Identification of the plasmid-encoded immunity protein for colicin E1 in the inner membrane of *Escherichia coli*

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A set of plasmids containing portions of the Col E1 plasmid were transformed into recA$^-$ cells. These cells, after UV irradiation, only incorporate labelled amino acids into plasmid-encoded proteins. UV-irradiated cells label a 14.5 kDa band if they are phenotypically immune to colicin E1, and do not contain this band if they are sensitive to colicin E1. We conclude that the 14.5 kDa protein is the colicin E1 immunity protein. When the inner and outer membranes of these cells are fractionated, the labelled band appears in the inner membrane. The immunity protein must be an intrinsic inner membrane protein, confirming the predictions made by hydrophobicity calculations from primary sequence data.

Maxicell *Col E1 plasmid* *Immunity protein* *Hydrophobicity calculation*

1. INTRODUCTION

The Col plasmids are a class of *Escherichia coli* plasmids that instruct the synthesis of proteins called colicins that are lethal to bacteria of the same or similar groups. Each Col plasmid must also code for a corresponding protein that protects the cell from the killing action of the colicin. This additional protein is said to confer colicin immunity.

The mechanisms of many colicin immunities are not yet elucidated, but it is known that the interactions are always highly specific. For example, the E1 class of colicins (Ia, Ib, E1, A, and K) all seem to kill sensitive cells in the same manner: dissipation of transmembrane potential by formation of a rather non-specific channel in the inner membrane [1]. There is no cross-reactivity between different colicins and their immunity proteins. Colicins Ia and Ib share the same outer membrane receptor, the same killing mechanism and even some partial sequence homology, yet their respective immunity proteins are specific for only one of the 2 molecules [2]. Immunity for E1, A and K colicins is also specific.

The work of Weaver et al. [2] on the colicin Ia system has allowed them to identify the colicin Ia immunity protein as a polypeptide of approx. 14.5 kDa residing as a component of the inner membrane of *E. coli*. This is a plausible result since it must interact with the colicin Ia protein whose target is the inner membrane.

Previous attempts at identifying the size and location of the colicin E1 immunity protein have proved inconclusive. Polypeptides ranging from 6.5 to 14.3 kDa have been tentatively identified as the immunity protein using such techniques as minicells and in vitro synthesis [3–5].

We have taken advantage of 2 results in our search for the colicin E1 immunity protein. The first is the availability of the elegant maxicell technique of Sancar et al. [6] which allows one to label exclusively plasmid-encoded proteins. The second is the availability of some well-characterized plasmids containing portions of the Col E1 plasmid that confer a variety of phenotypes.

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Furthermore, the availability of nucleotide sequence data for the plasmids we use offers us additional predictive capability. By reading a proposed amino acid sequence from the gene sequence, we can use hydrophobicity calculations [7] to garner information on the possible cellular locations of these plasmid proteins. Using these tools we have positively identified the colicin E1 immunity protein and determined its cellular location in the *E. coli* inner membrane.

2. MATERIALS AND METHODS

2.1. *Bacterial strains and plasmids*

Δ-21 [sup Δ recA-21 (thr^- leu^- mal^- sor^- $recA^-$)] is a derivative of *E. coli* K-12 via AB1157 from G. Walker. The plasmid entitled p8 is pDMS630 from J. Inselburg and is a derivative of the Col E1 plasmid containing a *Tn3* transposon that confers ampicillin resistance. p8 is col^+ imm^+ amp^R. p215 is pHSG215 also from J. Inselburg and is col^- imm^+ tet^R.

p4 was obtained from p215 by in vitro mutagenesis with 1 M hydroxylamine (pH 6.0), 0.6 M NaCl. Samples were removed at 45-min intervals and quenched with 0.7 M Tris-HCl (pH 8.0), 0.8 M NaCl. DNA was precipitated and resuspended in TEC buffer. p4 is col^- imm^- tet^R, and was selected from transformants on tetracycline and colicin-containing LB plates.

2.2. *Materials*

[^{35}S]Methionine (>1200 Ci/mmol) was purchased from Amersham. Antibiotics, D-cycloserine and lysozyme were purchased from Sigma. Enhance© was purchased from New England Nuclear.

2.3. *Methods*

Maxicells were prepared by the modified procedure of Sancar et al. [6].

The separation of inner and outer membranes basically followed the procedure of Ito et al. [8]. All procedures were done at 0–4°C. Membranes were resuspended in Tris buffer and homogenized for electrophoresis. The purity of inner and outer membrane fractions was checked using marker enzyme assays as described in Ito et al. [8].

