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Three Transport Systems for the Osmoprotectant Glycine Betaine Operate in *Bacillus subtilis*: Characterization of OpuD

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The accumulation of the osmoprotectant glycine betaine from exogenous sources provides a high degree of osmotic tolerance to *Bacillus subtilis*. We have identified, through functional complementation of an *Escherichia coli* mutant defective in glycine betaine uptake, a new glycine betaine transport system from *B. subtilis*. The DNA sequence of a 2,310-bp segment of the cloned region revealed a single gene (*opuD*) whose product (OpuD) was essential for glycine betaine uptake and osmoprotection in *E. coli*. The *opuD* gene encodes a hydrophobic 56.13-kDa protein (512 amino acid residues). OpuD shows a significant degree of sequence identity to the choline transporter BetT and the carnitine transporter CaiT from *E. coli* and a BetT-like protein from *Haemophilus influenzae*. These membrane proteins form a family of transporters involved in the uptake of trimethylammonium compounds. The OpuD-mediated glycine betaine transport activity in *B. subtilis* is controlled by the environmental osmolarity. High osmolarity stimulates de novo synthesis of OpuD and activates preexisting OpuD proteins to achieve maximal glycine betaine uptake activity. An *opuD* mutant was constructed by marker replacement, and the OpuD-mediated glycine betaine uptake activity was compared with that of the previously identified multicomponent OpuA and OpuC (ProU) glycine betaine uptake systems. In addition, a set of mutants was constructed, each of which synthesized only one of the three glycine betaine uptake systems. These mutants were used to determine the kinetic parameters for glycine betaine transport through OpuA, OpuC, and OpuD. Each of these uptake systems shows high substrate affinity, with K_m values in the low micromolar range, which should allow *B. subtilis* to efficiently acquire the osmoprotectant from the environment. The systems differed in their contribution to the overall glycine betaine accumulation and osmoprotection. A triple *opuA*, *opuC*, and *opuD* mutant strain was isolated, and it showed no glycine betaine uptake activity, demonstrating that three transport systems for this osmoprotectant operate in *B. subtilis*.

Bacillus subtilis faces in its natural habitat, the upper layers of the soil, frequent changes in the availability of water. Such alterations in the osmolarity of the environment profoundly influence the physiology of the bacterial cell (8, 19, 30, 32). High external osmolarity and, in particular, a sudden osmotic upshock will cause a decrease in turgor, changes in cell volume, and an increase in the intracellular solute concentration, resulting in the cessation of growth and cell division (42, 43). To offset these deleterious effects, *B. subtilis* initiates a two-step adaptation reaction to restore the disturbed water balance. Initially, large amounts of K^+ are accumulated after an osmotic upshock via turgor-sensitive transport systems (42). Subsequently, the intracellular concentration of proline is strongly increased through de novo synthesis (42, 43). Proline is a compatible solute that can be amassed to high intracellular concentrations without producing adverse effects on essential cellular functions (8, 30, 32). In contrast to high concentrations of inorganic salts, compatible solutes stabilize the quaternary structure of proteins, protect the integrity of cell components, and do not disturb protein-DNA interactions (2).

The accumulation of proline under high-osmolarity growth conditions through de novo synthesis is a slow process, requiring several hours to reach an intracellular concentration that is sufficient for osmoprotection (42). The accumulation of glycine betaine, an osmoprotectant that is widely found in the environment (8, 30, 33), is a much more efficient response to high-osmolarity stress (4). Glycine betaine is metabolically inert in *B. subtilis* under both high- and low-osmolarity growth conditions (4), and its intracellular accumulation can be accomplished through either synthesis (4) or direct uptake from the environment (23, 29, 42). Synthesis requires the presence of choline in the growth medium, and this precursor is then enzymatically converted in a two-step oxidation process into glycine betaine (4, 5).

Multiple transport systems are involved in the uptake of glycine betaine in *B. subtilis* (23). Two systems, OpuA (osmoprotectant uptake) and OpuC (ProU), have recently been identified (22, 23, 29) as members of the superfamily of prokaryotic and eukaryotic transporters, known as ATP-binding cassette (ABC) uptake systems or traffic ATPases (9, 20). The OpuA system comprises three components: OpuAA, an ATPase; OpuAB, an integral protein of the cytoplasmic membrane; and OpuAC, an extracellular substrate-binding protein that is anchored in the cytoplasmic membrane via a lipid modification (23). The OpuC (ProU) glycine betaine uptake system is related to OpuA (22) but contains an additional integral inner membrane component (29). Both OpuA (23) and OpuC (ProU) (29) exhibit structural and functional similarities to the binding protein-dependent glycine betaine transport system ProU from *Escherichia coli* (15, 30).

Since no studies involving mutants with defects in both OpuA and OpuC (ProU) have been reported, it is difficult to assess the individual contributions of each system to osmoprotection in *B. subtilis* and to evaluate the kinetic properties of these transport systems. Furthermore, it has not been possible to determine whether other glycine betaine transport systems

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operate in *B. subtilis*. We report here the characterization of a new glycine betaine uptake system (OpuD) that consists of only one component. OpuD represents a new type of bacterial glycine betaine uptake system and is a member of a small family of transport proteins involved in the accumulation of trimethylammonium compounds. We have also constructed a set of mutants with defects in the *opuA*, *opuC*, and *opuD* loci, and these strains have enabled us to characterize the kinetic properties of the three glycine betaine transport systems operating in *B. subtilis.*

MATERIALS AND METHODS

Media, chemicals, and growth conditions. Rich and minimal media for the growth of *E. coli* and *B. subtilis* strains were as previously described (23). The osmotic strength of media was increased by the addition of NaCl from highly concentrated stock solutions, and the osmolarity of the growth media was determined with a vapor pressure osmometer (model 5,500; Wescor Inc., Logan, Utah). The osmolarity of Spizizen's minimal medium (SMM) was 340 mosmol/kg of water. The antibiotics kanamycin, tetracycline, and spectinomycin were used in solid and liquid media for *B. subtilis* strains at final concentrations of 5, 15, and 100 μg/ml, respectively. The antibiotics ampicillin, kanamycin, tetracycline, and chloramphenicol were used with *E. coli* cultures at final concentrations of 100, 50, 5, and 30 μg/ml, respectively. Radiolabeled [1-^{14}C]glycine betaine (55 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc., St. Louis, Mo. [*methyl*-^{14}C]choline (53 mCi/mmol) and L-[*N-methyl*-^{14}C]carnitine (53 mCi/mmol) were obtained from DuPont de Nemours GmbH (Dreieich, Germany). [α-^{35}S]dATP (1,000 Ci/mmol) was from Amersham Buchler (Braunschweig, Germany).

