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Plasmid-Determined Immunity of *Escherichia coli* K-12 to Colicin Ia Is Mediated by a Plasmid-Encoded Membrane Protein

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The colicin Ia structural (*cia*) and immunity (*iia*) genes of plasmid pColIa-CA53 have been cloned into the cloning vector pBR322. These two genes are closely linked, and both of them can be isolated on a deoxyribonucleic acid fragment approximately 4,800 base pairs long. An analysis of the polypeptides synthesized in ultraviolet-irradiated cells containing these cloned genes led to the conclusion that the *iia* gene product is a polypeptide with a molecular weight of approximately 14,500. Insertion of transposon Tn*5* into the *iia* gene led to a concomitant loss of the immune phenotype and the ability to produce this protein. Fractionation of ultraviolet-irradiated cells harboring a plasmid carrying the *iia* gene showed that the immunity protein is a component of the inner (cytoplasmic) membrane. Furthermore, the mechanism of immunity to colicin Ia appears to operate at the level of the cytoplasmic membrane. This conclusion is based on our finding that membrane vesicles prepared from colicin Ia-immune cells could be depolarized by colicins E1 and Ib but not by colicin Ia.

Although the mechanism by which colicins E1, Ia, Ib, and K inhibit energy transduction in treated cells has been elucidated (13, 20, 27, 41, 48), it is not known how a cell which harbors a particular plasmid encoding any one of these colicins is able to protect itself specifically against the colicin which it produces while it remains sensitive to heterologous colicins of identical mode of action. This phenomenon, termed colicin immunity, is a general property of colicinogenic cells (22).

With regard to colicins E2 and E3 and cloacin DF13, the mechanism of immunity is better understood. Each of these bacteriocins, whose targets are intracellular (colicin E2 is a DNase, whereas colicin E3 and cloacin DF13 act on ribosomes, leading to cleavage of 16S rRNA), interacts specifically with a small cytoplasmic protein (immunity protein), which is encoded by the same plasmid that determines that colicin (8, 17, 40, 43). Thus, cells harboring the ColE2 plasmid are able to neutralize the activity of both endogenous and exogenous colicin E2 but not colicin E3 and vice versa. In the case of colicins E1, K, Ia, and Ib, all of which act by forming aqueous channels in bacterial cytoplasmic membranes, no such immunity protein has been isolated. Although several studies have suggested that the plasmid ColE1 gene which determines immunity to colicin E1 encodes a polypeptide with a molecular weight of approximately 13,000 to 14,500 (10, 16, 35, 37), no information is available concerning the possible role of this polypeptide in mediating immunity. In the cases which have been examined (colicins E2, E3, Ia, Ib, and E1), there is convincing evidence that immunity is not mediated by change in the ability of colicinogenic cells to adsorb colicin to cognate outer membrane receptors (24, 32).

Whatever the immunity mechanism, the molecular interactions involved must be highly specific. For example, strains harboring the ColIa-CA53 plasmid are immune to colicin Ia yet sensitive to colicin Ib and vice versa (B. A. D. Stocker, Microb. Genet. Bull. **23**:11, 1965), even though these colicins share many physical and chemical properties, exhibit extensive but not complete sequence homology in their primary structures, and have common antigenic determinants (21). Furthermore, both colicin Ia and colicin Ib absorb to the same bacterial receptor (24) and act by depolarizing cytoplasmic membranes through the formation of aqueous channels (27, 48). No bacterial mutant which is insensitive to one colicin but not the other has been described (3, 7). In view of these similarities, it seems reasonable that immunity to each of these colicins does not involve a plasmid-determined alteration in some step in its mode of action sequence but derives from some specific interaction between a plasmid-encoded

gene product and the colicin molecules. In this paper we describe the identification and subcellular localization of the pColIa immunity gene product.

MATERIALS AND METHODS

Strains and media. Strain JK16 (*Escherichia coli* K-12 WE110 *rspL* ColIa-CA53) was the source of both the ColIa-CA53 plasmid and colicin Ia. Colicin Ib was prepared from strain JK20 (*E. coli* K-12 W3110 *rspL* ColIb-P9) (25). Colicin E1 was a kind gift from W. Cramer (Purdue University, West Lafayette, Ind.). Strain CSR603 (CGSC 5830; relevant markers, *phr*, *recA*, and *uvrA*), which was used to identify plasmid-determined polypeptides, was obtained from B. Bachmann. Strain D6434, which was used to propagate phage λ, was supplied by P. Bassford (University of North Carolina, Chapel Hill). All other experiments were performed with strain JK362 (*E. coli* K-12 294 *endA hsdR thi pro*), which was obtained from K. Backman (Massachusetts Institute of Technology, Cambridge), or its colicin-resistant derivative, strain JK381.

M63 and M9 salts media and TB and LB broths were prepared as described by Miller (34). For selection or screening of antibiotic-resistant bacteria, ampicillin, tetracycline hydrochloride, and kanamycin sulfate (all from Sigma Chemical Co., St. Louis, Mo.) were included in agar (LB broth containing 1.5% [wt/wt] agar) at concentrations of 20 μg/ml. The sulfur-free Hershey salts medium (51) that was used in experiments with UV-irradiated cells contained 0.1 M Tris-hydrochloride (pH 7.4), 0.09 M NaCl, 0.04 M KCl, 0.02 M NH_4Cl, 0.64 mM KH_2PO_4, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, and 0.2% (wt/vol) glucose. For strain CSR603, 100 μg of threonine per ml, 100 μg of leucine per ml, 200 μ of proline per ml, 200 μg of arginine per ml, and 1 μg of vitamin B_1 per ml were included.

Colicin assays. To determine the capacity of whole cells to produce colicin, cultures were stabbed into tryptone plates (1.5% agar) containing 0.5 μg of mitomycin C (Calbiochem, La Jolla, Calif.) per ml. After incubation at 37°C for 12 to 18 h, the cells were killed by chloroform vapors, and the plates were overlaid with 2 to 3 ml of tryptone soft agar (0.7% agar) containing 2 to 3 drops of an overnight culture of strain JK362. Colicin-producing colonies gave a clear killing zone around each cell colony. Immunity to colicin Ia was defined as insensitivity of a strain to colicin Ia but sensitivity to colicin Ib-P9. In all cases immunity was complete in that all immune strains were insensitive to colicin Ia at even the highest concentrations tested.

