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Pseudomonas syringae BetT Is a Low-Affinity Choline Transporter That Is Responsible for Superior Osmoprotection by Choline over Glycine Betaine[▽]

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The plant pathogen *Pseudomonas syringae* derives better osmoprotection from choline than from glycine betaine, unlike most bacteria that have been characterized. In this report, we identified a betaine/carnitine/choline family transporter (BCCT) in *P. syringae* pv. tomato strain DC3000 that mediates the transport of choline and acetylcholine. This transporter has a particularly low affinity (K_m of 876 μM) and high capacity (V_{max} of 80 nmol/min/mg of protein) for choline transport relative to other known BCCTs. Although BetT activity increased in response to hyperosmolarity, BetT mediated significant uptake under low-osmolarity conditions, suggesting a role in transport for both osmoprotection and catabolism. Growth studies with mutants deficient in BetT and other choline transporters demonstrated that BetT was responsible for the superior osmoprotection conferred to *P. syringae* by choline over glycine betaine when these compounds were provided at high concentrations (>100 μM). These results suggest that *P. syringae* has evolved to survive in relatively choline-rich habitats, a prediction that is supported by the common association of *P. syringae* with plants and the widespread production of choline, but genus- and species-specific production of glycine betaine, by plants. Among the three putative BCCT family transporters in *Pseudomonas aeruginosa* and six in *Pseudomonas putida*, different transporters were predicted to function based on similarity to *Escherichia coli* BetT than to *P. syringae* BetT. Functional *P. putida* and *P. aeruginosa* transporters were identified, and their possession of a long C-terminal tail suggested an osmoregulatory function for this tail; this function was confirmed for *P. syringae* BetT using deletion derivatives.

The most widely distributed strategy of bacterial osmoadaptation is the accumulation of compatible solutes, which are compounds that are compatible with normal cellular function even at high cellular concentrations (43). This accumulation can be through de novo synthesis or through uptake of osmoprotectant compounds from the environment. These osmoprotectant compounds can function directly as compatible solutes, as with glycine betaine, or can serve as precursors, as occurs with choline, which is converted to glycine betaine after uptake. Accumulation via uptake is generally favored, because it is far less energetically expensive than de novo synthesis.

At present, three distinct types of transport systems are known to mediate the uptake of osmoprotectants in bacteria. The first and largest group contains the binding protein-dependent ATP-binding cassette (ABC) transporters, which include the recently identified OpuC transporter in *Pseudomonas syringae* (10) and ProU in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (7), as well as *Erwinia chrysanthemi* OusB (11), *Bacillus subtilis* OpuA (34) and OpuB and OpuC (33), *Lactococcus lactis* BusA/OpuA (45), and *Listeria monocytogenes* Gbu and OpuC (2). The second group contains the secondary transporters in the betaine-choline-carnitine transporter (BCCT) family and major facilitator superfamily (MFS). The BCCT family is large and includes BetT (37) and CaiT (17) in *E. coli*, OpuD in *B. subtilis* (32), BetM and EctM in *Marinococcus halophilus* (63), ButA in *Tetragenococcus halophila* (3), BetS in *Sinorhizobium meliloti* (6), BetP, EctP, and LcoP in *Corynebacterium glutamicum* (48, 59), BetL in *L. monocytogenes* (57), and CudT in *Staphylococcus xylosus* (51). In contrast, the major facilitator superfamily is represented by only ProP in *S. enterica* serovar Typhimurium and *E. coli* (8) and OusA in *E. chrysanthemi* (23), each of which is a single-component transporter. The third group contains only the Tea uptake system of the halophilic species *Halomonas elongate*; this is a tripartite ATP-independent periplasmic transporter (24). In general, the ABC and MFS transporters exhibit broad substrate specificities, whereas BCCTs exhibit a narrow specificity (11).

Here, we report on the characterization of a BCCT choline transporter in *P. syringae.* Transporters of the BCCT family transport molecules with a quaternary ammonium group [$R\text{-}N^{+}(CH_3)_3$]. They are typically energized by proton motive force or sodium motive force, with the exception of the catabolic carnitine transporter CaiT, which is a carnitine:γ-butyrobetaine antiporter (64). All BCCTs characterized to date possess 12 putative transmembrane regions, with their hydrophilic N and C termini located in the cytoplasm. The lengths of the N and C termini vary among the BCCTs, and the C termini appear to function in osmoregulation. This was suggested by the finding that C-terminal deletions in *E. coli* BetT and *C. glutamicum* BetP eliminated activation by hyperosmolarity, although BetT was locked in a low-activity state (61) and BetP was locked in a high-activity state (47).

Choline and phosphorylcholine are ubiquitous in plants as

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TABLE 1. Plasmids and strains used in this study

