Sequence of an Osmotically Inducible Lipoprotein Gene

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The osmB gene of Escherichia coli, whose expression is induced by elevated osmolarity, was cloned and physically mapped to a 0.65-kilobase-pair NsiI-HincII DNA fragment at 28 min on the E. coli chromosome. The OsmB protein was identified in minicells expressing the cloned gene. The nucleotide sequence of a 652-base-pair chromosomal DNA fragment containing the osmB gene was determined. The open reading frame encodes a 72-residue polypeptide with an M, of 6,949. This reading frame was confirmed by sequencing the fusion joint of an osmB::TnphoA gene fusion. The amino-terminal amino acid sequence of the open reading frame is consistent with reported signal sequences of exported proteins. The sequence around the putative signal sequence cleavage site, Leu-Ser-Ala-Cys-Ser-Asn, is highly homologous to the consensus sequence surrounding the processing site of bacterial lipoproteins. The presence of a lipid moiety on the protein was confirmed by demonstrating the incorporation of radioactive palmitic acid and inhibition of processing by globomycin. Preliminary localization of the authentic OsmB protein was determined in minicells harboring a plasmid that carries the NsiI-HincII fragment; it was primarily in the outer membrane. Surprisingly, an osmB mutant carrying the osmB::TnphoA insertion mutation was more resistant to the inhibition of metabolism by high osmolarity than the parent strain was.

Escherichia coli and other enteric bacteria are capable of surviving a wide range of external osmotic pressures (17, 22, 23). Our goal is to analyze the cellular response to an increase in osmotic pressure by identifying genes activated by elevated osmolarity and determining the contribution of their gene products to the adaptive process. Cellular activities already known to be osmotically dependent include the porin composition of the outer membrane and the expression and activity of transport systems for the major osmolytes K+, glycine betaine, proline, and choline. The kdp operon, encoding a high-affinity potassium transport system, is regulated at the transcriptional level by osmotic pressure and potassium availability (for a recent review, see reference 12). Transport of glycine betaine is mediated by two distinct transport systems encoded by proP and proU. ProP mediates relatively low-affinity transport (3), while proU specifies a high-affinity transport system whose synthesis is induced only when cells are grown at high osmolarity (4, 11, 14, 28).

The search for additional osmoregulated genes has made use of genetic fusions, a valuable technique for detecting genes subject to a common regulatory stimulus (33). For instance, Mu d(lac) bacteriophage insertions can create Lac+ fusions that respond to environmental stresses, such as UV radiation, phosphate starvation, or heat shock (33). A number of laboratories have used lac fusions to detect genes activated by elevated osmolarity in growth medium. The researchers obtained fusions to proP, proU, kdp, and the porin gene ompC, but no new osmoregulated genes were found (3, 12, 14, 16, 17). This was surprising since at least 20 proteins are induced in E. coli by osmotic shock (1; our unpublished data). The majority of these proteins are in the cell envelope; little is known about their functions and how they might aid the cell in coping with osmotic stress.

The transposon TnphoA (26) can be used to specifically detect bacterial genes that code for cell envelope proteins. TnphoA inserts into a bacterial chromosomal gene and, when in the proper frame, will fuse alkaline phosphatase to the amino terminus of the protein product of that gene. Alkaline phosphatase is enzymatically active only if fused to a sequence that promotes export of the protein into the envelope of the cell (27). The TnphoA approach can be used to search for genes regulated by osmolarity in E. coli by selecting fusion strains that produce higher alkaline phosphatase activity when grown in medium with high osmolarity than in medium with low osmotic pressure.

Nine different TnphoA insertion mutants with osmotically inducible alkaline phosphatase activity have been isolated (15). Among these, strain CLG2 carrying a TnphoA fusion to osmB was selected for the present study. We have cloned and sequenced osmB and found that it encodes a small hydrophobic protein, which has a good putative signal sequence followed by a region with a high degree of homology with the consensus sequence for lipoprotein cleavage. The presence of the lipid moiety has been confirmed through studies of wild-type OsmB and a fusion protein produced from the osmB::TnphoA gene.

MATERIALS AND METHODS

Bacterial strains, plages, and plasmids. The bacterial strains, plages, and plasmids used in this study are shown in Table 1.

Chemicals and media. Cells were grown in M9 minimal medium (29) containing thiamine (1 μg/ml) and 0.2% glucose. Cells for minicells or plasmid preparations were grown in LB (29) at 37°C. Ampicillin, kanamycin, and tetracycline were added at concentrations of 50, 50, and 12 μg/ml respectively, for growth of the bacteria carrying the plasmid and for transduction with phages P1 or M13K07. The compound 3-bromo-4-chloro-3-indoly phosphate (XP), an indicator dye which gives a blue color when cleaved by alkaline phosphatase, was used at a concentration of 40 μg/ml. XP was purchased from Sigma Chemical Co., St. Louis, Mo.

