JOURNAL OF BACTERIOLOGY, Oct. 1985, p. 237–244
0021-9193/85/100237-08$02.00/0

Vol. 164, No. 1

Localization of the Immunity Protein-Reactive Domain in Unmodified and Chemically Modified COOH-Terminal Peptides of Colicin E1

L. J. BISHOP, E. S. BJES, V. L. DAVIDSON,† AND W. A. CRAMER*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Received 15 March 1985/Accepted 3 July 1985

The region of the colicin E1 polypeptide that interacts with immunity protein has been localized to a 168-residue COOH-terminal peptide. This is the length of a proteolytically generated peptide fragment of colicin E1 against which *imm*$^+$ function can be demonstrated in osmotically shocked cells. The role of particular amino acids of the COOH-terminal peptide in the expression of the immune phenotype was studied. Chemical modification showed that the two histidine residues (His 427 and His 440) and the single cysteine residue (Cys 505) present in the COOH-terminal peptide were not necessary for the colicin-immunity protein interaction. The immunity protein was localized in the cytoplasmic membrane fraction, consistent with previous work of others on the colicin Ia immunity protein and the prediction from the immunity protein amino acid sequence that it is a hydrophobic protein. The distribution of hydrophobic residues along the immunity polypeptide was calculated.

The 4.2-megadalton ColE1 plasmid codes for both colicin E1, a 57,000-M_r-dalton bactericidal protein that acts by deenergizing the cytoplasmic membrane of sensitive *Escherichia coli*, and an immunity protein that protects cells against the colicin (3) but that has not yet been isolated and purified. Colicin E1 first binds to the vitamin B_{12} receptor, the *bfe* gene product, located in the bacterial outer membrane (OM) (14). The colicin subsequently forms a channel in the cytoplasmic membrane that is sufficiently permeable to small ions to collapse the membrane potential resulting in cell death (10, 12). The mechanism by which the immunity protein prevents cell death in response to exogenous colicin is not understood.

The immunity protein has not been found to complex with extracellular colicin E1 as do the immunity proteins for colicins E_2 and E_3, which also use the vitamin B_{12} receptor (14). Association of the respective immunity proteins with colicins E2 and E3 results in the formation of complexes that are inactive in vitro (16, 36). Cellular immunity to colicins E2, E3, or colicin Ia, whose mode of action is similar to that of colicin E1, was shown not to affect affinity for the receptor (19, 22).

Molecular details of immunity in the channel-forming colicins have been studied most thoroughly for the I colicins (25, 42). Colicin Ib, which uses the same OM receptor as does colicin Ia, can kill strains immune to colicin Ia (18), showing that the immunity protein is specific to the colicin and does not involve a general change in the character of the cell that would confer immunity to all colicins. The colicin Ia immunity phenotype has been demonstrated in inner membrane vesicles, and a 14,000- to 15,000-M_r polypeptide associated with immunity has been localized in the inner membrane (IM) fraction of UV-irradiated cells (42). It was shown that the immunity protein recognizes the COOH-terminal half of the colicin Ia molecule by examining the survival of Ia-immune strains to Ia-Ib hybrid molecules (25).

The present study was undertaken to define more precisely the immunity protein recognition domain of colicin E1, to examine the role of particular amino acids in the immune response, and to test the hypothesis that, by analogy to the colicin Ia immunity system, the immunity protein to colicin E1 operates at the level of the cytoplasmic membrane. The immunity protein-reactive domain of the colicin E1 molecule was investigated with COOH-terminal peptide fragments of colicin E1 that have previously been defined and characterized (7–9, 11). Digestion of colicin E1 with trypsin or thermolysin yields COOH-terminal fragments of 20,000 and 18,000 M_r, respectively, which encompass the channel-forming domain (7, 9, 11) and perhaps part of that involved in receptor binding (7). If immunity operates at the level of the cytoplasmic membrane, the immunity phenotype should be displayed by osmotically shocked cells lacking a complete OM. Such shocked cells have been used previously to test the activity of colicin peptide fragments that did not exhibit killing activity in vivo because of the OM barrier (31, 39).

MATERIALS AND METHODS

Strains and media. *E. coli* JC411(ColE1) grown in L broth was the source of colicin E1. *E. coli* W3110 *bfe*$^-$ lacking a functional OM receptor for colicin E1, obtained from M. Nomura (University of Wisconsin, Madison), was used for the osmotic shock experiments. The plasmid pHSG215, containing the *imm* locus, was isolated from the strain Y-mel (*E. coli* K-12 F$^-$ *mel-1*), supplied by J. Suit and S. E. Luria (Massachusetts Institute of Technology, Cambridge). The subcellular localization of plasmid-encoded proteins was determined in strain DR1984, obtained from M. Rudinski (Purdue University, West Lafayette, Ind.).

Plasmid isolation and transformation. Plasmid pHSG215 (Tc^r) was prepared from a late-log-phase culture of strain Y-mel grown in L broth containing 5 g of NaCl per liter, 5 g

* Corresponding author.
† Present address: Molecular Biology Division, VA Medical Center, San Francisco, CA 94121.

of yeast extract per liter, 3 g of Casamino Acids (Difco Laboratories, Detroit, Mich.) per liter, and 0.0125 g of tetracycline per liter. The plasmid was extracted by the alkaline lysis method (4) before centrifugation in a cesium chloride gradient. Cells of strains W3110 *bfe*$^-$ DR1984 were transformed by the calcium chloride procedure (23) with the plasmids pHSG215 and pBR322 (Tc^r Ap^r), supplied by S. Gelvin (Purdue University).

Construction of plasmids isogenic except for the *imm* gene. The immunity protein structural gene was isolated from the plasmid pBR324 (5) purified from K12-294 (a gift from P. van den Elzen, Free University, Amsterdam) as described above. The plasmid was digested with the restriction endonucleases *Eco*RI (300 U/ml; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and *Nru*I (300 U/ml; Boehringer Mannheim) in 50 mM Tris hydrochloride (pH 7.2)–50 mM NaCl–50 mM KCl–10 mM $MgCl_2$–5 mM β-mercaptoethanol–0.2 μg of bovine serum albumin per ml at 37°C for 20 min. The digest was subjected to agarose gel electrophoresis on a 1% gel containing 1 μg of ethidium bromide per ml at 50 V for 1 h, and the 480-base-pair fragment including the immunity protein gene was then electroeluted to DEAE paper (50 V, 10 min). The DNA was washed from the paper by incubation with 1.5 M NaCl–10 mM Tris hydrochloride (pH 7.5)–1 mM EDTA at 4°C for 18 h. The paper fragments were removed by centrifugation through glass wool. The ethidium bromide was extracted with isoamyl alcohol, and the DNA was then ethanol precipitated. The fragment was blunt ended by the T4 polymerase end-labeling system (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) according to the instructions of the manufacturer.

