Topological analysis of the lysine-specific permease of *Escherichia coli*

Jeri Ellis, Arthur Carlin, Chris Steffes, Jianhua Wu, Jiyang Liu and Barry P. Rosen

*Escherichia coli* accumulates lysine via two systems, one specific for lysine (LysP) and a second inhibited by arginine or ornithine (LAO). The *lysP* gene encodes a polypeptide of 489 residues. A topological analysis of the LysP protein was performed using gene fusions. Random in-frame fusions of the *lysP* gene with the *lacZ* or *blaM* genes were generated. Site-directed mutagenesis was also used to generate additional *blaM* fusions at specific locations in the *lysP* gene. Two methods were used to alleviate the problem of lethal expression of some *lysP::blaM* fusions. First, ternary fusions were constructed in which the *arsD* gene was fused at the 5' end of the *lysP* gene and the *blaM* gene fused at specific sites within the *lysP* gene. In these plasmids *lysP* expression was controlled by the *ars* promoter. Secondly, an *E. coli* strain with a *pcnB* mutation was used with some fusions to maintain the plasmids at a reduced copy number. From analysis of 30 gene fusions, a topological model of the LysP protein is proposed in which the protein has 12 membrane-spanning regions, with the N- and C-termini in the cytosol.

**Keywords:** lysine transport, permeases, gene fusions, ternary fusions, membrane protein topology

**INTRODUCTION**

Multiple systems exist for the transport of the basic amino acids arginine, lysine, and ornithine in *Escherichia coli*. The arginine-specific system transports only arginine (Rosen, 1971, 1973). The lysine-arginine-ornithine (LAO) system transports lysine and ornithine and is inhibited by arginine (Rosen, 1971). A third system, the lysine-specific permease (LysP), is inhibited by the lysine analogue S-(β-amino-ethyl)-γ-cysteine (thiosine) but not by ornithine or arginine (Rosen, 1971). The *lysP* gene has recently been cloned and sequenced (Steffes et al., 1992). A fourth lysine permease, encoded by the *cadB* gene, is proposed to be a lysine/cadaverine exchanger (Meng & Bennett, 1992).

The membrane topology of bacterial membrane proteins can be examined using gene fusions (Broome-Smith et al., 1990; Manoil, 1990; Hennessey & Broome-Smith, 1993). Several types of gene fusions were used in this study. In-frame fusions of the *lacZ* gene which encodes the enzyme β-galactosidase give information in which high β-galactosidase activity indicates fusion in the coding sequence for the cytosolic region of a membrane protein (Froshauer et al., 1988). β-Lactamase (*blaM*) fusions give complementary information; chimeras with fusions in the sequence for the periplasmic regions of the membrane protein result in resistance to high concentrations of ampicillin (*Ap*) (Broome-Smith & Spratt, 1986). Production of β-lactamase in the cytosol results in *Ap* in patches of cells on solid media, so that *blaM* fusions also provide information on the localization of membrane elements on the cytosolic side of the membrane. It cannot be assumed that the presence of the reporter does not affect the localization of the chimera (Hennessey & Broome-Smith, 1993). On the whole, however, the method gives empirically consistent results with a growing number of membrane proteins. For that reason conclusions based on the use of fusions of the genes for membrane proteins with the genes for spatial reporter proteins have been accepted with the realization that the reasoning is somewhat circular.

