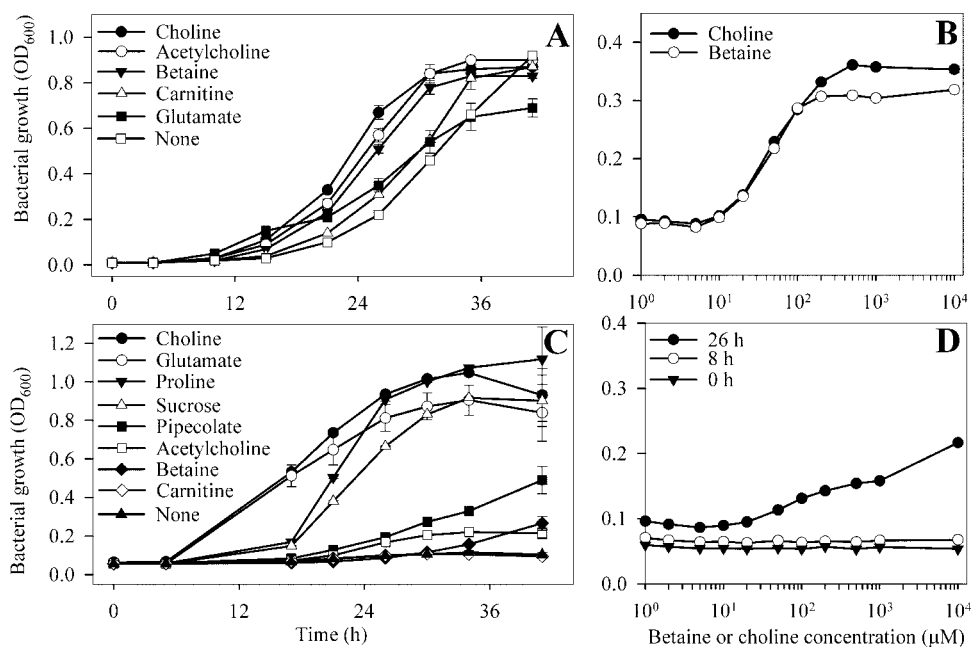
FIG. 2. Abilities of various exogenous compounds to serve as osmoprotectants or carbon sources for *P. syringae* pv. tomato DC3000. The OD_{600} values of DC3000 cultures are shown for growth in MinA medium amended with 0.3 M of NaCl and 1 mM of each of the indicated compounds (A), after 8 h of growth in MinA medium amended with NaCl at 0.4 M and glycine betaine and choline at the indicated concentrations (B), and for growth in glucose-free MinA medium amended with various compounds provided as a sole C source (10 mM) (C). The (D) OD_{600} values are shown for DC3000 $\Delta opuCA$ cultures after 0, 8, and 26 h in MinA medium amended with 0.4 M NaCl and glycine betaine at the indicated concentrations. Values are means ± SEM ($n = 3$).

sole C source for DC3000. These results indicate that DC3000 can transport and likely accumulate several osmoprotection-active compounds, despite its ability to catabolize them in the absence of osmotic stress, suggesting that DC3000 can regulate their catabolism under hyperosmotic conditions. No growth was observed on trehalose, taurine, or ectoine (data not shown), providing evidence against their transport or catabolism.

Superior osmoprotection by choline is correlated with a higher capacity for choline uptake by DC3000. Because choline must be converted to glycine betaine to confer cellular osmoprotection (51), the superior growth of DC3000 with choline (≥100 μM) at high osmolarity suggested that more choline than betaine was transported at these concentrations. Uptake

studies confirmed this prediction (Fig. 3). Specifically, the initial uptake rate for glycine betaine by salt-stressed DC3000 cells reached a plateau of approximately 22 nmol/min/mg protein at glycine betaine concentrations as low as 20 μM (Fig. 3A), whereas the initial uptake rate of choline was approximately twice this at a choline concentration of 100 μM and reached a plateau of approximately 80 nmol/min/mg protein with increasing choline concentrations (Fig. 3A). As expected, the initial uptake rates of both choline and glycine betaine were not significantly increased over the range of substrate concentrations tested in the absence of hyperosmotic stress. The uptake profiles for glycine betaine suggested the presence of at least one high-affinity, relatively low-capacity betaine transporter, whereas the uptake kinetics for choline suggested
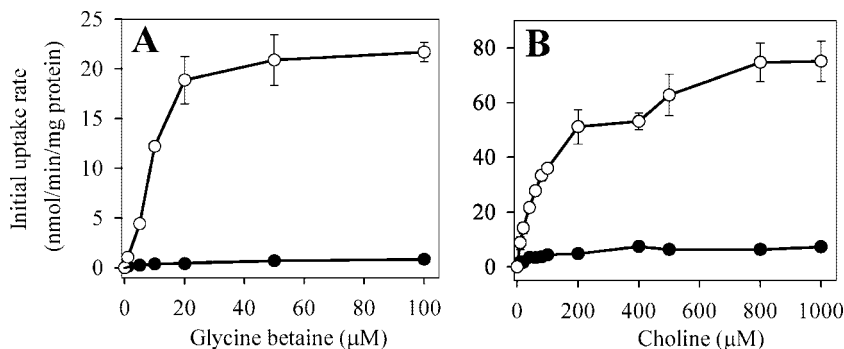

