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Gene *envY* of *Escherichia coli* K-12 Affects Thermoregulation of Major Porin Expression

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The temperature-dependent expression of OmpF and OmpC, the major channel-forming proteins of the *Escherichia coli* K-12 outer membrane, was studied. In wild-type cells, decreasing growth temperatures resulted in increased amounts of OmpF protein and correspondingly decreased quantities of OmpC protein. Bacteria deleted for the 13-min chromosomal region did not exhibit this temperature-dependent fluctuation in porin proteins. Plasmid pML22, which consists of pBR322 containing a 0.5-megadalton *E. coli* chromosomal DNA insert, complemented the thermoregulatory defect. The regulatory gene was named *envY*. In minicells, pML22 directed the synthesis of an envelope polypeptide (EnvY) having an apparent molecular weight of 25,000. The EnvY protein was synthesized in minicells in greater amounts at 27°C than at 37°C, and a reducing agent was necessary in the solubilization buffer for its subsequent detection on polyacrylamide gels. The results describe the initial characterization of a regulatory system which, along with proteins of the *ompB* operon, the cyclic AMP system, and the *tolC* gene product, is involved in a complex network affecting major porin expression.

The OmpC and OmpF proteins of *Escherichia coli* K-12 are outer membrane constituents that function as general diffusion pore proteins (porins) (for reviews, see references 22 and 34). The proteins have molecular weights of 38,306 (OmpC; 30) and 37,083 (OmpF; 13), and their structural genes appear to have evolved from a common ancestral gene. The relative amounts of these two proteins are somewhat strain dependent and vary with cultural conditions such as growth medium and growth temperature (2, 21); a direct correlation between medium osmolarity and the amount of OmpC exists (47). Despite fluctuations in the ratio of OmpF to OmpC, the total number of these porin proteins in membrane remains constant (21) at approximately 100,000 copies per cell (22).

Studies on porin expression regulation have chiefly focused on osmolarity effects. Osmoregulation of OmpC and OmpF is mediated primarily by the *ompB* operon, which maps at approximately 75 min and consists of two genes, *ompR* and *envZ* (1, 12, 31, 37). The OmpR protein has a molecular weight of 32,489, and amino acid sequence data suggest that it is a soluble protein (51). The *ompR* gene product is solely concerned with regulation of the major porin proteins, whereas the EnvZ protein, a 43,963-dalton polypeptide thought to reside in the envelope (31), is pleiotropic, affecting a variety of exported polypeptides whose production is influenced by environmental factors (23, 48, 49).

Several models have been proposed to explain how the *ompB* operon controls porin biosynthesis. In the model of Hall and Silhavy (11), OmpR functions as a positive regulator of OmpF and OmpC transcription, and the *envZ* gene product is an envelope protein which senses the environment and influences porin synthesis by controlling the multimerization of the OmpR protein. In a modification of this model, the EnvZ protein regulates OmpR synthesis (17). Most recently, Ozawa and Mizushima (38) have suggested that OmpF synthesis is regulated by OmpR and that OmpF acts as a negative regulator of OmpC expression.

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The above models for porin regulation were developed from studies on the effect of growth medium or osmolarity on OmpF and OmpC expression; the effects of temperature have been almost completely ignored since the initial report by Lugtenberg et al. (21). We show here that fluctuations in porin ratios with growth temperature are controlled by a second locus, distinct from the *ompB* operon.

MATERIALS AND METHODS

Bacterial strains and plasmids. Sources and relevant characteristics of *E. coli* K-12 strains used are listed in Table 1. Plasmids pMC44 (3, 10) and pGGC110 (43) were obtained from A. Markovitz and pBR322 was supplied by J. R. Walker.

Media and growth conditions. Unless noted otherwise cells were grown overnight with aeration in L broth (24). For osmoregulation experiments, L broth (0.086 M NaCl) was modified to contain 0.3 M NaCl or no NaCl. M9 medium (27) supplemented with either 0.2% glucose or 0.2% maltose was used for suppression or induction of LamB. For transduction and transformations, L plates (L broth with 1.5% agar) containing 25 μg of tetracycline per ml were used as the selective medium.

Genetic techniques. Plasmid DNA was obtained from cells by forming spheroplasts with lysozyme and EDTA, lysing the spheroplasts with 1% sodium dodecyl sulfate in 0.2 N NaOH, and precipitating the plasmid DNA with ethanol (4).