In-frame *lysP::blaM* and *lysP::lacZ* fusions were produced within the *lysP* gene by exonuclease digestion from the 3' end of the *lysP* gene *in vitro* followed by ligation to the 5' end of the *lacZ* gene or the 5' end of the portion of the *blaM* gene encoding the mature form of the enzyme. With this random procedure fusions were found to cluster in some regions, with no fusions in other regions of the membrane proteins.
lysP gene. Oligonucleotide-directed deletion mutagenesis was used to construct additional fusions (Boyd et al., 1987). Oligonucleotides were synthesized in which half was complementary to specific regions of the lysP gene and half complementary to the start of the mature form of the blaM gene. Directed mutagenesis using these oligonucleotides allowed isolation of specifically placed fusions without the introduction of additional codons. This approach was successful for some regions, but there were specific regions where no strains could be isolated containing the construct, perhaps because production of the chimeras was lethal. We had previously devised a ternary fusion procedure to obtain fusions in regions that might produce lethality (Wu et al., 1992). Ternary fusions are composed of parts of three genes. First, a small hydrophilic protein serves as a cytosolic anchor when fused to the N-terminus of the membrane protein, which is the second part of the ternary fusion. The third part is the localization reporter. Four ternary fusions with the lysP gene were constructed. A portion of the arsD gene was used for the 5' end, and for the localization marker the blaM gene was used. The final fusions were isolated using this approach. Expression of these ternary fusions is tightly regulated under control of the ars promoter, with Apβ ternary fusions observed only at submaximal concentrations of inducer. Finally, expression of several fusions could only be observed in strains with a pcmB mutation that reduces the copy number of pBR322-derived plasmids (Lopilato et al., 1986).

METHODS

Materials. Restriction enzymes and nucleic acid modifying enzymes were obtained from Bethesda Research Laboratories. Antibodies to β-lactamase were purchased from 5 → 3 Prime. Oligonucleotides were synthesized in the Macromolecular Core Facility of Wayne State University School of Medicine. All other chemicals were obtained from commercial sources.

Strains and plasmids. E. coli strains and plasmids used in this study are described in Table 1.

Media and growth conditions. Cells were grown in LB medium (Miller, 1972) at 37°C. Kanamycin was added to 50 μg ml⁻¹, ampicillin to 0.1 mg ml⁻¹ unless otherwise noted. Sodium arsenite was used at the indicated concentrations as an inducer of ternary fusions.

DNA manipulations. The conditions for plasmid isolation, DNA restriction endonuclease analysis, ligation, transformation and sequencing have all been described previously (Sambrook et al., 1989). Plasmid DNA was prepared using a Wizard DNA purification system (Promega). Small-scale purification of bacterial chromosomal DNA was done as described by Lewington et al. (1987). Bacteriophage λ preparation, restriction endonuclease digestions, Klenow fragment fill-in and ligations were performed as described by Sambrook et al. (1989). One-step preparation of competent cells and plasmid transformations was performed by the method of Chung et al. (1989). DNA fragments were separated by electrophoresis on 1% (w/v) agarose gels. The desired fragment was recovered from a low-melting-point agarose gel and purified by a Wizard clean-up system (Promega). The fusion junctions between the lysP and arsD, blaM or lacZ genes were determined by dideoxy sequencing of the double-stranded plasmid DNA. Double-stranded DNA was denatured with 0.2 M NaOH and 0.2 mM Na2EDTA for 30 min at 37°C. The sequencing assays (Sanger et al., 1977) were performed with a Sequenase Version 2.0 system (USB).

Isolation of lysP::lacZ gene fusions. The lysP::lacZ gene fusions were constructed in vitro. Plasmid pBPR20 was digested with restriction endonucleases EcoRI and HindIII, and a 2.7 kb fragment containing the lysP gene was cloned into plasmid pMB1069 that had been digested with both EcoRI and HindIII, yielding plasmid pJEL. To create in-frame lysP::lacZ fusions plasmid pJEL was linearized by digestion at the unique SnaBI site in lysP. A series of staggered deletions in the lysP gene was performed by digestion of the linearized DNA with exonuclease III for varying lengths of time (Sambrook et al., 1989). The products were pooled and digested with HindIII to remove the region 5' to the start of the lacZ gene. The ends of the fragments were filled in with the Klenow fragment of DNA polymerase I. The resulting linear fragments were ligated with T4 DNA ligase to create fusions of truncated lysP gene with the start of the lacZ gene. The plasmid mixture was electroporated into cells of E. coli strain MC1000 (lacZ). Blue colonies were selected on solid LB medium containing X-Gal. Cells with fusions were selected on LB medium containing ampicillin and confirmed by digestion with EcoRI and HindIII. Loss of the HindIII site was indicative of successful fusion. β-Galactosidase activity was assayed at 37°C essentially as described by Miller (1972). Portions (0.2 ml) of an exponential phase culture were mixed with 18 ml buffer consisting of 60 mM Na2HPO4, 40 mM Na2HPO4, 10 mM KCl, 1 mM MgSO4, 50 mM β-mercapto-ethanol, pH 8.0, 50 μl 0.1% SDS and 50 μl chloroform. Reactions were started by the addition of 0.4 ml 0.4% o-nitrophenylgalactoside. After centrifugation in a microfuge, the A405 of the supernatant was measured. A unit of activity is defined as 1 pmol substrate hydrolysed min⁻¹ per OD600 unit of cells using a molar extinction coefficient of 4860 for o-nitrophenol.