Bacterial strains and construction of *B. subtilis* mutants. The *B. subtilis* gene libraries constructed in the course of this study were propagated in *E. coli* DH5α (GIBCO BRL, Eggenstein, Germany). Strain MKH13 [Δ(*betTIBA*)*U169* Δ(*putPA*)*101* Δ(*proP*)*2* and Δ(*proU*)*608*] is deficient in glycine betaine uptake and synthesis (17). *B. subtilis* BKB4 [Δ(*opuA*::*neo*)*1*] and BKB7 [Δ(*opuA*::*tet*)*2*] are derivatives of JH642 (*trpC2 pheA1*) (BGSC 1A96) (23). Cells of *B. subtilis* were transformed with linear DNA fragments by the two-step transformation procedure (18). The *B. subtilis* mutant RMKB1 [Δ(*opuA*::*neo*)*1* (*opuD*::*tet*)*1*] was isolated by transforming strain BKB4 with linearized DNA of plasmid pRMK10 (Fig. 1) and selecting for tetracycline-resistant transformants. Strain RMKB2 [Δ(*opuA*::*tet*)*2*] Δ(*opuD*::*neo*)*2*] was constructed by transforming BKB7 with linearized DNA of plasmid pRMK36 (Fig. 1) and selecting for kanamycin-resistant colonies. Strain RMKB7 [Δ(*opuD*::*neo*)*2*] was isolated by transforming linearized DNA of plasmid pRMK36 (Fig. 1) into strain JH642 and selecting for kanamycin-resistant transformants. Strain JBB33 carries the *opuC-9* mutation, which is closely linked to a mini-Tn*10* (Spc^r) insertion (22). The *opuC-9* mutation was introduced into strains JH642, BKB7, and RMKB7 by transforming with chromosomal DNA of strain JBB33 and selecting for spectinomycin-resistant colonies. This yielded strains RMKB12 [*opuC-9*], RMKB8 [Δ(*opuA*::*tet*)*2 opuC-9*], and RMKB9 [Δ(*opuD*::*neo*)*2 opuC-9*]. Strain RMKB8 [Δ(*opuA*::*tet*)*2 opuC-9*] was transformed with chromosomal DNA of the Δ(*opuD*::*neo*)*2* strain RMKB7, yielding the triple mutant strain RMKB11 [Δ(*opuA*::*tet*)*2* Δ(*opuD*::*neo*)*2 opuC-9*].

Methods used with nucleic acids. Routine manipulation of plasmid DNA, the construction of recombinant plasmids, the isolation of chromosomal DNA from *B. subtilis*, and the detection of homologous sequences by Southern hybridization were all carried out by standard techniques (36). The DNA sequence of the *opuD* region was determined by the method of Sanger et al. (37) with the Sequenase 2.0 kit (U.S. Biochemical Corp., Braunschweig, Germany). A library of chromosomal DNA segments of the strain BKB4 [Δ(*opuA*::*neo*)*1*] was prepared by partial cleaving of chromosomal DNA with *Sau*3A. The resulting restriction fragments were separated by electrophoresis, and DNA segments in the range between 2 and 10 kb were eluted from the agarose gel and cloned into the *Bam*HI site of plasmid pACYC177 (Kn^r and Ap^r) (34). A second gene library was constructed in the same way by using a complete *Hin*dIII digest of chromosomal DNA from strain BKB4 and plasmid pHSG575 (Cm^r) (40) as the vector. The DNA of the recombinant plasmids was propagated in DH5α and used to transform strain MKH13 to search for osmotolerant clones capable of growing on minimal medium A (MMA) minimal agar plates containing 0.8 M NaCl and 5 mM glycine betaine (23).

Transport assays. Uptake of glycine betaine by bacterial cells was measured by using radiolabeled [1-^{14}C]glycine betaine as the substrate as described by Kempf and Bremer (23) except that cells were maintained at 37°C during the transport assay. For the kinetic studies, the glycine betaine concentration in the uptake assay was varied from 1 to 70 μM. To study the activation and induction of glycine betaine transport activity through OpuD, cells of strain RMKB8 were grown in SMM to mid-exponential phase (optical density at 578 nm = 0.57). The culture was then divided into three portions. One culture was treated with 100 μg of chloramphenicol per ml at time zero, and samples were assayed for [1-^{14}C] glycine betaine uptake at various time intervals. The treated culture and one of the untreated cultures were subjected to a sudden osmotic upshock after 28 min by adding NaCl from a concentrated stock solution (4 M NaCl in SMM) to the growth medium to a final concentration of 0.4 M.

Computer analysis. DNA and protein sequences were assembled and analyzed with the Lasergene program (DNASTAR, Ltd., London, United Kingdom) on an Apple Macintosh computer. Searches for homologies were performed at the National Center for Biotechnology Information with the BLAST programs (1).

Nucleotide sequence accession number. The nucleotide sequence of *opuD* and its flanking sequences have been deposited in GenBank and have been assigned the accession number U50082.

RESULTS

Cloning of the *opuD* gene by functional complementation. The mutant strain MKH13 lacks both the ProU and ProP glycine betaine uptake systems and hence cannot grow on high-osmolarity minimal plates containing this osmoprotectant (17). We have capitalized on the growth phenotype of strain MKH13 by using functional complementation to search for *B. subtilis* genes that could correct the deficiency in glycine betaine uptake. A gene library of partial *Sau*3A restriction fragments from chromosomal DNA of strain BKB4 [Δ(*opuA*::*neo*)*1*] in the low-copy-number plasmid pACYC177 (Kn^r Ap^r) was transformed into MKH13, and the resulting transformants (approximately 30,000 colonies) were searched for osmotolerant clones by replica plating on high-osmolarity minimal plates containing glycine betaine. Two strains that could grow under these selective conditions were found, and each carried a plasmid (pRMK1) with the same chromosomal DNA segment (Fig. 1A). In a separate experiment, strain MKH13 was transformed with a gene library of *Hin*dIII fragments cloned into the low-copy-number plasmid pHSG575 (Cm^r). Among approximately 30,000 Cm^r colonies, one strain that grew on the selection plates was detected. The plasmid (pRMK2) harbored by this strain carried an approximately 6.2-kb *Hin*dIII DNA fragment. Restriction analysis and Southern hybridization experiments (data not shown) revealed that the cloned chromosomal fragments present in plasmid pRMK1 were contained in the larger pRMK2 fragment (Fig. 1A). This physical analysis also showed that an additional DNA segment of approximately 1.4 kb was present in plasmid pRMK2 in the region that is common between pRMK1 and pRMK2. Southern hybridization of the chromosomal DNA present in pRMK2 to genomic DNA of strain BKB4 revealed that the extra 1.4-kb DNA segment found in plasmid pRMK2 did not exist in the *B. subtilis* chromosome and, therefore, must have been acquired during the cloning process in *E. coli.*