FIG. 3. Profiles for uptake of glycine betaine (A) and choline (B) by *P. syringae* pv. tomato DC3000 in 1/2-21C medium that was unamended (●) or amended with 0.2 M of NaCl (○). Log-phase cells were suspended in 1/2-21C medium containing [14C]glycine betaine or [14C]choline at various concentrations. Values are the means ± SEM ($n = 4$).
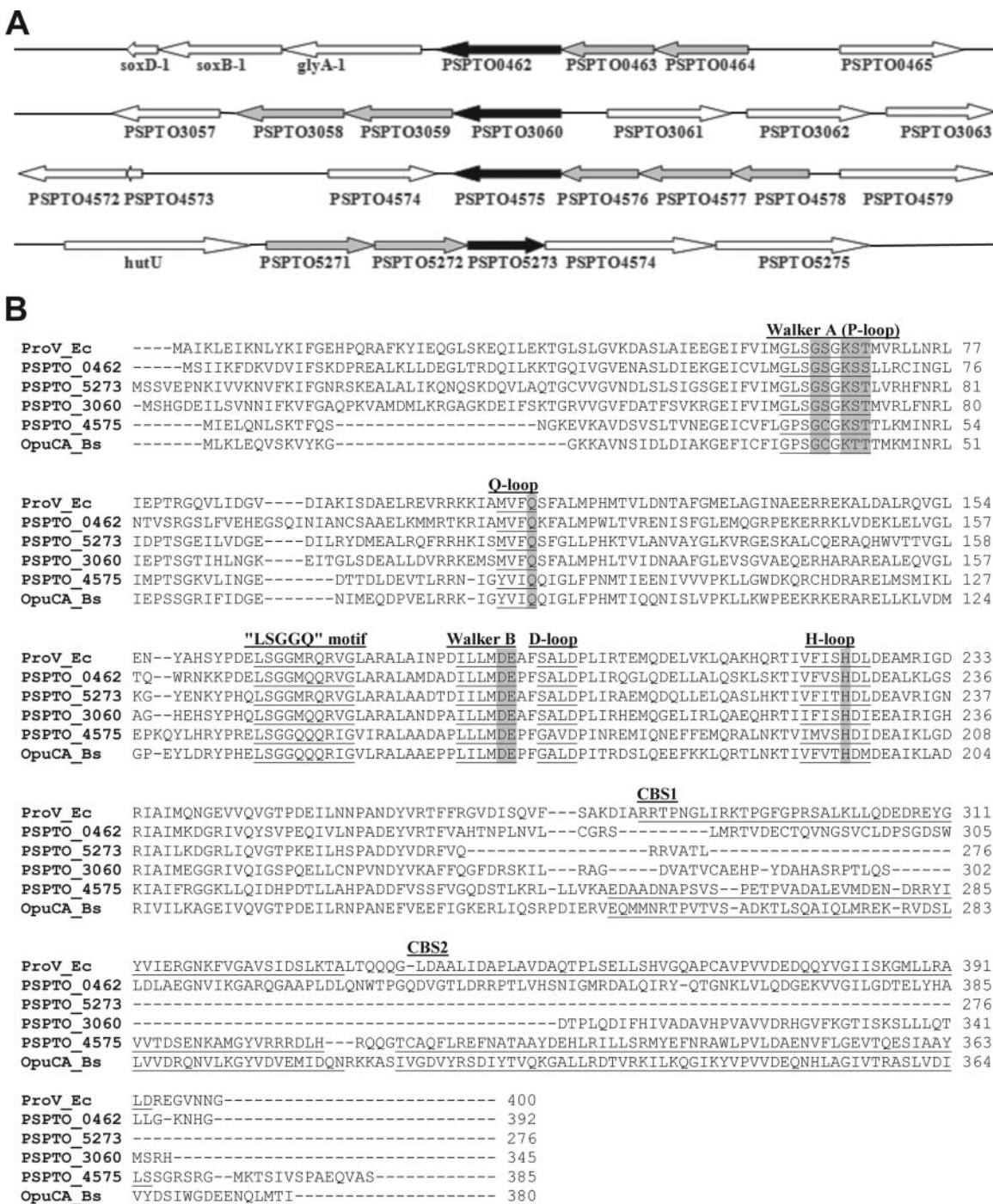
FIG. 4. The organization (A) and ATPase alignment (B) of four putative ABC transporter systems in *P. syringae* pv. tomato DC3000. Open reading frames that are predicted as the ABC transporter system are depicted in gray; the open reading frames that are predicted as the ATPase subunits are depicted in black. Alignment of 47 sequences of putative ABC transporters were performed using ClustalW (http://www.ebi.ac.uk /clustalw/); only the following sequences are shown: PSPTO_0462 (AAO54006), PSPTO_3060 (AAO56549), PSPTO_4575 (AAO58021), and PSPTO_5273 (AAO58699) as well as ProV from *E. coli* (ProV_Ec; AAA24427) and OpuCA from *B. subtilis* (OpuCA_Bs; O34992) for comparison. Conserved nucleotide-binding protein motifs, including the Walker A (or P-loop), Q-loop, Walker B, D-loop, and H-loop motifs, and the signature sequence for the nucleotide-binding protein of ABC transporters, the "LSGGQ" motif, are underlined and labeled. ATP-binding sites are shaded.

the presence of one or more low-affinity transporters with an overall high capacity for uptake.

Only one of four putative transporters examined in DC3000 was functional as an osmoregulatory ABC transporter. Using