Construction of random lysP::blaM gene fusions. To create a set of in-frame fusions between the lysP and blaM genes, plasmid pBPR2 (lysP::phoA) was digested with both BamHI and DraI, and a 1.2 kb fragment containing the lysP::phoA gene fusion was cloned into plasmid pBS633 that was previously digested with both BamHI and BalI, yielding plasmid pJBB. The plasmid was linearized by digestion at the unique BclI restriction site located at nucleotide 483 of the lysP gene, and the digest was treated with exonuclease III to generate a random series of lysP deletions. The pooled products were digested with PmlII to remove sequences 5' to the blaM gene, and the ends filled in with the Klenow fragment of DNA polymerase I. The resulting linear fragments were circularized by ligation with T4 DNA ligase, producing fusions between the first 351 bp of the lysP gene and the mature form of the blaM gene.

To create a plasmid containing the lysP gene upstream of the blaM gene, plasmid pJHW101 was digested with BamHI-SalI and a 2592 bp BamHI-SalI fragment from plasmid pBPR2 containing the entire lysP gene was inserted by ligation. Following transformation of the ligation mixture into E. coli JM109 with selection for kanamycin resistance (Km'), the resulting plasmid, pBPR100, was used to create lysP::blaM fusions over the first 1197 bp of the lysP gene. The plasmid was linearized by digestion with SmaI followed by exonuclease III digestion to generate a nested series of lysP deletions. The DNA was digested with SalI, the ends filled in with the Klenow fragment of DNA polymerase I and circularized by intramolecular ligation. E. coli strain HB101 was transformed with the ligation mixture, and Km' transformants selected. In-frame
## Table 1. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Genotype and phenotype</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E. coli strain</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HB101</td>
<td>supE44 hsdS20(rMamλ) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mil-1</td>
<td>Sambrook et al. (1989)</td>
</tr>
<tr>
<td>HMS174(DE3)</td>
<td>HMS174(F' recA rif') lysogen with integration of T7 RNA polymerase gene under control of lacUV5 promoter</td>
<td>Studier &amp; Moffatt (1986)</td>
</tr>
<tr>
<td>CC863</td>
<td>pncB Δ(lac) y 74galE galK Δ(ara-leu) thi rpsE pheA20 angE rpoB penB zed::Tn10</td>
<td>Lopilato et al. (1986)</td>
</tr>
<tr>
<td>BMH 71-18 mutS</td>
<td>thi supE Δ(lac-proAB), [mutS::Tn10] [F' proA'B' lacI5ZAM15]</td>
<td>Promega</td>
</tr>
<tr>
<td>MC1000</td>
<td>F- araD139 Δ(araABC-leu)7679 galU galK Δ(lac) X74 rpsL thi</td>
<td>Casadaban &amp; Cohen (1980)</td>
</tr>
<tr>
<td><strong>Plasmid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBPR2</td>
<td>pBR322 with 6·1 kb BamHI ltp::pheA insert</td>
<td>Steffes et al. (1992)</td>
</tr>
<tr>
<td>pBPR20</td>
<td>pT7-5 expression vector with 2·7 kb BamHI ltp insert</td>
<td>Steffes et al. (1992)</td>
</tr>
<tr>
<td>pMLB1069</td>
<td>pBR322 Δ(rev) lacZY'</td>
<td>Solomon et al. (1989)</td>
</tr>
<tr>
<td>pJBS633</td>
<td>blaM gene fusion vector</td>
<td>Broome-Smith &amp; Spratt (1986)</td>
</tr>
<tr>
<td>pBB</td>
<td>pJBS633 digested with BamHI/BalI and ligated with 1·2 kb BamHI/DraI insert from pBPR2 containing ltp</td>
<td>This study</td>
</tr>
<tr>
<td>pJEL</td>
<td>pMLB1069 containing the EcoRI–HindIII fragment from pBPR20 containing the ltp gene fragment 5' to the lacZ gene</td>
<td>This study</td>
</tr>
<tr>
<td>pJE series</td>
<td>Fusions of ltp to blaM in the first 351 bp region of ltp generated by random gene fusions</td>
<td>This study</td>
</tr>
<tr>
<td>pJHW101</td>
<td>blaM fusion vector containing the ars operon</td>
<td>Wu et al. (1992)</td>
</tr>
<tr>
<td>pBPR100</td>
<td>BamHI–SalI fragment containing 2·7 kb ltp gene cloned into pJHW101 placing ltp 3' to the first 1457 bp of arsA; blaM is 3' to the ltp fragment</td>
<td>This study</td>
</tr>
<tr>
<td>pBPR series</td>
<td>Fusions of ltp–blaM generated by random gene fusions and site-directed mutagenesis</td>
<td>This study</td>
</tr>
<tr>
<td>pBPR1500</td>
<td>Deletion of 1500 bp from pBPR100 between arsA and ltp by SphI digestion</td>
<td>This study</td>
</tr>
<tr>
<td>pWZ67</td>
<td>Contains fusion of the first 67 amino acids of arsD to the N-terminus of ltp</td>
<td>This study</td>
</tr>
<tr>
<td>pDLB series</td>
<td>Ternary gene fusions of arsD–ltp–blaM</td>
<td>This study</td>
</tr>
<tr>
<td>pYC18</td>
<td>ltp–blaM fusion in pJEl18 cloned into a pT7-5 overexpression vector</td>
<td>This study</td>
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</table>

