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OpuA, an Osmotically Regulated Binding Protein-dependent Transport System for the Osmoprotectant Glycine Betaine in *Bacillus subtilis**

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Bettina Kempf and Erhard Bremer‡

From the Max-Planck-Institute for Terrestrial Microbiology, D-35043 Marburg, Germany

Exogenously provided glycine betaine can efficiently protect *Bacillus subtilis* from the detrimental effects of high osmolarity environments. Through functional complementation of an *Escherichia coli* mutant deficient in glycine betaine uptake with a gene library from *B. subtilis*, we have identified a multicomponent glycine betaine transport system, OpuA. Uptake of radiolabeled glycine betaine in *B. subtilis* was found to be osmotically stimulated and was strongly decreased in a mutant strain lacking the OpuA transport system. DNA sequence analysis revealed that the components of the OpuA system are encoded by an operon (*opuA*) comprising three structural genes: *opuAA*, *opuAB*, and *opuAC*. The products of these genes exhibit features characteristic for binding protein-dependent transport systems and in particular show homology to the glycine betaine uptake system ProU from *E. coli*. Expression of the *opuA* operon is under osmotic control. The transcriptional initiation sites of *opuA* were mapped by high resolution primer extension analysis, and two *opuA* mRNAs were detected that differed by 38 base pairs at their 5′ ends. Synthesis of the shorter transcript was strongly increased in cells grown at high osmolarity, whereas the amount of the longer transcript did not vary in response to medium osmolarity. Physical and genetic mapping experiments allowed the positioning the *opuA* operon at 25° on the genetic map of *B. subtilis*.

Monitoring and adapting to changes in environmental conditions are critical processes that determine the survival of microorganisms and their successful long term competition for a given habitat. In its soil environment, *Bacillus subtilis* encounters often osmotic challenges due to frequent variations in the availability of water. Since the cell envelope is permeable to water, drying and wetting of the soil alters the osmotic conditions and hence triggers the flux of water across the cell membrane. Active and timely adaptation reactions are thus required to avoid cell lysis under low osmolarity or dehydration of the cytoplasm under high osmolarity growth conditions (1, 2). Despite the importance of changes in the environmental osmolarity for growth and survival of *B. subtilis*, the specific physiological and genetic adaptation mechanisms to such environmental challenges are in general rather poorly understood.

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‡ To whom correspondence should be addressed: Max-Planck Institut für Terrestrische Mikrobiologie, Karl-von-Frisch Str., D-35043 Marburg, Germany. Tel.: 49-6421-286681; Fax: 49-6421-285833; E-mail: bremer@mailer.uni-marburg.de.

The exposure of *B. subtilis* to a hypersaline environment triggers the induction of a set of general and salt-specific stress proteins, indicating that increased salt concentration alerts the cell to adverse growth conditions (3). The expression of a number of genes in this general stress regulon is determined by the alternative transcription factor σ^B, which serves to control a regulatory network responsive to stationary phase signals and growth-limiting conditions (4–6). However, *B. subtilis* mutant strains lacking the σ^B protein are not at a survival disadvantage compared with the wild type when exposed to osmotic shock or extreme desiccation under laboratory conditions (6). Therefore, it is uncertain whether members of the σ^B-controlled general stress regulon play a direct role in the adaptation of *B. subtilis* to high osmolarity environments.

A central part of the physiological response of *B. subtilis* to high osmolarity stress is the intracellular accumulation of inorganic and organic osmolytes that serve to counterbalance intracellular *versus* extracellular osmolarity and consequently help to maintain a turgor optimal for cell growth. An increase in medium osmolarity stimulates turgor-sensitive transport systems that mediate rapid accumulation of K^+ in the cell, which, in turn, restores turgor and permits cell growth to resume (7, 8). This initial reaction is followed by a cellular response that replaces ionic osmolytes, which are deleterious at high concentrations, with organic osmolytes, which are more compatible with the normal physiological and structural requirements of the bacterial cell (1, 2). In *B. subtilis* proline is the predominant organic osmolyte synthesized in defined medium by cells exposed to a hypersaline environment (7). However, several hours are required to reach a proline level that is sufficient for osmoprotection, leaving the cell at a growth disadvantage in harsh high osmolarity environments (9). *B. subtilis* can more efficiently respond to high osmolarity by accumulating glycine betaine (9). This potent osmoprotectant is widely found in nature and has been adopted across the microbial, plant, and animal kingdoms as an effective compatible solute (1, 2). Glycine betaine can be synthesized by *B. subtilis* from its precursor choline or taken up directly from the environment (7, 9, 10). A strong increase in the growth rate and the proliferation under environmental conditions that are otherwise strongly inhibitory for *B. subtilis* can be attained when glycine betaine can be directly accumulated from the growth medium (7, 9, 10). The presence of uptake systems for glycine betaine has been reported for a variety of Gram-negative and several Gram-positive bacteria (11–15), but the details of such transport systems have been studied at the molecular level only in *Escherichia coli* and *Salmonella typhimurium*. Here, two glycine betaine transport systems, ProP and ProU, have been characterized (1, 2, 16–18).

In *B. subtilis* the importance of exogenously provided glycine betaine for the efficient adaptation to a high osmolarity environment is firmly established, but the route of glycine betaine

uptake in this model system for Gram-positive bacteria is unknown. We have begun to characterize the mechanisms of glycine betaine uptake in *B. subtilis*, and we report in this paper the identification and analysis of a binding protein-dependent transport system (OpuA) for this osmoprotectant.

EXPERIMENTAL PROCEDURES

Bacterial Strains and Plasmids—The *B. subtilis* strain JH642 (*trpC2, pheA1*; BGSC[1] 1A96), a derivative of the *B. subtilis* wild-type strain 168, was constructed by J. A. Hoch and was obtained from M. Marahiel. Strain MO1099 (*trpC2, pheA1, amyE::ery*; BGSC 1A717) has been described (19). Strain TIBS57 (*amyE3, aroI10*; BGSC 1A474) was used for the genetic mapping of the *opuA* operon. The *B. subtilis* strains BKB4 (Δ(*opuA::neo*)*1*]) and BKB7 (Δ(*opuA::tet*)*2*) are derivatives of JH642 and were constructed by transforming JH642 with *Eco*RI restriction fragments isolated from plasmids pBKB11 (Δ(*opuA::neo*)*1*; Fig. 1), and pBKB52 (Δ(*opuA::tet*)*2*; Fig. 1), respectively, and selecting for kanamycin-resistant (BKB4) or tetracycline-resistant (BKB7) colonies on LB agar plates. The *E. coli* K-12 strain MKH13 (Δ(*putPA*) *101* Δ(*proP*)*2*Δ(*proU*) *608*) is a derivative of strain MC4100 (20) and is entirely deficient in glycine betaine uptake (21). The *E. coli* B strain BL21(λDE3) was employed for the selective expression of genes under T7φ10 control (22). The low copy number plasmid pHSG575 (23) was used for the construction of a *B. subtilis* gene library and for the construction of a number of subclones carrying various segments of the *opuA* region (Fig. 1). Strain DH5α (Life Technologies, Inc.) was used to propagate the *B. subtilis* gene library. The low copy number T7 expression plasmids pPD100 and pPD101 (24) were used to express the genes of the *opuA* operon under the control of the T7φ10 promoter. Plasmid pJL29 is a pBR322-derived vector for the construction of protein fusions to a truncated *'lacZ* gene (25); it was used here for the isolation of the Φ(*opuAA-lacZ*)*hyb1* hybrid gene. The *E. coli-B. subtilis* shuttle vector pRB373 has been described (26). DNA cartridges encoding genes conferring resistance to kanamycine or tetracycline were isolated from plasmids pAT21 and pBEST307, respectively (27, 28).

Growth Conditions, Media, and Chemicals—Bacteria were grown aerobically at 37 °C in LB, MMA glucose (0.2%), or Spizizen's minimal medium (SMM) supplemented with 0.5% glucose, 20 mg/liter L-tryptophan, 18 mg/liter L-phenylalanine, and a solution of trace elements (29, 30). The osmolarity of the various media was increased by addition of NaCl from a highly concentrated (5 M) stock solution. The osmolarity of media was determined with a vapor pressure osmometer (model 5500; Wescor Inc., Logan, UT). Expression of the *opuA* genes under T7φ10 control was carried out in cells grown in M9 minimal medium (29) supplemented with 0.2% casaminoacids. To select derivatives of strain MKH13 synthesizing glycine betaine transporters encoded by cloned *B. subtilis* DNA, we used MMA minimal agar plates containing 0.2% glucose as the carbon source, 0.8 M NaCl and 1 mM glycine betaine. Plates for the detection of extracellular α-amylase (AmyE) activity contained 1% starch in LB agar plates. Production of AmyE by *B. subtilis* strains was detected by flooding the colonies grown on LB starch plates with Gram's iodine stain (0.5% (w/v) iodine, 1% (w/v) potassium iodide) for 1 min and scoring for zones of clearing around the colony after decanting the stain (31). The AroI phenotype of *B. subtilis* strains was tested by scoring the growth of colonies on SMM minimal plates either lacking or containing 20 mg/liter L-tryptophan, 18 mg/liter L-phenylalanine, and 20 mg/liter L-tyrosine; AroI$^-$ strains can not grow on minimal plates lacking these aromatic amino acids. The antibiotics ampicillin, chloramphenicol, tetracycline, and kanamycin were used in *E. coli* at a final concentration of 100, 30, 5, and 15 μg/ml, respectively. Kanamycin, tetracycline, and erythromycin were used in *B. subtilis* at a final concentration of 5, 10, and 0.5 μg/ml, respectively. The cyclic peptide antibiotic globomycin, a specific inhibitor of signal peptidase II (32), was kindly provided by M. Inukai from Sankyo Pharmaceutical Co. (Japan). A stock solution (10 mg/ml) of globomycin was prepared in dimethyl sulfoxide and added to cultures to a final concentration of 120 μg/ml. Radiolabeled [1-^{14}C]glycine betaine (55 mCi/mmol) was purchased from ARC (American Radiolabeled Chemicals Inc., St. Louis, MO), radiolabeled [^{35}S]methionine (523 mCi/mmol) was obtained from ICN (Meckenheim, Germany), and [^{35}S]dATP (1000 Ci/mmol) was from Amersham Corp.