Materials. [1H]palmitic acid (specific activity, 50 Ci/mmol) and [35S]dATP (specific activity, 100 Ci/mmol) were pur-
TABLE 1. Bacterial strains and plasmids

| Strain or plasmid | Genotype | Reference or source |
|-------------------|----------|---------------------|
| E. coli strains   |          |                     |
| MC4100            | F- araD139 Δ(arg-lac)U169 rpsL150 relA fbbB5301 pT525 deoC1 | Casadaban (5) |
| MPh2              | MC4100 Δ(brm-2-phoA-proC) | Laboratory collection |
| MPh2'             | F- araD139 Δ(arg-lac)U169 rpsL150 relA fbbB5301 pT525 deoC1 Δ(brrQ-phoA-proC) osmB411::TnphoA | This study |
| CLG2              | F- araD139 Δ(arg-lac)U169 rpsL150 relA fbbB5301 pT525 deoC1 Δ(brrQ-phoA-proC) osmB416::TnphoA | This study |
| JJ2               | F- araD139 Δ(arg-lac)U169 rpsL150 relA fbbB5301 pT525 deoC1 Δ(brrQ-phoA-proC) osmB417::TnphoA | This study |
| P678-54           | F- thr-1 ara-13 leu-6, aux-8 tonA2 lacY1 minA glutU4 gal-6 minB rpsL135 maldA1 yra-7 met-2 thi-1 | Josephine (20) |
| CLG190            | araD139 Δ(ara-leu)7697 lacX74 galU galK penB ada::Tn10 recA1 (F' lacP lacZ lctY) | Laboratory collection |
| K38               | HfrC (λ) | Tabor (38) |
| K38-2             | HfrC (λ) pGP1-2 pJJ24-19U | This study |
| Plasmids          |          |                     |
| pSB118            | pUC18 derivative | Vidal-Ingiigliardi (37) |
| pTZ18U and pTZ19U | pUC vector derivative with T7 promoter and Fl origin | USB |
| pGP1-2            | T7 RNA polymerase under λ pl promoter | Tabor (38) |

Minicell isolation and labeling. Cells were grown in LB broth containing ampicillin (50 μg/ml) until 9 h into stationary phase. Minicell isolation by differential centrifugation and filtration through a GF/F glass fiber filter (Whitman, Inc., Clifton, N.J.) followed the procedure of Christen et al. (8). Harvested minicells were suspended in M9 medium containing 0.2% glyceral. After preincubation of the minicells for 10 min at 37°C, 50 μCi of [35S]Trans was added to each 1-ml sample of cells and incubation continued for an additional 45 min. For osmotic stress treatment, salt was added during the 10-min preincubation. Labeled minicells were harvested by centrifugation in a microcentrifuge for 5 min and suspended in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, the gel was stained with Coomassie blue, destained in 10% acetic acid–5% glyceral for 30 min, dried at 60°C, and exposed to X-ray film (XAR-5 film; Eastman Kodak Co., Rochester, N.Y.) at ~70°C.

Exclusive labeling of OsmB protein by the T7 RNA polymerase-promoter system. To overproduce OsmB, recombinant plasmid pJJ24-19U was constructed as follows. The BamHI-HindIII fragment of pCG242 was subcloned into the multi-cloning site of the pTZ19U vector to insert the fragment in the same orientation as that of the T7 promoter. E. coli K38 carries plasmid pGP1-2 (Table 1) encoding T7 RNA polymerase under the control of the temperature-sensitive c1857 and λ pl promoter. This strain was transformed with pJJ24-19U, resulting in strain K38-2. The T7 RNA polymerase produced at high temperature allows the exclusive expression in trans of plasmid-encoded genes by specific and strong transcription from the T7 promoter.

Cellular proteins were labeled in strain K38-2 with [35S]Trans or [3H]palmitic acid by the procedure of Tabor and Richardson (38). Strains K38-2, containing pGP1-2, and pJJ24-19U were grown in LB containing kanamycin and ampicillin at 30°C to an optical density of 0.25 at 600 nm. A 3-ml quantity of sample was removed and washed once with M9 medium. The pellet was suspended with 3 ml of M9 medium to which 0.3 ml of methionine assay medium (Difco Laboratories, Detroit, Mich.) was added. Cells were incubated at 30°C for 30 min before the temperature was shifted to 42°C. After the cells were incubated for 20 min at 42°C, rifampin was added to a final concentration of 200 μg/ml, and cells were incubated for an additional 15 min at 42°C. Cells were then shifted to 30°C for 30 min of preincubation and then labeled for 20 min with 30 μCi of [35S]Trans per ml. To examine the effect of globomycin, 200 μg of the antibiotic per ml was added when the cells were downshifted to 30°C. Labeled cells were lysed by three cycles of freezing and thawing, briefly sonicated, and centrifuged for 2 h in a microfuge to pellet membranes, leaving cytosolic and periplasmic fractions in the supernatant. The [35S]methionine-labeled membrane fractions were suspended in 100 μl of 10 mM Tris buffer (pH 7.5).

The [3H]palmitic acid labeling was done by the same method except that 10 μCi of [3H]palmitic acid per ml was added to cells in M9 plus 1% Casamino Acids for 2 h of labeling. To remove [3H]palmitate incorporated into phospholipids, membrane fractions were lipid extracted as follows. The membrane fraction was suspended in 0.2 ml of cold sodium phosphate buffer (pH 7.0), and then 20 μl of 4 M NaCl was added. After centrifugation, the pellet was suspended with 0.5 ml of chloroform-methanol (2:1, vol/vol) and shaken for 20 min at room temperature, and the solvent was removed by centrifugation for 30 min at 4°C. This solvent extraction was repeated three times, and the pellet was...
suspended with 50 μl of 10 mM Tris buffer (pH 7.0). Membrane proteins were separated by SDS-PAGE (21) or electrophoresis as described by Swank and Munkres (34).

