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Mechanism of Export of Colicin E1 and Colicin E3

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The mechanism of export of colicins E1 and E3 was examined. Neither colicin E1, colicin E3, nor colicin E3 immunity protein appears to be synthesized as a precursor protein with an amino-terminal extension. Instead, the colicins, as well as the colicin E3 immunity protein, appear to leave the cells where they are made, long after their synthesis, by a nonspecific mechanism which results in increased permeability of the producing cells. Induction of ColE3-containing cells with mitomycin C leads to actual lysis of those cells, at some time after synthesis of the colicin E3 and its immunity protein has been completed. Induction of ColE1-containing cells results in increased permeability of the cells, but not in actual lysis, and most of the colicin E1 produced never leaves the producing cells. Intracellular proteins such as elongation factor G can be found outside of colicinogenic cells after mitomycin C induction, along with the colicin. Until substantial increases in permeability occur, most of the colicin remains cell associated, in the soluble cytosol, rather than in a membrane-associated form.

Colicins are protein antibiotics produced by strains of *Escherichia coli* which carry specific plasmids called Col factors (6, 8, 18). Colicins have traditionally been regarded as exported or secreted proteins. Colicinogenic colonies release colicin, which causes a halo, or zone of inhibition of growth, of an indicator lawn spread over them (6, 14). The standard method of purifying colicins E1, E2, and E3 has been to isolate the colicins from 1 M salt washes of mitomycin C-induced colicinogenic cells (7, 26). In the case of E2 and E3, the extracellular colicins are always found in 1:1 complexes with their respective immunity proteins (13, 25). The immunity proteins have been shown in vitro to prevent the enzymatic action of E2 and E3, and presumably that is their function in the cells in which they are made. Additional free immunity protein can also be isolated from 1 M NaCl washes of colicinogenic cells, so these proteins can also be described as secretory proteins like the colicins (12, 27).

Recently, several procaryotic proteins which are transported through the cytoplasmic membrane have been shown to be synthesized as precursors, with an amino-terminal extension that is cleaved cotranslationally while the protein is transported through the membrane (3, 10, 11, 22). This mechanism, set forth in the signal hypothesis (2), is similar to that which has been shown to apply for a number of eucaryotic secretory proteins (5, 16).

We originally set out to determine whether colicins E1 and E3 are secreted by this mechanism, i.e., whether they are synthesized as precursors with a signal peptide that is cleaved during transport of the colicins across the membrane. However, since our results ruled out this mechanism for secretion for the colicins, we investigated other possible means by which these colicins could leave the cells where they are synthesized. We conclude that the majority of the colicin leaves those cells via an increase in cell permeability or actual lysis, long after its synthesis. Until the induced cells become permeable and the colicin appears in the culture medium, most of the colicin can be found associated with the cells, in the soluble cell cytosol, rather than in a membrane-associated form. These results are in substantial agreement with the recent results of Mock and Schwartz concerning the export of colicin E3 (19).

MATERIALS AND METHODS

Colicin-producing strains were W3110(ColE1) from the Yale *E. coli* Genetic Stock Center and W3110(ColE3) from D. Helinski. The colicin-sensitive indicator was a streptomycin-resistant derivative of W3110 from M. Nomura.

Covalently closed, circular ColE1 DNA was a generous gift of Gerald Vovis. ColE3 DNA was prepared from sodium dodecyl sulfate (SDS) lysates of 6.4-liter overnight cultures of W3110(ColE3) in M9-Casamino Acids medium (4), as previously described for purification of phage f1 replicative form I DNA (9, 20), except that centrifugation in sucrose was at 22,000 rpm for 16 h in the Spinco SW27 rotor.

Synthesis of L-[^{35}S]-*N*-formyl-methionyl tRNA and the in vitro coupled transcription-translation system for synthesis of colicin were essentially as previously described for the in vitro synthesis of phage f1-coded proteins (20). ColE1 or ColE3 DNA was present in the in vitro reactions at 56 or 15 μg/ml, respectively. In some instances, 0.3 mM guanosine 5'-diphosphate 3'-diphosphate (ppGpp) was present in the in vitro reactions to stimulate synthesis of the colicin (17). In vitro products were precipitated with the appropriate rabbit antisera to colicins E1, E3, or E3 immunity protein, prepared as described previously (13). The antigen-antibody complexes were precipitated with *Staphylococcus aureus* by the procedure of Kessler (15) with modifications of Chang et al. (3). Fixed *Staphylococcus* cells were the gift of Chung Nan Chang.

