

# Lipoprotein from the Osmoregulated ABC Transport System OpuA of *Bacillus subtilis*: Purification of the Glycine Betaine Binding Protein and Characterization of a Functional Lipidless Mutant

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**The OpuA transport system of *Bacillus subtilis* functions as a high-affinity uptake system for the osmoprotectant glycine betaine. It is a member of the ABC transporter superfamily and consists of an ATPase (OpuAA), an integral membrane protein (OpuAB), and a hydrophilic polypeptide (OpuAC) that shows the signature sequence of lipoproteins (B. Kempf and E. Bremer, J. Biol. Chem. 270:16701–16713, 1995). The OpuAC protein might thus serve as an extracellular substrate binding protein anchored in the cytoplasmic membrane via a lipid modification at an amino-terminal cysteine residue. A *malE-opuAC* hybrid gene was constructed and used to purify a lipidless OpuAC protein. The purified protein bound radiolabeled glycine betaine avidly and exhibited a  $K_D$  of 6  $\mu$ M for this ligand, demonstrating that OpuAC indeed functions as the substrate binding protein for the *B. subtilis* OpuA system. We have selectively expressed the *opuAC* gene under T7  $\phi$ 10 control in *Escherichia coli* and have demonstrated through its metabolic labeling with [<sup>3</sup>H]palmitic acid that OpuAC is a lipoprotein. A mutant expressing an OpuAC protein in which the amino-terminal cysteine residue was changed to an alanine (OpuAC-3) was constructed by oligonucleotide site-directed mutagenesis. The OpuAC-3 protein was not acylated by [<sup>3</sup>H]palmitic acid, and part of it was secreted into the periplasmic space of *E. coli*, where it could be released from the cells by cold osmotic shock. The *opuAC-3* mutation was recombined into an otherwise wild-type *opuA* operon in the chromosome of *B. subtilis*. Unexpectedly, this mutant OpuAC system still functioned efficiently for glycine betaine acquisition *in vivo* under high-osmolarity growth conditions. In addition, the mutant OpuA transporter exhibited kinetic parameters similar to that of the wild-type system. Our data suggest that the lipidless OpuAC-3 protein is held in the cytoplasmic membrane of *B. subtilis* via its uncleaved hydrophobic signal peptide.**

ATP binding cassette (ABC) transporters or traffic ATPases form a large superfamily of structurally related uptake and export systems that are found in all three kingdoms (3, 6, 19). The characteristic feature of these transporters is the obligatory coupling of ATP hydrolysis to substrate translocation. Binding-protein-dependent uptake systems are a subfamily of these ABC transporters and have been intensively characterized in gram-negative bacteria (6). The periplasmic substrate binding protein is usually present in large excess (31) and serves to capture the substrate with high affinity and deliver it to the membrane-bound transport complex. The high substrate affinity and the ability of binding-protein-dependent transport systems to accumulate the substrate within the cell against a steep concentration gradient allow the efficient scavenging of nutrients and ions, even when these compounds are present in minute concentrations in the environment (6).

The periplasmic location of the key component of the microbial ABC transporters originally suggested that binding-protein-dependent uptake systems were present only in gram-negative bacteria. However, this type of transport system is also prevalent in gram-positive bacteria (2, 12, 14), and recent reports indicate its presence in archaea as well (22, 54). It has been proposed that to compensate for the absence of an outer membrane, gram-positive microorganisms possess a lipid modification at the amino-terminal cysteine residue of the mature substrate binding protein that would anchor it in the cytoplas-

mic membrane and prevent its loss into the surrounding medium (14, 35). Lipoproteins are initially synthesized as lipid-modified precursors, and their proteolytic maturation is mediated by a lipoprotein-specific signal peptidase (8, 45), which can be specifically inhibited by the antibiotic globomycin (21).

We have recently identified a binding-protein-dependent ABC transport system (OpuA) in *Bacillus subtilis* in connection with our studies on the adaptation of this gram-positive soil bacterium to high-osmolarity environments (26). The OpuA system is the dominating transporter for the osmoprotectant glycine betaine in *B. subtilis* (24). The high-level intracellular accumulation of this trimethylammonium compound confers a high degree of osmotic tolerance and allows the growth of *B. subtilis* under conditions that are otherwise strongly inhibitory for its proliferation (5, 24, 26, 30, 51). The OpuA system displays the minimal configuration of bacterial ABC transporters (3, 6, 19) and consists of three components: an ATPase (OpuAA), an integral membrane protein (OpuAB), and a hydrophilic polypeptide (OpuAC), which likely functions as the substrate binding protein (26). In analogy with biochemically well-studied binding-protein-dependent transport systems, the OpuAA ATPase and the OpuAB translocase are likely to function as homodimers (6). OpuA is both structurally and functionally related to the ProU transport systems for glycine betaine from *Escherichia coli* and *Salmonella typhimurium*, each of which features a soluble, periplasmic substrate binding protein (4, 20, 33). The ATPases and integral membrane components of both the OpuA and ProU systems show a considerable degree of sequence identity, whereas the substrate binding proteins are only distantly related (16, 26).

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TABLE 1. *B. subtilis* strains used

Strain <sup>a</sup>	Relevant genotype	Reference or construction <sup>b</sup>
BKB4	$\Delta(\text{opuA-amhX::neo})1$	26
BKB7	$\Delta(\text{opuA::tet})2$	26
BKB13	$(\text{amhX::erm})1 \text{ opuA}^+$	27
JBB51	$\text{opuB-20::Tn10 (spc) opuC-21}$	23
RMKB2	$\Delta(\text{opuA::tet})2 \Delta(\text{opuD::neo})2$	24
BKB14	$(\text{amhX::erm})1 \text{ opuAC-3}$	pBKB82→BKB4 = Em <sup>r</sup> Kn <sup>s</sup>
BKB17	$\text{opuB-20::Tn10 (spc) opuC-21}$ $\Delta(\text{opuD::neo})2$	RMKB2→JBB51 = Kn <sup>r</sup>
BKB18	$\text{opuB-20::Tn10 (spc) opuC-21}$ $\Delta(\text{opuD::neo})2$ $\Delta(\text{opuA::tet})2$	BKB7→BKB17 = Tc <sup>r</sup>
BKB20	$\text{opuB-20::Tn10 (spc) opuC-21}$ $\Delta(\text{opuD::neo})2$ $(\text{amhX::erm})1 \text{ opuA}^+$	BKB13→BKB18 = Em <sup>r</sup>
BKB21	$\text{opuB-20::Tn10 (spc) opuC-21}$ $\Delta(\text{opuD::neo})2$ $(\text{amhX::erm})1 \text{ opuAC-3}$	BKB14→BKB18 = Em <sup>r</sup>

<sup>a</sup> All *B. subtilis* strains are derivatives of strain JH642 (*trpC2 pheA1*; BGSC 1A96).

<sup>b</sup> An arrow indicates transformation.

