

## Sequence of an Osmotically Inducible Lipoprotein Gene

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Received 6 July 1988/Accepted 17 October 1988

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  $M_r$  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<sup>+</sup>, 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<sup>+</sup> 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, phages, and plasmids.** The bacterial strains, phages, 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  $\mu$ g/ml) and 0.2% glycerol. 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  $\mu$ g/ml, respectively, for growth of the bacteria carrying the plasmid and for transduction with phages P1 or M13K07. The compound 5-bromo-4-chloro-3-indoyl phosphate (XP), an indicator dye which gives a blue color when cleaved by alkaline phosphatase, was used at a concentration of 40  $\mu$ g/ml. XP was purchased from Sigma Chemical Co., St. Louis, Mo.

**Materials.** [<sup>3</sup>H]palmitic acid (specific activity, 50 Ci/mmol) and [<sup>35</sup>S]dATP (specific activity, 100 Ci/mmol) were pur-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype	Reference or source
<i>E. coli</i> strains		
MC4100	F <sup>-</sup> <i>araD139</i> $\Delta$ ( <i>arg-lac</i> ) <i>U169</i> <i>rpsL150 relA flbB5301 ptsF25</i> <i>deoC1</i>	Casadaban (5)
MPh2	MC4100 $\Delta$ ( <i>brnQ-phoA-proC</i> )	Laboratory collection
MPh2'	MPh2 <i>phoA proC</i> <sup>+</sup>	This study
CLG2	F <sup>-</sup> <i>araD139</i> $\Delta$ ( <i>arg-lac</i> ) <i>U169</i> <i>rpsL150 relA flbB5301 ptsF25</i> <i>deoC1</i> $\Delta$ ( <i>brnQ-phoA-proC</i> ) <i>osmB411::Tnp<sub>phoA</sub></i>	Gutierrez (15)
JJ2	CLG2 <i>phoA proC</i> <sup>+</sup>	This study
CLG4	F <sup>-</sup> <i>araD139</i> $\Delta$ ( <i>arg-lac</i> ) <i>U169</i> <i>rpsL150 relA flbB5301 ptsF25</i> <i>deoC1</i> $\Delta$ ( <i>brnQ-phoA-proC</i> ) <i>osmD467::Tnp<sub>phoA</sub></i>	Gutierrez (15)
JJ4	CLG4 <i>phoA proC</i> <sup>+</sup>	This study
P678-54	F <sup>-</sup> <i>thr-1 ara-13 leu-6 azi-8</i> <i>tonA2 lacY1 minA glnU44</i> <i>gal-6 minB2 rpsL135 malA1</i> <i>xyl-7 mtl-2 thi-1</i>	Josephine (20)
CLG190	<i>araD139 (ara-leu)7697 lacX74</i> <i>galU galK pcnB zad::Tn10</i> <i>recA1</i> (F <sup>-</sup> <i>lacI<sup>a</sup> lacZ lacY</i> )	Laboratory collection
K38	HfrC ( $\lambda$ )	Tabor (38)
K38-2	HfrC ( $\lambda$ ) pGP1-2 pJJ242-19U	This study
Plasmids		
pSB118	pUC18 derivative	Vidal-Ingigliardi (37)
pTZ18U and pTZ19U	pUC vector derivative with T7 promoter and fl origin	USB
pGP1-2	T7 RNA polymerase under $\lambda$ <i>p<sub>L</sub></i> promoter	Tabor (38)

chased from Amersham Corp., Arlington Heights, Ill. [<sup>35</sup>S]Trans (specific activity of labeled methionine, 1,205 Ci/mmol; ICN Radiochemicals, Irvine, Calif.), [<sup>3</sup>H]uridine (specific activity, 16 Ci/mmol), and [<sup>14</sup>C]phenylalanine (specific activity, 450 mCi/mmol) were purchased from ICN Pharmaceuticals Inc., Irvine, Calif. Globomycin was a gift from Sankyo Co. Ltd., Tokyo, Japan. The sequencing kit, Sequenase, and GeneScribe-Z vectors were purchased from United States Biochemical Corp. (USB), Cleveland, Ohio. Restriction enzymes and T4 DNA ligase were from New England BioLabs, Inc., Beverly, Mass. and Boehringer Mannheim Biochemicals, Indianapolis, Ind.