Plasmid isolation. Plasmid ColIa-CA53 was prepared from a late-log-phase culture of strain JK16 grown in TB medium (34) supplemented with 20 mM glucose. The plasmid was extracted as described by Hansen and Olsen (14), except that excess polyethylene glycol was removed by gel filtration on Bio-Gel A50 (Bio-Rad Laboratories, Richmond, Calif.) before the sample was subjected to dye-buoyant density centrifugation in a cesium chloride gradient containing propidium diiodide for isolation of supercoiled DNA (2). Plasmid DNAs from strains carrying pBR322 or its derivatives were extracted by the cleared lysate method of Clewell and Helinski after amplification of plasmid DNA by chloramphenicol treatment (6). Supercoiled DNA was prepared as described above. Rapid analyses of plasmid DNAs from small volumes of cultures were performed by the method of Kahn et al. (19).

Restriction, ligation, and transformation. Usually, digestion of DNA with restriction endonucleases was carried out under the conditions suggested by the commercial enzyme source (Bethesda Research Laboratories, Rockville, Md.; New England Biolabs; or Boehringer Mannheim Corp., Indianapolis, Ind.). Digestion reactions were stopped by heating for 5 to 10 min at 65°C. Ligation of DNA fragments was performed in 6 mM Tris-hydrochloride (pH 7.9)–6 mM $MgCl_2$–6 mM β-mercaptoethanol–1 mM ATP by using T4 DNA ligase, as directed by the supplier (New England Biolabs or Boehringer Mannheim). Transformation of competent cells and selection of transformed bacteria with the desired phenotype were done as previously described (2).

Electrophoresis of DNA. The sizes and digestion patterns of DNA fragments were analyzed by gel electrophoresis in 0.8 or 1.0% agarose and 5, 8, or 12% polyacrylamide slab gels run in Tris-borate buffer (2).

Mapping of restriction sites. Restriction sites were mapped by examining the sizes of singly or doubly digested whole plasmid or purified fragments.

Tn*5* mutagenesis. The source of the transposon was bacteriophage λ::Tn*5* (c1857 *rex*::Tn*5* Oam29 Pam80 *b*221), which was obtained from P. Bassford. Strain D6434 was used for propagating λ::Tn*5* by the procedure described by Miller (34). A 1-ml amount of strain CSR603(pAR6) growing exponentially in LB broth containing 0.2% (wt/vol) maltose at 37°C (approximately 2×10^8 cells) was mixed with 1 ml of λ::Tn*5* (multiplicity of infection, 10) in the presence of 10 mM $MgSO_4$. Incubation at 37°C was continued for 30 min. Samples (1×10^7 to 2×10^7 cells) were spread onto plates containing LB agar supplemented with kanamycin and incubated at 42°C overnight. Approximately 100 colonies were found per plate; this resulted in a total of approximately 1,200 independent Tn*5* insertions into chromosomal or plasmid DNA. The colonies were then washed off the plates with a small volume of LB broth, and the pooled washes were used to inoculate 500 ml of TB broth. After growth at 37°C and amplification of plasmid DNA by chloramphenicol treatment, plasmid DNA was isolated as described above.

Identification of plasmid-encoded polypeptides. Plasmid-encoded polypeptides were identified in vivo by the method of Sancar et al. (39). Strain CSR603 carrying the plasmid under investigation was grown at 37°C with shaking to midexponential phase (90 to 100 Klett units; no. 42 filter) in M9 medium containing 1% (wt/vol) Casamino Acids, 1 μg of thiamine per ml, and 0.2% (wt/vol) glucose. Samples (10 ml) were spread evenly in the bottom of a disposable plastic petri dish (diameter, 100 mm) and irradiated with 20 J of UV light per m^2. The irradiated cells were incubated with shaking at 37°C for 1 h, and then D-cycloserine (final concentration, 200 μg/ml; Sigma) was added to kill any growing cells. Incubation was

continued overnight at 37°C. The survivors, which were assayed after overnight incubation, generally amounted to no more than 100 cells per ml, as determined by plating samples onto LB agar. The cells were then harvested, washed twice with an equal volume of sulfur-free Hershey salts medium (see above), and resuspended in 0.5 volume of the same medium. The cells were starved for sulfur by incubating them for 1 h at 37°C, and then they were labeled with 5 μCi of [^{35}S]methionine (1,300 Ci/mmol) per ml for 1 h. Except in the case of preparations that were destined for fractionation, harvested labeled cells were suspended in 50 mM Tris-hydrochloride (pH 8.0) (0.1 ml per original ml of growing cells) and stored frozen at −20°C.

Fractionation of irradiated labeled cells. A 50-ml culture of strain CSR603(pCA6) was grown in M9 medium, irradiated in 10-ml portions, and labeled with [^{35}S]methionine as described above. Cells were converted to spheroplasts and were separated into soluble and membrane fractions by using a modification of previously described procedures (49, 50).

Harvested irradiated labeled cells were suspended in 1 ml of 0.2 M Tris-hydrochloride (pH 8.0) at room temperature, and 1 mM EDTA (pH 7.6; potassium salt) was added. After 1 min, 1 ml of 0.2 M Tris-hydrochloride (pH 8.0)–1 M sucrose was added, and this was followed 1.5 min later by the addition of 20 μl of a freshly prepared lysozyme solution (6 mg/ml). After 1 min the cells were subjected to a mild osmotic shock by the addition of an equal volume (2 ml) of distilled water and further incubation for 30 min at room temperature. Then 10 mM $MgCl_2$ was added, and the spheroplasts were collected by centrifugation at 5,000 × *g* for 10 min. The resulting pellet was suspended in 0.5 ml of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) buffer (Sigma) adjusted to pH 7.4 with NaOH. The spheroplasts were disrupted by one passage through an ice-cold French pressure cell at 12,000 lb/in^2. After the addition of 20 μg of RNase A (10 μl of a 2-mg/ml solution), the unbroken spheroplasts and debris were removed by low-speed centrifugation (1,000 × *g*) for 30 min. The supernatant fraction was incubated on ice overnight.

On the next day, ^{125}I-labeled colicin Ib (33,000 cpm; 0.26 μg [24]) was added to the supernatant, and the mixture was incubated at 37°C for 10 min. The entire sample (0.5 ml) was then loaded on top of a sucrose step gradient (1 ml of 2.02 M sucrose, 1.5 ml of 1.44 M sucrose, and 2.5 ml of 0.77 M sucrose, all in 10 mM HEPES buffer) in a cellulose nitrate centrifuge tube. Centrifugation was for 4 h at 40,000 rpm in a Beckman SW50.1 rotor at 4°C. After completion of the centrifugation, the gradient, which contained two visible opaque bands, was fractionated from the bottom by collecting 0.22-ml samples.

