

## Chromosomal mutations of *Escherichia coli* that alter expression of conjugative plasmid functions

(*cpx* genes/F-pili/conjugal donor activity/surface exclusion/outer membrane)

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**ABSTRACT** We have identified two chromosomal genes of *Escherichia coli* K12 that are required for the expression of conjugative plasmid functions in the presence of normal plasmid DNA. Hfr cells with mutations in both of these genes are resistant to donor-specific bacteriophage and defective as conjugal donors. These characteristics can be attributed to the inability of mutant Hfr cells to elaborate F-pili, surface organelles required both for conjugal donor ability and for sensitivity to donor-specific bacteriophages. Mutant cells are also defective in surface exclusion, the property of donor cells to act as poor conjugal recipients. This defect can be attributed in part to a reduction in the amount of the F-plasmid *traT* gene product in the outer membrane of mutant cells; this protein is one of two plasmid gene products required for the full expression of surface exclusion. We have designated the chromosomal genes identified by these mutations as *cpxA* and *cpxB*; the mnemonic *cpx* signifying conjugative plasmid expression.

*Escherichia coli* strains carrying F-plasmid DNA or DNA of other conjugative plasmids exhibit various properties that distinguish them from strains lacking such DNA (1, 2). Principal among these properties is the capacity to transfer DNA to recipient cells by conjugation. In addition, donor strains are themselves poor conjugal recipients, a phenomenon described as surface exclusion.

The expression and control of donor properties require F-plasmid genes located within or near the 30-kilobase *tra* region (1-7). Strains carrying mutationally altered plasmids have been used to identify the genes in this region that are required for the formation of F-pili, filamentous organelles responsible for establishing cell-cell contact leading to DNA transfer, and those apparently required for DNA transfer itself (1, 2, 7). In addition, two *tra* genes, *traS* and *traT*, are required for the full expression of surface exclusion (6).

Cellular components determined by chromosomal genes are also likely to be important in conjugative plasmid function, because the expression of donor properties is strongly dependent on the physiological state of donor cells (8-10). Chromosomal mutations have been reported to alter donor properties (11-14), but specific components required for the expression of plasmid *tra* genes or for the function of *tra* gene products have not been identified.

Mutations that abolish donor properties of cells carrying normal F-plasmid DNA must be chromosomal and can be used to identify chromosomal gene products necessary for the synthesis or function of *tra* proteins. In this communication we report a method to isolate such mutants and the phenotypic characterization of one class of them.

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## MATERIALS AND METHODS

**Bacterial Strains and Bacteriophage.** Bacterial strains and their sources are listed in Table 1. Bacteriophages Q $\beta$  and R17 were obtained from Joan Steitz.  $\lambda$ NK55 (b<sub>221</sub>, CI857, 0am-29, C<sub>III</sub>::Tn10; ref. 19) was obtained from Robert Bender.

**Media and Growth Conditions.** Bacteria were routinely grown aerobically in nutrient broth (1% Bacto-tryptone/0.1% yeast extract/0.8% NaCl/0.2% glucose) supplemented with 40  $\mu$ g of thymidine per ml. Cell densities were measured by OD<sub>660</sub> or by viable count. Transconjugants were selected on agar plates containing minimal medium E (20) with appropriate nutritional supplements (40  $\mu$ g/ml). Nalidixic acid (20  $\mu$ g/ml) or streptomycin (100  $\mu$ g/ml) was included as a counterselective agent. Solid media contained 15 g of agar per liter.

**Mutagenesis and Screening for Q $\beta$ -Resistant Mutants.** These methods were essentially as described (21, 22), except that the concentration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was 8  $\mu$ g/ml. Under these conditions survival of the *recA* strain AE3087 (see Table 1) was about 1%.

**Electron Microscopy.** Cells were grown to an optical density of 1 at 41°C or 34°C. RNA bacteriophage R17 was added at a multiplicity of 200-1000 plaque-forming units per cell and incubation was continued for 10 min at the appropriate temperature. A drop of the infected cell culture was placed on a Formvar-coated copper grid for 15-30 min at room temperature. The grid was then rinsed with two drops of a solution containing 0.36% sucrose and stained with two drops of 1% phosphotungstate (pH 7.4). Excess stain was removed immediately with filter paper. The grids were examined in a Siemens Elmiskop 1A electron microscope.