Methods Used with Nucleic Acids—Routine manipulations of plasmid DNA, the isolation of chromosomal DNA from *B. subtilis*, and the detection of homologous sequences by Southern hybridization were all carried out by standard techniques (33). Sequencing of double-stranded plasmid DNA and of single-stranded DNA segments cloned in M13BM20 or M13BM21 (Boehringer Mannheim) was carried out using the Sequenase 2.0 kit (U.S. Biochemical Corp.) and the conditions recommended by the supplier. Sequencing reactions were primed with a number of synthetic oligonucleotide primers spaced along the *opuA* region. The entire 5.2-kb *Eco*RI insert present in plasmid pBKB1 (Fig. 1) was used to generate a probe by random nucleotide labeling using DIG-dUTP (DIG DNA labeling and detection kit, Boehringer Mannheim). Hybridization of the labeled DNA fragment to *Eco*RI- and *Pst*I-digested chromosomal DNA isolated from various *B. subtilis* strains was performed according to the instructions of the supplier, and the hybridization products were detected using LumiPhos 530 (U.S. Biochemical Corp.). Total RNA was prepared from cultures grown to mid-log phase (A_{578} = 0.5) in either LB medium or LB medium of high osmolarity (0.5 M NaCl added) of the *B. subtilis* strain JH642 carrying the *opuAA-lacZ* fusion plasmid pBKB56 essentially as described by Völker *et al.* (5). The RNA was further purified by passage through a Quiagen tip-100 column as suggested by the supplier (Diagen, Düsseldorf, Germany). The total amount of RNA isolated was spectrophotometrically (A_{260}) determined; an A_{260} of 1 corresponds to approximately 40 μg/ml RNA (33). For the primer extension reaction, a synthetic primer (5'-GAACTGCCTTCTTTGTTTGTTTCCC-3'), complementary to the *opuAA* mRNA (position 283–307 bp; see Fig. 3) was hybridized with 5 μg of RNA and extended with avian myeloblastosis virus reverse transcriptase (U.S. Biochemical Corp.) in the presence of radiolabeled [^{35}S]dATP at a final concentration of 1 μCi/ml. The size of the reaction products was determined on a 4% DNA sequencing gel under denaturing conditions and visualized by autoradiography. A sequencing ladder produced by using the same primer was run on the same sequencing gel to determine the precise 5' ends of the *opuA* mRNAs. Transformation of competent *B. subtilis* cells with plasmids and linear DNA fragments was done according to routine procedures (31).

Construction of Plasmids—A library of chromosomal DNA segments from the *B. subtilis* wild-type strain JH642 was prepared by cleaving chromosomal DNA with *Eco*RI and ligating the resulting restriction fragments into the *Eco*RI site in the polylinker of the *lacZ* α-complementing plasmid pHSG575. The DNA of the recombinant plasmids was transformed into strain DH5α, and colonies were selected on LB plates containing chloramphenicol, isopropyl-1-thio-β-D-galactopyranoside (1 mM), and X-gal (40 μg/ml). Approximately 90% of the obtained transformants (40,000 colonies) carried plasmids with cloned *B. subtilis* DNA as judged from their LacZ$^-$ phenotype. All colonies were pooled and grown for 2 h in LB medium with chloramphenicol; the plasmid DNA was then extracted and used to transform the *E. coli* strain MKH13. Plasmids pBKB13, pBKB14, pBKB17, pBKB18, pBKB38, and pBKB46 were constructed by deleting defined restriction fragments from the *opuA*$^+$ plasmids pBKB1 and religating the plasmid backbones (Fig. 1). Plasmids pBKB15, pBKB35, and pBKB39 were isolated by cloning appropriate restriction fragments isolated from plasmid pBKB1 into the vector pHSG575 (Fig. 1). Plasmid pBKB33 carries the entire *opuA* operon on a 4-kb *Eco*RI-*Eco*RV restriction fragment (Fig. 1) that has been cloned into the polylinker sequence of the T7φ10 expression vector pPD100, thus positioning *opuA* under T7φ10 control. The same restriction fragment was inserted in the reverse orientation with respect to the T7φ10 promoter present in the vector pPD101, yielding plasmid pBKB34. Plasmid pBKB44, which expresses the *opuAA*$^+$ *opuAB*$^+$ genes under T7φ10 control was constructed by deleting a 636-bp *Nsi*I fragment carrying most of the *opuAC* gene (Fig. 1) from the *opuA*$^+$ plasmid pBKB33. To achieve expression of the *opuAA*$^+$ gene under T7φ10 control, a 1.7-kb *Eco*RI-*Hpa*I restriction fragment (Fig. 1) was inserted into the vector pPD100, yielding plasmid pBKB43. A plasmid expressing the *opuAC*$^+$ gene under T7φ10 control was constructed by isolating a 1.4-kb *Apa*LI-*Not*I fragment from pBKB1; the overhanging ends of the restriction fragments were filled in with Klenow enzyme and then ligated into the *Sma*I site of plasmid pPD100. Plasmids positioning the *opuAC* gene under T7φ10 control (pBKB58) or aligning it in the reverse orientation with the T7φ10 promoter (pBKB57) were identified by restriction analysis. To construct an *opuAA-lacZ* gene fusion, a 1.3-kb *Eco*RI-*Sna*BI restriction fragment from pBKB1 (Fig. 1) was cloned into the *Eco*RI and *Sma*I sites of the *'lacZ* fusion vector pJL29, yielding plasmid pBKB54. In this plasmid, the reading frames of *opuAA* and *'lacZ* are properly aligned across the *Sna*BI and *Sma*I junction, thus generating a hybrid protein fusion, Φ(*opuAA-lacZ*)*hyb1*. The entire hybrid gene was transferred from plasmid pBKB54 on a 4.4-kb *Eco*RI-*Dra*I restriction fragment into the *E. coli-B. subtilis* shuttle vector pRB373, which had been cut

[1] The abbreviations used are: BGSC, *Bacillus* Genetic Stock Center; bp, base pair(s); kb, kilobase pair(s); MMA, minimal medium A; SMM, Spizizen's minimal medium.

with *Eco*RI and *Sma*I; this construction resulted in plasmid pBKB56.

Transport Assays for Radiolabeled Glycine Betaine—Uptake of glycine betaine in *B. subtilis* and *E. coli* was measured using [1-^{14}C]glycine betaine (55 mCi/mmol) as a substrate. The cells were grown to mid-exponential phase (A_{578} = 0.15–0.5) in minimal medium with glucose as the carbon source and used immediately for the transport assay. *E. coli* strains were grown in MMA or MMA with 0.2 M NaCl, and *B. subtilis* strains were grown in SMM or SMM with 0.4 M NaCl. The uptake assay contained [1-^{14}C]glycine betaine at a final substrate concentration of 10 μM (5.5 mCi/mmol) in a total reaction volume of 2 ml. Samples (0.3 ml) were taken at various times and filtered through 0.45 μm-pore-size filters (Schleicher and Schuell GmbH, Dassel, Germany). The cells were washed with 20 ml of isotonic minimal salts, and the radioactivity retained on the filters was determined in a scintillation counter. Protein concentrations were determined from total cell extracts using the Bio-Rad protein assay with acetylated bovine serum albumin as the standard. The cell extracts were prepared by passing the *B. subtilis* cells five times through a French press cell at 103,000 kilopascals.

Preparation of Total Cell Extracts, SDS-Polyacrylamide Gel Electrophoresis, and Immunological Detection of the OpuAA'-β-Galactosidase Hybrid Protein—Cultures (20 ml in a 100-ml Erlenmeyer flask) of strain JH642 carrying the *opuAA-lacZ* fusion plasmid pBKB56 were grown overnight at 37 °C in LB medium or LB medium with 0.5 M NaCl. The optical density (A_{578}) of the cultures was determined and adjusted to A_{578}=5. From each culture, 2-ml portions were withdrawn, the cells were collected by centrifugation and resuspended in 150 μl of TE (10 mM Tris-HCl, 1 mM EDTA, pH = 8.0), and 15 μl of lysozyme (10 mg/ml in H_2O) was added. The cell suspension was then incubated for 8 min at 37 °C in a water bath, 50 μl of 4-fold concentrated sample buffer (final concentration, 0.06 M Tris, pH 6.8, 5% SDS, 10% glycerol, 3% dithiothreitol, 0.001% bromphenol blue) was added, and the cells were lysed by incubation for 5 min at 95 °C. To reduce the viscosity of the cell extract, 2 μl of benzon nuclease (Merck) was added and incubated for 10 min at 37 °C, followed by another short (5-min) incubation at 95 °C. Aliquots of the cell extracts were then immediately loaded onto 7% SDS-polyacrylamide gels (34), and the proteins were visualized by staining with Coomassie Brilliant Blue (33). For the immunological detection of the OpuAA'-β-galactosidase hybrid protein, total cellular extracts were electrophoretically separated on 7% SDS-polyacrylamide gels and transferred onto a sheet of Immobilon (Millipore, Eschwege, Germany). The bound proteins were probed with a rabbit β-galactosidase antiserum, and the antigen-antibody complexes were visualized with a second goat anti-rabbit immunoglobulin G alkaline phosphatase-conjugated antibody (Sigma) using 5-bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium chloride (Boehringer Mannheim) as substrates.

Expression of the opuA Gene Products under T7 Control—Plasmids carrying various genes from the *opuA* operon under the transcriptional control of the T7φ10 promoter were transformed into strain BL21(λDE3) to selectively visualize the *opuA*-encoded proteins. These plasmids are pBKB33 (*opuAA*$^+$ *opuAB*$^+$ *opuAC*$^+$), *pBKB44* (*opuAA*$^+$ *opuAB*$^+$), *pBKB43* (*opuAA*$^+$), and pBKB58 (*opuAC*$^+$). Plasmids pBKB34 and pBKB57 carrying the *opuA*$^+$ operon and *opuAC*$^+$ gene, respectively, improperly aligned with respect to the T7 promoter were used as controls. Cultures (20 ml in 100-ml Erlenmeyer flasks) of strain BL21(λDE3) carrying the various plasmids were grown in M9 minimal medium with 0.2% casaminoacids to mid-log phase (A_{578} = 0.5–0.7); the cells were washed with M9 minimal medium, resuspended in 20 ml of M9 minimal medium supplemented with 0.2% methionine assay medium (Difco), and grown for 1 h at 37 °C. T7φ10-mediated gene expression was initiated by adding isopropyl-1-thio-β-D-galactopyranoside to a final substrate concentration of 1 mM, after 30 min rifampicin was added (200 μg/ml) to inhibit the *E. coli* RNA polymerase. After a 1-h incubation at 37 °C, 1-ml portions of the cultures were withdrawn, the proteins were labeled for three min with [^{35}S]methionine (final concentration, 5.2 nCi/ml), and the cells were collected by centrifugation and resuspended in 50 μl of SDS-sample buffer. Portions (30 μl) of the cell extracts were loaded onto 12% SDS-polyacrylamide gels, the proteins were electrophoretically separated, and radiolabeled peptides were visualized by autoradiography. To inhibit signal peptidase II, the antibiotic globomycin was added to the appropriate cultures 10 min prior to the radiolabeling of the proteins.

