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Mutations in Genes *cpxA* and *cpxB* Alter the Protein Composition of *Escherichia coli* Inner and Outer Membranes

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Mutations in chromosomal genes *cpxA* and *cpxB* altered the protein composition of the inner and outer bacterial membranes. Electrophoretic analyses of membrane proteins from isogenic strains differing only at their *cpx* loci and of spontaneous *cpxA*$^+$ revertants of a *cpxA cpxB* double mutant showed that the alterations define a pattern that is uniquely attributable to the *cpx* mutations. Two major outer membrane proteins, the OmpF matrix porin and the murein lipoprotein, were deficient or absent from the outer membrane of mutant cells, whereas the quantities of two other major outer membrane proteins, the OmpC matrix porin and the OmpA protein, were not significantly altered. The *cpx* mutations did not generally alter the functional or chemical properties of the cell envelope. In the electron microscope, mutant cells appeared ovoid, but individual cells showed no surface irregularities to suggest gross defects in the cell envelope. These observations suggest that the primary effect of the mutations is to alter selectively the synthesis or translocation of certain envelope proteins.

We initially identified chromosomal genes *cpxA* and *cpxB* of *Escherichia coli* K-12 by mutations causing a temperature-sensitive reduction in DNA donor activity and surface exclusion when the mutant cells contained normal F-plasmid DNA (22). *cpx* mutations also cause a temperature-sensitive auxotrophy for isoleucine and valine (23). A *cpxA* mutation by itself reduces DNA donor activity and impairs growth in the absence of isoleucine and valine, but both defects are more severe in *cpxA cpxB* double mutants. A *cpxB* mutation by itself is cryptic (13, 24). Neither *cpxA* (87.8 min; 23, 31) nor *cpxB* (41 min; 23) maps near any *ilv* locus or, since both genes are chromosomal, near any of the F-plasmid *tra* genes.

It is not evident what DNA donor activity and the synthesis of isoleucine and valine have in common. It seems unlikely that they share a common genetic regulatory mechanism or that a protein directly required for one process is also required for the other. Another hypothesis is that the *cpx* mutations alter a structure or event that is itself required for diverse cellular functions, for example, the cell envelope or its biogenesis. Conjugation is clearly a cell surface phenomenon, and most of the F-plasmid *tra* gene products have been assigned an envelope location (20). Moreover, membrane interactions have been implicated in the synthesis of isoleucine and valine or its control (4, 6).

The experiments described below examine the effects of the *cpx* mutations on the cell envelope. Our results show that the *cpx* mutations alter the protein composition of the cell envelope. We propose that this is their primary effect.

MATERIALS AND METHODS

Bacterial strains, media, and growth conditions. The bacterial strains used in this study are described in Table 1. Cells were grown in nutrient broth at 41°C unless otherwise indicated. Cell growth was monitored by the optical density of the cultures at 660 nm. Bacteriophages C21, U3, and K20 were provided by Paul Manning.

Membrane isolation. Inner and outer membranes were isolated from 200 ml of exponentially growing cells, essentially as described by Osborn et al. (27). Spheroplasts were prepared with 100 μg of lysozyme per ml in 1 mM EDTA and broken by 90 s of sonication (at 70 W) with the Branson Sonifier. Inner and outer membranes were separated by equilibrium sedimentation in a 30 to 60% sucrose gradient. Two inner membrane bands, L1 and L2, were pooled to obtain analytical data; no M band material was observed. Cross-contamination of each membrane fraction with material from the other was minimal, as reflected by sodium dodecyl sulfate (SDS)-gel electrophoresis of the proteins in the separated fractions and by their 2-keto-3-deoxyoctonate (KDO) contents (outer mem-

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TABLE 1. *E. coli* K-12 strains[a]

Strain	Relevant genotype	Source or reference
AE1031	Hfr *cpxA*$^+$ *cpxB*$^+$ *zeb-1*::Tn*10*	23
AE1010	Hfr *cpxA*$^+$ *cpxB1*	22
AE1019	Hfr *cpxA2 cpxB1*	22
AE1018	Hfr *cpxA1 cpxB1*	22
AE1028	Hfr *cpxA1 cpxB*$^+$ *zeb-1*::Tn*10*	From AE1018 by P1 transduction (21)
AE1072	Hfr *cpxA*$^+$ *cpxB1*	23; spontaneous Ilv$^+$ (*cpxA*$^+$) revertant of AE1019
AE1066	Hfr *cpxA*$^+$ *cpxB1*	23; spontaneous Ilv$^+$ (*cpxA*$^+$) revertant of AE1019
AE1063	Hfr *cpxA2 cpxB1*	23; spontaneous Ilv$^+$ pseudorevertant of AE1019
AE1002	Hfr *cpxA*$^+$ *cpxB*$^+$ *argH1 rpoB*	Arg$^-$ Rif^r transductant of KL14 (CGSC 4294)[b]
AE1121	Hfr *cpxA2 cpxB1* *argH*$^+$	From AE1002 by P1 transductions