The amount of ^{125}I-labeled colicin Ib radioactivity (a marker for the outer membrane [24]) in each fraction was determined directly with a gamma counter, and the amount of ^{35}S was determined by spotting 5 μl of each fraction onto glass fiber filters and counting in a liquid scintillation spectrometer. NADH oxidase activity (a marker for the inner membrane [36]) was determined after 0.2 ml of each fraction was added to 2.3 ml of 10 mM HEPES buffer; 5 mM NADH (disodium salt; grade III; Sigma) freshly prepared in 10 mM Tris-hydrochloride (pH 8.0) was then added, and oxygen utilization at 37°C was followed by using an oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio). For calculations we used a value of 470 ng-atoms of oxygen per ml of air-saturated buffer (5).

The total protein content of each diluted sample was determined after sodium deoxycholate-trichloroacetic acid precipitation (1). This was necessary due to interference by sucrose and HEPES buffer in the assay of protein by the method of Lowry et al. (31).

To prepare for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the protein in a 600-μl sample of each fraction (containing 15 μg of ovalbumin carrier) was precipitated by slowly adding 2 volumes of cold acetone. After incubation on ice for 30 min, the resulting precipitates were collected by centrifugation at 10,000 × *g* for 15 min, dried under a vacuum, and suspended in 50 μl of a 1:1 mixture of SDS sample buffer and 50 mM Tris-hydrochloride (pH 8.0).

Vesicles. Cytoplasmic membrane vesicles were prepared from strains as previously described (46). The medium used for growing the cells was M63 medium containing 0.3% (wt/vol) yeast extract and 0.2% (wt/vol) glucose 6-phosphate. Incorporation of colicins into these vesicles was performed by the freeze-thaw method (46). The accumulation of [^{3}H]-triphenylmethyl phosphonium ions ($TPMP^+$; 80 Ci/mol; final concentration, 0.15 mM; New England Nuclear Corp., Boston, Mass.) was driven by 20 mM ascorbate (potassium salt; pH 7.0; Sigma)–0.1 mM phenazine methosulfate (Sigma) under a stream of oxygen. Protein was determined by the method of Lowry et al. (31), using bovine serum albumin as a standard.

SDS-PAGE. Polypeptides were separated by slab SDS-PAGE (gel length, 200 mm; gel thickness, 0.75 mm), using minor modifications of the method described by Laemmli (28). The buffer in the running gel contained 0.375 M Tris-hydrochloride (pH 8.8), 0.1% (wt/vol) SDS, 0.025% *N,N,N',N'*-tetramethylethylenediamine, and 0.2 mg of ammonium persulfate per ml. The linear gradient running gel contained 12.5 to 15% acrylamide (0.63 to 0.75% cross-linker) in a 0 to 20% (wt/vol) sucrose gradient for stabilization. The stacking gel contained 0.125 M Tris-hydrochloride (pH 6.8), 0.1% SDS, 0.04% *N,N,N',N'*-tetramethylethylenediamine, 0.3 mg of persulfate per ml, and 6% acrylamide (with 0.30% cross-linker).

Protein samples were processed by adding equal volumes of SDS sample buffer (0.167 M Tris-hydrochloride, pH 6.8, 33% [vol/vol] glycerol, 3.33% [wt/vol] SDS, 0.5 M 2-mercaptoethanol, and a trace of bromophenol blue) and incubating these preparations in a boiling water bath for 3 min. Whole-cell samples (UV-irradiated cells) were mixed with equal volumes of SDS sample buffer and incubated for 5 min in a boiling water bath before being centrifuged at 12,000 × *g* for 5 min to remove insoluble material. Samples were loaded into the wells of the stacking gel under the running buffer.

Molecular standards included the following: ^{14}C-la-

beled methyl derivatives obtained from New England Nuclear Corp., phosphorylase β (molecular weight, 92,500), bovine serum albumin (69,000), ovalbumin (46,000), and carbonic anhydrase (30,000). In addition, [2,3-^{3}H]propionyl-labeled lysozyme (molecular weight, 14,300; prepared by T. Davis, University of Illinois, Urbana) was included. Depending on the expected length of the film exposure, 1 to 10,000 cpm of the ^{14}C-labeled proteins and 5 to 30,000 cpm of the ^{3}H-labeled lysozyme were included per gel.

Gels were run at 9 to 15 mA and 4°C in buffer containing 0.025 M Tris base, 0.192 M glycine, and 0.1% (wt/vol) SDS. Electrophoresis was terminated when the dye front reached the bottom of the gel (8 to 16 h). Then the gel was soaked in 0.5 M sodium salicylate for 30 min at room temperature and dried without rinsing (4). The Kodak X-Omat XR-5 film used in the experiments designed to analyze the fractions from UV-irradiated cells was sensitized by the pre-flash method of Laskey and Mills (29). This allowed quantitation of the relative amounts of labeled polypeptides. Gels were exposed to film at −80°C for 1 to 4 days before development, as recommended by the manufacturer. The fluorograms of the gels containing the samples from the experiment in which fractions from UV-irradiated cells were used were scanned with a densitometer (E-C Apparatus Corp.) attached to a strip chart recorder (Heath-Schlumberger). The individual peaks were cut out and weighed to determine the amount of each polypeptide. Since the large number of samples analyzed required the use of three separate films, small differences in exposure or development were corrected for by normalizing the data to an identical amount of ^{3}H-labeled lysozyme which was run on each of the three gels; 1 U was defined as 100 times the ratio of the amount of material found in the peak corresponding to the TET or IMM protein divided by the amount of material in the ^{3}H-labeled lysozyme peak.

RESULTS

Cloning of *cia* and *iia* genes. Plasmid ColIa-CA53 DNA was purified by dye-buoyant density gradient centrifugation and was digested with restriction endonuclease *Eco*RI. The resulting fragments were ligated into the single *Eco*RI site of the plasmid vector pBR322, and the resulting mixture was transformed into strain JK362 with selection for tetracycline resistance. Of the 34 tetracycline-resistant clones isolated, 8 produced colicin Ia. These same eight clones were not sensitive to colicin Ia, yet they were fully sensitive to colicin Ib; thus, these clones were scored as colicin Ia immune. One of these clones was chosen for further study.