Plasmid DNA was digested with *Eco*RI as described by Polisky et al. (39). Digestion of plasmid DNA with *Pst*I was for 3 h in 20 mM Tris-hydrochloride (pH 7.5)–10 mM $MgCl_2$–50 mM NaCl at 30°C. Ligation of enzymatically digested DNA was done at 15°C in 50 mM Tris-hydrochloride (pH 7.8)–10 mM $MgCl_2$–20 mM dithiothreitol–1 mM ATP with 200 U of T4 polynucleotide ligase.

Transduction of RW193 with a P1 lysate (42) was according to Willetts et al. (50). Transformation of bacterial cells with plasmid DNA was done by heat shocking a mixture of DNA and cells that had been made competent by suspension first in 0.1 M $MgCl_2$ and then in 0.1 M $CaCl_2$ (5).

Membrane isolation. Total cell envelope was prepared by

TABLE 1. *E. coli* K-12 strains

Strain	Genotype	Source/ reference
RW193	F⁻ *proC leu6 trpE38 entA thi*	16
ML1931	RW193 *lamB60*::Tn*10*	This study
UT400, UT1500, UT2300, UT4400, UT5600, UT6100	*fep* derivatives of RW193	7, 28
PS170	UT2300 *ompC*::Tn*5*	C. L. Pickett
PS160	UT2300 *ompF*::Tn*5*	C. L. Pickett
pop3208	*araD139* Δ*(arg-lac)205 flbB5301 ptsF25 relA1 rpsL150 lamB204 deoC1*	Coli Genetic Stock Center
MB1	As pop3208 except *lamB60*::Tn*10*	Coli Genetic Stock Center
P678-54	*thr leu thi supE lacY tonA gal mal xyl ara mtl minA minB*	29

the method of Inouye and Guthrie (14). The inner membrane was then solubilized by sodium lauryl sarcosinate, and the outer membrane was collected by centrifugation at 100,000 × *g* for 30 min (9).

Electrophoresis. Electrophoresis of DNA was carried out in 0.7% agarose (Bio-Rad Laboratories, Richmond, Calif.) with E buffer (40 mM Tris, 2 mM EDTA adjusted to pH 7.9 with acetic acid). Two gel systems were used in the electrophoresis of membrane proteins. The Lugtenberg system has been described (20) and utilizes 11% polyacrylamide, 0.2% sodium dodecyl sulfate, and 0.375 M Tris-hydrochloride buffer, pH 8.8. The urea gel system is a modification (23) of gel system D of Pugsley and Schnaitman (40) and typically consisted of 7% polyacrylamide, 0.1% sodium dodecyl sulfate, and 8 M urea in a 0.375 M Tris-hydrochloride buffer, pH 8.8. For electrophoresis of minicell proteins the urea gel system was altered to contain 10% polyacrylamide. The stacking gel and chamber buffer for all electrophoresis systems were according to Lugtenberg et al. (20). Samples (15 to 20 μg of protein as assayed by the method of Lowry et al. [18] or 100,000 cpm for ^{35}S-labeled protein) were solubilized as described by Laemmli (15). Staining and destaining were done as described previously (8). Autoradiographs were made with Kodak XRP-1 X-ray film (Eastman Kodak Co., Rochester, N.Y.).

Preparation, labeling, and fractionation of minicells. Minicells were isolated and labeled with [^{35}S]methionine (100 μCi/ml, final concentration) as described before (29). Minicells were incubated for 50 min before labeling at 27°C to reduce the amount of long-lived mRNA. The total envelope was obtained as described above, and the corresponding supernatant was used as the soluble fraction.

RESULTS

Effect of growth temperature on outer membrane proteins. Growth of *E. coli* RW193 cells at various temperatures resulted in fluctuations in the amounts of at least four outer membrane proteins (Fig. 1). As previously reported by Lugtenberg et al. (21), the quantity of OmpC protein increased with increased growth temperature and there was a concomitant decrease in the amount of OmpF protein. These temperature-dependent fluctuations in OmpF and OmpC protein levels were independent of growth phase (data not shown). A third protein later identified as LamB (see below) was present in greater amounts in stationary-phase cells grown at 27°C than in those grown at 37°C; the amount present in 20°C-grown cells varied and sometimes equaled the amount seen at 27°C. Little of this protein was seen regardless of growth temperature when cells were harvested in early to mid-log phase (data not shown). The fourth protein is OmpT, which has also been designated protein *a* and protein 3b, and its absence at low temperatures is well documented (21, 25, 43).