[a] Except for strains AE1002 and AE1121, all of the strains used in these experiments are derivatives of AE2000, whose complete genotype is given in reference 22. The genealogy and specific methods of construction for all of these strains are available (J. McEwen, Ph.D. thesis, Albert Einstein College of Medicine, Bronx, N.Y., 1980).

[b] Obtained from Barbara Bachmann, Coli Genetic Stock Center, Yale University, New Haven, Conn.

brane/inner membrane ≅ 10) and NADH oxidase activities (inner membrane/outer membrane > 30).

Isolation of matrix-associated proteins. The procedure for the differential isolation of matrix-associated proteins was essentially as described by Rosenbusch (30). Exponentially growing cells (200 ml) at an optical density of 0.6 were collected by centrifugation, washed once with 25 ml of 10 mM Tris-hydrochloride (pH 7.3), and suspended in 4 ml of extraction buffer (10 mM Tris-hydrochloride [pH 7.3], 2% [wt/vol] SDS, 5% [vol/vol] 2-mercaptoethanol, 10% [vol/vol] glycerol). The extract was heated at 60°C for 30 min to solubilize proteins not associated with the matrix. The insoluble matrix and its associated proteins were pelleted by 60 min of centrifugation at 35,000 rpm in a Beckman type 40 Ti rotor and suspended in 200 μl of electrophoresis sample buffer.

Polyacrylamide gel electrophoresis. Membrane proteins at a concentration of 1 mg/ml were boiled for 5 min in an electrophoresis sample buffer containing 62.5 mM Tris-hydrochloride (pH 6.8), 5% (vol/vol) 2-mercaptoethanol, 2% (wt/vol) SDS, and 7.5% (vol/vol) glycerol. Samples containing 20 μg (outer membrane) or 30 μg (inner membrane) of protein were resolved by electrophoresis in the discontinuous buffer system of Laemmli (17) through a 3% acrylamide stacking gel and a 12.5% acrylamide resolving gel. After electrophoresis, the gels were fixed and stained as described by Fairbanks et al. (9).

Phospholipid analysis. Isogenic *cpxA*$^+$ (strain AE1010) and *cpxA1* (strain AE1018) cells, both containing the *cpxB1* mutation, were maintained in exponential growth in 200 ml of nutrient broth at 41°C for seven generations in the presence of 1.25 μCi of [^{32}P]PO_4^- per ml. The cells, containing about 15% of the total radioactivity, were harvested in the cold by centrifugation at 8,000 × *g* for 10 min, suspended in 5 ml of nutrient broth at 4°C, and collected by centrifugation. Phospholipids were extracted and analyzed by two-dimensional thin-layer chromatography in solvents 2 and 4, as described by Ames (2).

Other methods. Antibiotic sensitivities were estimated from zones of growth inhibition on agar containing antibiotic medium 3 (Difco Laboratories). The antibiotics were applied on paper disks (BBL Microbiology Systems). Deoxycholate sensitivities were estimated from growth inhibition in antibiotic medium 3, buffered at pH 7.5. Protein was determined as described by Lowry et al. (18). The KDO content and NADH oxidase activity were measured as described by Osborn (26) and Osborn et al. (27), respectively. The lipopolysaccharide content was estimated from the KDO content, assuming 0.5 μmol of KDO per mg of LPS (26). Buoyant densities at 5°C were estimated from refractive indexes of sucrose gradient fractions. Genetic methods and enzyme assays were as previously described (22–24). Penicillin-binding proteins were detected as described by Spratt (32).

Materials. Materials have been described elsewhere (23, 24) or were obtained from standard commercial sources.

