JOURNAL OF BACTERIOLOGY, Apr. 1983, p. 375–382
0021-9193/83/040375-08$02.00/0

Vol. 154, No. 1

Synthesis of Outer Membrane Proteins in *cpxA cpxB* Mutants of *Escherichia coli* K-12

JOAN McEWEN, LIDIA SAMBUCETTI, AND PHILIP M. SILVERMAN*

Department of Molecular Biology, Albert Einstein College of Medicine, Bronx, New York 10461

Received 18 November 1982/Accepted 25 January 1983

Two major proteins, the murein lipoprotein and the OmpF matrix porin, are deficient in the outer membrane of *cpxA cpxB* mutants of *Escherichia coli* K-12. We present evidence that the *cpx* mutations prevent or retard the translocation of these proteins to the outer membrane. The mutations had no effect on the rate of lipoprotein synthesis. Mutant cells labeled for 5 min with radioactive arginine accumulated as much lipoprotein as otherwise isogenic $cpxA^+$ $cpxB^+$ cells. This lipoprotein accumulated as such; no material synthesized in mutant cells and reactive with antilipoprotein antibodies had the electrophoretic mobility of prolipoprotein. Hence, the initial stages of prolipoprotein insertion into the inner membrane leading to its cleavage to lipoprotein appeared normal. However, after a long labeling interval, mutant cells were deficient in free lipoprotein and lacked lipoprotein covalently bound to peptidoglycan, suggesting that little if any of the lipoprotein synthesized in mutant cells reaches the outer membrane. Immunoreactive OmpF protein could also be detected in extracts of mutant cells labeled for 5 min, but the amount that accumulated was severalfold less in mutant cells than in $cpxA^+$ $cpxB^+$ cells. Analysis of β-galactosidase synthesis from *ompF-lacZ* fusion genes showed this difference to be the result of a reduced rate of *ompF* transcription in mutant cells. Even so, little or none of the OmpF protein synthesized in mutant cells was incorporated into the outer membrane.

Mutations in *Escherichia coli* K-12 genes *cpxA* and *cpxB* together alter different cellular functions that otherwise have no relation to each other. Thus, mutant cells are defective in the expression of DNA donor activity and surface exclusion when they contain the conjugative plasmid F (16, 18) and are deficient in the synthesis of isoleucine and valine (17, 25). We suggested that the *cpxA* and *cpxB* mutations alter a structure that is itself required for diverse cellular functions, and we identified this structure as the cell envelope by showing that the *cpxA* and *cpxB* mutations selectively alter the protein composition of the inner and outer bacterial membranes (15).

A critical question is how the *cpx* mutations selectively affect cell envelope protein composition. If the mutations act on the cell envelope proteins themselves, rather than on the expression of cell envelope protein genes, their effects suggest that the *cpx* gene products are required to organize and sort certain envelope proteins, most likely in the envelope itself.

The TraJ protein, an outer membrane protein encoded by the F-plasmid, fails to accumulate in mutant cells, even though the *traJ* promoter and *traJ* mRNA translation initiation sequences remain active (23). These observations suggested that the *cpx* mutations affect the TraJ protein itself rather than its synthesis. However, as the TraJ protein is a quantitatively minor cell envelope component (1, 12, 14, 19), we have been unable to evaluate directly the effects of the *cpx* mutations on its synthesis and translocation to the outer membrane. We have identified acetohydroxyacid synthase I as the only enzyme required for isoleucine and valine synthesis whose function is affected by the *cpxA* and *cpxB* mutations (17, 25), but this enzyme is not itself an integral membrane protein and the effect of the *cpx* mutations on its function in the cell may be indirect (25).

Two quantitatively major outer membrane proteins, the murein lipoprotein and the OmpF matrix porin, are also deficient in mutant cells (15). The murein lipoprotein is the most abundant protein in *E. coli* by number of molecules per cell (3). It is synthesized as a larger precursor (prolipoprotein), which is covalently modified to yield lipoprotein itself (26, 30). The kinetics of prolipoprotein synthesis, modification, and translocation to the outer membrane suggest that the entire sequence normally occurs rapidly and, except for peptide bond formation, in the cell envelope itself (13). About one-third of the lipoprotein molecules in the outer membrane

are covalently linked to the underlying peptidoglycan (3, 8).

There is no evidence that prolipoprotein synthesis is actively regulated (21). The lipoprotein deficiency in the outer membrane of *cpxA cpxB* mutant cells therefore seemed more likely to be the result of a defect in the post-translational stages of prolipoprotein processing or lipoprotein translocation to the outer membrane. In this communication, we show that lipoprotein synthesis occurs normally in *cpxA cpxB* mutant cells. Our results suggest that these *cpx* mutations affect an early stage in the translocation of lipoprotein to the outer membrane. The effect of the *cpx* mutations on the OmpF protein is more complex because transcription of the *ompF* gene is reduced severalfold in mutant cells. Even so, OmpF protein that is synthesized in mutant cells fails to reach the outer membrane.