Fusions of the ltp gene to the coding region of the mature form of β-lactamase were identified by applying approximately 5 x 10⁶ cells in patches to LB agar containing 50 µg ampicillin ml⁻¹. Positive strains were screened for resistance in single colonies or patches using 100 µg ampicillin ml⁻¹ on LB agar.

**Construction of gene fusions by site-directed deletion mutagenesis.** Oligonucleotide-directed deletion mutagenesis using the Altered Sites in vitro Mutagenesis system (Promega) was utilized to construct fusions at specific sites within the ltp gene (Boyd et al., 1987). Fusions at amino acids 154, 440 and 489 were created using 42mer synthetic oligonucleotides in which the 5' end of each oligonucleotide contained a 21 bp sequence complementary to the beginning of the blaM gene in pBPR100. The remaining 21 nucleotides of each contained a sequence complementary to the desired fusion site in ltp. The oligonucleotides were phosphorylated with T4 polynucleotide kinase. Single-strand DNA was generated from the phagemid pBPR100 using helper phage R408 and annealed with a 25-fold molar excess of the phosphorylated oligonucleotide. The mixture was heated to 70 °C for 5 min, allowing complete denaturation, then slowly cooled to room temperature to allow annealing. The complementary strand containing the mutation was resynthesized using T4 DNA polymerase and T4 DNA ligase. This mixture was then transformed by electroporation into a repair deficient E. coli strain, BMH 71-18 (mutS), and allowed to grow in LB medium with shaking overnight at 37 °C. Plasmid DNA was isolated from pooled transformants and digested with SalI to linearize unmutagenized plasmids, reducing their transformation efficiency. The mixture was used to transform cells of E. coli strain JM109, with selection for Km'.