This clone harbored plasmid pJK1. When isolated and transformed into strain JK362, this plasmid conferred resistance to tetracycline and ampicillin, as well as the ability to produce colicin Ia and Ia immunity. Characterization of plasmid pJK1 showed that it contained two *Eco*RI fragments approximately 6,500 base pairs (bp) long that originated from pColIa-CA53, as well as the 4,360-bp fragment of the pBR322 vector. To generate a plasmid containing only one pColIa-CA53-derived *Eco*RI fragment, we subjected pJK1 to a round of *Eco*RI digestion and ligation and transformed it into strain JK362. One colicin-producing clone contained a plasmid, which was designated pJK5.

Upon cleavage with *Eco*RI, pJK5 yielded DNA fragments which were approximately 6,600 (pColIa-CA53 derived) and 4,360 (vector derived) bp long. The important features of the restriction map of pJK5 which were used for subsequent subcloning are a single *Kpn*I site and the two mapped *Cla*I sites (Fig. 1). An additional, unmapped *Cla*I site resides in the region between the rightmost *Cla*I and *Eco*RI sites. Although an additional *Cla*I site is located in the vector, pBR322 has no recognition sequence for *Kpn*I.

To determine the importance of maintaining the continuity of the DNA sequence across the *Kpn*I site in either colicinogeny or immunity, we inserted the small 1,521-bp *Kpn*I fragment of bacteriophage λ DNA (45) into the unique *Kpn*I site of pJK5. This was accomplished by codigestion of phage λ and pJK5 DNA with *Kpn*I, followed by ligation and transformation with selection for tetracyline resistance. One of the resulting clones was immune to colicin Ia, although it did not produce colicin. An analysis of the plasmid (designated pKP37) (Fig. 1) in this strain showed that the expected insertion event had occurred; that is, restriction of pKP37 with *Kpn*I yielded both a 1,521-bp fragment and a fragment which was indistinguishable from

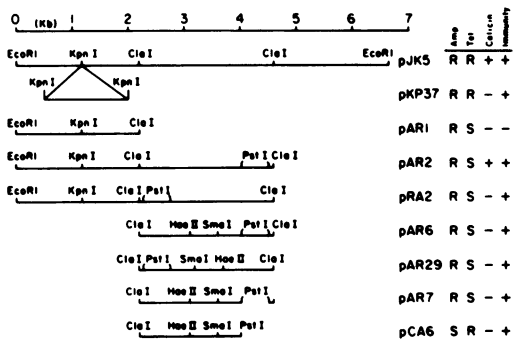

FIG. 1. *Structure of cloned pColIa-CA53 DNA. Cloning procedures are described in the text. For each plasmid, the ends of the fragment shown are joined by pBR322 DNA sequences such that the tet gene lies immediately to the right and the bla gene lies immediately to the left of the ends shown. (Plasmid pCA6 is an exception [see text and Fig. 2].) Plasmid phenotypes and partial restriction maps were determined as described in the text. Amp, Ampicillin; Tet, tetracycline; R, resistant; S, sensitive; Kb, kilobases.*

*Kpn*I-treated pJK5. The simplest explanation for these results is that the *Kpn*I site in pJK5 is either in the colicin Ia structural gene (*cia*) or in a gene or regulatory sequence involved in the expression of this gene. Since the insertion had no effect on immunity, it is likely that expression of the immunity gene (*iia*) is either under control of a promoter which is separate from the promoter controlling *cia* or that both *cia* and *iia* genes are under a common promoter, with *iia* as the promoter proximal gene.

Plasmids resulting from deletions of various *Cla*I fragments of pJK5 were isolated. Plasmid pJK5 was digested partially with *Cla*I and transformed directly into strain JK362 (without prior ligation) with selection for ampicillin resistance. This was necessary since the unique *Cla*I site in pBR322 is probably within the promoter for the tetracycline resistance gene at position 23 of the previously published sequence (44). Characterization of the resulting clones led to the identification of two useful plasmids, pAR1 and pAR2 (Fig. 1). Although strains carrying pAR2 produced colicin and were immune, pAR1 conferred neither phenotype. As expected, neither phenotype determined resistance to tetracycline.

Digestion of pAR2 with *Cla*I followed by ligation led to the generation of pRA2, which conferred immunity but not colicin production. Restriction analysis of pRA2 showed that it was identical to pAR2, except that the smaller *Cla*I fragment was in the opposite orientation. These results demonstrated that the continuity of the leftmost *Cla*I site in pJK5 and pAR2 is necessary for colicin production. Furthermore, the internal *Cla*I fragment in pJK5 must carry all of the information necessary to determine colicin immunity.

The internal (immunity-determining) 2,400-bp *Cla*I fragment of pAR2 was next cloned into the *Cla*I site of pBR322. The ampicillin-resistant transformants which carried either of the resulting plasmids (pAR6 and pAR29) were colicin Ia immune and, as expected, sensitive to tetracycline. Restriction analyses showed that these plasmids differed only in the orientation of the cloned *Cla*I fragment (Fig. 1).

We found that deletion of the region limited by the two indicated *Pst*I sites in pAR6 led to the construction of plasmid pAR7, which was fully able to confer immunity. This plasmid was derived by *Pst*I restriction of pAR6 followed by ligation and transformation with selection for ampicillin. This selection insured religation of the *Pst*I-generated ends within the β-lactamase gene of the pBR322 vector. It should be pointed out the region between the indicated *Pst*I sites in pAR6 contain two additional but unmapped *Pst*I sites.

In view of our attempt to identify the immunity gene product (see below), we thought it advisable to prepare a plasmid that contained the immunity gene but did not express β-lactamase. To construct such a plasmid, the *Pst*I-*Eco*RI fragment of pAR6 which determined immunity was ligated to *Eco*RI/*Pst*I-restricted pBR322, and the mixture was transformed into strain JK362 with selection for tetracycline resistance (Fig. 2). Characterization of the resulting clones showed that one of them contained a plasmid having the structure shown in Fig. 2. Cells containing this plasmid were tetracycline resistant, ampicillin sensitive, and immune to colicin Ia.

It should be noted that in the cases tested (pJK1, pJK5, and pAR2) colicin Ia production was inducible by mitomycin C. Although the induced levels of colicin made in strains carrying the cloned colicin Ia structural gene were approximately fourfold higher than the levels in induced cells harboring pColIa-CA53, we did not determine whether this difference reflected an influence of cloning on gene expression per se. With regard to immunity, pColIa-CA53 and all of the constructed plasmids carrying the cloned *iia*$^+$ gene were equally immune at all colicin concentrations tested.