Identification of LamB. Another protein in addition to OmpF was found in increased quantities after growth at low temperatures. Like OmpF and OmpC, the apparent molecular weight of this protein differed in the two gel electrophoresis systems used. The protein had a molecular weight of approximately 50,000 in the gel system of Lugtenberg et al. (20) and is probably the same protein previously identified as pilin (21). Its apparent molecular weight and that the protein was maltose inducible and catabolite repressible (Fig. 2, lanes 5 and 6) suggested that the protein could be LamB, an outer membrane protein involved in the uptake of maltose and maltodextrins (46) and the bacteriophage λ receptor (41). To test this idea, outer membrane proteins from two *lamB* mutants (pop3208 and MB1) were examined. Both mutants lacked the 50K protein (Fig. 2, lanes 2 and 3). Finally, the *lamB*::Tn*10* mutation of MB1 was moved into RW193 by P1 transduction. The resulting transductant (ML1931) lacked

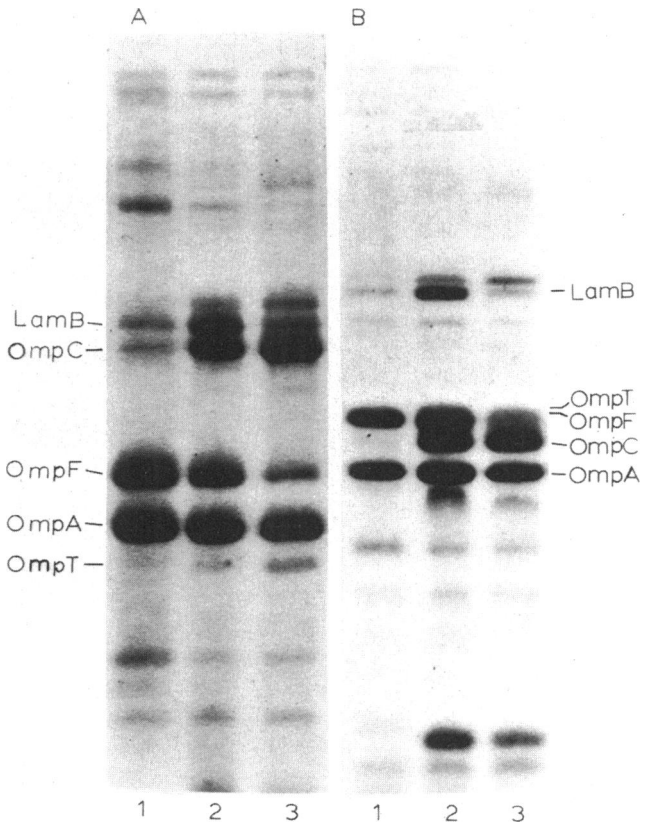

FIG. 1. Effect of temperature on outer membrane protein profiles of RW193. Electrophoresis was performed with the 8 M urea system (A) and the Lugtenberg system (B). Outer membranes from cells grown at 20, 27, and 37°C are shown in lanes 1, 2, and 3, respectively.

this same protein (Fig. 2, lane 4). Lugtenberg (personal communication) has also determined that the polypeptide initially identified as pilin is LamB.

Identification of mutants defective in OmpF, OmpC, and LamB protein thermoregulation. We previously described mutants of RW193 which lack the outer membrane protein FepA and presented evidence that many of these mutants arose from deletion mutations (26). Some of these mutants, which map in the 13-min region of the chromosome, also lack OmpT protein (7). One of these mutants, UT5600, was found to be defective in thermoregulation of OmpF, OmpC, and LamB (Fig. 3). Screening of additional *fepA* deletion mutants disclosed that UT4400, but not UT400, UT1500, UT2300, or UT6100, was also defective in thermoregulation. Osmoregulation of the major porin proteins was unaffected in UT5600 (data not shown).