Inspection of the *opuAC* DNA sequence indicates that the OpuAC protein is initially synthesized as a precursor molecule, pro-OpuAC, with a signal sequence of 20 amino acid residues (26). The residues in the vicinity of the putative processing site (Leu-Ala-Ala-Cys) perfectly match the signature sequence for lipoproteins, suggesting that the amino-terminal Cys residue of the mature protein is covalently modified by the typical ester-linked and amide-linked acylation of lipoproteins (8, 45). Processing of the OpuAC precursor in the heterologous host *E. coli* can be inhibited by globomycin, and the finding that OpuAC is essential for the OpuA-mediated glycine betaine uptake (26) suggests that this protein functions as an extracellular substrate binding protein tethered to the *B. subtilis* cytoplasmic membrane via a lipid anchor.

In this study, we have used metabolic labeling with radiolabeled palmitate and site-directed mutagenesis to conclusively demonstrate that OpuAC is indeed a lipoprotein. In addition, we provide a functional analysis of the purified OpuAC protein and show that it serves as the high-affinity glycine betaine binding protein for the *B. subtilis* OpuA system.

#### MATERIALS AND METHODS

**Growth conditions, media, and chemicals.** Rich and minimal media for the growth of *B. subtilis* and *E. coli* strains were as previously described (26). All cultures were grown aerobically at 37°C. Growth of *B. subtilis* strains in low- and high-osmolarity media was performed in Spizizen's minimal medium (SMM). For growth curves, 75 ml of prewarmed medium in 500-ml Erlenmeyer flasks was inoculated with fresh overnight cultures to an optical density at 578 nm (OD<sub>578</sub>) of 0.15 and incubated in a water bath shaker at 37°C at 220 rpm. The osmolarity of SMM was raised by the addition of NaCl from concentrated stock solutions. The antibiotics ampicillin and chloramphenicol were used with *E. coli* strains at final concentrations of 100 and 30 µg/ml, respectively. Erythromycin, kanamycin, spectinomycin, and tetracycline were used with *B. subtilis* strains at final concentrations of 0.4, 5, 100, and 10 µg/ml, respectively. Radiolabeled [<sup>1-14</sup>C]glycine betaine (55 mCi/mmol) was purchased from American Radiolabeled Chemicals Inc., St. Louis, Mo. [<sup>9,10(n)-3</sup>H]palmitic acid (toluene solution; 52.0 mCi/mmol) and [<sup>35</sup>S]dATP (>1,000 Ci/mmol) were purchased from Amersham Buchler (Braunschweig, Germany).

**Bacterial strains, plasmids, and construction of *B. subtilis* mutants.** All *B. subtilis* strains used in this study are derivatives of the wild-type strain JH642 (*trpC2, pheA1*; BGSC 1A96) and are listed in Table 1. The *E. coli* K-12 strain MKH13 [*putPA(101) Δ(proP)2 Δ(proU)608*], a derivative of MC4100, is entirely deficient in glycine betaine uptake and lacks the *proU*-encoded glycine betaine binding protein (ProX) (18). The *E. coli* B strain BL21(λDE3) was used for the selective expression of genes under the control of the T7 φ10 promoter (44). The low-copy-number cloning vector pHSG575 (48) was used for the construction of several plasmids carrying different *opuA* alleles. The low-copy-number T7 φ10

expression vector pPD100 (11) was used to express the wild-type and mutant OpuAC proteins. The *malE* fusion vector pMAL-c2 (New England Biolabs, Schwalbach, Germany) (17) was used to construct the *malE-opuAC* hybrid gene. The DNA cartridge encoding the gene that confers resistance to erythromycin was isolated from plasmid pBKB80 (27). The filamentous bacteriophage M13BM20 (Boehringer Mannheim, Mannheim, Germany) was used as a vector for site-directed mutagenesis experiments.

**Methods used with nucleic acids and construction of plasmids.** Manipulations of plasmid DNA and the isolation of chromosomal DNA from *B. subtilis* were done according to routine procedures (40). Sequencing reactions of single-stranded M13 DNA, double-stranded plasmid DNA, and quick-chilled PCR products were done with a Sequenase 2.0 kit and 7-deaza-dGTP as recommended by the supplier (U.S. Biochemical Corp., Braunschweig, Germany). Plasmids pBKB1 (*opuA*<sup>+</sup>), pBKB52 [ $\Delta(\text{opuA::tet})2$ ], pBKB58 (T7 φ10-*opuA*<sup>+</sup>), and pBKB81 [ $(\text{amhX::erm})1$ ] are derivatives of the low-copy-number cloning vector pHSG575 (48) and have been described elsewhere (26, 27). For the construction of a *malE-opuAC* fusion, we first amplified the *opuAC* coding region from chromosomal DNA of strain JH642 by PCR, using a set of primers (5'-CCGGGAGTACTGGTTCAGAAAACATGAAAATG-3', bp 2395 to 2416 in the *opuA* operon; 5'-GGGCCTGCAGTTACTTCTTCAAATCTTC-3', bp 3213 to 3196 in the *opuA* operon) (26) with synthetic restriction recognition sequences (underlined). The PCR fragment was cut with *ScaI* and *PstI* and inserted into the *malE* fusion vector pMAL-c2 which had been cleaved with *XmnI* and *PstI*. The resulting plasmid, pBKB76, carries an in-frame *malE-opuAC* translational fusion under the control of the *tac* promoter. This *malE-opuAC* hybrid gene lacks the coding region for the *opuAC* signal sequence and carries a threonine codon (ACT) instead of a cysteine codon (TGC) at the beginning of the *opuAC* reading frame.

Site-directed mutagenesis of the *opuAC* gene was carried out with the Sculptor in vitro mutagenesis system (Amersham Buchler). The switch from a cysteine (TGC) to an alanine (GCC) codon was performed with a synthetic oligonucleotide (5'-CACTTGCGGCTGCCGGTTCAGAAAAC-3'; mismatches underlined), using single-stranded DNA of an M13BM20 clone carrying the *ApaLI-NotI* fragment from the *opuA*<sup>+</sup> plasmid pBKB1 (Fig. 1) as a template. After the mutagenesis reaction, the entire *ApaLI-NotI* fragment was sequenced, and only the primer-derived *opuAC-3* mutation was present. Plasmid pBKB79 [*opuAC-3*] was obtained by ligating a 1.3-kb *BstXI-NotI* restriction fragment from the mutant M13 clone into the [ $\Delta(\text{opuA::tet})2$ ] plasmid pBKB52 which had been cleaved with the same enzymes. The resulting plasmid, pBKB79, thus differs from the wild-type plasmid pBKB1 (Fig. 1) only by the presence of the *opuAC-3* mutation. Plasmid pBKB82 [*opuAC-3 (amhX::erm)1*] (Fig. 1) was constructed by insertion of the erythromycin resistance cassette obtained as a *SmaI-EcoRV* fragment from pBKB80 into the blunt-ended *NotI* restriction site present in pBKB79 (Fig. 1). The presence of the *opuAC-3* allele in pertinent plasmids and strains was verified by sequencing either double-stranded plasmid DNA or PCR products derived from the chromosomes of the appropriate strains. The T7 φ10 expression plasmid pBKB83 (*opuAC-3*<sup>+</sup> [Fig. 1]) was constructed by ligation of the *ApaLI-NotI* restriction fragment from pBKB79 into plasmid pPD100.