Computer Work—Analysis of nucleotide sequences from the *opuA* region and the alignment and analysis of protein sequences was performed with the Lasergene program from DNA Star (DNASTAR, Ltd., London) on an Apple Macintosh II computer. Multiple sequence alignments were done at the National Center for Biotechnology Information (NCBI) using the BLAST programs and the current versions of the available data bases (November, 1994) (35). The nucleotide sequence of the *opuA* region (see Fig. 3) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number U17292.

RESULTS

Cloning of the Structural Genes for a Glycine Betaine Transport System—To clone genes from *B. subtilis* that code for glycine betaine transporters, we capitalized on the growth properties of the *E. coli* mutant strain MKH13. This strain is defective for glycine betaine synthesis and also lacks the glycine betaine transport systems, ProP and ProU (21). Therefore, it is severely impaired in its ability to cope with a high osmolarity environment and, in contrast to its parental strain MC4100, cannot grow in high osmolarity media containing the osmoprotectant glycine betaine. We reasoned that it should be possible to functionally complement the deficiency of strain MKH13 in glycine betaine uptake with the appropriate *B. subtilis* genes. A gene bank of *Eco*RI restriction fragments was prepared from chromosomal DNA of the *B. subtilis* strain JH642 in the low copy number cloning vector pHSG575 (Cm^r) and transformed into MKH13 by selecting for Cm^r colonies on LB agar plates. These transformants were then replica-plated on high osmolarity minimal plates (MMA agar plates with 0.8 M NaCl) containing 1 mM glycine betaine to search for MKH13 derivatives that could grow under these selective conditions. Such strains were readily obtained, and each of the 46 MKH13 derivatives analyzed carried the same pHSG575-derived plasmid containing a 5.2-kb *Eco*RI restriction fragment. A restriction map of one of these plasmids, pBKB1, is shown in Fig. 1.

The ability of strain MKH13(pBKB1) to grow at high osmolarity in the presence of 1 mM glycine betaine was shown to be dependent on the presence of plasmid pBKB1 by retransformation into strain MKH13. There was no growth of MKH13-(pBKB1) in high osmolarity minimal medium (MMA agar plates with 0.8 M NaCl) lacking this osmoprotectant. Osmoprotection by glycine betaine requires the intracellular accumulation of this compound (1, 2). We therefore measured the initial [1-^{14}C]glycine betaine uptake in cultures of strain MKH13-(pBKB1) grown in low osmolarity or high osmolarity minimal media at a final substrate concentration of 10 μM. Glycine betaine transport activity was readily detectable in cultures of MKH13(pBKB1), and we found that this transport activity was under osmotic control (Fig. 2*A*). Thus, plasmid pBKB1 encodes an osmotically controlled uptake system for glycine betaine from *B. subtilis*. We designate this glycine betaine transporter as OpuA (osmo protectant uptake) and refer to its structural gene(s) as *opuA*.

To identify the approximate position of the *opuA* gene(s) within the cloned DNA segment from the *B. subtilis* chromosome, we subcloned defined restriction fragments from plasmid pBKB1 into the low copy number vector pHSG575 and also constructed a number of deletion derivatives of pBKB1 (Fig. 1). Each of these plasmids was introduced by transformation into strain MKH13, and the ability of these transformants to grow in high osmolarity minimal media in the presence of 1 mM glycine betaine was tested. The results from these complementation experiments are summarized in Fig. 1. It is apparent that a large portion of the cloned 5.2-kb *Eco*RI fragment is required to mediate glycine betaine uptake activity. Thus, OpuA is most likely a multicomponent glycine betaine transport system.

Mutations in opuA Strongly Impair Glycine Betaine Transport Activity in B. subtilis—OpuA mediates glycine betaine uptake in *E. coli*. To investigate the role of the *opuA*-encoded transport system for glycine betaine transport in *B. subtilis*, we constructed two *opuA* mutations on plasmid pBKB1 and then

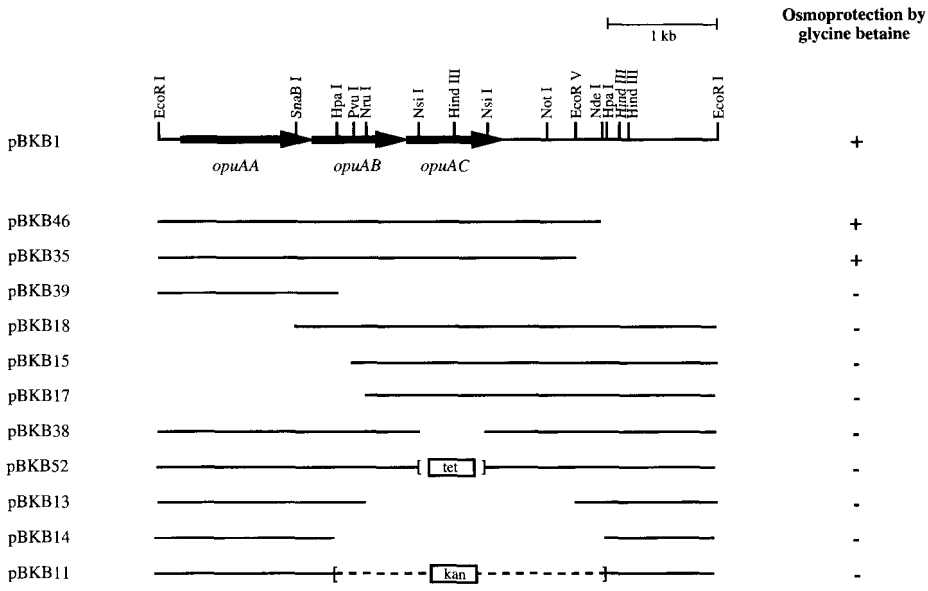

FIG. 1. **Physical and genetic organization of the cloned *opuA* region.** A map of a plasmid, pBKB1, which carries the entire *opuA* operon, is shown. The exact positions and transcriptional orientations of the *opuAA*, *opuAB*, and *opuAC* genes were inferred from DNA sequence analysis. The extent of the DNA segment present in the various deletion derivatives and subclones of plasmid pBKB1 are indicated by the *lines*. Plasmids pBKB11 and pBKB52 are deletion derivatives of pBKB1 and carry, in addition, gene cartridges encoding kanamycin (*kan*) or tetracycline (*tet*) resistance, respectively. The *kan* and *tet* gene cartridges are not drawn to scale. Osmoprotection by glycine betaine was assayed by monitoring the growth of the *E. coli* mutant strain MKH13 lacking both the ProP and ProU glycine betaine transport systems and harboring the various pBKB1-derived plasmids on glucose minimal plates containing 0.8 M NaCl and 1 mM glycine betaine. Growth of the strains was scored after 3 days of incubation at 37 °C.

recombined them by homologous recombination into the *B. subtilis* chromosome. For the construction of the Δ(*opuA*::*neo*)*1* mutation, an *Hpa*I restriction fragment was deleted from plasmid pBKB1 and substituted by a gene cartridge encoding a kanamycin determinant, yielding pBKB11 (Fig. 1). The Δ(*opuA*::*tet*)*2* mutation was constructed in an analogous way by removing an *Nsi*I DNA fragment from plasmid pBKB1 and inserting a tetracycline resistance gene as the selective marker, resulting in plasmid pBKB52 (Fig. 1). Both *opuA* mutations destroyed the plasmid pBKB1-encoded glycine betaine uptake activity in the *E. coli* strain MKH13 (Fig. 1). We isolated the Δ(*opuA*::*neo*)*1* and the Δ(*opuA*::*tet*)*2* constructs from plasmids pBKB11 and pBKB52, respectively, as *Eco*RI restriction fragments and then used this DNA to transform the *B. subtilis* strain JH642 to either kanamycin resistance (Δ(*opuA*::*neo*)*1*) or tetracycline resistance (Δ(*opuA*::*tet*)*2*). One transformant from each experiment was purified, and the proper integration of the Δ(*opuA*::*neo*)*1* (strain BKB4) or Δ(*opuA*::*tet*)*2* (strain BKB7) mutation into the *B. subtilis* genome via a double recombinational cross-over event was proven by Southern hybridization using a DNA probe derived from plasmid pBKB1 (data not shown).

We measured the initial uptake activity for radiolabeled [1-^{14}C]glycine betaine of the *opuA*$^+$ *B. subtilis* strain JH642 and its Δ*opuA* derivatives BKB4 and BKB7 in cells grown in low osmolarity and high osmolarity media with low substrate concentration (10 μM). An efficient and osmotically stimulated glycine betaine transport activity was present in the wild-type strain JH642 (Fig. 2*B*). In contrast, both *opuA* mutations strongly impaired glycine betaine uptake; this is documented in Fig. 2*B* for the Δ(*opuA*::*neo*)*1* deletion present in strain BKB4. Thus, *opuA* encodes an osmotically controlled glycine betaine transport system in *B. subtilis*. We note that neither of the chromosomal *opuA* deletions present in the *B. subtilis* strains BKB4 and BKB7 abolish glycine betaine uptake entirely (Fig. 2*B*). Thus, besides OpuA, at least one additional glycine betaine transporter must exist in *B. subtilis*, and the pattern of glycine betaine uptake in the *opuA* mutants indicates that this transport activity is also under osmotic control (Fig. 2*B*). In the above described glycine betaine uptake experiments, the high osmolarity media were prepared by adding NaCl to the growth media. In analogous transport assays in which NaCl was replaced by an isoosmolar concentration of KCl, glucose, or maltose, glycine betaine uptake activity in strains JH642 (*opuA*$^+$) and BKB4 (Δ(*opuA*::*neo*)*1*) was stimulated to an extent similar to that shown in Fig. 2*B* (data not shown). Thus, stimulation of glycine betaine transport in *B. subtilis* growing in a high osmolarity environment is a true osmotic effect since it can be triggered with either ionic or nonionic osmolytes.

opuA Encodes a Binding Protein-dependent Transport System—To characterize the nature of the *opuA*-encoded glycine betaine transporter more closely, we determined the DNA sequence of a 3.4-kb DNA segment from pBKB1 that covers the region necessary for glycine betaine uptake activity (Figs. 1 and 3). Analysis of the sequenced DNA segment revealed the presence of three open reading frames that are oriented in the same direction and constitute the *opuA* locus (Fig. 3). Downstream of *opuA*, a region is present that harbors a DNA structure with dyad symmetry. This inverted repeat is bracketed by runs of AT base pairs, indicating that it possibly could function as a Rho-independent bidirectional transcriptional terminator (36). The *opuA* locus consists of three structural genes (*opuAA*, *opuAB*, and *opuAC*), and their tight physical organization strongly suggests that they are genetically organized in an operon. The intergenic region between the *opuAA* stop codon (TAA) and the ATG start codon of the *opuAB* gene is only one nucleotide in length, and the ribosome-binding site for *opuAB* is thus present in the preceding *opuAA* coding region (Fig. 3). The genetic organization of the *opuAB* and the *opuAC* junction is even more tightly spaced; here, the ATG start codon of *opuAC* is part of the TGA stop codon for *opuAB* (Fig. 3). Each of the three genes is preceded at an appropriate distance by a potential ribosome-binding site, which for the *opuAB* and *opuAC* genes is entirely contained in the coding region of the preceding gene (Fig. 3).