**Donor Activity.** Donor activity was measured by transfer of the F'116 *zzf*::Tn10 plasmid (Table 1) from otherwise isogenic *cpxA* mutant and *cpxA*<sup>+</sup> cells. Donor strains AE3016 (*cpxA*<sup>+</sup>) and AE3018 (*cpxA1*) were grown to an optical density of 0.5-1 at 34°C or 41°C and then incubated for 60 min with a 5-fold excess of AE2004 recipient cells. Mating mixtures were then vortex mixed, washed once by centrifugation, and spread on minimal plates selective for cells with the Nal<sup>r</sup>, Tet<sup>r</sup>, Thy<sup>+</sup> phenotype of transconjugants. In addition, donor cell concentrations at the end of the experiment were determined by viable count on LB-streptomycin plates. The number of transconjugants per donor cell (expressed as a *percentage*) was calculated from these data.

**Surface Exclusion.** Hfr H donor cells and AE2000 (F-*cpxA*<sup>+</sup>), AE1010 (Hfr *cpxA*<sup>+</sup>), AE1018 (Hfr *cpxA1*), or AE1019 (Hfr *cpxA2*) recipient cells were grown to an optical density of 0.5-0.8 in nutrient broth at 41°C or 34°C. They were

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Table 1. Bacterial strains\*

Strain	Genotype <sup>†</sup>	Source or comment
AE3087	F'116 <i>thyA</i> <sup>+</sup> /[a], <i>metB1</i> , <i>rpsL104</i> , <i>recA1</i> , <i>thyA23</i>	J. Chase; CGSC <sup>‡</sup> 4254
KN401	Same as AE3087, except <i>cpxA1</i>	This study; Q $\beta$ -resistant mutant of AE3087
KN312	Same as AE3087, except <i>cpxA2</i>	This study; Q $\beta$ -resistant mutant of AE3087
AE2000	F <sup>-</sup> [a], <i>metB1</i> , <i>rpsL104</i> , <i>thyA</i>	This study; spontaneous Thy <sup>-</sup> derivative of JC411 (CGSC <sup>‡</sup> strain 4274)
AE1010	Hfr, otherwise same as AE2000	This study; spontaneous Hfr from an F <sup>+</sup> derivative of AE2000; selected for early transfer of <i>thr</i>
AE1018	Hfr, same as AE1010, except <i>metB</i> <sup>+</sup> , <i>cpxA1</i>	This study; <i>metB</i> <sup>+</sup> , <i>cpxA1</i> transductant of AE1010
AE1019	Same as AE1018, except <i>cpxA2</i>	This study; <i>metB</i> <sup>+</sup> , <i>cpxA2</i> transductant of AE1010
AE3016	F'116 <i>zff::Tn10</i> , <i>thyA</i> <sup>+</sup> /[a], <i>metB1</i> , <i>rpsL104</i> , <i>recA1</i> , <i>thyA</i>	This study; <i>recA</i> derivative of AE2000 carrying the F'116 <i>zff::Tn10</i> plasmid <sup>§</sup>
AE3018	Same as AE3016, except <i>metB</i> <sup>+</sup> , <i>cpxA1</i>	This study; constructed from a <i>metB</i> <sup>+</sup> , <i>cpxA1</i> transductant of AE2000
AE2004	[a], <i>metB1</i> , <i>thyA</i> , <i>nalA</i>	This study; spontaneous Thy <sup>-</sup> Nal <sup>R</sup> derivative of JC355 (CGSC <sup>‡</sup> strain 869)
M2121	pSC101 in JC3272	P. Manning; refs. 15 and 16
M1889	pRS31 in JC3272	P. Manning; refs. 15 and 16
KL262	F <sup>-</sup> <i>thi-1</i> , <i>tyrA2</i> , <i>pyrD34</i> , <i>trp-45</i> , <i>thyA33</i> , <i>recA1</i> , <i>galK35</i> , <i>malA1</i> , <i>xyl-7</i> , <i>mtl-2</i> , <i>rpsL118</i>	CGSC <sup>‡</sup> strain 4322

\* All strains are derivatives of *E. coli* K12. Unless otherwise indicated, they were constructed by methods described in ref. 17.