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**Table 2.** β-Galactosidase activity of *E. coli* strain MC1000 (*lacZ*) expressing *lysP::lacZ* fusions

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>LysP fusion site (amino acyl residue)</th>
<th>Junction sequence*</th>
<th>β-Galactosidase activity†</th>
</tr>
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<tr>
<td>None</td>
<td>–</td>
<td>GCC TCT GGA GCT TGG GGG</td>
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</tr>
<tr>
<td>pJEL40</td>
<td>40</td>
<td>TCG TTT GCA GCT TGG GGG</td>
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</tr>
<tr>
<td>pJEL83</td>
<td>83</td>
<td>GAG GGA ACA GCT TGG GGG</td>
<td></td>
</tr>
<tr>
<td>pJEL221</td>
<td>221</td>
<td>GAT CCG GCA GCT TGG GGG</td>
<td></td>
</tr>
<tr>
<td>pJEL232</td>
<td>232</td>
<td>TCT GCG GCA GCT TGG GGG</td>
<td></td>
</tr>
<tr>
<td>pJEL235</td>
<td>235</td>
<td>GCGTCTACAGCTTGG</td>
<td></td>
</tr>
<tr>
<td>pJEL297</td>
<td>297</td>
<td>AAA GCG CCA GCT TGG GGG</td>
<td></td>
</tr>
<tr>
<td>pJEL320</td>
<td>320</td>
<td>GGATCGTGATAGCTTGG</td>
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</tr>
<tr>
<td>pJEL333</td>
<td>333</td>
<td>CGC TTC CGA GCT TGG GGG</td>
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<td>pJEL342</td>
<td>342</td>
<td>GCC TCT ACA GCT TGG GGG</td>
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<tr>
<td>pJEL345</td>
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<td>AAA GCC CCA GCT TGG GGG</td>
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<tr>
<td>pJEL351</td>
<td>351</td>
<td>TCG CTTG GCA GCT TGG GGG</td>
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<tr>
<td>pJEL353</td>
<td>353</td>
<td>GCG GTG CCA GCT TGG GGG</td>
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<tr>
<td>pJEL355</td>
<td>355</td>
<td>CTG TAT GCA GCT TGG GGG</td>
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<tr>
<td>pJEL397</td>
<td>397</td>
<td>GCG TCT GCA GCT TGG GGG</td>
<td></td>
</tr>
</tbody>
</table>

* The sequence from *lysP* is underlined.
† Assays of β-galactosidase activity were performed using cells permeabilized with SDS and chloroform as described under Methods. Activity is expressed as µmol substrate hydrolysed min⁻¹ per OD₆₀₀ unit of cells.

**Construction of ternary fusions.** Using the above methods fusions were not obtained in three regions of the *lysP* gene corresponding to amino acid residues 127, 189, 272 and 280 of the LysP protein. One possibility was that expression of the fusion genes was toxic to the cells, resulting in loss of the plasmid. Ternary fusions have proved useful for analysis of the topology of selected membrane-spanning regions of the ArsB protein where potentially toxic chimeras were produced (Wu et al., 1992). Ternary fusions between the *arsD*, *bsP* and *blaM* genes were created from plasmid pBPRA1500, which contains the *lysP* gene between the *arsD* and *blaM* genes. Site-directed deletion mutagenesis was performed using a 42mer oligonucleotide that would produce a gene fusion comprising the first 67 codons of the *arsD* gene fused to the second codon of the *lysP* gene. Plasmid DNA was isolated from pooled transformants and digested with *SphI* to linearize unmutagenized plasmids, reducing their transformation efficiency. Transformants were selected for Km' resistance. In the resulting plasmid, pWZ67, expression of the *arsD::lysP* fusion was under control of the tightly repressed *ars* promoter.