MATERIALS AND METHODS

Bacterial strains and media. All strains are derivatives of *E. coli* K-12. Hfr strains AE1031 (*cpxA*$^+$ *cpxB*$^+$) and AE1019 (*cpxA2 cpxB1*) have been described previously (16, 18), as have strains MC4100 (*ompF*$^+$), MH513 [ϕ(*ompF'-lacZ*$^+$)16-3], and MH621 [ϕ(*ompF-lacZ*)16-21(Hyb)] (5). The *cpxA2 cpxB1* derivatives of these strains were constructed in three steps. First, the *metB1* allele was introduced into each strain by P1 transduction. The P1 donor was *metB1 rpoB* (Rif^r). Rifampicin-resistant transductants were screened for methionine auxotrophy. Next, the *cpxB1* allele was introduced into each strain by P1 cotransduction with the *zeb-1*::Tn*10* insertion (18). Since the *cpxB1* mutation is cryptic in a *cpxA*$^+$ strain, several tetracycline-resistant transductants were used as recipients in a third P1 transduction with a lysate from a *metB*$^+$ *cpxA2* strain. Met$^+$ recombinants were selected and screened for Ilv$^-$ phenotype at 41°C, as previously described (18).

Unless otherwise indicated, cells were grown aerobically at 41°C, the nonpermissive temperature for the *cpxA2*(Ts) allele, in Vogel-Bonner minimal medium supplemented as appropriate for each strain. Cell growth was monitored by optical density at 660 nm.

Cell-labeling protocols. For 5-min [^{14}C]arginine labeling, strains AE1031 and AE1019 (both arginine auxotrophs) were grown to stationary phase at 34°C, diluted 40-fold in fresh medium, and grown for four generations at 41°C to an optical density of 0.4 to 0.5. The cells in 10 ml of each culture were collected by centrifugation, suspended in 10 ml of medium lacking arginine, and incubated for 5 min at 41°C to deplete internal arginine pools. The cells (8 ml) were then labeled for 5 min with L-[*U*-^{14}C]arginine (5 μCi/ml; 0.34 Ci/mmol). Incorporation was terminated by adding unlabeled arginine (final concentration, 300 μg/ml) and chilling the cultures rapidly in a solid CO_2-ethanol bath.

For 90-min [^{14}C]arginine labeling, strains AE1031 and AE1019 were grown as described above. After three generations of growth at 41°C (optical density, 0.2), the cells were transferred by centrifugation to minimal medium (8 ml) containing 3 μg of unlabeled arginine per ml and 5 μCi of L-[*U*-^{14}C]arginine (0.34 Ci/mmol) per ml; the final arginine concentration in the medium was 6.2 μg/ml. Incubation at 41°C was then continued for 90 min (about 1.5 generations).

Incorporation of radioactivity into acid-precipitable material was linear over the 5-min labeling interval for both strains and for AE1019 over the 90-min interval. Strain AE1031 stopped net incorporation between 60 and 90 min at 41°C.

AE1031 cells labeled for 90 min with [^{3}H]arginine were prepared exactly as described above for [^{14}C]arginine labeling, except that L-[5(n)-^{3}H]arginine (100 μCi/ml; 24 Ci/mmol) replaced [^{14}C]arginine, and unlabeled arginine was added to a final concentration of 7 μg/ml. The same volume (8 ml) of these labeled cells was added to each of the cultures labeled with [^{14}C]arginine before analysis.

For [^{35}S]methionine labeling, AE1031 and AE1019 were prepared as described above, except that cell growth was at 41°C throughout. At an optical density of 0.5, the cultures were transferred by centrifugation to minimal medium (5 ml) lacking methionine, incubated for 5 min at 41°C, and then labeled for 15 min by addition of 0.25 ml of a mixture containing unlabeled methionine (10 μg/ml) and [^{35}S]methionine (200 μCi/ml; 1,300 Ci/mmol). When appropriate, globomycin was added to AE1031 cells at a concentration of 250 μg/ml 5 min before addition of radioactive methionine. After labeling, cells were chilled in an ice bath and harvested by centrifugation at 4°C and 10,000 × *g* for 5 min.

Analysis of labeled cells. Crude cell envelopes from cells labeled with [^{35}S]methionine were isolated as described previously by Osborn et al. (22), washed once by centrifugation at 4°C with 2 ml of 1.5 mM EDTA (pH 7.5), suspended in 0.15 ml of solution containing 62 mM Tris-hydrochloride (pH 6.8), 1% (wt/vol) sodium dodecyl sulfate, and 5% (vol/vol) glycerol, and heated at 95°C for 20 min. A portion of each sample (20 μl) was diluted 10-fold with a buffer solution containing 50 mM Tris-hydrochloride (pH 8), 0.1 mM EDTA, 0.15 M NaCl, and 2% (vol/vol) Triton X-100. Rabbit antilipoprotein antiserum (60 μl) was added to each sample, and the samples were incubated at 37°C for 2 h and then at 4°C for 18 h after addition of 320 μg of goat anti-rabbit immunoglobulin G immunoglobulin. The immune precipitates were collected by a 5-min centrifugation in a microcentrifuge, washed four times, each time with 1 ml of the Triton buffer described above, and dissolved in 55 μl of electrophoresis sample buffer by heating at 95°C for 20 min. The proteins were separated by electrophoresis in the gel system described by Ito et al. (11). After electrophoresis, the gel was fixed in 20% trichloroacetic acid at 4°C for 30 min and then in 30% methanol–10% acetic acid–10% trichloroacetic acid at room temperature. The gel was stained and destained as described previously by Fairbanks et al. (4), treated with En^{3}Hance according to the manufacturer's instructions, dried in vacuo at 60°C, and exposed to Kodak X-Omat (XAR-5) film at −80°C for 7 days.