the Transporter Protein Analysis Database in 2004 (43), we identified four putative complete ABC transporter systems in DC3000 with glycine betaine or choline as a predicted substrate: PSPTO_0462–PSPTO_0464, PSPTO_3058–PSPTO_3060, and
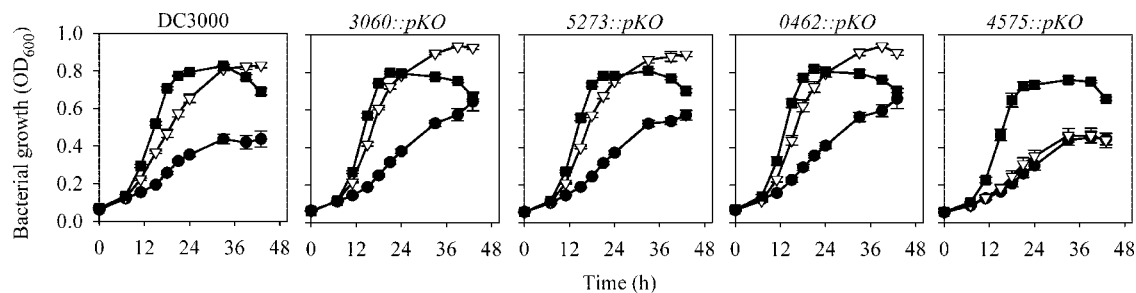
FIG. 5. Growth of DC3000 and various mutants in 1/2-21C medium that lacked succinate and contained 0.3 M of NaCl and was unamended (●), amended with 1 mM of choline (■), or amended with 1 mM of glycine betaine (▽). Mutants are designated by their PSPTO numbers. Values are the means ± SEM for three to six replicates. pKO, pKnockout.

PSPTO_5271–PSPTO_5273 with glycine betaine and proline as predicted substrates and PSPTO_4575–PSPTO_4578 with glycine betaine, choline, and carnitine as predicted substrates (Fig. 4A). We also identified one putative secondary transporter for choline, PSPTO_5269 (not shown). Sequence alignment of the predicted substrate-binding components, ATPases, and permease components with known osmoregulatory transporters indicated that the highest level of conservation was among the ATPase components and the lowest level was among the substrate-binding components, consistent with previous studies (16). The predicted amino acid sequence of the ATPase PSPTO_4575 contained tandem CBS domains on the C terminus (Expect value [E] = 4×10^{-16}), which are domains that have been associated with osmoregulatory function in *L. lactis* OpuA (7). The other three predicted ATPases lacked these domains ($E \geq 0.49$).

Mutants containing a pKnockout insertion in the ATPase gene of each of the four putative ABC transporters were constructed. Growth in 1/2-21C medium showed that the loss of these transporters did not affect DC3000 fitness in the absence of hyperosmolarity (data not shown) or under hyperosmotic conditions (0.3 M NaCl) in the absence of osmoprotectants (Fig. 5). Mutants inactivated in PSPTO_3060, PSPTO_5273, and PSPTO_0462 were not detectably altered in their growth when glycine betaine or choline was added. In contrast, the PSPTO_4575 mutant was dramatically reduced in growth in a glycine betaine-amended medium (Fig. 5), suggesting that a transporter system involving the PSPTO_4575 protein was the primary or sole betaine transporter in DC3000 under these conditions.

The rates for uptake of [^{14}C]glycine betaine and [^{14}C]choline into DC3000 and the pKnockout mutants were compared

in the presence and absence of 0.2 M of NaCl (Fig. 6). The mutants 3060::pKO, 5273::pKO, and 0462::pKO were similar to DC3000 in their uptake rates for both substrates under the hyperosmotic conditions. They were also similar under the nonhyperosmotic conditions, with the exception of mutant 0462::pKO, which was reduced in its choline uptake rate in the basal medium, suggesting a role in uptake for catabolism. 4575::pKO exhibited uptake rates that were reduced by at least 99% for glycine betaine and 35% for choline under the hyperosmotic conditions.

In addition to an ATPase (PSPTO_4575), the PSPTO_4575–PSPTO_4578 genes were predicted to encode two permeases (PSPTO_4576 and PSPTO_4578) and one periplasmic binding protein (PSPTO_4577), with PSPTO_4578 as the first gene in the putative operon (Fig. 4A). Among the homologs of the ATPase that have been functionally characterized, the PSPTO_4575 protein shared the greatest sequence identity with the ATPase components of the gram-positive bacterial transporters OpuC of *Listeria monocytogenes* and OpuC and OpuB of *Bacillus subtilis* (OpuCA and OpuBA; 45 to 46% identity) (20, 29) and much less sequence similarity with the ATPase component of the gram-negative transporters ProU of *E. coli* (ProV; 36% identity) (38) and OusB of *Erwinia chrysanthemi* (OusBV; 35% identity) (14). To date, functional OpuC-type transporters have been characterized from gram-positive bacteria only. Loci encoding the OpuC transporters in *L. monocytogenes* and *B. subtilis*, as well as the OpuB transporter of *B. subtilis*, contain the same gene arrangement as PSPTO_4575–PSPTO_4578, including two permease genes, but occur in reverse order relative to the DC3000 locus (4, 30). Hereafter, we refer to the transporter encoded by the
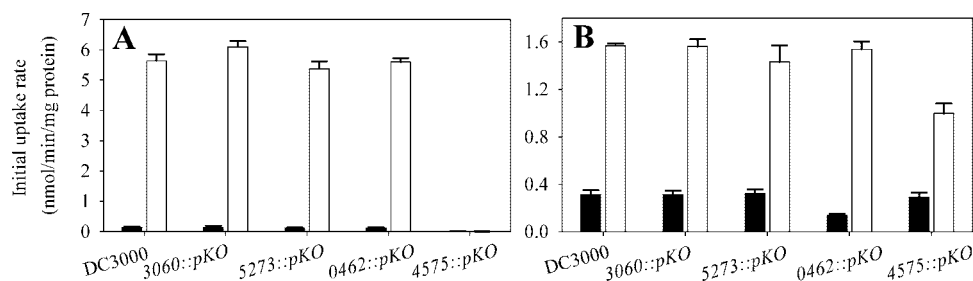