The vector pKK223-3 (P-L Biochemicals, Inc., Milwaukee, Wis.) was digested with the restriction endonuclease *Sma*I (200 U/ml; Boehringer Mannheim) in 20 mM KCl–6 mM Tris hydrochloride (pH 8.0)–6 mM β-mercaptoethanol at 25°C for 30 min. The *Sma*I was inactivated by heating to 65°C for 10 min, and the digest was extracted twice with 1:1 phenol-chloroform followed by three extractions with diethyl ether. The linearized vector was precipitated by the addition of sodium acetate and ethanol and was redissolved in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA at a concentration of 200 μg/ml. *Sma*I-cut pKK223-3 was mixed with the blunt-ended *imm* gene fragment in a 3:1 molar ratio and was ligated (T4 ligase; Boehringer Mannheim) in 20 mM Tris hydrochloride (pH 8.0)–10 mM $MgCl_2$–10 mM dithiothreitol–0.6 mM ATP–15% polyethylene glycol (Sigma Chemical Co., St. Louis, Mo.) at 12°C for 70 min followed by 4°C for 18 h. The high-molecular-weight concatemers formed by this ligation were isolated by centrifugation and cleaved to unit length by digestion with the restriction endonuclease *Pst*I (200 U/ml; Boehringer Mannheim) in 50 mM NaCl–10 mM Tris hydrochloride (pH 7.5)–10 mM $MgCl_2$–1 mM dithiothreitol at 37°C for 1 h. After heat inactivation of the *Pst*I at 65°C for 15 min, the digest was extracted twice with 1:1 phenol-chloroform and twice again with diethyl ether. The resultant products were diluted fourfold and were ligated (T4 ligase; Boehringer Mannheim) as above except that polyethylene glycol was not added. The ligase was heat inactivated at 65°C for 15 min and extracted with phenol-chloroform and diethyl ether as described above, and the DNA was precipitated by the addition of sodium acetate and ethanol. After being redissolved in 10 mM Tris hydrochloride (pH 8.0)–1 mM EDTA, any plasmid that did not contain an insert, i.e., any still containing a site for the restriction endonuclease *Sma*I, was linearized by digestion with *Sma*I as described above. The digest was transformed into *E. coli* JM83 and DR1984, which were made competent by the calcium chloride procedure (23). A portion of the transformation mix was inoculated into 10 ml of L broth containing 37 μg of ampicillin per ml and was grown at 37°C overnight. The cells were diluted 10-fold the following morning, allowed to grow for 1 h at 37°C, and subjected to selection against colicin E1 (36 ng/ml). The resultant culture was diluted and plated onto 1.4% agar plates containing 37 μg of ampicillin per ml. Fifteen resultant single colonies were selected for further analysis. The resultant clones were screened for the presence of the immunity protein insert by digestion with *Bam*HI (as described above) and 1% agarose gel electrophoresis. The clones were screened for the orientation of the insert relative to the *tac* promoter by digestion of DNA isolated as described from 16 insert-containing clones with the restriction endonucleases *Pst*I and *Sau*3A (150 and 100 U/ml; Boehringer Mannheim) under conditions described for *Pst*I digestion and 1.3% agarose gel electrophoresis. One clone with the immunity protein gene inserted in the opposite orientation relative to the *tac* promoter was selected for this study.

Preparation of colicin E1 and its proteolytic fragments. Colicin E1 was prepared from strain JC411 by the method of Cleveland et al. (9). The purified colicin was dialyzed against 100 mM potassium phosphate (pH 7.0) and stored at 4°C. The 20,000-M_r COOH-terminal tryptic peptide of colicin E1 was prepared by digestion of ~15 mg of colicin E1 (trypsin-colicin, 1:250 molar ratio, 26°C) and was purified by gel permeation through a 2.5- by 10-cm Sephadex G-100 column as previously described (11). The 18,000-M_r COOH-terminal thermolytic peptide was prepared by digestion of colicin E1 with a 1:125 (wt/wt) ratio of thermolysin to colicin and was purified by gel permeation through a 1- by 25-cm Sephadex G-75 column as previously described (7, 31). Eluted thermolytic and tryptic peptides were detected by A_{280}, and the purity of the eluted peptides was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with 12.5% acrylamide and 0.33% bisacrylamide.

Cysteine modification of colicin E1. Colicin E1 in 0.1 M potassium phosphate (pH 7.0) was mixed with 8 M urea to yield a final urea concentration of 6 M, and the pH of the solution was then adjusted to 8.0 with NaOH. The colicin E1 in urea was allowed to incubate with 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB, Sigma; colicin-DTNB [1:100]) for 16 h at 22°C. Upon sulfhydryl group modification there is a stoichiometric release of thionitrobenzoate anion, which was monitored at 412 nm ($\varepsilon_M = 1.36 \times 10^4$ M^{-1} cm^{-1}) to determine the concentration of reactive cysteine residues (26). Unmodified controls were incubated as described above with 6 M urea. DTNB-modified and control colicin E1 were dialyzed three times against 1 liter of 0.1 M potassium phosphate (pH 7.0) before assay of activity.

Histidine modification of the proteolytic fragment of colicin E1. The COOH-terminal tryptic peptide (0.65 mg/ml) in 0.1 M potassium phosphate (pH 7.0) was incubated at 25°C with 0.12 mM diethylpyrocarbonate (DEP) for 20 min. DEP was first diluted into anhydrous ethanol, and the effective concentration was determined by reaction with 10 mM imidazole (pH 7.5) (27). The modification reaction was monitored spectrophotometrically between 320 and 230 nm, and the extent of the reaction was calculated from the extinction coefficient (ε_M = 3,200 M^{-1} cm^{-1}) of the carbethoxy histidyl residues at 238 nm (28).