Cells labeled for 5 min with [^{14}C]arginine and mixed with cells labeled with [^{3}H]arginine were collected and washed once by centrifugation in the cold with 0.5 ml of 10 mM sodium phosphate buffer (pH 7.2) containing 0.9% NaCl. The cells were thoroughly suspended in phosphate buffer as above without NaCl and sonicated

at full energy with the microtip of a Kontes Ultramicrosonic Cell Disruptor until the suspension cleared. Each sample was then adjusted to 1% sodium dodecyl sulfate, 10% (vol/vol) glycerol, and 1% (vol/vol) 2-mercaptoethanol and heated at 70°C for 20 min. Matrix porin and lipoprotein were immunoprecipitated, without further fractionation, as described previously by Inouye et al. (9). Radioactive proteins were dissolved in 55 μl of electrophoresis sample buffer and separated by gel electrophoresis. When appropriate, the gels were dried without preparation for exposure of ^{3}H radioactivity and exposed to Kodak X-Omat (XAR-5) film for autoradiography. Otherwise, the wet gels were sliced into 1-mm segments and processed for liquid scintillation spectrometry (24).

Crude cell envelopes were isolated from cells labeled with [^{14}C]arginine for 90 min essentially as described previously by Osborn et al. (22), including treatment with 100 μg of lysozyme per ml for 30 min at 4°C before sonication. The crude cell envelope pellets were suspended in 175 μl of 10 mM sodium phosphate buffer (pH 7.2)–1% sodium dodecyl sulfate–10% glycerol–1% 2-mercaptoethanol and analyzed by immunoprecipitation, gel electrophoresis, and liquid scintillation spectrometry as described above.

Unless otherwise specified, gel electrophoresis was carried out as previously described (15).

Materials. Radioactive arginine and methionine were purchased from Amersham Corp., Arlington Heights, Ill. Globomycin and rabbit antisera prepared against cell envelope proteins from *E. coli* K-12 were obtained from M. Inouye. Goat anti-rabbit immunoglobulin G immunoglobulin was purchased from Cappel Laboratories, Downingtown, Pa. All other materials were obtained from standard commercial sources.

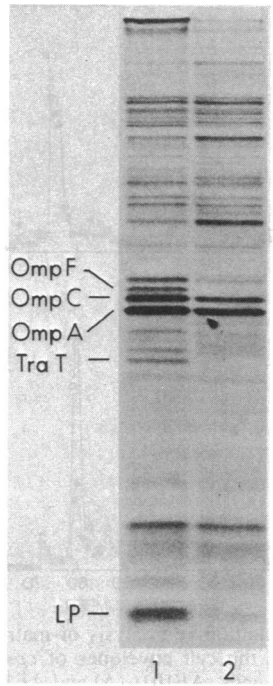

FIG. 1. Analysis of cell envelope proteins from strains AE1031 (*cpxA*$^{+}$ *cpxB*$^{+}$; lane 1) and AE1019 (*cpxA2 cpxB1*; lane 2). Both strains were labeled with [^{14}C]arginine for 90 min, cell envelope fractions were isolated, and radioactive cell envelope proteins were identified by gel electrophoresis and autoradiography. LP, Lipoprotein.

RESULTS

Quantitative analysis of lipoprotein and OmpF protein in *cpxA cpxB* mutant cell envelope. Strains AE1031 (*cpxA*$^{+}$ *cpxB*$^{+}$) and AE1019 (*cpxA2 cpxB1*) were labeled with [^{14}C]arginine for 90 min at 41°C. Each culture was then mixed with an equal volume of an AE1031 culture similarly labeled with [^{3}H]arginine, and cell envelope fractions were isolated, after brief lysozyme treatment, by sonication and differential centrifugation. Qualitative comparison of the ^{14}C-labeled cell envelope proteins by gel electrophoresis and autoradiography confirmed our previous observation that the mutant cell outer membrane is deficient in murein lipoprotein, the OmpF matrix porin, the F-plasmid *tra* gene products, and several other envelope proteins (15). The amounts of OmpC matrix porin and the OmpA protein were unaffected by the mutations (Fig. 1). For a quantitative analysis, porin proteins and murein lipoprotein were isolated by immunoprecipitation and separated by gel electrophoresis. The gels were sliced, and the amount of ^{3}H and ^{14}C radioactivity in the different slices was measured by liquid scintillation spectrometry (Fig. 2). The ^{3}H-labeled proteins, which served both as recovery and mobility standards, migrated to three regions of the gel. The most rapidly migrating component was the free form of murein lipoprotein. The material at the top of the resolving gel probably contains lipoprotein covalently bound to large peptidoglycan fragments (10, 29). The third region (M) contained the OmpF and OmpC matrix porins and a small amount of OmpA protein. These components were not resolved when the gels were sliced for measurement of radioactivity; they are indicated in Fig. 1, which shows total cell envelope proteins from cells labeled for 90 min, and in Fig. 5, which shows porin proteins immunoprecipitated from unfractionated extracts of cells labeled for 5 min.

As expected, the patterns of ^{14}C and ^{3}H radioactivity from the *cpxA*$^{+}$ *cpxB*$^{+}$ strain AE1031 were congruent (Fig. 2A). In contrast, the patterns from [^{3}H]arginine-labeled AE1031 and [^{14}C]arginine-labeled AE1019, a *cpxA2 cpxB1* double mutant, were different in all three regions of the gel (Fig. 2B).