FIG. 6. Uptake of radiolabeled glycine betaine (A) or choline (B) by *P. syringae* pv. tomato DC3000 and various mutants in 1/2-21C medium in the absence (solid bars) or presence (open bars) of 0.2 M of NaCl. Radiolabeled substrates were provided at a concentration of 5 μM. Values are the means ± SEM ($n = 3$).
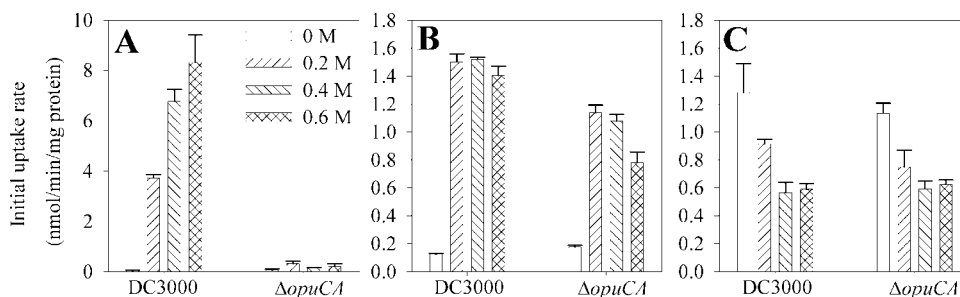
FIG. 7. Effect of NaCl concentration on the uptake of radiolabeled glycine betaine (A), choline (B), or proline (C) by *P. syringae* pv. tomato DC3000 and the Δ*opuCA* mutant. Uptake was examined in 1/2-21C medium in the presence of 0, 0.2, 0.4, and 0.6 M of NaCl. Radiolabeled substrates were provided at a concentration of 5 μM. Values are the means ± SEM (*n* = 3).

PSPTO_4575–PSPTO_4578 locus as the OpuC transporter and the ATPase component encoded by PSPTO_4575 as OpuCA, by convention with previous nomenclature (20, 29).

To minimize possible pleiotropic effects associated with an insertion mutation, a deletion mutant that lacked the complete *opuCA* gene was constructed. The effect of hyperosmolarity on the rate of uptake by DC3000 and the Δ*opuCA* mutant was evaluated for [^{14}C]glycine betaine and [^{14}C]choline as well as [^{14}C]proline, which did not function as an osmoprotectant for this strain. The rate of glycine betaine uptake increased with increasing NaCl concentration for DC3000 (Fig. 7A) but was dramatically lower in the presence of 0.8 M or 1 M NaCl (data not shown). This was consistent with its inability to grow at NaCl concentrations greater than 0.7 M in the presence or absence of an osmoprotectant. The Δ*opuCA* mutant was reduced at least 95% in [^{14}C]glycine betaine uptake at all NaCl concentrations (Fig. 7A) and exhibited a smaller but detectable reduction in [^{14}C]choline uptake (Fig. 7B). The rate of [^{14}C]proline uptake by DC3000 decreased under hyperosmotic conditions (Fig. 7C), consistent with the lack of osmoprotection by proline, and was not affected by the Δ*opuCA* mutation.

Under hyperosmotic conditions, the Δ*opuCA* mutant exhibited low but detectable growth when glycine betaine was provided at concentrations of ≥50 μM, but not at concentrations of <50 μM (Fig. 2D), suggesting that this mutant has residual betaine uptake activity and therefore that OpuC is the primary, but not the sole, transporter for glycine betaine in DC3000. Similarly, the Δ*opuCA* mutant exhibited residual choline up-

take activity (Fig. 7B) and better growth in the presence than in the absence of choline (Fig. 5), indicating that OpuC is one of multiple transporters for choline in DC3000.

OpuC is a high-affinity betaine transporter and a low-affinity choline transporter with relatively broad substrate specificity. To evaluate the physical properties of OpuC, a fragment containing the PSPTO_4575–PSPTO_4578 locus was identified in a DC3000 genomic library that was generated using the broad-host-range vector pME6041. The resulting plasmid, pME*opuC*, was introduced into *E. coli* strain MKH13, which lacked the PutP, ProP, and ProU transport systems and thus was unable to transport glycine betaine; this strain also lacked BetT and thus was unable to transport choline (31). Plasmid pME*opuC* restored glycine betaine uptake activity to MKH13 based on an osmoprotection assay in which the pME*opuC*- but not the pME6041-containing strain grew in M63 minimal medium amended with 0.5 M of NaCl and 1 mM of glycine betaine (data not shown). Choline did not provide osmoprotection to MKH13(pME*opuC*), consistent with the fact that MKH13 lacks the *betAB* genes necessary to convert choline into its osmoprotective form, glycine betaine, and thus cannot derive osmoprotection from choline, regardless of its ability to transport it.

The kinetic properties of OpuC were identified using the heterologously expressed *opuC* locus, as has been done for similar transporters (10, 14). The apparent K_m for glycine betaine uptake by OpuC was 3.7 ± 0.3 μM, with a V_{max} of 34.3 ± 0.6 nmol/min/mg of protein (Fig. 8A), indicating that
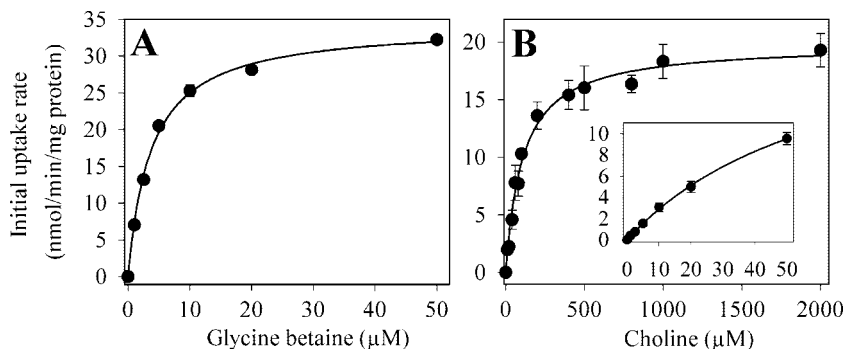

FIG. 8. Kinetics of OpuC-mediated uptake of glycine betaine (A) and choline (B) in *E. coli* MKH13(pME*opuC*). Log-phase cells grown in M63 medium amended with 0.5 M of NaCl were suspended in 50 mM of phosphate buffer (pH 7) containing 0.5 M of NaCl, 0.2% glucose, and [^{14}C]glycine betaine or [^{14}C]choline at various concentrations. The results are means ± SEM (*n* = 3).