**DNA sequencing.** DNA sequencing utilized the dideoxy chain termination method (32). The Sequenase (USB) method for short-fragment sequencing was applied directly to denatured double-stranded DNA of plasmids pJJ242 and pJJ243 by the protocol of Promega Biotec, Madison, Wis. These two plasmids carry the chromosomal DNA inserts in the pTZ18U and pTZ19U vectors, respectively. For the determination of the *osmB-phoA* fusion joints, denatured double-stranded DNA of a plasmid carrying the fusion was hybridized with a 13-mer primer specific for the *phoA* sequence. The fusion joint was deduced from the Tnp<sub>phoA</sub> left end sequence (26). For long-fragment sequencing, single-stranded DNA was prepared from the multifunctional pTZ vectors and sequenced by the USB protocol. Electrophoresis was performed with polyacrylamide field gradient gels (0.4 mm thick) of 42 and 30 cm at 2,000 V. Sequence data were processed on a computer (IBM Instruments, Inc., Danbury, Conn.) with Microgenie software.

**Minicell isolation and labeling.** Cells were grown in LB broth containing ampicillin (50  $\mu$ g/ml) until 9 h into stationary phase. Minicell isolation by differential centrifugation and filtration through a GF/F glass fiber filter (Whatman, Inc., Clifton, N.J.) followed the procedure of Christen et al. (8). Harvested minicells were suspended in M9 medium containing 0.2% glycerol. After preincubation of the minicells for 10 min at 37°C, 50  $\mu$ Ci of [<sup>35</sup>S]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% glycerol 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 pJJ242-19U was constructed as follows. The *Bam*HI–*Hind*III 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 *cI857* and  $\lambda$  *p<sub>L</sub>* promoter. This strain was transformed with pJJ242-19U, 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 [<sup>35</sup>S]Trans or [<sup>3</sup>H]palmitic acid by the procedure of Tabor and Richardson (38). Strains K38-2, containing pGP1-2, and pJJ242-19U 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  $\mu$ g/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  $\mu$ Ci of [<sup>35</sup>S]Trans per ml. To examine the effect of globomycin, 200  $\mu$ g 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 [<sup>35</sup>S]methionine-labeled membrane fractions were suspended in 100  $\mu$ l of 10 mM Tris buffer (pH 7.5).

The [<sup>3</sup>H]palmitic acid labeling was done by the same method except that 10  $\mu$ Ci of [<sup>3</sup>H]palmitic acid per ml was added to cells in M9 plus 1% Casamino Acids for 2 h of labeling. To remove [<sup>3</sup>H]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  $\mu$ l 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  $\mu$ l 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 JJ2 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  $\mu$ Ci of [<sup>35</sup>S]Trans per ml and incubated for 15 min at 37°C. Globomycin treatment involved its addition to 200  $\mu$ g/ml at this step. The membrane fractions were isolated as described above.

In the case of [<sup>3</sup>H]palmitic acid labeling, 5 ml of JJ2 culture in M9 glycerol plus 1% Casamino Acids medium was osmotically stressed as above and then labeled with 6  $\mu$ Ci of [<sup>3</sup>H]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  $\mu$ l of 10 mM Tris buffer (pH 7.5), and then mixed vigorously with 50  $\mu$ l 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  $\mu$ Ci of [<sup>35</sup>S]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- $\mu$ l samples of exponentially growing cells were pulse-labeled for 4 min with 20  $\mu$ l of 0.22 mM [<sup>14</sup>C]phenylalanine (225 mCi/mmol) in M9 medium. Protein synthesis was stopped by the addition of 500  $\mu$ l 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- $\mu$ l samples of exponentially growing cells were taken every 30 min and added to temperature-equilibrated plastic tubes containing 10  $\mu$ l of radioactive [<sup>3</sup>H]uridine (10  $\mu$ Ci) 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- $\mu$ l samples were incubated with 20  $\mu$ l of [<sup>14</sup>C]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  $\mu$ m; 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  $\mu$ g 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*<sup>+</sup> 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 ColE1-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::Tn10* 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 (kbp) *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