The deduced amino acid sequences of the *opuAA*, *opuAB*, and *opuAC* genes exhibit features characteristic for binding protein-dependent transport systems (37, 38) and, in particular, show striking homology to the components of the glycine betaine binding protein-dependent transport system ProU from *E. coli* (18). The *opuAA* gene encodes a hydrophilic protein of 418 amino acid residues (M_r 46,473), and a comparison of the OpuAA protein with protein sequences present in the data libraries revealed strong sequence identities to many prokaryotic and eukaryotic proteins involved in ATP hydrolysis. Such a close relatedness in the amino acid sequence is a hallmark of the energizing components of binding protein-dependent trans-

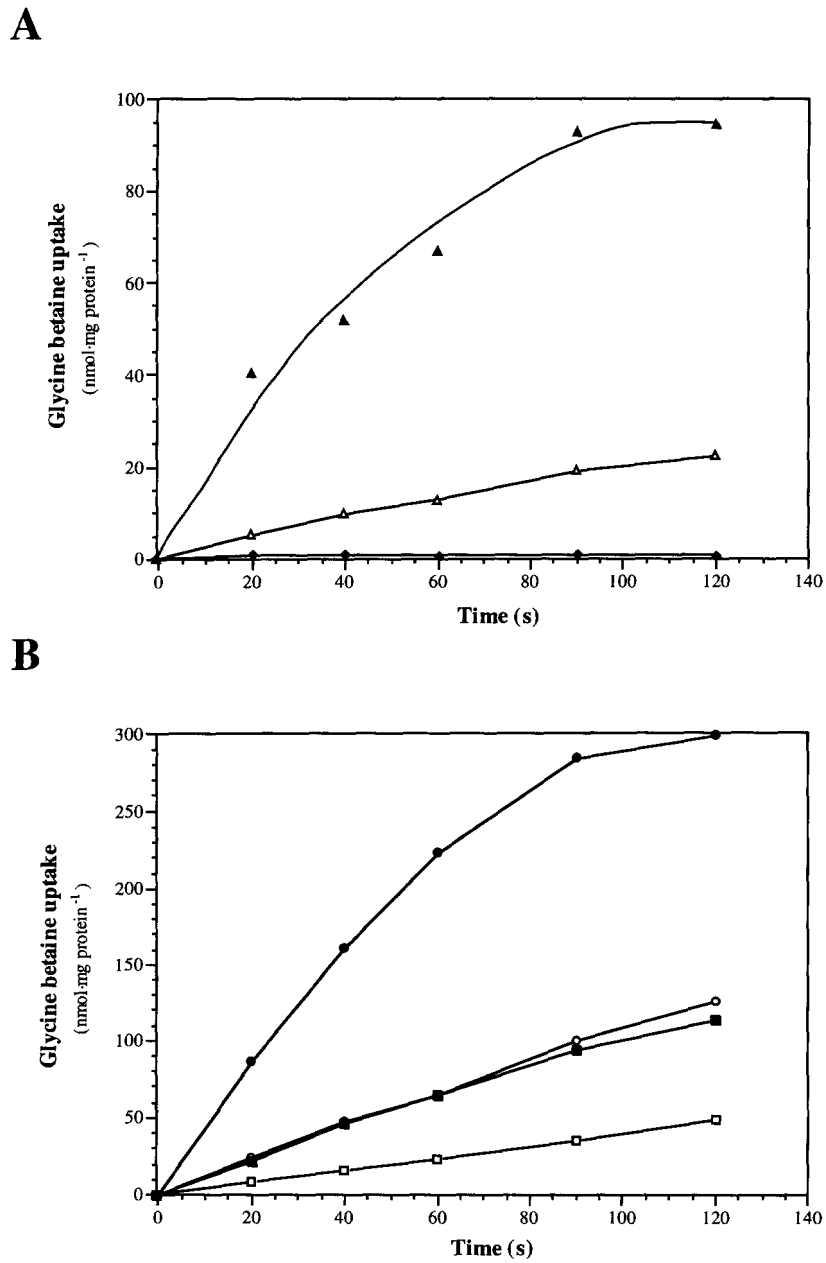

FIG. 2. **OpuA-mediated glycine betaine transport.** Uptake of radiolabeled [1-^{14}C]glycine betaine was assayed in low and high osmolarity grown cultures at a final substrate concentration of 10 μM. *A*, the *E. coli* mutant strain MKH13 (Pro-P$^-$ ProU$^-$) harboring plasmid pBKB1 (*opuA*$^+$) was grown in MMA (△) or MMA with 0.2 M NaCl (▲) to mid-log phase and assayed for glycine betaine uptake. Strain MKH13 carrying the vector plasmid pHSG575 grown in MMA with 0.2 M NaCl (◆) was used as a control. *B*, the *B. subtilis* strains JH642 (*opuA*$^+$) (○, ●) and BKB4 (*opuA*$^-$) (□, ■) were grown in SMM (○, □) or SMM with 0.4 M NaCl (●, ■) to mid-log phase, and glycine betaine uptake was then determined.

port systems (37, 38). The alignment of the OpuAA protein with the corresponding polypeptide (ProV) from the ProU system from both *E. coli* and *S. typhimurium* is shown in Fig. 4*A*. Approximately 58% of the amino acid residues are identical among all three proteins, and only a single gap needs to be introduced to achieve a good alignment of the protein sequences over their entire length. Sequence conservation is particularly apparent in the N-terminal half of the OpuAA and ProV proteins and is pronounced around the Walker A and B ATP-binding motifs (Fig. 4*A*).

The *opuAB* reading frame codes for a quite hydrophobic polypeptide (M_r 30,250) that is homologous to the integral inner membrane protein ProW of the *E. coli* ProU transport system. Analysis of the topology of the *E. coli* ProW protein with *phoA* and *lacZ* fusions has revealed that ProW has seven transmembrane segments with the carboxyl terminus in the cytoplasm and the amino terminus in the periplasm (39).[2] The ProW and OpuAB proteins show extensive sequence homology (47% identity) over their entire length and can be aligned without introducing a single gap into the amino acid sequence. Thus, the topology of OpuAB and ProW appears to be similar. The OpuAB protein (282 amino acids) is considerably smaller than ProW (354 amino acids). Most of the reduced size of OpuAB can be ascribed to a deletion removing 55 amino acids present in the N-terminal region of ProW thought to be exposed in the periplasmic space (39).[2] A small segment (amino acids 183–203) of OpuAB displays limited homology to integral inner membrane components of other binding protein-dependent transport systems from both Gram-negative and Gram-positive bacteria (40). It has been speculated that these residues contribute to an interaction site for the ATPases of the binding protein-dependent transporters (37, 38).

The last gene in the operon, *opuAC*, encodes a 293-amino acid residue hydrophilic protein with a predicted M_r of 32,218. The OpuAC protein is likely the substrate-binding protein component for the OpuA glycine betaine transport system. Its homologue, ProX, in the *E. coli* ProU system is a periplasmic protein (17, 41) that is initially synthesized with an N-terminal

[2] M. Haardt and E. Bremer, unpublished results.