Growth of the plasmid-containing derivatives of MKH13 in high-osmolarity media was dependent on the presence of glycine betaine, indicating that plasmids pRMK1 and pRMK2 encode an uptake system for this osmoprotectant. This was confirmed by measuring the initial rate of [1-^{14}C]glycine betaine uptake in cultures of MKH13 (pRMK1) and MKH13 (pRMK2). We carried out a deletion analysis of the cloned DNA segment in plasmid pRMK1 and also inserted antibiotic resistance cassettes into various restriction sites of plasmids pRMK1 and pRMK2 (Fig. 1B). This allowed us to localize the approximate position of the gene encoding the glycine betaine transport activity. The size of this fragment (approximately 1.9 kb) suggests that this new glycine betaine transport system is encoded by a single gene; we refer to this gene in the following as *opuD* (osmoprotectant uptake) and to its gene product as the OpuD protein.

Nucleotide sequence of *opuD*. For the DNA sequence analysis of the *opuD* gene, we sequenced the entire insert of pRMK6 since it carried the shortest cloned chromosomal region from *B. subtilis* required for glycine betaine transport activity (Fig. 1B). Inspection of the sequenced 1,939-bp DNA region revealed the presence of a 1,479-bp open reading frame

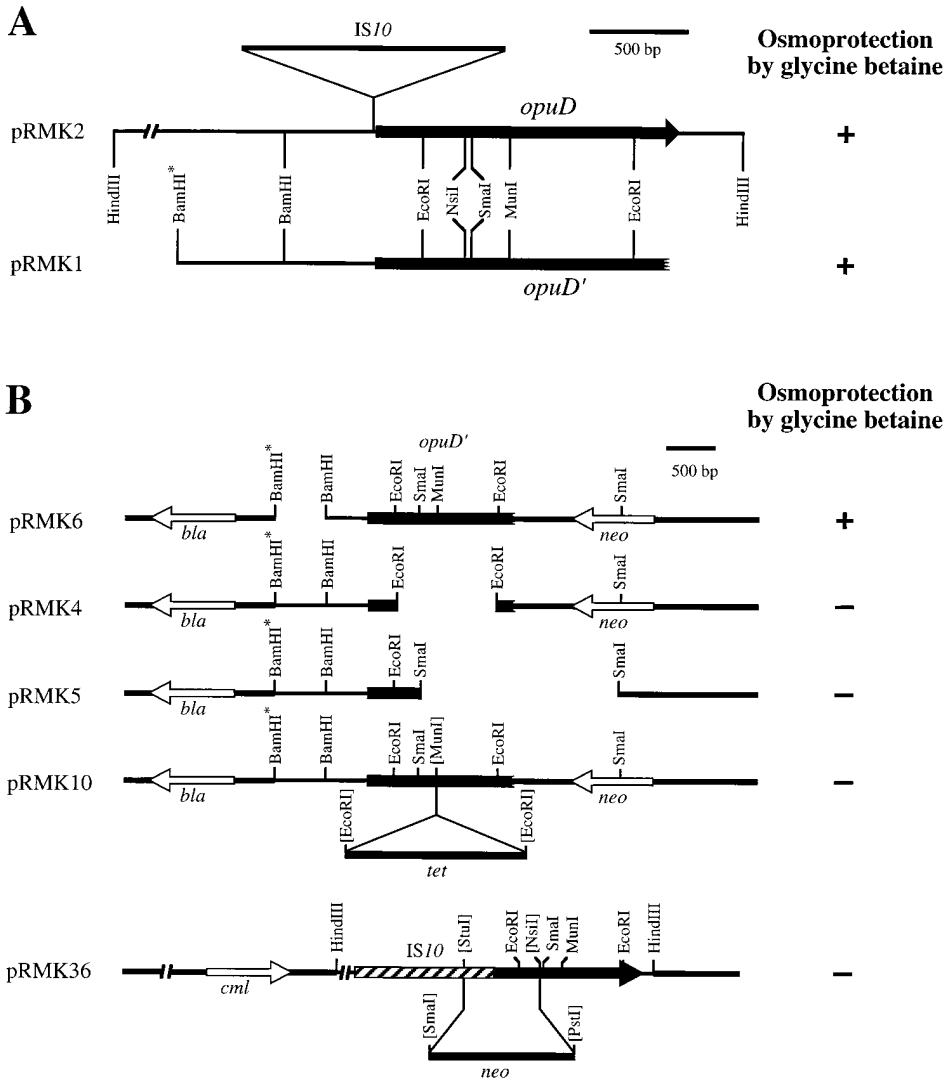

FIG. 1. Physical and genetic organization of the cloned *B. subtilis* DNA segment carrying *opuD*. (A) Plasmid pRMK1 was isolated from a gene bank of partial *Sau*3A fragments inserted into the *Bam*HI site of vector pACYC177. Plasmid pRMK2 was isolated from a gene library of *Hin*dIII restriction fragments inserted into the *Hin*dIII site of plasmid pHSG575. Plasmid pRMK2 carries an IS*10* insertion, and the *opuD* gene carried on plasmid pRMK1 is truncated at its 3′ end. (B) Plasmids pRMK6, pRMK4, and pRMK5 are deletion derivatives of pRMK1; plasmid pRMK10 is also derived from pRMK1 but carries a tetracycline (*tet*) resistance cassette inserted into the *Mun*I site present in *opuD*. Plasmid pRMK36 is a deletion derivative of pRMK2 and carries a kanamycin (*neo*) resistance cassette which replaces part of the 5′ region of *opuD*. Restriction sites destroyed in the course of the cloning are shown in brackets. The *Bam*HI site marked with an asterisk was created by cloning of the genomic *Sau*3A DNA fragment into the *Bam*HI site of plasmid pACYC177. Osmoprotection (growth, +; no growth, −) by glycine betaine was scored by monitoring the growth of *E. coli* MKH13 harboring the indicated plasmids on high-osmolarity minimal plates (MMA with 0.8 M NaCl) in the presence of 5 mM glycine betaine. Growth of the strains was scored after 3 days of incubation at 37°C.