Identification of the *iia* gene product. The polypeptides encoded by the plasmid constructed as described above were identified by using the system of Sancar et al. (39) (Fig. 3). Whereas UV-treated strain CSR603 cells containing pBR322 synthesized a 37,000-dalton *tet* product (TET protein) and a 30,000-dalton *bla*

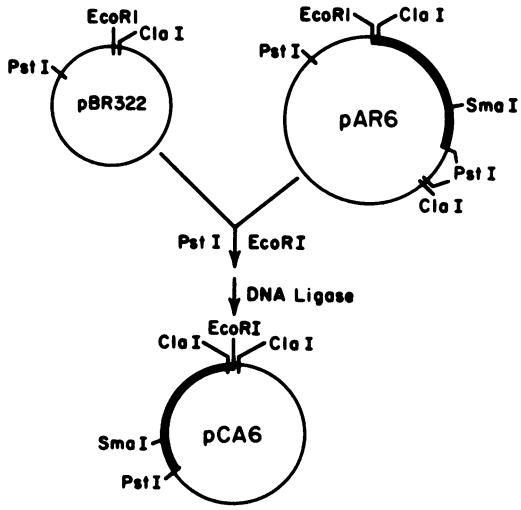

FIG. 2. *Construction of pCA6. The shaded region indicates the part of pAR6 that was cloned into the PstI-EcoRI site of pBR322 to produce pCA6.*

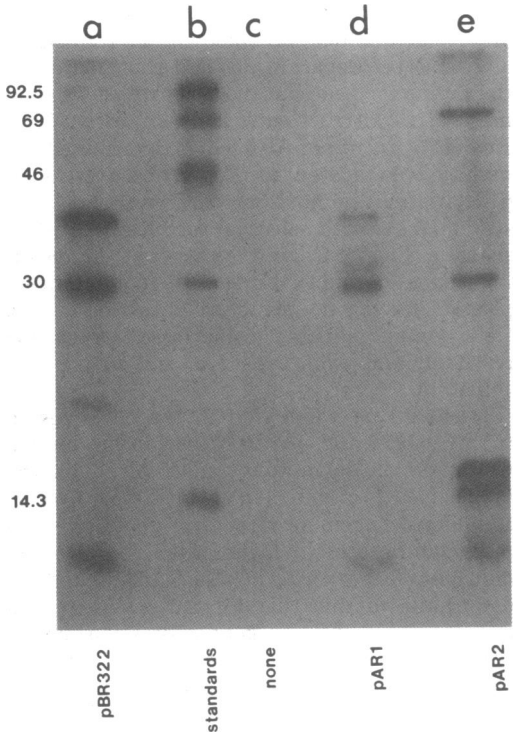

FIG. 3. *Polypeptides encoded by pBR322, pAR1, and pAR2. Labeled UV-irradiated cells were prepared and analyzed by SDS-PAGE autoradiography as described in the text. The molecular weights (×10³) of the protein standards are shown at the left. Approximately 3 μl of cell material was applied to each lane.*

product (β-lactamase) (39), as well as unidentified polypeptides having molecular weights of about 23,000 and 12,000, cells containing no plasmid produced no labeled polypeptides (Fig. 3, lane c). As expected, cells harboring pAR2 produced β-lactamase, but not the TET protein. In addition, this plasmid determined polypeptides with molecular weight of 69,000, 15,500, and 14,500. The 69,000-dalton polypeptide was probably colicin Ia, which is known to migrate to exactly this position in this gel system. Furthermore, this polypeptide was absent from cells containing plasmids which did not determine colicin Ia production. For example, this polypeptide (as well as the 15,500- and 14,500-dalton proteins) were absent from cells harboring pAR1. It is possible that the 37,000-dalton protein observed in this lane resulted from low-level expression of the *tet* gene or was a truncated colicin Ia protein. As expected, pAR1 determined β-lactamase. Figure 4 shows the polypeptides synthesized in UV-treated cells containing pKP37. As Fig. 4 shows, the 69,000-dalton polypeptide was absent. This provides further evidence that this protein is colicin Ia.

As described above (Fig. 3), pAR2 encoded pColIa-CA53-derived polypeptides with molecular weights of 69,000, 15,500, and 14,500. In contrast, although plasmids pAR6 and pAR29 determined vector-derived β-lactamase and the 12,000-dalton polypeptide, they encoded only the 14,500-dalton polypeptide (Fig. 4). The apparent extra bands found in the case of pAR29 resulted from the fact that the sample applied to this lane contained more radioactivity than the samples applied to other lanes. Finally, pCA6 directed the synthesis of vector-derived TET and 12,000-dalton proteins and the 14,500-dalton polypeptide that was determined by all *iia*⁺ plasmids. The additional minor band that migrated as a protein with a molecular weight of 19,000 was not identified.

The complete correspondence between the ability of a particular plasmid to confer colicin Ia immunity and its ability to direct the synthesis of a 14,500-dalton polypeptide strongly suggests that this protein is responsible for the colicin-immune phenotype. Completely analogous results were obtained in experiments in which we analyzed plasmid-directed proteins synthesized in an in vitro transcription-translation system (C. A. Weaver, Ph.D. thesis, University of Illinois, Urbana, 1981).

Isolation of Tn*5* insertions into the *iia* gene. To confirm the identification of the *iia* gene product, we examined the results of Tn*5*

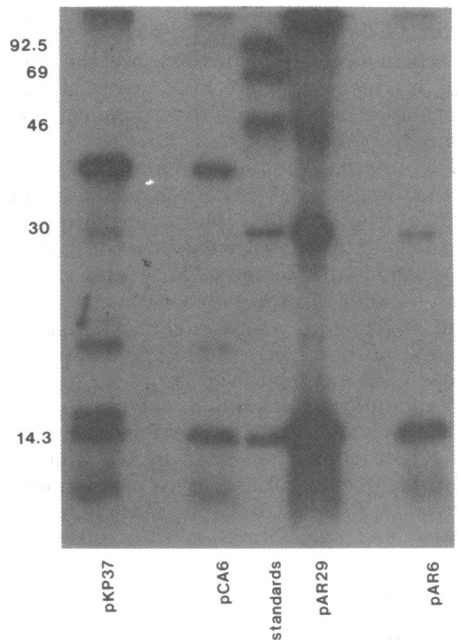

FIG. 4. *Polypeptides encoded by pKP37, pAR6, pAR29, and pCA6. Analyses were performed as described in the legend to Fig. 3.*

insertions into the DNA sequence that determine immunity. This was accomplished by mixing strain CSR603 carrying pAR6 with bacteriophage λ::Tn*5* and then spreading the resulting mixture onto plates containing kanamycin. Since this phage can undergo neither lytic development nor lysogenic development in this host, colonies that appear on the plates containing kanamycin must derive from transposition of the Tn*5* element from phage DNA to host DNA. Transformation of the plasmid DNA isolated from such cells into a new host (in this case strain JK362) with selection for kanamycin resistance should lead to the isolation of clones harboring derivatives of pAR6 containing a Tn*5* insertion. Using this procedure, we isolated several strains containing pAR6 *iia*::Tn*5* and, for control purposes, pAR6 *bla*::Tn*5* plasmids. Using the established restriction sites in Tn*5* (18, 52), we determined the precise site of insertion of this transposon into pAR6 (Fig. 5). As Fig. 5 shows, pCA33 (nonimmune phenotype) arose by insertion into the 2,400-bp *Cla*I fragment of pAR6, which, as previously shown, confers the colicin Ia immunity phenotype. On the other hand, pCA35 (ampicillin-sensitive phenotype) arose by insertion into vector sequences known to determine β-lactamase production.

