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

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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 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 of 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 (L, 2).

The exposure of B. subtilis to a hypersaline environment triggers the induction of a set of general and salt-specific stress genes, 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 oσ2, 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 oσ2 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 oσ2-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 osmoles 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 osmoles, which are deleterious at high concentrations, with organic osmoles, 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

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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 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::EcoR; BGSC 1A717) has been described (19). Strain TIB857 (amyE3, aro10; BGSC 1A4744) was used for the genetic mapping of the *opaA* operon. The *B. subtilis* strains *BBK4* (Δ*opa:A::neo*) and *BBK7* (Δ*opa:A::tet*) are derivatives of *JH642* and were constructed by transforming *JH642* with EcoRI restriction fragments isolated from plasmids pBBK11 (Δ*opa:A::neo*; Fig. 1), and pBBK52 (Δ*opa:A::tet*; Fig. 1), respectively, and selecting for kanamycin-resistant (BBK4) or tetracycline-resistant (BBK7) colonies on LB agar plates. The *E. coli* K-12 strain MKH13 (*ApuP* 101 *Δspo1P2*3 [proU] 608) is a derivative of strain MC1000 (20) and is entirely deficient in glycine betaine uptake (21). The *E. coli* B strain RB211 was derived for the selection of *B. subtilis* strains carrying the T7d10 control (22). The low copy number plasmid pBS0575 (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 *opaA* 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 for the expression of the *opaA* operon under the control of the T7d10 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 *opuAA-opuB-yhb* hybrid gene. The *E. coli* B subtilis shuttle vector pBB373 has been described (26). DNA cartrides encoding genes ferring resistance to kanamycin 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, MMB medium for ES, or S. spizizen’s minimal medium. The growth conditions were maintained until late log phase. AroI phenotype of *B. subtilis* strains was detected by flooding the colonies grown on LB agar plates with 0.2% L-phenylalanine, and 20 mg/liter L-tyrosine; Aror: strains can not grow in minimal medium (29). A stock solution (10 mg/ml) of globomycin was prepared in a final concentration of 0.5 M NaCl added. The osmolarity (0.5 M NaCl) of the medium was determined with a vapor pressure osmometer (model 5500; Wescor Inc., Logan, UT). Expression of the DNA, we used MMA minimal agar plates containing 0.2% glucose as the carbon source, 0.8 M NaCl and 1 mM glycine betaine.

**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 double-stranded DNA template method (18). Partial restriction fragments were identified using a hybridization procedure 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 EcoRI insert present in plasmid pBBK1 (Fig. 1) was used to generate a probe by random nucleotide labeling using DIG-UTP (DIG DNA labeling and detection kit, Boehringer Mannheim). The labeled DNA fragment to EcoRI- and PstI-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 in LB medium, or LB medium of high osmolarity (0.5 M NaCl added) of the *B. subtilis* strain *JH642* carrying the *opaAA-lacZ* fusion plasmid pBBK66 essentially as described by Volker 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 estimated. A stock solution (10 mg/ml) of globomycin was prepared in 40 μg/ml RNA (33). For the primer extension reaction, a synthetic primer (5’-GAAATCTCTTCTTGTTGTTTTG-3’), complementary to the *opaA* 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 [α-32 P]deoxycytidine monophosphate (34). The 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 *opaA* 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 EcoRI and ligating the resulting restriction fragments into the EcoRI site in the polylinker of the lacZ α-complementing plasmid pHSG575 (35). 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 pBBK13, pBBK14, pBBK17, pBBK18, pBBK38, and pBBK46 were constructed by deleting defined restriction fragments from the *opaA* plasmids pBBK1 and religating the plasmid backbone (Fig. 1). Plasmids pBBK15 and pBBK33 were constructed by using appropriate restriction fragments isolated from plasmid pBBK1 into the vector pHS575 (Fig. 1). Plasmid pBBK33 carries the entire *opaA* operon on a 4-kb EcoRI-EcoRV restriction fragment (Fig. 1) that has been cloned into the polylinker sequence of the T7d10 expression vector pPD100, thus positioning *opaAA* under T7d10 control. The same restriction fragment was inserted in the reverse orientation with respect to the T7d10 promoter present in the vector pPD101, yielding plasmid pBBK34. Plasmid pBBK44, which expresses the opuAA*" opuAB* genes under T7d10 control was constructed by deleting a 656-bp NsiI fragment from pBBK3 (Fig. 1). The remaining 5′ end of the *opaAC* gene (Fig. 1) from the *opaA* plasmid pBBK35. To achieve expression of the *opaA* gene under T7d10 control, a 1.7-kb EcoRI-Hpall restriction fragment (Fig. 1) was inserted into the vector pPD100, yielding plasmid pBBK43. A plasmid expressing the *opaAC* gene under T7d10 control was constructed by isolating a 1.4-kb ApaLI-NcoI fragment from pBBK7; the overhanging ends of the restriction fragments were filled in with Klenow enzyme and then ligated into the *Smal* site of plasmid pPD100. Plasmids positioning the *opaAC* gene under T7d10 control (pBBK58) or aligning it in the reverse orientation with the T7d10 promoter (pBBK57) were identified by restriction analysis. To construct an *opaAA-lacZ* gene fusion, a 1.9-kb EcoRI restriction fragment from pHS575 (Fig. 1) was cloned into the vector EcoRI and *Smal* sites of the *lacZ* fusion vector pJL29, yielding plasmid pBBK54. In this plasmid, the reading frames of *opaAA* and *lacZ* are properly aligned across the *Snml* and *SmaI* junction, thus generating a hybrid protein fusion, *opuAA-lacZDhby1*. The entire hybrid gene was transferred to the *E. coli* B subtilis shuttle vector pRBS375, which had been cut with an internal restriction fragment.
with EcoRI and Smal; this construction resulted in plasmid pBBK56.