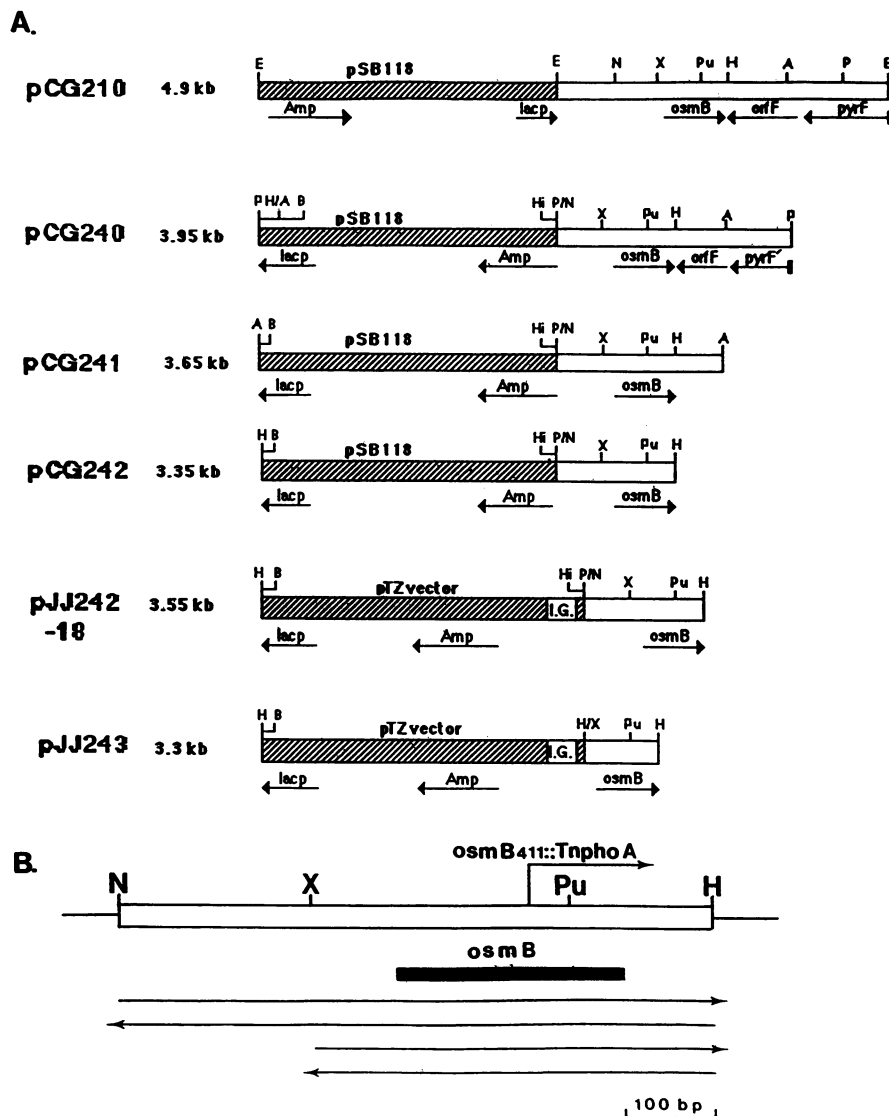


FIG. 1. (A) *osmB* plasmids. The plasmids pCG210, pCG240, pCG241, pCG242, pJJ242-18, and pJJ243 were constructed as described in Results. pCG241 and pCG242 were derived from pCG240 by deleting a 300-bp *AccI* fragment and a 600-bp *HincII* fragment, respectively. pJJ242-19 has the opposite transcriptional 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 [<sup>35</sup>S]methionine-labeled minicells. Two polypeptides, with  $M_r$ 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 osmolality; the 15K protein was completely repressed, and  $\beta$ -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  $M_r$  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  $\approx 15,000$ - $M_r$  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  $\approx 15,000$ - $M_r$  *orfF* gene product from pCG240 must use the upstream *lac* promoter

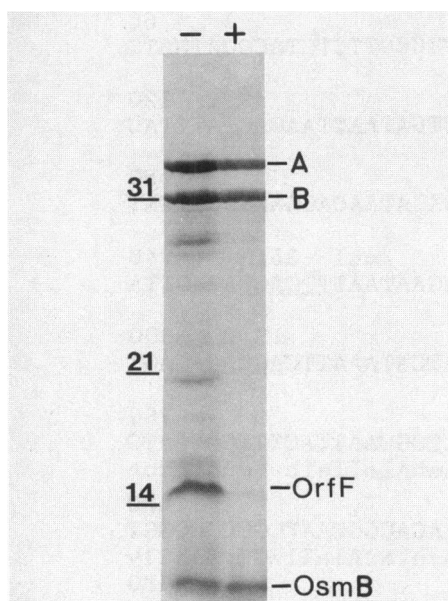


FIG. 2. Identification of the OsmB protein. Labeled proteins were synthesized by minicells carrying pCG210 plasmid. Cells were grown in M9 medium supplemented with 0.3 M NaCl (+) or without salt (-). A and B indicate the precursor and mature forms of  $\beta$ -lactamase, respectively. The molecular weight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

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 *NsiI-HincII* DNA fragment.

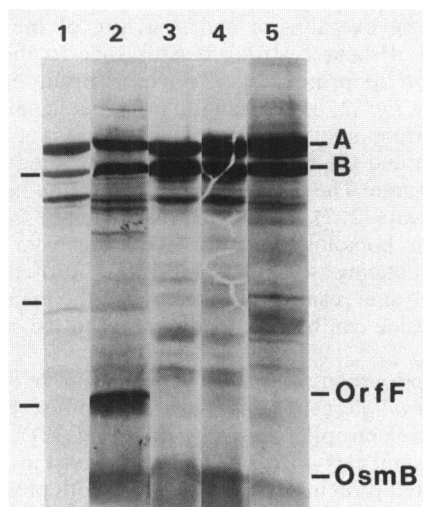