**Preparation of total-cell extracts, SDS-polyacrylamide gel electrophoresis, and immunological detection of the OpuAC protein.** Total-cell extracts from *B. subtilis* and *E. coli* cultures were prepared as described previously (26). For the immunological detection of the OpuAC and OpuAC-3 proteins, bacterial cell extracts or protein preparations were electrophoretically separated on sodium dodecyl sulfate (SDS)-13.5% polyacrylamide gels (29) and transferred (49) onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Eschwege, Germany). The bound proteins were then probed with a 1:5,000 dilution of a rabbit OpuAC antiserum. The OpuAC antibody complexes were subsequently visualized with a second goat anti-rabbit immunoglobulin G-alkaline phosphatase-conjugated antibody (Sigma, Deisenhofen, Germany), using 5-bromo-4-chloro-3-indolylphosphate and nitrobluetetrazolium chloride (Boehringer Mannheim) as substrates. Protein concentrations were determined by using the Bio-Rad protein assay, with bovine serum albumin as the standard.

**Expression and labeling of the OpuAC and OpuAC-3 proteins.** *E. coli* BL21(λDE3), which carries the gene for the T7 RNA polymerase under control of the *lacPO* promoter (44), was used as the host to express the *opuAC* and *opuAC-3* genes under T7 φ10 control (11, 26). For labeling of the wild-type OpuAC and mutant OpuAC-3 proteins with [<sup>9,10(n)-3</sup>H]palmitic acid, we used the procedure of Ehlert et al. (13), with slight modifications. Strains BL21 (pPD100), BL21(pBKB58), and BL21(pBKB83) were grown in M9 minimal medium with chloramphenicol, 0.2% glucose, and 0.2% Casamino Acids. When the cultures reached mid-exponential phase (OD<sub>578</sub> = 0.4), 10-ml portions were transferred to sterile 100-ml Erlenmeyer flasks in which 50 µCi of a toluene solution of [<sup>9,10(n)-3</sup>H]palmitic acid had been evaporated under a gentle nitrogen stream. Expression of the plasmid-encoded genes was induced by adding 1 mM isopropylthiogalactopyranoside (IPTG) to the culture and further shaking of the flasks at 37°C in a water bath. After 45 min, rifampin was added to a final concentration of 200 µg/ml for another 45 min to inhibit the synthesis of host proteins. Thereafter, the cells were harvested by centrifugation (10 min at 850 × g), washed once with 50 mM sodium phosphate (pH 7.4)-10 mM MgCl<sub>2</sub>-12% sucrose, and resuspended in 120 µl of 20 mM Tris-HCl (pH 8.0)-1 mM EDTA-1% SDS. Lysis of the cells was completed by incubating the suspension for 10 min at 95°C. After cooling to room temperature, 1 µl of benzonuclease

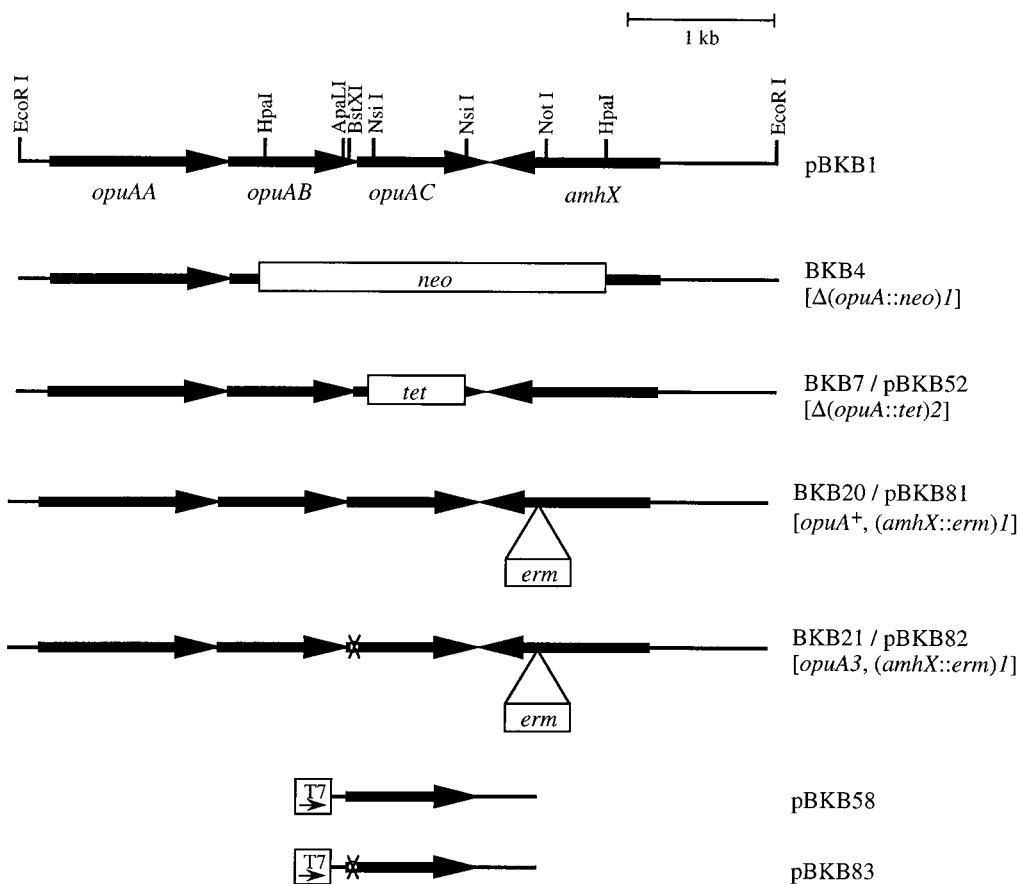


FIG. 1. Genetic and physical organization of the *opuA* region. The upper part shows the positions and directions of transcription of the three structural genes of the *opuA* operon (*opuAA*, *opuAB*, and *opuAC*) (26) and the flanking amidohydrolase *amhX* gene (27) together with some restriction sites. The lower part shows the *opuA* mutant alleles and plasmids used in this study. Plasmids pBKB58 and pBKB83 were used to express the *opuAC* and *opuAC-3* genes under T7  $\phi$ 10 control. The small cross indicates the position of the *opuAC-3* mutation; the *neo*, *tet*, and *erm* antibiotic resistance cassettes are not drawn to scale.