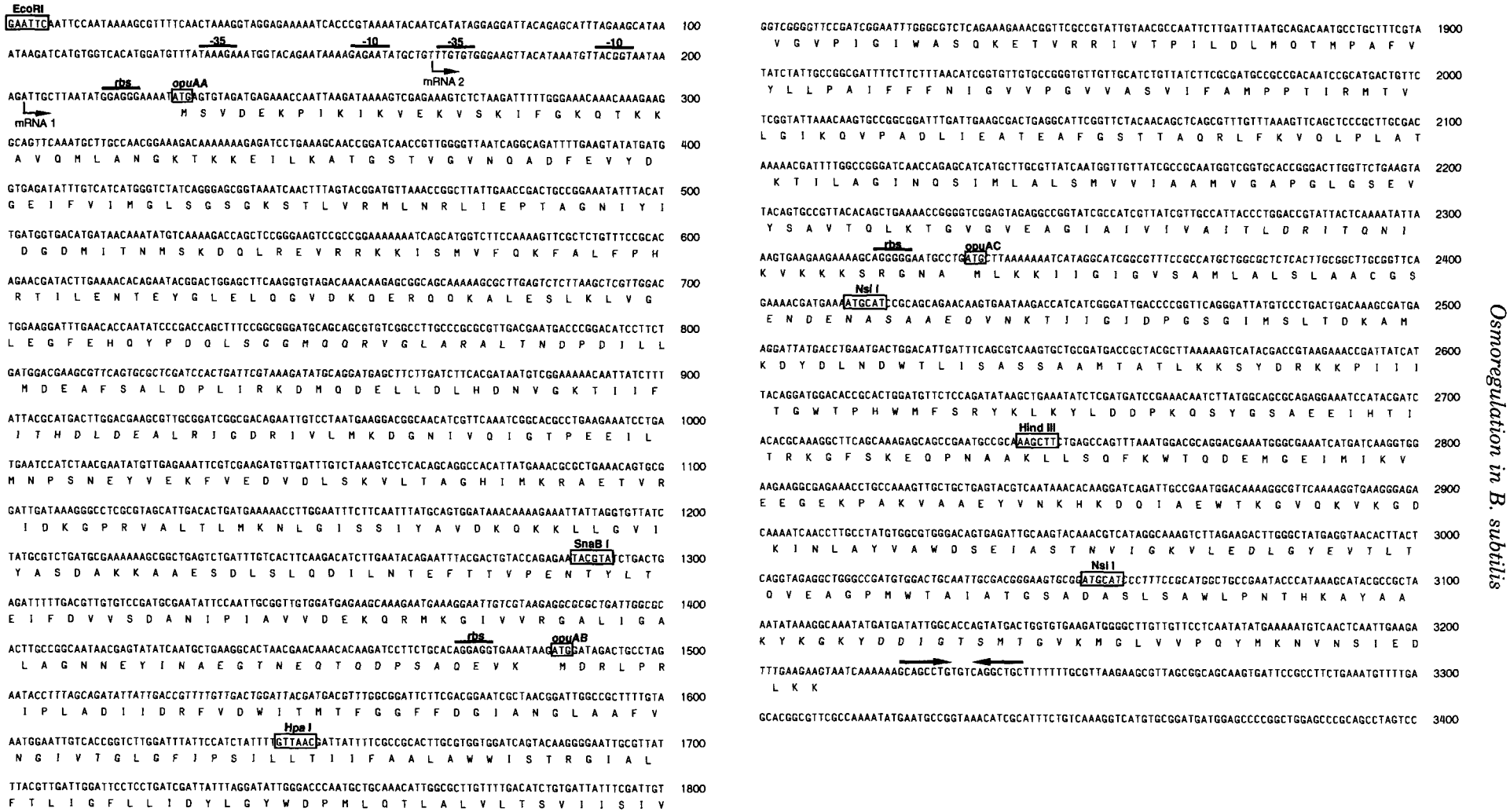

FIG. 3. **Nucleotide sequence of the *opuA* operon.** The determined DNA sequence of the *opuA* operon and the deduced amino acid sequences of the OpuAA, OpuAB, and OpuAC proteins are shown. The proposed ATG start codons for these genes are *boxed*, and putative ribosome-binding sites (*rbs*) are *overlined*. The transcription initiation sites for the *opuA* mRNAs (mRNA-1, mRNA-2) are indicated by *arrows*, and the corresponding putative −10 and −35 regions are marked. An inverted repeat downstream from the *opuAC* gene is indicated by a pair of *arrows*. The positions of recognition sites for a number of restriction enzymes are *boxed*.

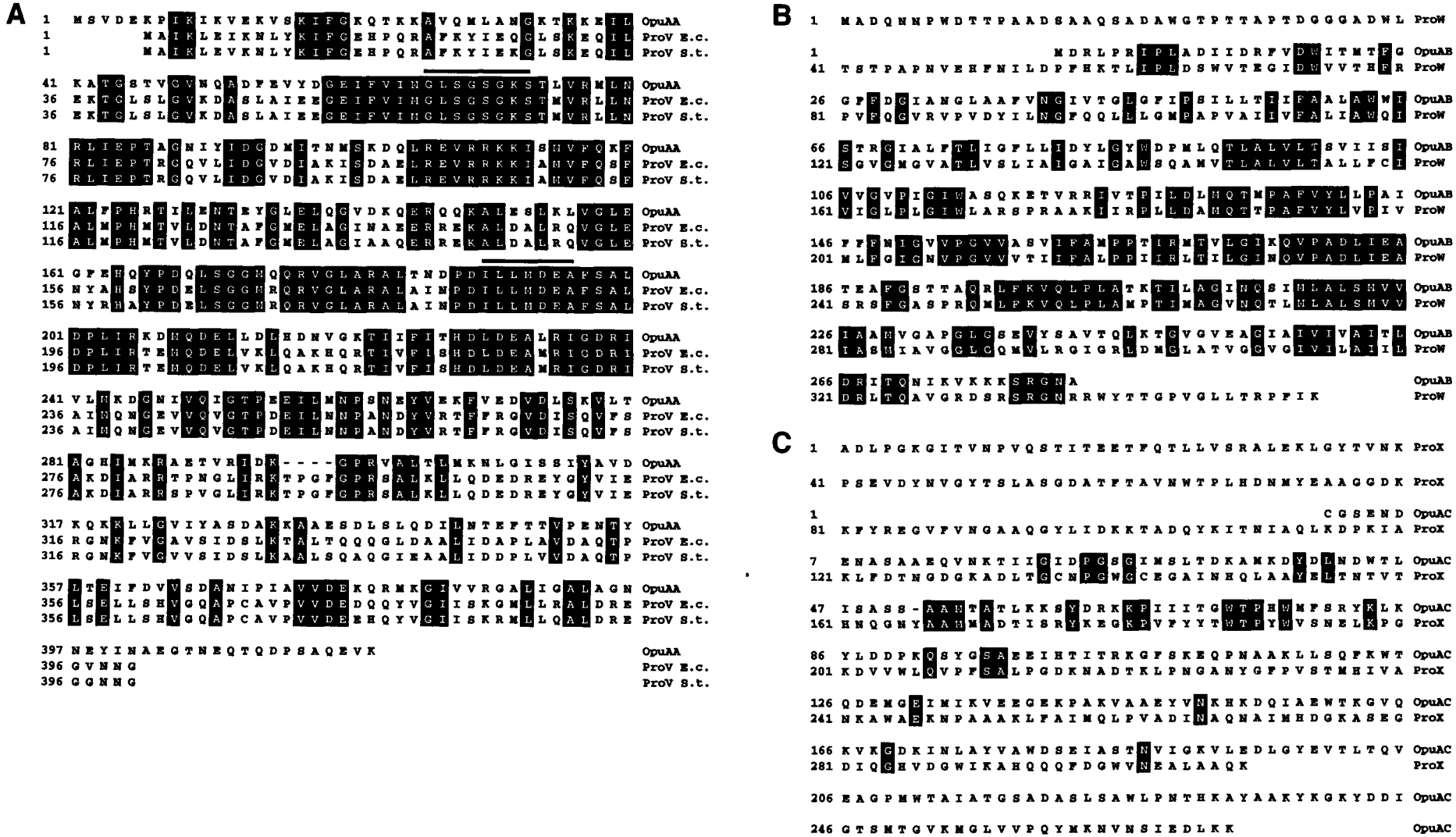

FIG. 4. **Alignment of the sequences of the components of the OpuA transport system with those of ProU.** *A*, the amino acid sequence of the *opuA*-encoded ATPase from *B. subtilis* is compared with the homologous protein ProV from *E. coli* (18) and *S. typhimurium* (64). The regions of the three proteins corresponding to the Walker A and B ATP-binding motifs are *overlined*. *B*, comparison of the OpuAB protein from *B. subtilis* and the corresponding ProW protein from *E. coli*. *C*, alignment of the sequences of the processed glycine betaine-binding protein ProX from *E. coli* and the OpuAC protein from *B. subtilis*.

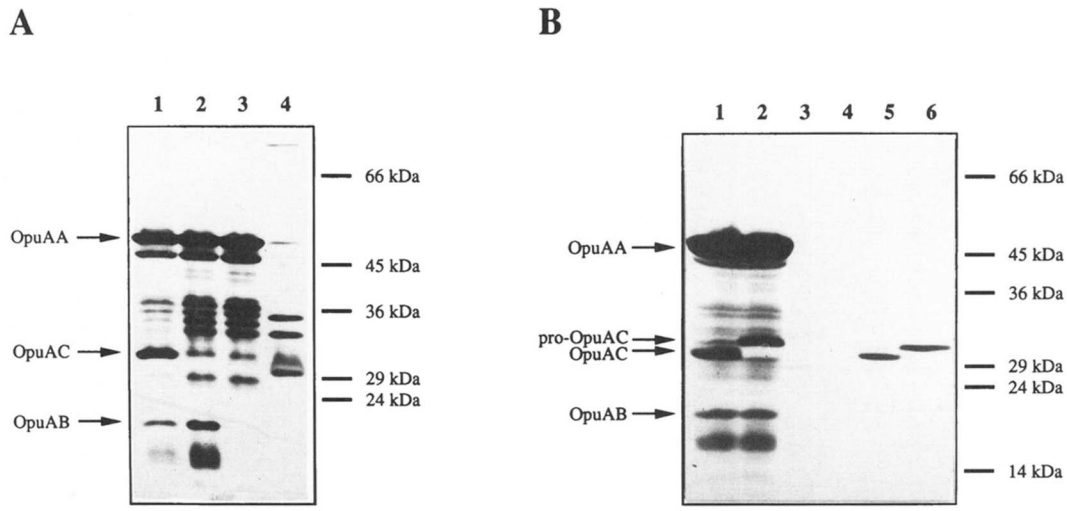

FIG. 5. **Expression of the *opuA*-encoded proteins under the control of the T7ϕ10 promoter.** *A*, T7ϕ10-mediated gene expression and radiolabeling of proteins was performed in derivatives of strain BL21(λDE3) carrying plasmids pBKB33 (*opuAA*$^+$, *opuAB*$^+$, *opuAC*$^+$) (*lane 1*), pBKB44 (*opuAA*$^+$, *opuAB*$^+$) (*lane 2*), pBKB43 (*opuAA*$^+$) (*lane 3*), and pBKB34 (*opuA*$^+$, but improperly aligned with respect to the T7ϕ10 promoter) (*lane 4*). *B*, effect of the antibiotic globomycin on the processing of the pro-OpuAC protein. T7ϕ10-mediated gene expression and radiolabeling of proteins was performed in derivatives of strain BL21(λDE3) carrying plasmids pBKB33 (*opuAA*$^+$, *opuAB*$^+$, *opuAC*$^+$) (*lanes 1* and *2*), pBKB57 (*opuAC*$^+$, but improperly aligned with respect to the T7ϕ10 promoter) (*lanes 3* and *4*), and pBKB58 (*opuAC*$^+$) (*lanes 5* and *6*). The samples of *lanes 2*, *4*, and *6* were treated with globomycin. The proteins were electrophoretically separated on a 12% SDS-polyacrylamide gel and visualized by autoradiography. The positions of the OpuAA, OpuAB, OpuAC, and pro-OpuAC proteins are indicated by *arrows*. The molecular mass of marker proteins is indicated on the *right*.

signal sequence extension (18). The first 20 amino acids of the *opuAC*-encoded protein exhibit the features of a secreted protein and show the characteristic signatures of a lipoprotein signal sequence. There is a positively charged N-terminal end, followed by a highly hydrophobic stretch of amino acids and a string of amino acids (Leu-Ala-Ala-Cys) that conforms to the consensus sequence recognized by signal peptidase II (42, 43). As a rule, the cysteine residue constitutes the N terminus of the proteolytically processed protein and is modified through the covalent attachment of lipids. This lipid modification of the N terminus serves to anchor the extracellular protein in the cytoplasmic membrane, and such lipoproteins have been described as substrate-binding proteins for a number of binding protein-dependent transport systems in Gram-positive bacteria (42, 43). The substrate binding proteins, ProX and OpuAC, show the least sequence conservation (33% identity in a 46-amino acid segment) among the components of the ProU and OpuA transport systems (Fig. 4*C*). Only a limited number of residues in the central part of the OpuAC and ProX proteins can be aligned, whereas the N-terminal and C-terminal ends of both proteins are entirely different (Fig. 4*C*).