FIG. 3. Localization of the *osmB* coding sequence. Proteins synthesized by minicells carrying the indicated plasmids were labeled with [ $^{35}$ S]Trans and analyzed by SDS-PAGE. An autoradiograph of the gel is shown here. Lane 1, pTZ18U; lane 2, pCG240; lane 3, pCG241; lane 4, pCG242; lane 5, pJJ243. A, B, and molecular weight markers (indicated by bars to the left of the gel) are as defined in the legend to Fig. 2.

***osmB* nucleotide sequence.** The DNA fragment containing the *osmB* gene was subcloned into the pTZ18U and pTZ19U vectors for single- and double-strand DNA sequencing. The *NsiI-HincII* DNA fragment had been introduced into the multilinker site of the pSB118 vector, allowing a *HindIII-BamHI* fragment including the *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 *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 *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 *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 *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 *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 *osmB* gene expression was repressed by glucose in the medium (unpublished results).

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

**Derived protein sequence of the *osmB* gene product.** The *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 *osmB* with the cleavage site of the major lipoprotein speci-



FIG. 4. Nucleotide sequence of the *osmB* region. The sequence of the antisense strand is shown with numbering starting from the 5' end. B411 indicates the fusion joint. A putative Shine-Dalgarno sequence (S. D.) is shown. Possible -10 and -35 regions are underlined. The arrows below the sequence indicate regions of dyad symmetry.