that begins with an ATG start codon at position 460 bp (Fig. 2). This start codon is preceded by a DNA sequence complementary to the 3′ end of the 16S rRNA of *B. subtilis* (3′-UCUUU CCUCCACUAG-5′ [38]) and is thus likely to function as the ribosome-binding site of *opuD*. Surprisingly, there was no stop codon in the *opuD* reading frame before sequences from the vector plasmid pACYC177 were found, indicating that plasmids pRMK1 and pRMK6 (Fig. 1) encode truncated *opuD* genes. To complete the nucleotide sequence of *opuD*, we determined the DNA sequence of a 0.55-kb *Eco*RI-*Hin*dIII restriction fragment from plasmid pRMK2 that should contain the 3′ end of *opuD* (Fig. 1A). When this sequence was attached to the DNA sequence determined from pRMK6, we found that the *opuD* reading frame continued for 19 additional codons before a TAA stop codon was encountered. The *opuD* reading frame is thus 1,536 bp long. Downstream of the *opuD* stop codon, we found an extended inverted repeat (ΔG [25°C] = −118 kJ) that can form a stem-loop structure that could possibly function as a factor-independent transcription termination signal (31) for the *opuD* transcript (Fig. 2). Upstream of *opuD* we found the 3′ end of an incomplete open reading frame with the same transcriptional orientation as the *opuD* gene (Fig. 2). A data bank search revealed that the protein encoded by the incomplete open reading frame was homologous to hypothetical proteins (YHIN) with unknown functions from both *E. coli* and *Haemophilus influenzae* (11).

Features of the *opuD*-encoded gene product. The *opuD* gene encodes a 512-residue protein (OpuD) with a calculated molecular mass of 56.13 kDa. The amino terminus of OpuD does not show features characteristic of bacterial signal sequences,

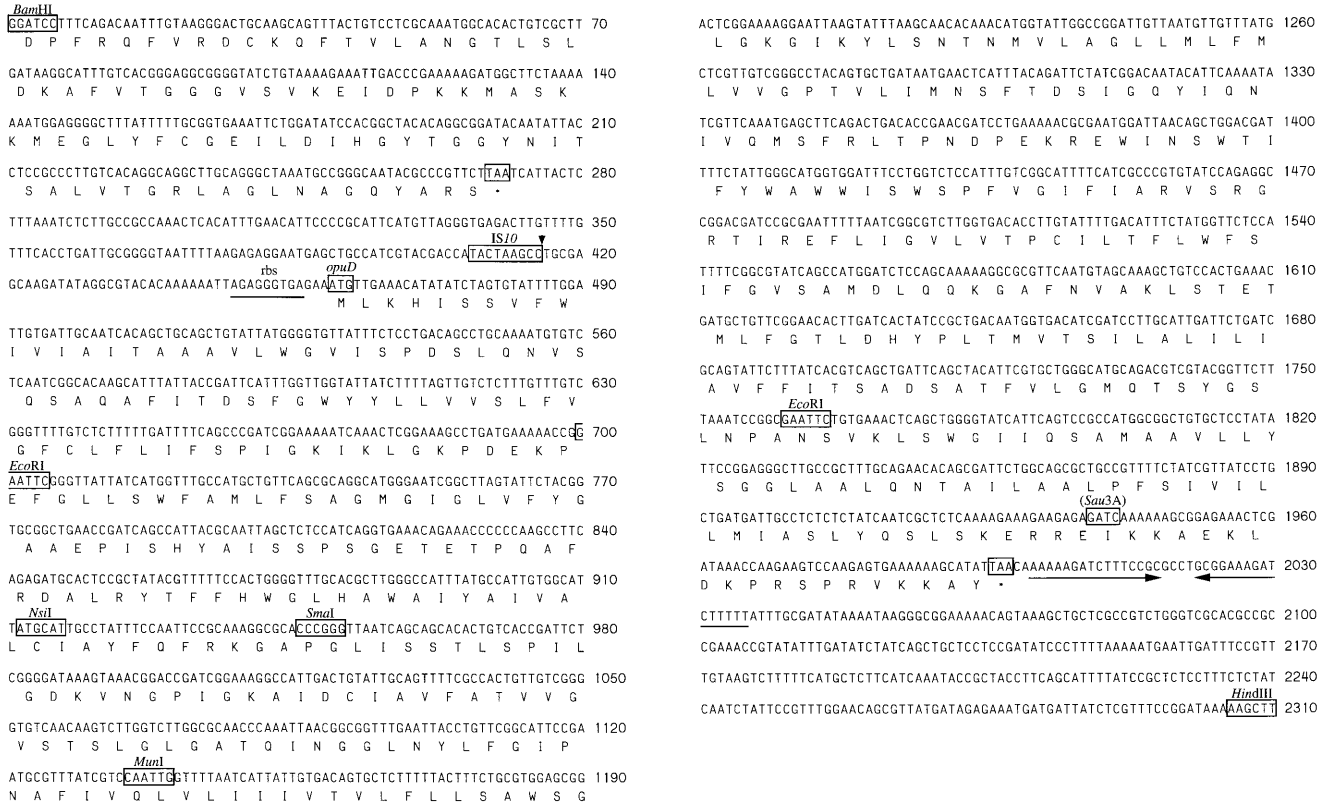

FIG. 2. Nucleotide sequence of the *opuD* region. The determined DNA sequence of the *opuD* region and the deduced amino acid sequences for the OpuD protein and for the truncated YHIN-like protein upstream of *opuD* are shown. The putative ATG start codon of *opuD* is boxed, and the potential ribosome-binding site is underlined. An inverted repeat downstream of *opuD* that could possibly function in transcription termination is shown by a pair of arrows. Recognition sites for several enzymes are indicated; the indicated *Sau*3A site marks the end of the truncated *opuD* gene carried by plasmid pRMK1 (Fig. 1). The site where the IS*10* element is inserted in plasmid pRMK2 is marked with an arrowhead, and the DNA sequence duplicated during the integration of IS*10* is boxed.