An everted membrane vesicle fraction from *E. coli* BL15 was prepared as described (3) and was the generous gift of Chung Nan Chang. Incubations in the presence of this membrane fraction also contained 12 mM Nikkol (octaethylene glycol dodecyl ether; Nikko Chemicals Co., Tokyo, Japan), a nonionic detergent that has been shown to increase the efficiency of processing of precursor proteins by the membrane fraction (3).

Membranes and cytosol were prepared essentially as described by Osborn and Munson (21), except that inner and outer membranes were not separated. W3110(ColE1) and W3110(ColE3) were grown in M9 medium supplemented with yeast extract and Casamino Acids (7). Portions of each culture were induced with 0.5 μg of mitomycin C per ml, 1 h for W3110(ColE3), and 1.5 h for W3110(ColE1). Cells were harvested, resuspended without washing, and made into spheroplasts as described (21). Lysis of the spheroplasts by osmotic shock (21) was selected as the method least likely to displace loosely associated colicin from the membranes. The total membrane fraction was then recovered by making lysates 5 mM in $MgCl_2$ and centrifuging for 30 min at 50,000 rpm in a Beckman type 60 Ti rotor. The membrane pellets were suspended in 5% sucrose–5 mM Tris-chloride–5 mM EDTA, pH 8. The cytosol was concentrated by packing it, in a dialysis bag, in dry Aquacide II-A (Calbiochem).

Slab gels for analysis of in vitro products and in vivo colicin production contained 15% acrylamide, 0.2% bisacrylamide, 8 M urea, and SDS as previously described (13). Gel sample buffer contained 0.0625 M Tris-chloride (pH 6.8)–10% glycerol–5% β-mercaptoethanol–3% SDS–0.001% bromophenol blue.

Colicin E3 was purified by a modification (12) of the method of Herschman and Helinski (7). Colicin E1 was purified by a modification of the method of Schwartz and Helinski (26), as follows. A 40-liter amount of a culture of W3110(ColE1) was induced for 3 h with 0.5 μg of mitomycin C (Sigma Chemical Co.) per ml. The cells were pelleted and washed twice, for 15 min per wash, with 400 ml of 1 M NaCl–TM (TM buffer: 50 mM Tris-chloride, pH 7.6, 30 mM NH_4Cl, 10 mM magnesium acetate). The dialyzed wash was precipitated with ammonium sulfate (26) and then applied to a column of DEAE-Sephadex A-50 (4.5 by 12.6 cm) which had been equilibrated in TM. The colicin was eluted from the column with a 500-ml linear gradient from TM to 0.5 M KCl–TM. The peak containing the colicin activity, as assayed by spot tests on sensitive cells (12), was further purified by chromatography on CM-Sephadex C-50. A column (4.5 by 12.6 cm) was equilibrated with Tris-borate buffer, pH 8.5 (0.089 M Trizma base–0.089 M boric acid), and the colicin was eluted with a 500-ml linear gradient from 0 to 0.5 M KCl in the same buffer. The resulting colicin was about 75% pure, as judged by SDS gel electrophoresis. The method of Rice and Means (23), as adapted by Sander (24), was used to label purified colicin E1 and E3 in vitro with [^{14}C]formaldehyde (New England Nuclear Corp.).

Purified *E. coli* elongation factor G (EF-G) and purified rabbit anti-EF-G immunoglobulin G (IgG) were the generous gifts of P.-C. Tai.

Dialyzed salt washes from induced and uninduced cultures, as well as the supernatant media from those cultures, which were to be analyzed by Ouchterlony double diffusion, were first concentrated by packing them, in dialysis bags, in dry Aquacide II-A.