Strain or plasmid	Description or relevant genotype	Reference or source
Escherichia coli strains		
DH5α	Host for cloning	Invitrogen
MG1655	Host for mutagenesis	
MKH13	Δ*putPA-101* Δ*proP2* Δ*proU*::*spc-608* Δ*betTIBA*; Sp^r	
P. syringae pv. tomato strains		
DC3000	Wild type; Rf^r	44
DC-*betT*::pKO	DC3000 PSPTO_5269::pKnockout-Ω; Rf^r Sp^r	This work
DCΔ*betT*	DC3000 Δ*betT*; Rf^r	This work
DCΔ*opuC*	DC3000 Δ*opuCA*; Rf^r	10
GB1	DC3000 Δ*betT* Δ*opuCA*; Rf^r	This work
GB2	DC3000 Δ*betT* ΔPSPTO_0462-PSPTO_0464; Rf^r	This work
GB3	DC3000 Δ*opuCA* ΔPSPTO_0462-PSPTO_0464; Rf^r	This work
GB4	DC3000 Δ*betT* Δ*opuCA* ΔPSPTO_0462-PSPTO_0464; Rf^r	This work
Plasmids		
pKnockout-Ω	Suicide vector for rapid gene inactivation in *P. syringae*; Rf^r Sm^r Sp^r	66
pKD13	Template for *kan* cassette flanked by *FLP* recombination target sites; Ap^r Km^r	13
pKD46	Encodes λ Red recombinase; *repA101ts*; Ap^r	13
pFlp2	Encodes Flp recombinase, suicide vector in *P. syringae*; Ap^r	27
pRK2013	RP4 transfer functions for mobilization; Km^r	16
pRK2073	pRK2013 with Tn*7* in the *kan* gene; Sm^r Sp^r	5
pKO5269	pKnockout-Ω containing 1,634-bp PCR-amplified fragment with partial PSPTO_5269	This work
pME6041	*E. coli-Pseudomonas* shuttle and cloning vector; $oriV_{pVSI}$ $oriV_{p15A}$ oriT; Km^r	26
pME*opuC*	pME6041 containing 9.6-kb genomic fragment with PSPTO_4575-PSPTO_4578; Km^r	10
pME*betT*	pME6041 containing 7.7-kb EcoRV genomic fragment with PSPTO_5269 and its flanking regions (bases 5,986,969 to 5,994,741 in the DC3000 genome); Km^r	This work

intermediates and degradation products of the membrane lipid phosphatidylcholine, whereas glycine betaine is produced in detectable amounts by some plant species but not others (60). As a pathogen and epiphyte of plants, *P. syringae* has likely evolved mechanisms to exploit plant-derived compounds as part of its adaptive response to low water availability and other stresses. We previously reported that *P. syringae* is distinct from most other characterized bacteria in its ability to derive better osmoprotection from choline than from glycine betaine, and our results indicated that this resulted from more rapid uptake of choline, particularly in the presence of high concentrations of these compounds (10). We found that *P. syringae* mutants that were lacking the ABC family OpuC transporter lost their ability to use glycine betaine for osmoprotection but were only slightly reduced in their use of choline (10), suggesting that one or more additional transporters may contribute to the rapid choline uptake. Here, we report on a BCCT, designated BetT, that is responsible for this superior osmoprotection by choline. This is the first functional characterization of a BCCT in a pseudomonad and is the first BCCT shown to have a low affinity for choline, a property that suggests adaptation to choline-rich environments. Furthermore, a comparative study with other pseudomonads shows the usefulness of *P. syringae* BetT for identifying functional BCCTs in those organisms.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used are listed in Table 1. *P. syringae* strains were grown at 28°C, whereas *Escherichia coli* strains harboring pKD46 were grown at 30°C, and all other *E. coli* were grown at 37°C. *P. syringae* strains were grown at 28°C in King's B medium (35), MinA medium (42), or the low-osmoticum medium [1/2]-21C (10, 25) containing 2 mM of succinate, unless otherwise indicated. *E. coli* was grown in LB (42) or M63 medium (56) supplemented with vitamin B_1 (0.0005%). Antibiotics were used at the following concentrations (in μg ml^{-1}): ampicillin, 100; kanamycin, 50 for *P. syringae* or 20 for *E. coli*; rifampin, 100; spectinomycin, 60.

Mutagenesis of *P. syringae* pv. tomato DC3000. The suicide vector pKnockout-Ω was used to generate an insertional mutation. A PCR product, which had been amplified using the primers DC5269-1 (5′-TCATCTGCACCTGGTAGATGAACG-3′) and DC5269-2 (5′-GGTCGGCTGGTATTACATGATGGT-3′), was cloned into XcmI-digested pKnockout-Ω to generate the plasmid pKO5269, which was then introduced into DC3000 through a triparental mating with pRK2073 (16). Insertional mutations were confirmed using PCR. To create a mutant with an internal deletion in PSPTO_5269, PCR amplification of pKD13 was performed using the primer set 5269H3 (5′-GTTGCCATCGCCGGTGTCGACAAGGGCGTGCGCGTGGTGTAGGCTGGAGCTGCTTC) and 5269H4 (CGTCGACAGCACCACAGTGCCCGAATCGGCCGACGTATTCCGGGGATCCGTCGACC-3′) to form a product that contained a *kan* cassette surrounded by *FRT* sites and by 36-bp regions that share sequence similarity with PSPTO_5269. This chimeric fragment was introduced into MG1655(pKD46, pKO5269) by electroporation to replace a 492-bp region of PSPTO_5269 in pKO5269 using lambda Red recombinase-mediated recombination (13). Upon PCR confirmation of successful replacement, the plasmid containing the marked deletion was mobilized into DC3000 via triparental mating with pRK2073 with Rf Km selection. Deletion mutants were identified as Rf^r Km^r Sp^s colonies and were confirmed by PCR. The *kan* cassette was excised by introducing pFlp2 (27), which was later cured using sucrose (5%) counterselection, and excision was confirmed by PCR. The resulting mutant, DCΔ*betT*, was then used to generate a Δ*betT* Δ*opuCA* double deletion mutant by introducing a plasmid containing a *kan*-marked deletion of *opuCA* (10) and screening for the integration of the marked deletion with subsequent pFlp2-mediated excision of the *kan* cassette.

Osmoprotection assays. Late-log-phase cells were inoculated to a density of 10^7 cells/ml and grown at 28°C with shaking. Growth was monitored either in test tubes based on the optical density at 600 nm (OD_{600}) or in microtiter plates based on optical density measurements at both 630 nm and 450 nm, with subsequent conversion of the OD_{630}/OD_{450} values to OD_{600} values, as described previously (10).