RESULTS

Envelope protein composition of *cpxA* mutants. Inner and outer membranes from essentially isogenic strains differing only at their *cpx* loci and grown at the nonpermissive temperature (41°C) were separated from each other, and their proteins were examined by gel electrophoresis. This analysis revealed several differences between the *cpxA*$^+$ *cpxB*$^+$ strain AE1031 and the *cpxA1 cpxB1* strain AE1018 (Fig. 1). Some proteins in the outer (Fig. 1A) or inner (Fig. 1B) membrane fractions from the *cpxA*$^+$ *cpxB*$^+$ cells were absent or quantitatively diminished in the corresponding fraction from the *cpxA1 cpxB1* cells (unfilled arrowheads). Among the deficient outer membrane proteins were the murein lipoprotein (labeled LP in the figure) and, as we have noted before (22), the F-plasmid TraT protein. The TraT protein is the most abundant *tra* gene product in the outer membrane and the only one detectable by these experiments (1, 20, 22, 25). Other proteins were more abundant in the envelope from *cpxA1 cpxB1* cells (filled arrowheads). Proteins indicated in Fig. 1 are likewise indicated for comparison in subsequent figures.

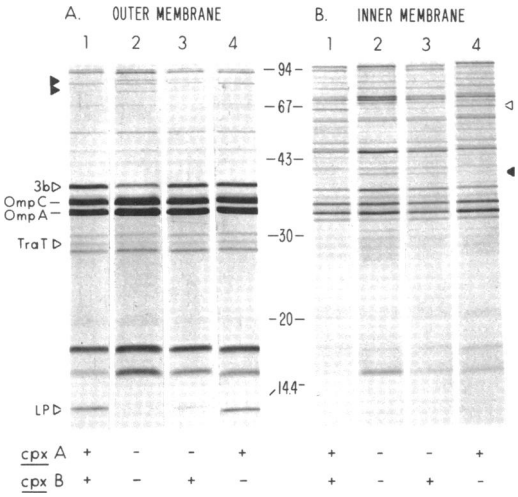

FIG. 1. Effect of *cpxA1* and *cpxB1* mutations on envelope protein composition. Outer (A) and inner (B) membrane proteins were prepared and separated by gel electrophoresis as described in the text. The genotypes of the different strains are indicated beneath the figure: +, a wild-type allele; −, a mutant allele. The strains were AE1031 (Hfr *cpxA*$^+$ *cpxB*$^+$), AE1018 (Hfr *cpxA1 cpxB1*), AE1028 (Hfr *cpxA1 cpxB*$^+$), and AE1010 (Hfr *cpxA*$^+$ *cpxB1*). The numbers between (A) and (B) are the molecular weights (× 10^3) of marker proteins with the indicated electrophoretic mobilities. Open arrowheads indicate proteins that were deficient in mutant cells, and filled arrowheads indicate proteins that were more abundant (see the text). The nomenclature for outer membrane proteins follows the suggestions of Reeves (29) and Osborn and Wu (28). Protein 3b has been described by Lugtenberg et al. (19) and by Manning and Reeves (21), and its structural gene has been isolated (10). The electrophoretic mobilities of the OmpC and OmpF matrix porins (Fig. 2) were determined by comparison with outer membrane proteins of strains lacking one or the other porin structural gene owing to an insertion mutation (12). The mobility of the F-plasmid TraT protein was determined as previously described (22). The mobility of the murein lipoprotein (LP) was determined by comparison with that of radioactive lipoprotein prepared by immune precipitation (15).

Another difference between *cpxA*$^+$ *cpxB*$^+$ and *cpxA cpxB* double-mutant strains can be seen in the region of the matrix porins of cells grown at 41°C in Vogel-Bonner minimal medium. In the experiment shown (Fig. 2), envelopes from the two strains grown in this medium and in nutrient broth were subjected to differential SDS extraction to yield a fraction enriched in matrix-associated proteins (30). The fraction from the *cpxA*$^+$ *cpxB*$^+$ strain contained both the OmpF and OmpC matrix porins when the cells were grown in minimal medium, but only the OmpC porin when they were grown in nutrient broth. In contrast, the fraction from the *cpxA cpxB* double-mutant strain contained only the OmpC protein when the cells were grown in either medium. Apparently, AE1010 and its derivatives make little if any OmpF matrix porin at 41°C when they are grown in nutrient broth.

With the exception of the TraT protein, the mutations identically altered the envelope protein composition of F$^-$ and Hfr cells (data not shown). The mutations did not substantially alter the pattern of soluble (cytoplasmic plus periplasmic) proteins resolved by one-dimensional gel electrophoresis (data not shown). The envelope protein compositions of mutant and wild-type cells grown at 34°C were more nearly similar, although some quantitative differences, for example in the amounts of murein lipoprotein and OmpF matrix porin, persisted.

