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Overproduction of NlpE, a New Outer Membrane Lipoprotein, Suppresses the Toxicity of Periplasmic LacZ by Activation of the Cpx Signal Transduction Pathway

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The LamB-LacZ-PhoA tripartite fusion protein is secreted to the periplasm, where it exerts a toxicity of unknown origin during high-level synthesis in the presence of the inducer maltose, a phenotype referred to as maltose sensitivity. We selected multicopy suppressors of this toxicity that allow growth of the tripartite fusion strains in the presence of maltose. Mapping and subclone analysis of the suppressor locus identified a previously uncharacterized chromosomal region at 4.7 min that is responsible for suppression. DNA sequence analysis revealed a new gene with the potential to code for a protein of 236 amino acids with a predicted molecular mass of 25,829 Da. The gene product contains an amino-terminal signal sequence to direct the protein for secretion and a consensus lipoprotein modification sequence. As predicted from the sequence, the suppressor protein is labeled with [^{3}H]palmitate and is localized to the outer membrane. Accordingly, the gene has been named *nlpE* (for new lipoprotein E). Increased expression of NlpE suppresses the maltose sensitivity of tripartite fusion strains and also the extracytoplasmic toxicities conferred by a mutant outer membrane protein, LamBA23D. Suppression occurs by activation of the Cpx two-component signal transduction pathway. This pathway controls the expression of the periplasmic protease DegP and other factors that can combat certain types of extracytoplasmic stress.

The analysis of protein secretion in the gram-negative bacterium *Escherichia coli* has been aided by gene fusion technology (2, 24). Hybrid proteins containing amino-terminal domains derived from secreted periplasmic or outer membrane proteins fused to an active LacZ (β-galactosidase) domain have proven useful for studying secretion. Fusion of MalE (periplasmic maltose-binding protein) or LamB (outer membrane maltoporin or λ receptor) to the normally cytoplasmic protein LacZ confers a toxicity upon host strains during conditions of high-level synthesis. Strains harboring these fusions lyse during growth in the presence of the inducer maltose, a phenotype referred to as maltose sensitivity (1, 26). Intragenic mutations that relieve the toxicity of these proteins alter the amino-terminal signal sequence and prevent entry into the secretory pathway (8). By using this approach, the essential features of the signal sequence were defined. These hybrid proteins also show abnormally low levels of LacZ activity under noninducing conditions. The low activity presumably results from secretion of the hybrid protein to the periplasm, where it is inactivated by intermolecular disulfide bond formation (27). Hence, signal sequence mutations increase LacZ activity by preventing secretion and subsequent inactivation. Additionally, extragenic mutations that impair secretion increase retention of the hybrid protein in the cytoplasm, resulting in increased LacZ activity. This approach identified several of the *sec* genes which encode components of the secretory machinery (2, 24).

New variants of the standard *lamB-lacZ* hybrid that bestow novel phenotypes were constructed (27). The maltose sensitivity conferred by the original LamB-LacZ hybrid protein is exerted in the cytoplasm and results from an inhibition of the secretion machinery during high-level synthesis of the hybrid protein. This consequence of LamB-LacZ overproduction is called hybrid protein jamming. Truncation of the LacZ domain by fusion of PhoA or by introduction of the late nonsense mutation X90 has created a new class of model proteins, LamB-LacZ-PhoA and LamB-LacZX90, that do not inhibit the secretion machinery at growth temperatures of 34°C or higher. Even though these proteins do not jam, they impart a maltose sensitivity that is indistinguishable from that of LamB-LacZ. This novel maltose sensitivity results from secretion of the X90 and tripartite fusion proteins to the periplasm, where they form disulfide-bonded aggregates.

Another novel envelope associated toxicity is conferred by a mutant outer membrane protein, LamBA23D, that contains a defect in the signal sequence cleavage site (4). The precursor form of LamBA23D is poorly processed by leader peptidase (LepA) and accumulates in the bacterial envelope. The accumulation of unprocessed LamBA23D creates a permeability defect as evidenced by increased sensitivity to detergent (4) and the antibiotic amikacin (5a). The cause of this toxicity may be related to targeting of the mutant precursor to the outer membrane, but it is not known how this leads to manifestation of the observed defects.

To learn more about the nature of the extracytoplasmic toxicities of LamBA23D, LamB-LacZX90, and LamB-LacZ-PhoA, suppressors of their toxicities were selected. A class of suppressors that combats the toxic effects of all these proteins was discovered. These suppressor mutations encode activated alleles of the previously characterized gene *cpxA* and consequently have been referred to as *cpxA** (18). CpxA and CpxR form the sensor and response regulator components of the well-characterized family of bacterial two-component regulatory systems (7, 32). These systems utilize an inner membrane protein to sense an environmental parameter and communi-

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cate the information via a kinase/phosphatase activity to a response regulator (29). The response regulator is often a DNA-binding protein that can elicit an alteration in transcription. *cpxA** alleles function in part by increasing the expression of *degP* (6), the gene specifying a major periplasmic protease (17, 30). Increased synthesis of DegP and another unknown factor(s) renders *cpxA** strains resistant to high-level synthesis of the toxic envelope proteins (5a). Accordingly, CpxA is thought to control the expression of genes that can combat extracytoplasmic stress.

Here we describe the isolation of a previously uncharacterized gene which can confer maltose resistance to *lamB-lacZ-phoA* strains when it is expressed from a high-copy-number plasmid. Reminiscent of *cpxA** suppressors, high-level expression of this gene can also combat the toxicities exhibited by *lamBA23D* strains. These results provide new information about the functioning of the Cpx signal transduction pathway.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *E. coli* K-12 strains used in this study are all derivatives of strain MC4100 (5). The parent of tripartite fusion strains is WBS1106 {MC4100 Δ*phoA532* Φ(*lamB-lacZ-phoA*) Hyb1-1 [λp1(209)]} (27). The parent of *lamBA23D* strains is JHC285 (4). The *cpxR*::*spc* allele (6) and the *degP*::Tn*10* allele (laboratory collection) were transduced into different backgrounds by P1. Strain DH5α was used as a host for plasmids during cloning procedures (23). The *drpA* plasmid, pKLF2, is described elsewhere (33). The plasmid library used in this study was obtained from Susan Gottesman and contains *Sau*3A partially digested chromosomal DNA from a MC4100Δ*lon* strain cloned at the *Bam*HI site of pBR322. Plasmids pLD130 and pLD111 are two of the identical maltose-resistant suppressor plasmids isolated from the library. Six other suppressor plasmids were analyzed, but all of them appeared identical to pLD130 and pLD111 following restriction analysis. pND18, which contains *nlpE* cloned under the control of the arabinose promoter in the vector pBAD18, is described elsewhere (6).