Cloning of a gene that regulates temperature-dependent fluctuations of some outer membrane proteins. Plasmid pMC44, which carries some of the DNA that is missing in UT5600, including the structural gene for OmpT (10, 43), complemented the thermoregulation defect of UT5600. The

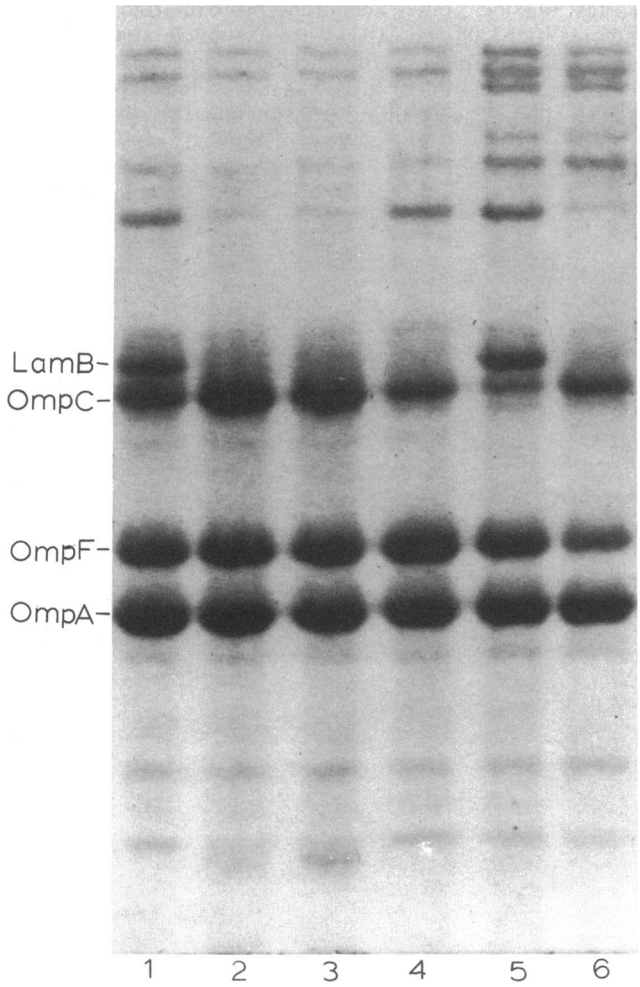

FIG. 2. Identification of the LamB polypeptide. Outer membranes were prepared from cells grown at 27°C to stationary phase in L broth (lanes 1 to 4), maltose minimal (lane 5), and glucose minimal (lane 6) media. Proteins were resolved with the urea gel system. Strains examined were: (1) RW193; (2) pop3802; (3) MB1; (4) ML1931; (5 and 6) RW193.

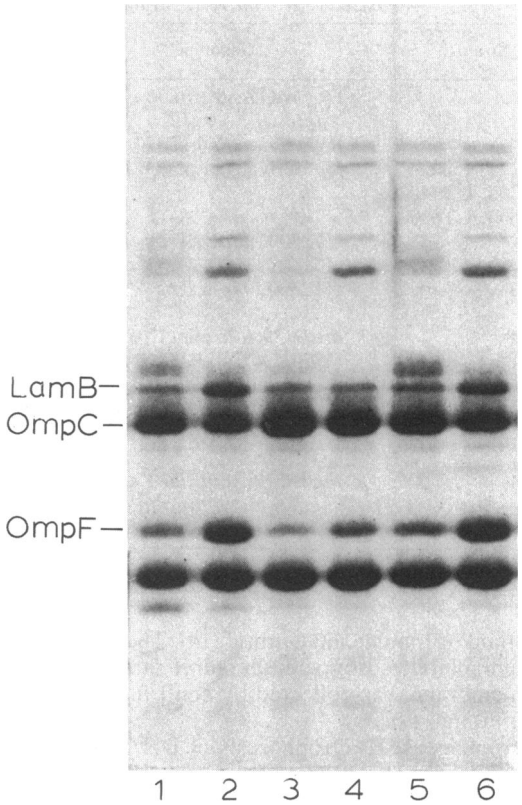

FIG. 3. Lack of temperature-dependent porin fluctuation in strain UT5600 and complementation by plasmid pML22. Electrophoresis was with the 8 M urea system. Strains examined and growth temperatures were: (1) RW193, 37°C; (2) RW193, 27°C; (3) UT5600, 37°C; (4) UT5600, 27°C; (5) UT5600(pML22), 37°C; (6) UT5600(pML22), 27°C.