In the region of the gel containing the matrix proteins, the entire peak of ^{14}C radioactivity shifted toward the right edge of the peak of ^{3}H radioactivity, reflecting the absence of [^{14}C]ar-

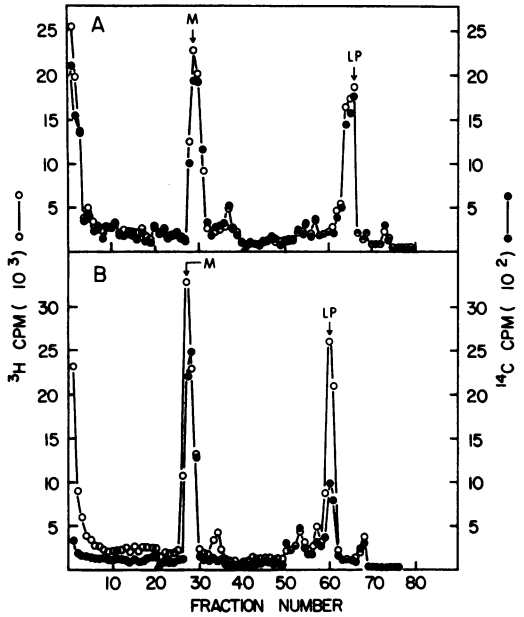

FIG. 2. Quantitative analysis of matrix porin and lipoprotein in the cell envelopes of *cpxA cpxB* and *cpxA*$^+$ *cpxB*$^+$ cells. AE1031 (A) and AE1019 (B) cells were labeled with [^{14}C]arginine as described in the legend to Fig. 1 and mixed with AE1031 cells labeled with [^{3}H]arginine. Preparation of cell envelopes with lysozyme, immunoprecipitation with an antiserum containing antibodies against lipoprotein (LP) and matrix porins (M), gel electrophoresis, and analysis by liquid scintillation spectrometry were as described in the text. Electrophoresis was from left to right.

ginine-labeled OmpF protein in the mutant cell envelope (Fig. 1).

Essentially no ^{14}C radioactivity was detected at the top of the gel, suggesting that mutant cells lack lipoprotein covalently bound to peptidoglycan. Some free lipoprotein was detected. The amount was quantitated by comparing the ^{14}C/^{3}H ratio in free lipoprotein with the corresponding ratio in total protein, measured after sonication of the cells (see above). This comparison showed that the mutant cell envelope contained 27% of the amount of radioactive free lipoprotein in the *cpxA*$^+$ *cpxB*$^+$ cell envelope. This is a minimum estimate of the effect of the mutations since the *cpxA*$^+$ *cpxB*$^+$ cells in this experiment stopped net incorporation of radioactivity between 60 and 90 min, and the estimate does not include the effect of the mutations on lipoprotein covalently bound to peptidoglycan.

Quantitative analysis of lipoprotein synthesis in *cpxA cpxB* mutant cells. Strain AE1031 (*cpxA*$^+$ *cpxB*$^+$) and AE1019 (*cpxA2 cpxB1*) cells were labeled with [^{14}C]arginine for 5 min and mixed with AE1031 cells labeled for 90 min with [^{3}H]arginine, before the preparation of cell extracts by sonication. Matrix proteins and murein lipoprotein were then immunoprecipitated from the mixed cell extracts, without purification of cell envelope fractions, the radioactive proteins were separated by gel electrophoresis, and radioactivity was determined by liquid scintillation spectrometry (Fig. 3). In this experiment, no radioactivity was detected at the top of the resolving gel, owing to the fact that no lysozyme was used and the murein-bound lipoprotein remained with very large peptidoglycan fragments that failed to enter the gel. The ^{14}C/^{3}H ratios of free lipoprotein, in comparison to the ratios of total protein, showed that *cpxA*$^+$ *cpxB*$^+$ (Fig. 3A) and *cpxA2 cpxB1* cells (Fig. 3B) accumulated the same amount of lipoprotein over the 5-min labeling interval. This result shows that lipoprotein synthesis is not affected by the *cpxA* and *cpxB* mutations.

The murein lipoproteins that accumulated in mutant and *cpx*$^+$ cells had indistinguishable electrophoretic mobilities (see Fig. 2 and 3), suggesting that in both cases prolipoprotein was cleaved to lipoprotein. To confirm this in a gel system known to resolve lipoprotein and prolipoprotein, we used the 19% acrylamide–6 M urea gel system described by Ito et al. (11). Strains AE1031 (*cpxA*$^+$ *cpxB*$^+$) and AE1019

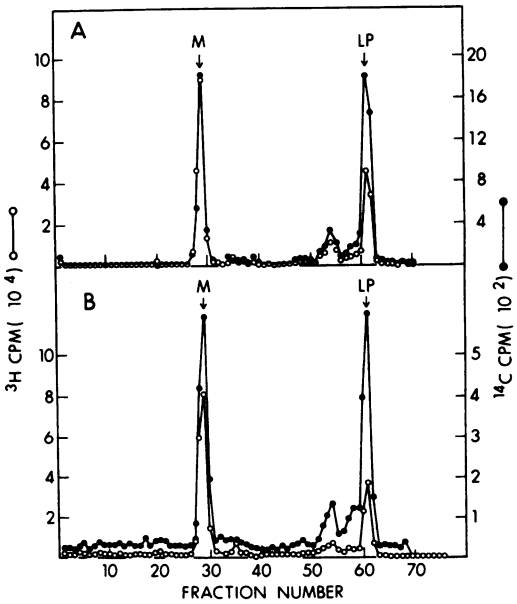

FIG. 3. Quantitative analysis of lipoprotein synthesis in *cpxA cpxB* and *cpxA*$^+$ *cpxB*$^+$ cells. AE1031 and AE1019 cells were labeled with [^{14}C]arginine for 5 min and mixed with AE1031 cells labeled with [^{3}H]arginine. Preparation of unfractionated cell extracts (without lysozyme), immunoprecipitation, gel electrophoresis, and analysis were as described in the legend to Fig. 2 and in the text. Electrophoresis was from left to right. Abbreviations are as in Fig. 2.