Survival of osmotically shocked cells. *E. coli* W3110 *bfe*$^-$ cells were grown and subjected to osmotic shock with the

omission of EDTA (30). Cells were plasmolyzed in 20% (wt/vol) sucrose–33 mM Tris hydrochloride (pH 7.1) and were centrifuged at 13,000 × *g* for 10 min at 4°C. Colicin E1 or COOH-terminal colicin peptide, in volumes specified below (see Fig. 1 through 4), was placed on the wall of the tube containing the well-drained, plasmolyzed cell pellet. Ice-cold $MgCl_2$ (3×10^{-4} M, 4 ml) was rapidly pipetted into the tube during vortexing. Osmotically shocked cells were then incubated at 0°C for 15 min, diluted into L broth, and spread onto L broth plus 1.5% agar plates. To assay survival in the absence of colicin or derived peptides, buffer or colicin that had been heat denatured by boiling for 10 min was placed on the wall of the tube.

Subcellular localization of plasmid-encoded protein. The localization of the plasmid-encoded immunity protein was identified in the maxicell strain DR1984 by the method of Sancar et al. (34). After 1 h of sulfur starvation in M-9 medium, 10-ml cultures of UV-irradiated and cycloserine-treated (100 μg/ml) maxicells were labeled by a 1-h incubation with 10 μCi of [^{35}S]methionine per ml, harvested by centrifugation at 4,000 × *g* for 10 min, washed with sulfur-free buffer, and resuspended in 0.5 ml of 0.75 M sucrose–10 mM Tris acetate (pH 8.0). Lysozyme was added to a final concentration of 6 μg of original cell culture per ml, and the cell suspension was incubated on ice for 5 min. Spheroplasts were made by the gradual addition of 1 ml of 1.5 mM EDTA over a 5-min period, followed by disruption by osmotic shock as the cell suspension was poured into 6 ml of ice-cold H_2O, stirred for 10 min, and then centrifuged at 1,200 × *g* for 10 min to remove unlysed cells in the presence of phenylmethylsulfonyl fluoride (0.5 mM) as a protease inhibitor. Membranes were sedimented by centrifugation at 80,000 × *g* for 4 h or (Fig. 2C) for 2 h. The supernatant, containing periplasmic and cytoplasmic proteins, was precipitated by the addition of bovine serum albumin (100 μg) and trichloroacetic acid, and then centrifuged at 12,000 × *g* for 30 min. The pellet was washed in acetone, dried under N_2, and dissolved in 10 mM Tris-NaOH (pH 8.0). For solubilization with sodium lauryl sarcosinate (sarcosyl; Sigma), the membrane pellet was washed with 10 mM Tris acetate (pH 8.0)–5 mM EDTA, sedimented as above, frozen overnight, and suspended in the above wash buffer with the addition of sarkosyl to a final concentration of 1% (15). For solubilization with Triton X-100, the pellet was washed with 10 mM Tris acetate (pH 8.0)–30 mM $MgCl_2$, sedimented, frozen overnight, and resuspended with 2% Triton X-100 (13). The membranes were incubated at room temperature in the presence of detergent for 30 min to solubilize the IMs, and the OM was sedimented by centrifugation at 80,000 × *g* for 4 h. Protein samples were prepared by boiling in a solubilizing mix containing 8 M urea and 4% sodium dodecyl sulfate before analysis on sodium dodecyl sulfate gels containing 15% polyacrylamide.

RESULTS

Expression of immunity against COOH-terminal peptides. Cells of strain W3110 *bfe*$^-$ harboring either pHSG215 (*imm*$^+$) or pBR322 (*imm*$^-$) were killed by colicin E1, colicin COOH-terminal peptide, or chemically modified COOH-terminal peptide, if present during osmotic shock (Fig. 1). In all cases, colicin E1 or its COOH-terminal peptides were capable of killing cells under conditions of osmotic shock in nonimmune strains, with a high degree of protection against lethality afforded by the presence of the immunity protein. Plasmid pBR322, coding for tetracycline and ampicillin resistance proteins but not the immunity protein, had no effect on colicin E1 immunity when compared with strains harboring no plasmid (data not shown).

Immunity to the channel-forming domain of colicin E1 is demonstrated in Fig. 1A and B. W3110(pBR322 [*imm*$^-$]) cells that were shocked in the presence of 10 nM, 100 nM, and 1 μM colicin E1 exhibited survival levels of 55, 6.5, and 0.37%, respectively, whereas the corresponding survival levels of similarly shocked W3110(pHSG215 [*imm*$^+$]) cells were 95, 50, and 25% (Fig. 1A). The survival levels of cells shocked in the presence of 10 nM, 100 nM, and 1 μM COOH-terminal colicin peptide prepared by proteolytic cleavage with thermolysin (7) were 2, 0.2, and 0.01%, respectively, for W3110(pBR322), compared with 85, 60, and 1.5% for W3110(pHSG215) (Fig. 1B). Also apparent was the inability of immunity protein to protect against high levels of colicin E1, analogous to the immunity breakdown observed at high concentrations of membrane-depolarizing colicin Ia or Ib (21). Survival levels of cells shocked in the presence of a slightly larger 20,000-M_r peptide prepared by cleavage of colicin with trypsin (11), 1 and 50% for W3110(pBR322) and W3110(pHSG215), respectively, in the presence of 1 μM peptide, were higher than those shocked in the presence of colicin E1 or thermolytic peptide (Fig. 1C).

Chemical modification of COOH-peptide and effect on immunity. Histidine and cysteine residues could be involved in specific association of the colicin and immunity proteins through hydrogen bonding or disulfide bridges, respectively. Chemical modification by DEP of the two histidines His 427 and His 440 in the channel-forming domain did not significantly alter the lethal effect of the tryptic peptide or protection by the immunity protein, implying no critical role for these residues in the immune response (Fig. 1D). Chemical modification of the single cysteine molecule, Cys 505, with the Ellman reagent DTNB after denaturation in urea resulted in a small decrease in activity of the tryptic peptide in W3110(pBR322), but did not inhibit protection by the immunity protein in W3110(pHSG215), and urea treatment of the peptide followed by dialysis before assay of activity had no effect on activity (Fig. 1E).