Identification of the opuA Gene Products—To identify the proteins encoded by the *opuA* operon, we used the T7 RNA polymerase and T7ϕ10 promoter expression system (22, 24). We constructed a set of T7 expression plasmids carrying either the entire *opuA* operon (*opuAA*, *opuAB*, *opuAC*; pBKB33), the first two structural genes (*opuAA*, *opuAB*; pBKB44), or just the first gene of the *opuA* locus (*opuAA*; pBKB43). These plasmids were transformed into the *E. coli* strain BL21(λDE3), which carries a chromosomal copy of the structural gene for the T7 RNA polymerase under the control of the *lacPO* regulatory sequences (22). T7ϕ10 promoter-mediated expression of the various *opuA*-encoded genes was initiated by adding isopropyl-1-thio-β-D-galactopyranoside to the cultures, and the translated proteins were then labeled with [^{35}S]methionine. We were able to express the *opuA*-encoded proteins under T7ϕ10 control in *E. coli,* but many degradation products of the proteins were visible (Fig. 5*A*), indicating that the OpuA proteins from *B. subtilis* were relatively unstable when produced in the heterologous *E. coli* host. Such protein instability has also been observed when components for the binding protein-dependent iron-hydroxamate transport system from *B. subtilis* were expressed in *E. coli* under T7ϕ10 control (44). A comparison of the plasmid pBKB33-, pBKB44-, and pBKB43-encoded proteins allowed us to visualize and identify the components of the OpuA system. The *opuA*$^+$ plasmid pBKB33 directed the synthesis of three proteins with a apparent molecular mass of 47,000 daltons (OpuAA), 24,000 daltons (OpuAB), and 30,500 daltons (OpuAC). The 30,500-dalton protein was absent when the *opuAA*$^+$ *opuAB*$^+$ plasmid pBKB44 was used to mediate gene expression, thus identifying this polypeptide as the product of the *opuAC* gene. The same protein is synthesized in strain BL21(λDE3) carrying just the *opuAC* gene under T7ϕ10 control on plasmid pBKB58 (Fig. 5*B*, *lane 5*). A 47,000-dalton protein was produced in cells expressing only *opuAA* from plasmid pBKB43, (Fig. 5*A*, *lane 3*), thus identifying this polypeptide as the OpuAA protein. Both the 47,000-dalton protein (OpuAA) and the 24,000-dalton protein were synthesized when the *opuAA*$^+$ *opuAB*$^+$ genes (plasmid pBKB44) were expressed under T7ϕ10 control (Fig. 5*A*, *lane 2*). Thus, the 24,000-dalton protein must be OpuAB. None of these *opuA*-encoded proteins were produced when an *opuA*$^+$-containing restriction fragment was cloned into the T7 expression vector in an orientation reversed with respect to that present in plasmid pBKB33 (Fig. 5*A*; compare *lanes 1* and *4*). The apparent molecular masses of the OpuAA (47,000-dalton) and the OpuAC (30,500-dalton) proteins compare favorably with the molecular masses deduced for OpuAA (46,473 daltons) and OpuAC (30,235 daltons for the proteolytically processed but unmodified polypeptide) from the DNA sequence (Fig. 3) of their structural genes. In contrast, the apparent molecular mass of the OpuAB protein (24,000 daltons) as calculated from its electrophoretic mobility on a 12% SDS-polyacrylamide gel, deviates considerably from the molecular mass predicted for this protein from the *opuAB* DNA sequence (30,250 daltons). The OpuAB protein constitutes a quite hydrophobic integral membrane protein, and its apparent electrophoretic behavior is therefore not too surprising.

OpuAC Probably Is a Lipoprotein—As outlined above, the OpuAC protein is likely to carry lipid modifications at its N

terminus, anchoring it in the membrane. One characteristic feature of such lipoproteins is the inhibition of the proteolytical processing of their signal sequence by the cyclic peptide antibiotic globomycin (32, 45). To test whether OpuAC is indeed a lipoprotein, we expressed the entire *opuA* operon and the *opuAC* gene alone under T7ϕ10 control in the presence or absence of globomycin. The presence of globomycin inhibited completely the processing of the OpuAC protein and resulted in the accumulation of its precursor molecule, pro-OpuAC (Fig. 5*B*). In contrast, globomycin had no influence of the electrophoretic mobility of the OpuAA and OpuAB proteins (Fig. 5*B*, *lanes 1* and *2*). Thus, the selective block imposed by globomycin on pro-OpuAC processing strongly indicates that OpuAC is a lipoprotein with an amino-terminal cysteine-lipid anchor for the mature protein.

Osmoregulation of opuA Expression—The OpuA-mediated glycine betaine transport activity is osmotically modulated (Fig. 2). To test whether this was due (at least in part) to osmotic control of *opuA* transcription and to identify the *opuA* promoter(s), we mapped the transcription initiation sites by primer extension analysis. A 1.3-kb *Eco*RI-*Sna*BI restriction fragment carrying the *opuA* upstream region and most of the *opuAA* coding sequence was used to construct a Φ(*opuAA-lacZ*)*hyb1* protein fusion in the *E. coli-B. subtilis* shuttle vector pRB373. The resulting plasmid, pBKB56, was transformed into the *B. subtilis* strain JH642 to increase the gene dosage for the *opuA* regulatory region. Total RNA was then prepared from log-phase cultures grown either in LB medium or in LB medium with increased osmolarity (LB + 0.5 M NaCl). An *opuA*-specific primer was annealed to the RNA isolated from the low and high osmolarity grown cells and extended with avian myeloblastosis virus reverse transcriptase in the presence of [^{35}S]dATP. The reaction products were then separated on a DNA sequencing gel and visualized by autoradiography. Two *opuA*-specific mRNA species were detected that differed in size (38 bp) at their 5′ ends (Fig. 6*C*). Synthesis of the shorter transcript (mRNA-1) is under osmotic control, and the amount of this mRNA increases strongly in high osmolarity grown cells. In contrast, production of the longer transcript (mRNA-2) was not influenced by the osmolarity of the growth medium (Fig. 6*C*). Thus, expression of the *opuA* operon is mediated by two promoters that respond differently to changes in medium osmolarity. Inspection of the DNA sequence upstream of the initiation sites of mRNA-1 and mRNA-2 revealed the presence of putative −35 (consensus sequence: TTGACA) and −10 (consensus sequence: TATAAT) sequences that could possibly constitute promoters recognized by a form of RNA polymerase complexed with the main vegetative σ factor (σ^A) of *B. subtilis* (Fig. 6*A*) (46).

We monitored the influence of medium osmolarity on *opuA* expression with the aid of the Φ(*opuAA-lacZ*) *hyb1* hybrid gene present on plasmid pBKB56. This protein fusion encodes a hybrid protein that carries at its amino terminus a large segment of the OpuAA protein and at its carboxyl terminus an almost complete β-galactosidase. To test whether synthesis of the OpuAA′-βGal hybrid protein was under osmotic control, we grew the *B. subtilis* strain JH642(pBKB56) overnight in LB medium and LB medium with 0.5 M NaCl, prepared total cell extracts, and separated the proteins electrophoretically on a 7% SDS-polyacrylamide gel (Fig. 7*A*). Consistent with the influence of medium osmolarity on *opuA*-directed transcription, we detected a strong increase in the production of the hybrid OpuAA′-βGal protein in high osmolarity grown cells. This hybrid protein cross-reacted with an antiserum directed against β-galactosidase (Fig. 7*B*). We observed that increased synthesis of the large OpuAA′-βGal hybrid protein resulted in the formation of insoluble aggregates, which displayed no β-galactosidase activity. The protein from the *E. coli* ProU system, ProV, analogous in function to OpuAA from *B. subtilis*, is a membrane-associated protein, and the clumping of a ProV′-βGal hybrid protein has also been reported (47).

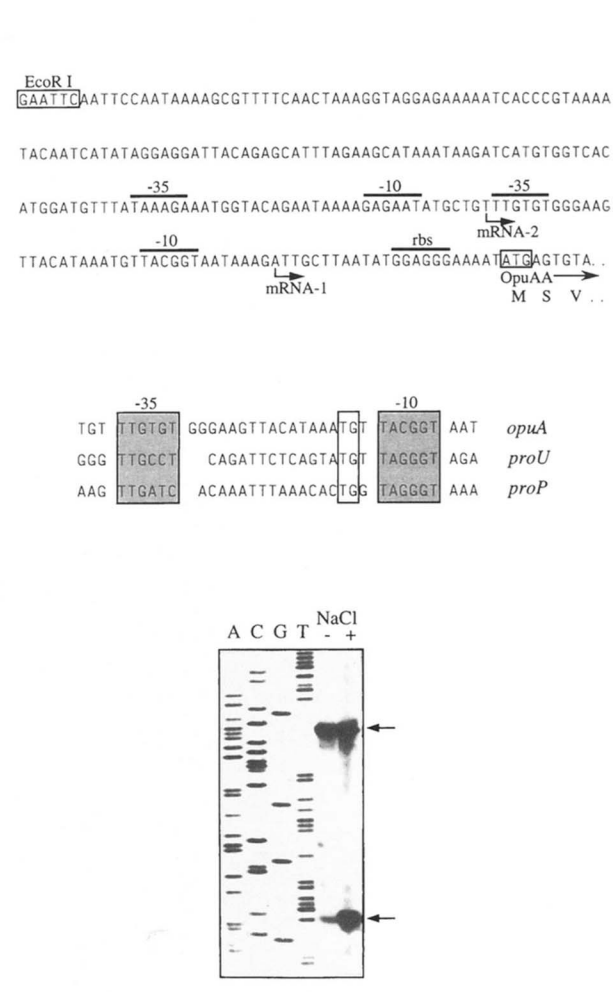

FIG. 6. **Structure of the *opuA* regulatory region and mapping of the *opuA* transcription initiation sites.** *A*, DNA sequence of the *opuA* regulatory region. Putative −10 and −35 sequences and the ribosome-binding site (*rbs*) for the *opuAA* gene are *overlined*. The exact positions of the transcription initiation sites for the *opuA* mRNA-2 and for the *opuA* mRNA-1 are indicated by *arrows*. *B*, comparison of the promoter regions of the osmoregulated *proU* and *proP* from *E. coli* with those of the osmotically controlled *opuA* P1 promoter from *B. subtilis*. *C*, mapping of the start sites for the *opuA* mRNAs. Total RNA was prepared from cells of strain JH642(pBKB56) grown in LB or LB with 0.5 M NaCl, hybridized to a primer complementary to the *opuA* mRNA, and extended with reverse transcriptase in the presence of radiolabeled [^{35}S]dATP. DNA sequencing reactions primed with the same synthetic oligonucleotide used for the primer extension reaction were employed as a standard to size the *opuA* mRNAs.