Also shown in Fig. 1 are the individual effects of the *cpxA1* and *cpxB1* mutations. The *cpxA1* mutation by itself altered the protein composition of the cell envelope in a manner qualitatively similar to the effect of the *cpxA1* and *cpxB1* mutations together, but quantitatively less extreme. The *cpxB1* mutation by itself had no discernible effect on envelope protein composition.

Several experiments established that the pattern of alterations in envelope protein composition is the result only of genetic differences at the *cpx* loci. We first examined the effect of two independently obtained *cpxA* mutant alleles in the *cpxB1* background of strain AE1010. Envelope proteins from the *cpxA1 cpxB1* strain AE1018 are shown in Fig. 1, and those from the *cpxA2 cpxB1* strain AE1019 are shown in Fig. 3. Both *cpxA* alleles caused the same set of alterations. Also shown in Fig. 3 are the envelope proteins from two spontaneous *cpxA*$^+$ revertants of AE1019 (23). In both, the reversion event mapped to a locus cotransducible by P1

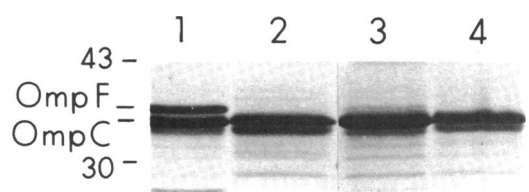

FIG. 2. Matrix porin proteins from strains AE1031 (*cpxA*$^+$ *cpxB*$^+$; lanes 1 and 3) and AE1019 (*cpxA2 cpxB1*; lanes 2 and 4) grown at 41°C in nutrient broth (lanes 3 and 4) or in Vogel-Bonner minimal medium (lanes 1 and 2). The numbers indicate the molecular weights (× 10^3) of marker proteins with the indicated electrophoretic mobilities. The material migrating slightly faster than the OmpC matrix porin is not immunologically related to either the OmpF or the OmpC porin.

TABLE 2. Properties of spontaneous Ilv$^+$ revertants of AE1019

Strain[a]	*cpx* genotype[a]	Aceto-hydroxy-acid synthase I[b]	Bacterio-phage Qβ sensi-tivity[c]	Surface exclusion[d]
AE1031	*cpxA*$^+$ *cpxB*$^+$	23.8	S	2,000
AE1019	*cpxA2 cpxB1*	6.6	R	1.5
AE1066	*cpxA*$^+$ *cpxB1*	28.2	S	1,454
AE1072	*cpxA*$^+$ *cpxB1*	28.0	S	2,400
AE1063	*cpxA2 cpxB1*	22.2	R	7.5

[a] See Table 1. AE1066, AE1072, and AE1063 are spontaneous Ilv$^+$ revertants of AE1019.

[b] Nanomoles of acetolactate formed per minute per milligram of protein (24).

[c] S, Sensitive to Qβ; R, resistant to Qβ (22).

[d] Leu$^+$ Str^r recombinants formed with an F$^-$ recipient/Leu$^+$ Str^r recombinants formed with the indicated Hfr recipient (22, 23).

with *metB* and *argH* in a manner indistinguishable from *cpxA* itself (23) and completely restored sensitivity to donor-specific bacteriophages and surface exclusion, as well as the level of acetohydroxyacid synthase I (Table 2), which is the only Ilv biosynthetic enzyme affected by the *cpx* mutations (24, 33). The reversions also essentially restored the envelope protein composition to the pattern characteristic of *cpxA*$^+$ *cpxB*$^+$ cells (Fig. 3).

Lanes 5 in Fig. 3 show the inner and outer membrane proteins from an Ilv$^+$ pseudorevertant of AE1019, designated AE1063. The site of the reversion event in AE1063 is not linked by P1 transduction to either *cpxA* or *cpxB* (23). The reversion event restored acetohydroxyacid synthase I function, but failed to restore donor-specific phage sensitivity or surface exclusion (Table 2). The pattern of outer membrane proteins of this pseudorevertant was indistinguishable from that of its parent, AE1019 (Fig. 3A). The pattern of inner membrane proteins also resembled that of AE1019, except for two prominent proteins that were absent or much less abundant in the inner membrane of AE1019, of the two *cpxA*$^+$ revertants of AE1019, or of the *cpxA*$^+$ *cpxB*$^+$ strain AE1031 (Fig. 3B).