TABLE 2. Abilities of various compounds to inhibit the uptake of choline and glycine betaine by OpuC expressed in *E. coli* MKH13(pME*opuC*)

Competitor	% inhibition of uptake of indicated compound with unlabeled competitor provided at indicated final concn[a]		
	Glycine betaine		Choline (1 mM)
	100 μM	1 mM	
Glycine betaine	86	97	99
Proline betaine	65	94	99
Carnitine	38	82	98
Choline	17	68	95
Acetylcholine	7	36	87
Phosphorylcholine	9	12	66
Proline	−1	−3	41
Ectoine	4	−6	25
DL-Pipecolate	−4	2	26
Trehalose	0	−18	−5
Glycine	−4	13	−20

[a] Cells were grown in M63 medium amended with 0.5 M of NaCl, and uptake was realized with 10 μM of [^{14}C]glycine betaine or 10 μM of [^{14}C]choline. The results are expressed as percents inhibition of uptake and are means for three independent experiments with variations of less than 15%. The uptake rates in the absence of competitors were 1.5 nmol/min/mg protein for choline and 19 nmol/min/mg protein for glycine betaine.

OpuC mediates high-affinity transport of glycine betaine. OpuC uptake of choline did not saturate in the substrate range from 1 to 50 μM (Fig. 8B, inset) but did over the range from 0 to 2,000 μM (Fig. 8B). The apparent K_m for choline uptake by OpuC was 113 ± 13 μM, with a V_{max} of 20 ± 0.7 nmol/min/mg of protein, indicating that OpuC mediates low-affinity transport of choline.

In competition assays with MKH13(pME*opuC*) for evaluating the substrate specificity of OpuC, [^{14}C]glycine betaine uptake was inhibited 86 and 65% by the addition of unlabeled glycine betaine and proline betaine, respectively, when these competitors were added at a 10-fold-higher concentration than the radiolabeled glycine betaine and 97 and 94%, respectively, when they were added at a 100-fold excess (Table 2). Carnitine, choline, and acetylcholine had an intermediate effect on [^{14}C]glycine betaine uptake, whereas phosphorylcholine, proline, ectoine, DL-pipecolate, trehalose, and glycine did not significantly affect [^{14}C]glycine betaine uptake, even when provided at a 100-fold excess (Table 2). Glycine betaine, proline betaine, carnitine, choline, acetylcholine, and to a lesser extent phosphorylcholine also inhibited [^{14}C]choline uptake when added at a 100-fold excess (Table 2). [^{14}C]choline uptake appeared to be modestly inhibited by proline, ectoine, and DL-pipecolate, but this inhibition is consistent with our observation that [^{14}C]choline transport was much more easily inhibited by other compounds than was glycine betaine transport. One possible explanation for this is that choline binding to the OpuC periplasmic binding component is weaker than glycine betaine binding, making choline uptake more easily inhibited by competitors.

Taken together, these results confirm that OpuC mediates the uptake of multiple substrates, including glycine betaine, choline, proline betaine, carnitine, and acetylcholine. Although phosphorylcholine served as an effective osmoprotectant for DC3000, we propose that phosphorylcholine itself was not a substrate for uptake but rather was converted to choline by a periplasmic phosphorylcholine phosphatase and thus was taken up as choline. Such a phosphatase has been identified as PchP in *P. aeruginosa* (36) and has been suggested by the presence of a homolog in DC3000 (the PSPTO_0436 protein). The absence of this enzyme in *E. coli* strains, including strain MKH13, may explain the lack or scarcity of competition by phosphorylcholine for betaine and choline uptake.

The presence of CBS domains correlated with osmoregulatory function by DC3000 OpuC and by transporters in other pseudomonads. Small protein modules known as CBS domains are common in many proteins, including a variety of transporters such as the ATPase component of ABC transporters, and generally are present as tandem pairs. Similar to the ATPase of the *L. lactis* OpuA transporter, which is the only osmoregulatory ABC transporter for which the CBS domains have been functionally examined (7), the ATPase of the DC3000 OpuC transporter has a C-terminal tail adjacent to the tandem CBS domains, which are joined by a linker region (Fig. 9). To evaluate the requirement for these domains and the C-terminal tail in OpuC transporter function, we generated various C-terminal deletion constructs (Fig. 9). Multiple constructs were generated to address ambiguity in the assignment of individual amino acids to the CBS domains (27). Whereas the full-length *opuCA* construct restored growth of the Δ*opuCA* mutant under hyperosmotic stress conditions in the presence of glycine betaine or choline, only the deletion constructs that contained complete CBS1 and CBS2 domains, but not necessarily a C-terminal tail, promoted growth under similar conditions (Fig. 9). These data demonstrate that the C-terminal tail was not required for OpuC function and that the CBS2 domain, and possibly both CBS domains, may be critical to its function.

The correlation between the presence of CBS domains and osmoregulatory function in the four DC3000 transporters examined prompted us to examine this correlation in transporters from other bacterial species. All of the transporters known to have osmoregulatory activity possess tandem CBS domains in their ATPase component, with E values of ≤4 × 10^{-5} for these domains, whereas those without these domains did not exhibit osmoregulatory activity (Table 3). The predicted *E. coli* transporter YehX is interesting in that it is closely related to the PSPTO_4575 protein (Fig. 10) and is induced by osmotic stress (12), but it does not appear to function in osmoprotection, based on its presence in the glycine betaine/choline transport-deficient mutant MKH13. The lack of tandem CBS domains at the YehX C terminus may explain its lack of osmoregulatory activity. Similarly, although the *L. monocytogenes* Lmo1421-associated transporter was predicted to transport choline based on its homology to *B. subtilis* OpuB (47), this transporter was recently found to lack osmoregulatory activity (2, 53). Other ATPases of characterized ABC transporters known to transport choline (ChoV) (19), ectoine (EhuA) (28), proline betaine (PrbV) (1), and histidine and glycine betaine (HutV/HisV) (8) were not activated by hyperosmolarity and lacked CBS domains; instead, these were induced by their substrates, suggesting a role in catabolism.