<sup>†</sup> The symbol [a] denotes the following genotype: *leu-6*, *his-1*, *argG6*, *lacY1*, *malA1*, *xyl-7*, *mtl-2*, *gal-6*, *tonA2*, *tsx-1*, *supE44*, *cpxB1*.

<sup>‡</sup> *Coli* Genetic Stock Center.

<sup>§</sup> The plasmid F'116 *zff::Tn10* is a derivative of F'116 carrying the Tn10 transposon, which confers resistance to tetracycline (18). To construct this plasmid, a strain carrying F'116 was mutagenized with  $\lambda$ NK55 in conditions preventing lysogenization or replication of the bacteriophage (19). Tn10 insertions in F'116 were isolated after transfer of tetracycline resistance by conjugation to an appropriate recipient strain.

mixed in a volume ratio of 1:4 (donor/recipient) and incubated with slow shaking for 15 min at 34°C or 41°C. The number of Leu<sup>+</sup> Str<sup>R</sup> recombinants was determined after dilution and Vortex mixing to disrupt mating aggregates.

**Membrane Isolation.** Outer membrane was isolated from 200 ml of exponentially growing cells as described by Osborn *et al.* (23). Cross-contamination with inner membrane material was  $\leq$ 10%, as estimated from the activity of NADH oxidase, an inner membrane marker (23), in the outer membrane fractions.

**NaDodSO<sub>4</sub>/Polyacrylamide Gel Electrophoresis.** Purified outer membrane fractions at 1 mg of protein per ml were boiled for 5 min in a solution containing 62.5 mM Tris-HCl (pH 6.8), 5% (vol/vol) 2-mercaptoethanol, 2% (wt/vol) NaDodSO<sub>4</sub>, and 7.5% (vol/vol) glycerol. Samples containing 20  $\mu$ g of protein were resolved by electrophoresis in the discontinuous buffer system of Laemmli (24) through a 3% acrylamide stacking gel and a 12.5% acrylamide resolving gel. After electrophoresis, gels were fixed and stained as described by Fairbanks *et al.* (25).

**Analytical Methods.** Protein was determined as described by Lowry *et al.* (26) and NADH oxidase activity, as described by Osborn *et al.* (23). The optical density of bacterial cultures was measured at 660 nm.

## RESULTS

### Chromosomal Mutants Resistant to Bacteriophage Q $\beta$ .

We identified chromosomal mutations affecting F-plasmid functions by employing criteria previously used to identify F-plasmid mutations that prevent the formation of F-pili. These criteria include resistance to donor-specific bacteriophages, such as Q $\beta$ , which adsorb to F-pili, and loss of conjugal donor activity (21, 27, 28). Approximately 1000 Q $\beta$ -resistant colonies of the F' strain AE3087 were identified as described in *Materials and Methods*. Five of these were temperature-sensitive, plating Q $\beta$  at 34°C but not at 41°C, and these were examined further to determine whether the mutations were in chromosomal or plasmid DNA. Strain KN401 is an example of a chromosomal

mutant (Fig. 1). The F-plasmid in KN401, present during mutagenesis of its parental strain, could be replaced with the cognate but unmutagenized plasmid without restoring sensitivity to Q $\beta$  (Fig. 1 A and B); conversely, the plasmid in the mutant appeared normal when transferred at the permissive temperature to a different genetic background (Fig. 1C). A second, independently isolated mutant (KN312) had the same properties as KN401 (Table 1).

A detailed genetic analysis of KN401 and KN312 will be published elsewhere. Each is the result of a revertible mutation in a gene that is 37% cotransducible with *metB*, near 87 min