Finally, we examined the effect of the *cpx* mutations in a different genetic background. The *cpxB1* and *cpxA2* alleles were placed by P1 transduction in strain AE1002, a derivative of Hfr strain KL14, whose ancestral stock differs from that of AE1010 (3). The mutations caused essentially the same set of alterations in envelope protein composition in either genetic background (cf. Fig. 1 and 4). The effect of the mutations on the TraT protein and OmpF matrix porin was more evident in the KL14 derivative grown in nutrient broth at 41°C than in the

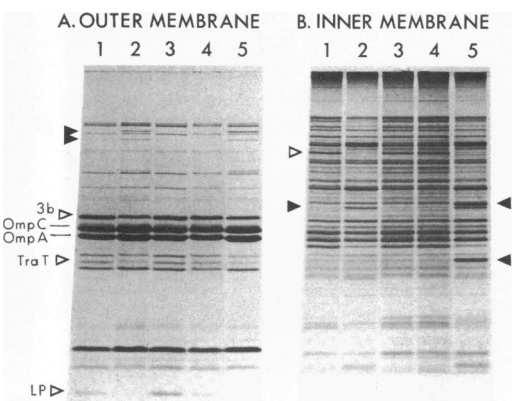

FIG. 3. Effect of the *cpxA2* mutation and reversion to *cpxA*$^+$ on envelope protein composition. See Fig. 1 for comparison. The bacterial strains were AE1031 (Hfr *cpxA*$^+$ *cpxB*$^+$; lanes 1), AE1019 (Hfr *cpxA2 cpxB1*; lanes 2), AE1066 (Hfr *cpxA*$^+$ *cpxB1*; lanes 3), AE1072 (Hfr *cpxA*$^+$ *cpxB1*; lanes 4), and AE1063 (Hfr *cpxA2 cpxB1*; lanes 5); AE1066, AE1072, and AE1063 are spontaneous Ilv$^+$ revertants of AE1019 (see the text).

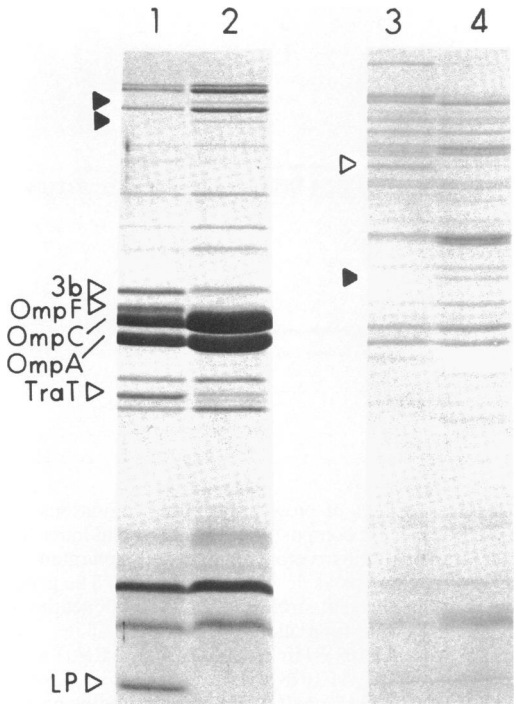

FIG. 4. Effect of the *cpxA2 cpxB1* mutations in an Hfr strain derived from KL14. See Fig. 1 and 2 for comparison. Lanes 1 and 2 show outer membrane proteins of strain AE1002 (Hfr *cpxA*$^+$ *cpxB*$^+$; lane 1) and AE1121 (Hfr *cpxA2 cpxB1*; lane 2); lanes 3 and 4 show inner membrane proteins from these two strains.

AE1010 derivatives grown under the same conditions.

Functional and chemical properties of the mutant cell envelope. A variety of experiments indicated that the functional and bulk chemical properties of the cell envelope were not grossly altered by the *cpx* mutations. Since the results of these experiments were largely negative, insofar as Cpx$^-$ strains (AE1018 or AE1019) and Cpx$^+$ strains (AE1010 or AE1031) appeared similar, they will only be summarized.