Cellular localization of the immunity protein. The localization of the immunity protein was approached by labeling maxicells with [^{35}S]methionine and examining the distribution of labeled plasmid-encoded proteins in cellular fractions separated by centrifugation and the use of two different detergent systems, Sarkosyl and Triton X-100, to separate IM and OM fractions (13, 15). The separation, as judged by the polypeptide pattern in lanes 1 and 2 (IM) and 3 and 4 (OM) of Coomassie blue-stained polypeptides in fractions separated with sarcosyl (Fig. 2A), is similar to that previously reported by others (1, 35). The IM and OM fractions show a different distribution of polypeptides, with a larger number of the IM fraction. Given the large number of polypeptides, however, and the lack of resolution of one-dimensional gels, the quality of the separation should be judged by examination of polypeptides with known locations. Such a group of polypeptides is provided by the tetracycline resistance gene product (37,000 M_r [34]), which is located in the IM (24), and secreted β-lactamase responsible for ampicillin resistance (31,000 and 28,000 M_r in plasmid pBR322 and 28,000 M_r in pKK223-3 [6, 37]), which is located in the periplasm (24). Incorrectly processed β-lactamase should be localized in the IM. Examination of the plasmid-encoded proteins in fluorograms (Fig. 2B, C, and E) shows that the *tet* protein is almost exclusively found in the IM fraction, as compared with the OM, and that β-lactamase is found in the soluble fraction (Fig. 2C), as predicted for

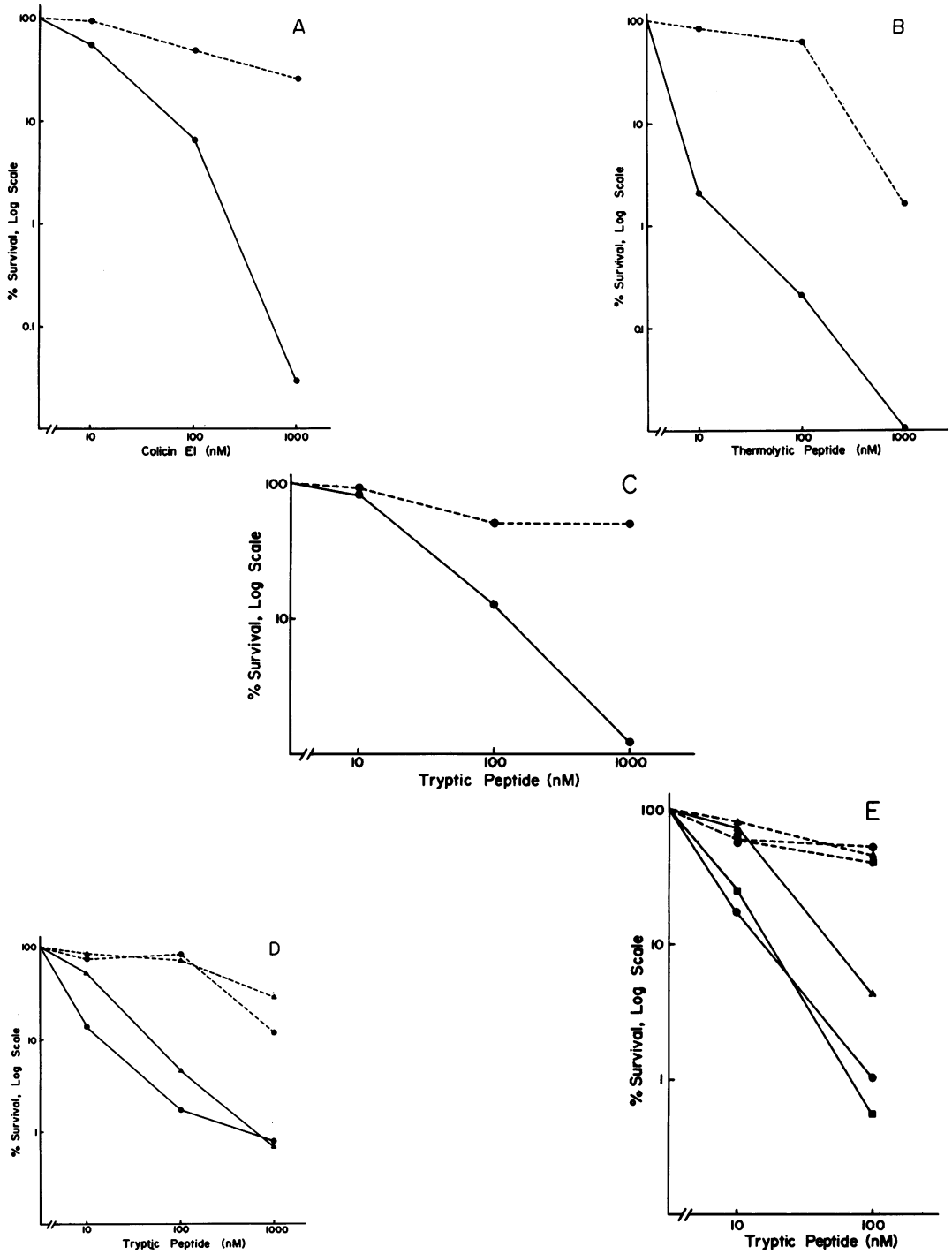

FIG. 1. Effect of *imm* protein on cell sensitivity to colicin COOH-terminal peptides. Survival levels are plotted of W3110(pBR322 [*imm*$^-$]) (——) and W3110(pHSG215 [*imm*$^+$]) (– – –) cells subjected to osmotic shock in the presence of the following peptides in 100 mM potassium phosphate (pH 7.0), with the volume of buffer noted in parentheses. (A) Colicin E1 (80 μl). (B) 18,000-M_r thermolytic peptide (80 μl). (C) 20,000-M_r tryptic peptide (45 μl). (D) DEP-modified (▲) and unmodified (●) tryptic peptide (120 μl). (E) DTNB-modified (▲), urea-treated (■), and untreated (●) tryptic peptide (50 and 75 μl for untreated and urea-treated peptide). All survival levels are expressed relative to those (typically 25 to 50%) of cells shocked in the presence of the appropriate heat-denatured peptide (1 μM).