Media and chemicals. All growth media used have been described previously (25) with the exception of glycerol minimal medium, which contained 0.4% glycerol and 0.5% Luria broth. We purchased [^{3}H]palmitate (1 mCi/0.2 ml, 55 Ci/mM) from NEN Research Products, Du Pont Co., Boston, Mass., and Kodak XAR film from Eastman Kodak, Rochester, N.Y. 5-Bromo-4-chloro-3-indolylphosphate (X-Phos) was purchased from Calbiochem, LaJolla, Calif. The *E. coli* gene-mapping membrane was purchased from TaKaRa Biomedical, Shiga, Japan. Difco Laboratories, Detroit, Mich., and BBL, Cockeysville, Md., supplied antibiotic disks for sensitivity tests. Reagents for enhanced chemiluminescence detection, random-prime labeling, and DNA hybridization were obtained from Amersham, Arlington Heights, Ill. All DNA restriction enzymes, β-agarase I, T4 DNA ligase, and Klenow polymerase were purchased from New England Biolabs, Beverly, Mass. Reagents for sequence analysis were obtained from United States Biochemical, Cleveland, Ohio.

DNA techniques. Competent cells were prepared for plasmid transformation by standard techniques. Sequence analysis of pLD404 was performed with the *Eco*RI-*Sty*I DNA fragment from pLD404. Purification and sequencing of this fragment were performed as described previously for PCR products (20, 22). Oligonucleotide primers were obtained from the Princeton University Syn/Seq Facility for sequencing and PCR amplification of DNA. For mapping analysis, a randomly primed DNA probe of pLD404 was prepared, hybridized, washed, and detected on the gene-mapping membrane by enhanced chemiluminescence as described by the supplier of these reagents. PCR amplification of DNA from a single bacterial colony has been described previously (22). Subclones of pLD130 were created by digestion with the restriction enzyme whose sites defined the boundaries of the region to be deleted. The plasmid was then ligated by standard techniques to create the deletions.

Construction of the *nlpE* null mutation. 5′ and 3′ regions of *nlpE* were amplified by PCR with primers containing restriction sites for cloning into a vector with a temperature-sensitive replication origin (pMAK705) (12). The primers for amplification of the 5′ region were NLPEF (5′GGAATTCCGAC GACCCACGC3′) and DNLP1 (5′CGGATCCAGCTTGTCAGCGG3′). The primers for amplification of the 3′ region were DRPABAK (5′CCGCGTCTT CAGCACTTCC3′) and DNLP2 (5′CGGATCCCGATACGGCAGGG3′). pMAK705 was partially cut with *Eco*RI and then cut to completion with *Bam*HI. The large plasmid from this digest was gel purified as described previously (20) and ethanol precipitated by standard techniques, as were all DNA fragments prior to ligation. DNA amplified from the 5′ region of *nlpE* was cut with *Eco*RI and *Bam*HI and purified. These fragments were then ligated under standard conditions and transformed into DH5α at 30°C. This construct, pBS6, and DNA amplified from the 3′ region were cut with *Bam*HI and *Hin*dIII, purified, ligated, and transformed. This construct, pBS7, and the *spc* interposon-containing plasmid, pHP45Ω (9), were cut with *Bam*HI, and the appropriate fragments were purified, ligated, and transformed. This final construct, pBS8, contains the polar *spc* interposon flanked by *nlpE* DNA. This plasmid was transformed into MC4100 at 42°C by selecting resistance to spectinomycin to ensure cointegrate formation. The transformants were purified three times at 42°C and screened for cold resistance and ampicillin sensitivity to confirm resolution of the cointegrate. The chromosomal deletion/insertion at *nlpE* was confirmed by PCR amplification and restriction enzyme analysis. P1-mediated generalized transduction was used to move this allele into different genetic backgrounds.

Disk sensitivity assays. Strains for maltose sensitivity tests were grown to saturation in glycerol minimal medium containing ampicillin (50 μg/ml), pelleted, and resuspended in 1/2 volume of M63. Maltose sensitivity was measured against 10 μl of maltose solution (concentrations are given in Tables 1 and 2) on a filter paper disk atop a lawn of the test strain (100 μl) in F-top agar (3 ml) on glycerol minimal medium plates. The antibiotic and detergent sensitivities of the *nlpE*::*spc* and isogenic *nlpE*$^+$ strains were compared on Luria broth agar with lawns of cells (100 μl) in Luria broth top agar (3 ml). Strains for this test were grown to saturation in Luria broth and added directly to the top agar. The following drug-containing disks (Difco and BBL) and detergents (10 μl per disk) were used for this analysis: chloramphenicol, kanamycin, gentamicin, rifampin, sulfathiazole, moxalactam, penicillin G, nafcillin, oxytetracycline, bacitracin, novobiocin, neomycin, cephalothin, clindamycin, chlorotetracycline, tetracycline, trimethoprin, amikacin, polymyxin B, cloxacillin, naladixic acid, 10% sodium dodecyl sulfate (SDS), and 20% deoxycholate. All assays were performed at 37°C. Results are expressed as the diameter of growth inhibition after subtraction of the diameter of the filter paper disk in millimeters. Standard concentrations of antibiotics were included in the top agar to maintain plasmids when appropriate.

Palmitate labeling. Overnight cultures were diluted 1:50 into 50 ml of glycerol minimal medium containing ampicillin and grown at 37°C to an A_{600} of approximately 0.2 to 0.3. Arabinose was then added to a final concentration of 0.4%. The cultures were incubated for 15 min at 37°C, 250 μCi of [^{3}H]palmitate (50 μl) was added, and the cultures were grown for a further 3 h at 37°C. The cultures were then collected on ice.

Isolation and fractionation of total membranes. A 50-ml portion of either palmitate-labeled or unlabeled cells was pelleted, resuspended in 25 ml of 50 mM Tris (pH 7.5) (buffer), repelleted, and suspended in 2 ml of buffer with the following additives: 2 μl of RNase I (2 mg/ml), 2 μl of DNase I (1 mg/ml), 2 μl of leupeptin (5 mg/ml), 4 μl of aprotinin (10 mg/ml), 1 μl of pepstatin A (1 M), and 20 μl of phenylmethylsulfonyl fluoride (100 mM in ethanol) (all from Sigma). The cells were lysed by two passages through a French pressure cell press (diameter, 3/8-in. [0.95 cm]) at 15,000 lb/in^2. Samples were centrifuged at 5,000 rpm for 15 min in a Beckman SS34 rotor to pellet unlysed cells and debris. The supernatant was centrifuged in a TLA100.2 rotor in a Beckman Optima TL ultracentrifuge at 100,000 rpm for 20 min. The supernatant was saved as a soluble fraction; the pellet made up the total membrane fraction. The membranes were resuspended in 100 μl of buffer and loaded onto a sucrose step gradient consisting of 0.3 ml of 70% and 0.75 ml of 53% sucrose–50 mM Tris (pH 7.5). The gradients were centrifuged at 100,000 rpm for 65 min in the TLA100.2 rotor with the acceleration and deceleration set to 5. We observed bands of inner (upper) and outer (lower) membrane in the gradient following centrifugation and collected them as 100-μl fractions. All manipulations were performed on ice and at 4°C whenever possible.