Polyacrylamide gel electrophoresis was performed in the presence of SDS using the discontinuous buffer system of Laemmli [9]. For autoradiography, gels were treated for 1 h with Enhance followed by 1 h in 10% acetic acid/2% glycerol, then dried under vacuum with heating and exposed 2–3 days to Kodak X-ray film at −70°C.

3. RESULTS AND DISCUSSION

Using the maxicell technique, we can successfully label plasmid proteins with [^{35}S]methionine and leave chromosomal proteins unlabelled. Δ-21 maxicells that contain no plasmid incorporate no label into protein (not shown).

p4 is a hydroxylamine-mutagenized derivative of p215 that is phenotypically non-immune (i.e. colicin-sensitive). Cells containing either p4 or p215 are tetracycline-resistant. A 37 kDa protein has been previously identified in pBR322-containing maxicells as the tetracycline-resistance protein [6] and that band is clearly seen in both p4 and p215 maxicells. There are a few other faint bands in both strains, but the significant difference is the presence of a major band in p215 cells at approx. 14.5 kDa that is totally absent in p4 cells. This band, which is also present in p8 cells, must correspond to the immunity protein.

Maxicells containing p8 appear to incorporate label into a large array of proteins, far greater than could be coded for on the 11.6 kilobases of the p8 plasmid. Using rabbit anti-colicin E1 antibodies we have found that colicin-producing cells contain proteins with colicin E1 antigenic determinants over a range of molecular masses up to about 60 kDa (not shown). We interpret this as a set of degradation products and proteolytic fragments of the colicin molecule that are naturally occurring in colicin producers. Thus maxicells containing p8 should label a whole variety of colicin fragments, and this is the observed result.

Additionally, p8 maxicells must express labelled ampicillin resistance gene products which are characteristically seen in the 28–31 kDa range. p8 cells are also phenotypically immune, and so must contain the labelled immunity protein.

Inner and outer membranes were isolated from [^{35}S]methionine-labelled maxicells as outlined in section 2. Protein content was determined by the method of Lowry et al. [10] and equal amounts of protein of inner and outer membranes of p4, p8

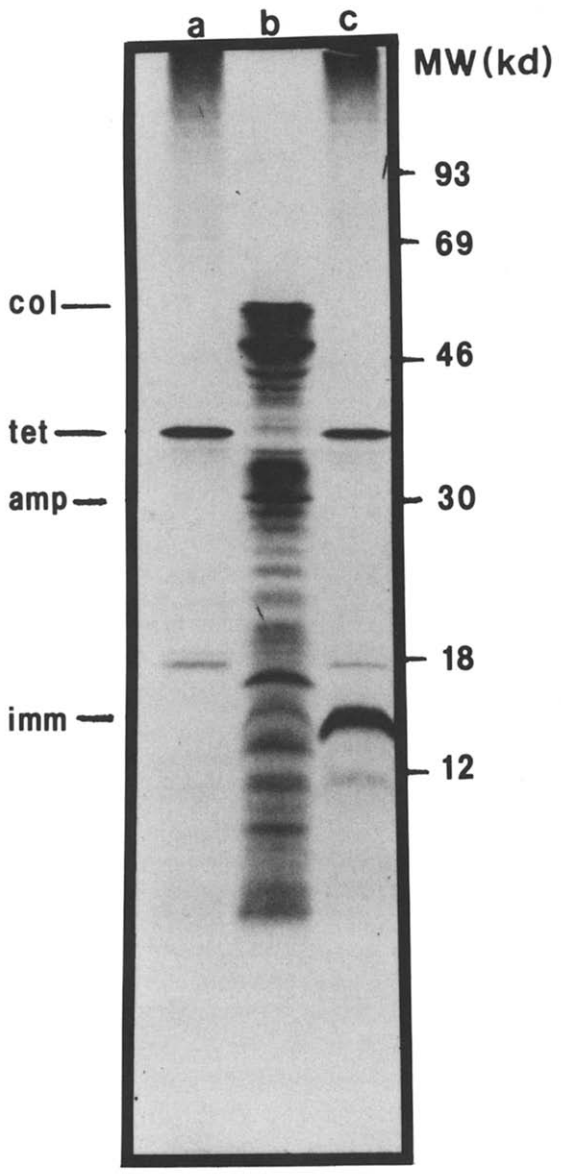

Fig.1. [^{35}S]Methionine incorporation into plasmid-coded proteins in maxicells. Maxicells were UV-irradiated, then labelled with [^{35}S]methionine. Cells were subjected to SDS-PAGE followed by autoradiography as described in text. Lanes: (a) Δ-21 maxicells containing p4, (b) Δ-21 (p8), (c) Δ-21 (p215).

and p215 cells were subjected to SDS-PAGE. The results are seen in fig.2.