Isotopic labeling of the OsmB-PhoA fusion. Strain J12 carrying the osmB411-phoA fusion was grown in M9-glycerol medium and osmotically stressed by the gradual addition of NaCl to a final concentration of 0.4 M, followed by growth for 15 min at 37°C. Samples (2 ml) of culture were removed and labeled by the addition of 50 μCi of [35S]Trans per ml and incubated for 15 min at 37°C. Globomycin treatment involved its addition to 200 μg/ml at this step. The membrane fractions were isolated as described above.

In the case of [3H]palmitic acid labeling, 5 ml of J12 culture in M9 glycerol plus 1% Casamino Acids medium was osmotically stressed as above and then labeled with 6 μCi of [3H]palmitic acid per ml, followed by growth for 2 h at 37°C. The membrane fraction and phospholipid extraction were the same as described above.

Sarkosyl extraction of membrane fractionation. The membrane fraction was isolated as described above, suspended in 50 μl of 10 mM Tris buffer (pH 7.5), and then mixed vigorously with 50 μl of 4% Sarkosyl NL-30 (CIBA-GEIGY Corp., Summit, N.J.). After 2 h of microcentrifugation, the Sarkosyl-soluble supernatant fraction was removed and proteins were precipitated with 10% trichloroacetic acid. After standing on ice for 2 h, the precipitate was collected by centrifugation, washed with acetone twice, dried at room temperature, and suspended in 33 mM Tris buffer (pH 7.5). The Sarkosyl-insoluble pellet was washed and suspended in 33 mM Tris buffer (pH 7.5). Equivalent counts per minute of each fraction were loaded onto SDS-PAGE gels.

DNA-directed cell-free protein synthesis. Cell-free coupled transcription-translation was performed by using a commercial kit (Amersham), according to the instructions supplied. Proteins synthesized in vitro were labeled by using 600 μCi of [35S]Trans per ml for 1 h at 37°C and then were subjected to SDS-PAGE (21).

Assay for protein and nucleotide syntheses by labeling. To measure the rate of protein synthesis, 100-μl samples of exponentially growing cells were pulse-labeled for 4 min with 20 μl of 0.22 mM [14C]phenylalanine (225 mCi/mmol) in M9 medium. Protein synthesis was stopped by the addition of 500 μl of cold 10% trichloroacetic acid, and the mixture was placed on ice for 1 h. After precipitation, the mixture was filtered through premoistened glass microfiber filters GF/C (2 cm; Whatman), washed three times with 1 ml of 0.1 M HCl, and washed again with 1 ml of 95% ethanol and the dried filters were counted in a liquid scintillation counter (Beckman Instruments, Inc., Fullerton, Calif.).

To measure the rate of polynucleotide synthesis, 100-μl samples of exponentially growing cells were taken every 30 min and added to temperature-equilibrated plastic tubes containing 10 μl of radioactive [3H]uridine (10 μCi) in M9 medium. Incubation was continued for 4 min and the reaction was terminated with trichloroacetic acid as described above in the protein synthesis assay.

For the amino acid transport assay, exponentially growing cells were mixed with chloramphenicol to a concentration of 0.125 mg/ml and 100-μl samples were incubated with 20 μl of [14C]phenylalanine (same concentration as that in the protein synthesis assay) for periods from 0 to 70 s. The transport period was terminated by rapid dilution with 2.5 ml of 0.1 M NaCl or 0.6 M NaCl for cells grown in standard or salt-supplemented medium, respectively. The mixtures were filtered through premoistened nitrocellulose filters (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.) and washed with 2.5 ml of the appropriate NaCl solution. The dried filters were counted as described above.

Genetic procedures. Transformation was done by the method of Maniatis et al. (25). Standard procedures were used for growth of bacteria and generalized transduction with phage P1 (29).

Selection for recombination of the osmB::TnphoA fusion onto plasmids carrying the osmB gene was based on the assumption that amplification of the TnphoA sequences on a multicopy plasmid would result in increased resistance to kanamycin. Fusion strain CLG2 transformed with pDK07 or pCG240, which carry the intact osmB gene, was spread onto LB agar containing XP and 300 μg of kanamycin per ml. Large, dark blue colonies which appeared after 24 h of incubation at 37°C were streaked out, and plasmids were prepared from these clones. In all the tested cases, restriction analysis revealed a mixture of two plasmids, the original one and a recombinant plasmid carrying the TnphoA insertion. Strain CLG190 was transformed with this mixture, and plasmids were prepared from purified PhoA" transformants. Restriction analysis was carried out to verify that only one recombinant plasmid was present. CLG190 carries recA1 and a pcnB mutation which reduces the copy number of the ColEl-derived plasmids (24). This was necessary because osmB-phoA fusions were very deleterious when present on high-copy-number plasmids. Plasmids carrying a TnphoA insertion may have occurred by transposition of TnphoA, rather than recombination of the gene fusion. To ensure that this was not the case, we always analyzed at least four independent recombinant candidates. All the plasmids we obtained from a given fusion were indistinguishable and were assumed to result from recombination.