**Transport Assays for Radiolabeled Glycine Betaine—Uptake of glycine betaine in *B. subtilis* and *E. coli* was measured using [1-14C]glycine betaine (55 mCi/mmol) as a substrate. The cells were grown to mid-exponential phase (A600 = 0.15-0.5) in minimal medium with glucose as the carbon source and used immediately for the transport assay. *E. coli* cells were grown in MMA or MMA with 0.2 M NaCl (0.2 M NaCl) as the carbon source and used immediately for the transport assay.

When radiolabeled glycine betaine was added to the cell suspensions, the cell suspensions were incubated at 37 °C for 30 min. The uptake of glycine betaine was determined by measuring the radioactivity retained on the filters. The filters were washed with 50 mL of J.LM-pore-size filters (Schleicher and Schuell GmbH, Dassel, Germany) and the radioactivity retained on the filters was determined in a scintillation counter.

**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 *B. subtilis* carrying the opuAA'-β-galactosidase fusion plasmid pBBK56 were grown overnight at 37 °C in LB medium or LB medium with 0.5 mM NaCl. The optical density (A578) of the cultures was determined and adjusted to A578 = 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 TE) was added. The cells were then incubated for 10 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 benzonase nuclease (Merck) was added and incubated for 10 min at 37 °C, followed by another short (5-min) incubation at 95 °C.

**Expression of the opuA Gene Products under T7 Control—** Plasmids carrying various genes from the *opuA* operon under the transcriptional control of the T7 promoter were introduced into strain BL21(ADE3) to selectively visualize the *opuA*-encoded proteins. These plasmids are pBBK33 (opuAA' opuAB' opuAC'), pBBK44 (opuAA' opuAB' opuAC'), pBBK43 (opuAA'), and pBBK58 (opuAA'). Plasmids pBBK34 and pBBK57 carrying the *opuA'* operon and *opuA'-gene, respectively, improperly aligned with respect to the T7 promoter were used as controls. Controls (20 ml in 100-ml Erlenmeyer flasks) of strain BL21(ADE3) carrying the various plasmids were grown in M9 minimal medium with 0.2% casamino acids to mid-log phase (A600 = 0.5–0.7); the cells were washed with M9 minimal medium, resuspended in 20 μL of M9 minimal medium supplemented with 0.2% methionine assay medium, and 15 μL of radiolabeled 1-μl sample was added to each sample. The mediated transport of glycine betaine 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 [35S]methionine (final concentration, 52 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 cycloheximide was added to the appropriate cultures 10 min prior to the radiolabeling of the proteins.

**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 osmoprotective glycine betaine. We reasoned that it should be possible to functionally complement the deficiency of strain MKH13 in glycine betaine uptake with the appropriate *opuA* genes. A gene bank of EcoRI restriction fragments was prepared from chromosomal DNA of the *B. subtilis* strain JH642 in the low copy number cloning vector pHSG575 (Cm') and transformed into MKH13 by selecting for Cm' colonies on LB agar plates. These transformants were then replica-plated on high osmolarity minimal plates (MMA agar plates with 0.8 mM 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 EcoRI restriction fragment. A restriction map of one of these plasmids, pBBK1, is shown in Fig. 1.