Transport assays. [*methyl*-^{14}C]choline (specific activity of 55 mCi/mmol) was 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

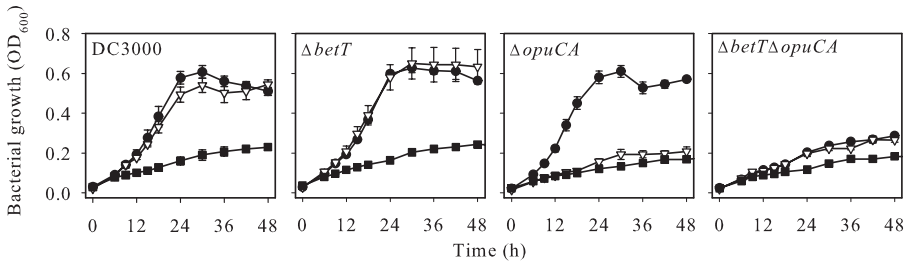

FIG. 1. Growth of DC3000 and DC3000 mutants carrying deletions in the *betT*, *opuCA*, or both *betT* and *opuCA* genes in [1/2]-21C medium that contained 0.3 M of NaCl and was unamended (■), amended with choline (1 mM) (●), or amended with glycine betaine (1 mM) (▽). Values are the means ± SEM ($n = 3$) and are representative of three experiments.

described by Ko et al. (36). *P. syringae* cells were grown in MinA medium to an OD_{600} of 0.3 to 0.5, and *E. coli* cells were grown in M63 medium to an OD_{600} of 0.6 to 0.7. Cells were washed and suspended in fresh growth medium, unless otherwise indicated, to an OD_{600} of 0.1 to 0.2 for evaluation of glycine betaine uptake and to an OD_{600} of 1 for evaluation of choline uptake. Following introduction of NaCl, cells were incubated at 28°C for 2 to 6 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 for 2 min or 5 min for evaluation of glycine betaine or choline uptake, respectively, and terminating uptake by centrifugation at 13,000 × *g*. The supernatant was immediately removed from each pellet. Unless indicated otherwise, the cells were washed with 1 ml of medium that was the same osmolarity as the incubation medium, and the cells were suspended in 1 ml of ScintiVerse BD (Fisher Scientific, Fair Lawn, NJ). The amount of 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, radiolabeled choline was used at final concentrations of 1 to 4,000 or 8,000 μM (specific activity ranged from 0.6 to 1,199 μCi/mmol). For competition experiments, unlabeled compounds were used at final concentrations of 1 mM and 10 mM, whereas [*methyl*-^{14}C]choline was used at a final concentration of 100 μM (with a specific activity of 550 μCi/mmol). The protein content of cell suspensions was determined using the 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_5269 deletion derivatives. To construct C-terminal deletions in the PSPTO_5269 protein, the PSPTO_5269 gene was amplified by PCR using one upstream primer, 5′-CCCGCTCAAGCAAGCGTCAT-3′, with each of four downstream primers with sequence similarity to distinct endpoints within the PSPTO_5269 gene and containing the original or a supplemental stop codon (in bold): 5269R1 (5′-**TCA**ACGCACCAGATGCAGAAAC-3′), 5269R2 (5′-**TCA**CAGAAACTGCATATGCCGTTCGTAC-3′), 5269R3 (5′-**TCA**GTCGTTGATGACTTGCTCCTTGGT-3′), and 5269R4 (5′-**TCA**GCGGCGGTTGTTCAACTGTTTG-3′). The resulting fragments were cloned into the EcoRV site of pME6041 and were introduced into the DC3000 mutant GB4 (Table 1). The abilities of the cloned genes to complement the PSPTO_5269 deletion in GB4 were evaluated based on growth on MinA medium containing 0.5 M of NaCl and 2 mM of choline.

Identification of osmoregulatory BCCT genes from other pseudomonads. A genomic library using PvuII-restricted genomic DNA of *Pseudomonas putida* KT2440 was constructed in the same manner as a *Pseudomonas aeruginosa* PAO1 library described previously (10). A DNA fragment encoding the putative BCCT from *P. syringae* pv. syringae B728a, Psyr_4827, was amplified by PCR using the primers 4827F1 (5′-GTACCAGGTTGGAGTTGGCGATC-3′) and 4827R2 (5′-ATAATCGTCAGGGAGATCGCGGAA-3′) and was cloned into the EcoRV site of pME6041.

RESULTS

The BetT transporter contributes to choline-based osmoprotection and to choline uptake in DC3000. In a previous study, we found that inactivation of the ABC family transporter OpuC in *P. syringae* pv. tomato strain DC3000 reduced but did not eliminate choline uptake activity under hyperosmotic conditions (10), suggesting the existence of one or more additional osmoregulatory transporters for choline in DC3000. Using the Transporter Protein Analysis Database in 2004 (49), we identified one additional putative choline transporter in DC3000, and this was the BCCT encoded by the PSPTO _5269 locus. The predicted PSPTO _5269 protein shares 44% sequence similarity with the *E. coli* BetT transporter (37) and is hereafter referred to as BetT. Whereas the *betT* gene in *E. coli* is adjacent to the *betIBA* locus, which encodes a regulator and enzymes required for the conversion of choline to the compatible solute glycine betaine, the *betT* gene in DC3000 is located far from the putative *betIBA* genes (PSPTO_0440-PSPTO_0443).