gene responsible for the complementation, which we termed *envY*, was subcloned into vector pBR322 (Fig. 4). The new plasmid, pML22, did not carry *ompT* but complemented the UT5600 thermoregulation defect (Fig. 3). Plasmid pGGC110, which resulted from the same cloning procedure that gave rise to pML22, carries *ompT* (43) but did not complement the thermoregulation defect of UT5600 (data not shown). (The *envY* product was not necessary for the temperature-dependent fluctuation in OmpT levels; the OmpT produced under the direction of pGGC110 in UT5600 was more abundant at 37°C than at 27°C). Plasmid pMC44 contains a 2-megadalton fragment of the *E. coli* chromosome (3); this is distributed such that pGGC110 contains 1.5 megadaltons of the insert (43) and pML22 contains the remaining 0.5 megadalton.

Identification of a protein produced by pML22. Plasmid pML22 directed the synthesis of one polypeptide in minicells (Fig. 5); the polypeptide (EnvY protein) had a molecular weight of approximately 25,000 and was produced in minicells labeled at 27°C but not at 37°C. In some experiments the 25K polypeptide was produced at 37°C, but the amount was never equal to that synthesized at 27°C. In contrast, growth temperature had little effect on the abundance of pBR322-directed synthesis of β-lactamase. The reducing agent β-mercaptoethanol was required in the solubilization buffer for visualization of the 25K polypeptide. When <5% β-mercaptoethanol was used, either a smear or nothing was seen in the area of autoradiographs where the EnvY protein was usually found.