Physical and Genetic Mapping of the opuA Operon—A comparison of the nucleotide sequence of the 3.4-kb *opuA* region (Fig. 3) to the DNA data base revealed two short DNA sequences of 81 and 31 bp that matched (with the exception of a single mismatch in each DNA segment) the DNA sequence from 2910–2991 bp and 3339–3370 bp, respectively (Fig. 3). These matching sequences are located upstream of the *amyE* gene at 25° on the *B. subtilis* genetic map (48). They represent junction points of repeating units amplified in a mutant strain showing hyperproduction of an extracellular α-amylase (AmyE) and increased resistance to the antibiotic tunicamycin (*tmrB*) (49, 50). The identification of these junction point sequences within the *opuA* region suggested that the *opuA*

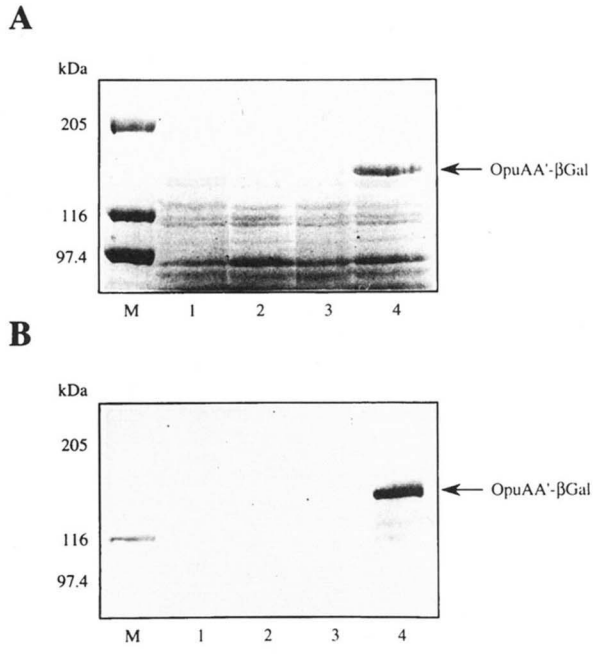

FIG. 7. **Osmoregulated expression of a *opuAA-lacZ* protein fusion in *B. subtilis*.** *A*, SDS-polyacrylamide gel electrophoresis of total protein extracts from the *B. subtilis* strain JH642 containing plasmids pRB373 (vector; *lanes 1* and *2*) or plasmid pBKB56 ((Φ(*opuAA-lacZ*) *hyb1*); *lanes 3* and *4*) grown in LB medium or LB medium with 0.5 M NaCl. The position of the OpuAA′-βGal hybrid protein is indicated by the *arrow*, and the molecular mass of marker proteins (*M*) is indicated on the *left*. Only the upper portion of the gel is shown; the proteins were stained with Coomassie Brilliant Blue. *B*, proteins of the samples displayed in *A* (*lanes 1-4*) and the marker proteins (*M*) containing β-galactosidase were electrophoretically separated by SDS-polyacrylamide gel electrophoresis, transferred to a nylon membrane, and reacted with an antiserum against β-galactosidase.

operon is located in the vicinity of the *amyE* gene. We therefore carried out both physical and genetic mapping experiments to test this assumption and to position the *opuA* operon on the *B. subtilis* genetic map.

Using a DNA probe covering *opuA* (Fig. 8*A*), we performed Southern hybridization experiments with chromosomal DNA prepared from strains JH642 (*opuA*$^+$ *amyE*$^+$), BKB4 (Δ(*opuA*::*neo*)*1 amyE*$^+$), and MO1099 (*opuA*$^+$ Δ(*amyE*::*ery*)). As expected, the *opuA* probe hybridized to a single 5.2-kb *Eco*RI fragment in chromosomal digests of the *opuA*$^+$ strains JH642 and MO1099 but recognized a smaller *Eco*RI restriction fragment (4 kb) in the chromosomal digest of the Δ(*opuA*::*neo*)*1* strain BKB4 (Fig. 8*B*). Thus, the DNA probe used detects specifically the *opuA* region in the *B. subtilis* genome. Two closely spaced *Pst*I restriction sites are present in the *amyE* gene (Fig. 8*A*), both of which were removed during the construction of the Δ(*amyE*::*ery*) mutation (19) (Fig. 8*A*). The *opuA* DNA probe detected an approximately 12-kb *Pst*I restriction fragment in a chromosomal digest of strain JH642 (and a correspondingly smaller 10.8-kb restriction fragment from strain BKB4), but an approximately 20-kb *Pst*I fragment was found in the Δ(*amyE*::*ery*) strain MO1099 (Fig. 8*B*). The larger size of the hybridizing chromosomal *Pst*I restriction fragment from strain MO1099 results from the fusion of two adjacent *Pst*I fragments (Fig. 8*A*). Taken together, these data show that *opuA* and *amyE* are physically located close to one another. The *amyE* gene has been positioned by DNA hybridization next to the end of a *Not*I restriction fragment on the physical map of the *B. subtilis* chromosome (51). Consistent with this previous report, we found a *Not*I restriction site downstream of the *opuA* operon (Fig. 1).

The linkage between *opuA* and *amyE* was also apparent when we performed a genetic mapping experiment using the DNA transformation technique. Chromosomal DNA from

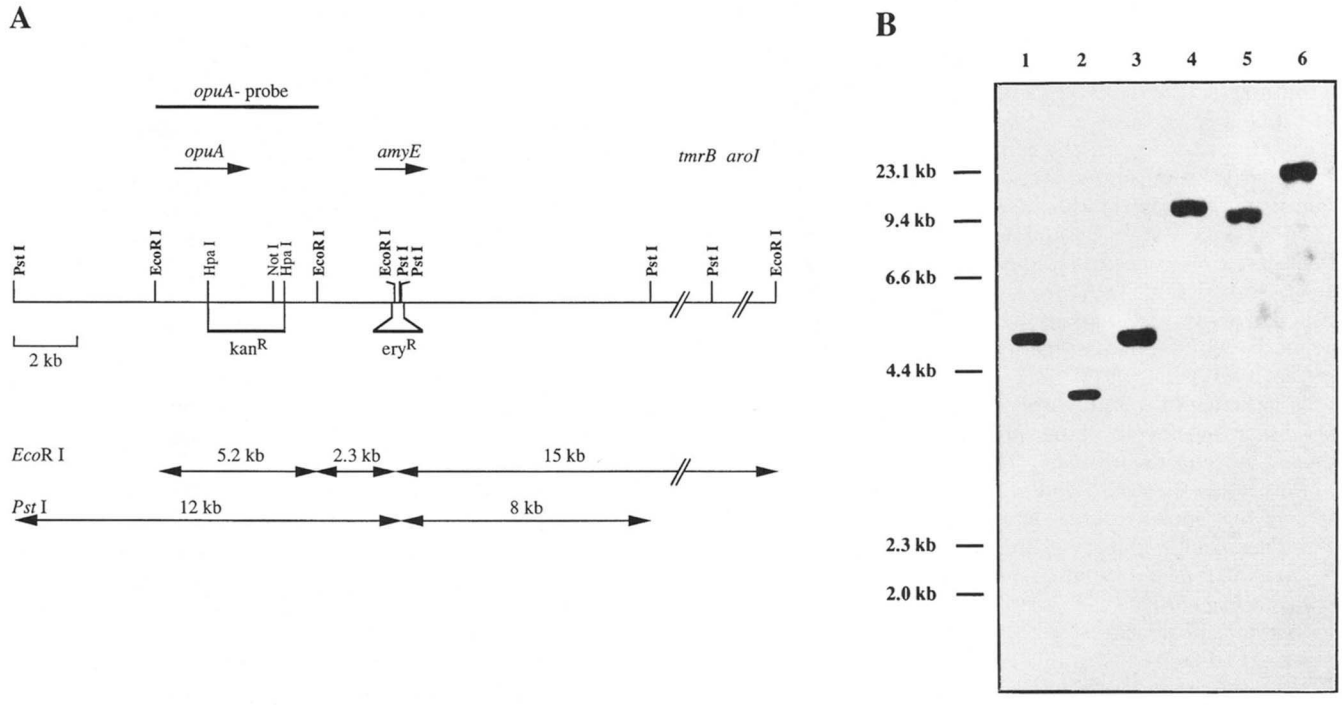

FIG. 8. **Physical mapping of the *opuA* operon.** *A*, restriction map and genetic organization of the *opuA*, *amyE*, *tmrB*, and *aroI* regions around 25° on the *B. subtilis* genetic map. The genetic and physical data for this diagram were compiled from the literature (48–50). All *Eco*RI, *Not*I, and *Pst*I sites are shown, but only the relevant *Hpa*I sites are indicated. The positions of the Δ(*opuA*::*neo*)*1* deletion in strain BKB4 and the Δ(*amyE*::*ery*) mutation in strain MO1099 are marked. The locations and directions of transcription of the *opuA* operon and the *amyE* gene are indicated by *arrows*, and the position of the *Eco*RI restriction fragment used as an *opuA*-specific probe in Southern hybridization experiments are indicated. *B*, Southern blot of chromosomal DNA of strains JH642 (*lanes 1* and *4*), BKB4 (*lanes 2* and *5*), and MO1099 (*lanes 3* and *6*) cut with *Eco*RI (*lanes 1*, *2*, and *3*) or *Pst*I (*lanes 4*, *5*, and *6*), respectively. The position of DNA standard fragments is shown on the *left*.

strain BKB4 (Δ(*opuA*::*neo*)*1 amyE*$^+$ *aroI*$^+$) was used to transform the *B. subtilis* strain TIBS57 (*opuA*$^+$ *amyE3 aroI10*) to kanamycin resistance. This latter strain was used as the recipient because it carries both an *amyE* mutation and an alteration in the *aroI*, gene which is closely linked to the *tmrB* locus (Fig. 8*A*). Transformants of strain TIBS57 were selected on LB agar plates containing kanamycin and were then tested for both their AmyE phenotype on starch-containing agar plates and their AroI phenotype on minimal plates lacking the aromatic amino acids Tyr, Phe, and Trp. From 197 kanamycin-resistant transformants characterized, 176 (89%) were found to be AmyE$^+$ and AroI$^-$, attesting to the tight genetic linkage between the *opuA* operon and the *amyE* gene. Consistent with the expected greater genetic distance between *opuA* and *aroI* (Fig. 8*A*), only a minor portion (21 of 197) of the kanamycin-resistant transformants of strain TIBS57 had acquired simultaneously both the *amyE*$^+$ and *aroI*$^+$ genes from strain BKB4. Taken together, these physical and genetic mapping experiments allow us to unambiguously position the *opuA* operon at 25° on the *B. subtilis* genetic map, and we conclude from the physical map of *opuA* that this operon is transcribed in a clockwise fashion on the *B. subtilis* chromosome.