A pKnockout-Ω insertional mutant of *betT*, designated DC-*betT*::*pKO*, derived a similar growth benefit from choline and glycine betaine in a hyperosmotic medium (data not shown), in contrast to the better growth of DC3000 with choline than with glycine betaine (10). A deletion mutant lacking a 492-bp internal region of the *betT* gene was similarly attenuated in choline-derived osmoprotection (Fig. 1). A Δ*betT* Δ*opuCA* double mutant did not derive significant osmoprotection from glycine betaine, consistent with the behavior of an Δ*opuCA* mutant and the known role of OpuC as the primary osmoregulatory glycine betaine transporter in DC3000 (10). In contrast to the Δ*opuCA* or Δ*betT* mutants, however, the Δ*betT* Δ*opuCA* mutant did not derive significant osmoprotection from choline (Fig. 1), indicating that BetT as well as OpuC contributes to osmoregulatory choline uptake. The Δ*betT* Δ*opuCA* mutant exhibited reduced osmoprotection by carnitine, acetylcholine, and phosphorylcholine (1 mM), whereas the *betT* mutant did not (data not shown), which is consistent with OpuC transport of these compounds (10). The fact that the Δ*betT* Δ*opuCA* mutant grew slightly better under hyperosmotic conditions in the presence of choline or glycine betaine than in their absence (Fig. 1) suggests the existence of at least one more transporter for these compounds.

To verify that the mutations in *betT* impacted choline uptake, we measured [^{14}C]choline uptake by these mutants in the presence and absence of hyperosmotic stress in MinA medium, which supports better growth of DC3000 than [1/2]-21C medium under hyperosmotic conditions. All of the strains had a low initial rate of choline uptake in the absence of hyperosmotic stress, whereas only the Δ*betT* and Δ*betT* Δ*opuCA* mutants exhibited a lower initial rate of uptake than DC3000

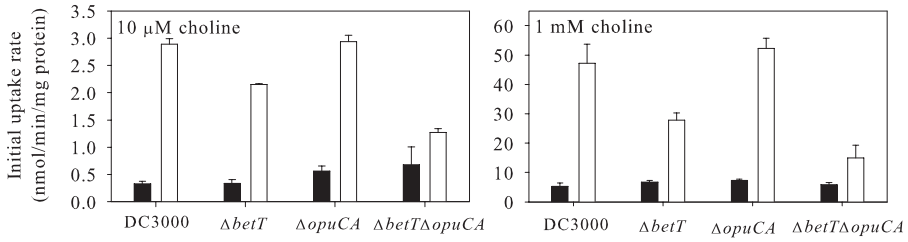

FIG. 2. Uptake of radiolabeled choline by DC3000 and various mutants in MinA medium in the absence (solid bars) or presence (open bars) of 0.5 M of NaCl. Values are the means ± SEM (n = 3).

under hyperosmotic conditions, with the Δ*betT* and Δ*betT* Δ*opuCA* mutants exhibiting reductions of 25 to 50% and 71 to 90%, respectively (Fig. 2). Under these conditions, OpuC clearly contributed less than BetT to the initial rate of choline uptake. The residual uptake activity by the Δ*betT* Δ*opuCA* mutant also suggested the existence of at least one additional transporter for these compounds. Similar results were obtained when [1/2]-21C medium was used for the uptake studies (data not shown). The larger reduction in choline uptake activity observed in the presence of 1 mM than 10 μM choline (Fig. 2) suggests that BetT is a low-affinity choline transporter.

BetT is a low-affinity, high-capacity osmoregulatory transporter with narrow substrate specificity. The kinetic properties of *P. syringae* pv. tomato strain DC3000 BetT were assayed using the heterologously expressed *betT* locus in *E. coli* MKH13, as has been done for other transporters (6, 11). This strain lacks the PutP, ProP, and ProU transport systems and thus is unable to transport glycine betaine and choline. For these experiments, the DC3000 *betT* gene and its flanking regions were identified in a DC3000 genomic library that had been constructed through the ligation of EcoRV-digested genomic DNA with the broad-host-range vector pME6041, which had been treated with EcoRV and dephosphorylated with Antarctic phosphatase (New England Biolabs, Ipswich, MA). The plasmid, pME*betT*, enabled choline uptake (Fig. 3A) but not glycine betaine uptake (Fig. 3B) by MKH13, in contrast to pME*opuC*, which enabled choline and glycine betaine uptake as expected (Fig. 3). Choline did not provide osmoprotection to MKH13(pME*betT*), consistent with the absence of the *betBA* genes necessary to convert choline into its osmoprotective form, glycine betaine, in MKH13.

The initial choline uptake rate increased linearly as the choline concentration increased from 0 to 50 μM (data not shown), but a range of choline concentrations as high as 8 mM was required to estimate the kinetic properties of BetT (Fig. 4). In the presence of NaCl (0.5 M), BetT had a K_m of 876 ± 67 μM and a V_{max} of 80 ± 2.2 nmol/min/mg of protein (means ± standard errors of the means [SEM]). These values are dramatically higher than those of other BCCTs (6, 32, 48).

The substrate specificity of BetT was analyzed by measuring the extent to which putative substrates were able to compete for uptake with radiolabeled choline in the presence of NaCl. [^{14}C]choline uptake was inhibited 84% and 40% by the addition of 100- and 10-fold higher concentrations, respectively, of unlabeled acetylcholine than radiolabeled choline (Table 2). Betaine aldehyde, an intermediate in the oxidation of choline to glycine betaine, was also a good competitor (data not shown). Phosphorylcholine, a choline analog that has been shown to be abundant in the xylem sap of the *P. syringae* pv. tomato host plant tomato (40), was a weak competitor when provided at a 10-fold excess (Table 2). Higher concentrations of phosphorylcholine resulted in excessive precipitation in the assay buffer and thus were not tested. In contrast, glycine betaine and proline (Table 2), as well as carnitine, ectoine, pipecolate, and proline betaine (data not shown), were not competitors, even when present at a 100-fold excess over radiolabeled choline.