Figure 6 shows an analysis of the polypeptides encoded by pAR6 (*iia*$^+$ *bla*$^+$), pCA33 (*iia*::Tn*5* *bla*$^+$), and pCA35 (*iia*$^+$ *bla*::Tn*5*). pCA33 and pCA35 specified polypeptides with molecular weights of 59,000, 44,000, 26,000, 19,000, and 13,500 which were not determined by pAR6. These polypeptides are presumably the polypeptides coded for by Tn*5* DNA sequences (38). The 14,500-dalton protein was absent from the pattern of pCA33 (*iia*::Tn*5*)-encoded polypeptides. The fact that this protein is specified by pCA35 (*iia*$^+$) strongly supports the conclusion that this protein is responsible for immunity to colicin Ia. It should be noted that since the gel system used in this analysis did not clearly resolve β-lactamase (28,000 daltons) from kanamycin phosphotransferase (26,000 daltons), we were not able to use this method to confirm the expected absence of β-lactamase production in the strain carrying pCA35 (*bla*::Tn*5*).

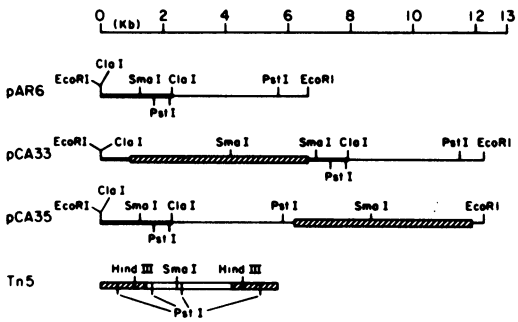

FIG. 5. *Restriction maps of pAR6, pCA33, and pCA35. Parental pAR6 and Tn5 are included for comparison. In the map of pAR6, the cross-hatching represents the cloned sequence. In the map of pCA33, the cross-hatching represents Tn5 sequences inserted into the cloned fragment. In the map of pCA35, the cross-hatching represents Tn5 sequences inserted into vector DNA. In the map of Tn5, the cross-hatching indicates the inverted repeat region of the transposon. Kb, Kilobases.*

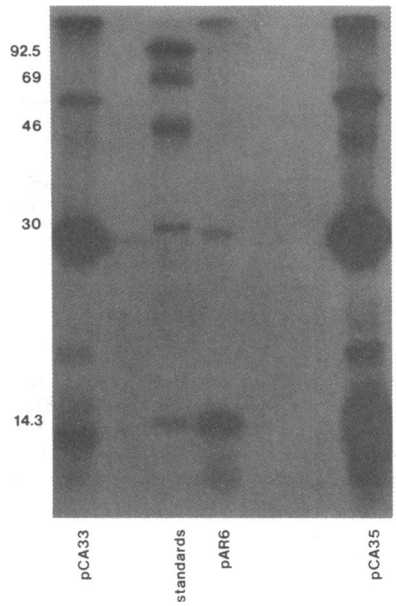

FIG. 6. *Polypeptides encoded by pAR6, pCA33, and pCA35. Analyses were performed as described in the legend to Fig. 3.*

Demonstration of colicin immunity in membrane vesicles. We wanted to determine whether colicin Ia immunity could be expressed in vitro at the level of the cytoplasmic membrane. Membrane vesicles prepared from strains harboring pKP37 (*cia*$^-$ *iia*$^+$) and pBR322 were, therefore, treated with colicins Ia, Ib, and E1 and then assayed for the ability to generate a membrane potential (accumulate TPMP$^+$) after the electron donor pair ascorbate and phenazine methosulfate was added. As Fig. 7 shows, all three colicins inhibited TPMP$^+$ uptake in control vesicles, whereas vesicles prepared from the colicin Ia-immune strain were sensitive to colicins E1 and Ib but not to colicin Ia.

Figure 8 shows the response of vesicles prepared from strain JK381 harboring either pKP37, pAR6, or pBR322 to various amounts of colicin Ia or Ib. Again, vesicles from colicin Ia-immune strains were much less sensitive to colicin Ia. The observed higher activity of colicin Ib against control vesicles was not sufficient to

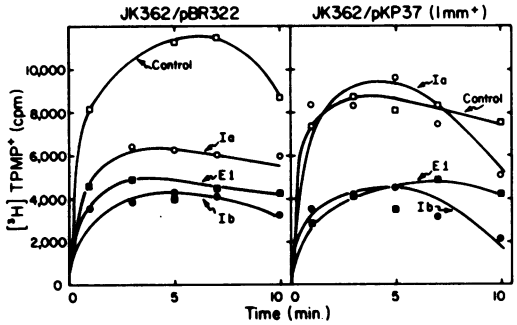

FIG. 7. *Effects of colicins Ia, Ib, and E1 on vesicles prepared from sensitive and colicin Ia-immune strains. Vesicles from strains JK362(pBR322) and JK362(pKP37) were prepared and assayed for ascorbate-phenazine methosulfate-driven TPMP+ uptake as described in the text. For each time point colicin Ia (1.12 μg), Ib (1.1 μg), or E1 (1.0 μg) was added to 50 μl of a vesicle preparation before a freeze-thaw cycle. JK362(pBR322) and JK362(pKP37) vesicle concentrations were 2.1 and 2.74 mg/ml, respectively.*