(*cpxA2 cpxB1*) were labeled with [^{35}S]methionine for 15 min, and crude cell envelopes from the labeled cells were immunoprecipitated with antilipoprotein antiserum (lacking antibodies reactive with the matrix porins). The electrophoretic mobilities of the immunoreactive lipoproteins from *cpxA*$^+$ *cpxB*$^+$ and *cpxA2 cpxB1* cells were indistinguishable; in neither case was there detectable accumulation of material with the mobility of prolipoprotein (Fig. 4).

OmpF protein synthesis in *cpxA cpxB* mutant cells. We could not use ^{14}C/^{3}H ratios to estimate the effect of the *cpx* mutations on OmpF protein synthesis, since the electrophoretic resolution of the OmpF protein from the more abundant OmpC protein, evident upon autoradiography (Fig. 1), was lost when the gels were sliced (Fig. 2 and 3). We therefore prepared an autoradiogram from a companion gel to the one shown in Fig. 3. Densitometric analysis of that autoradiogram indicated that, in comparison with *cpxA*$^+$ *cpxB*$^+$ cells, mutant cells accumulated several-fold less OmpF protein during the 5-min labeling interval (Fig. 5). This result could be attributed to an effect of the *cpx* mutations on *ompF* gene expression or, because 5 min is still long relative to the kinetics of OmpF protein synthesis (2), to very rapid turnover of the OmpF protein. To resolve this ambiguity, we determined the effect of the *cpxA2* and *cpxB1* mutations on β-galactosidase levels in strains containing either an *ompF*::*lacZ* operon fusion or an *ompF*::*lacZ*

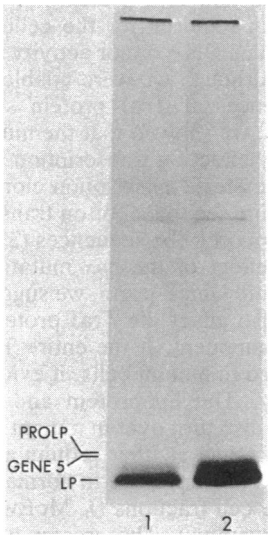

FIG. 4. Electrophoretic mobility of lipoprotein (LP) synthesized in a *cpxA cpxB* mutant. Cell labeling with [^{35}S]methionine, extraction, immunoprecipitation, and gel electrophoresis were as described in the text. The antiserum used in this experiment lacked antibodies to the matrix porins. Lane 1: Strain AE1031 (*cpxA*$^+$ *cpxB*$^+$); lane 2: strain AE1019 (*cpxA2 cpxB1*). The different intensities are the result of poor recovery of lipoprotein from strain AE1031 in this experiment, since the whole-cell envelope preparations used for these immunoprecipitations contained equal quantities of lipoprotein, as shown by gel electrophoresis (data not shown). The mobilities of bacteriophage fd gene 5 protein (M_r = 9,700) and prolipoprotein (PROLP) are indicated for comparison. The mobility of prolipoprotein in this electrophoretic system was determined by analysis of crude cell envelopes from AE1031 cells labeled with [^{35}S]methionine in the presence of globomycin, which inhibits the enzymatic cleavage of prolipoprotein to lipoprotein (10).

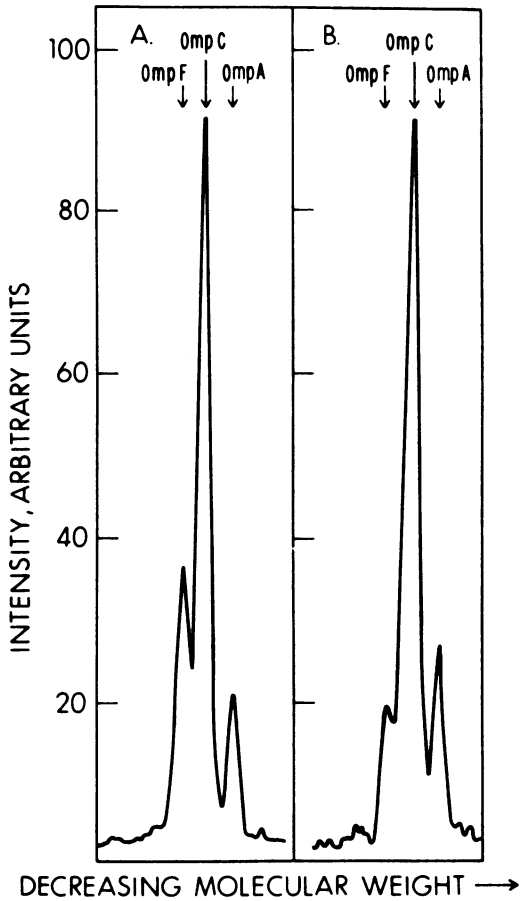

FIG. 5. Analysis of OmpF protein synthesis in *cpxA cpxB* and *cpxA*$^+$ *cpxB*$^+$ cells. A portion of the immunoprecipitated proteins analyzed in Fig. 3 was run on a companion gel prepared as described for that figure. After electrophoresis, the gel was dried without preparation for fluorography. An autoradiogram was prepared from the dried gel, and the regions containing the matrix porins were scanned densitometrically. A control experiment showed that under these conditions, the proteins labeled with [^{3}H]arginine do not expose the film. Hence, the scan showed only the proteins labeled with [^{14}C]arginine over the 5-min labeling interval. (A) Strain AE1031 (*cpxA*$^+$ *cpxB*$^+$); (B) strain AE1019 (*cpxA2 cpxB1*).