Ternary fusions between the *arsD::lysP* fusion gene and the *blaM* gene for the mature form of β-lactamase were created by site-directed deletion mutagenesis using 42mer oligonucleotides. The oligonucleotides contained 21 nucleotides complementary to specific sequences in the *lysP* gene and 21 nucleotides complementary to the start of the *blaM* gene. Four ternary fusions were constructed, encoding chimeras at residues 127, 189, 272 and 280 of the LysP protein. In addition, the chimeras would have the first 67 residues of the ArsD protein and the entire mature β-lactamase. Induction of these ternary fusions with 1–2 µM arsenite in cells of *E. coli* strain JM109 resulted in very poor growth, even in the absence of ampicillin, and no growth was observed with 5 µM arsenite, presumably...
due to lethality of the chimeras. When the ternary fusion plasmids were transformed to *E. coli* strain CC863 (pmb) to maintain these pBR322-derived plasmids at a reduced copy number (Lopilo et al., 1986), arsenite-inducible ApR was observed. However, different levels of induction were found to be optimal for expression of different constructs. While 1 mM arsenite was sufficient to induce ApR of the *lysP::blaM* fusion at position 189 of the *lysP* protein, 10 mM arsenite was needed for strains encoding the chimeras at *lysP* residues 272 and 280, and 50 mM was required for expression of the chimera at *lysP* residue 127. The reason for these differences is unexplained.

**PAGE and immunological blotting.** Cell cultures were prepared by boiling in SDS sample gel buffer for 5 min. SDS-PAGE was performed as described by Laemmli (1970). Immunological blotting (Gershoni & Palade, 1983) was performed using antibodies to β-lactamase (5′ → 3′ Prime) at a 1:2000 dilution to detect the β-lactamase-containing chimeric proteins.

**N-terminal sequence analysis of the *lysP* protein.** The N-terminal sequence of the *lysP* protein was determined using a *lysP–β-lactamase* chimeric protein isolated from cells carrying plasmid pYC18. This plasmid has a *lysP::blaM* fusion gene encoding the N-terminal 18 amino acids of the *lysP* protein under control of the T7 phage promoter. Production of T7 RNA polymerase was induced in *E. coli* strain HMS714DE3 by the addition of 0.4 mM IPTG, producing high level production of the chimeric protein. Induced cells (200 ml) were harvested by centrifugation at 4 °C and suspended in 2 ml PBS (0.136 M NaCl, 2.68 mM KCl, 5.37 mM Na₂HPO₄, 1.76 mM KH₂PO₄). The cells were lysed by a single passage through a French press at 20000 p.s.i. (138 MPa). Inclusion bodies were isolated by low speed centrifugation. The membrane and cytosolic fractions were separated by centrifugation at 150000 *g* for 1 h. The cellular location of the chimera was determined by immunoblotting using antibodies to β-lactamase. The chimeric protein was identified in the cytosolic fraction as a polypeptide. From a separate gel the chimeric band was transferred electrophoretically to a polyvinylidene difluoride membrane (Matsuda, 1987). The membrane was stained with 0.2% Coomassie blue, destained with a solution of 45% (v/v) methanol and 10% (v/v) acetic acid, and washed extensively with HPLC grade water. The membrane was dried, and the band corresponding to the chimeric protein was excised and subjected to N-terminal sequence analysis using a gas phase sequencer in the Macromolecular Core Facility of Wayne State University.

**RESULTS**

**Characterization of *lysP::lacZ* fusions**

Fourteen in-frame *lysP::lacZ* fusions were isolated by screening for blue colonies on solid medium contain X-Gal. Each strain exhibited high levels of β-galactosidase activity, consistent with a cytosolic location of β-galactosidase (Table 2). From DNA sequencing the locations were determined to correspond to fusions at residues 40, 83, 221, 232, 235, 297, 302, 333, 342, 345, 351, 353, 355 and 397 of the *lysP* protein. The locations of these and other fusions in the *lysP* protein are shown in Fig. 1.

**Characterization of *lysP::blaM* fusions**

Nine in-frame *lysP::blaM* fusions were obtained using exonuclease digestion from the 3′ end of the *lysP* gene. From DNA sequence analysis the location of the fusions corresponded to residues 15, 18, 45, 66, 118, 310, 344, 356 and 369 of the *lysP* protein (Table 3). Using site-directed deletion mutagenesis, three additional fusions were created at locations corresponding to residues 154, 440 and 489 of the *lysP* protein. Even using site-directed deletion mutagenesis, fusions could not be obtained in regions of the *lysP* gene corresponding to residues 127, 189, 272 and 280 of the *lysP* protein using the native *lysP* gene, presumably because production of the chimeras was lethal. For that reason ternary fusions were created by site-directed deletion mutagenesis at these four locations.