indicating that OpuD is not a secreted protein. A hydrophobicity plot according to the method of Kyte and Doolittle (27) of OpuD revealed an alteration of hydrophobic and hydrophilic segments that is characteristic for integral membrane proteins and suggests the presence of 12 transmembrane segments, each with a length of approximately 20 amino acid residues. Application of the positive-inside rule (41) to the OpuD sequence suggests that both the amino and carboxy termini are located in the cytoplasm. This results in an asymmetric distribution of the 34 positively charged residues in OpuD; the majority (27 residues) are present in cytoplasmic loops, and only seven residues are located in extracellular loops. In contrast, there is no strong charge bias in the distribution of the negatively charged residues in OpuD. A total of 13 negatively charged residues are present in extracellular segments of the protein, and 11 are located in cytoplasmic loops. A noteworthy feature of OpuD is its highly hydrophilic and strongly charged carboxy-terminal end: 15 of 28 amino acid residues are charged residues (Fig. 3). The OpuD protein contains four cysteine residues (Cys-60, Cys-152, Cys-188, and Cys-352), each of which occurs in predicted transmembrane spans (segments 2, 4, 5, and 9 [Fig. 3]), and there are no charged residues present in any of the proposed 12 transmembrane segments of OpuD.

OpuD is a member of a trimethylammonium transporter family. A search for proteins homologous to OpuD in the databases revealed significant sequence identities to two other *E. coli* transport proteins that are also involved in the uptake of trimethylammonium compounds. One of these, BetT, is a high-affinity choline transport system that functions in the osmoregulatory choline glycine betaine synthesis pathway (28). The second protein is CaiT, a transporter for carnitine and structurally related trimethylammonium compounds that are used by *E. coli* under anaerobic growth conditions as alternative terminal electron acceptors (10). OpuD exhibits 23.8% sequence identity to CaiT and 35.5% identity to BetT. Additionally, high identities (39.6%) were also detected to a BetT-like protein from *H. influenzae* (11). The substrates of the OpuD, CaiT, and BetT (*E. coli*) proteins (glycine betaine, carnitine, and choline, respectively) have a common structure; each has a fully methylated quaternary ammonium group. Thus OpuD, CaiT, and BetT (*E. coli*) form a family of transporters involved in the uptake of trimethylammonium compounds. The BetT protein from *H. influenzae* is likely to be part of this protein family, although its substrate(s) remains to be experimentally determined. Multiple alignments of the OpuD, CaiT, BetT (*E. coli*) and BetT (*H. influenzae*) proteins revealed that they are related to each other over the entire length of their sequence (Fig. 3). Blocks of well-conserved amino acid residues are noticeable, and many of them correspond to predicted transmembrane regions of OpuD. However, the strongly conserved regions are also found in parts of both extracellular and cytoplasmic loops. A stretch of 32 amino acid residues corresponding to amino acid residues 312 to 343 in the OpuD sequence is highly conserved among the four proteins (Fig. 3). This region comprises the eighth transmembrane segment (312 to 332

FIG. 3. Alignment of OpuD with the amino acid sequences of carnitine and choline transporters. The amino acid sequence of OpuD from *B. subtilis* is aligned with those of CaiT from *E. coli* and the BetT protein from *E. coli* and a BetT-like protein from *H. influenzae*. Amino acid residues identical in at least two of the protein sequences are marked. The positions of the 12 presumed transmembrane spans of OpuD are indicated.

amino acid residues) of OpuD and the entire cytoplasmic connecting loop (10 amino acid residues) to the ninth transmembrane segment (Fig. 3). It is tempting to speculate that this segment of the OpuD, CaiT, BetT (*E. coli*), and BetT (*H. influenzae*) proteins either is an important structural determinant or plays a role in substrate binding and translocation across the membrane.

Plasmid pRMK2 carries a copy of the insertion element IS*10*. During the cloning of *opuD*, plasmid pRMK2 acquired a small portion of extra DNA material (1.4 kb) from the chromosome of the *E. coli* host strain MKH13 (Fig. 1). DNA sequence analysis of both the left and right junctions unambiguously identified the inserted DNA segment as a copy of the transposable element IS*10*, which can be a significant source of spontaneous mutations (6, 25). This element had inserted at position 415 bp, 44 bp upstream of the *opuD* start codon (Fig. 2), and thus likely affects *opuD* expression. A 9-bp duplication of the *opuD* DNA sequence was found at the integration site, a feature typical for the insertion process of IS*10* (25). IS*10* exhibits a certain target specificity, giving rise to IS*10* hot spots (3). Such a preferred target sequence is also present at the insertion site of IS*10* in *opuD* (Fig. 2). The *opuD* clones recovered by functional complementation of strain MKH13 either carried an IS*10* element in the vicinity of the *opuD* start codon (pRMK2) (Fig. 1A) or were truncated at their 3′ end (pRMK1) (Fig. 1A). Apparently, the synthesis of the *B. subtilis* OpuD protein was not well tolerated by the heterologous *E. coli* host even when low-copy-number plasmids were used for the cloning of the *opuD* gene.

Construction of a *B. subtilis opuD* mutation. To characterize the contribution made by OpuD to glycine betaine uptake in *B. subtilis*, we constructed two *opuD* mutations. In the (*opuD*::*tet*)*1* allele, the *opuD* reading frame was disrupted by inserting a tetracycline resistance cassette into the unique *Mun*I restriction site (plasmid pRMK10) (Fig. 1B). In the Δ(*opuD*::*neo*)*2* mutation, the 5′ end of *opuD* was removed and replaced with a kanamycin resistance cassette (plasmid pRMK36) (Fig. 1B). Both plasmid-encoded *opuD* mutations abolished glycine betaine transport and osmoprotection of strains MKH13 (pRMK10) and MKH13 (pRMK36). The (*opuD*::*tet*)*1* and Δ(*opuD*::*neo*)*2* mutations were transferred by marker replacement into the chromosome of the *B. subtilis* wild-type strain JH642, and the presence of the mutant *opuD* alleles in strain RMKB13 [(*opuD*::*tet*)*1*] and strain RMKB7 [Δ(*opuD*::*neo*)*2*] was verified by Southern hybridization.