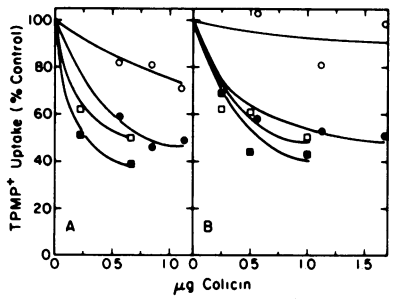

FIG. 8. *Immunity specificity in vesicles prepared from strains containing pKP37 and pAR6. Vesicles were prepared as described in the text. (A) Vesicles (50 μl) from strains JK381(pBR322) (2.38 mg/ml) (● and ■) and JK381(pKP37) (2.45 mg/ml) (○ and □) were assayed for [3H]TPMP+ uptake after a single freeze-thaw cycle in the presence of different amounts of colicin Ia (● and ○) or colicin Ib (■ and □). [3H]-TPMP+ uptake determined in the absence of colicin (100% level) was 8,430 cpm for vesicles from strain JK381(pBR322) and 7,180 cpm for vesicles from strain JK381(pKP37). (B) Vesicles (50 μl) from strain JK381(pBR322) (2.3 mg/ml) (● and ■) and strain JK3819(pAR6) (1.80 mg/ml) (○ and □) were assayed for [3H]TPMP+ uptake after a single freeze-thaw cycle in the presence of different amounts of colicin Ia (● and ○) or colicin Ib (■ and □). [3H]TPMP+ uptake determined in the absence of colicin (100% level) was 10,160 cpm for vesicles from strain JK381(pBR322) and 5,100 cpm for vesicles from strain JK3819(pAR6). For the experiments shown in this figure, the amounts of TPMP+ uptake were determined from the averages of the absolute amounts of accumulated substrate at the 3-, 5-, and 7-min time points of individual uptake experiments.*

account for the differences obtained with immune vesicles. The fact that inhibition was not complete even at high colicin concentrations is consistent with the results of our previous studies (46, 48). It should be noted that whole cells of strain JK381 are resistant to colicins Ia and Ib due to a lack of specific colicin I receptors. The sensitivity of vesicles derived from this strain, as well as from strain JK381(pBR322), to colicins Ia and Ib is consistent with our previous finding that outer membrane receptors are not required for colicin I action on membrane vesicles (46, 48).

Subcellular localization of the immunity protein. Our finding that specific colicin Ia immunity is a property of membrane vesicles suggested that the immunity protein is associated with the cytoplasmic membrane. However, experiments in which we compared the SDS-PAGE profiles of proteins in vesicles prepared from immune and nonimmune strains failed to reveal the presence of the 14,500-dalton polypeptide previously identified as the immunity protein. As an alternative approach, labeled UV-irradiated cells were fractionated into soluble and inner and outer membrane fractions, and the identities of the labeled polypeptides found in each fraction were ascertained by SDS-PAGE.

To do this, labeled UV-irradiated cells from a strain carrying pCA6 were converted to spheroplasts and then disrupted in a French pressure cell. The resulting lysate was subjected to sucrose gradient centrifugation to separate the subcellular fractions. Colicin Ib binding was used as an outer membrane marker (25), and NADH oxidase activity was used to identify inner membrane material (36). A typical profile is shown in Fig. 9. Whereas ^{125}I-labeled colicin Ib binding (i.e., outer membrane material) was distributed almost entirely in a single peak, NADH oxidase activity (i.e., inner membrane material) was distributed approximately equally in two regions of the gradient. These results suggest that although inner membrane material was substantially free of outer membrane material, the peak of outer membrane material did contain substantial amounts of inner membrane material. Out inability to obtain purer outer membrane material by this method may reflect the known difficulty in fractionating the cell envelopes of stationary-phase cells (49).

^{35}S radioactivity was distributed in three peaks, which were centered at fractions 4, 10, and 24. The amount of radioactive material associated with inner and outer membrane material paralleled the distribution of NADH oxidase, as would be expected if the labeled protein

species were associated with the inner membrane. The ^{35}S-labeled material present in the peak at the top of the gradient was not precipitated with trichloroacetic acid and thus was probably free ^{35}S[S]methionine. When the specific activity of NADH oxidase was determined across the gradient (Fig. 9B), there was an evident peak, which corresponded to the position of a peak of total protein and ^{35}S radioactivity.

To determine the distributions of the TET and immunity proteins across the gradient, a sample from each fraction was subjected to SDS-PAGE, followed by fluorography. Each protein was then quantitated by a microdensitometer analysis of the developed fluorogram. As Fig. 10 shows, the distributions of both proteins exactly followed the pattern of inner membrane material (Fig. 9A). In fact, their total distributions, as well as their specific activity profiles, were identical to the distribution and profile of NADH oxidase. When the ratio of total immunity protein to total NADH oxidase or colicin Ib binding activity was calculated for each fraction, the immunity protein followed NADH oxidase activity, but not colicin Ib binding (Fig. 10C). These results, together with independent reports that the TET protein resides in the cytoplasmic membrane (12, 33), lead to the conclusion that the colicin Ia immunity protein is a component of the bacterial inner membrane.

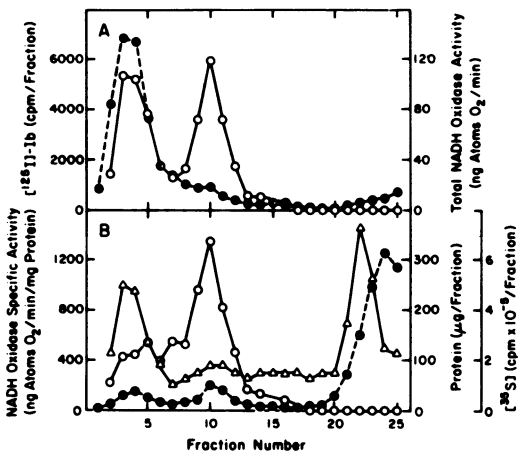

FIG. 9. *Separation of labeled maxicells of strain CSR603(pCA6) into soluble, inner membrane, and outer membrane fractions. Broken spheroplasts from [^{35}S]methionine-labeled maxicells from strain CSR603(pCA6) were subjected to sucrose density gradient centrifugation as described in the text. Sedimentation was from right to left. (A) Symbols: ●, ^{125}I-labeled colicin Ib binding; ○, NADH oxidase activity. (B) Symbols: △, total protein; ●, total ^{35}S; ○, NADH oxidase specific activity.*