Interestingly, the uptake of 10 μM of [^{14}C]choline, rather than of 100 μM as shown in Table 2, was inhibited only 55% by a 100-fold excess of choline and was not inhibited by acetyl-

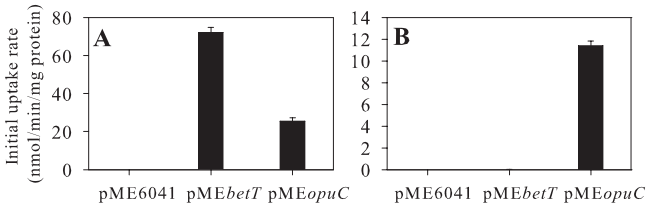

FIG. 3. Functional complementation of MKH13 by the plasmids pME6041, pME*betT*, and pME*opuC* as indicated by the restoration of uptake of choline (A) and glycine betaine (B) in M63 medium in the presence of NaCl (0.5 M) and glycine betaine (5 μM) or choline (0.5 mM). Values are means ± SEM (n = 6).

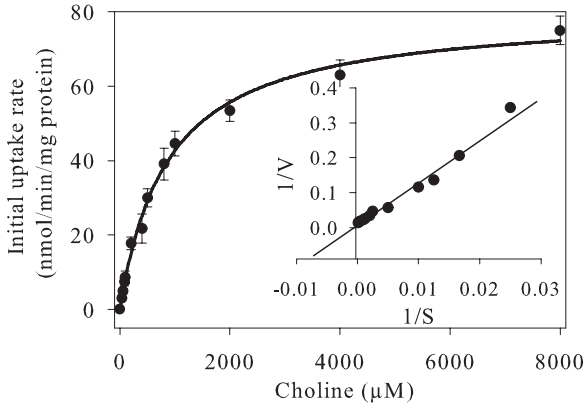

FIG. 4. Kinetics of BetT-mediated choline uptake in *E. coli* MKH13(pME*betT*) in the presence of 0.5 M of NaCl. Cells grown in M63 medium amended with 0.5 M of NaCl were suspended in 50 mM phosphate buffer containing 0.5 M of NaCl, 0.2% glucose and [*methyl*-^{14}C]choline at various concentrations. The results are means ± SEM (n = 6).

TABLE 2. Abilities of various compounds to inhibit the uptake of choline by BetT expressed in *E. coli* MKH13(pME*betT*)[a]

Chemical	Concn (mM)	% Inhibition of [^{14}C]choline uptake
Choline	10	95
Choline	1	75
Acetylcholine	10	84
Acetylcholine	1	40
Phosphorylcholine	1	10
Glycine betaine	10	0
Proline	10	0

[a] Cells were grown in M63 medium amended with 0.5 M NaCl, and uptake was realized with 100 μM [^{14}C]choline. The results are expressed as percent inhibition of uptake and are means of three independent experiments with variations of less than 10%. The uptake rate in the absence of competitors was 8.0 nmol/min/mg of protein.

choline or phosphorylcholine. This inability of substrates to inhibit the BetT-mediated uptake of low concentrations of choline contrasts sharply with the strong inhibition of OpuC-mediated uptake of low concentrations of choline by substrates (10). This lack of inhibition may represent a unique property of this BCCT.

Contrary to choline uptake by the *E. coli* BetT transporter and glycine betaine uptake by the *S. meliloti* BetS transporter, choline uptake by the *P. syringae* pv. tomato BetT transporter did not require sodium (Fig. 5A). The BetT transporter expressed by MKH13(pME*betT*) transported choline in a Na^+-free buffer amended with KCl to confer hyperosmotic stress, although this transport was at a slightly reduced rate. The DC3000 BetT transporter is therefore not a Na^+ symporter.

BetT-mediated choline uptake increased dramatically not only in DC3000 in the presence of NaCl (Fig. 2) but also in MKH13(pME*betT*) in the presence of NaCl and the protein synthesis inhibitor chloramphenicol (Fig. 5B). This increased activity thus resulted, at least in part, from activation of the BetT transporter by hyperosmotic stress. Although activation was observed at NaCl concentrations up to at least 0.8 M, NaCl concentrations of 0.4 to 0.6 M caused maximal activity (Fig. 5B).

As a species, *P. syringae* is well adapted for growth in the

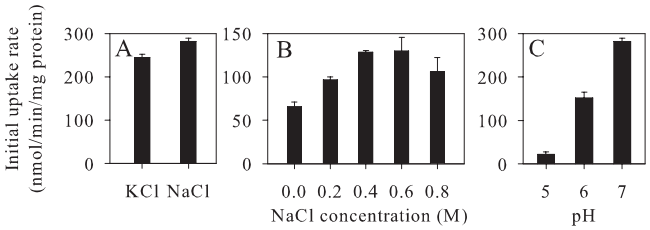

FIG. 5. Rate of BetT-mediated uptake of choline by *E. coli* MKH13(pME*betT*) cells that were suspended in 50 mM phosphate buffer (A and C) or [1/2]-21C (B) containing 0.4% glucose and 50 μM of chloramphenicol to inhibit protein synthesis. The cell suspensions were also amended with either NaCl or KCl (0.8 M) (A), various concentrations of NaCl (B), or NaCl (0.8 M) at the indicated pH (C), and uptake of radiolabeled choline (1 mM) was evaluated. During the uptake assays, the radiolabel was measured in unwashed rather than washed cell pellets, and this contributed to relatively high background radiolabel counts. Values are means ± SEM (n = 4), and results are representative of two independent experiments.