RESULTS

When ColE1 DNA was used to direct synthesis in an in vitro coupled transcription-translation system, the in vitro product (Fig. 1, lane f) was indistinguishable, on 15% acrylamide–8 M urea–SDS gels, from colicin E1 synthesized in vivo (Fig. 1), and the in vitro product had colicin activity when assayed by spot testing on colicin-sensitive indicator cells. In the presence of an *E. coli* membrane vesicle fraction previously shown to cleave the precursors for phage f1 coat protein (3) and *E. coli* alkaline phosphatase (C. N. Chang, H. Inouye, and P. Model, unpublished data), the in vitro colicin E1, radioactively labeled with [^{35}S]methionine, ran at the same position on the gel as that synthesized in the absence of membranes (Fig. 1, lanes f through i). The in vitro product synthesized in the presence of membranes consistently had about one-half the in vivo colicin activity of reactions done without membranes. Since the intensities of the corresponding colicin bands on the autoradiograms was about equal (Fig. 1), we believe the reduction in measurable activity is due to binding of newly synthesized colicin to the membrane vesicles in those reactions. Cloacin DF13, which is closely related to colicin E3, has been shown to bind to inner membrane vesicles (B. Oudega, Ph.D. thesis, Free University of Amsterdam, The Netherlands, 1978).

When [^{35}S]-*N*-formyl-methionyl-tRNA was the only radiolabeled component in the cell-free system, the colicin E1 synthesized in the presence of the membrane fraction plus Nikkol was also indistinguishable from that synthesized in the absence of membranes (Fig. 1, lanes b

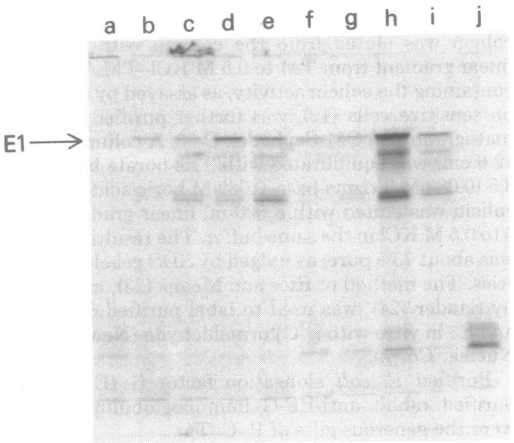

FIG. 1. *Autoradiogram of gel of immune-precipitated colicin E1 synthesized in vitro. (a–e) Label for in vitro synthesis was [^{35}S]-N-formyl-methionyl-tRNA; (f–j) label for in vitro synthesis was [^{35}S]methionine; (a and j) background synthesis, reactions contained no added DNA. All other reactions contained the following, in addition to ColE1 DNA and components specified in the text: (b) no additions; (c) membranes plus 12 mM Nikkol; (d) 0.3 mM ppGpp; (e) membranes plus 12 mM Nikkol and ppGpp; (f) no additions; (g) membranes plus 12 mM Nikkol; (h) 0.3 mM ppGpp; (i) membranes plus 12 mM Nikkol plus 0.3 mM ppGpp; arrow denotes position of in vivo colicin E1 marker run on same gel.*

through e). Under the same conditions, the precursor for phage f1 coat protein is cleaved by a component of the membrane fraction, the amino-terminal radiolabeled signal peptide can be found running near the dye front on the gel, and radioactively labeled material at the position of the full-length precursor protein disappears (3). Since there is no removal of *N*-terminal label by the membrane vesicles and no shift in the position of internally [^{35}S]methionine-labeled colicin E1 synthesized in the presence of the membranes, we conclude that colicin E1 is not synthesized as a precursor protein like other *E. coli* secretory proteins.

Although, in this coupled system, the in vitro synthesis of colicin E3 is much less efficient than the synthesis of colicin E1, there is also no evidence for synthesis of colicin E3 as a precursor. Colicin E3 immunity protein synthesized in vitro in a system identical to that described for colicin E1 also appears not to be made as a precursor with an amino-terminal extension (results not shown).

Thus, the in vitro results indicate that neither colicins E1 nor E3, nor colicin E3 immunity protein is a secretory protein which is transported through the cell membrane by means of a precursor. We therefore determined the location of these colicins at various times after induction of colicinogenic cultures with mitomycin C to clarify the mechanism by which these proteins leave the cells where they are made.