well-separated fractions. The different forms of β-lactamase were found in the soluble and IM fractions, with negligible amounts in the OM component (Fig. 2B). The separation with Triton X-100 gave a larger yield of OM relative to IM (Fig. 2D). In this case, some forms of β-lactamase were seen in the OM fraction, but definitely less than were seen in the IM, and the dominant 30,000-M_r form was again seen in the soluble fraction (Fig. 2E). Unidentified bands in the

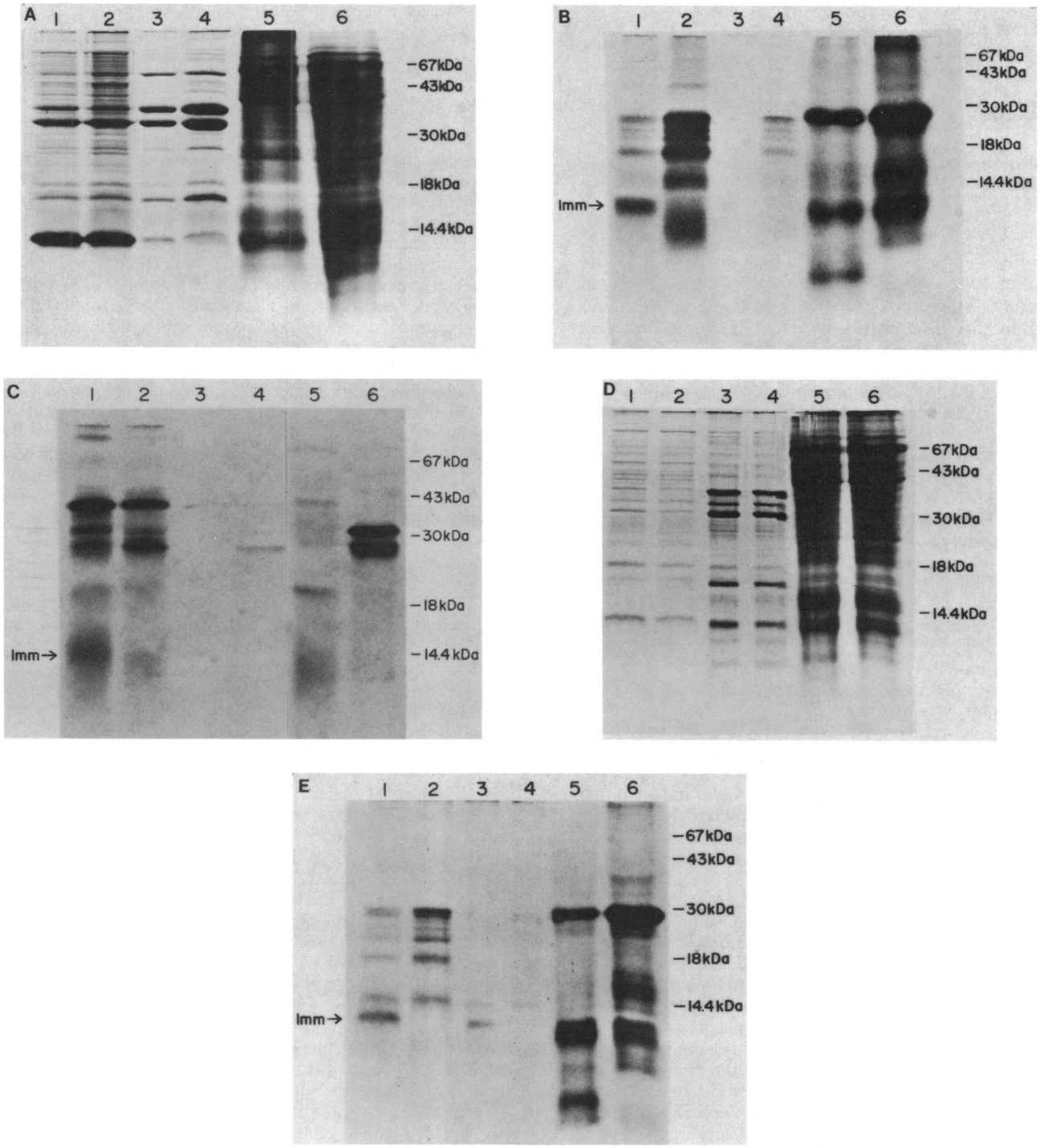

FIG. 2. Localization of immunity protein by [^{35}S]methionine labeling of maxicell strain DR1984 and subcellular fractionation, using different detergents (sarcosyl or Triton X-100) to solubilize IM and OM. (A through C) sarcosyl solubilization (15): Coomassie blue-stained sodium dodecyl sulfate gel of total protein (A) and fluorogram (B) of plasmid-encoded proteins labeled with [^{35}S]methionine in strains harboring pKK-223-3 (*imm*$^-$) and pLJB1 (*imm*$^+$), isogenic except for the *imm* gene. For panels A and B and for C through E (described below), lanes 1 (*imm*$^+$), and 2 (*imm*$^-$) contain the IM-enriched fraction; lanes 3 (*imm*$^+$) and 4 (*imm*$^-$) contain the OM-enriched fraction, and lanes 5 (*imm*$^+$ and 6 (*imm*$^-$) contain the cytoplasmic-periplasmic fraction. (C) Fluorogram of [^{35}S]methionine plasmid-encoded protein harboring pHSG215 (*imm*$^+$) or pBR322 (*imm*$^-$). (D and E) Solubilization by Triton X-100 (13): lanes of Coomassie blue-stained gel (D) and fluorogram (E) of plasmids isogenic except for *imm* gene are as in A and B. In panels A and B, lanes 1 and 2, corresponding to IM, and 5 and 6, corresponding to soluble fractions, were loaded with equal amounts of protein (80 μg), and OM fractions (3 and 4) were loaded with 12 and 20 μg, respectively. Lanes corresponding to different fractions in C through E were loaded in proportion to recovery yield of each fraction from the original culture. KDa, Kilodaltons.

```
                10                  20                  30
M S L R Y Y I K N I L F G L Y C T L I Y I Y L I T K N S E G

                40                  50                  60
Y Y F L V S D K M L Y A I V I S T I L C P Y S K Y A I E Y I

                70                  80                  90
A F N F I K K D F F E R R K N L N N A P V A K L N L F M L Y

                100                 110
N L L C L V L A I P F G L L G L F I S I K N N
```

FIG. 3. Sequence of colicin E1 immunity protein (27, 32).