(Benzonase; Merck, Darmstadt, Germany) was added, and the samples were incubated at 37°C for 10 min to reduce their viscosity. After a 10-min centrifugation in an Eppendorf centrifuge (13,000 rpm), 100  $\mu$ l of the supernatant was removed and added to 1 ml of ice-cold acetone. After incubation at 4°C overnight, the samples were recentrifuged and the delipidated protein pellet was washed in 50% ice-cold ethanol. Finally, the proteins were solubilized in 50  $\mu$ l of a 1% SDS solution and kept at 50°C for 5 min. The radioactivity of the various samples was determined by scintillation counting, and portions (corresponding to approximately 800,000 cpm) were mixed with an appropriate amount of concentrated SDS-sample buffer (40). The proteins were separated on an SDS-12% polyacrylamide gel and visualized by fluorography, using Amplify (Amersham Buchler). Periplasmic proteins were prepared from IPTG-induced (26) cultures of *E. coli* BL21(pBKB58 [*opuAC*<sup>+</sup>]) and BL21(pBKB83 [*opuAC-3*]) according to the cold osmotic shock procedure of Neu and Heppel (34).

**Inhibition of pro-OpuAC processing in *B. subtilis* by globomycin.** Strains JH642 (*opuAC*<sup>+</sup>) and BKB21 (*opuAC-3*) were grown in SMM with 0.4 M NaCl at 37°C to mid-exponential phase ( $OD_{578} = 0.5$ ), and the antibiotic globomycin was then added to a final concentration of 200  $\mu$ g/ml. The cultures were grown for additional 30 min, the cells were harvested by centrifugation, and OpuAC-related proteins were visualized by Western blotting using the polyclonal OpuAC antiserum.

**Overproduction and purification of the OpuAC protein and preparation of an OpuAC antiserum.** Plasmid pBKB76 carrying the maltose binding protein (MBP)-OpuAC protein fusion was transformed into *E. coli* MKH13, which lacks the entire *proU* operon (18). Expression of the hybrid gene in a 2-liter culture ( $OD_{578} = 0.5$ ) of MKH13(pBKB76) grown in LB with 0.2% glucose was induced by the addition of IPTG (final concentration, 0.3 mM). After 2 h, the cells were harvested by centrifugation, resuspended in 20 ml of column buffer (20 mM Tris-HCl [pH 7.4], 200 mM NaCl, 1 mM EDTA), and disrupted by four passages through a French pressure cell (10<sup>8</sup> Pa). Cellular debris was removed by centrifugation (30 min) at 3,000  $\times$  g. Eight milliliters of the supernatant was loaded onto a 15-ml amylose column (1.6 by 20 cm). The column was washed with column buffer until the  $OD_{280}$  reached the baseline, and the hybrid truncated

MBP (MBP<sup>\*</sup>)-OpuAC protein was eluted from the amylose resin with column buffer containing 10 mM maltose; approximately 25 mg of fusion protein was isolated. In total, 75 mg of fusion protein was proteolytically cleaved by incubation with 60  $\mu$ g of factor Xa (1 h at room temperature and subsequent incubation at 4°C for 16 h). The preparation was extensively dialyzed against 5 liters of buffer A (10 mM Tris-HCl [pH 8], 25 mM NaCl) and was then passed over a MonoQ 10/10 column (Pharmacia, Freiburg, Germany). From 75 mg of proteolytically cleaved MBP<sup>\*</sup>-OpuAC protein loaded onto the MonoQ column, approximately 15 mg of pure OpuAC protein was found in the column flowthrough. The MBP<sup>\*</sup> protein and the factor Xa protease eluted from the column at concentrations of 150 and 400 mM NaCl in buffer A, respectively. The OpuAC protein was extensively dialyzed against 5 liters of 10 mM Tris-HCl (pH 7.4) and stored at -20°C. To obtain an antiserum against the OpuAC protein, 80  $\mu$ g of pure OpuAC protein was dissolved in 150  $\mu$ l of phosphate-buffered saline, mixed with 150  $\mu$ l of Freund's complete adjuvant (Behringwerke AG, Marburg, Germany), and injected into a rabbit at days 1, 2, 3, 7, 21, and 35. The animal was bled at day 44, and the quality of the OpuAC antiserum was tested by Western blotting using OpuAC<sup>+</sup> and OpuAC<sup>-</sup> extracts of *B. subtilis* cells.

**Binding tests with purified OpuAC protein.** The ammonium sulfate precipitation technique of Richarme and Kepes (38) was used to study the substrate binding properties of the OpuAC protein. For competition experiments, the concentration of OpuAC or the MBP<sup>\*</sup>-OpuAC fusion protein was held at a 5  $\mu$ M, and the proteins were incubated with 5  $\mu$ M [<sup>14</sup>C]glycine betaine and various concentrations (0.5  $\mu$ M to 2 mM) of unlabeled glycine betaine, choline, or proline. All experiments were done in a volume of 0.1 ml of 10 mM Tris-HCl (pH 7.4). The samples were equilibrated for 5 min at room temperature, and the proteins were then precipitated by the addition of 0.9 ml of an ice-cold, saturated ammonium sulfate solution. After a 3-min incubation on ice, the mixture was filtered through wet, 0.45- $\mu$ m-pore-size filters (ME25; Schleicher & Schuell, Dassel, Germany) and washed twice with 10 ml of an ice-cold, saturated ammonium sulfate solution. The radioactivity retained on the filters was measured by liquid scintillation counting. To determine the  $K_D$  of the binding reaction, the final concentration of the OpuAC protein was 5  $\mu$ M and the concentration of its

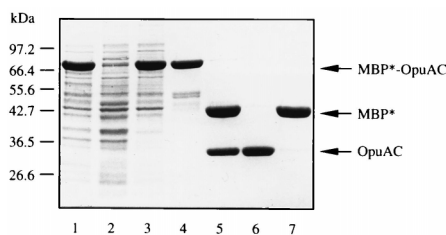


FIG. 2. Purification of the OpuAC glycine betaine binding protein. Expression of the *malE-opuAC* fusion in exponentially growing cells of *E. coli* MKH13(pBKB76) was induced by the addition of 0.3 mM IPTG. Lane 1, induced cell extract; lane 2, uninduced cell extract; lane 3, induced extract after passage through a French pressure cell; lane 4, MBP\*-OpuAC fusion protein eluted from the amylose column with maltose; lane 5, MBP\* and OpuAC proteins after cleavage of the hybrid MBP\*-OpuAC fusion protein with factor Xa; lane 6, OpuAC protein after chromatography on MonoQ; lane 7, MBP\* protein after chromatography on MonoQ. The proteins were electrophoretically separated on an SDS-12% polyacrylamide gel and visualized by staining with Coomassie brilliant blue; 5  $\mu$ g of protein was loaded per lane. MBP\* is a functional fragment of the *malE*-encoded MBP.

substrate [ $^{14}$ C]glycine betaine was varied between 1 and 100  $\mu$ M. For these tests, substrate concentrations greater than 10  $\mu$ M were obtained by adding unlabeled glycine betaine to a fixed concentration (5  $\mu$ M) of radiolabeled glycine betaine. The measured radioactivity was appropriately corrected.

**Transport assays.** Uptake of [ $^{14}$ C]glycine betaine by bacterial cells grown in SMM or SMM with 0.4 M NaCl was measured as described previously (26) except that the cells were maintained at 37°C during the transport assays. For the kinetic studies, the glycine betaine concentration in the uptake assays was varied between 1 and 70  $\mu$ M.