Based on the presence of CBS domains and the conserved ABC transporter domains shown in Fig. 4B, we predicted that the following ATPase components contribute to osmoregula-
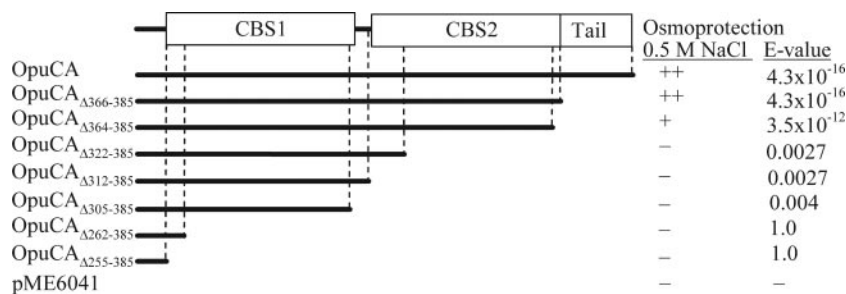
FIG. 9. Complementation of a Δ*opuCA* deletion mutant of DC3000 by pME6041 containing *opuCA* or various deletion constructs that were predicted to encode truncated OpuCA derivatives. The Δ*opuCA* deletion mutant also contained deletions in PSPTO_5269, which encodes a putative secondary transporter for choline, and PSPTO_0462, which encodes a putative transporter for choline catabolism. Osmoprotection function was based on the ability to grow on MinA medium amended with NaCl (0.5 M) and glycine betaine and choline (2 mM each). The E values for the tandem CBS domains were obtained using the Pfam HMM database (http://pfam.janelia.org/hmmsearch.shtml).

tory transport in the *Pseudomonas* species for which genomic sequences are available: Psyr4249 in *P. syringae* pv. syringae B728a, PA3891 in *P. aeruginosa* PAO1, PP0868 in *P. putida* KT2440, PSPPH4276 in *P. syringae* pv. phaseolicola 1448A, PFL0868 in *P. fluorescens* Pf-5, and PSEEN1040 in *Pseudomonas entomophila* L48. Each of these is only one of several ABC transporters predicted to transport choline or glycine betaine by the Transporter Protein Analysis Database in 2004 (43). We tested this prediction for *P. syringae* pv. syringae B728a and *P. aeruginosa* PAO1 by screening genomic libraries of these strains for clones that conferred enhanced growth upon a DC3000 mutant that contained a Δ*opuCA* deletion as well as deletions in PSPTO_5269, which encodes a possible betaine/

choline/carnitine transporter, and PSPTO_0462, which encodes a possible choline transporter for catabolic uptake (Fig. 6B). The sequence of the complementing clones indicated that transporters involving Psyr4249 from *P. syringae* pv. syringae strain B728a and PA3891 from *P. aeruginosa* PAO1 were functional, as predicted.

DISCUSSION

The uptake of exogenous compounds that confer osmoprotection may be important to the plant pathogen *P. syringae* in its natural, often water-limited habitat, aerial plant leaves. In this study, we began to characterize the transporters required

TABLE 3. Comparison of characterized and putative osmoregulatory ATPases for their osmoregulatory function and the presence and nature of CBS domains and a C-terminal tail

Organism	ATPase	Fn[b]	Result for indicated region[a]			
			CBS[c]	CBS1[d]	CBS2[d]	C-terminal tail[d]
E. coli K-12	ProV	+	4×10^{-5}	+ (6:10, 56)	− (7:2, 52)	0 (1:1, 7)
S. enterica serovar Typhimurium LT2	ProV	+	5×10^{-9}	+ (7:10, 59)	− (7:2, 52)	0 (1:1, 7)
E. chrysanthemi	OusBV	+	3×10^{-11}	+ (7:13, 59)	− (5:1, 52)	− (2:1, 7)
L. lactis	OpuAA	+	1×10^{-13}	+ (6:9, 56)	− (7:4, 52)	− (11:2, 17)
L. monocytogenes	GbuA	+	5×10^{-18}	+ (7:11, 56)	− (6:4, 52)	− (1:0, 8)
B. subtilis	OpuAA	+	5×10^{-20}	+ (6:11, 57)	− (8:4, 54)	− (5:1, 24)
B. subtilis	OpuCA	+	5×10^{-25}	+ (7:10, 55)	+ (5:8, 53)	− (4:0, 14)
B. subtilis	OpuBA	+	2×10^{-25}	+ (7:10, 54)	+ (8:9, 53)	− (3:1, 14)
L. monocytogenes	OpuCA	+	3×10^{-26}	− (8:7, 55)	+ (7:10, 53)	− (10:1, 31)
P. syringae DC3000	PSPTO_4575	+	4×10^{-16}	− (12:7, 53)	− (8:4, 53)	+ (1:3, 20)
P. syringae B728a	Psyr4249	+	4×10^{-16}	− (12:7, 53)	− (8:4, 53)	+ (2:3, 23)
P. aeruginosa PAO1	PA3891	+	1×10^{-19}	− (10:9, 53)	− (7:3, 53)	+ (1:3, 20)
E. coli MC4100	YehX	−	0.42	None	None	None
L. monocytogenes	Lmo1421	−	0.41	None	None	None
S. meliloti 1021	ChoV	−	0.14	None	None	None
S. meliloti 1021	EhuA	−	>1.0	None	None	None
S. meliloti 1021	PrbV	−	>1.0	None	None	None
S. meliloti 5000	HisV/HutV	−	>1.0	None	None	None
P. syringae DC3000	PSPTO_5273	−	>1.0	None	None	None
P. syringae DC3000	PSPTO_0462	−	>1.0	None	None	None
P. syringae DC3000	PSPTO_3060	−	0.49	None	None	None