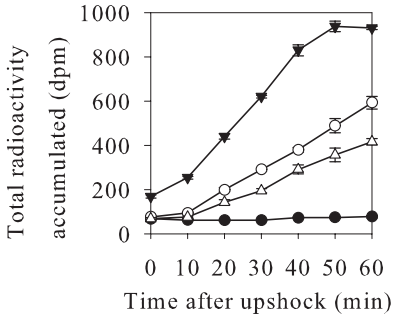

FIG. 6. Accumulation of radiolabel by DC3000 mutants GB3 (circles) and GB2 (triangles and inverted triangles) during transport of [^{14}C]choline (open symbols) and [^{14}C]glycine betaine (filled symbols). Cells of GB3 (*betT*$^+$ Δ*opuCA* ΔPSPTO_0462-PSPTO_0464) and GB2 (*opuC*$^+$ Δ*betT* ΔPSPTO_0462-PSPTO_0464) grown in [1/2]-21C medium were subjected to an osmotic upshock (0.4 M of NaCl) in the presence of radiolabeled choline or glycine betaine (1 mM). The results are means ± SEM (n = 3).

intercellular spaces of plant leaves. The pH of the apoplastic fluid in these spaces is estimated to be 5.5 to 7 (30). To evaluate if BetT transporter activity is optimally adapted to these pH levels, we measured the initial rate of choline uptake by BetT in MKH13(pME*betT*) under various pH levels in the presence of chloramphenicol (Fig. 5C). Surprisingly, choline uptake was strongly reduced by lowering the pH below 7 and thus was not optimal at the lower pH values estimated for the intercellular spaces of leaves (Fig. 5C). Further acidification to pH 4 completely eliminated detectable uptake activity (data not shown).

Choline uptake by BetT is responsible for the superior osmoprotection of choline over glycine betaine for DC3000. To evaluate the relative contributions of BetT and OpuC to the uptake of choline and glycine betaine, we constructed the DC3000 mutants GB3 and GB2, which lack OpuC and BetT, respectively, as well as the transporter encoded by PSPTO_0462-PSPTO_0464, which is involved in the uptake of choline and glycine betaine for catabolism (C. Chen and G. A. Beattie, unpublished data). We examined the total accumulation of radiolabeled choline and glycine betaine following osmotic upshock. Choline was immediately taken up by the BetT transporter, whereas glycine betaine or choline uptake by the OpuC transporter was not significant until at least 20 min after the upshock (Fig. 6). The level of radiolabel accumulation by the BetT transporter remained higher than that by the OpuC transporter for at least 60 min.

We also compared the growth of these transporter mutants in the presence of 0.4 M of NaCl and various concentrations of choline or glycine betaine. Although cells with the BetT and OpuC transporters did not show detectable differences in growth with choline in the first 4 h after inoculation (Fig. 7A), by 8 h cells with the BetT transporter had grown significantly more than those with OpuC transporter in the presence of high concentrations (>100 μM) of choline or glycine betaine (Fig. 7B). Cells with the OpuC transporter grew better in the presence of glycine betaine than choline; however, they grew slower, as evidenced by a lower OD_{600} at 8 and 12 h, than those with the BetT transporter in the presence of high concentrations of choline (Fig. 7B and C). The superior osmoprotection

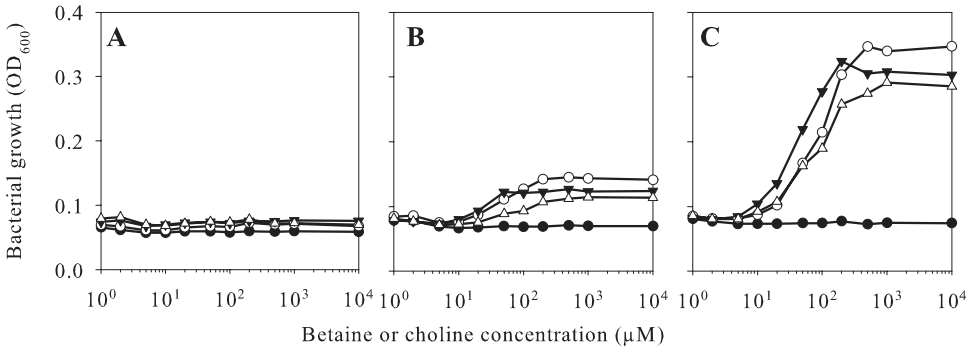

FIG. 7. Osmoprotection mediated by the BetT (circles) and OpuC (triangles and inverted triangles) transporters at various concentrations of choline (open symbols) or glycine betaine (filled symbols), with bacterial growth reflected in the OD_{600} of the cultures measured at 4 h (A), 8 h (B), and 12 h (C). The DC3000 transporter mutants GB3 and GB2, described in the legend to Fig. 6, were used. Values are means ± SEM (n = 4), but the error bars are within the symbols.

resulting from BetT-mediated choline transport over OpuC-mediated glycine betaine transport lessened over time after 12 h due to all of the cultures reaching their maximal densities.

Taken together, these uptake and growth data demonstrate that the rapid choline uptake by the BetT transporter is responsible for the superior osmoprotection conferred by high concentrations of choline to *P. syringae*.