## RESULTS

**Purification of the OpuAC protein.** To investigate the biochemical properties of the OpuAC protein, we made use of the MBP fusion technique (17). We used for the construction of a *malE-opuAC* hybrid a truncated *malE* gene product lacking its signal sequence (MBP\*) and fused the *opuAC* coding region to the *malE* frame in such a way that the resulting hybrid protein lacked the OpuAC signal sequence peptide and the cysteine residue required for lipid modification. The fusion of both genes was engineered such that a cleavage site for the protease Xa was present at the MBP\*-OpuAC junction. The *malE-opuAC* plasmid pBKB76 was introduced into *E. coli* MKH13, which lacks the entire *proU* operon (18), so that a possible contamination of the MBP\*-OpuAC preparation with the *proX*-encoded glycine betaine binding protein was avoided. The MBP\*-OpuAC fusion protein was purified by affinity chromatography on an amylose resin, and the MBP\* and OpuAC portions were liberated from the hybrid protein after proteolytic cleavage with factor Xa (Fig. 2). Separation of the two polypeptides and their further purification was then achieved by ion-exchange chromatography on a MonoQ column, yielding essentially homogeneous preparations of both the OpuAC and MBP\* proteins (Fig. 2).

**Determination of the dissociation constant of the OpuAC protein for glycine betaine.** To demonstrate the function of OpuAC as the substrate binding protein for the OpuA-mediated glycine betaine transport, we characterized the binding of radiolabeled glycine betaine to the purified OpuAC protein. For these experiments, we used the ammonium sulfate precipitation technique of Richarme and Kepes (38) to study the interaction of the substrate binding protein with its ligand. The purified OpuAC protein was incubated at room temperature with [ $^{14}$ C]glycine betaine for 5 min. The proteins were then precipitated by the addition of a cold saturated ammonium sulfate solution and were collected by filtration. The OpuAC protein bound glycine betaine avidly, whereas there was no

binding of radiolabeled glycine betaine by the purified MBP\*. Neither proline nor choline was able to compete with radiolabeled glycine betaine for the binding to the purified OpuAC protein (data not shown). Interestingly, the MBP\*-OpuAC hybrid protein also bound [ $^{14}$ C]glycine betaine effectively and showed substrate binding characteristics virtually identical to those of the purified OpuAC protein. This observation suggests that the MBP\* and OpuAC moieties of the hybrid protein properly fold as functionally independent domains in the MBP\*-OpuAC fusion protein.

To determine the dissociation constant ( $K_D$ ) of OpuAC for its substrate (38), we measured the binding of [ $^{14}$ C]glycine betaine over a wide range of concentrations (1 to 100  $\mu$ M), using a fixed amount of OpuAC (5  $\mu$ M) in the binding assay. Substrate binding of OpuAC showed saturation kinetics. Even at substrate concentrations as low as 1  $\mu$ M, radiolabeled glycine betaine was effectively retained by the OpuAC protein. By assuming that OpuAC possesses a single binding site for glycine betaine, we calculate a  $K_D$  of approximately 6  $\mu$ M for this substrate. This value attests to the high affinity of the OpuA system for its substrate and closely matches that of the Michaelis constant ( $K_m = 2.4$  to 5  $\mu$ M) measured in vivo for the OpuA-mediated uptake of the osmoprotectant glycine betaine (24, 25).

**Labeling of OpuAC with [ $^3$ H]palmitate.** To directly demonstrate that OpuAC is a lipoprotein, we attempted to metabolically label it with [ $^3$ H]palmitic acid. For these experiments, we used a low-copy-number expression vector carrying the *opuAC*<sup>+</sup> gene (pBKB58) under the control of the inducible T7  $\phi$ 10 promoter (Fig. 1). The T7 RNA polymerase/T7  $\phi$ 10 promoter system was chosen since it allows the selective expression of *opuAC* and the simultaneous inhibition of synthesis of the host proteins by the addition of rifampin to the cultures (44). Induction of T7  $\phi$ 10-mediated *opuAC* expression in *E. coli* BL21(pBKB58) resulted in the synthesis of an approximately 30-kDa polypeptide, which corresponds to the OpuAC protein (26) (Fig. 3A, lanes 4 and 5). Labeling of the cultures by the addition of [ $^3$ H]palmitic acid led to the production of a

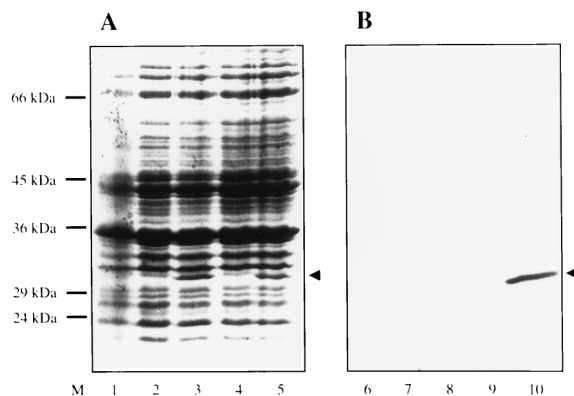


FIG. 3. Expression of OpuAC and OpuAC-3 under the control of the T7  $\phi$ 10 promoter and labeling with [ $^{9,10(n)-3}$ H]palmitic acid. T7  $\phi$ 10-mediated expression of the *opuAC* and *opuAC-3* genes was performed in derivatives of *E. coli* BL21 carrying the vector pPD100 (induced with 1 mM IPTG [lanes 1 and 6]), the *opuAC-3*<sup>+</sup> plasmid pBKB83 (uninduced [lanes 2 and 7] or induced [lanes 3 and 8]), or the *opuAC*<sup>+</sup> plasmid pBKB58 (uninduced [lanes 4 and 9] or induced [lanes 5 and 10]). After induction, the cultures were split into two portions and the proteins were electrophoretically separated on SDS-13.5% polyacrylamide gels. (A) The proteins from the cell extract were visualized by staining with Coomassie brilliant blue. (B) Fluorograph of the proteins from the [ $^{9,10(n)-3}$ H]palmitic acid-labeled cell culture; the film was exposed overnight. A longer exposure (10 days) of the gel revealed the presence of several other host-encoded lipoproteins.