The homology of the *B. subtilis* OpuD protein to the CaiT and BetT proteins from *E. coli* (Fig. 3) prompted us to test whether OpuD would also transport carnitine or choline in addition to glycine betaine. Uptake of radiolabeled carnitine and choline was determined in *E. coli* MKH13 carrying the

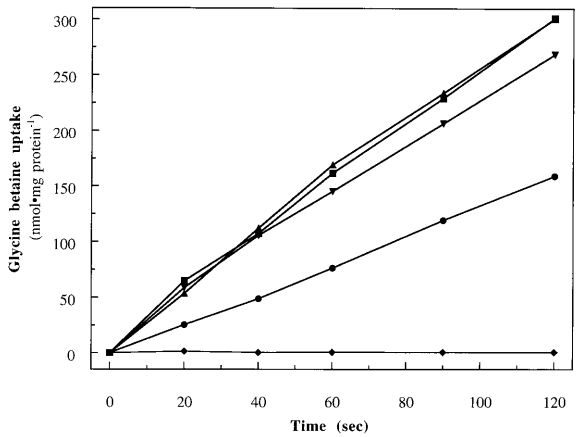

FIG. 4. Glycine betaine uptake is mediated by three uptake systems. Cultures of the wild-type strain JH642 (*opuA*$^+$ *opuC*$^+$ *opuD*$^+$) (■) and its mutant derivatives strains BKB7 (*opuA opuC*$^+$ *opuD*$^+$) (●), RMKB7 (*opuA*$^+$ *opuC*$^+$ *opuD*) (▲), RMKB12 (*opuA*$^+$ *opuC opuD*$^+$) (▼), and RMKB11 (*opuA opuC opuD*) (◆) were grown in SMM with 0.4 M NaCl to mid-log phase and assayed for glycine betaine uptake at a final substrate concentration of 10 μM.

opuD$^+$ plasmid pRMK2 at a final substrate concentration of 10 μM. No carnitine or choline uptake was detectable in cultures of MKH13 (pRMK2) grown in low-osmolarity (MMA) or high-osmolarity (MMA with 0.2 M NaCl) media whereas glycine betaine transport was readily detectable under these growth conditions. We also measured the uptake of radiolabeled choline and carnitine in the *opuD* mutant strain RMKB13, but there was no reduction in the transport of these quaternary ammonium compounds in comparison with the *opuD*$^+$ parent strain JH642 (data not shown). Thus, the OpuD protein does not mediate either carnitine or choline uptake.

Glycine betaine uptake in *B. subtilis* is mediated by three transport systems. Glycine betaine uptake in a *B. subtilis* wild-type strain is stimulated in cultures grown in high-osmolarity media (23). We measured glycine betaine transport in the *opuD* mutant strains RMKB13 and RMKB7 grown in SMM with 0.4 M NaCl at a final glycine betaine concentration of 10 μM and compared it with that of the *opuD*$^+$ strain JH642. There was essentially no difference in the initial rate of glycine betaine uptake between these strains (Fig. 4). Thus, in an otherwise wild-type background, loss of the OpuD system has no appreciable effect on the overall glycine betaine uptake activity. We also measured the initial rate of glycine betaine uptake in strains lacking either the OpuA (strain BKB7) or the OpuC (strain RMKB12) transport systems under the conditions described above for OpuD. Loss of the OpuA system resulted in a strong decrease in glycine betaine uptake, whereas in an *opuC* mutant, transport was only moderately affected (Fig. 4). Thus, at a low substrate concentration the activity of the OpuA system dominates that of OpuC and OpuD.

To evaluate the distinctive contribution of the OpuA, OpuC, and OpuD transporters to the overall glycine betaine transport activity and their kinetic parameters, mutant strains which synthesize just one of these transporters are needed. We therefore constructed such a set of *B. subtilis* mutants and also isolated a strain defective in all three glycine betaine transport systems. In the triple mutant strain RMKB11 [Δ(*opuA*::*tet*)*2 opuC-9* Δ(*opuD*::*neo*)*2*], glycine betaine uptake activity was completely abolished (Fig. 4), indicating that no glycine betaine transport systems other than OpuA, OpuC, and OpuD operate in *B. subtilis*. Glycine betaine uptake mediated by the OpuA, OpuC, and OpuD systems was osmotically stimulated and contributed additively to the overall uptake activity observed in the wild-type strain JH642 (data not shown).

TABLE 1. Kinetic parameters of the glycine betaine uptake systems OpuA, OpuC, and OpuD from *B. subtilis*[a]

System and growth condition	K_m (μM)	V_{max} (nmol min^{-1} mg of protein^{-1})
OpuA		
−	2.4	110
+	2.4	282
OpuC		
−	5.1	41
+	6	65
OpuD		
−	9.5	16
+	13	61

[a] The K_m and V_{max} values were determined at 37°C in cultures grown either in SMM medium (uninduced condition [−]) or in SMM medium with 0.4 M NaCl (induced condition [+]). The following strains were used: RMKB9 (OpuA$^+$ OpuC$^-$ OpuD$^-$), RMKB2 (OpuA$^-$ OpuC$^+$ OpuD$^-$), and RMKB8 (OpuA$^-$ OpuC$^-$ OpuD$^+$).

Kinetics of glycine betaine transport through OpuA, OpuC, and OpuD. We determined the kinetic parameters of glycine betaine uptake via the various transport systems. Strains RMKB8 (*opuA opuC opuD*$^+$), RMKB9 (*opuA*$^+$ *opuC opuD*), and RMKB2 (*opuA opuC*$^+$ *opuD*) were grown in low- or high-osmolarity media, and the initial velocities of [1-^{14}C]glycine betaine uptake were determined over a wide range of substrate concentrations. Glycine betaine uptake through OpuA, OpuC, and OpuD showed saturation kinetics. The determined K_m and V_{max} values for these transport systems are summarized in Table 1. Each of these three glycine betaine transporters recognizes its substrate with high affinity, with K_m values in the micromolar range. Their affinity for glycine betaine is little affected by the osmolarity of the growth medium; however, high osmolarity stimulates the maximal velocity of glycine betaine uptake through each system (Table 1).