RESULTS

Cloning of the osmB region. osmB belongs to a group of genes whose expression is stimulated when the osmotic pressure of the medium is elevated. These genes were originally identified by the construction of fusions with the gene for alkaline phosphatase (phoA) by random insertion of the transposon TnphoA. osmB has been mapped at 28 min on the E. coli chromosome (15). The osmB411::TnphoA fusion is almost 100% cotransducible with a pyrF::Tnl0 insertion in a transduction experiment with the bacteriophage P1. We took advantage of this close linkage to more precisely locate and clone the osmB gene. Donovan and Kushner (10) reported the construction of pDK07, a plasmid on which they have cloned a 15-kilobase-pair (kb) E. coli chromosomal DNA fragment carrying the pyrF gene. We transformed CLG2, a strain containing the chromosomal osmB411::TnphoA fusion, with pDK07 and recombined the TnphoA insertion onto this plasmid by selecting for kanamycin hyperresistant recombinants (see Material and Methods).

As a result of restriction mapping of the resulting recombinant plasmid, the TnphoA insertion site appeared to lie within a 2.2-kbp EcoRI fragment of pDK07. The plasmid pCG210 (Fig. 1A) was constructed by subcloning this fragment into the vector pSB118 (37). pCG240 was constructed by further subcloning a 1.25-kbp NsiI-PstI fragment, which is internal to the 2.2-kbp EcoRI fragment, into pSB118. We found that the chromosomal osmB411::TnphoA insertion could recombine with both pCG210 and pCG240. The site of this insertion, approximately 50 bp to the left of the PvuII restriction enzyme site, is shown in Fig. 1B. Therefore, osmB is located between pyrF and rnb, very close to pyrF. The orientation of the TnphoA insertion indicates that the
FIG. 1. (A) osmB plasmids. The plasmids pCG210, pCG240, pCG241, pCG242, pJJ242-18, and pJJ243 were constructed as described in Results. pCG241 and pCG242 were derived from pCG240 by deleting a 300-bp AccI fragment and a 600-bp HincII fragment, respectively. pJJ242-19 has the opposite transcriptional direction of that of pJJ242-18. (B) Strategy and restriction sites used for sequencing the osmB gene. The cloned region that was sequenced, (■) the site of osmB411::TnphoA insertion, and the osmB structural gene (●) are shown. Arrows indicate the sequencing direction by the dideoxynucleotide chain termination method. Abbreviations: A, AccI; B, BamHI; E, EcoRI; H, HincII; Hi, HindIII; N, NsiI; P, PstI; Pu, PvuII; X, XmnI; Amp, ampicillin; I.G., intergenic region.

direction of transcription of osmB was opposite that of pyrF, i.e., counterclockwise on the chromosome of E. coli.

Identification of the osmB gene product. To identify the osmB gene product, the minicell-producing strain P678-54 was first transformed with plasmid pCG210. Polypeptides encoded by this plasmid were identified by SDS-PAGE of [35S]methionine-labeled minicells. Two polypeptides, with Mr's of 15,000 and 10,000, were expressed from the chromosomal insert of pCG210 when the minicells were incubated in standard M9 medium (Fig. 2, lane 1). When the minicells were subjected to salt stress by the addition of 0.3 M NaCl, the 10,000-molecular-weight polypeptide (10K polypeptide) was still strongly expressed. In contrast, synthesis of the other plasmid products was inhibited at high osmolarity; the 15K protein was completely repressed, and β-lactamase expression from the vector amp gene was significantly reduced. The differential effect of salt stress on the plasmid products suggested that the 10K protein was the product of the osmotically inducible osmB gene.

Plasmids pCG240, pCG241, and pCG242 carry progressively smaller portions of the chromosomal insert (Fig. 1A). A polypeptide forming a broad band with an Mr of approximately 10,000 was synthesized from all three plasmids (Fig. 3), indicating that its coding region is in the NsiI-HincII fragment of about 0.6 kbp. The ≈15,000-Mr polypeptide was synthesized only in minicells carrying pCG240. This polypeptide was previously shown to be specified by orfF, a gene adjacent to pyrF and cotranscribed with pyrF (36). Since construction of pCG240 utilized a PstI restriction cut that removed the pyrF promoter as well as about half of the pyrF structural gene, synthesis of the ≈15,000-Mr orfF gene product from pCG240 must use the upstream lac promoter.
on the vector. This promoter was in the proper orientation to promote transcription of orfF. Gene expression from these three plasmids showed the same response to increased osmotic pressure as that observed with pCG210 (data not shown), indicating that sequences required for osmoregulation of osmB, as well as the region coding for the structural gene, are present on the 0.6-kbp \textit{NsiI-HincII} DNA fragment.

\textbf{FIG. 2.} Identification of the OsmB protein. Labeled proteins were synthesized by minicells carrying pCG210 plasmid. Cells were grown in M9 medium supplemented with 0.3 M NaCl (+) or without salt (−). A and B indicate the precursor and mature forms of \( \beta \)-lactamase, respectively. The molecular weight markers shown to the left of the gel are carbonic anhydrase (31K), soybean trypsin inhibitor (21K), and lysozyme (14.4K).