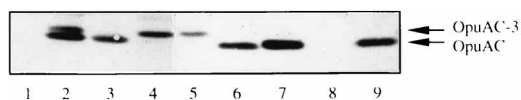


FIG. 4. Immunological detection of the wild-type and mutant OpuAC proteins. The wild-type and mutant *opuAC* genes were expressed under the control of the T7  $\phi$ 10 promoter in *E. coli* BL21 carrying plasmid pBKB58 (*opuAC*<sup>+</sup>) or pBKB83 (*opuAC-3*). The proteins were electrophoretically separated on an SDS-13.5% polyacrylamide gel, blotted onto a polyvinylidene difluoride membrane, and probed with a polyclonal anti-OpuAC antiserum. Lane 1, BL21 (pPD100 [expression vector]), total-cell extract; lane 2, BL21(pBKB83), total-cell extract; lane 3, BL21(pBKB83), osmotic shock fluid; lane 4, BL21(pBKB83), osmotically shocked cells; lane 5, *B. subtilis* BKB21 (*opuAC-3*), total-cell extract; lane 6, *B. subtilis* JH642 (wild type), total-cell extract; lane 7, BL21(pBKB58), total-cell extract; lane 8, BL21(pBKB58), osmotic shock fluid; lane 9, BL21 (pBKB58), osmotically shocked cells. Since the OpuAC antiserum cross-reacts with the mutant OpuAC-3 protein at a strongly reduced level, the amount of protein loaded onto the gel was adjusted to allow a clear detection of the different protein species in cells synthesizing the OpuAC-3 protein. The protein samples loaded in lanes 6 to 9 were 50-fold diluted in comparison to those loaded in lanes 1 to 5.

single labeled protein with an apparent molecular mass of 30 kDa in the induced cells but not in the uninduced culture (Fig. 3B, lanes 9 and 10). It is thus apparent that the OpuAC protein is covalently modified *in vivo* with a lipid.

To demonstrate that the mature OpuAC protein was acylated at the amino-terminal cysteine residue, we used site-directed mutagenesis to replace the codon for this cysteine (TGC) with a codon (GCC) for an alanine residue. The resulting mutant *opuAC* gene (*opuAC-3*) was cloned in the T7  $\phi$ 10 expression vector pPD100 (11), yielding plasmid pBKB83 (Fig. 1). The OpuAC-3 protein was produced in induced cultures of BL21(pBKB83) in amounts comparable to that of the wild-type OpuAC protein (Fig. 3A, lanes 3 and 4). However, it was not metabolically labeled with [<sup>3</sup>H]palmitic acid (Fig. 3B, lanes 7 and 8). Taken together, these data provide strong evidence that the *B. subtilis* OpuAC protein is a lipoprotein with a lipid modification at its amino-terminal cysteine residue.

**Export of the mutant OpuAC-3 protein into the periplasm of *E. coli*.** The precursors of lipoproteins are specifically cleaved by signal peptidase II (8, 45). Proteolytic cleavage of the OpuAC precursor by this protease is suggested by the inhibition of pro-OpuAC processing in the presence of the peptide antibiotic globomycin (26). The above-described replacement of the amino-terminal cysteine residue of OpuAC by the amino acid alanine in the mutant OpuAC-3 protein should result in the switching of the cleavage site for signal peptidase II (Leu-Ala-Ala-Cys) (8, 45) for one that should be recognized by signal peptidase I (Leu-Ala-Ala-Ala) (50). Indeed, in contrast to the processing of the wild-type OpuAC protein, cleavage of the pro-OpuAC-3 protein was no longer inhibited by globomycin in *E. coli* (data not shown).

Processing of the lipidless pro-OpuAC-3 protein by signal peptidase I should result in a periplasmic localization of the mutant protein in *E. coli*. We expressed the *opuAC-3* gene under T7  $\phi$ 10 control in *E. coli* BL21 and monitored OpuAC-3 production in Western blot experiments using a polyclonal antiserum (Fig. 4). We detected two cross-reacting bands for the OpuAC-3 polypeptide in total-cell extracts (Fig. 4, lane 2). A cold osmotic shock (34) quantitatively released the electrophoretically faster-migrating form from the cells, suggesting a periplasmic location for this protein species (Fig. 4, lane 3). In contrast, the slower-migrating cross-reacting band remained exclusively associated with the cells (Fig. 4, lane 4). Thus, these two protein species are likely to represent the unprocessed precursor and the mature form of OpuAC-3.

Fractionation experiments in *E. coli* cells expressing the

wild-type *opuAC* gene yielded a pattern different from that of the mutant OpuAC-3 protein. Only one cross-reacting protein species was observed in cell extracts of strain BL21(pBKB58), and its electrophoretic mobility was identical to that of the OpuAC protein synthesized by *B. subtilis* (Fig. 4, lanes 6 and 7). As expected from the anchorage of the wild-type OpuAC protein by its lipid tail in the cytoplasmic membrane, none of the OpuAC protein was released from the periplasm of *E. coli* by the cold osmotic shock procedure (Fig. 4, lanes 8 and 9).

We conclude from these data that the loss of the lipid modification of the cysteine residue in the OpuAC-3 protein and the concomitant switch in the processing site from the lipoprotein-specific signal peptidase II to signal peptidase I allows export of the *B. subtilis* OpuAC-3 protein into the periplasmic space of the heterologous *E. coli* host. Processing of the OpuAC-3 precursor is inefficient, and it appears that the precursor of OpuAC-3 remains tethered to the cytoplasmic membrane via its hydrophobic signal sequence peptide.

**Construction and characterization of a chromosomal *opuAC-3* mutant.** To study the influence of the *opuAC-3* allele on the glycine betaine transport activity of the OpuA system, we used homologous recombination to introduce this mutation into the chromosome of *B. subtilis*. For this purpose, we first transferred the *opuAC-3* allele into an *opuA*<sup>+</sup> plasmid by recombinant DNA procedures and then inserted an erythromycin resistance cassette as a selectable marker into *amhX*, a nonessential amidohydrolase gene (27) located downstream of the *opuA* operon (Fig. 1). DNA of the resulting plasmid (pBKB82) was linearized and transformed into the  $\Delta$ (*opuA::neo*)1 mutant strain BKB4 (Fig. 1), and erythromycin-resistant transformants were selected. The use of this acceptor strain allowed us to identify erythromycin-resistant, kanamycin-sensitive recombinants in which the  $\Delta$ (*opuA::neo*)1 deletion of BKB4 was replaced by *opuA* sequences from pBKB82 (*opuAC-3*). The resulting strain thus carries the *opuAC-3* allele in an otherwise wild-type *opuA* operon (Fig. 1). The expected physical structure of the mutant chromosomal *opuA* locus was verified by PCR amplifications using primers flanking the *opuA* operon, and the presence of the *opuAC-3* mutation in the *B. subtilis* chromosome was proven by direct DNA sequence analysis of appropriate PCR products (data not shown).