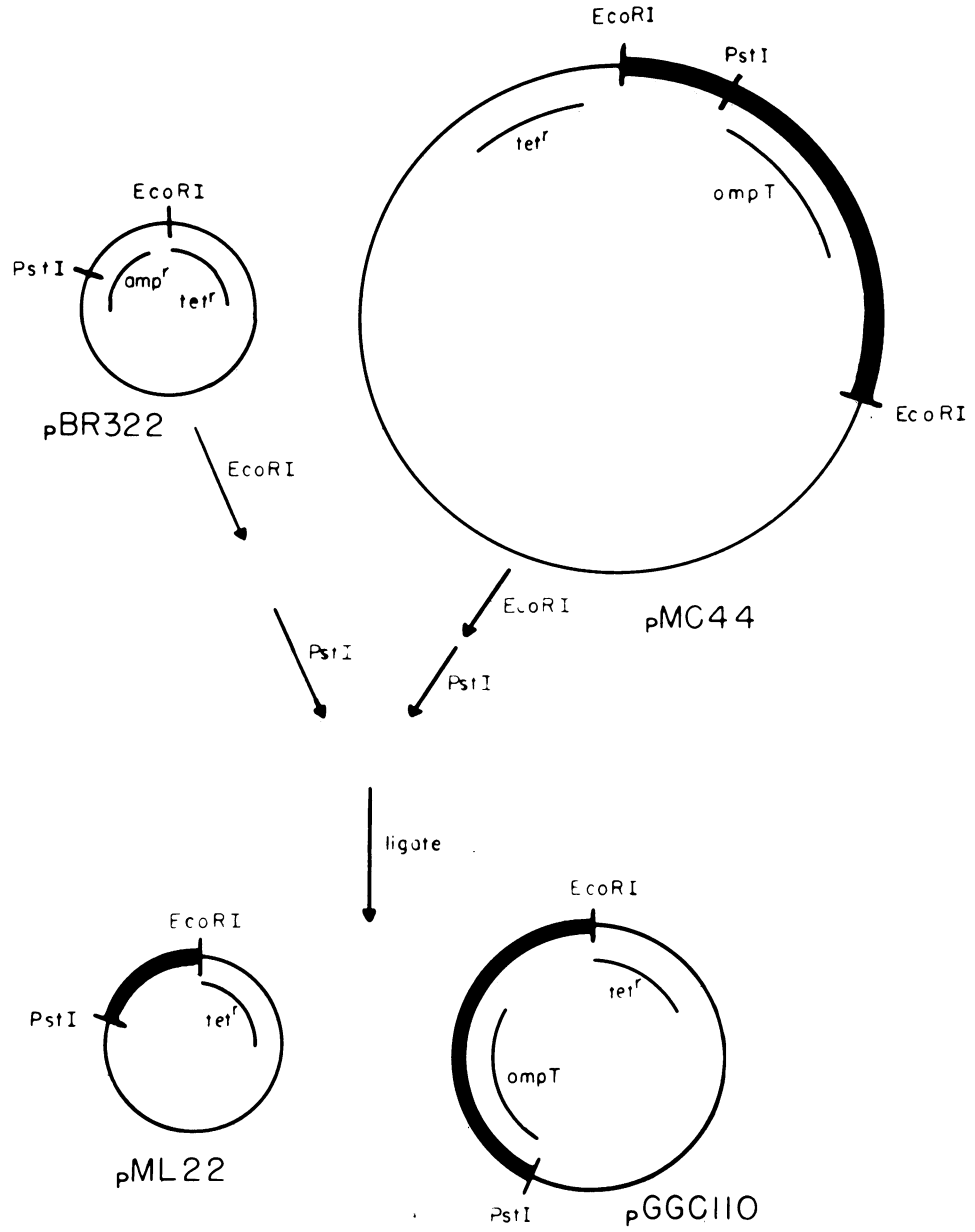

FIG. 4. Construction of pML22 and pGGC110. Bold lines are chromosomal DNA.

Localization of the EnvY polypeptide. Minicells harboring pML22 were labeled with [^{35}S]methionine, harvested, and separated into soluble and membrane fractions (Fig. 6). The pML22 product was present in the membrane fraction. No indication of processing of EnvY was observed; i.e., no cytoplasmic or higher-molecular-weight precursor of the 25K polypeptide was noted.

Attempts to further localize EnvY protein were unsuccessful. Fractionation of minicell envelopes by the detergents Sarkosyl (9) and Triton X-100 (44) indicated that EnvY protein was an outer membrane component. However, EnvY protein was found in inner and outer membrane and the soluble fraction by isopycnic sucrose density gradient analysis (36).

Temperature-dependent regulation in porin mutants. OmpC protein synthesis has been described as constitutive with respect to osmoregulation in strains carrying a mutation in the structural gene *ompF* (38). To test whether *ompF* mutations also render OmpC expression independent of thermoregulation, mutants with Tn*5* insertions in *ompF* or *ompC* were grown at 27 and 37°C and their outer membranes were examined (Fig. 7). Thermoregulation of the porins was unaffected by the mutations. OmpC in the *ompF* mutant PS160 fluctuated as in the parental strain UT2300 (lanes 1 to 4), and OmpF expression was normal in the *ompC* mutant PS170 (lanes 5 and 6). The results shown in lanes 7 to 12 confirmed in these strains the observations of Ozawa and Mizushima (38). Osmoregulation of OmpF was normal despite the introduction of an *ompC* mutation. In contrast, medium osmolarity did not affect OmpC expression in cells carrying an *ompF* mutation; OmpC appeared at fully induced levels in L broth modified to contain no NaCl or 0.3 M NaCl.

DISCUSSION

Control of major porin expression involves interplay among the products of several widely dispersed loci. In addition to the previously described effects of *ompR* and *envZ* mutations, strains bearing *tolC* mutations lack OmpF protein (33). The TolC protein, an outer membrane component, apparently performs a post-transcriptional function necessary for the appearance of OmpF protein in outer membrane (32). Also, expression of OmpF protein is low and OmpC protein is correspondingly elevated in cells having either adenylate cyclase (*cya*) or cyclic AMP receptor protein (*crp*) mutations (30, 45). These effects of the cyclic AMP system were found to be independent of growth temperature (data not shown). We described here an additional locus involved in porin regulation. The data confirmed and extended the previous findings of Lugtenberg et al. (21) that the levels of proteins OmpF and OmpC in the outer membrane vary with growth temperature. The new locus was also shown to control the thermoregulation of LamB protein in stationary-phase cells. LamB, like OmpF and OmpC, is a porin (19). The PhoE protein is an inducible general pore protein (22) that is related to OmpF and OmpC in its primary amino acid sequence and its DNA sequence (30). The effect of the thermoregulatory locus on PhoE could not be determined, however, because of difficulties in identifying this protein in the strains used here.