\textbf{osmB nucleotide sequence.} The DNA fragment containing the \textit{osmB} gene was subcloned into the pTZ18U and pTZ19U vectors for single- and double-strand DNA sequencing. The \textit{NsiI-HincII} DNA fragment had been introduced into the multilinker site of the pSB118 vector, allowing a \textit{HindIII-BamHI} fragment including the \textit{NsiI-HincII} DNA to be subcloned into the multilinker sites of these vectors, forming plasmids pJJ242-18 and pJJ242-19. The nucleotide sequence of the 652-bp \textit{NsiI-HincII} DNA fragment was determined by the dideoxy chain-termination method (32). The sequencing strategy is shown in Fig. 1B, and the nucleotide sequence of the region including \textit{osmB} is shown in Fig. 4. The nucleotide sequence was translated into amino acids in all three frames on both strands. Only one open reading frame, encoding a polypeptide of 72 amino acids, is free of termination codons, while many termination codons occur throughout the entire region in the other reading frames on both strands. There is a second potential initiation codon at codon 8 within the putative \textit{osmB} reading frame, which would encode a polypeptide of 65 amino acids. However, only the 72-amino-acid open reading frame indicated on Fig. 4 has an upstream consensus ribosome-binding site.

The proposed reading frame was confirmed by sequencing the \textit{osmB411::TnphoA} gene fusion. The fusion joint is within the putative coding sequence at nucleotide 445 and would produce a hybrid protein containing the first 47 residues of OsmB fused, in phase, to alkaline phosphatase.

The open reading frame includes a purine-rich ribosome-binding site (Shine-Dalgarno sequence) between position 291 and 298. A Pribnow box and −35 region, characteristic of \textit{E. coli} promoters, are located at positions 249 through 254 (−TATTAT−) and positions 228 through 233 (−TTCACC−), respectively. Two regions of dyad symmetry exist downstream from the TAA stop codon: a stretch of 12 base pairs centered 18 bases downstream and a larger region 107 bases downstream, at position 630. The latter region has been suggested as the transcription termination site of orfF (38). A sequence homologous to the consensus sequence for catabolite activator protein binding (31) was not found in the 200-bp upstream region even though \textit{osmB} gene expression was repressed by glucose in the medium (unpublished results).

For further confirmation that the indicated open reading frame is \textit{osmB}, the 423-bp \textit{XmnI-HincII} fragment was subcloned into the \textit{HincII} site of the pTZ18U vector to form recombinant plasmid pJJ243, which was then expressed in minicells. This \textit{XmnI} site is immediately upstream of the putative −35 promoter region. The 10K protein was expressed in minicells harboring pJJ243 (Fig. 3).

\textbf{Derived protein sequence of the osmB gene product.} The \textit{osmB} open reading frame would encode a polypeptide with a molecular mass of 6,949 daltons. The protein contains a potential signal sequence; there are two positively charged lysine residues at positions 6 and 7, followed by a stretch of 15 hydrophobic amino acids, ending in an Ala-Cys sequence at positions 23 and 24. Cleavage between these two amino acids would generate a predicted mature protein of 49 amino acids with cysteine at the amino terminal. The sequence surrounding the putative cleavage site is highly homologous to the consensus processing site found in bacterial lipoproteins (39). These exported proteins are produced as precursors with a signal peptide linked to the amino termini. The signal sequences are cleaved by signal peptidase II (39) during secretion, and the amino-terminal cysteine is modified by addition of lipid. Figure 5 compares this region in \textit{osmB} with the cleavage site of the major lipoprotein speci-
fied by lpp and indicates the variations in the consensus sequence that have been found in other bacterial lipoproteins (39). A common feature of all these lipoproteins is the sequence Leu-X-Y-Cys at the COOH-terminal end of the signal sequence, where X and Y are predominantly small amino acids. The putative cleavage site for osmB, Leu-Ser-ALA-Cys, conforms to this pattern.

In fact, the overall size and structure of the predicted OsmB (Fig. 4) bear a strong resemblance to those of the major E. coli lipoprotein (also known as Braun lipoprotein) specified by lpp (2, 19). In addition to the signal sequence and processing site, the molecular masses of OsmB (7 kilodaltons) and the major lipoprotein (7.2 kilodaltons) are nearly the same. The C-terminal domain of lpp has obvious tandem repeats (2, 7). For osmB, the sequence from Thr-34 to Leu-48 is homologous to the sequence from Thr-53 to Ile-66, introducing some spacing. Both proteins end in C-terminal lysine residues. In the case of the major lipoprotein, this lysine can be covalently bound to the peptidoglycan.

The protein product of osmB produced in vivo, either in minicells or whole cells, forms a broad, diffuse band when subjected to electrophoresis on Laemmli gels (21). However, when a plasmid carrying osmB was expressed in an in vitro DNA-directed protein synthesis system, both precursor and mature forms of OsmB appear as discrete bands (Fig. 6A). This suggests that the diffuse behavior on gels may be a result of posttranslational modification by lipid.