SDS-polyacrylamide gel electrophoresis (PAGE) and autoradiography. Previously described methods were used (28). The volume of loading buffer added per milliliter of pelleted cells was calculated by the following formula: volume (milliliters) = A_{600}/5. Samples prepared following isolation of membrane fractions were resuspended in loading buffer to approximate the dilution obtained by the above formula. The inner and outer membrane samples obtained from the fractionation procedure were resuspended in an equal volume of loading buffer that contained no glycerol.

Nucleotide sequence accession number. The nucleotide and deduced amino acid sequences of *nlpE* have been deposited in GenBank under accession number U18345.

RESULTS

Rationale. High-level synthesis of LamB-LacZ-PhoA during growth in the presence of the inducer maltose exerts a periplasmic toxicity that results in cell lysis. This protein forms high-molecular-weight aggregates in the periplasm of dying cells, but the precise cause of toxicity remains a mystery. Perhaps periplasmic LacZ titrates some essential periplasmic function(s). Expression of the gene encoding this titrated function from a multicopy plasmid might overcome lethality by providing an excess of the inactivated function. Alternatively, proteins that can combat the toxic effects of this periplasmic protein could be identified in the same manner. Therefore, multicopy suppression of the maltose-sensitive *lamB-lacZ-*

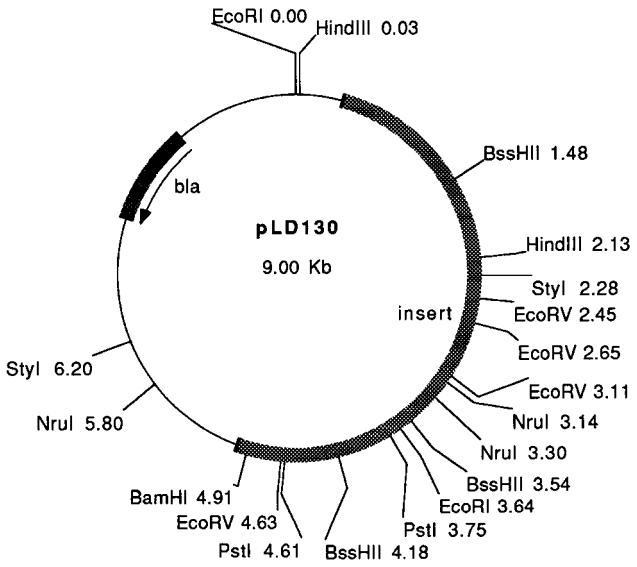

FIG. 1. Restriction map of pLD130. Important restriction sites of the genomic insert (shaded band) and plasmid backbone are shown. Positions of sites are approximate.

phoA strain should provide clues about the nature of this toxicity or how cells cope with periplasmic stress.

Selection and identification of a suppressor plasmid. We performed multicopy suppression with a library of cloned chromosomal DNA. This library (*Sau*3A partially digested chromosomal DNA cloned into the *Bam*HI site of pBR322) was transformed into the maltose-sensitive *lamB-lacZ-phoA* strain, WBS1106. Following phenotypic expression, the cells were plated directly onto maltose minimal agar containing ampicillin and X-Phos, a chromogenic indicator of alkaline phosphatase (PhoA) activity. After 2 days of incubation at 37°C, lawns of transformants grew on this agar because of the ability of the strain to initially grow on maltose medium. Replica printing of these lawns onto maltose minimal agar containing ampicillin and X-Phos provided additional selection for maltose-resistant mutants. Mutations that prevent synthesis or secretion of LamB-LacZ-PhoA appeared white on the indicator medium, thus providing an easy way to avoid this class of maltose-resistant suppressors. Following purification of 91 maltose-resistant and PhoA$^+$ isolates, plasmid DNA was prepared and transformed into WBS1106 to confirm the plasmid linkage of the suppressor. A total of 76 plasmid-borne suppressors were identified in this manner. Approximately 1 in 2,400 transformants contained a suppressor plasmid.

Eight plasmids that conferred maltose resistance were subjected to restriction enzyme analysis to determine if they had common restriction fragments. All eight plasmids examined in this manner contained the same restriction fragments (data not shown). Further restriction analysis of one of these plasmids (pLD130) was performed, and a map showing the approximate location of relevant restriction sites is shown in Fig. 1.

Mapping. We determined the physical map location of the cloned DNA from a suppressor plasmid by DNA hybridization techniques. A gene-mapping membrane containing DNA from the overlapping, ordered clones of the Kohara phage library is available commercially. Randomly primed labeled DNA was created from a suppressor plasmid and hybridized to the gene-mapping membrane (see Materials and Methods). Plasmid-derived DNA hybridized to Kohara phages 122 and 123 (14), localizing the suppressor gene at 4.7 to 4.9 min on the chromosome (21). The similarity between restriction sites of the plasmid insert and those reported for this genomic region (21) confirmed the map location.

These mapping data suggested that our suppressor plasmid, pLD130, probably contained the previously characterized *drpA* gene (33). A *drpA* temperature-sensitive mutant and the original *drpA* plasmid were obtained to determine if this gene was responsible for suppression. Unlike the suppressor plasmid, the original *drpA* plasmid, pKLF2, does not contain any DNA extending to the left of the *Hin*dIII (H) site (Fig. 2A). The temperature sensitivity of the *drpA* strain was complemented by pLD130 as well as pKLF2. However, pKLF2 did not confer maltose resistance; consequently, this provides evidence that the region to the left of the *Hin*dIII site is responsible for suppression by pLD130 (Fig. 2A).

To demonstrate directly that the region downstream of *drpA*, to the left of the *Hin*dIII site, is responsible for suppression, we created subclones of pLD130. Subclones were created