Cpx$^-$ cells were no more sensitive to novobiocin, ampicillin, tetracycline, chloramphenicol, nalidixic acid, kanamycin, or deoxycholate than were Cpx$^+$ cells. Cpx$^+$ and Cpx$^-$ strains were both resistant to bacteriophage C21 and sensitive to bacteriophage U3, a pattern characteristic of *E. coli* K-12 strains with normal core lipopolysaccharide synthesis (5, 14). Routine analyses of envelope fractions from Cpx$^+$ and Cpx$^-$ strains showed no striking differences in buoyant density, lipopolysaccharide content (0.3 to 0.4 mg/mg of outer membrane protein), phospholipid composition (80% phosphatidylethanolamine, 11% phosphatidylglycerol, and 6% to 8% cardiolipin), penicillin-binding proteins (data not shown), or inner membrane NADH oxidase activity (0.6 to 0.9 μmol of NADH oxidized per min per mg of inner membrane protein). Both Cpx$^+$ and Cpx$^-$ outer membranes were positive for heptose by the cysteine-hydrochloride test (8).

On LB agar plates (23), Cpx$^-$ cells were resistant at 41°C and sensitive at 34°C to bacteriophage K20, whereas Cpx$^+$ cells were sensitive at both temperatures. K20 is serologically related to T2 (13) and requires outer membrane OmpF protein as its receptor (P. Manning and P. Reeves, personal communication). Hence, the temperature-sensitive K20 resistance of Cpx$^-$ cells is consistent with the absence of OmpF protein in their outer membrane when the cells are at 41°C. We used LB medium for these experiments because it contains no glucose and less NaCl than nutrient broth, conditions that favor the synthesis of OmpF matrix porin (12). In addition, Cpx$^-$ cells appeared more sensitive to EDTA at 41°C than did Cpx$^+$ cells, especially during growth on a solid medium. This property has been associated with a lipoprotein deficiency (34).

In the electron microscope, Cpx$^-$ cells grown at 41°C appeared ovoid, but otherwise they were not grossly deformed or irregular in shape (Fig. 5). Note also the absence of F-pili in the Cpx$^-$ culture incubated at 41°C.

DISCUSSION

The *cpxA* and *cpxB* mutations selectively altered the protein composition of the cell envelope. The alterations, although complex, defined a characteristic pattern, as demonstrated by the analysis of essentially isogenic strains carrying different *cpxA* alleles, of genealogically distinct strains carrying the same *cpxA* and *cpxB* alleles, and of spontaneous *cpxA*$^+$ revertants. Moreover, the effect on envelope protein composition of a *cpxA* mutation alone and a *cpxB* mutation alone followed the pattern that we established for their individual effects on the expression of DNA transfer functions in F′ or Hfr strains and on isoleucine and valine synthesis (23, 24): a *cpxA* mutation by itself produces a mutant phenotype that is exacerbated by the *cpxB1* mutation, whereas the *cpxB1* mutation by itself is cryptic.

The alteration in envelope protein composition appears to be the primary effect of the *cpxA* and *cpxB* mutations. They did not alter other major components of the envelope, nor did they lead to gross abnormalities that would have been reflected in altered permeability to hydrophobic antibiotics, such as novobiocin, increased sensitivity to the detergent deoxycholate, or obvious irregularities in cell morphology, such as membrane "blebbing." Our analysis would not have detected differences in phospholipid fatty acid composition or in a small population of lipopolysaccharide molecules. However, changes of this kind are unlikely to be the primary effect of the mutations, given the selective character of the differences in protein composition and the fact that the mutations affect both inner and outer membrane proteins.

We propose that the diverse cellular processes affected by the *cpx* mutations have in common a requirement for cell envelope components whose functions as such are defective in mutant cells. In the case of the F-dependent activities, this component appears to be the plasmid-encoded *traJ* gene product (L. Sambucetti, L. Eoyang, and P. M. Silverman, J. Mol. Biol., in press), a 24,000-molecular-weight outer membrane protein (20, 25). Since the TraJ protein is essential for the efficient transcription of most of the other *tra* genes (1, 35), *cpxA cpxB* mutant cells fail to synthesize normal levels of other *tra* gene products, as shown above for the TraT protein.