We probed the synthesis of the wild-type OpuAC and the OpuAC-3 proteins in Western blot experiments using total protein extracts from *B. subtilis* cultures grown to mid-log phase. The polyclonal OpuAC antiserum reacted in the wild-type strain JH642 with a protein of the expected size (approximately 30 kDa) (26) that was absent in a strain carrying a deletion of the *opuAC* gene [ $\Delta$ (*opuA::tet*)2 (Fig. 1)] (Fig. 5, lanes 1 and 5). In the strain synthesizing the OpuAC-3 protein, the antiserum detected a polypeptide with an electrophoretic

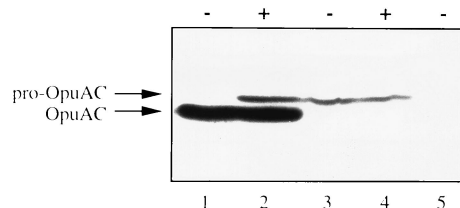


FIG. 5. Globomycin has no influence on the processing of the mutant OpuAC-3 protein *in vivo*. Mid-exponential-phase cultures of strains JH642 (*opuAC*<sup>+</sup>) (lanes 1 and 2) and BKB21 (*opuAC-3*) (lanes 3 and 4) were grown in the presence (+) or absence (-) of globomycin, and proteins related to OpuAC were visualized by Western blotting with a polyclonal OpuAC antiserum. The  $\Delta$ (*opuA::tet*)2 strain BKB18 (lane 5) served as a control.

mobility lower than that of the OpuAC protein (Fig. 5, lane 3). The size of this cross-reacting protein corresponded to that of the precursor of OpuAC-3 synthesized in the heterologous *E. coli* host (Fig. 4; compare lanes 4 and 5), suggesting that this *B. subtilis* protein species represents uncleaved pro-OpuAC-3. We did not detect any protein species in total-cell extracts that could correspond to the processed form of OpuAC-3. Since this protein species will not carry a lipid modification tethering it to the cytoplasmic membrane, one might expect to find it in the culture supernatant. However, despite repeated attempts, we were unable to detect the OpuAC-3 protein or a proteolytic degradation product in lyophilized samples of the growth medium. These data suggest either that the pro-OpuAC-3 precursor is not processed in *B. subtilis* at all or that the mature OpuAC-3 protein is so unstable that it cannot be detected in the Western blotting experiments. We separated total-cell extracts from the *opuA*<sup>+</sup> and *opuAC-3* strains BKB20 and BKB21 by high-speed ultracentrifugation into soluble and membrane-cell envelope fractions and probed them with the OpuAC antiserum in Western blotting experiments. The major portion of both the wild-type OpuAC and the mutant OpuAC-3 proteins were found in the 180,000 × *g* pellet (data not shown), suggesting that both proteins are located in the cell membrane. Both protein species were also present in the supernatant, indicating that the mechanical disruption of the bacterial cells in a French press partially releases the membrane-tethered polypeptides from the cytoplasmic membrane.

To demonstrate that the mutant OpuAC-3 protein was no longer a lipoprotein in *B. subtilis* as well, we tested the influence of globomycin on the processing of both the wild-type and mutant OpuAC proteins. Addition of globomycin to the *opuAC*<sup>+</sup> strain JH642 resulted in the accumulation of the biosynthetic precursor of OpuAC, whereas globomycin had no effect on the OpuAC-3 protein (Fig. 5). The electrophoretic mobility of the OpuAC-3 polypeptide corresponded to that of the pro-OpuAC protein (Fig. 5), thus providing further evidence that OpuAC is indeed a lipoprotein in *B. subtilis* and for the notion that the signal peptide of the mutant OpuAC-3 protein is not removed in the authentic host strain.

**The lipidless OpuAC-3 mutant protein functions in glycine betaine uptake in vivo.** To study whether the mutant form of the OpuAC glycine betaine binding protein still functioned in OpuA-mediated glycine betaine uptake, we performed growth experiments with osmotically stressed cells in the absence or presence of the osmoprotectant glycine betaine. Since multiple glycine betaine uptake systems operate in *B. subtilis* (24, 26, 30), we first constructed an isogenic pair of strains synthesizing either the wild-type OpuA (strain BKB20) or the OpuA-3 (strain BKB21) systems as the only glycine betaine transporter. Both strains were propagated in minimal medium of low (SMM) or high (SMM with 1.2 M NaCl) osmolarity, and their growth was monitored by measuring the optical density of the cultures. High osmolarity strongly impaired the growth of both strains, and the presence of 1 mM glycine betaine in the growth medium greatly relieved this growth inhibition to similar extents (Fig. 6). Hence, the *opuAC-3* allele must encode a functional glycine betaine binding protein. The presence of such a substrate binding protein is absolutely required for the functioning of the OpuA system (26) since growth inhibition by high osmolarity cannot be relieved by glycine betaine in strain BKB18 [*opuAA*<sup>+</sup> *opuAB*<sup>+</sup>  $\Delta$ (*opuAC::tet*)2], which possesses the OpuAA and OpuAB components of the OpuA transporter but lacks the OpuAC substrate binding protein (Fig. 6).

**Kinetic parameters of the mutant OpuA-3 transport system.** To compare the transport characteristics of the mutant OpuA-3 system with those of the wild-type transporter, we deter-

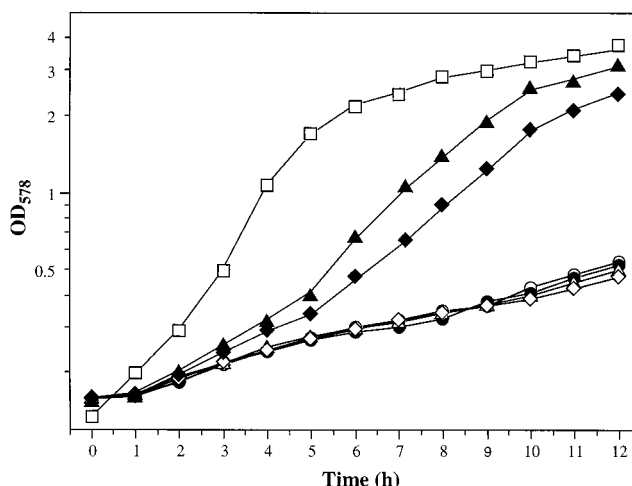


FIG. 6. The mutant OpuAC-3 protein functions in glycine betaine uptake. Cultures (75 ml in 500-ml Erlenmeyer flasks) were inoculated from fresh overnight cultures of *B. subtilis* BKB18 [ $\Delta$ (*opuA::tet*)2] (○ and ●), BKB20 (*opuA*<sup>+</sup>) (△ and ▲), and BKB21 (*opuAC-3*) (◇ and ◆). The cultures were grown in SMM (□), in SMM with 1.2 M NaCl (○, △, and ◇), and in SMM with 1.2 M NaCl in the presence of 1 mM glycine betaine (●, ▲, and ◆) in a shaking water bath (220 rpm) at 37°C. For the clarity of presentation, only one of the three identical growth curves of strains BKB18, BKB20, and BKB21 in SMM is shown.

mined the kinetic parameters for glycine betaine uptake in the *opuAC-3* strain BKB21. BKB21 was grown in SMM or SMM with 0.4 M NaCl, and the initial velocities of [<sup>1-14</sup>C]glycine betaine uptake were determined over a wide range (1 to 70 μM) of substrate concentrations. Glycine betaine transport via the mutant OpuA-3 system showed saturation kinetics. We determined  $K_m$  and  $V_{max}$  values of 4.1 μM and 112 nmol min<sup>-1</sup> mg of protein<sup>-1</sup> for the culture grown in SMM and 5 μM and 207 nmol min<sup>-1</sup> mg of protein<sup>-1</sup> for the culture grown in SMM with 0.4 M NaCl. These kinetic parameters closely match those previously determined for the OpuA system:  $K_m$  = 2.4 μM and  $V_{max}$  = 110 nmol min<sup>-1</sup> mg of protein<sup>-1</sup> in SMM and  $K_m$  = 2.4 μM and  $V_{max}$  = 282 nmol min<sup>-1</sup> mg of protein<sup>-1</sup> in SMM with 0.4 M NaCl (24). Hence, the mutant OpuA-3 transport system retains high affinity for its substrate, and its transport capacity for glycine betaine is only moderately reduced in comparison to the wild-type OpuA transporter.