The observation that a bacterial strain (UT5600) with a large deletion in the 13-min region of the chromosome failed to exhibit thermoregulation provided the opportunity to screen for and clone the thermoregulatory gene. This gene, which we have named *envY*, appears to specify an envelope protein with an apparent molecular weight of approximately 25,000. Evidence that the 25K polypeptide is the *envY* product is twofold. The 0.5-megadalton bacterial DNA insert in pML22 can direct the synthesis of only 28,000 daltons of protein (6); therefore, the coding capacity of cloned DNA in pML22 is not adequate to specify another polypeptide. Additional supporting evidence is provided by the observation that there is a direct temperature-dependent relationship between EnvY protein synthesis and OmpF protein expression.

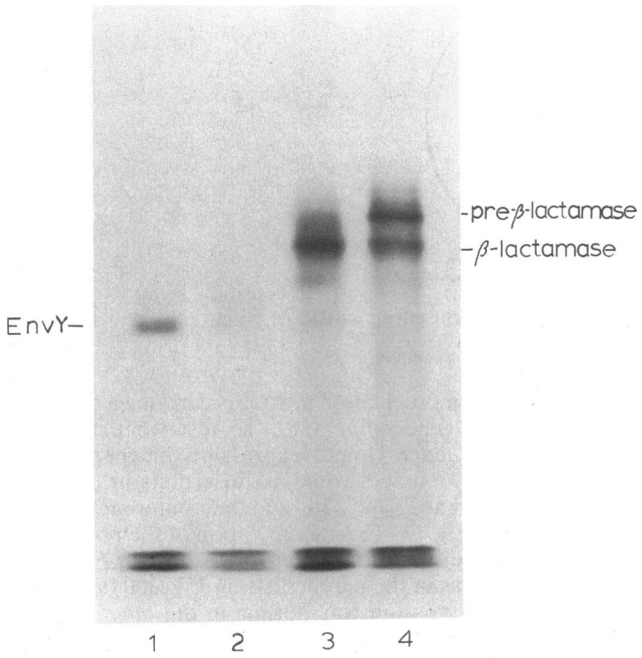

FIG. 5. Autoradiogram of [^{35}S]methionine-labeled polypeptides synthesized in minicells by plasmid pML22 or pBR322. Minicell proteins were electrophoresed on a 10% polyacrylamide–8 M urea gel. Plasmids harbored by the minicells and growth temperatures were as follows: (1) pML22, 27°C; (2) pML22, 37°C; (3) pBR322, 27°C; (4) pBR322, 37°C.

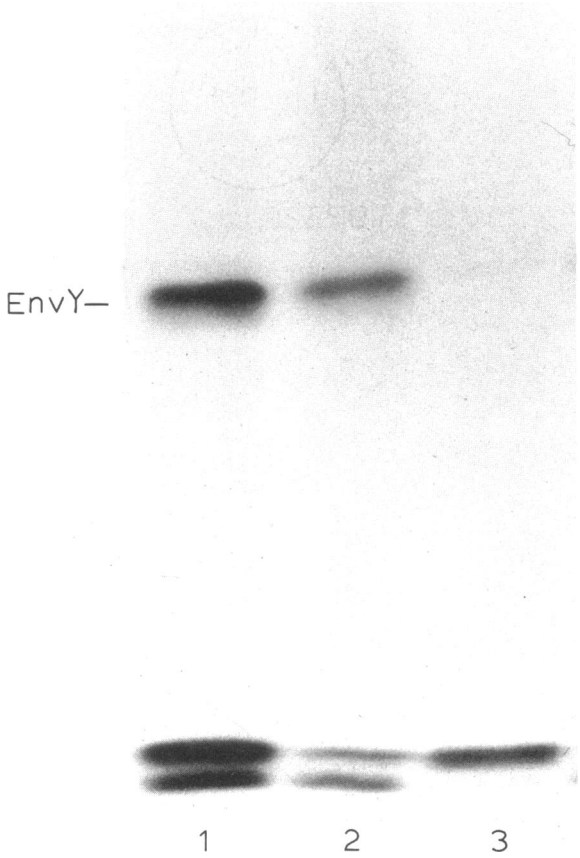

FIG. 6. Autoradiogram of [^{35}S]methionine-labeled soluble and envelope polypeptides synthesized at 27°C in minicells by plasmid pML22. Electrophoresis was as in the legend to Fig. 5. Lanes are: (1) whole minicells; (2) total envelope; (3) soluble fraction.