TABLE 1. *ompF* gene expression in a *cpxA cpxB* mutant[a]

ompF allele	*cpx* alleles	β-galactosidase activity[b]
φ(*ompF'-lacZ*$^+$)16-3	*cpxA*$^+$ *cpxB*$^+$	77
	cpxA2 cpxB1	27
φ(*ompF-lacZ*)16-21(Hyb)	*cpxA*$^+$ *cpxB*$^+$	370
	cpxA2 cpxB1	63

[a] Bacterial strains are described in the text. Overnight cultures were grown at 41°C in Vogel-Bonner minimal medium. Each strain was then diluted 20-fold into the same medium at 41°C and incubated with aeration until the cultures reached optical densities of 0.5 to 0.8.

[b] Enzyme activity with *o*-nitrophenyl-β-D-galactoside as substrate was measured, and activity units per optical density unit of culture turbidity are expressed as described previously by Hall and Silhavy (5).

protein fusion. Any effect of the *cpx* mutations on *ompF* gene expression should be reflected in the level of β-galactosidase in these strains.

To carry out these experiments, we constructed the *cpxA2 cpxB1* derivatives of the *ompF-lacZ* operon fusion strain MH513 and the *ompF-lacZ* protein fusion strain MH621 (see reference 5 and above). The constructions relied on the Ilv$^-$ phenotype of *cpxA2 cpxB1* double mutants (17). We also constructed the *cpxA2 cpxB1* derivative of strain MC4100, the parent of both MH153 and MH621, and examined its cell envelope protein composition by gel electrophoresis as previously described (15). The *cpx* mutations in the genetic background of MC4100 reduced the amounts of the OmpF porin and murein lipoprotein in the cell envelope, whereas they had no effect on the amounts of OmpC porin or OmpA protein (data not shown). This experiment confirms and extends our previous observation that the effects of the *cpxA* and *cpxB* mutations are expressed in different *E. coli* K-12 genetic backgrounds.

The effect of the *cpx* mutations on *ompF* gene expression is shown in Table 1. The steady-state levels of β-galactosidase in mutant strains containing the *ompF-lacZ* operon fusion or the *ompF-lacZ* protein fusion were reduced three- or sixfold, respectively, in comparison with the isogenic *cpxA*$^+$ *cpxB*$^+$ strains. This difference is similar to that estimated by autoradiography of immunoprecipitates (see above) and indicates that the *cpx* mutations reduce *ompF* gene expression. As described previously by Hall and Silhavy (5), β-galactosidase levels were higher in the protein fusion strains than in the operon fusion strains, perhaps owing to the utilization in the former of the strong *ompF* translation initiation sequences. However, the *cpx* mutations had a somewhat greater effect on β-galactosidase levels in the protein fusion strains, perhaps owing to slow turnover of the fusion protein in mutant cells.

The electrophoretic mobilities of the OmpF proteins accumulating in *cpxA2 cpxB1* and *cpxA*$^+$ *cpxB*$^+$ cells over a 5-min labeling interval were indistinguishable (Fig. 5), suggesting that the OmpF leader peptide was cleaved in the mutant. However, we have not established whether or not our gels will separate pro-OmpF protein from OmpF protein.

DISCUSSION

This and our previous report (15) show that both the lipoprotein and the OmpF protein are synthesized in *cpxA cpxB* mutant cells in excess of their amounts in the cell envelope. We infer from our results that these *cpx* mutations affect the post-translational processing, translocation, or stability of these proteins as cell envelope components. Mutant cells also fail to accumulate the F-plasmid TraJ protein, an outer membrane protein required for the cellular expression of conjugal DNA donor activity and surface exclusion. Although we were unable to provide direct evidence for TraJ protein synthesis in mutant cells, we showed that the mutations had no inhibitory effect on transcription initiation at the *traJ* promoter, transcription elongation into the *traJ* coding sequence, or on translation initiation from *traJ* mRNA sequences (23). By analogy to the effect of the *cpx* mutations on the lipoprotein and OmpF porin, we suggest that the mutations also affect the TraJ protein as a cell envelope component. If the entire TraJ protein is synthesized in mutant cells, it evidently turns over rapidly. The lipoprotein and the OmpF protein may also turn over in mutant cells, albeit more slowly, since neither protein accumulates in the mutant cell envelope to normal levels (15) or in soluble cell fractions (J. McEwen, unpublished observations). The greater effect of the *cpx* mutations of β-galactosidase levels in the *ompF-lacZ* protein fusion strain than in the *ompF-lacZ* operon fusion strain may refelect this slow turnover.