23,000-M_r (IM fraction) and 12,000-M_r (soluble fraction) regions have been previously reported by other workers (42; M. Rudinski, personal communication).

According to the nucleotide sequence, the molecular weight of the immunity protein is 13.3 kilodaltons. Comparing the odd-numbered lanes (1, 3, and 5) corresponding to *imm*$^+$ plasmid with the even-numbered lanes (2, 4, and 6) for *imm*$^-$, a well-defined band at 13,000 M_r was seen in the sarcosyl-separated IM fraction of Fig. 2B, where protein products of plasmids isogenic except for *imm* were compared. Figure 2C compares the two plasmids used in the osmotic shock and chemical modification experiments of Fig. 1, which were not isogenic. A broad band at approximately 13,000 M_r was again seen in the sarcosyl-separated IM fraction of the *imm*$^+$ pHSG215 plasmid, and a diffuse band of somewhat lower molecular weight was seen in the IM fraction of the *imm*$^-$ pBR322 (Fig. 2C). It was concluded that the immunity protein is localized in the IM fraction. A very low-molecular-weight polypeptide, the smallest on the fluorogram, was also seen in the *imm*$^+$ lane (lane 5) of the soluble fraction from both sarcosyl- and Triton X-100-separated material (Fig. 2D and E, respectively). This polypeptide is either derived from proteolysis of the *imm* protein or is possibly a product of transcription under the *tac* promoter. The 12,000-M_r band also seen in the soluble fraction (Fig. 2D and E) was not related to the immunity protein since this band was found in the *imm*$^-$ and the *imm*$^+$ lanes (lanes 6 and 5).

Putative amino acid sequence of the colicin E1 immunity protein. Analysis of the amino acid sequence of the E1 immunity protein derived from the nucleotide sequence predicts that the immunity polypeptide has properties of a membrane protein. The gene coding for colicin E1 immunity has been identified and localized in a 1.6-kilobase-pair plasmid, PA02, derived from the ColE1 plasmid (32). When the nucleotide sequence of PA02 is read in each direction in each of the three possible reading frames, the two largest protein sequences are a 45-residue polypeptide that is probably responsible for cell lysis and release of colicin after induction of colicin production (38), and a 113-residue protein corresponding to a nucleotide sequence previously identified as coding for the immunity protein (32). The primary amino acid sequence of the 113-residue polypeptide is shown in Fig. 3. The following evidence implies that the sequence shown in Fig. 3 is that of the *imm* gene product. Its size is consistent with the observation that a 360-base-pair mRNA is synthesized in vitro from PA02 (29). The choice of an initiation site used in the present work is the same as that proposed by Patient (33) to initiate transcription of an *imm* gene product. The molecular weight of this protein, 13.3 kilodaltons, is consistent with the observation in *E. coli* minicells that colicin E1 immunity is correlated with the presence of an ~13,000- to 14,000-M_r polypeptide (17; Fig. 2B, D, and E). The amino acid sequence of the immunity protein is generally hydrophobic, consistent with its localization in the cytoplasmic membrane.

DISCUSSION

The mechanism of action of the immunity protein on the group of membrane-depolarization colicins has yet to be elucidated. There is no decrease in adsorption of colicins to OM receptors in immune strains (19, 22). The colicin Ia immunity protein and phenotype were found to be located in the IM fraction. By analogy, one would predict that the colicin E1 immunity protein would be located in the IM, the target of colicin E1 action. The immune phenotype should then be demonstrable in osmotically shocked cells, which have been used previously in studies of colicin action (31, 39), and which allow a bypass of receptor-mediated OM resistance to colicin action without release of cytoplasmic or IM constituents (30). Cells tolerant to colicin E3 because of changes in OM proteins can be killed by this colicin under conditions of osmotic shock, whereas IM-mediated tolerance to colicin L cannot be overcome by the shock (39). Thus, it has been documented that conditions of osmotic shock can bypass OM receptor-mediated resistance or tolerance to colicin action, but not tolerance mediated by the cytoplasmic membrane. In the present work the immune phenotype could be demonstrated in response to colicin E1 addition in osmotically shocked cells harboring the plasmid pHSG215 (*imm*$^+$). This plasmid, which codes for the colicin E1 immunity protein and for ampicillin resistance, was selected to avoid the complicating effects of endogenous colicin E1 and lysis protein production (38). To localize the region of interaction with immunity protein in the colicin polypeptide, defined COOH-terminal peptides from the channel-forming domain were used to challenge shocked cells.

The channel-forming region was found to be localized in COOH-terminal tryptic (11), thermolytic (7), or cyanogen bromide (9) peptides with 20,000, 18,000, or 16,000 M_r, respectively. The activities of two thermolytic fragments containing the colicin E1 COOH terminus "Th 1," the 18,000-M_r fragment used in Fig. 1B, and "Th 2," 39,000 M_r, have been studied previously in shocked *bfe*$^-$ cells (31). Both fragments lack in vivo activity but possess killing ability under conditions of osmotic shock. The Th 1 peptide, like the tryptic peptide, lacks the receptor-binding function but contains the ionophoretic domain. Th 2 contains the receptor-binding and ionophoretic domains, and its lack of ability to kill in vivo has led to the proposal that the missing N-terminal third of the molecule is utilized in translocation of colicin E1 to the IM (31). The immunity-reactive domain of colicin Ib has been shown to reside in the COOH-terminal half of the protein (25), which has been inferred, although not yet proved, to contain the channel-forming domain of colicin Ib. The present study has further localized the immunity recognition domain of colicin E1 to a 168-residue region spanned by the thermolytic peptide Th 1 defined by Ohno-Iwashita and Imahori (31), extending from N-terminal amino acid residue Ile 345 to Lys 512 of the 522-residue colicin polypeptide.