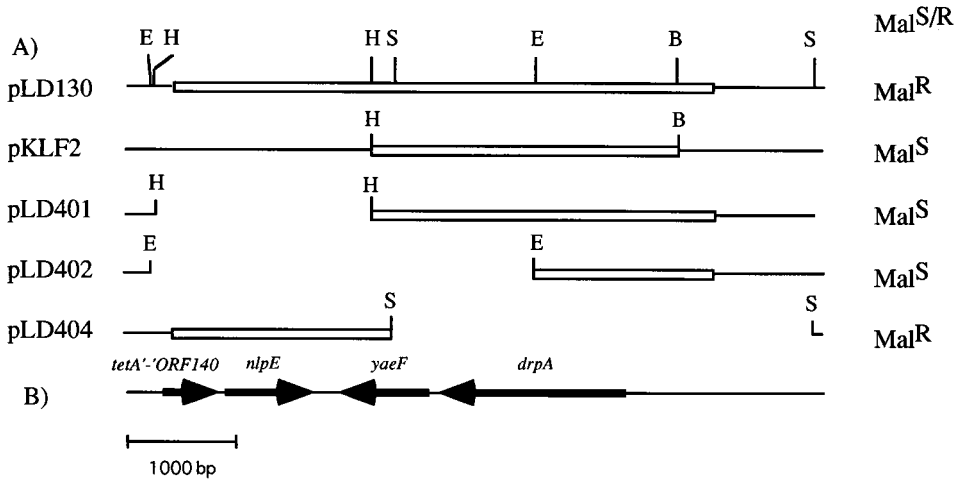

FIG. 2. Restriction map and suppressor activity of plasmid clones. (A) The subclones pLD401, pLD402, and pLD404 were created by removal of the DNA represented by the gaps from pLD130 and religating. pKLF2 is the original *drpA* clone (33). Abbreviations of restriction enzyme sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; S, *Sty*I. The effect on maltose sensitivity was determined in WBS1106. Mal^R, no growth inhibition was observed in the disk assay (see Materials and Methods); Mal^S, a wild-type zone of growth inhibition was observed. (B) For clarity, the approximate positions of genes in this chromosomal region are shown (see the text).

by deleting parts of the genomic insert (see Materials and Methods), and the ability of each to confer maltose resistance was assayed (Fig. 2A). One subclone, pLD404, containing approximately 1,800 bp of DNA downstream of *drpA*, conferred the suppressor phenotype and was subjected to DNA sequence analysis. This region contains the complete coding sequence for a new gene that we are calling *nlpE* and two other partial open reading frames (ORFs) described below (Fig. 2B).

Sequence analysis of the suppressor region. Both strands of DNA from the insert region of pLD404 were sequenced by the dideoxy method (Fig. 3A). The sequence of the insert contains three significant ORFs that could potentially encode a gene product responsible for suppression (Fig. 3). First, an ORF downstream of the *drpA* gene spans the *Hin*dIII and *Sty*I restriction sites (Fig. 3, *'yaeF* [ORF292]). The 5′ region of this ORF is found on the *drpA* sequence (33) (Fig. 2); therefore, the complete gene is not present in our suppressor plasmid. It is therefore likely that one of the other ORFs mediates suppression. A complete ORF of 236 amino acids (*nlpE*, see below) is located downstream of a partial ORF of 124 amino acids at the other end of the genomic insert. In the accompanying paper by Gupta et al., the partial ORF is reported to be composed of 140 amino acids and as such will be called ORF140 (11). The proximity of the stop codon of ORF140 and the translational start site of *nlpE*, as well as the lack of any obvious promoter in this region, suggest that the two genes are normally found in an operon. The 5′ end of this operon is absent in the suppressor plasmid, but ORF140 is situated such that the tetracycline resistance protein (TetA) from pBR322 is fused to it in frame (Fig. 2 and 3B). This *tetA* hybrid gene is transcribed constitutively from the *tetA* promoter (3, 31), thus providing transcription of *nlpE.*

Since there was only one complete ORF present on our plasmid, *nlpE*, we focused our attention on this gene. Close examination of the translated protein sequence revealed several interesting features (10). First, the protein appeared to contain an amino-terminal signal sequence to direct it for secretion from the cytoplasm through the general secretory pathway. Second, the protein contains a lipoprotein modification sequence of LMGC, at the carboxy-terminal end of the presumed signal sequence. This sequence defines the cleavage site for signal peptidase II (*lsp*); cleavage occurs between G and C. This cysteine is modified with a diacylglyceryl moiety prior to the cleavage of lipoprotein precursors by signal peptidase II, thus resulting in a mature protein with diacylglyceryl-cysteine at the amino terminus (13). Such cleavage products undergo further posttranslational modification at the amino terminus, i.e., N-acylation (13). If these sequences in NlpE are functional, the protein will be secreted to the envelope with incorporated palmitate. The toxicity of LamB-LacZ-PhoA is exerted in the envelope, the presumed location of NlpE. Accordingly, *nlpE* became the primary candidate for mediating suppression by pLD404.

Identification of the suppressor protein. To facilitate identification of the suppressor gene, we cloned *nlpE* into the arabinose-inducible expression vector pBAD18. The construction of this plasmid (pND18), which encodes *nlpE*, is described elsewhere (6). This plasmid confers maltose resistance to *lamB-lacZ-phoA*-containing strains, thus proving that increased expression of *nlpE* mediates suppression (Table 1). The amount of arabinose used to induce expression is critical to the ability of this plasmid to suppress. Maltose sensitivity is not suppressed during conditions of high-level expression (0.2% arabinose) or low-level synthesis (0% arabinose) (Table 1). The level of NlpE that is produced from our original suppressor plasmid (pLD130) was fortuitously appropriate for suppression.

To prove that the lipoprotein modification sequence of *nlpE* is functional, we labeled cells with [^{3}H]palmitate to see if pND18 overexpressed a palmitate-modified protein. Cells containing either pND18 or the control plasmid pBAD18 were labeled with [^{3}H]palmitate to specifically label cellular lipoproteins as described in Materials and Methods. Whole-cell, soluble, total-membrane, inner membrane, and outer membrane fractions from each strain reveal the subcellular distribution of the overexpressed protein (see Materials and Methods). Figure 4 shows that the pND18-containing cells overexpress a protein that incorporates [^{3}H]palmitate. This protein has an apparent molecular mass of 25,000 Da, which is in close agreement with the predicted molecular mass of 23,722 Da for the mature protein. Both the inner and outer membrane fractions contain this protein when overproduced, as well as several apparent degradation products. In contrast, a rare lipoprotein with the same apparent molecular weight as the overexpressed protein is found only in the outer membrane fraction of the control vector cells. These data suggest that the gene responsible for suppression encodes a rare outer membrane lipoprotein. We propose that this gene be named *nlpE*, for new lipoprotein E.

A mechanism of suppression. We have previously reported that NlpE overproduction provides a strong signal to induce the expression of *degP* and that induction requires functional *cpxA* and *cpxR* (6). Suppression of the maltose sensitivity of *lamB-lacZ-phoA* strains by NlpE overproduction absolutely requires *cpxR* (Table 2). CpxA and CpxR form the sensor and response regulator components of the well-characterized bacterial two-component regulatory systems (see Introduction). Therefore, NlpE multicopy suppression functions by activation of the Cpx two-component regulatory system.