The ability of strain MKH13(pBBK1) to grow at high osmolarity in the presence of 1 mM glycine betaine was shown to be dependent on the presence of plasmid pBBK1 by retransformation into strain MKH13. There was no growth of MKH13 (pBBK1) in high osmolarity minimal medium (MMA agar plates with 0.8 mM NaCl) lacking this osmoprotectant. Osmoprotection by glycine betaine requires the intracellular accumulation of this compound (1, 2). We therefore measured the initial [1-14C]glycine betaine uptake in cultures of strain MKH13 (pBBK1) grown in low osmolarity or high osmolarity minimal media. A final substrate concentration of 10 μM. Glycine betaine transport activity was readily detectable in cultures of MKH13 (pBBK1), and we found that this transport activity was under osmotic control (Fig. 2A). Thus, plasmid pBBK1 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 pBBK1 into the low copy number vector pHSG575 and also constructed a number of deletion derivatives of pBBK1 (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 EcoRI 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 pBBK1 and then
glycine betaine transport activity was present in the wild-type strain JH642. Thus, the chromosomal tations destroyed the plasmid pBKBl-encoded glycine betaine transporter more closely, determining the DNA sequence present in the various deletion derivatives and subclones of plasmid pBKBl. These derivatives were isolated by homologous recombination into the B. subtilis chromosome. For the construction of the ∆opiAA::neoI mutation, an HpaI restriction fragment was deleted from plasmid pBKBl and substituted by a gene cartridge encoding a kanamycin determinant, yielding pBKBl11 (Fig. 1). The ∆opiAA::tet2 mutation was constructed in an analogous way by removing a NsiI DNA fragment from plasmid pBKBl1 and inserting a tetracycline resistance gene as the selective marker, resulting in plasmid pBKBl12. In both cases, the deletion was confirmed by Southern hybridization using a DNA probe derived from pBKBl11 and pBKBl12, respectively, as templates. The proper integration of the deletion in pBKBl and pBKBl11 was confirmed additionally by EcoRI restriction fragments and then used this DNA to transform the B. subtilis strain JH642 to either kanamycin resistance (ΔopiAA::neoI) or tetracycline resistance (ΔopiAA::tet2). One transformant from each experiment was purified, and the proper integration of the deletion in strain BKB4 or ΔopiAA::tet2 (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 pBKBl11 (data not shown).

We measured the initial uptake activity for radiolabeled [1-14C]glycine betaine of the opuA+ B. subtilis strain JH642 and its ∆opiAA derivatives BKB4 and BKB7 in cells grown in low osmolarity and high osmolarity media with low osmolarity concentration (10 μM). An efficient and osmotically stimulated glycine betaine uptake was present in the wild-type strain JH642 (Fig. 2B). In contrast, both ∆opiAA mutations strongly impaired glycine betaine uptake; this is documented in Fig. 2B for the ∆opiAA::neoI 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. 2B). Thus, besides OpuA, at least one additional glycine betaine transporter must exist in B. subtilis, and the pattern of glycine betaine uptake in the ∆opiAA mutants indicates that this transport activity is also under osmotic control (Fig. 2B). 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 (ΔopiAA::neoI) was stimulated to an extent similar to that shown in Fig. 2B (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 pBKBl 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 (Mr, 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-
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A

FIG. 2. OpuA-mediated glycine betaine transport. Uptake of radiolabeled [1-14C]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− ProU−) harboring plasmid pBK1 (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.

B

The opuAB reading frame codes for a quite hydrophobic polypeptide (M, 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). 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). 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, of 32,218. The OpuAC protein is likely the substrate-binding 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 a pair of arrows, and the corresponding putative -10 and -35 regions are marked. An inverted repeat downstream from the opuA 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.
signal sequence extension (18). The first 20 amino acids of the opuA-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. 4C). 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. 4C).