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

Consensus sequence	-L-X-Y-↓-C-
<i>osmB</i> protein	S L S A C S N W S K
Major lipoprotein( <i>lpp</i> )	L L A G C S S N A K
Other substitutions*	A S A G A N S S
	V V A N S N N
	I N Q L Q G
	G L Y R
	D V T
	I

FIG. 5. Putative cleavage site of OsmB signal sequence compared with consensus cleavage sequence of lipoproteins from gram-negative bacteria. Signal peptide cleavage sites and consensus sequences are indicated by the arrow and boldface letters, respectively. The asterisk for other substitutions indicates that the work was done by others (6, 7, 9, 30, 35, 39).

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



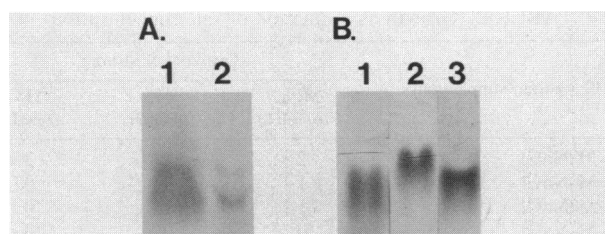


FIG. 6. Protein products of *osmB*. (A) *osmB* expression in vivo and in vitro. Lane 1, OsmB produced in vivo under control of a T7 promoter-T7 RNA polymerase expression system. Lane 2, OsmB produced by in vitro DNA-directed protein synthesis. OsmB labeled with [ $^{35}$ S]Trans was subjected to SDS-PAGE on a Laemmli gel (21). The autoradiogram of the gel is shown. (B) Incorporation of [ $^3$ H]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, [ $^{35}$ S]methionine labeling; lane 2, [ $^{35}$ S]methionine labeling with 200  $\mu$ g of globomycin per ml; lane 3, [ $^3$ H]palmitate labeling.

amplify its expression to detect lipid labeling. Amplification was achieved by placing *osmB* under the control of a T7 promoter by using the exclusive expression system of Tabor and Richardson (38). The *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 pGP1-2, that expresses T7 RNA polymerase under temperature-sensitive control. When the temperature was raised to 42°C, the T7 polymerase was synthesized and *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 [ $^{35}$ S]methionine in lane 1. A parallel labeling with [ $^3$ H]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 *osmB411::TnpA* gene was first identified by [ $^{35}$ S]methionine labeling of whole cells. A new, membrane-associated protein with an  $M_r \approx 50,000$  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 *osmB8136-TnpA* gene, which migrates with an  $M_r \approx 48,000$  (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

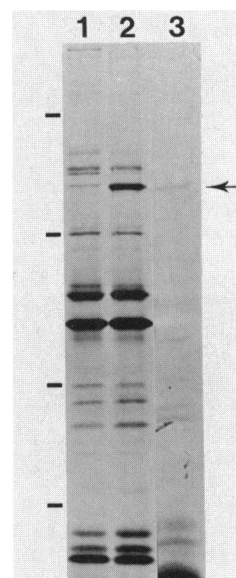


FIG. 7. Lipid modification of an OsmB-PhoA fusion. Strain JJ2 (*osmB411::TnpA*) was labeled with the appropriate isotope, and membranes were isolated and analyzed by SDS-PAGE (21). The position of the OsmB-PhoA hybrid protein is indicated by the arrow. Lanes 1 and 2, [ $^{35}$ S]Trans-labeled membrane proteins. Cells were grown in M9 medium alone (lane 1) or supplemented with 0.4 M NaCl (lane 2). Lane 3, [ $^3$ H]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).

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 *osmB411::TnpA* 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 *osmB* gene specifies a lipoprotein.

**Preliminary localization of OsmB.** The amino-terminal sequence of the *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.