Previous work from our laboratory has identified *cpxA** alleles as genetic suppressors of LamB-LacZ-PhoA- and LamBA 23D-mediated toxicities (see Introduction). These *cpxA** alleles completely abolish the extracytoplasmic toxicities conferred by these model proteins. With the tripartite fusion, these suppressors function by increasing the expression of the periplasmic protease DegP, which enhances the degradation of the toxic protein (5a). We thought it likely that overproduction of NlpE would function by a similar mechanism, since suppression is dependent on *cpxR*. Consistent with this hypothesis, NlpE production from a suppressor plasmid enhances the degradation of LamB-LacZ-PhoA (data not shown). As with *cpxA** suppression, enhanced degradation requires *degP*; however, considerable suppression of the maltose sensitivity of *lamB-lacZ-phoA* strains occurs in a *degP* null background (Table 2). This suggests the presence of other factors, besides DegP, that are regulated by the Cpx pathway to suppress maltose sensitivity.

We have also found that NlpE overproduction significantly decreases the SDS- and amikacin-sensitive phenotypes of *lamBA23D* strains. Moreover, this effect is *cpxR* dependent. NlpE therefore mediates suppression through the Cpx pathway by regulating the expression of factors that can combat these extracytoplasmic toxicities.

Induction of DegP does not always confer suppression. Overproduction of outer membrane proteins, as well as other insults to the bacterial envelope, is known to increase *degP* synthesis (6, 19). This induction of *degP*, however, does not function through the Cpx pathway (6). We tested the ability of overexpression of the outer membrane protein OmpF, which strongly induces *degP*, to suppress the maltose sensitivity of *lamB-lacZ-phoA* strains. We observed no differences in the

A

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Sau3A
1    GATCACCGCCATTCGTGCGCAGGGCGCGGGCGGGCAGCATGTTAATAAGACCTCAACGGCTATTCATCTGCGTTTTGACATTCGGGCGTCCAGCCTGCCA
     >I  T  A  I  R  A  Q  G  A  G  G  Q  H  V  N  K  T  S  T  A  I  H  L  R  F  D  I  R  A  S  S  L  P
      ORF140

101  GAGTATTACAAAGAGCGTCTGCTCGCCGCCAGCCATCATTTGATCAGCAGTGATGGCGTGATTGTCATTAAGGCACAGGAATACCGCAGTCAGGAACTGA
     E  Y  Y  K  E  R  L  L  A  A  S  H  H  L  I  S  S  D  G  V  I  V  I  K  A  Q  E  Y  R  S  Q  E  L  N

201  ACCGCGAAGCAGCTCTGGCCCGGCTGGTGGCTATGATTAAAGAATTAACAACAGAAAAAAAAGCCCGACGACCCACGCGGCCCACCCGTGCATCGAAAGA
       R  E  A  A  L  A  R  L  V  A  M  I  K  E  L  T  T  E  K  K  A  R  R  P  T  R  P  T  R  A  S  K  E

                                                                                          S.D.
301  GCGCAGGCTGGCATCGAAAGCACAAAAATCAAGCGTGAAGGCGATGCGCGGCAAAGTGCGCAGCGGTCGGGAATAAAAAGAAGGAATGGATGGTGAAAAA
       R  R  L  A  S  K  A  Q  K  S  S  V  K  A  M  R  G  K  V  R  S  G  R  E  *                 M  V  K  K
                                                                                                  nlpE

401  AGCGATAGTGACAGCGATGGCTGTAATCAGCCTCTTTACTCTGATGGGATGTAATAATCGGGCCGAAGTCGATACGCTTTCTCCGGCGCAGGCTGCCGAA
       A  I  V  T  A  M  A  V  I  S  L  F  T  L  M  G  C  N  N  R  A  E  V  D  T  L  S  P  A  Q  A  A  E

501  CTGAAACCGATGCCGCAAAGTTGGCGCGGCGTGCTGCCGTGTGCCGATTGCGAAGGAATCGAAACCTCTCTGTTCCTCGAAAAAGACGGAACATGGGTGA
       L  K  P  M  P  Q  S  W  R  G  V  L  P  C  A  D  C  E  G  I  E  T  S  L  F  L  E  K  D  G  T  W  V  M

601  TGAATGAGCGTTATCTCGGTGCTCGTGAAGAACCTTCCTCCTTCGCTTCCTACGGTACATGGGCGCGAACCGCTGACAAGCTGGTATTAACCGACAGCAA
       N  E  R  Y  L  G  A  R  E  E  P  S  S  F  A  S  Y  G  T  W  A  R  T  A  D  K  L  V  L  T  D  S  K

701  AGGTGAAAAGTCATATTATCGGGCGAAAGGCGATGCGCTGGAGATGCTCGATCGTGAAGGCAATCCGATTGAATCGCAGTTCAACTATACGCTGGAAGCG
       G  E  K  S  Y  Y  R  A  K  G  D  A  L  E  M  L  D  R  E  G  N  P  I  E  S  Q  F  N  Y  T  L  E  A
                                                SPIM

801  GCACAATCCAGTTTACCTATGACGCCGATGACCCTGCGGGGCATGTATTTTTATATGGCTGATGCGGCGACCTTCACTGATTGCGCGACCGGAAAACGTT
       A  Q  S  S  L  P  M  T  P  M  T  L  R  G  M  Y  F  Y  M  A  D  A  A  T  F  T  D  C  A  T  G  K  R  F

                                          BssHII
901  TCATGGTAGCGAATAACGCAGAGCTGGAGCGTAGCTACCTGGCTGCGCGCGGTCACAGTGAAAAACCGGTGTTACTGTCAGTAGAAGGTCACTTTACGCT
       M  V  A  N  N  A  E  L  E  R  S  Y  L  A  A  R  G  H  S  E  K  P  V  L  L  S  V  E  G  H  F  T  L

1001 TGAGGGTAATCCGGATACCGGTGCGCCGACTAAAGTATTGGCACCCGATACGGCAGGGAAATTTTACCCCAACCAGGATTGCAGTAGTTTGGGGCAGTAA
       E  G  N  P  D  T  G  A  P  T  K  V  L  A  P  D  T  A  G  K  F  Y  P  N  Q  D  C  S  S  L  G  Q  *

1101 CCCGTCTTGAGACAGAAACAAACGCAAAACAGGCCAGAAGGATATATTTCAACATTTTGAATTTGCACGTTTTTTGTAGGCCGGATAAGGCGTTTACGCC

1201 GCATCCGGCAATGGTGCTCAACGCCTGATGCGACGCTGGCGCGTCTTATCATGCCTACAACCCCCCTCATACCTTAAGGCGGATAAGGCATTTACTTTAT

1301 CACCGGGTCAAACCAACAAAGCGACCCGCCTTGATATAAATCCCAGGCTTCAGGTGCCCAACATACTGTAACTGCGTTTCTGGCTTAAACGCAGAGACAT
       *  R  T  L  G  V  F  R  G  A  K  I  Y  I  G  P  K  L  H  G  V  Y  Q  L  Q  T  E  P  K  F  A  S  V