[a] Values in parentheses are ratios of the anionic residue number/cationic residue number and the total numbers of amino acids in the indicated domain or tail.
[b] The ability (+) or inability (−) to mediate the uptake of osmoprotectant compounds in response to increasing osmolality is indicated. Functional information was obtained from *E. coli* ProV (21), *S. enterica* serovar Typhimurium ProV (49), OusBV (14); *L. lactis* OpuAA (52); GbuA (3); *B. subtilis* OpuAA (31); *B. subtilis* OpuCA (30); OpuBA (30); *L. monocytogenes* OpuCA (4, 54); PSPTO_4575, Psyr4249, PA3891, and PP0868 (this study); YehX (lack of uptake in MKH13, which contains an intact *yehX*); Lmo1421 (3, 53); ChoV (19); EhuA (28); PrbV (1); HisV/HutV (8); and PSPTO_5273, PSPTO_0462, and PSPTO_3060 (this study).
[c] E values for the tandem CBS domains were obtained by using the Pfam HMM database (http://pfam.janelia.org/hmmsearch.shtml).
[d] The predicted net charge of the CBS1 domain, CBS2 domain, or C-terminal tail based on the number of cationic or anionic residues, with + indicating more cationic residues, − indicating more anionic residues, and 0 indicating equal numbers.
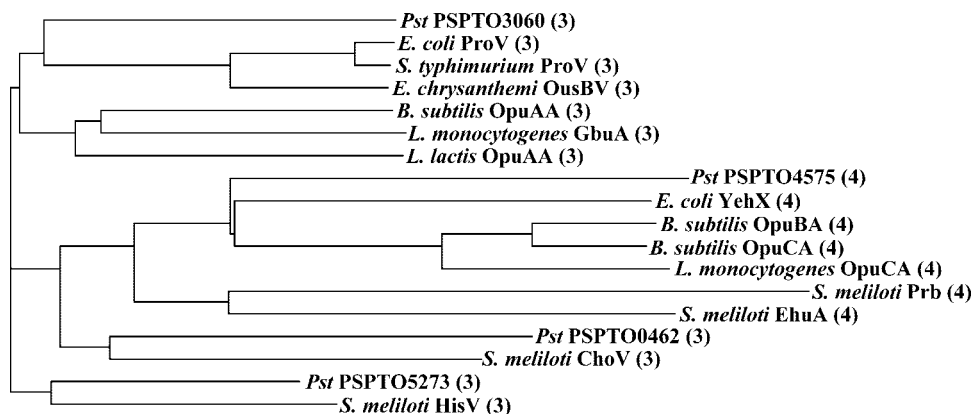
FIG. 10. A neighbor-joining tree for the ATPase components of the glycine betaine and choline ABC transporters that have been functionally characterized in prokaryotes and those examined in this study. The osmoregulated *E. coli* YehX protein was included for comparison, although it is not functional (12). The numbers in parentheses indicate the number of genes in the locus encoding each transporter. The proteins that are shown include AAO56549 (*P. syringae* pv. tomato PSPTO_3060), AAC75724 (*E. coli* ProV), AAL21694 (*Salmonella enterica* serovar Typhimurium ProV), AAQ06630 (*E. chrysanthemi* OusBV), P4692 (*B. subtilis* OpuAA), CAC99092 (*Listeria monocytogenes* GbuA), AAF37878 (*L. lactis* OpuAA), AAO58021 (*P. syringae* pv. tomato PSPTO_4575), AAA60492 (*E. coli* YehX), Q45460 (*B. subtilis* OpuBA), O34992 (*B. subtilis* OpuCA), CAC99506 (*L. monocytogenes* OpuCA), CAC46836 (*S. meliloti* Prb), CAC48813 (*S. meliloti* EhuA, which transports ectoine and glycine betaine [28]), AAO54006 (*P. syringae* pv. tomato PSPTO_0462), CAC46980 (*S. meliloti* ChoV), AAO58699 (*P. syringae* pv. tomato PSPTO_5273), and CAC47281 (*S. meliloti* HisV, which transports histidine and glycine betaine [8]). Sequences were aligned using ClustalW (13), and a guide tree was constructed by the neighbor-joining method.

for the uptake of osmoprotectant compounds by *P. syringae* pv. tomato strain DC3000, and in doing so, we characterized the first osmoregulatory ABC transporter in a *Pseudomonas* species, the *P. syringae* pv. tomato OpuC transporter. Surprisingly, this transporter shows greater similarity to the osmoprotectant transporters that have been characterized in gram-positive bacteria than to those in gram-negative bacteria (Fig. 10), with the strongest similarity to the OpuC transporters of *L. monocytogenes* and *B. subtilis* and the OpuB transporter of *B. subtilis* (20, 30). In contrast, the *P. syringae* pv. tomato homolog of the well-characterized ProU transporter from *E. coli* and *S. enterica* serovar Typhimurium, encoded by PSPTO_3058–PSPTO_3060, did not function in osmoprotection. The *P. syringae* pv. tomato OpuC transporter was similar to the *B. subtilis* and *L. monocytogenes* OpuC transporters in having two distinct putative permeases (OpuCB and OpuCD) rather than one, like the *E. coli* ProU (38) and *Erwinia chrysanthemi* OusB (14) transporters (Fig. 10). *P. syringae* pv. tomato OpuC was also similar in exhibiting a broad substrate specificity and a relatively high transport capacity for glycine betaine (V_{max} values of 34 and 65 nmol/min/mg of protein for *P. syringae* pv. tomato and *B. subtilis* OpuC, respectively) but had a lower affinity and transport capacity for choline (30). One or more additional transporters in DC3000 appear to contribute to choline uptake by this strain.