**Evidence for lipid modification of OsmB.** Incorporation of radioactive palmitic acid into OsmB would provide direct evidence for a lipid moiety on the protein. As a result of the small amount of OsmB in the cell, it was necessary to
amplify its expression to detect lipid labeling. Amplification was achieved by placing \textit{osmB} under the control of a T7 promoter by using the exclusive expression system of Tabor and Richardson (38). The \textit{osmB} structural gene was inserted into pTZ19U in the same orientation as that of the T7 promoter, creating recombinant plasmid pJJ242-19U. This plasmid was transformed into strain K38, carrying plasmid pGPI-2, that expresses T7 RNA polymerase under temperature-sensitive control. When the temperature was raised to 42°C, the T7 polymerase was synthesized and \textit{osmB} gene was overexpressed. Membrane fractions were then isolated as described in Materials and Methods and the proteins were separated on a gel as described by Swank and Munkres (34). The autoradiogram in Fig. 6B shows labeling of OsmB with [3H]methionine in lane 1. A parallel labeling with [3H]palmitate is shown in lane 3 of Fig. 6B, demonstrating incorporation of fatty acid into mature OsmB protein.

An OsmB-PhoA fusion protein gene was also shown to be lipid modified. The fusion protein produced from the chromosomal \textit{osmB411::TnphoA} gene was first identified by [35S]methionine labeling of whole cells. A new, membrane-associated protein with an \(M_w \approx 50,000\) was strongly induced when cells carrying the fusion gene were grown in medium supplemented with 0.4 M NaCl (Fig. 7, lanes 1 and 2). The \(M_w\) of this protein is close to the 51,397 predicted from the DNA sequence (Fig. 4), and the membrane localization is in agreement with the finding that all of the fusion alkaline phosphatase activity was membrane associated (data not shown). Finally, we have confirmed that this is the OsmB-PhoA fusion by demonstrating cross-reaction with anti-alkaline phosphatase antibodies on Western blots (immunoblots) (data not shown). Lane 3 (Fig. 7) shows incorporation of palmitic acid into a band with the same mobility as that of hybrid protein. Palmitate was also incorporated into the protein produced from the \textit{osmB8136-TnphoA} gene, which migrates with an \(M_w \approx 48,000\) (data not shown). Comigration of palmitate-labeled bands with two different hybrids is further evidence for modification of OsmB-PhoA fusions.

Globomycin is a cyclic peptide antibiotic that blocks the maturation of major and minor lipoproteins from precursor to mature forms by inhibiting the action of signal peptidase II (39). Treatment of cells with globomycin results in the accumulation of pro-lipoproteins retaining the signal peptide. Both the wild-type OsmB and the OsmB-PhoA fusion accumulated as larger, putative precursor forms as a result of globomycin treatment. OsmB expressed from the T7 promoter migrated as a somewhat larger protein when globomycin was present (Fig. 6, lane 2). When the strain carrying the \textit{osmB411::TnphoA} chromosomal fusion was treated with the antibiotic, production of the mature protein was blocked and a larger protein accumulated (Fig. 8). The \(M_w\) difference between the two forms was about 2,000, i.e., the expected size of the signal peptide. The apparent inhibition of processing by globomycin is further support for the prediction, based on DNA sequence homologies, that the \textit{osmB} gene specifies a lipoprotein.

\textbf{Preliminary localization of OsmB.} The amino-terminal sequence of the \textit{osmB} product and other analogies to the major lipoprotein suggest that OsmB is an exported protein, possibly to the outer membrane. The subcellular localization of OsmB was examined in minicells carrying plasmid pCG242.

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure6.png}
\caption{Protein products of \textit{osmB}. (A) \textit{osmB} expression in vivo and in vitro. Lane 1, OsmB produced in vivo under control of a T7 promoter-T7 RNA polymerase expression system. Lane 2, OsmB produced by in vitro DNA-directed protein synthesis. OsmB labeled with [3H]palmitate and inhibition of processing by globomycin. OsmB was synthesized by the T7 promoter/T7 RNA polymerase system. The labeled proteins were separated on a gel as described by Swank and Munkres (34). Lane 1, [3H]methionine labeling; lane 2, [35S]methionine labeling with 200 μg of globomycin per ml; lane 3, [3H]palmitate labeling.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure7.png}
\caption{Lipid modification of an OsmB-PhoA fusion. Strain JJ2 (\textit{osmB411::TnphoA}) was labeled with the appropriate isotope, and membranes were isolated and analyzed by SDS-PAGE (21). The position of the OsmB-PhoA hybrid protein is indicated by the arrow. Lanes 1 and 2, [35S]Trans-labeled membrane proteins. Cells were grown in M9 medium alone (lane 1) or supplemented with 0.4 M NaCl (lane 2). Lane 3, [3H]palmitate-labeled membrane proteins from cells grown in M9 medium supplemented with 0.4 M NaCl. The molecular weight markers (indicated by bars to the left of the gel) are bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (31K), and soybean trypsin inhibitor (21K).}
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\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Effect of globomycin on OsmB-PhoA. Strain JJ2 (\textit{osmB411::TnphoA}) was labeled with [35S]TranS in different media, and membrane fractions were analyzed by SDS-PAGE. Lane 1, M9 medium; lane 2, M9 plus 0.4 M NaCl; lane 3, M9 plus 0.4 M NaCl in the presence of 200 μg of globomycin per ml.}
\end{figure}
After labeling, minicells harboring pCG242 were lysed either by freeze-thawing or treating with lysozyme as described in Materials and Methods and the envelope fraction was removed by centrifugation. The remaining soluble fraction includes both periplasmic and cytoplasmic contents. Figure 9 shows that all of the OsmB structural protein was localized to the envelope fraction. The envelope fraction was then treated with the detergent Sarkosyl NL-30 and the detergent-soluble and -insoluble fractions were analyzed by SDS-PAGE and autoradiography. A substantial portion of the OsmB protein was found in the Sarkosyl-insoluble fraction. The same fractionation pattern was obtained from the membranes of whole cells in which OsmB was overproduced through the T7 polymerase system (data not shown). The effectiveness of the fractions was monitored by Coomassie blue staining of the gels. The major outer membrane proteins OmpF, OmpC, and OmpA were entirely in the Sarkosyl-insoluble fractions after detergent extraction (data not shown). These data suggest that OsmB may be an outer membrane protein.