1401 CACCAATACGCATATGCATCAGATCGGCAGGGCTAATCCAGCCCGATTGCGCCAGTGTAAGTGGATGCCCGGCTTTGGCAAAGGCATCCGTGACAAATTC
     D  G  I  R  M  H  M  L  D  A  P  S  I  W  G  S  Q  A  L  T  L  P  H  G  A  K  A  F  A  D  T  V  F  E

1501 CGAACAAAACCACGACTTTTTGTCTCCTTCGCCCACACTGCTTAACTGCGCTTTCGCCAGGCCGCTGACGCACTGTTGGCGAAAATCCTCGGAGAACGGA
       S  C  F  W  S  K  K  D  G  E  G  V  S  S  L  Q  A  K  A  L  G  S  V  C  Q  Q  R  F  D  E  S  F  P

1601 TTCAGTGAGCACATCTGGCGAGTCACCATAAAGGGAATAAATTCGACAATGCCGCGATAGTTATAACCGCTATCTTTGATTTTATTGGCAAACGCGGTGA
     N  L  S  C  M  Q  R  T  V  M  F  P  I  F  E  V  I  G  R  Y  N  Y  G  S  D  K  I  K  N  A  F  A  T

                                                HindIII
1701 TATCTGTGGCTTGTTGCGGGGTAAGATCCGGGACTCGTAAGACGAAAAGCTTATCACTATGCTTCATCGCTTTTTTAAGGGAAACGA
     I  D  T  A  Q  Q  P  T  L  D  P  V  R  L  V  F  L  K  D  S  H  K  M  A  K  K  L  S  V  <
                                                                                  yaeF (COMP.)
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B

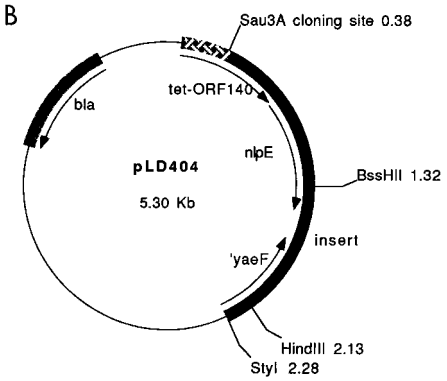

FIG. 3. DNA sequence and ORFs from the chromosomal region responsible for suppression. (A) The DNA sequence, ORFs, and other relevant features of the uncharacterized region from pLD404 (GenBank accession number, U18345). The coding strand of *nlpE* is shown. The complementary strand codes for *yaeF* (ORF292). SPIM marks the start of the serine protease inhibitor motif (10), and S.D. marks the position of the Shine-Dalgarno ribosome-binding site. The underlined amino acids mark the lipoprotein modification sequence. (B) Illustration of the orientation of the ORFs with respect to plasmid-encoded genes. All positions are approximate.

TABLE 1. NlpE confers maltose resistance to *lamB-lacZ-phoA* strains[a]

Plasmid and arabinose concn (%) in culture[b]	Diam of growth inhibition (mm) in following arabinose concn (%) in top agar[c]:		
	0	0.05	0.2
pND18			
0	9	10	10
0.05	8	0	8[d]
0.2	9	14	11
pBAD18			
0	ND[e]	ND	ND
0.05	11	10	8
0.2	11	9	9

[a] Sensitivity was quantitated as described in Materials and Methods for 2% maltose. Results from a typical experiment are shown.
[b] Overnight cultures of the test strains were grown in the given concentration of arabinose.
[c] Test strains were plated in F-top agar containing the given concentration of arabinose.
[d] A hazy zone of growth inhibition was observed; all other zones were clear.
[e] ND, not determined.

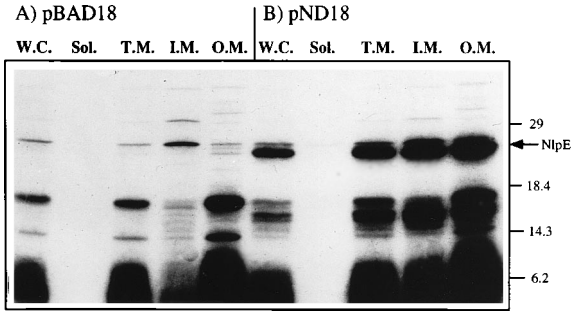

FIG. 4. Palmitate labeling of NlpE. (A) Cellular fractions from cultures transformed with the parental plasmid pBAD18. (B) Cellular fractions from cultures transformed with a *nlpE*-producing plasmid, pND18. Whole-cell (W.C.), soluble cytoplasmic and periplasmic contents (Sol.), total-membrane (T.M.), inner membrane (I.M.), and outer membrane (O.M.) fractions were created from [^{3}H]palmitate-labeled cultures and subjected to SDS-PAGE (see Materials and Methods).

diameters of growth inhibition by maltose for a *lamB-lacZ-phoA* fusion strain transformed with the OmpF plasmid or a control vector. These results demonstrate that the induction of *degP* by this mechanism is insufficient to combat the toxicity of periplasmic LacZ.

Characterization of an *nlpE* null mutant. Because NlpE-mediated suppression works through the Cpx signal transduction pathway, NlpE may normally function in this pathway. To test the normal, wild-type function of NlpE, we created a null mutation in the gene. A disrupted copy of *nlpE* was constructed on a plasmid that could be integrated into the chromosome at *nlpE*. Resolution of this cointegrate leaves the disrupted copy of *nlpE* in the chromosome (see Materials and Methods). The resulting *nlpE*::*spc* allele has the potential to code for the first 98 amino acids of the NlpE precursor protein. However, the deletion/substitution caused by the spectinomycin interposon (9) removes a significant portion of the coding sequence. This disruption has been confirmed by PCR analysis and presumably creates a null allele of *nlpE.*

Characterization of the *nlpE*::*spc* mutant strain has not revealed any remarkable phenotypes. The *nlpE*::*spc* mutation does not confer noticeable growth defects during growth on all media tested at any temperature (23 to 44°C). The maltose sensitivity of *lamB-lacZ-phoA* strains is not affected by the *nlpE* null mutation. No differences were observed in the sensitivities of the *nlpE*::*spc* strain to 21 different antibiotics, SDS, and deoxycholate (see Materials and Methods), thus providing evidence that the mutation does not alter the permeability properties of cells. We prepared whole-cell, soluble, total-membrane, inner membrane, and outer membrane fractions from the isogenic *nlpE*::*spc* and *nlpE*$^+$ strains and found no differences following SDS-PAGE of these samples (data not shown). Also, we observed no effect of the *nlpE*::*spc* insertion on the induction of *degP* in *cpxA** mutant backgrounds or with other treatments that are known to induce *degP*, such as outer membrane protein overproduction (data not shown). This analysis does not rule out the possibility that NlpE functions to stimulate the Cpx pathway under certain conditions. However, since no conditions other than NlpE overproduction are known to stimulate the Cpx pathway, tests of epistasis are impossible at this time. The accompanying paper by Gupta et al. suggests a role for this newly identified lipoprotein in copper transport and homeostasis (11).