The EnvY protein is unusual; to our knowledge it is the only *E. coli* envelope protein that requires the presence of a reducing agent in the solubilization buffer if it is to subsequently be detected in polyacrylamide gels. The precise molecular weight of protein EnvY is uncertain for several reasons. The presence of urea in sodium dodecyl sulfate-polyacrylamide gels alters the mobility of many envelope proteins (cf. Fig. 1A and B). In the Lugtenberg gel system EnvY had an apparent molecular weight of 28,000 (data not shown). EnvY may be identical to M4, a 28,000-dalton protein specified by pMC44 (10; A Markovitz, personal

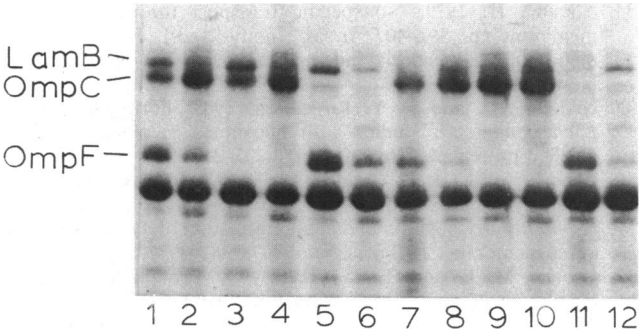

FIG. 7. Temperature- and osmolarity-dependent porin fluctuation in *ompF* and *ompC* mutants. Outer membranes were electrophoresed with the 8 M urea system. The gel patterns shown in lanes 1 to 6 are from cells grown in L broth, and the patterns shown in lanes 7 to 12 are from cells grown at 37°C in L broth modified to contain no NaCl or 0.3 M NaCl. For lanes 1 to 6, strains examined and growth temperatures were: (1) UT2300, 27°C; (2) UT2300, 37°C; (3) PS160 (*ompF*), 27°C; (4) PS160, 37°C; (5) PS170 (*ompC*), 27°C; (6) PS170, 37°C. For lanes 7 to 12, strains examined and media were: (7) UT2300, no NaCl; (8) UT2300, 0.3 M NaCl; (9) PS160, no NaCl; (10) PS160, 0.3 M NaCl; (11) PS170, no NaCl; (12) PS170, 0.3 M NaCl.

communication). Also, the close correspondence in size between the maximum coding capacity of the pML22 insert and the observed apparent molecular weight of *EnvY* suggests the possibility that the insert contains only the amino-terminal portion of the polypeptide and that this portion is all that is necessary for activity. We think, however, that the entire *envY* gene is present on pML22 as pMC44, the larger plasmid from which pML22 was derived, directs the synthesis of a polypeptide that comigrates with the protein specified by pML22 (data not shown).

The *envY* gene was found on the same 2-megadalton *E. coli* DNA fragment that contains *ompT*. On the most recent *E. coli* linkage map (1), which includes substantial alterations in the positions of genes in the 12 to 14-min region, *envY* is therefore located at approximately 12.9 min. It is interesting that the structural genes for two envelope proteins, one which appears primarily at high temperatures (OmpT) and one (EnvY) which is most prevalent in minicells at low temperatures, are closely linked. Phage LP81 has a low plating efficiency on strains missing this region (7), and it was speculated that OmpT comprises at least part of its receptor. However, LP81 fails to infect UT5600(pMC44) so neither the OmpT nor the EnvY protein, alone or together, provides the missing function.

The EnvY polypeptide is, like OmpR, dispensable under laboratory growth conditions, and the advantages of regulating OmpF and OmpC remain speculative. The OmpF pore is larger than the channel composed of OmpC protein (35). Therefore, in *E. coli* K-12, the EnvY protein determines that larger channels are present in cells growing at lower temperatures and that, when such cells are in stationary phase, an additional channel, that formed by the LamB polypeptide, is present. The major porins are expressed in low-osmolarity medium much as they are at low temperatures. To determine the relative significance of thermal and osmoregulation, RW193 cells were grown at 27°C in high-osmolarity (0.3 M NaCl) L medium. An intermediate effect was observed; the amount of OmpF protein was less than that present in cells grown at 27°C in L broth but more than that in cells grown at 37°C in L medium or L medium modified to contain 0.3 M NaCl. The OmpC protein was expressed in a complementary pattern (data not shown).

How EnvY functions and interacts with other porin regulatory components is not known. Porin expression is affected by systems operating at both transcriptional (*ompB* operon and cyclic AMP system) and post-transcriptional (*tolC*) levels. We are now determining at which level *envY* acts.

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