Colicinogenic cells, at mid- to late-logarithmic growth were induced with 0.5 μg of mitomycin C per ml. One hour after addition of mitomycin C to W3110(ColE3) and 3 h after induction of W3110(ColE1), the cells were harvested and washed for 5 min in a Virtis blender with 1 M NaCl–TM. The cells were then harvested, and a portion of the salt-washed pellet was lysed by sonicating it in SDS-containing gel sample buffer. Portions of the 1 M NaCl-washed cells were also broken by sonication without SDS, to avoid inactivation of the colicin by SDS. The salt washes and sonic extracts, as well as the original supernatant medium, were assayed for colicin activity by spot testing on sensitive cells (12), and portions were also analyzed by SDS–urea slab gel electrophoresis. As controls, uninduced colicinogenic cells and mitomycin C-induced non-colicinogenic cells were also analyzed (Table 1 and Fig. 2 and 3).

As measured by the intensity of the stained colicin bands on gels of samples from approximately corresponding amounts of culture, some colicin can be found in the 1 M NaCl washes, but the majority remains in the lysates. There is a fairly good correlation between the relative intensities of the stained colicin bands on the gels (Fig. 2 and 3) and the amount of activity in the spot test assay (Table 1). It should be pointed out here that the "lysates" of 1 M salt-washed cells (Fig. 2, lanes d, i, and k; Fig. 3, lanes e, i, and l) contained membranes as well as cytosol from those cells. The only fractionation was to separate those proteins that washed off in 1 M salt.

Since the majority of the colicin appeared to remain associated with the cells, and was not washed off in 1 M NaCl, it was necessary to determine whether the cell-associated colicin was membrane-associated, but not accessible to the high-salt wash or whether, in fact, the newly synthesized colicin was not membrane bound. We therefore prepared membrane and cytosol fractions from colicinogenic cultures. Since the cells for these preparations were not washed before spheroplasting and lysis, any colicin associated with either side of either the inner or outer membrane should appear in the membrane fraction. Membrane and cytosol fractions were assayed for colicin activity, and portions from approximately equivalent amounts of cells were analyzed on SDS–urea gels (Table 1; Fig. 2, lanes a, b, f, and g; and Fig. 3, lanes b, c, f, and

TABLE 1. *Localization of colicin activity in mitomycin C-induced colicinogenic cultures*[a]

Colicin	Time in mitomycin (h)	Fraction[b]	U in fraction	% of total activity
E1	1.5	Membranes	5×10^4	2
E1	1.5	Cytosol	1.5×10^6	98
E1	3	Lysate	2×10^6	97
E1	3	Wash	6×10^4	3
E1	3	Medium	8×10^3	0.3
E1	16	Lysate	8×10^6	85
E1	16	Wash	6×10^5	6
E1	16	Medium	8×10^5	8.5
E3	1	Membranes	5×10^4	3
E3	1	Cytosol	3×10^6	97
E3	1	Lysate	8×10^6	91
E3	1	Wash	6×10^5	7
E3	1	Medium	2×10^5	2
E3	3	Lysate	8×10^5	9
E3	3	Wash	6×10^5	6
E3	3	Medium	8×10^6	85
E3	16	Lysate	2×10^5	2
E3	16	Wash	6×10^4	0.7
E3	16	Medium	8×10^6	97

[a] Activity was assayed by spot testing dilutions on colicin-sensitive indicator lawns. Uninduced cultures generally had 10 to 100 times less colicin activity than their mitomycin C-induced counterparts. No colicin activity was ever detected in non-colicinogenic control cultures. Membranes and cytosol were fractionated as described in the text.

[b] Lysates were from 1 M NaCl-washed cells, broken by sonication. Wash fractions were 1 M NaCl washes (5 min in Virtis blender) of cells from induced cultures. Medium was supernatant culture medium from which cells were harvested after the specified induction period.

g). Only 2% of the induced colicin E1 activity and 3% of the induced colicin E3 activity was membrane associated (Table 1). Those relative amounts determined by in vivo activity correspond roughly to the relative intensities of the stained colicin bands on the gels. We conclude from these experiments that at early times after induction, most of the colicin is not membrane bound, and is not removed from the cells by washing with 1 M salt.