Acetohydroxyacid synthase I is the only Ilv biosynthetic enzyme affected by the *cpx* mutations (24, 33). The enzyme is synthesized in mutant cells, but is either inactive in vivo or uncoupled from the overall Ilv biosynthetic pathways (33). The enzyme is unstable in mutant cells at temperatures above 37°C, and this accounts for the reduced level of activity in mutant cell extracts (33). However, this instability is not the primary effect of the *cpx* mutations on acetohydroxyacid synthase I in vivo, since mutant

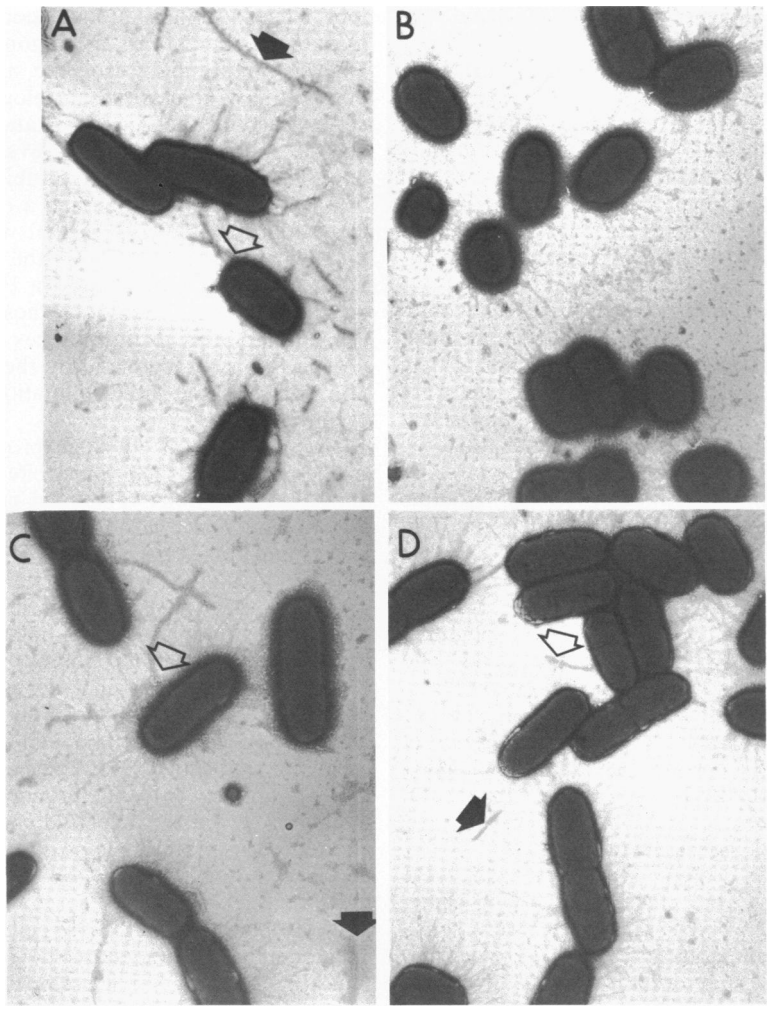

FIG. 5. Electron microscopy of mutant and *cpx*$^+$ cells grown at different temperatures. The cells were incubated with RNA bacteriophage R17 to identify F-pili and prepared for electron microscopy as previously described (21). The open arrowheads indicate cell-bound F-pili, and the filled arrowheads indicate detached F-pili in the medium. (A) Hfr *cpxA*$^+$ *cpxB1* strain AE1010, 41°C (×7,500); (B) Hfr *cpxA2 cpxB1* strain AE1019, 41°C (×8,200); (C) as in (A), except the cells were grown at 34°C (×8,000); (D) as in (B), except the cells were grown at 34°C (×8,000).

cells containing substantial quantities of active enzyme, owing to a multicopy plasmid containing the structural gene for the enzyme, *ilvB*, remain auxotrophic for isoleucine and valine at 41°C (33). Purification of acetohydroxyacid synthase I does not indicate that the enzyme is itself an integral membrane component (11; Eoyang and Silverman, unpublished data). However, the inner membrane protein composition of AE1063, the spontaneous Ilv$^+$ pseudorevertant of the *cpxA2 cpxB1* strain AE1019, supports previous observations suggesting an inner membrane involvement in acetohydroxyacid synthase I function in vivo (4, 6).

It remains to be determined how the *cpx* mutations affect envelope protein composition so selectively. If the mutations act on the envelope protein themselves, rather than on the expression of envelope protein genes, their effects suggest that some inner and outer membrane proteins are organized and sorted during envelope biogenesis by a common process that depends on the *cpx* gene products. Other studies have suggested analogous levels of organization for some periplasmic and outer membrane proteins (7, 16).

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