DISCUSSION

The LamB-LacZ-PhoA fusion protein is secreted efficiently to the periplasm, where it exerts a pronounced cellular toxicity (27). We suspect that in this location, LacZ titrates some essential periplasmic function(s). To identify genes whose products can combat this toxicity when expressed at elevated levels, we used the technique of multicopy suppression. Using this method, we have identified a new gene, *nlpE*, that maps to 4.7 min and encodes a rare 24-kDa outer membrane lipoprotein. We have shown that NlpE overproduction causes suppression by activating the Cpx two-component signal transduction pathway. This regulatory system contains the membrane receptor kinase CpxA and the response regulator CpxR (see Introduction). *degP* is one of the known downstream targets in the Cpx pathway (6), and tests of epistasis confirm that all of these gene products are important for suppression. Increased levels of this periplasmic protease enhance the degradation of the LamB-LacZ-PhoA fusion protein, thus preventing its accumulation to toxic levels. Mutations that activate the Cpx pathway (*cpxA**) suppress the toxicity associated with periplasmic LacZ by a similar mechanism (5a).

A mutation such as *lamBA23D*, which hinders the removal of the signal sequence from an outer membrane precursor protein, confers growth defects as well (4). In this case, we

TABLE 2. NlpE-mediated suppression is dependent on *cpxR* and *degP*[a]

Relevant genotype	Diam of growth inhibition (mm) in:	
	2% maltose	10% maltose
pLD404 (*nlpE*$^+$)		
lamB-lacZ-phoA	0	0
lamB-lacZ-phoA cpxR::*spc*	14	21
lamB-lacZ-phoA degP::Tn*10*	0	12
pBR322 (control)		
lamB-lacZ-phoA	9	16
lamB-lacZ-phoA cpxR::*spc*	11	19
lamB-lacZ-phoA degP::Tn*10*	10	17

[a] Sensitivity was quantitated as described in Materials and Methods for two different concentrations of maltose. Results from a typical experiment are shown.

suspect that the toxicity results from the aborted attempt by the cell to target the mutant precursor protein to the outer membrane prior to its release from the cytoplasmic membrane. The *cpxA** mutations suppress this growth defect (5a). As expected, overexpression of NlpE suppresses the defects of *lamBA23D* strains. We conclude from all of these results that high levels of NlpE activate the CpxA kinase. This, in turn, leads to increased levels of CpxR-phosphate, which activates *degP* transcription.

When expressed at high levels, a fraction of the NlpE molecules may fail to fold and/or assemble in the outer membrane correctly. Indeed, the fractionation experiments shown in Fig. 4 support this view. Accordingly, we have considered the possibility that CpxA senses overexpressed NlpE as "junk." We think this explanation unlikely because overexpression of other outer membrane proteins, even other lipoproteins, does not cause Cpx-mediated *degP* induction (6), nor does it cause suppression. This suggests a normal interaction between NlpE and CpxA. This interaction could be direct; alternatively, NlpE may perform some function that CpxA monitors.

To address the critical question of NlpE function, we have constructed a chromosomal *nlpE* insertion mutation. Although some *nlpE* sequences remain in this construct, we think it likely that the mutation abolishes NlpE function. Careful examination of the *nlpE* mutant strain did not reveal any useful phenotypes. In the accompanying paper, Gupta et al. report that our *nlpE* null mutant exhibits an increased sensitivity to copper, and sequence analysis reveals a potential heavy-metal binding site in NlpE (11). These data could indicate a role for NlpE in copper homeostasis. Alternatively, it may be that the copper sensitivity observed in the *nlpE* mutant reflects the inability of the cell to mount an appropriate stress response.

NlpE may function to sense outer membrane stress and communicate this information directly to CpxA. Such a model is attractive because it seems likely that cells have a mechanism to monitor outer membrane composition. However, a direct test of this model requires a more complete understanding of the natural signals that activate the Cpx pathway. At present, the only known stimulus is overproduction of NlpE, and, obviously, this signal cannot be used to test the involvement of NlpE.

Examination of the NlpE sequence reveals a serine protease inhibitor motif starting at amino acid 99 of the mature protein (Fig. 4A). Although the location of this motif in NlpE is somewhat unusual (15), it is tempting to speculate that it may be important. It is possible that NlpE functions normally to regulate the activity of DegP and other periplasmic serine proteases (16). This model is especially attractive because it offers a plausible explanation for the dosage dependence of NlpE for suppression of LamB-LacZ-PhoA. This observation is paradoxical, because high levels of NlpE induce DegP synthesis (6) but no suppression is observed (Table 1). At moderate levels of NlpE overexpression, increased amounts of DegP produced by Cpx-mediated induction are sufficient to degrade the toxic fusion protein. However, high levels of NlpE may specifically interfere with DegP activity and/or the activity of the other factors (see below) that contribute to suppression. Tests of this model require a more careful examination of this motif.

An alternative explanation for the dosage dependence of NlpE for suppression posits that very high levels of NlpE simply saturate the enzymatic activity of DegP. This nonspecific saturation could also explain why overexpression of other outer membrane proteins, which induce *degP* by a different mechanism, fails to suppress. Here again, DegP may be effectively titrated by the high levels of outer membrane protein.

We do not at present understand why induction of *degP* by the Cpx system allows suppression of LamB-LacZ-PhoA but similar *degP* induction by overexpression of *ompF* does not. Here again, it is possible that high levels of outer membrane proteins saturate the enzymatic activity of DegP. Perhaps the cell cannot deal effectively with the stress caused by overproducing both an outer membrane protein and LamB-LacZ-PhoA. On the other hand, since *degP* is controlled by two different signaling pathways (6), it may be that the two regulons contain distinct but overlapping sets of genes. Other genes in the Cpx regulon may contribute substantially to suppression.

As noted above, tests of epistasis demonstrate the importance of DegP for Cpx-mediated suppression of LamB-LacZ-PhoA. However, we think it likely that the Cpx two-component system controls expression of other important genes as well. Significant suppression by NlpE is still observed in strains that lack DegP but not in strains that lack CpxA/R (Table 2). In suppressor strains that lack DegP, the LamB-LacZ-PhoA fusion protein is stabilized but is less toxic than are comparable levels of fusion protein in wild-type strains. This strongly suggests that the Cpx pathway regulates the synthesis of some other factors that can combat this periplasmic toxicity by a mechanism that does not involve degradation. Identification of these genes and their products may provide important information regarding this periplasmic stress response and, perhaps, the mechanisms employed to fold and target noncytoplasmic proteins in general.