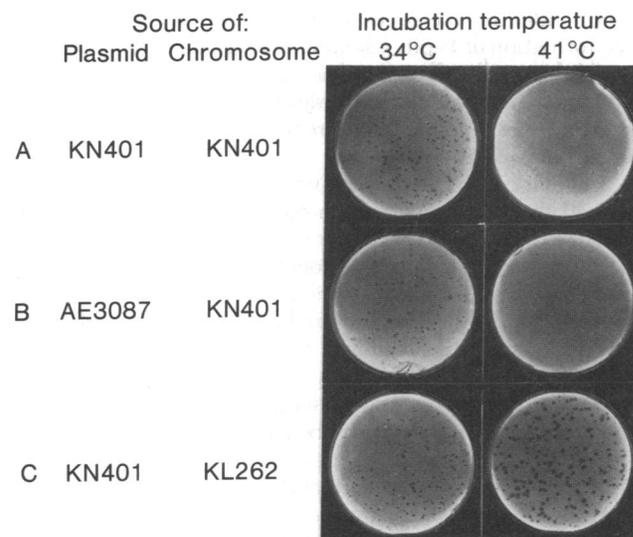


FIG. 1. A chromosomal mutant resistant to Q $\beta$ . RNA bacteriophage Q $\beta$  was plated at 34°C and at 41°C on temperature-sensitive mutant KN401 (A); a strain derived from KN401 by curing it of its original plasmid, which was present during mutagenesis, and replacing it with the cognate but unmutagenized plasmid from AE3087 (B); and strain KL262 containing the original plasmid present in mutant strain KN401 (C).

on the current *E. coli* K12 genetic map. However, the mutant phenotype also requires a mutation in a second gene that is cotransducible with *eda* and *faddD*, near 41 min on the genetic map. This mutation is already present in strain AE3087 [a derivative of KL110 (29)] and in several laboratory strains related to it (e.g., JC411; see ref. 29 for the genealogy of these strains); however, it is cryptic in the absence of the second mutation.

To designate chromosomal mutations as distinct from plasmid mutations with the same phenotype, we suggest that chromosomal genes identified by such mutations be referred to as *cpx* (conjugative plasmid expression). Hence, we refer to the two genes we have identified as *cpxA* (87 min) and *cpxB* (41 min). The studies reported here describe the effects of *cpxA* mutations in a *cpxB* mutant background on the expression of F-plasmid *tra* gene functions in otherwise isogenic strains (Table 1).

**Effect of *cpxA1* Mutation on F-pili Formation.** At 41°C *cpxA* mutants formed Q $\beta$  infectious centers and adsorbed Q $\beta$  at low levels, not exceeding a few percent of those observed with parental cells. In the few mutant cells that could be infected by Q $\beta$  at 41°C, the yield of progeny virus per infected cell was comparable to the yield from parental cells (data not shown). These properties suggested that the mutation blocks an extracellular stage of Q $\beta$  infection. Accordingly, we examined *cpxA*<sup>+</sup> and *cpxA* mutant cells in the electron microscope for the presence of F-pili capable of adsorbing RNA bacteriophage R17. More than 60% of the *cpxA*<sup>+</sup> Hfr cells grown at 41°C had at least one attached F-pilus that adsorbed R17 over its entire length. In addition, detached F-pili, which retain phage-adsorbing activity, accumulated in the medium. Similar results were obtained when *cpxA*<sup>+</sup> cells were grown at 34°C. In contrast, few (<1%) of the mutant cells grown at the nonpermissive temperature had attached F-pili, nor did detached F-pili accumulate in the medium. At 34°C about 9% of mutant cells had attached F-pili, and detached F-pili appeared in the medium. Though relatively few mutant cells grown at 34°C have attached F-pili at a given time, they are fully sensitive to Q $\beta$ , as measured by plaque assay (Fig. 1A). Most or all mutant cells must therefore form functional F-pili at 34°C over the interval of the plaque assay.

Conjugal donor activity was also used to measure functional F-pili, because this assay is independent of RNA bacteriophage adsorption. As shown (Table 2), the donor activities of mutant cells grown at 34°C or 41°C were substantially less than those of *cpxA*<sup>+</sup> cells. In addition, the donor activity of mutant cells was 10-fold less at 41°C than at 34°C, and is therefore temperature sensitive.

The inability of mutant cells to elaborate F-pili at 41°C cannot be attributed to loss of plasmid DNA, because *cpxA* F' strains grown at 41°C become sensitive to Q $\beta$  when shifted to 34°C. Moreover, as shown above, the mutant phenotype is expressed in Hfr strains, in which a loss of F-DNA seems unlikely.