The nature of the interaction between the COOH-terminal domain of the colicin E1 and immunity protein molecule is not known. A chemical modification study was done of particular amino acids in the colicin COOH-terminal peptides that are present in low stoichiometry and that could participate in H bonding or disulfide formation with the

immunity protein. Only two histidines, His 427 and His 440, are present in the COOH peptide, and these can be converted with DEP to spectrophotometrically defined carbethoxy histidyl residues. The one cysteine molecule, Cys 505 in the COOH-terminal colicin peptide, could form disulfides with the immunity protein with a proposed sequence containing three cysteines (Fig. 3). The DEP- and DTNB-modified tryptic peptides, previously shown not to be significantly altered in ionophoretic activity (L. J. Bishop, V. L. Davidson, and W. A. Cramer, Biophys. J. 45:62a, 1984), were employed to examine any possible role of the two histidines or single cysteine contained in the COOH-terminal peptide in the immune response. These residues were found not to be necessary for the interaction of the colicin E1 with its immunity protein.

Subcellular localization of the immunity protein demonstrated that it was present in the IM fraction of envelopes solubilized by two different detergent systems. The distribution of hydrophobic residues in the membrane-bound immunity protein was determined based on the amino acid sequence by the algorithm and free-energy values of Kyte and Doolittle (20). A plot of this function reveals the presence of three hydrophobic domains (Fig. 4). A similar result was obtained with the amino acid transfer free-energy values of other workers (2, 41). The hydrophobicity index function shown in Fig. 4 contrasts with those obtained for the immunity proteins of colicin E3 and cloacin DF13 (40), bacteriocins that inhibit protein synthesis in membrane-free systems in vitro. The amino acid sequences of the latter immunity proteins are hydrophilic and contain no apparent membrane-associated domain, which can be seen by inspection of the sequence or calculated as in Fig. 4 (data not shown). For the colicin E1 immunity protein, residues 1 through 54 and 81 through 113 are nonpolar (Fig. 4) and therefore are assumed to be membrane associated. Segments 1 through 25 and 34 through 54 are each of sufficient length to span the membrane. Residues 66 through 80 compose a hydrophilic domain that presumably extends into either the cytoplasm or the periplasm, depending upon the orientation of the protein in the IM. This hydrophilic domain contains an especially highly polar sequence of nine residues (66 through 74; K-K-D-F-F-E-R-R-K) in which there are seven charged residues and a net charge of +3. This unique group of charged residues may serve as an anchoring group for the membrane protein.

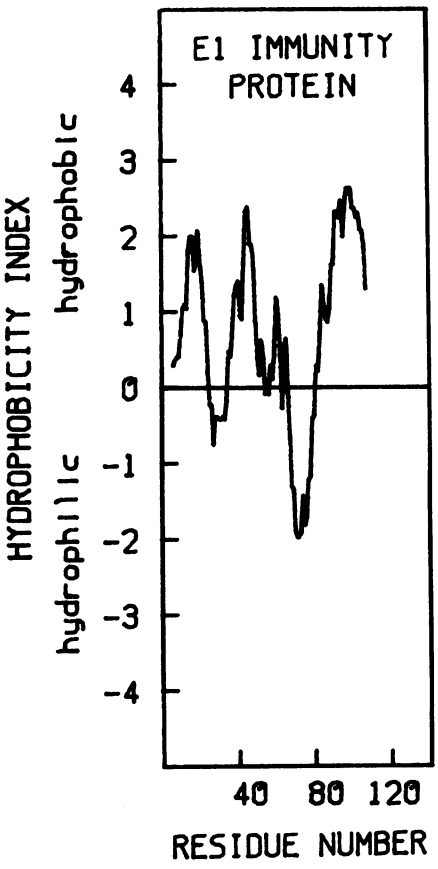

FIG. 4. Hydrophobicity index function of the colicin E1 immunity protein. The index was calculated with a sliding averaged interval of 11 amino acids according to the algorithm and relative amino acid free-energy transfer values of Kyte and Doolittle (20) and the program adaptations discussed elsewhere (43). Ordinate values above and below the base line correspond to net hydrophobic or hydrophilic sequences, respectively.

ACKNOWLEDGMENTS

This research was supported by Public Health Service grant GM-18457 from the National Institutes of Health.

We thank W. R. Widger and K. R. Brunden for the development of computer programs used in this work, Mark Rudinski for helpful discussions, and Sheryl Kelly and Marge Miles for assistance in the preparation of this manuscript.

LITERATURE CITED

1. **Achtman, M., P. A. Manning, C. Edelbluth, and P. Herrlich.** 1979. Export without proteolytic processing of inner and outer membrane proteins encoded by F sex factor *tra* cistrons in *Escherichia coli* minicells. Proc. Natl. Acad. Sci. USA **76**:4837–4841.
2. **Argos, P., J. K. M. Rao, and P. A. Hargrave.** 1982. Structural prediction of membrane-bound proteins. Eur. J. Biochem. **128**:565–575.
3. **Bazaral, M., and D. R. Helinski.** 1968. Circular DNA forms of colicinogenic factors E1, E2 and E3 from *Escherichia coli*. J. Mol. Biol. **36**:185–194.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Bolivar, F.** 1979. Molecular cloning vectors derived from the ColE1 type plasmid pMB1. Life Sci. **25**:807–818.
6. **Brosius, J., and A. Holy.** 1984. Regulation of ribosomal RNA promoters with a synthetic *lac* operator. Proc. Natl. Acad. Sci. USA **81**:6929–6933.
7. **Brunden, K. R., W. A. Cramer, and F. S. Cohen.** 1984. Purification of a small receptor-binding peptide from the central region of the colicin E1 molecule. J. Biol. Chem. **259**:190–196.
8. **Bullock, J. O., F. S. Cohen, J. R. Dankert, and W. A. Cramer.** 1983. Comparison of the macroscopic and single channel conductance properties of colicin E1 and its COOH-terminal tryptic peptide. J. Biol. Chem. **258**:9908–9912.
9. **Cleveland, B. M., S. Slatin, A. Finkelstein, and C. Levinthal.** 1983. Structure-function relationships for a voltage-dependent ion channel: properties of C-terminal fragments of colicin E1. Proc. Natl. Acad. Sci. USA **80**:3706–3710.
10. **Cramer, W. A., J. R. Dankert, and Y. Uratani.** 1983. The membrane channel-forming bacteriocidal protein, colicin E1. Biochim. Biophys. Acta **737**:173–193.
11. **Dankert, J. R., Y. Uratani, C. Grabau, W. A. Cramer, and M. Hermodson.** 1982. On a domain structure of colicin E1. J. Biol. Chem. **257**:3857–3863.
12. **Davidson, V. L., K. R. Brunden, W. A. Cramer, and F. S. Cohen.** 1984. Studies on the mechanism of action of channel-forming colicins using artificial membranes. J. Membr. Biol.