Cells harbouring the *bla* fusions were screened for resistance to ampicillin. Nine strains were resistant to less than 50 μg ampicillin ml⁻¹ and only exhibited growth in patch at 50 μg ampicillin ml⁻¹, indicative of a cytosolic localization of the β-lactamase moiety. These strains contained fusions at amino acids 15, 18, 66, 118, 154, 310, 344, 356 and 489. Seven strains were resistant to at least 100 μg ampicillin ml⁻¹ as demonstrated by single colony growth, consistent with a periplasmic localization of β-lactamase. These strains contained fusions corresponding to residues 45, 127, 189, 272, 280, 369 and 440 of the *lysP* protein (Table 3). Note that the fusion at residue 489...
**Table 3. Phenotype of *E. coli* strains expressing lysP::blaM fusions**

<table>
<thead>
<tr>
<th>Plasmid*</th>
<th>LysP fusion site (amino acyl residue)</th>
<th>Junction sequence†</th>
<th>Ampicillin resistance phenotype‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>pJE15</td>
<td>15</td>
<td>TTA CGC CGT CTG CGT</td>
<td>Patch</td>
</tr>
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<td>pJE18</td>
<td>18</td>
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<td>Patch</td>
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<td>pJE45</td>
<td>45</td>
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<td>Single colony</td>
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<td>pJE66</td>
<td>66</td>
<td>TTC CTG ATG CTG CGT</td>
<td>Patch</td>
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<td>pJE118</td>
<td>118</td>
<td>GCA GCT CAG CTG CGT</td>
<td>Patch</td>
</tr>
<tr>
<td>pDLB127</td>
<td>127</td>
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<td>Single colony</td>
</tr>
<tr>
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<td>154</td>
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<td>Patch</td>
</tr>
<tr>
<td>pDLB189</td>
<td>189</td>
<td>CCT GCG GGC CTG CGT</td>
<td>Single colony</td>
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<tr>
<td>pDLB272</td>
<td>272</td>
<td>AGC CTG CTG CTG CGT</td>
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<td>489</td>
<td>GAT AAG AAA CTG CGT CAC</td>
<td>Patch</td>
</tr>
</tbody>
</table>

* pDLB plasmids were in *E. coli* strain CC863 (pmB); others were in strain JM109.
† The sequence from ~SP is underlined.
‡ Single colony refers to the ability to form single colonies on LB plates containing 100 μg ampicillin ml⁻¹. Patch refers to the inability to form single colonies, but the ability to form a small patch of confluent cells on LB plates containing 100 μg ampicillin ml⁻¹.

produces a chimera containing the entire LysP protein, allowing localization of the C-terminus in the cytosol.

**N-terminal sequence of the LysP protein**

The LysP-β-lactamase chimeric protein was isolated from cells carrying plasmid pYC18. This chimera, which contains the N-terminal 18 amino acids of the LysP protein, was found exclusively in the soluble fraction. To confirm that the chimeric protein actually contained the N-terminus of the LysP protein, it was subjected to six cycles of gas phase sequencing. The sequence VSETKT was obtained, and only a single N-terminal sequence observed. This corresponds exactly to the second through seventh residues of the LysP protein predicted from the nucleotide sequence (Steffes et al., 1992). An N-terminal methionine was not observed.

**DISCUSSION**

From the hydropathic profile of the LysP protein there are 12 regions of 18 or more amino acid residues in length with a hydropathy index greater than 1.5 (Steffes et al., 1992), indicative of possible membrane-spanning α-helices (Kyte & Doolittle, 1982). However experimental data are required to test the topology predicted from the hydropathic analysis. A genetic approach was used to determine the number and topological arrangement of the membrane-spanning regions of the LysP protein. In-frame gene fusions were constructed between the lysP
Membrane topology of LysP

**Fig. 2.** Topological model of the LysP protein. The model proposes 12 membrane-spanning α-helices joined by six periplasmic loops (P1–P6) and five cytoplasmic loops (C1–C5). The N- and C-termini are suggested to be cytosolic. The precise placement of each residue cannot be assigned from the data. The suggested placement has glycyl or prolyl residues placed in turn regions where possible. Acid (■) and basic (◉) residues are indicated.