At least one and probably several inner membrane proteins are deficient in *cpxA cpxB* mutant cells (15), but we have not established whether or not these proteins are synthesized.

The *cpx* mutations had no effect at all on lipoprotein synthesis over at least a 5-min interval. Lipoprotein synthesized in mutant cells accumulated in the cell envelope and was indistinguishable in electrophoretic mobility from lipoprotein synthesized in *cpxA*$^+$ *cpxB*$^+$ cells. Specifically, it did not accumulate as prolipoprotein. These results imply that prolipoprotein synthesized in mutant cells inserts in the inner membrane and is processed at least to the stage

where it is cleaved to lipoprotein (26, 30). It is not yet clear how far translocation of lipoprotein in mutant cells proceeds past this point. Normally, prolipoprotein is processed in the inner membrane to fully modified lipoprotein, which is then translocated to the outer membrane; both of these events occur very rapidly (13, 30). Covalent attachment of lipoprotein to the peptidoglycan is a slower process and normally occurs in the outer membrane (8). However, experiments with the antibiotic globomycin indicate that the COOH-terminal of prolipoprotein can become accessible for covalent attachment to the peptidoglycan even when the protein as a whole fractionates with the inner membrane (7), presumably held there by its uncleaved leader peptide. In view of this observation and the absence of peptidoglycan-bound lipoprotein in *cpxA cpxB* mutant cells after a long labeling interval, we speculate that the *cpx* mutations affect an early stage of lipoprotein translocation, such that at least the COOH-terminal part of the protein remains in the inner membrane.

The effects of the *cpx* mutations on the OmpF protein are complex. The mutations reduced *ompF* gene expression, but not enough to account for the virtual absence of the OmpF protein from the outer membrane. A sensitive indicator for the absence of the OmpF protein is the resistance of *cpxA cpxB* mutant cells to K20, a bacteriophage that requires OmpF protein as a receptor (15). By analogy to bacteriophage λ receptors, which can be reduced from several thousand to as few as two receptors per cell with only a 10-fold reduction in the efficiency of λ plating (2), *cpx* mutant cells must contain few, if any, K20 receptors to be resistant to this bacteriophage (efficiency of K20 plating is $\sim 10^{-3}$). The biochemical data indicate that the lack of these receptors is a quantitative reflection of the lack of OmpF protein in the outer membrane. It seems unlikely that a three- or even sixfold reduction in *ompF* gene expression could account for such a severe effect, especially in view of the fact that immunoreactive OmpF protein could be found in mutant cells labeled for 5 min.

The mechanism by which the *cpx* mutations reduce *ompF* gene expression is not clear. One possibility is that the *cpx* mutations act indirectly by interfering with the operation of other genetic loci involved in matrix porin regulation. Mutations at the *envZ* or *tolC* loci, for example, reduce *ompF* expression relative to *ompC* expression (5, 6, 20). Moreover, like *cpxA* and *cpxB* mutations, *tolC* mutations do not reduce *ompF* expression enough to account for its absence in the outer membrane of the *tolC* mutants (20). This hypothesis could also explain some of the pleiotropic effects of the *cpx* mutations on envelope protein composition (15), since *envZ* and *tolC* mutations are also pleiotropic in that respect (20, 27, 28). However, the correspondence between the envelope proteins affected by *envZ* or *tolC* mutations on the one hand, and by the *cpx* mutation on the other, has not been examined systematically. Alternatively, the *cpx* mutations may directly affect the amount of OmpF in the outer membrane, perhaps by a mechanism similar to that suggested above for the effect of the mutations on lipoprotein. In this case, the rate of *ompF* transcription could be sensitive to the level of OmpF protein translocation intermediates that may accumulate in *cpx* mutants.

ACKNOWLEDGMENTS

We thank T. Silhavy for bacterial strains and M. Inouye for antisera and globomycin.

This work was supported by Public Health Service grants CA-13330, HDO-7154, and GM-11301 from the National Institutes of Health. P.S. acknowledges support as an Established Investigator of the American Heart Association and an Irma T. Hirschl Career Investigator.

LITERATURE CITED

1. **Achtman, M., P. Manning, C. Edelbluth, and P. Herrlich.** 1979. Export without proteolytic processing of inner and outer membrane proteins encoded by F sex factor *tra* cistrons in *Escherichia coli* minicells. Proc. Natl. Acad. Sci. U.S.A. **76**:4837–4841.
2. **Crowlesmith, I., K. Gamon, and U. Henning.** 1981. Precursor proteins are intermediates *in vivo* in the synthesis of two major outer membrane proteins, the OmpA and OmpF proteins, of *Escherichia coli* K12. Eur. J. Biochem. **113**:375–380.
3. **DiRienzo, J., K. Nakamura, and M. Inouye.** 1978. The outer membrane proteins of gram-negative bacteria: biosynthesis, assembly and function. Annu. Rev. Biochem. **47**:481–532.
4. **Fairbanks, G., T. Steck, and F. Wallach.** 1971. Electrophoretic analysis of the major polypeptides of the human erythrocyte membrane. Biochemistry **10**:2606–2617.
5. **Hall, M., and T. Silhavy.** 1981. The *ompB* locus and the regulation of the major outer membrane porin proteins of *Escherichia coli* K12. J. Mol. Biol. **146**:23–43.
6. **Hall, M., and T. Silhavy.** 1981. Genetic analysis of the *ompB* locus in *Escherichia coli* K12. J. Mol. Biol. **151**:1–16.
7. **Ichihara, S., M. Hussain, and S. Mizushima.** 1981. Mechanism of export of outer membrane lipoproteins through the cytoplasmic membrane in *Escherichia coli*. J. Biol. Chem. **257**:495–500.
8. **Inouye, M., J. Shaw, and C. Chan.** 1972. The assembly of a structural lipoprotein in the envelope of *Escherichia coli*. J. Biol. Chem. **247**:8154–8159.
9. **Inouye, S., K. Takeishi, N. Lee, M. DeMartini, A. Hirashima, and M. Inouye.** 1976. Lipoprotein from the outer membrane of *Escherichia coli*: purification, paracrystallization, and some properties of its free form. J. Bacteriol. **127**:555–563.
10. **Inukai, M., M. Takeuchi, K. Shimizu, and M. Arai.** 1978. Mechanism of action of globomycin. J. Antibiot. **31**:1203–1205.
11. **Ito, K., T. Date, and W. Wickner.** 1980. Synthesis, assembly into the cytoplasmic membrane, and proteolytic processing of the precursor of coliphage M13 coat protein. J. Biol. Chem. **255**:2123–2130.
12. **Kennedy, N., L. Beutin, M. Achtman, R. Skurray, U. Rahmsdorf, and P. Herrlich.** 1977. Conjugation proteins