## DISCUSSION

The data presented here demonstrate that the *B. subtilis* OpuAC protein functions as a high-affinity substrate binding protein for glycine betaine. The soil bacterium *B. subtilis* employs this trimethylammonium compound as a metabolically inert and effective osmoprotectant (5) and amasses it under laboratory growth conditions from glycine betaine-containing minimal media (1 mM) to an intracellular level of 700 mM after a moderate osmotic upshock with 0.4 M NaCl (51). The supply of glycine betaine is likely to be highly variable in the soil (15), and *B. subtilis* thus has to rely on effective transport systems to ensure physiologically adequate intracellular concentrations. The ABC transporter OpuA is the dominating uptake system for glycine betaine in *B. subtilis*, and with its high affinity and large transport capacity (24), it is well suited for the effective scavenging of this osmoprotectant from the environment. The high-affinity ligand-binding protein OpuAC is essential for the functioning of the OpuA transport system (26).

Substrate binding proteins provide the primary interaction

site for the ligand with the uptake system in bacterial ABC transporters. Affinities in the low micromolar range are typical for periplasmic substrate binding proteins from gram-negative bacteria (3, 6, 19). The purified *B. subtilis* OpuAC protein bound glycine betaine with a dissociation constant of 6  $\mu\text{M}$ , an affinity closely matching that of the glycine betaine binding protein (ProX) from *E. coli* (4, 33) and *S. typhimurium* (20). Both the *opuAC*- and *proX*-encoded polypeptides thus recognize the same substrate with comparably high affinities despite the low degree of homology in their amino acid sequences (26).

ABC transporters are used by *B. subtilis* not only for glycine betaine uptake (24, 26, 30) but also for the transport of peptides (28, 32, 37, 39), iron-hydroxamate (42), phosphate (47), and sugars (52). Genetic studies have established the importance of the presumed substrate binding proteins for the functioning of such ABC transport systems (26, 37). Our analysis of the purified OpuAC protein supports the conclusions derived from these studies and is the first example where the expected function of a *B. subtilis* substrate binding protein was directly demonstrated by biochemical analysis. The ligand binding proteins from ABC transporters of gram-negative bacteria can be readily obtained from the periplasmic space, thus greatly favoring their biochemical and biophysical analysis (6). Comparable studies of the substrate binding proteins of gram-positive bacteria are complicated by their lipid modification (9, 41), and we have therefore taken a gene fusion approach to purify large quantities of lipidless OpuAC in a functional form.

The presence of the signature sequence for lipoproteins (8) in the OpuAC biosynthetic precursor and the inhibition of pro-OpuAC processing in *E. coli* by the antibiotic globomycin suggested that this polypeptide is modified by a lipid (26). Metabolic labeling of OpuAC by [ $^3\text{H}$ ]palmitate in *E. coli* and the properties of a mutant OpuAC protein lacking the cysteine residue implicated in acylation now provide direct evidence for the nature of OpuAC as a lipoprotein. Furthermore, processing of pro-OpuAC was also inhibited in *B. subtilis* by globomycin. The cellular machinery that mediates lipid modification and maturation of lipoproteins in gram-positive bacteria appears to be essentially the same as that operating in gram-negative microorganisms (8, 53). We therefore take our data as evidence that the OpuAC protein is a lipoprotein tethered to the *B. subtilis* cytoplasmic membrane and functions as an extracellular, high-affinity glycine betaine binding protein. This type of cellular attachment appears to be generally used by gram-positive bacteria to compensate for the absence of an outer membrane (1, 14, 35, 37). Although in certain cases substrate binding proteins have been recovered in acylated form from culture supernatants from *Streptococcus mutans*, *S. pneumoniae*, and *B. subtilis* (36, 37, 46), it is entirely unclear whether these extracellular protein species function in substrate recognition and uptake. We did not detect the OpuAC protein in culture supernatants using a highly specific antiserum.

Remarkably, the lipidless OpuAC mutant (*opuAC-3*) characterized in this study still functioned in the OpuA-mediated glycine betaine uptake and osmoprotection. Glycine betaine uptake in this mutant strain remained more effective than uptake mediated by the two other glycine betaine transporters of *B. subtilis*, a secondary and an ABC transport system (24, 30). The in vitro-constructed *opuAC-3* mutation creates a consensus sequence for the proteolytic processing of pro-OpuAC by signal peptidase I from *E. coli* (50), and one would thus have predicted the efficient processing of the biosynthetic precursor and the secretion of the mature OpuAC-3 protein into the periplasmic space. However, processing of pro-OpuAC-3 in *E. coli* proved to be inefficient, and only part of the synthesized

protein was secreted into the periplasm, where it could be released by cold osmotic shock. A substantial portion of pro-OpuAC-3 remained unprocessed and was found associated with the *E. coli* cells. Pro-OpuAC-3 was also present in the cells of *B. subtilis* and fractionated with the cytoplasmic membrane, but there was no cross-reacting material that could correspond to the mature OpuAC-3 protein either associated with the cells or in the supernatant. We currently can only speculate as to why the proteolytic cleavage of the pro-OpuAC-3 protein is so inefficient. Sequences beyond the cleavage site can have a profound influence on the efficiency of precursor processing and the export of the mature protein in *B. subtilis* (43). Subtle alterations in the vicinity of the processing site can also result in decreased processing rates and can cause the tethering of the precursor molecules in the cytoplasmic membrane (7). Construction of such signal sequence mutants in the levansucrase gene of *B. subtilis* also resulted in a decreased production of this exoenzyme (7). Similar processes probably also operate for pro-OpuAC-3 production and processing, leading to decreased cellular levels of this protein and its anchoring in the cytoplasmic membrane by its uncleaved hydrophobic signal sequence.

The characteristics of the lipidless but functional OpuAC-3 protein are reminiscent of a *male* mutant that produces a precursor MBP which is translocated across the cytoplasmic membrane but is not cleaved by the signal peptidase. This *male* mutant strain exhibits a Mal<sup>+</sup> phenotype and transports maltose actively. The kinetic parameters of the mutant maltose transport system closely match those of the wild-type binding-protein-dependent maltose uptake system (10). We found that the mutant OpuA-3 system functions effectively in osmoprotection, transports glycine betaine efficiently, and displays uptake kinetics similar to those of the wild-type OpuA transporter. It thus appears that the tethering of substrate binding proteins via an uncleaved hydrophobic signal sequence still allows productive interactions with the membrane components of ABC transporters. Cell membrane attachment of extracellular substrate binding proteins through an uncleaved signal sequence therefore seems to be functionally equivalent to the tethering of these proteins in gram-positive bacteria via their usual lipid modification at the amino-terminal cysteine residue.

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