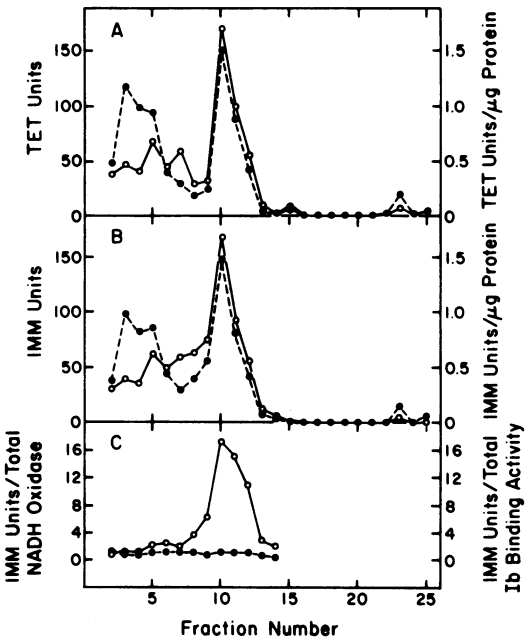

FIG. 10. *Quantitation of TET and immunity (IMM) proteins in soluble, inner membrane, and outer membrane maxicell fractions. TET and immunity proteins were quantitated as described in the text. (A) Symbols: ●, total TET units; ○, TET units normalized to total protein per fraction. (B) Symbols: ●, total IMM units; ○, IMM units normalized to total protein per fraction. (C) IMM units were normalized to inner membrane activity (total NADH oxidase) (●) and to outer membrane activity (^{125}I-labeled colicin Ib binding) (○). The normalized values for IMM units per ^{125}I counts per minute were multiplied by 100 to yield the data shown in this figure. This was done so that the values of IMM units normalized to both inner and outer membrane activities in fraction 2 were approximately 1.*

DISCUSSION

We found that the structural gene for colicin Ia (cia$^+$), as well as the gene which determines specific immunity to this colicin (*iia*$^+$), resides on a 4,800-bp *Eco*RI-*Pst*I fragment of plasmid pColIa-CA53. This same fragment encodes another polypeptide (15,500 daltons), which we have not identified yet. It is possible that this protein functions in the regulation of the inducible colicin Ia structural gene (22). Whether this protein represents a repressor of this system must await further analysis.

The close linkage of *cia* and *iia* genes was expected. It will be interesting to determine whether this close linkage is indicative of an arrangement of the *cia* and *iia* genes in an operon. Previous studies have indicated a close linkage between immunity and structural genes

for colicin E1 (9, 15, 41), cloacin DF13 (47), and colicin V (11).

The 14,000- to 14,500-dalton polypeptide which we identified is almost certainly the colicin Ia immunity protein (i.e., the product of the *iia* gene). This protein is encoded by all plasmids determining immunity to this colicin which have been constructed, and most convincingly, a plasmid that lost the ability to confer Ia immunity due to insertional inactivation by Tn*5* concomitantly lost the coding capacity for this polypeptide.

Although we were not able to obtain a complete separation of the inner and outer membranes, our data strongly suggest that the colicin Ia immunity protein is a component of the inner (cytoplasmic) membrane. This protein exhibits a profile across the membrane peaks which is identical to the profiles of two established inner membrane components, NADH oxidase and the TET protein. Furthermore, the profile of this protein is quite different from the profile of a known outer membrane marker (the colicin I receptor). Experiments showing that membrane vesicles prepared from colicin Ia-immune whole cells can be inactivated by colicin Ib but are immune to colicin Ia have provided further evidence that the immunity protein resides in this subcellular fraction.

We propose that the mechanism of immunity to colicin Ia involves some specific interaction between colicin and the immunity protein. An alternative mechanism involving modification of a cellular target seems much less likely. Studies on the modes of action of colicins Ia and Ib have failed to identify differences with regard to required cellular activities or final targets. Furthermore, no mutant which is insensitive to one of these colicins but not the other has been isolated (3, 7).

The exact mechanism of immunity remains to be determined. Of prime importance is a determination of whether the immunity protein acts stoichiometrically or catalytically. It is clear from previous studies (24, 30), as well as from the results presented here, that immunity does not operate at the level of the specific colicin Ia/Ib receptor. Preliminary experiments indicated that colicin Ia-immune membrane vesicles bind similar amounts of colicin Ia and colicin Ib, whether they are prepared from immune cells having colicin I outer membrane receptors or from immune cells lacking such receptors.

As a working hypothesis, we propose that an interaction of colicin Ia with the cytoplasmic membrane of an immune strain leads to an interaction between colicin and immunity protein. Structural differences between colicins Ia and Ib (21) and presumably between the colicin Ia and Ib immunity proteins would serve as the basis for immunity specificity. Just how the required interactions come about and subsequently prevent formation of stable colicin channels can be discussed only speculatively. The formation of such stoichiometric complexes might provide the explanation for the observation of immunity breakdown which is known to occur at high colicin Ia or Ib concentrations (30). It seems probable that such a breakdown would occur when the cytoplasmic membrane is challenged with an amount of colicin which is in excess of the amount of immunity protein.

Although colicins Ia and Ib are able to form channels in protein-free liposomes and artificial planar membranes, it is not known whether these proteins interact at random or at specific sites in the cytoplasmic membranes of treated cells. Such specificity might derive from interaction between the colicin and membrane components or result from directed orientation due to interactions between receptor-bound colicins at the outer membrane and the inner membrane. Indeed, we have proposed previously that the formation of apposition sites between inner and outer membranes is critical for the action of some colicins (23). In either case, immunity protein must somehow "find" the colicin. One possibility is that the immunity protein is so abundant in the cytoplasmic membrane that any association of colicin with this membrane leads to an interaction with the *iia* gene product. However, this possibility seems unlikely since the protein compositions of membrane vesicles prepared from immune and nonimmune cells are the same, as determined by SDS-PAGE (unpublished data). In particular, there has been no indication of a plasmid-determined polypeptide corresponding to the 14,500-dalton protein identified in UV-irradiated cells. Thus, the immunity protein must be a minor membrane component.

These considerations lead to the notion that if the colicin interacts with the inner membrane at random, the immunity protein must "search out" the colicin. Perhaps lateral diffusion of the *iia* gene product in the membrane is sufficient to bring this about. On the other hand, if colicin action involves interaction at a limited number of specific membrane sites, interaction between colicin and immunity protein could be assured if the immunity protein resided at precisely these sites in the membrane. In another kind of model, we envisage that the immunity protein affords the colicin an alternative, but innocuous, membrane binding site. Therefore, colicin molecules would be shunted from interactions, which would lead to channel formation in nonimmune cells.

These results mark an important step in elu-

cidating the mechanism by which plasmid pColIa confers immunity to colicin Ia. It seems likely that a common mechanism may be employed by all plasmids which confer immunity to those colicins whose mode of action involve the formation of channels in target cytoplasmic membranes.

ACKNOWLEDGMENTS

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