DISCUSSION

The uptake of glycine betaine confers a high level of osmotic tolerance in *B. subtilis* and thus is an important facet in the stress response of this soil microorganism to high osmolarity (9). Glycine betaine is a preferred osmoprotectant in *B. subtilis* because the endogenous accumulation of proline is strongly reduced under high osmolarity growth conditions when glycine betaine is present in the culture medium (7). The data presented here show that glycine betaine transport in *B. subtilis* is under osmotic control and involves at least two transport systems. We have characterized one of these transporters in some detail and identified it as a multicomponent, binding protein-dependent transport system, OpuA.

Bacterial binding protein-dependent transport systems are members of a superfamily of prokaryotic and eukaryotic transporters, known as ATP-binding cassette (ABC) transporters or traffic ATPases (37, 38). These transporters couple hydrolysis of ATP to nutrient and ion uptake or to the translocation of drugs, polysaccharides, peptides and proteins across biological membranes. Because the substrate for the *opuA*-encoded ABC uptake system is metabolically inert in *B. subtilis* and serves an osmoprotective function (9), OpuA can be classified as part of the cellular defense machinery that permits this soil microorganism to cope with high osmolarity environments. Binding protein-dependent transport systems exhibit a very high affinity toward their substrate and can mediate unidirectional solute accumulation against a steep concentration gradient (37, 38). Consequently, transporters such as the OpuA system are especially well suited to scavenge their substrate effectively from the environment even when it is present at a very low concentration and still attain a high intracellular level of the transported compound. Glycine betaine is synthesized by plants (52) and is brought in a varying supply into the habitat of *B. subtilis* through the degradation of plant tissues, thus necessitating effective mechanisms for the active acquisition of this important osmoprotectant.

Characteristic for the binding protein-dependent transport systems of Gram-negative bacteria is the presence of a soluble, ligand-binding, periplasmic protein that serves to capture the substrate and deliver it to the membrane-bound components. Binding of glycine betaine to the periplasmic ProX proteins (Fig. 9) from *E. coli* and *S. typhimurium* has been demonstrated directly (17, 41, 53). Since Gram-positive bacteria have no periplasm, it has been proposed that extracellular proteins

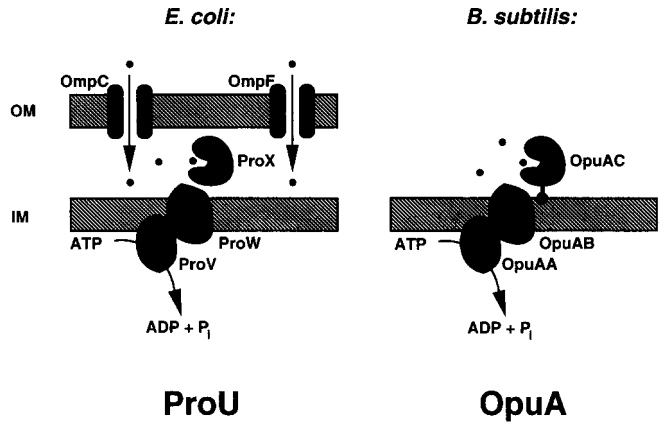

FIG. 9. **Model for the proposed organization of the binding protein-dependent glycine betaine transport systems ProU and OpuA.** Glycine betaine uptake through the ProU system in the Gram-negative bacterium *E. coli* is compared with that mediated by the OpuA system of the Gram-positive microorganism *B. subtilis*. The ProV and OpuAA proteins are ATP-binding subunits that couple ATP hydrolysis to the transport process. The ProW and OpuAB proteins are hydrophobic integral inner membrane (*IM*) proteins. The substrate-binding proteins of the transport systems are depicted within the periplasm (ProX) in *E. coli* and anchored to the membrane by an amino-terminal lipoyl moiety in *B. subtilis* (OpuAC). Permeation of glycine betaine (●) across the *E. coli* outer membrane (*OM*) occurs through the general diffusion pores OmpC and OmpF (65).

anchored via lipid modifications in the cytoplasmic membrane can serve the physiological function of periplasmic proteins from Gram-negative bacteria (42, 43). The components of the OpuA transport system show homology to those from the binding protein-dependent glycine betaine uptake system ProU from *E. coli* (Fig. 4). The amino acid sequence of the ATPases (OpuAA and ProV) and the integral inner membrane components (OpuAB and ProW) from both systems show extensive identity, but the substrate-binding proteins from the OpuA and ProU systems are not well conserved (Fig. 4). Such low level conservation of the ligand-binding proteins has also been observed for several other pairs of ABC transporters of Gram-negative and Gram-positive microorganisms (54). The OpuAC protein is essential for the OpuA-mediated glycine betaine transport in *B. subtilis* (Fig. 1), and its processing is inhibited by globomycin (Fig. 5*B*), indicating that OpuAC is a lipid-modified and extracellular substrate-binding protein. The overall organization and subunit composition of the *B. subtilis* OpuA system is shown in Fig. 9 and is compared with its counterpart, ProU, from the Gram-negative bacterium *E. coli*.

The Δ(*opuA*::*neo*)*1* mutation (Fig. 1) present in strain BKB4 removes both the genes for the substrate-binding protein (OpuAC) and the integral inner membrane component (OpuAB), thus inevitably destroying the functioning of the OpuA system entirely. A strongly reduced glycine betaine transport at low substrate concentration (10 μM) reflects the loss of the OpuA-mediated transport activity (Fig. 2*B*). However, the presence of the Δ(*opuA*::*neo*)*1* deletion does not completely abolish glycine betaine uptake and hence uncovers the existence of a second transport pathway for this osmoprotectant in *B. subtilis*. This second glycine betaine transport system is under osmotic control (Fig. 2*B*), implying that it is also involved in the defense against the deleterious effects of high osmolarity.

Experiments with a *opuAA-lacZ* protein fusion showed that the amount of OpuA is responsive to changes in the osmotic strength of the environment. High osmolarity growth conditions induce *opuA* expression, and two differently regulated promoters direct the transcription of the *opuA* operon; one is

osmotically controlled (*opuA* P-1), whereas the second (*opuA* P-2) does not respond to the osmotic stimulus (Fig. 6). The putative −10 and −35 regions of the *opuA* P-1 and *opuA* P-2 promoters show homology (Fig. 6*A*) to the consensus sequence of σ^A-dependent promoters (46), and hence both promoters are likely transcribed by an RNA polymerase complex containing the main vegetative σ factor (σ^A). The alternative transcription factor σ^B is an important regulatory element for a large network of stress proteins of *B. subtilis* whose synthesis increases after exposure of the bacterial cell to salt (4–6). We have tested glycine betaine uptake in several *sigB* mutants and found no difference from their *sigB*$^+$ parents, indicating that σ^B does not play a central role in the regulation of the glycine betaine uptake systems of *B. subtilis*.[3] The distance of 17 bp between the −35 and −10 boxes in the *opuA* P-2 promoter matches the ideal distance between −35 and −10 regions of σ^A-dependent promoters, whereas the osmoregulated *opuA* P-1 promoter has a suboptimal spacing of 18 bp (Fig. 6*A*). Although both *opuA* promoters can direct the synthesis of substantial amounts of mRNA (Fig. 6*C*), they do not conform closely to the −35 and −10 consensus sequences of σ^A-dependent promoters (Fig. 6, *A* and *B*). In particular, the osmoregulated *opuA* P-1 promoter is unusual since it contains in its −10 region a string of three consecutive GC base pairs. Interestingly, both the osmoregulated *proU* and *proP* promoters from *E. coli* also exhibit −10 regions rich in GC base pairs (Fig. 6*B*), and each of these promoters contains a TG motif characteristic for an extended −10 region that can partially compensate for inefficient −35 regions (55, 56). A point mutation in the *E. coli proU* −10 region altering one of the GC base pairs to an AT base pair increases the basal level of *proU* expression at low osmolarity but does not alter its osmotic regulation (47). It is thus likely that the unusual −10 region of the *B. subtilis opuA* P-1 promoter makes an important contribution to the low basal level of the *opuA* P-1 transcript in the absence of osmotic stress (Fig. 6*C*).

In addition to their unusual −10 regions, both the osmotically regulated *proU* and *opuA* P-1 promoters deviate in the length of their spacer regions between the −35 and −10 sequences from the consensus 17-bp distance and contain suboptimal spacings of 16 and 18 bp, respectively (Fig. 6*B*). Expression of the osmotically regulated *proU* operon from *E. coli* is sensitive to changes in DNA topology (57, 58). RNA polymerase appears to make specific contacts with both the −10 and −35 regions, and the relative orientation of these sequences is an important determinant for the efficiency of transcription initiation (59). Promoters with a 16- or 18-bp spacer sequence might therefore respond sensitively to environmentally controlled alterations in DNA topology, and both the *E. coli proU* and *B. subtilis opuA* P-1 promoters might thus be members of a class of DNA twist-sensitive promoters (60).

DNA sequences located upstream and downstream of the osmoregulated *E. coli proU* promoter and the nucleoid-associated DNA binding protein H-NS and HU contribute to the finely tuned genetic control of *proU* expression in response to changes in medium osmolarity (25, 58, 61–63). Our identification of the osmoregulated *opuA* P-1 promoter from *B. subtilis* is an important first step in identifying the DNA sequences required in *cis* to mediate osmotically controlled transcription and in unravelling the signal transduction pathway that allows *B. subtilis* to sense changes in the environmental osmolarity and convert this information into a genetic response that finally leads to increased *opuA* expression.

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[3] B. Kempf and E. Bremer, unpublished results.

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