Two fusions were obtained in what was predicted to be near the end of the first membrane-spanning region or in the first periplasmic loop, P1. Cells expressing the **lysP::lacZ** fusion at residue 40 of the LysP protein were strongly positive on solid media with X-Gal and exhibited the highest β-galactosidase activity of any fusion, suggesting that this region was located in the cytosol. On the other hand, cells expressing the **lysP::blaM** at LysP residue 45 exhibited resistance to ampicillin even at 200 μg ml⁻¹, and the chimera was membrane bound (data not shown), indicating a periplasmic location for this region. A separation by five residues is not sufficient to span the membrane. It is possible that residues 40 and 45 could both be within the first membrane-spanning region. However, the location of the site of fusion near the periplasmic side of the first membrane-spanning region coupled to the highly positive nature of the N-terminus could result in retardation in translocation of the first membrane-spanning sequence (von Heijne, 1989; Hennessy & Broome-Smith, 1993). Thus the phenotype of cells with the **lysP::lacZ** fusion at residue 40 could be anomalous.

With one exception, the activities of the other fusions were consistent with the proposed locations shown in Fig. 1. The exception is the **lysP::phoA** fusion used to identify the **lysP** gene (Steffes et al., 1992). This was identified as a light blue colony on plates containing the chromogenic alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate. The fusion site was found at residue 228 of the LysP protein, within the proposed C3 cytosolic loop. It is located between the site of **lysP::lacZ** fusions corresponding to LysP residues 221, 232 and 235. All three chimeras exhibited significant β-galactosidase activity, indicating that this region is most likely located in the cytosol. Similar results have been reported for

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**Note:** The text above is a natural representation of the document content, ensuring coherence and readability. The diagram is included as a visual aid to complement the textual explanation. The image contains a topological model of the LysP protein, illustrating 12 membrane-spanning α-helices and their respective loops. The N- and C-termini are suggested to be cytosolic, with specific residues marked for acid (■) and basic (◉) content.
malF::pboA fusions in the C2 cytosolic loop and may relate to the lack of positive charges between the membrane-spanning region and the fusion junction (Boyd & Beckwith, 1989.)

Comparison with homologues is also instructive. A number of related amino acid permeases have been identified (Steffes et al., 1992). In the bacterial homologues PheP (phenylalanine permease), TyrP (tyrosine permease) and AroP (aromatic amino acid permease), the third transmembrane helix has been predicted to occur at a region homologous to residues 83–107 in LysP (Pi et al., 1993). From the data presented here that region is contained within the first cytosolic loop (C1) of LysP. It seems unlikely that it would be a membrane-spanning sequence in homologous proteins; we would predict a similar cytosolic location for those regions of the other bacterial permeases. A difference between the prokaryotic and eukaryotic members of this family is the presence of additional sequences in the latter (Steffes et al., 1992). Specifically, the proline permease of Saccharomyces cerevisiae (Vandenbol et al., 1989) contains an additional five residues in a location corresponding to residue 76 of the LysP protein. The proline permease of Aspergillus nidulans (Sophianopoulou & Sezzucchio, 1989) contains four additional residues in the region of residue 126 of the LysP protein.

The arginine, histidine and proline permease of S. cerevisiae (Hoffmann, 1985; Tanaka & Fink, 1985; Vandenbol et al., 1989) and the proline permease of A. nidulans each contain 8–10 additional residues in a location corresponding to residue 197 of the LysP protein. Finally, the proline permeases of S. cerevisiae and A. nidulans have three additional residues in a location corresponding to residue 272 of the LysP protein. Since all of the homologues have amino acid transport activity, those extra sequences are the equivalent of insertions in the lysine permease. Insertions can be accommodated in extramembranous loops but are unlikely to occur in membrane-spanning α-helices. These support the conclusion for the location of the corresponding regions of the lysine permease in loops C1, P2, P3 and P4, respectively.

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