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*ₐ of 6 μM 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 φ10* control in *Escherichia coli* and have demonstrated through its metabolic labeling with [³H]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 [³H]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 OpuA 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 hydrophilic 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 cytoplasmic 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).
B. subtilis inoculated with fresh overnight cultures to an optical density at 578 nm (OD578) spectinomycin, and tetracycline were used with M. luteus. The antibiotics ampicillin and chloramphenicol were used with growth of B. subtilis E. coli, K-12 strain MG1655 (Braunschweig, Germany).

**MATERIALS AND METHODS**

**Growth conditions, media, and chemicals.** Rich and minimal media for the strains used are listed in Table 1. Relevant genotype Reference or construction

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Reference or construction</th>
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<tbody>
<tr>
<td>BKB4</td>
<td>Δ(opuAC-amhX::neo)</td>
<td>26</td>
</tr>
<tr>
<td>BKB7</td>
<td>Δ(opuAC::tetR)</td>
<td>26</td>
</tr>
<tr>
<td>BKB13</td>
<td>(amhX::erm) opuA*</td>
<td>27</td>
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<tr>
<td>JBB51</td>
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<tr>
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</tr>
<tr>
<td>BKB14</td>
<td>(amhX::erm) opuAC-3</td>
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<tr>
<td>BKB17</td>
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<tr>
<td>BKB21</td>
<td>opuA::Tn10l (opuA::eres)</td>
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*All B. subtilis strains are derivatives of strain JH6-42 (tpc2 pheA1; BGS 1A96). An arrow indicates transformation.

**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 OpuA-3 proteins, bacterial cell extracts or protein preparations were electroeluted separately from 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 anti-protein antisera. The OpuAC and OpuA-3 antibodies were visualized with a second goat anti-rabbit immunoglobulin G-alkaline phosphatase-conjugated antibody (Sigma, Deisenhofen, Germany), using 5-bromo-4-chloro-3-indolyphosphate 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 OpuA-3 proteins.** E. coli BL21(DE3), which carries the gene for the T7 DNA polymerase under control of the lacP promoter (44), was used as the host to express the opuAC and opuA-3 genes under T7 +10 control (11, 26). For labeling of the wild-type OpuAC and mutant OpuA-3 proteins with [9,10-(n)-H]palmitic acid, we used the procedure of Ehler et al. (13), with slight modifications. Strains BL21 (pPD100), BL21(pBB80), and BL21(pBB83) were grown in M9 minimal medium with chloramphenicol, 0.2% glucose, and 0.2% Casamino Acids. When the cultures reached mid-exponential phase (OD600 = 0.4), 10-ml portions were transferred to sterile 100-ml Erlenmeyer flasks in which 50 µCi of a solution of [9,10-(n)-H]palmitic acid had been evaporated under a gentle nitrogen stream. Expression of the plasmid-encoded genes was induced by adding 1 mM isopropylthiolgalactopyranoside (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 and samples were harvested 45 min to inhibit expression 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 MgCl2-12% 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 nucleon.
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 opuAC operon (opuAA, opuAB, and opuAC) (26) and the flanking amidohydrolase amnX gene (27) together with some restriction sites. The lower part shows the opuAC mutation and amnX. 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 µl of the supernatant was removed and added to 1 ml of ice-cold acetone. After incubation at 4°C overnight, the samples were re-centrifuged and the delipidated protein pellet was washed in 50% ice-cold ethanol. Finally, the proteins were solubilized in 50 µl 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 (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(pBKB58 [opuAC-3]) 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 JH662 (opuAC·) and BKE21 (opuAC·) 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 µg/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 at 37°C to mid-exponential phase (OD_{578} = 0.5), and the antibiotic globomycin was then added to a final concentration of 150 µg/ml. The cell suspension was harvested by centrifugation, (20 mM Tris-HCl [pH 7.4]), and the proteins were incubated with 5 µM 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-µm-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 µM and the concentration of its
substrate [1-14C]glycine betaine was varied between 1 and 100 μM. For these tests, substrate concentrations greater than 10 μM were obtained by adding unlabeled glycine betaine to a fixed concentration (5 μM) of radiolabeled glycine betaine. The measured radioactivity was appropriately corrected.

Transport assays. Uptake of [1-14C]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 μM.

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 pBBK76 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 protolytic 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 (36) to study the interaction of the substrate binding protein with its ligand. The purified OpuAC protein was incubated at room temperature with [1-14C]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 [1-14C]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 (Kd) of OpuAC for its substrate (38), we measured the binding of [1-14C]glycine betaine over a wide range of concentrations (1 to 100 μM), using a fixed amount of OpuAC (5 μM) in the binding assay. Substrate binding of OpuAC showed saturation kinetics. Even at substrate concentrations as low as 1 μM, 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 Kd of approximately 6 μM 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 (Km = 2.4 to 5 μM) measured in vivo for the OpuA-mediated uptake of the osmoprotectant glycine betaine (24, 25).