For the four *P. syringae* pv. tomato DC3000 transporters examined in this study, the presence of CBS domains was strongly correlated with osmoregulatory transport activity, suggesting that tandem CBS domains may be a predictor of osmoregulatory activity. This was supported by four lines of evidence. First, Biemans-Oldehinkel et al. (7) demonstrated that these domains are involved in osmosensing by *L. lactis* OpuA. Second, these domains were required for *P. syringae* pv. tomato OpuC-mediated uptake for osmoprotection. Third, without exception, the ATPase components of all of the pro-

karyotic osmoregulatory ABC transporters characterized to date possess full-length CBS domains. And fourth, we demonstrated osmoregulatory function for two transporters that were predicted to function based on the presence of CBS domains. Specifically, we identified Psyr4249 and PA3891 as components of the *P. syringae* pv. syringae strain B728a and *P. aeruginosa* strain PAO1 transporters that we predicted to function in osmoregulation. We verified the accuracy of this prediction by screening for genomic clones that could restore growth to a choline/betaine uptake-deficient DC3000 mutant under hyperosmotic conditions and showing that the complementing clones encoded Psyr4249 and PA3891. Previous studies that have presumed functionality for the transporter encoded by PSPTO_3058–PSPTO_3060 (45) illustrate the need for such predictive power.

In addition to tandem CBS domains, the ATPases of the osmoregulatory ABC transporters also have a C-terminal tail. We observed that this C-terminal tail was not required for osmoregulatory transporter function (Fig. 9). In *L. lactis* OpuAC, this 18-amino-acid tail is anionic and functions in modulating OpuA activity in response to ionic strength and ionic lipids (35). Deletion of this anionic tail resulted in mutants that were capable of uptake but required higher cytoplasmic ion concentrations to activate uptake (35). Mahmood et al. (35) present a model in which *L. lactis* OpuC activation requires disruption of the electrostatic interactions between a CBS domain and the membrane, presumably mediated by a cationic CBS domain surface and anionic lipids in the membrane, with the charged tail influencing the ionic strength required to disrupt these interactions. In contrast to the anionic C-terminal tail of *L. lactis* OpuA, the *Pseudomonas* OpuC-type transporters all have a cationic C-terminal tail and exhibit distinct charges by their CBS regions (Table 3). Given that the C-terminal tails differ in length and charge among CBS domain-containing transporters of diverse bacteria (35), the con-

served, cationic nature of the *Pseudomonas* species tails suggests the evolution of a *Pseudomonas*-specific approach for modulating the osmosensing activities of these transporters.

Our results do not exclude the possibility that the PSPTO_0462–PSPTO_0464, PSPTO_5271–PSPTO_5273, and PSPTO_3058–PSPTO_3060 loci encode transporters that function in uptake for catabolism. In fact, we now have evidence that PSPTO_0462–PSPTO_0464 contributes to the catabolic uptake of glycine betaine and choline (C. Chen and G. A. Beattie, unpublished data). This is consistent with the relative similarity of the PSPTO_0462 protein to the ChoV transporter of *S. meliloti* (Fig. 10), which functions in choline uptake for catabolism (19). The similarity of the PSPTO_5273 protein to *S. meliloti* HisV/HutV, which is involved in the uptake of histidine and glycine betaine under low osmolarity (8), suggests a possible role for PSPT5273 in catabolism, although this has not yet been examined. The similarity of the PSPTO_3060 protein to ProV of *E. coli* and *S. enterica* serovar Typhimurium, however, does not suggest a role in catabolism. Surprisingly, the PSPTO_3058–PSPTO_3060 locus is absent in the other *Pseudomonas* species strains for which complete genome sequence information is available, including two other *P. syringae* strains, suggesting that this locus is novel to DC3000 and possibly has been recently acquired.

In this study, we demonstrated that *P. syringae* pv. tomato DC3000 derived better osmoprotection from choline than from similar concentrations of glycine betaine when the compounds were provided at concentrations of 100 μM or higher; we have also observed this with two other *P. syringae* strains (data not shown). This contrasts with the relative levels of protection conferred by these compounds to *P. aeruginosa* and *P. putida* (18) and to most other bacterial genera examined. Superior osmoprotection by choline suggests that choline uptake is more efficient than glycine betaine uptake in DC3000 when these compounds are present at relatively high concentrations, particularly given that choline must be converted to glycine betaine to confer osmoprotection. Such efficiency may be reflected, in part, by the presence of a single primary transporter, OpuC, that transports glycine betaine under hyperosmotic conditions but at least two transporters that transport choline. The affinity of DC3000 for choline (K_m of 118 μM) and glycine betaine (K_m of 12 μM) also suggests that DC3000 is adapted to environments with low betaine and high choline concentrations. Interestingly, although the concentrations of glycine betaine and choline are not known in tomato and *Arabidopsis thaliana*, which are host plants for DC3000, previous studies suggest that plant-associated bacteria are likely to have greater access to choline than glycine betaine. In specific, glycine betaine accumulates in some plant species when they are drought or salinity stressed but is present in relatively few plant species in the absence of stress (44). In contrast, possibly due to its association with the synthesis and degradation of the major lipid component of plant membranes, phosphatidylcholine, choline is generally present at detectable levels in plant tissue (57). Choline may therefore be more widely available for uptake by plant-associated bacteria during periods of water limitation. Knowledge of the full complement of DC3000 osmoprotectant transporters, as has been initiated in this work, will allow us to test this hypothesis in future studies.

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