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 (opuA; pBKB43). These plasmids were transformed into the E. coli strain BL21(DE3), 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 [35S]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. 5A), 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(DE3) carrying just the opuAC gene under T7φ10 control on plasmid pBKB58 (Fig. 5B, lane 5). A 47,000-dalton protein was produced in cells expressing only opuAA from plasmid pBKB43 (Fig. 5A, lane 3), thus identifying this polypeptide as the OpuA 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. 5A, 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. 5A; 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 (46,475 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 proteolytic 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 opuAC operon and the opuAC gene alone under T76610 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. 5B). In contrast, globomycin had no influence of the electrophoretic mobility of the OpuAA and OpuAB proteins (Fig. 5B, 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 EcoRI-StuI 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, pBBK56, 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 osmolality (LB + 0.5 M NaCl). An opuA-specific primer was annealed to the RNA isolated from the low and high osmolality grown cells and extended with avian myeloblastosis virus reverse transcriptase in the presence of [35S]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. 6C). Synthesis of the shorter transcript (mRNA-1) is under osmotic control, and the amount of this mRNA increases strongly in high osmolality grown cells. In contrast, production of the longer transcript (mRNA-2) was not influenced by the osmolality of the growth medium (Fig. 6C). Thus, expression of the opuA operon is mediated by two promoters that respond differently to changes in medium osmolality. 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 (σ70) of B. subtilis (Fig. 6A) (46).

We monitored the influence of medium osmolality on opuA expression with the aid of the Φ(opuAA-lacZ)hyb1 hybrid gene present on plasmid pBBK56. 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/pBBK56 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. 7A). Consistent with the influence of medium osmolality on opuA-directed transcription, we detected a strong increase in the production of the hybrid OpuAA−βGal protein in high osmolality grown cells. This hybrid protein cross-reacted with an antiserum directed against β-galactosidase (Fig. 7B). 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).

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-amylace (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
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. 8A), we performed Southern hybridization experiments with chromosomal DNA prepared from strains JH642 (opuAΔamyE+), BKB4 (Δ(opuAΔneo)1 amyE+), and MO1099 (opuAΔamyE::ery). As expected, the opuA probe hybridized to a single 5.2-kb EcoRI fragment in chromosomal digests of the opuAΔ strains JH642 and MO1099 but recognized a smaller EcoRI restriction fragment (4 kb) in the chromosomal digest of the Δ(opuAΔneo)1 strain BKB4 (Fig. 8B). Thus, the DNA probe used detects specifically the opuA region in the B. subtilis genome. Two closely spaced PstI restriction sites are present in the amyE gene (Fig. 8A), both of which were removed during the construction of the Δ(amyE::ery) mutation (19) (Fig. 8A). The opuA DNA probe detected an approximately 12-kb PstI 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 PstI fragment was found in the Δ(amyE::ery) strain MO1099 (Fig. 8B). The larger size of the hybridizing chromosomal PstI restriction fragment from strain MO1099 results from the fusion of two adjacent PstI fragments (Fig. 8A). 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 NotI restriction fragment on the physical map of the B. subtilis chromosome (51). Consistent with this previous report, we found a NotI 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. 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 (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 Coomasie 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.

![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 EcoRI, NotI, and PstI sites are shown, but only the relevant HpaI sites are indicated. The positions of the Δ(opuAΔneo)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 EcoRI 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 EcoRI (lanes 1, 2, and 3) or PstI (lanes 4, 5, and 6), respectively. The position of DNA standard fragments is shown on the left.](image-url)
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. 8A). 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. 8A), 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 osmolality (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, OpU.

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 or ion uptake or to the translocation of charged, hydrophobic substrates 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), OpU can be classified as part of the cellular defense machinery that permits this soil microorganism to cope with high osmolality 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 OpU 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 (62) 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 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 OpU 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 (OpUB and ProW) from both systems show extensive identity, but the substrate-binding proteins from the OpU 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 OpU-mediated glycine betaine transport in B. subtilis (Fig. 1), and its processing is inhibited by globomycin (Fig. 5F), 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 OpU system entirely. A strongly reduced glycine betaine transport at low substrate concentration (10 μM) reflects the loss of the OpU-mediated transport activity (Fig. 2B). 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. 2B), implying that it is also involved in the defense against the deleterious effects of high osmolality.

Experiments with a opuA-lacZ protein fusion showed that the amount of OpuA is responsive to changes in the osmotic strength of the environment. High osmolality 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. 6A) to the consensus sequence of σ^P-dependent promoters (46), and hence both promoters are likely transcribed by an RNA polymerase complex containing the main vegetative σ^P factor (σ^P). The alternative transcription factor σ^R 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^R does not play a central role in the regulation of the glycine betaine uptake systems of *B. subtilis*. 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^P-dependent promoters, whereas the osmoregulated opuA P-1 promoter has a suboptimal spacing of 18 bp (Fig. 6A). Although both opuA promoters can direct the synthesis of substantial amounts of mRNA (Fig. 6C), they do not conform closely to the −35 and −10 consensus sequences of σ^P-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. 6B), 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 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. 6C).

In addition to their unusual −10 regions, both the osmoregulated 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. 6B). 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 −35 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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