**Effect of *cpxA* Mutations on Surface Exclusion.** Donor cells are generally poor conjugal recipients (10), a phenomenon referred to as entry (30) or surface exclusion (28). A quantitative expression of this phenomenon is the ratio of recombinants formed in a cross between a standard donor and an F<sup>-</sup> recipient to those formed in a cross between the same donor and an F<sup>+</sup> or Hfr recipient (6, 31). By this criterion mutations in the *cpxA* gene decrease surface exclusion (Table 3). The mutant allele derived from KN401, *cpxA1*, decreased surface exclusion 1:25 at 41°C and the allele derived from KN312, *cpxA2*, decreased exclusion 1:140. The relative effects of the mutations were comparable at 34°C, except that surface exclusion for all of the Hfr recipients was 4- to 7-fold greater at 34°C than at 41°C.

Table 2. Donor activity of *cpxA* mutant cells

Donor genotype	% donor activity*	
	34°C	41°C
<i>cpxA</i> <sup>+</sup>	(100)	(100)
<i>cpxA1</i>	3.7 ± 1.1	0.28 ± 0.15

\* For each temperature the donor activity of *cpxA*<sup>+</sup> cells was set at 100%. The activity ( $\pm$ SD) of *cpxA1* cells of three (34°C) or four (41°C) experiments is expressed relative to this value.

At present two F-plasmid *tra* genes, *traS* and *traT*, have been identified as necessary for the full expression of surface exclusion (6, 31). The proteins encoded by these two genes act independently of each other (6) and of gene products required for DNA donor activity (31). We therefore expected that *cpxA* mutations would decrease the synthesis or function of at least one of these proteins. We chose to examine first the product of the *traT* gene, *traTp*. Most *tra* gene products appear to be localized in the cell envelope (32), but *traTp* is the only one present in sufficient quantity to be reliably detected by conventional staining methods in NaDodSO<sub>4</sub>/polyacrylamide gels (6). In the cell envelope of a strain carrying plasmid pRS31, which includes the *EcoRI* restriction fragment of F DNA that contains the *traS* and *traT* genes (6), *traTp* is a prominent protein with a mobility corresponding to a molecular weight of 27,000. As expected, it is not present in the cell envelope from the same strain carrying the pSC101 cloning vector (Fig. 2). As shown (Fig. 3), *traTp* is also a relatively prominent component of purified outer membranes isolated from Hfr *cpxA*<sup>+</sup> cells. Outer membranes from isogenic F<sup>-</sup> cells contain a small amount of a protein with the same mobility as *traTp*, apparently a chromosomal gene product (Fig. 3; see also ref. 6).

Both the *cpxA1* (Fig. 3) and *cpxA2* (not shown) mutations decreased the amount of *traTp* in purified outer membranes from Hfr cells. The decreases due to the *cpxA1* mutations were 1:3–1:4 in cells grown at 41°C or at 34°C (Fig. 4). This result is in qualitative agreement with the data in Table 3, but the effect of the *cpxA1* mutation on the amount of *traTp* in the outer membrane is less than its effect on surface exclusion itself, suggesting a nonlinear relationship between these two parameters. Achtman *et al.* (6) reported a variation in surface exclusion with plasmid composition and copy number that they correlated with the amount of *traTp* in the cell envelope. Their correlation was linear only in *traS* mutants. It is therefore possible that *cpxA* mutations also affect the expression of the *traS* gene or the function of the *traS* protein. Because *traTp* and *traSp* function independently of each other (6, 32), an effect of the mutation on both proteins would lead to a nonlinear relationship between surface exclusion and either one of them.

## DISCUSSION

This study describes the isolation and characterization of *E. coli* mutants that are unable to express F-plasmid functions in the presence of normal F-plasmid DNA. The phenotype of these mutants is similar to that of certain plasmid mutants, designated Tra<sup>-</sup>. However, the new mutations are located in chromosomal genes for which we suggest the mnemonic *cpx* (conjugative plasmid expression). The mutants we have described identify two *cpx* genes, *cpxA* and *cpxB*, located at 87 min and 41 min, respectively, on the current genetic map (33) of *E. coli* K12 (unpublished results).

Other reports have described chromosomal mutants with altered F-plasmid functions (11–14). However, none of these mutants was obtained by selecting for this phenotype and none of the genes identified appears identical to either *cpxA* or *cpxB*.