In p4 and p215 cells we see strong bands corresponding to tetracycline-resistance protein, a known inner membrane protein. The enriched

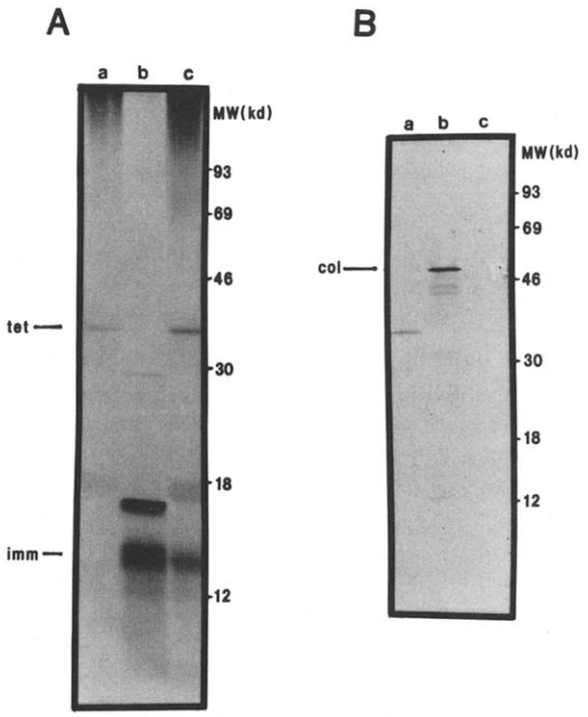

Fig.2. (A) Labelled polypeptides in maxicell inner membranes. (B) Labelled polypeptides in maxicell outer membranes. Inner and outer membrane extracts of [^{35}S]methionine-labelled maxicells were prepared as described in text and subjected to SDS-PAGE followed by autoradiography. Lanes are Δ-21 containing: (a) p4, (b) p8, (c) p215.

amount of the 14.5 kDa band in the inner membrane fraction supplies the best evidence so far that the immunity protein resides in the inner membrane.

In p8 we see a great reduction in the number of colicin fragments, although some intact colicin is still present. The ampicillin resistance proteins (including β-lactamase) are cytosolic and do not appear in membrane fractions.

Not surprisingly, very little label ends up in the outer membrane fraction. In p8, colicin (mostly intact) does show up in the outer membrane. The outer membrane contains the known receptor for the colicin E1 molecule [11], thus we expect to find some intact colicin associated with the outer membrane.

We have positively identified an inner membrane protein that occurs only in cells that are

phenotypically immune to colicin E1, and not in sensitive cells. We believe that this 14.5 kDa protein is the colicin E1 immunity protein.

The nucleotide sequence for the plasmids used here is available from pBR322 or pCol E1 data [12–14], and the amino acid sequence for the colicin E1 protein has been determined. It is known that almost all of the molecule is highly hydrophilic and there is a short region near the carboxy terminus that is hydrophobic which is included in the channel portion of the molecule (fig.3a). Thus we might expect to find endogenous colicin E1 in the cytosol and in the membrane fraction of maxicells.

The phenomenon of colicin degradation is not uncommon. The process of colicin killing itself may indeed involve the clipping of the 20 kDa carboxy-terminal fragment for insertion into the membrane. The action of internal bacterial proteases may be necessary for cell survival. In vitro experiments show that trypsin, chymotrypsin and other proteases degrade colicin leaving a 20 kDa fragment intact [15–18]. A 17 kDa band that may correspond to this colicin fragment is prevalent in p8 maxicells, both in whole cells and in inner membrane fractions (see figs 1 and 2). We are currently testing this possibility by using monoclonal antibodies against specific domains of the colicin E1 molecule [19].