Enhanced growth of osmB mutants under high-salt conditions. Strain JJ2, which carries the osmB411::TnPhoA insertion in the chromosomal gene, grew better than the parental strain MPH2' on all media. Most surprising was the observation that this osmB mutant grew much better under high-salt conditions. Table 2 compares the growth of the two strains. The parent MPH2' grew slowly when M9 medium was supplemented with 0.6 M NaCl salt and was unable to grow at all at higher salt concentrations. Addition of the osmoregulation glycerol betaine at 2 mM allowed poor growth of MPH2' in medium supplemented with 0.7 and 0.8 M NaCl. The mutant strain JJ2, on the other hand, could grow when salt concentrations as high as 0.8 M NaCl were added to M9 medium. Addition of 2 mM glycine betaine to medium supplemented with 0.8 M NaCl allowed strain JJ2 to grow at the same rate as that of the wild-type strain on M9 plus 0.6 M NaCl. Since we did not expect a mutant strain lacking an osmotically inducible gene product to grow better than the wild-type strain, we checked to determine whether the presence of the TnPhoA transposon might confer some growth advantage. In the mutant search that identified osmB, TnPhoA insertion mutants in nine different osmotically inducible genes were isolated (15). Strains carrying each of the other fusions grew more poorly than wild-type MPH2' under conditions of high osmosality. Strain JJ4 is a representative of this group; it carries an osmotically inducible TnPhoA insertion at 34.5 min on the E. coli genetic map and was salt sensitive for growth (Table 2). JJ4 could grow only with a salt concentration up to 0.4 M NaCl salt, and 2 mM glycine betaine addition did not rescue the growth at high osmosality. Therefore, the presence of the TnPhoA transposon did not confer a growth advantage on the osmB mutant.

Since the enhanced growth of strain JJ2 might be caused by participation of a second site mutation, the osm B411::TnPhoA was transduced into a clean background of the parent MPH2' by P1 transduction. This newly constructed strain also grew better than the wild type, as did two additional osmB' strains carrying the osmB755::TnPhoA or osmB8136::TnPhoA fusion genes.

The metabolic resistance to salt inhibition exhibited by strain JJ2 was investigated in more detail by measuring the rates of protein and polynucleotide syntheses. Addition of 0.5 M NaCl to M9 medium inhibited the rate of incorporation of [14C]phenylalanine into protein by 80% in the parent strain MPH2', relative to cells grown without salt addition. Protein synthesis in the mutant strain JJ2 was more resistant to salt inhibition. After 150 min, the rate of [14C]phenylalanine incorporation by JJ2 was about twofold greater than that of MPH2', that is, only 60% inhibited by salt addition.

The differences in amino acid incorporation between strains could be due to differences in the rate of protein synthesis or rate-limiting amino acid transport. We therefore measured the rate of [14C]phenylalanine transport in both

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**FIG. 9.** Localization of the OsmB protein in minicells. Minicells harboring pCG242 were fractionated into cytoplasm and membrane, and membranes were then divided into Sarkosyl-soluble and Sarkosyl-insoluble fractions. 35S-labeled proteins were separated by SDS-PAGE. An autoradiograph of the gel is shown here. Lanes 1 and 1', cytoplasmic proteins; lanes 2 and 2', total membrane proteins; lanes 3 and 3', detergent-soluble proteins; lanes 4 and 4', detergent-insoluble proteins. The prime (') indicates that the cells were grown in medium with high osmolality. A and B indicate the precursor and mature forms of β-lactamase, respectively. The molecular weight markers (indicated by bars to the left of the gel) are bovine serum albumin (66K), ovalbumin (45K), carbonic anhydrase (31K), soybean trypsin inhibitor (21K), and lysozyme (14.4K).