Homology of transporters in other pseudomonads to *P. syringae* BetT more accurately predicted function than homology to *E. coli* BetT. The three *P. syringae* strains for which the complete genome sequence is available, DC3000, B728a, and 1448A, each encode only a single BCCT family transporter. Other *Pseudomonas* species, however, have multiple genes exhibiting homology to BCCT family transporters, including the three putative BCCTs in *P. aeruginosa* PAO1, PA3933, PA5291, and PA5375, and the six in *P. putida* KT2440, PP_0229, PP_2692, PP_3957, PP_3628, PP_5061, and PP_5374. Only one gene within each of these sets exhibits a genetic arrangement like that of *E. coli betT*, i.e., is located near the *betBA* genes, which are required for converting choline to glycine betaine (54). Thus, based on this similarity to *E. coli betT*, the genes PP_5061 and PA5375 (annotated as *betT1*) were predicted to encode functional choline transporters (55, 62, 65). However, the BetT transporter from *P. syringae* pv. tomato DC3000 was more similar to PP_0229 and PA3933 (70 to 76% sequence identity with *P. syringae* BetT) than to PP_5061 or PA5375 (43 to 45% sequence identity with *P. syringae* BetT). To evaluate if sequence similarity to *P. syringae* BetT provided predictive power for functional choline transporters in other *Pseudomonas* species, we performed complementation studies in which genomic libraries of *P. aeruginosa* PAO1 and *P. putida* KT2440 were introduced into the DC3000 mutant GB4 (Table 1), which lacked BetT, OpuC, and the catabolic transporter PSPTO_0462-PSPTO_0464. Clones containing PP_0229 and PA3933 conferred growth on a choline-amended hyperosmotic medium (Table 3), demonstrating that these transporters functioned in choline uptake under these conditions. Additionally, clones containing PA5291 conferred growth on a glycine betaine-amended hyperosmotic medium. In contrast, clones containing locus PA5375 did not confer growth on choline- or glycine betaine-amended hyperosmotic medium, suggesting that PA5375 is not involved in osmoregulatory choline, or even glycine betaine, uptake. These results illustrate that, at least in this case, functional information on a transporter in a *Pseudomonas* species was a much more effective tool than functional information in *E. coli* for accurately predicting protein function in other pseudomonads and, in fact, was a required tool.

Working with BetT of *E. coli*, Tondervik and Strom (61) demonstrated the importance of the C terminus to osmoregulatory function. While *E. coli* BetT mutants carrying C-terminal deletions of one or two amino acid residues were not affected in osmotic activation, deletion of 6 or 12 amino acid residues significantly reduced activation by hyperosmolarity, with further deletions up to 101 residues reducing activity to a very low, noninducible state. Interestingly, in contrast to BCCTs in gram-positive bacteria, all characterized osmoregulatory BCCTs from gram-negative bacteria possess long C termini, ranging from 161 to 180 amino acid residues (Table 3), whereas two BCCTs, *E. coli* CaiT and *P. aeruginosa* BetT1, which possess C termini of only 10 and 29 amino acid residues, respectively, are not osmotically activated (Table 3).

To evaluate if the C terminus of the *P. syringae* DC3000 BetT transporter is required for its osmoregulatory function, we generated constructs lacking 0, 4, 16, or 44 of the C-terminal amino acid residues. Constructs encoding the full-length BetT or BetT lacking the terminal four amino acid residues restored the ability of the transporter-deficient GB4 mutant to grow on choline under high osmolarity, whereas constructs lacking the terminal 16 residues restored some growth and those lacking the terminal 44 residues restored none (Table 3).

DISCUSSION

Among the BCCTs characterized to date, *P. syringae* BetT is unusual in having a very low affinity for its substrate. For example, the K_m value for the only other characterized BCCT choline transporter is 8 μM (37), compared to 876 μM for *P. syringae* BetT. Similarly, *P. syringae* BetT exhibits a particularly high capacity for substrate transport with a V_{max} of 80 nmol/min/mg of protein for choline, compared to other BCCTs, such as *E. coli* BetT, which has a V_{max} of only 5 nmol/min/mg of protein for glycine betaine. These kinetics, combined with the specificity of BetT for choline and its analog acetylcholine,

TABLE 3. Comparison of some physical and functional properties of BCCT transporters and various deletion constructs characterized in gram-negative bacteria

Species	Transporter	Substrate[a]	Total length[b]	C-tail length[b]	Function[c]	Reference
E. coli	BetT	CHO	677	180	+	61
E. coli	BetU	GB, PB	667	167	+	39
H. influenzae	BetT	CHO	669	175	+	18
S. meliloti	BetS	GB, PB	706	171	+	6
P. syringae	PSPTO_5269	CHO	664	161	+	This study
P. syringae	BetT-0[d]	CHO	664	161	+	This study
P. syringae	BetT-4[d]	CHO	660	157	+	This study
P. syringae	BetT-16[d]	CHO	648	145	+/−	This study
P. syringae	BetT-44[d]	CHO	620	117	−	This study
P. syringae	Psyr_4827[e]	CHO	664	161	+	This study
P. aeruginosa	PA3933	CHO	653	165	+	This study
P. aeruginosa	PA5291	GB	661	169	+	This study
P. putida	PP_0229	CHO	653	167	+	This study
E. coli	CaiT	CARN	504	10	−	64
P. aeruginosa	PA5375[f]	ND	516	29	−	This study
V. cholerae	OpuD[g]	GB	540	16	ND	32

[a] GB, glycine betaine; ECT, ectoine; CHO, choline; PB, proline betaine; CARN, carnitine; ND, not determined.
[b] The length of the C-terminal tail of each transporter was predicted using the TMHMM method (http://www.cbs.dtu.dk/services/TMHMM/), which takes into account the expected hydrophobicity of transmembrane regions and the abundance of positively charged residues on the cytoplasmic side of the membrane. The number of amino acid residues in each transporter (total length) and C-terminal tail (C-tail length) is indicated.
[c] The ability (+), intermediate ability (+/−), or inability (–) to mediate the uptake of osmoprotectant compounds in response to hyperosmolarity is indicated. Transporters evaluated in this study exhibited growth (+), some growth (+/−), or no growth (–) on MinA agar medium containing 0.5 M NaCl and 2 mM choline when expressed in the DC3000 transporter-deficient mutant GB4.
[d] BetT-0, BetT-4, BetT-16, and BetT-44 refer to PSPTO_5269 lacking 0, 4, 16, or 44 amino acid residues at the C terminus, respectively.
[e] Psyr_4827 was cloned from *P. syringae* pv. syringae strain B728a.
[f] PA5375 did not confer osmoregulatory transport when expressed from its native promoter or from a constitutive promoter in GB4.
[g] OpuD of halophilic *V. cholerae* functions under high-salt conditions; however, its activity under low-osmolarity conditions has not been determined (31).

suggest that this strain encounters habitats that are relatively choline rich.