79:105–118.
13. **DePamphilis, M. L., and J. Adler.** 1971. Attachment of flagellar basal bodies to the cell envelope: specific attachment to the outer, lipopolysaccharide membrane and the cytoplasmic membrane. J. Bacteriol. **105**:396–407.
14. **Di Masi, D. R., J. C. White, C. A. Schnaitman, and C. Bradbeer.** 1973. Transport of vitamin B_{12} in *Escherichia coli*: common receptor sites for vitamin B_{12} and the E colicins on the outer membrane of the cell envelope. J. Bacteriol. **115**:506–513.
15. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115**:717–722.
16. **Hirose, A., J. Kumagai, and K. Imahori.** 1976. Dissociation and reconstruction of colicin E3 and immunity substance complex. J. Biochem. **79**:305–311.
17. **Inselburg, J., and B. Applebaum.** 1978. Proteins synthesized in minicells containing plasmid ColE1 and its mutants. J. Bacteriol. **133**:1444–1451.
18. **Konisky, J.** 1973. Chemistry of colicins, p. 41–58. *In* L. Hager, (ed.), Chemistry and function of colicins. Academic Press, Inc., New York.
19. **Konisky, J., and B. S. Cowell.** 1972. Interaction of colicin Ia with bacterial cells. J. Biol. Chem. **247**:6524–6529.
20. **Kyte, J., and R. Doolittle.** 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. **157**:105–132.
21. **Levisohn, R., J. Konisky, and M. Nomura.** 1968. Interaction of colicins with bacterial cells. IV. Immunity breakdown studies with colicins Ia and Ib. J. Bacteriol. **96**:811–821.
22. **Maeda, A., and M. Nomura.** 1966. Interaction of colicins with bacterial cells. I. Studies with radioactive colicins. J. Bacteriol. **91**:685–694.
23. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53**:159–162.
24. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 368–369. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
25. **Mankovich, J. A., P. Lai, N. Gokul, and J. Konisky.** 1984. Organization of the colicin Ib gene. J. Biol. Chem. **259**: 8764–8768.
26. **Means, G. E., and R. E. Feeney.** 1971. Chemical modification of proteins, p. 220. Holden-Day, Inc., San Francisco.
27. **Melchior, W. B., and D. Fahrney.** 1970. Ethoxyformylation of proteins. Reactions of ethoxyformic anhydride with α-chymotrypsin, pepsin, and pancreatic ribonuclease at pH 4. Biochemistry **9**:251–258.
28. **Miles, E. W.** 1977. Modification of histidyl residues in proteins by diethylpyrocarbonate. Methods Enzymol. **47**:441–442.
29. **Morita, M., and A. Oka.** 1979. The structure of a transcriptional unit of colicin E1 plasmid. Eur. J. Biochem. **97**:435–443
30. **Nossal, N. G., and T. A. Heppel.** 1966. The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241**:3055–3062.
31. **Ohno-Iwashita, Y., and K. Imahori.** 1982. Assignment of the functional loci in the colicin E1 molecule by characterization of its proteolytic fragments. J. Biol. Chem. **257**:6446–6451.
32. **Oka, A., N. Nomura, M. Morita, H. Sugisaki, K. Sugimoto, and M. Takanami.** 1979. Nucleotide sequence of small ColE1 derivatives: structure of the regions essential for autonomous replication and colicin E1 immunity. Mol. Gen. Genet. **172**:151–159.
33. **Patient, R. K.** 1979. Characterization of *in vitro* transcription initiation and termination sites in col E1 DNA. Nucleic Acids Res. **6**:2647–2665.
34. **Sancar, A., A. M. Hack, and W. D. Rupp.** 1979. Simple method for identification of plasmid-coded proteins. J. Bacteriol. **137**:692–693.
35. **Schnaitman, C.** 1970. Protein composition of the cell wall and cytoplasmic membrane of *Escherichia coli*. J. Biol. Chem. **104**:890–901.
36. **Sidikaro, J., and M. Nomura.** 1974. E2 immunity substance A. Proteins from E3 colicinogenic cells that accounts for their immunity to colicin E3. J. Biol. Chem. **249**:445–453.
37. **Stüber, D., and H. Bujard.** 1981. Organization of transcriptional signals in plasmids pBR322 and pACYC184. Proc. Natl. Acad. Sci. USA **78**:167–171.
38. **Suit, J. L., M.-L. J. Fan, J. F. Sabik, R. Labarre, and S. E. Luria.** 1983. Alternative forms of lethality in mitomycin c induced bacteria carrying col E1 plasmids. Proc. Natl. Acad. Sci. USA **80**:579–583.
39. **Tilby, M., I. Hindennach, and U. Henning.** 1978. Bypass of receptor-mediated resistance to colicin E3 in *Escherichia coli* K-12. J. Bacteriol. **136**:1189–1191.
40. **van den Elzen, P., W. Gaastra, C. E. Spelt, F. K. de Graaf, E. Veltkamp, and H. J. J. Nijkamp.** 1980. Molecular structure of the immunity gene and immunity protein of the bacteriocinogenic plasmid CloDF13. Nucleic Acids Res. **8**:4349–4363.
41. **Von Heijne, G., and C. Blomberg.** 1979. Transmembrane translocation of proteins—the direct transfer model. Eur. J. Biochem. **97**:175–181.
42. **Weaver, C. A., A. H. Redborg, and J. Konisky.** 1981. Plasmid-determined immunity of *Escherichia coli* K-12 to colicin Ia is mediated by a plasmid-encoded membrane protein. J. Bacteriol. **148**:817–828.
43. **Widger, W. R., W. A. Cramer, R. G. Herrmann, and A. Trebst.** 1984. Sequence homology and structural similarity between cytochrome *b* of mitochondrial complex III and the chloroplast b_6-*f* complex: position of the cytochrome *b* hemes in the membrane. Proc. Natl. Acad. Sci. USA **81**:674–678.