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**TABLE 2. Growth as a function of salt concentration**

| M9 medium supplement | MPH2' (osmB') | JJ2 (osmB) | JJ4 (osmD) |
|----------------------|--------------|------------|------------|
| 0.4 M NaCl           | 2            | 2          | 2          |
| 0.5 M NaCl           | 1            | 2          | 0          |
| 0.6 M NaCl           | 1            | 1          | 0          |
| 0.7 M NaCl           | 0            | 0.5        | 0          |
| 0.8 M NaCl           | 0            | 0.5        | 0          |
| 0.7 M NaCl + 2 mM GB | 0.5          | 1          | 0          |
| 0.8 M NaCl + 2 mM GB | 0.5          | 1          | 0          |

* The growth of each strain was determined by relative sizes of colonies after incubation in medium supplemented with various salt concentrations. The numbers 0, 0.5, 1, and 2 indicate no growth, poor growth, slow growth, and good growth, respectively.
strains as a function of the osmolarity of the medium. High osmolarity reduces the rate of phenylalanine transport by about half, but the initial rate of [14C]phenylalanine uptake was affected to the same extent in both parent and mutant strains. Therefore, the difference in the rate of amino acid incorporation between MPH2 and JJ2 does reflect a higher rate of protein synthesis in the osmB::TphoA strain.

The rate of [3H]uridine incorporation into polynucleotides was inhibited to the same extent in parent and mutant immediately following salt addition. However, polynucleotide synthesis by JJ2 recovered faster than that of MPH2. By 150 min after the addition of NaCl to 0.5 M, the rate of polynucleotide synthesis was about twofold greater in JJ2 than in MPH2. The higher rates of both protein and polynucleotide syntheses demonstrate the resistance of the metabolism of the osmB mutant to osmotic stress.

**DISCUSSION**

The transposon TphoA has been used to identify a number of osmotically inducible genes coding for exported proteins (15). The alkaline phosphatase activity from an osmB::TphoA protein fusion was induced three- to fourfold by supplementing K medium with 0.4 M NaCl or 0.6 M sucrose (15). In these experiments, the basal medium was M9 plus glycerol, which has an osmolality about 150 mosM greater than K medium. Nonetheless, we found a fivefold induction of alkaline phosphatase activity by the addition of 0.4 M NaCl. Strong osmotic induction of the fusion protein can also be seen in Fig. 7 and 8. However, the amount of fusion protein may underestimate the induction ratio since a β-galactosidase transcriptional fusion under the control of the osmB promoter shows a 10-fold induction under similar conditions (manuscript in preparation). This discrepancy could be due to instability of the OsmB-PhoA protein fusion.

We have now physically mapped the osmB gene and find that the overall size and structure of the predicted OsmB protein bear a strong resemblance to those of the major E. coli lipoprotein specified by lpp (2, 19) with respect to molecular weight, C-terminal lysine residue, signal sequence, consensus sequence at potential cleavage site, and internal tandem repeats. The high homology with the lpp product suggested that osmB specified a new lipoprotein, which was confirmed by direct observation. The main amino acid sequence difference between the lpp product and OsmB is in the C-terminal domain. Lpp has many charged residues and could potentially form amphipathic helical structures (2). The OsmB C-terminal domain is hydrophobic with antiparallel β-sheets as the most likely structure (18).

The major lipoprotein of E. coli is localized to the outer membrane, capable of providing a covalent linkage to the peptidoglycan through the C-terminal lysine residue (2, 19). Recent data from the laboratory of M. Inouye implicates the second amino acid of the mature protein as the determinant of final localization of the exported lipoprotein (40). When the amino acid following cysteine was serine, the protein was exported to the outer membrane. Simply changing that amino acid to aspartate resulted in an inner membrane destination. For OsmB, the amino acid following cysteine is serine. That fact, coupled with all the other analogies to the major lipoprotein, indicate that OsmB should be an outer membrane protein. The preliminary localization experiments reported here generally support that view, although our data indicated that OsmB was primarily, but not entirely, in the outer membrane. This unclear distribution could be due to several limitations inherent in these experiments. First, overproduction of outer membrane proteins from multicopy plasmids has been shown to cause accumulation of excess protein in the inner membrane (40). Second, any uncleaved precursor molecules in the inner membrane would be more heavily labeled with [35S] since the putative signal sequence contains three methionine residues. Last, the Sarkosyl fractionation method is indicative but not definitive. Density gradient separation of inner and outer membranes will be required for a final subcellular localization.

After maturation, the predicted OsmB protein has 49 amino acids, giving a Mw of 4,230 for the unmodified polypeptide. However, OsmB produced in minicells migrates on SDS-PAGE gels with an apparent molecular weight of 10,000. Anomalous migration in SDS-PAGE gels is characteristic of many lipoproteins. For instance, PAL is a 16K polypeptide although its M, is 20,000 (7) and the 36K lipoprotein had an M, of 54,000 on gels (35). Additionally, low-molecular-weight proteins frequently demonstrate abnormal mobility. For instance, relA and hok gene products (52 and 51 amino acids, respectively) migrate with apparent molecular weights of 11,000 to 12,000 (13).

Surprisingly, OsmB mutant strains grew better than the parent strain MPH2 under high-salt conditions. One can speculate that the osmotically inducible osmB lipoprotein may cross-link the outer membrane and peptidoglycan in a manner that restricts the growth rate during osmotic stress, although it might aid survival under these conditions.

Our studies indicate that OsmB can be added to the rapidly increasing list of lipoproteins in procaryotic and eucaryotic cells. The functions of most lipoproteins have not been identified nor have there been any studies of osmoregulation of total lipoprotein synthesis. The possibility of switching among lipoproteins to promote adaptation to elevated osmotic pressure exists and should be studied in the future.

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