Labeling of OpuAC with [3H]palmitate. To directly demonstrate that OpuAC is a lipoprotein, we attempted to metabolically label it with [3H]palmitic acid. For these experiments, we used a low-copy-number expression vector carrying the opuAC gene (pBKB58) under the control of the inducible T7 φ10 promoter (Fig. 1). The T7 RNA polymerase/T7 φ10 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 φ10-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 [3H]palmitic acid led to the production of a
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 -10 promoter in E. coli BL21 carrying plasmid pBBK88 (opuAC1) or pBBK83 (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-OpuA antibody. Lane 1, BL21 (pPD100 [expression vector]), total-cell extract; lane 2, BL21(pBBK83), total-cell extract; lane 3, BL21(pBBK83), osmotically shocked cells; lane 4, BL21(pBBK83), osmotically shocked cells; lane 5, B. subtilis BB21 (opuAC-3), total-cell extract; lane 6, B. subtilis JH642 (wild type), total-cell extract; lane 7, BL21(pBBK85), total-cell extract; lane 8, BL21(pBBK85), osmotically shocked cells; lane 9, BL21 (pBBK85), osmotically shocked cells. Since the OpuAC antisera 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 alanine residue. The resulting mutant opuAC gene (opuAC-3) was cloned in the T7 -10 expression vector pPD100 (11), yielding plasmid pBBK83 (Fig. 1). The OpuAC-3 protein was produced in induced cultures of BL21(pBBK83) in amounts comparable to that of the wild-type OpuAC protein (Fig. 3A, lanes 3 and 4). However, it was not metabolically labeled with [3H]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 II (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 -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 of the cells, suggesting a processing (pro) form of 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(pBBK88), 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+ plasmid by recombinant DNA procedures and then inserted a 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 (pBBk82) was linearized and transformed into the Δ(opuA::neo)2 mutant strain BK4 (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 Δ(opuA::neo)1 deletion of BK4 was replaced by opuA sequences from pBBk82 (opuAC3). 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 with 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 [Δ(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
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 opuAC-1 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-3 strain H642 resulted in the accumulation of the bio-synthetic 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 osmoregulatant 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+ opuAB- Δ(opuAC::tetR)], 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-

mined the kinetic parameters for glycine betaine uptake in the opuAC-3 system. BKB21. BKB21 was grown in SMM or SMM with 0.4 M NaCl, and the initial velocities of [1-14C]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⁻¹ mg⁻¹ of protein⁻¹ for the culture grown in SMM and 5 μM and 207 nmol min⁻¹ mg⁻¹ of protein⁻¹ 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⁻¹ mg⁻¹ of protein⁻¹ in SMM and K_m = 2.4 μM and V_max = 282 nmol min⁻¹ mg⁻¹ of protein⁻¹ 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 shock 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 \textit{B. subtilis} OpuAC protein bound glycine betaine with a dissociation constant of 6 \(\mu\)M, an affinity closely matching that of the glycine betaine binding protein (ProX) from \textit{E. coli} (4, 33) and \textit{S. typhimurium} (20). Both the \textit{opuAC}- and \textit{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 \textit{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 \textit{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 \textit{E. coli} by the antibiotic globomycin suggested that this polypeptide is modified by a lipid (26). Metabolic labeling of OpuAC by \(^{3}H\)palmitate in \textit{E. coli} and the properties of a mutant OpuAC protein lacking the cysteine residue implied in acylation now provide direct evidence for the nature of OpuAC as a lipoprotein. Furthermore, processing of pro-OpuAC was also inhibited in \textit{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 \textit{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 \textit{Streptococcus mutans}, \textit{S. pneumoniae}, and \textit{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 (\textit{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 \textit{B. subtilis}, a secondary and an ABC transport system (24, 30). The in vitro-constructed \textit{opuAC}-3 mutation creates a consensus sequence for the proteolytic processing of pro-OpuAC by signal peptidase I from \textit{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 \textit{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 \textit{E. coli} cells. Pro-OpuAC-3 was also present in the cells of \textit{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 \textit{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 levanuscrase gene of \textit{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 \textit{malE} mutant that produces a precursor MBP which is translocated across the cytoplasmic membrane but is not cleaved by the signal peptidase. This \textit{malE} mutant strain exhibits a Mal" 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 osmorefraction, 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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