Osmotic activation and induction of OpuD-mediated glycine betaine transport. To test whether the OpuD-mediated glycine betaine transport at high osmolarity (Table 1) depends on de novo protein synthesis or on osmotic activation of preexisting OpuD proteins, we measured glycine betaine uptake in the presence or absence of chloramphenicol. Exponentially growing cells of strain RMKB8 (*opuA opuC opuD*$^+$) were subjected to a sudden osmotic upshock by adding NaCl to the SMM growth medium to a final concentration of 0.4 M NaCl. There was an immediate stimulation of the rate of glycine betaine uptake after the osmotic upshock, and the rate of glycine betaine transport continued to rise over a period of 90 min (Fig. 5). There was also a rapid increase in glycine betaine uptake in the cultures that were pretreated with chloramphenicol approximately 30 min prior to the upshock, but in these cells, we observed no further increase in transport activity over that observed initially after the osmotic upshock (Fig. 5). These data suggest that the OpuD-mediated glycine betaine transport in cells grown at high osmolarity depends on both the activation of preexisting OpuD proteins and de novo protein synthesis, but further studies are required to unequivocally verify this conclusion.

Contribution of the individual transporters to osmoprotection by glycine betaine. To assess the contribution made by the OpuA, OpuC, and OpuD transport systems to osmoprotection conferred by glycine betaine (4), we grew the wild-type strain

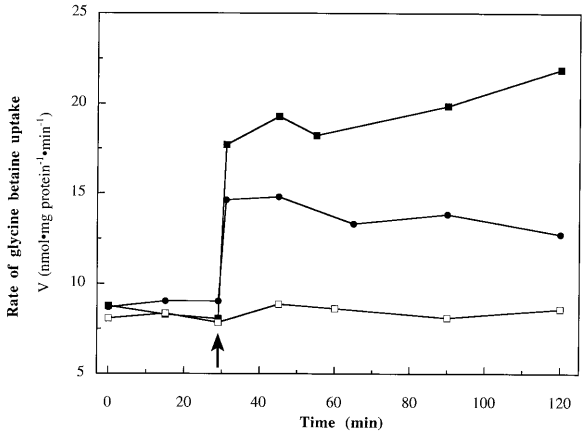

FIG. 5. Activation and induction of glycine betaine transport activity mediated by the OpuD system. Cells of strain RMKB8 (*opuA opuC opuD*$^+$) were grown in SMM to mid-exponential phase and then divided into three portions. Samples were taken at various time intervals and assayed for glycine betaine uptake activity. One culture was untreated (□); 100 μg of chloramphenicol per ml was added to the second culture (●) at time zero, and this culture was then subjected to a sudden osmotic upshock after 28 min (arrow) by adding NaCl to the growth medium to a final concentration of 0.4 M. The third culture (■) was also subjected to osmotic upshock but did not receive a chloramphenicol treatment. The rate of glycine betaine uptake was measured at a final substrate concentration of 10 μM.

and mutants synthesizing a single glycine betaine uptake system under high-osmolarity growth conditions. Growth of all strains was strongly impaired, but the addition of 1 mM glycine betaine to the medium largely alleviated growth inhibition in the wild-type strain JH642 (OpuA$^+$ OpuC$^+$ OpuD$^+$) (Fig. 6). The mutant strains were also protected from the negative effects of high osmolarity by glycine betaine but to different degrees (Fig. 6). Growth of the OpuA$^+$ strain RMKB9 (OpuA$^+$ OpuC$^-$ OpuD$^-$) was not different from that of the wild-type strain JH642 (Fig. 6), demonstrating that the OpuA system is physiologically the most important glycine betaine uptake system in *B. subtilis*. The OpuC and OpuD systems are clearly less efficient, as reflected by the reduced growth of the OpuC$^+$ (RMKB2) and OpuD$^+$ (RMKB8) strains in comparison with that of the OpuA$^+$ strain (RMKB9) in high-osmolarity medium in the presence of glycine betaine. A similar degree of osmoprotection by glycine betaine can be achieved in strains synthesizing either the OpuC or the OpuD transport system (Fig. 6) as expected from their similar kinetic parameters (Table 1). Growth of the triple mutant strain RMKB11 (OpuA$^-$ OpuC$^-$ OpuD$^-$) was not protected from the detrimental effects of high osmolarity by glycine betaine (Fig. 6).

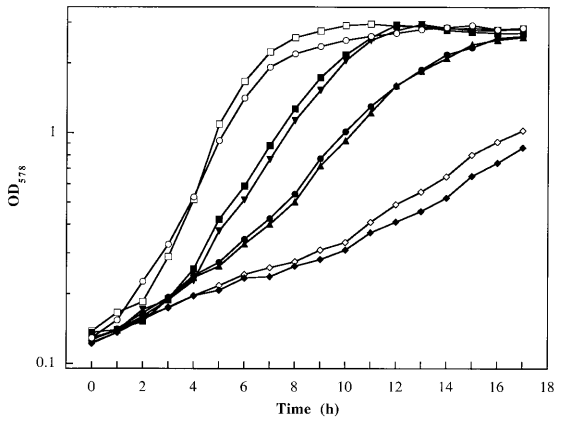

FIG. 6. Three glycine betaine transport systems contribute to osmoprotection. *B. subtilis* JH642 (*opuA*$^+$ *opuC*$^+$ *opuD*$^+$) (□, ■, and ◇), RMKB9 (*opuA*$^+$ *opuC opuD*) (▼), RMKB8 (*opuA opuC opuD*$^+$) (▲), RMKB2 (*opuA opuC*$^+$ *opuD*) (●), and RMKB11 (*opuA opuC opuD*) (◆ and ○) were grown in SMM, in SMM with 1.2 M NaCl, and in SMM with 1.2 M NaCl in the presence of 1 mM glycine betaine. For clarity of the presentation of the data, not all growth curves are documented. The growth of all strains in SMM was identical, and this is shown for the wild-type strain JH642 (*opuA*$^+$ *opuC*$^+$ *opuD*$^+$) (□) and the triple mutant strain RMKB11 (*opuA opuC opuD*) (○). Likewise, there was no growth difference between these strains when they were grown in high-osmolarity media (SMM with 1.2 M NaCl); a representative growth curve is documented for strain JH642 (◇). Curves for the growth in high-osmolarity medium in the presence of glycine betaine are shown for the wild type and each mutant strain (closed symbols). OD_{578}, optical density at 578 nm.