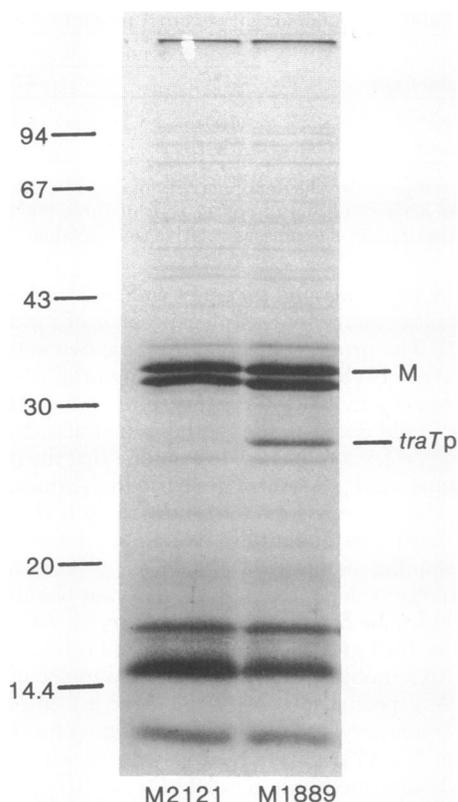


FIG. 2. Identification of *traTp* as an envelope protein. NaDodSO<sub>4</sub> gel electropherograms of envelope proteins from strain M2121, which carries pSC101, and strain M1889, which carries pRS31. Total envelope fractions were prepared by sonic oscillation of spheroplasts and high-speed sedimentation as described by Osborn *et al.* (23). Proteins were resolved by NaDodSO<sub>4</sub> gel electrophoresis and stained. The numbers to the left are the molecular weights (in kilodaltons) of standard proteins with the indicated mobilities. The product of the pRS31 *traT* gene (*traTp*) appears in the envelope of strain 1889, as indicated. The band designated M in this figure and in Fig. 4 contains matrix proteins and is indicated for comparison.

Iyer *et al.* (13) and Palchoudhury and Iyer (12) reported that the *dnaB43* mutation reduced F-pili formation and surface exclusion independent of its effect on DNA replication and growth. More recently, Lerner and Zinder (14) identified another chromosomal mutation in a gene they called *fex* (*f* expression) that reduced conjugal donor activity. They mapped *fex* close to 0 min on the *E. coli* K12 genetic map. In addition to these two reports, L. Beutin and M. Achtman (36) identified two chromosomal genes, *sfrA* (*sex factor regulation*) and *sfrB* required for the expression of F-plasmid functions. The *fex* and *sfrA* genes and a gene we refer to as *cpxC* (unpublished results) appear to be identical.

Table 3. Effect of *cpxA* mutations on surface exclusion

Recipient genotype	Recipient mating type	Surface exclusion*	
		41°C	34°C
<i>cpxA</i> <sup>+</sup>	F <sup>-</sup>	(1.0)	(1.0)
<i>cpxA</i> <sup>+</sup>	Hfr	563 <sup>†</sup>	2,111
<i>cpxA1</i>	Hfr	22	158
<i>cpxA2</i>	Hfr	4	19

\* Recombinants formed with F<sup>-</sup> recipient.

† Values are the means of three (41°C) or two (34°C) experiments.

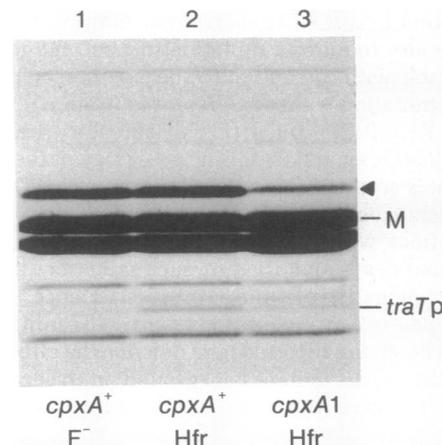


FIG. 3. Effect of a *cpxA* mutation on the amount of *traTp* in the outer membrane. Outer membrane fractions were prepared and the proteins were resolved by gel electrophoresis. Lanes: 1, strain AE2000; 2, strain AE1010; 3, strain AE1018. Only the relevant portion of each gel is shown. The mobility of the band labeled *traTp* was identical to the mobility of *traTp* in Fig. 2. The filled arrowhead shows another outer membrane protein affected by the *cpxA* mutation, as discussed further in the text.