P. syringae is commonly associated with plants, and plants generate choline by various pathways. Choline is synthesized as a precursor for the major plant membrane lipid phosphatidylcholine, with this synthesis occurring exclusively in the cytosol (12, 41). Choline can also be liberated from phosphatidylcholine through the action of phosopholipases such as phospholipase D (1). Members of the large and diverse phospholipase D family are widely distributed across plant species (reviewed in reference 4), with distinct members activated during growth (1) or in response to environmental stresses, such as nutrient deprivation (52), wounding (53), and infection by microorganisms (14, 46). The biosynthesis and degradation processes that yield choline contribute to the ubiquity and relative abundance of choline within plants (67). In contrast, the majority of plant species lack the enzymes required for glycine betaine synthesis, which occurs via oxidation of choline, and thus glycine betaine production is limited to a narrow range of species, many of which produce it primarily in response to environmental stress.

Free glycine betaine is more widely distributed than choline in many habitats, such as soils, sediments, and aquatic environments (19, 20). Both compounds can be exuded from plant roots, excreted by microorganisms, and released during the degradation of dead plants and microorganisms. Whereas glycine betaine is relatively stable in these environments, choline is readily oxidized into glycine betaine by soil and aquatic microorganisms (28) and also may be used in the generation of phosphatidylcholine for membrane synthesis by eukaryotic microorganisms and some prokaryotes (15). A greater abundance of glycine betaine than choline in these habitats is supported by the fact that most soil and aquatic bacteria examined derive better osmoprotection from glycine betaine than from choline (9, 19, 22, 29, 32, 50), and some can use only glycine betaine, as they lack enzymes required for conversion of choline to glycine betaine (21, 31). Furthermore, most of the BCCTs characterized to date in bacteria not associated with live hosts transport glycine betaine or proline betaine but not choline, as illustrated by the substrate specificity of OpuD of *Vibrio cholerae* (Table 3), BetT of *Aphanothece halophytica*, BetL of *Listeria monocytogenes*, BetP and EctP of *Corynebacterium glutamicum*, and OpuD of *Bacillus subtilis*. Among the few BCCTs that transport choline, almost all were identified in microorganisms that are associated with animal or plant hosts, including BetT of *P. syringae* and the BetT-like transporter in *Haemophilus influenzae* (Table 3), as well as CudT of the gram-positive species *Staphylococcus xylosus*.

All three *P. syringae* strains sequenced to date encode single putative BCCTs, whereas *P. putida* and *P. aeruginosa* have multiple putative BCCTs. Consequently, *P. syringae* served as a simpler model for evaluating BCCT function. The power of a comparative approach in which knowledge generated in *P. syringae* could be translated into knowledge in the more complex systems was demonstrated by the identification of functional transporters in *P. aeruginosa*. The *P. aeruginosa* BCCT that exhibited a genetic arrangement similar to that of the *bet* locus in *E. coli* K-12, namely, that it was adjacent to a putative *betIBA* locus, was previously annotated as *betT1* because it was presumed to encode a functional homolog of *E. coli* BetT. Here, we found that the *betT1* gene (PA5375) did not restore transport for choline or glycine betaine to a DC3000 mutant, even when it was fused to a promoter that exhibits constitutive

expression in DC3000 (Chen and Beattie, unpublished), whereas the transporter encoded by PA3933 transported choline for osmoprotection. Thus, sequence similarity to *P. syringae* BetT was a better predictor of function than similarity with *E. coli* genes and transporters. In addition to demonstrating the necessity of functional information in a *Pseudomonas* species for accurate gene predictions in other pseudomonads, this study demonstrated the utility of a *P. syringae* mutant deficient in all of its transporters for choline and glycine betaine as a tool for identifying functional *Pseudomonas* species transporters.

Although our results suggested PA5375 is not involved in the uptake of choline by *P. aeruginosa* under hyperosmotic conditions, we cannot rule out involvement in the uptake of choline-related substrates under other conditions. Recently, Son and colleagues (58) demonstrated that PA5375 was highly expressed when phosphatidylcholine was used as a carbon source during in vitro growth, suggesting that it may be involved in the uptake of phosphatidylcholine degradation products such as phosphorylcholine. Interestingly, PA5375 was predicted to have a cleavable type I signal peptide on its N terminus, suggestive of extracellular secretion (38), whereas the two *P. aeruginosa* BCCTs characterized in this study and all BCCTs characterized to date in other gram-negative bacteria lack this signal.

Our results support previous findings that the C termini of BCCTs have a role in osmoregulatory transport in gram-negative bacteria. This support lies in two findings: first, the function of the putative *P. aeruginosa* BCCTs was correlated with the presence of a long C-terminal tail, and second, deleting 44 amino acid residues from the C terminus of *P. syringae* BetT eliminated choline transport under hyperosmotic conditions. A requirement for a long C-terminal tail in osmoregulatory transport could be used to help predict which BCCTs in a given gram-negative bacterial strain are likely to function in uptake for osmoprotection rather than, or in addition to, uptake for catabolism. This is particularly important for organisms like *Oceanobacillus iheyensis* HTE831 and *Brevibacterium linens* BL2, for which the sequenced genomes suggest the presence of as many as eight putative BCCTs. Further characterization of such BCCTs should provide insight into the roles of these transporters in the ecology of these organisms.

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