DISCUSSION

Glycine betaine is synthesized by plants and is brought into the habitat of *B. subtilis* by root exudates and decaying plant material (13, 33). Its availability is likely to vary considerably in the upper layers of the soil, and consequently, effective mechanisms are required for the acquisition of this osmoprotectant from the environment. The data presented here show that three glycine betaine transport systems operate in *B. subtilis*. Two of the transporters, OpuA and OpuC (ProU), are multicomponent systems that belong to the ABC-type superfamily of transporters (22, 23, 29). In contrast, the OpuD system is a single-component transporter. Each of the OpuA, OpuC, and OpuD transporters is under osmotic control and actively participates in the stress reaction of *B. subtilis* to a high-osmolarity environment (Fig. 6). The properties of the triple mutant strain RMKB11 (*opuA opuC opuD*) show that no additional glycine betaine transporters of physiological relevance operate in *B. subtilis* (Fig. 4 and 6).

Two types of glycine betaine transport systems have been reported in bacteria. One group is related to the binding protein-dependent ProU system from *E. coli* (30), and this class comprises the *B. subtilis* OpuA and OpuC (ProU) systems (22, 23, 29). The second group is represented by the single-component ProP transporter from *E. coli* and the ProP-related OusA protein from the plant-pathogen *Erwinia chrysanthemi* (14). The amino acid sequence of the OpuD protein is not related to ProP or OusA, and hence, OpuD represents a new type of bacterial glycine betaine uptake system. The *opuD* sequence predicts a hydrophobic protein of 512 amino acid residues that is likely to form an integral membrane protein with 12 transmembrane spans, a structural feature found commonly in secondary transport systems (35). The OpuD protein, together with the choline transporter BetT (28) and the carnitine transporter CaiT (10) from *E. coli* and a BetT-like protein with undefined function from *H. influenzae* (11), forms a small family of transporters involved in the uptake of trimethylammonium compounds. The substrates of CaiT, BetT, and OpuD are structurally related, but OpuD does not exhibit any uptake activity for either carnitine or choline.

We found that both the ATPases and the integral membrane components of both the ABC-type transport systems OpuA and OpuC (ProU) from *B. subtilis* are related to each other. However, the presumed substrate-binding proteins of both systems, OpuAC and OpuCC (ProX), show only a low degree (12%) of sequence identity. There is ample evidence that the substrate-binding proteins of ABC-type transporters in gram-positive bacteria are extracellular lipoproteins that are tethered to the cytoplasmic membrane (7, 39). Indeed, we have previously reported that OpuAC shows features typical for

bacterial lipoproteins (23) and have recently also demonstrated that the purified OpuAC protein binds glycine betaine with high affinity (24). Lin and Hansen (29) have speculated that the presumed substrate-binding protein of the *B. subtilis* ProU (OpuC) system is anchored in the cytoplasmic membrane via a stretch of hydrophobic amino acids at the amino terminus of ProX (OpuCC). However, a lipid modification of the ProX (OpuCC) protein is suggested by the presence of a ProX (OpuCC) signal peptide sequence (29) that shows the characteristic signatures (Leu^{-3}-Ser-Gly-Cys^{+1}) for the proteolytic processing and lipid modification site of bacterial lipoproteins (7, 39).

Each of the three glycine betaine transport systems present in *B. subtilis* shows high substrate affinity, with K_m values in the low micromolar range (Table 1). This feature of the OpuA, OpuC, and OpuD systems should allow the bacterial cell to acquire glycine betaine from the environment even when this osmoprotectant is present at a very low concentration. The transport systems differ, however, in their contribution to the overall glycine betaine accumulation by the cell, as reflected by their different V_{max} values (Table 1). The ABC-type OpuA system is certainly the predominant glycine betaine transporter of *B. subtilis*. The uninduced level of transport activity at low substrate concentration exceeds greatly that of the osmotically induced OpuC and OpuD systems. The dominating function of OpuA for glycine betaine uptake is further illustrated by the growth properties of the $OpuA^+$ strain RMKB9 ($OpuC^-$ $OpuD^-$) which can grow with approximately the same efficiency as a wild-type strain producing all three uptake systems (Fig. 6). The OpuC system is also an ABC-type transporter, but its capacity for glycine betaine uptake is far less than that of OpuA and is similar to that of the single-component OpuD system under high-osmolarity growth conditions (Table 1). Consistent with the kinetic parameters of the OpuC and OpuD systems are the similar growth characteristics of the $OpuC^+$ strain RMKB2 ($OpuA^-$ $OpuD^-$) and the $OpuD^+$ strain RMKB8 ($OpuA^-$ $OpuC^-$) in high-osmolarity media containing glycine betaine (Fig. 6). The presence of multiple glycine betaine uptake systems in *B. subtilis* should enable the cell to react flexibly to a varying supply of this osmoprotectant in the habitat. In addition, the OpuA, OpuC, and OpuD transporters might have different affinities and substrate specificities for a range of osmoprotective compounds.

B. subtilis accumulates significant amounts of glycine betaine in low-osmolarity media through synthesis from choline (4) or through direct uptake from the environment (21, 42). The accumulation of this osmoprotectant at low osmolarity by gram-positive bacteria (4, 12, 16, 26) appears to be of general physiological importance. It is probably connected with the high turgor maintained by gram-positive microorganisms. The contribution to glycine betaine uptake made by the OpuA system under these low-osmolarity growth conditions exceeds by far that made by the OpuC and OpuD transporters. The high basal level in OpuA activity is a reflection of the dual transcriptional regulation of the *opuA* operon. It is expressed both from an osmotically inducible promoter and from transcription initiation signals that mediate constitutive expression of *opuA* in log-phase cells (23).

The data presented in this communication describe the identification of the full set of glycine betaine uptake systems operating in *B. subtilis* and report on the kinetic properties and physiological functions of the individual transporters. These data now form the base for detailed investigations on the substrate specificity for various osmoprotectants of the OpuA, OpuC, and OpuD transport systems; their genetic regulation by environmental factors; and the integration of these transporters into the cellular framework that allows *B. subtilis* to cope effectively with high-osmolarity stress.

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