The sequence which must correspond to the 37 kDa tetracycline-resistance protein codes for a protein whose hydrophobicity profile is that of a membrane protein (fig.3b). It contains long hydrophobic regions (membrane-spanning) with shorter interspersed regions of relative hydrophilicity. A similar look at the putative sequence for the colicin E1 immunity protein shows that it looks much like a membrane protein (fig.3c). This 113-amino-acid transcript is the only reasonably lengthened coding region in the known

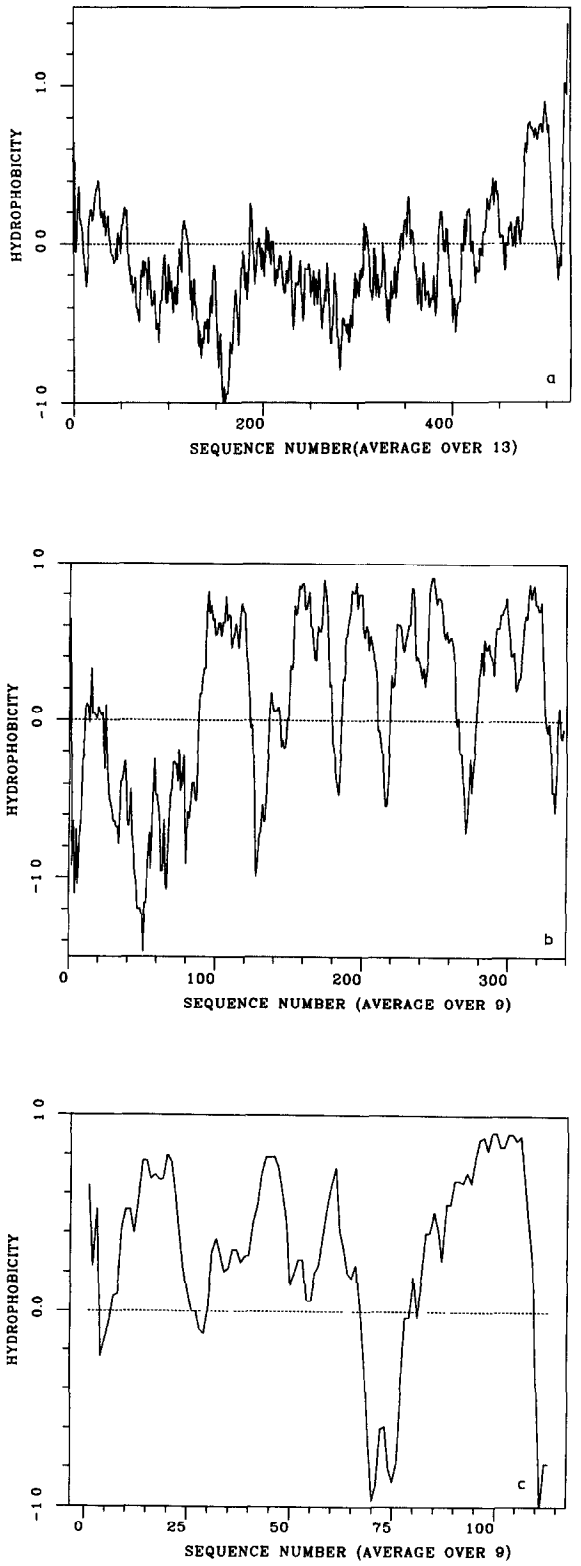

Fig.3. Hydrophobicity plots for 3 proteins from pCol E1 and pBR322, according to the method of Kyte and Doolittle [7]. Hydrophobicity values for individual amino acids are consensus values from Eisenberg [21] and are averaged over the stated number of residues. (a) Colicin E1 protein, (b) tetracycline resistance protein, (c) colicin E1 immunity protein.

area of immunity on the Col E1 plasmid and in the proper direction of transcription [20]. Its proposed molecular mass corresponds well to the value we report. Furthermore, a DNA fragment containing this coding region has been isolated and cloned into two new vectors to create new, immunity-conferring plasmids (Shanafelt, A., Goldman, K., Kastelein, R. and Kayalar, C., submitted).

Although the immunity protein is easily visualized by the maxicell technique, both Coomassie blue and silver staining of SDS gels fail to detect any extra bands in cells containing immunity-conferring plasmids (not shown). This indicates that the immunity protein is in relatively low abundance, and yet it is able to protect cells against large amounts of external colicin. This may imply a catalytic mode of action for the immunity protein, however, it is not known how much of the external colicin reaches the inner membrane. It has been hypothesized that very little actually does. Since the immunity protein has to act only against the colicin reaching the inner membrane, its action may still be stoichiometric. Experiments in finding temperature-sensitive mutants in immunity will serve to clarify the question.

We have isolated a 660-basepair fragment of the Col E1 plasmid that contains the entire putative immunity gene. Although this gene is already located on a plasmid, its expression is very low. We have cloned the fragment into a plasmid containing the regulatable lambda P_L promoter. We hope to purify large quantities of the immunity protein from this strain.

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