Both DNA donor activity and surface exclusion are decreased in *cpxA cpxB* mutants. Each of these properties requires expression of a different set of plasmid structural genes located in the *tra* region of plasmid DNA. Each requires the presence of a distinct cell envelope component determined by the products of these genes: F-pili, required for DNA donor activity as well as for sensitivity to donor-specific bacteriophage, and *traTp*, required along with the *traS* gene product for the full expression of surface exclusion (6). These components, and the corresponding donor cell properties, can be manifested independently of each other (6, 31). Hence, the *cpxA* and *cpxB* mutations must affect more than one *tra* gene product.

The mutants we have described were selected for resistance to Q $\beta$  and hence for defects in the formation of F-pili. As noted above, surface exclusion can be manifested independently of F-pili formation. Therefore, it is not obvious why surface exclusion should be decreased in mutant cells. One hypothesis is based on the fact that most *tra* genes of F, including both genes required for surface exclusion and several required for the formation of F-pili, are organized into a single transcriptional unit (34). This unit is thought to be positively regulated at the transcriptional level by the product of the *traJ* gene or a derivative of it (35). Chromosomal gene products might also regulate *tra* gene expression either as independent elements or as part of the *traJ* system. This hypothesis is supported by the observation that mutant cells carrying pRS31, which contains the *traT* and *traS* genes but probably not the genetic regulatory elements of the *tra* region, exhibit levels of surface exclusion indistinguishable from those of wild-type cells carrying the same plasmid (unpublished results).

As noted above (Fig. 3), alterations in cell envelope protein composition caused by the *cpxA* and *cpxB* mutations extend to proteins present both in donor and F<sup>-</sup> cells. These alterations involve both inner and outer membrane proteins (unpublished results). Possibly, interactions between one or more *tra* gene products and cell envelope components altered by the *cpxA* and *cpxB* mutations are essential for expression of the *tra* transcriptional unit. The product of the *traJ* gene is a good candidate because it appears to be essential for expression of most *tra* genes (35) and it is an envelope component (32). At present, the relationship, if any, between these two properties of *traJp* is not clear.

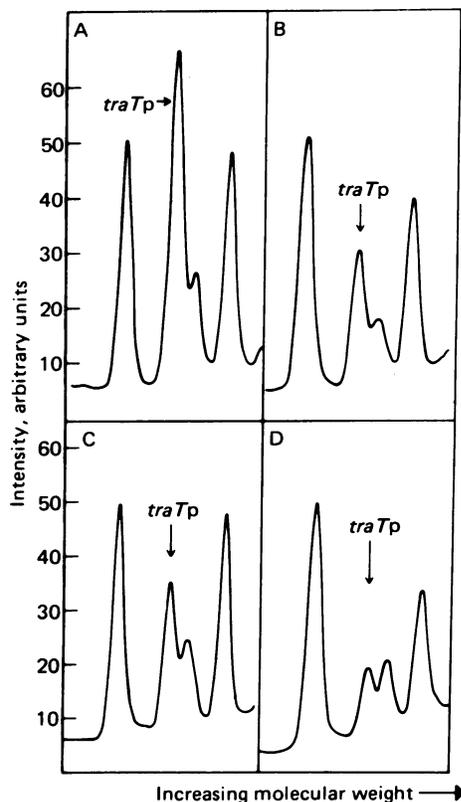


FIG. 4. Densitometric analysis of *traTp* content of outer membrane fractions. Outer membrane was isolated from strains AE1010 (A and B) and AE1018 (C and D) grown at 34°C (A and C) or 41°C (B and D), and the proteins were resolved by gel electrophoresis and stained. Densitometer tracings were prepared from the region containing *traTp* (see Fig. 3) by normalizing the peak intensity of the first band (lowest molecular weight protein) to 50. The normalization involves only slight adjustments ( $\pm 10\%$ ) when samples to be compared are derived from cells grown at the same temperature. The areas under the *traTp* peaks, obtained from an integrator tracing, were used to estimate the effect of the *cpxA1* mutation on the *traTp* content of the outer membrane at each growth temperature (see text). For this estimate, the amount of material with the electrophoretic mobility of *traTp* in the outer membrane of  $F^-$  cells was subtracted.

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