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REFERENCES

1. **Bassford, P. J., T. J. Silhavy, and J. R. Beckwith.** 1979. Use of gene fusions to study secretion of maltose-binding protein into *Escherichia coli* periplasm. J. Bacteriol. **139:**19–31.
2. **Bieker, K. L., G. J. Phillips, and T. J. Silhavy.** 1990. The *sec* and *prl* genes of *Escherichia coli*. J. Bioenerg. Biomembr. **22:**291–310.
3. **Bolivar, F., R. L. Rodriguez, P. J. Green, M. C. Betlach, H. L. Heyneker, H. W. Boyd, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
4. **Carlson, J. H., and T. J. Silhavy.** 1993. Signal sequence processing is required for the assembly of LamB trimers in the outer membrane of *Escherichia coli*. J. Bacteriol. **175:**3327–3334.
5. **Casadaban, M. J.** 1976. Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and mu. J. Mol. Biol. **104:**541–555.

5a. **Cosma, C. L., P. N. Danese, J. H. Carlson, T. J. Silhavy, and W. B. Snyder.** Activation of the Cpx signal transduction pathway of *Escherichia coli* suppresses the toxicity conferred by certain envelope associated stresses. Submitted for publication.

6. **Danese, P. N., W. B. Snyder, C. L. Cosma, L. J. B. Davis, and T. J. Silhavy.** 1995. The Cpx two-component signal transduction pathway of *Escherichia coli* regulates transcription of the gene specifying the stress-inducible periplasmic protease, DegP. Genes Dev. **9:**387–398.
7. **Dong, J., S. Iuchi, H.-S. Kwan, Z. Lu, and E. C. C. Lin.** 1993. The deduced amino-acid sequence of the cloned *cpxR* gene suggests the protein is the cognate regulator for the membrane sensor, CpxA, in a two-component signal transduction system of *Escherichia coli*. Gene **136:**227–230.
8. **Emr, S. D., and T. J. Silhavy.** 1980. Mutations affecting localization of an *Escherichia coli* outer membrane protein, the bacteriophage λ receptor. J. Mol. Biol. **141:**63–90.
9. **Fellay, R., J. Frey, and H. Krish.** 1987. Interposon mutagenesis of soil and water bacteria: a family of DNA fragments designed for in vitro insertional mutagenesis of Gram-negative bacteria. Gene **52:**147–154.

10. **Genetics Computer Group.** 1994. Motifs analysis. Program manual for the Wisconsin Package, version 8, September 1994. Genetics Computer Group, Madison, Wis.
11. **Gupta, S. D., B. T. O. Lee, J. Camakaris, and H. C. Wu.** 1995. Identification of *cutC* and *cutF* (*nlpE*) genes involved in copper tolerance in *Escherichia coli*. J. Bacteriol. **177:**4207–4215.
12. **Hamilton, C. M., M. Aldea, B. K. Washburn, P. Babitzke, and S. R. Kushner.** 1989. New method for generating deletions and gene replacements in *Escherichia coli*. J. Bacteriol. **171:**4617–4622.
13. **Hayashi, S., and H. C. Wu.** 1990. Lipoproteins in bacteria. J. Bioenerg. Biomembr. **22:**451–471.
14. **Kohara, Y., K. Akiyama, and K. Isono.** 1987. The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. Cell **50:**495–508.
15. **Laskowski, M., and I. Kato.** 1980. Protein inhibitors of proteinases. Annu. Rev. Biochem. **49:**593–626.
16. **Lazdunski, A. M.** 1989. Peptidases and proteases of *Escherichia coli* and *Salmonella typhimurium*. FEMS Microbiol. Rev. **63:**265–276.
17. **Lipinska, B., M. Zylicz, and C. Georgopoulos.** 1990. The HtrA (DegP) protein, essential for *Escherichia coli* survival at high temperatures, is an endopeptidase. J. Bacteriol. **172:**1791–1797.
18. **McEwen, J., and P. M. Silverman.** 1980. Chromosomal mutations of *Escherichia coli* K-12 that alter the expression of conjugative plasmid function. Proc. Natl. Acad. Sci. USA **77:**513–517.
19. **Mecsas, J., P. E. Rouviere, J. W. Erickson, T. J. Donohue, and C. A. Gross.** 1993. The activity of σ^E, an *Escherichia coli* heat-inducible σ-factor, is modulated by expression of outer membrane proteins. Genes Dev. **7:**2618–2628.
20. **Osborne, R. S., and T. J. Silhavy.** 1993. PrlA suppressor mutations cluster in regions corresponding to three distinct topological domains. EMBO J. **12:** 3391–3398.
21. **Rudd, K. E.** 1992. Alignment of *E. coli* DNA sequences to a revised, integrated genomic restriction map, p. 2.3–2.42. *In* J. Miller (ed.), A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Russo, F. D., J. M. Slauch, and T. J. Silhavy.** 1993. Mutations that affect separate functions of OmpR, the phosphorylated regulator of porin transcription in *Escherichia coli*. J. Mol. Biol. **231:**261–273.
23. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
24. **Schatz, P. J., and J. Beckwith.** 1990. Genetic analysis of protein export in *Escherichia coli*. Annu. Rev. Genet. **24:**215–248.
25. **Silhavy, T. J., M. L. Berman, and L. W. Enquist.** 1984. Experiments with gene fusions. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
26. **Silhavy, T. J., H. A. Shuman, J. Beckwith, and M. Schwartz.** 1977. Use of gene fusions to study outer membrane protein localization in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **74:**5411–5415.
27. **Snyder, W. B., and T. J. Silhavy.** 1995. β-Galactosidase is inactivated by intermolecular disulfide bonds and is toxic when secreted to the periplasm of *Escherichia coli*. J. Bacteriol. **177:**953–963.
28. **Stader, J., S. A. Benson, and T. J. Silhavy.** 1986. Kinetic analysis of *lamB* mutants suggests the signal sequence plays multiple roles in protein export. J. Biol. Chem. **261:**15075–15080.
29. **Stock, J. B., A. M. Stock, and J. M. Mottonen.** 1990. Signal transduction in bacteria. Nature (London) **344:**395–400.
30. **Strauch, K. L., K. Johnson, and J. Beckwith.** 1989. Characterization of *degP*, a gene required for proteolysis in the cell envelope and essential for growth of *Escherichia coli* at high temperature. J. Bacteriol. **171:**2689–2696.
31. **Unger, B., G. Klock, and W. Hillen.** 1984. Nucleotide sequence of the repressor gene of the RA1 tetracycline resistance determinant: structural and functional comparison with three related repressor genes. Nucleic Acids Res. **12:**7693–7703.
32. **Weber, R. F., and P. J. Silverman.** 1988. The Cpx proteins of Escherichia coli K-12: structure of the CpxA polypeptide as an inner membrane component. J. Mol. Biol. **203:**467–476.
33. **Zhou, Z., and M. Syvanen.** 1990. Identification and sequence of the *drpA* gene from *Escherichia coli*. J. Bacteriol. **172:**281–286.