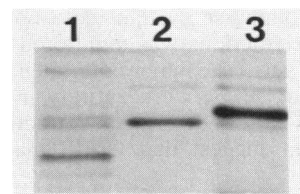


FIG. 8. Effect of globomycin on OsmB-PhoA. Strain JJ2 (*osmB411::TnpA*) was labeled with [ $^{35}$ S]Trans in different media, and membrane fractions were analyzed by SDS-PAGE. Lane 1, M9 medium; lane 2, M9 plus 0.4 M NaCl; lane 3, M9 plus 0.4 M NaCl in the presence of 200  $\mu$ g of globomycin per ml.

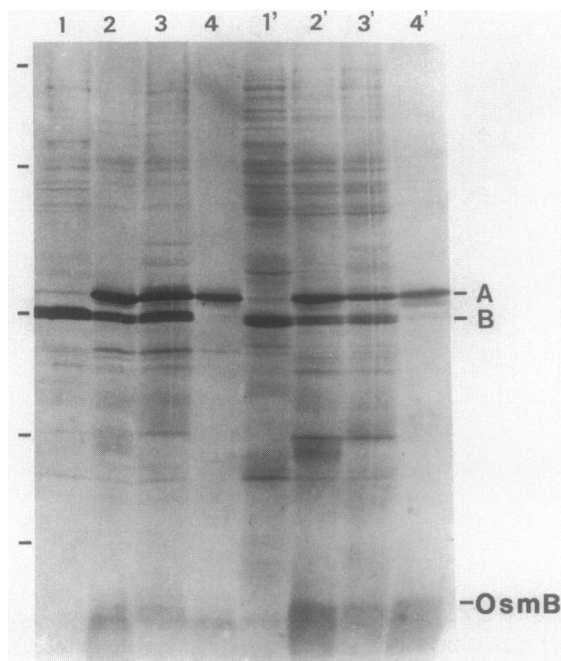


FIG. 9. Localization of the OsmB protein in minicells. Minicells harboring pCG242 were fractionated into cytoplasm and membrane, and membranes were then divided into Sarkosyl-soluble and Sarkosyl-insoluble fractions.  $^{35}\text{S}$ -labeled proteins were separated by SDS-PAGE. An autoradiograph of 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 osmolarity. A and B indicate the precursor and mature forms of  $\beta$ -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).

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

TABLE 2. Growth as a function of salt concentration

M9 medium supplement	Growth <sup>a</sup> of strain:		
	MPh2' ( <i>osmB</i> <sup>+</sup> )	JJ2 ( <i>osmB</i> )	JJ4 ( <i>osmD</i> )
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

<sup>a</sup> 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.

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 osmoprotectant glycine 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 osmolarity. 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 osmolarity. 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 *osmB411::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*<sup>+</sup> 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 [ $^{14}\text{C}$ ]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 [ $^{14}\text{C}$ ]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 [ $^{14}\text{C}$ ]phenylalanine transport in both



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 [ $^{14}\text{C}$ ]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::TnphoA* strain.

The rate of [ $^3\text{H}$ ]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 *TnphoA* has been used to identify a number of osmotically inducible genes coding for exported proteins (15). The alkaline phosphatase activity from an *osmB-TnphoA* 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 osmolarity 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  $\beta$ -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  $\beta$ -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  $^{35}\text{S}$  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  $M_w$  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  $M_r$  is 20,000 (7) and the 36K lipoprotein had an  $M_r$  of 54,000 on gels (35). Additionally, low-molecular-weight proteins frequently demonstrate abnormal mobility. For instance, *relF* and *hok* gene products (52 and 51 amino acids, respectively) migrate with apparent molecular weights of 11,000 to 12,000 (13).

Surprisingly, OsmB<sup>-</sup> 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.

### ACKNOWLEDGMENT

This work was supported by Public Health Service grant GM33778 from the National Institutes of Health to M. Villarejo.

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