encoded by the F sex factor. Nature (London) **270**:580–585.
13. **Lin, J., C. Giam, and H. Wu.** 1980. Assembly of the outer membrane lipoprotein in *Escherichia coli*. J. Biol. Chem. **255**:807–811.
14. **Manning, P., and M. Achtman.** 1979. Cell-to-cell interactions in conjugating *E. coli*: the involvement of the cell envelope, p. 409–447. *In* M. Inouye (ed.), Bacterial outer membranes. John Wiley & Sons, Inc., New York.
15. **McEwen, J., and P. M. Silverman.** 1982. Mutations in genes *cpxA* and *cpxB* alter the protein composition of *Escherichia coli* inner and outer membranes. J. Bacteriol. **151**:1553–1559.
16. **McEwen, J., and P. Silverman.** 1980. Chromosomal mutations of *Escherichia coli* that alter the expression of conjugative plasmid functions. Proc. Natl. Acad. Sci. U.S.A. **77**:513–517.
17. **McEwen, J., and P. Silverman.** 1980. Mutations in genes *cpxA* and *cpxB* of *Escherichia coli* K-12 cause a defect in isoleucine and valine syntheses. J. Bacteriol. **144**:68–73.
18. **McEwen, J., and P. Silverman.** 1980. Genetic analysis of *Escherichia coli* K-12 chromosomal mutants defective in expression of F-plasmid functions: identification of genes *cpxA* and *cpxB*. J. Bacteriol. **144**:60–67.
19. **Moore, D., B. A. Sowa, and K. Ippen-Ihler.** 1981. Location of an F-pilin pool in the inner membrane. J. Bacteriol. **146**:251–259.
20. **Morona, R., and P. Reeves.** 1982. The *tolC* locus of *Escherichia coli* affects the expression of three major outer membrane proteins. J. Bacteriol. **150**:1016–1023.
21. **Nakamura, K., and M. Inouye.** 1979. DNA sequence of the gene for the outer membrane lipoprotein of *E. coli*: an extremely AT-rich promoter. Cell **18**:1109–1117.
22. **Osborn, M., J. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of the assembly of the outer membrane of *Salmonella typhimurium*. J. Biol. Chem. **247**:3962–3972.
23. **Sambucetti, L., L. Eoyang, and P. Silverman.** 1982. Cellular control of conjugation in *Escherichia coli* K-12: effect of chromosomal *cpx* mutations of F-plasmid gene expression. J. Mol. Biol. **161**:13–31.
24. **Silverman, P., M.-O. Huh, and L. Sun.** 1974. Protein synthesis during zoospore germination in the aquatic phycomycete *Blastocladiella emersonii*. Dev. Biol. **40**:59–70.
25. **Sutton, A., T. Newman, J. McEwen, P. M. Silverman, and M. Freundlich.** 1982. Mutations in genes *cpxA* and *cpxB* of *Escherichia coli* K-12 cause a defect in acetohydroxyacid synthase I function in vivo. J. Bacteriol. **151**:976–982.
26. **Tokunaga, M., H. Tokunaga, and H. Wu.** 1982. Post-translational modification and processing of *Escherichia coli* prolipoprotein *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **79**:2255–2259.
27. **Wandersman, C., F. Moreno, and M. Schwartz.** 1980. Pleiotrophic mutations rendering *Escherichia coli* K-12 resistant to bacteriophage TP1. J. Bacteriol. **143**:1374–1383.
28. **Wanner, B. L., A. Sarthy, and J. Beckwith.** 1979. *Escherichia coli* pleiotropic mutant that reduces amounts of several periplasmic and outer membrane proteins. J. Bacteriol. **140**:229–239.
29. **Wensink, J., and B. Witholt.** 1981. Identification of different forms of the murein-bound lipoprotein found in isolated outer membranes of *Escherichia coli*. Eur. J. Biochem. **113**:349–357.
30. **Wu, H., J. Lin, P. Chattopadhyay, and H. Kanazawa.** 1980. Biosynthesis and assembly of murein lipoprotein in *Escherichia coli*. Ann. N.Y. Acad. Sci. **343**:368–383.