It appeared, moreover, that what little colicin was extractable with salt was accompanied by small but significant amounts of a large number of other proteins, many of which seemed to correspond to gel bands of cytosol proteins. This was particularly true for colicin E3 producers. Those proteins did not appear in 1 M salt washes of uninduced cells or induced, non-colicinogenic cells. The few proteins that did appear consistently in washes of uninduced cells were a specific subset of membrane proteins (compare lanes b and c of Fig. 2 and lanes c and d of Fig. 3). It seemed possible that the colicin which could be found in 1 M salt washes might result from an increase in permeability of the induced cells, or from actual lysis of some of the culture. We therefore looked for the extracellular appearance of EF-G, an intracellular, non-membrane-bound protein, which should not be found outside the cells unless they had become permeable. Salt washes from induced and uninduced cells were concentrated and analyzed by Ouchterlony double diffusion for reaction with purified rabbit anti-EF-G IgG (Fig. 4a). The washes from both the induced W3110(ColE1) and W3110(ColE3) contained material that precipitated with antibody to EF-G, whereas the wash from uninduced cells and a wash of induced non-colicinogenic W3110 did not. The medium from these cultures also contained colicin activity, about one-third the amount in the salt wash for colicin E3 and 10% of that in the wash for colicin E1 (Table 1), but EF-G was not detected in that medium. However, since the amount of EF-G in the washes was at the limit of sensitivity of the Ouchterlony precipitation assay, it is possible that slightly smaller amounts of EF-G did appear in the culture medium at these times after induction and were not detected by the immune assay.

Since colicin-producing cells cause halos in a lawn of sensitive bacteria, extracellular colicin must ultimately become diffusible, rather than remaining tightly associated with the producing cells. Colicinogenic cells were therefore induced overnight (16 h), and the localization of the colicin was determined as for the shorter induction periods. Although the total amount of colicin produced in 16 h is not significantly greater than what is made within 1 h (for colicin E3) or 3 h (for colicin E1, which seems to be made more slowly or later than colicin E3), the localization of that colicin is much different at the two times. Much more of the total colicin, particularly colicin E3, was free in the overnight culture medium, and there was a corresponding decrease in the colicin associated with the cell lysates (Table 1 and Fig. 5). A majority of the colicin E3 immunity protein was also in the medium, rather than remaining cell associated. There was also an increase in the number and amounts of extracellular proteins in both the salt wash and medium from the ColE1 culture, and in the medium from the overnight ColE3 culture (Fig. 5). This

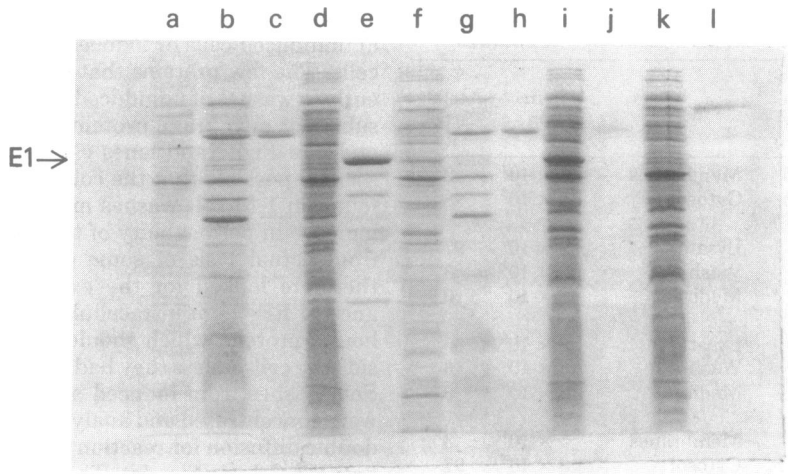

FIG. 2. *Polyacrylamide slab gel electrophoresis of fractions from colicin E1-producing cells and control non-colicinogenic cells, stained with Coomassie blue. Experiment A (lanes a, b, f, and g), all W3110(ColE1): (a) no mitomycin, cytosol; (b) no mitomycin, membranes; (f) 0.5 μg of mitomycin C per ml, 1.5 h, cytosol; (g) 0.5 μg of mitomycin C per ml, 1.5 h, membranes. Experiment B (lanes c, d, h, i, j, and k): (c) no mitomycin, 1 M NaCl wash of W3110(ColE1); (d) no mitomycin, SDS-lysate, W3110(ColE1); (h) 0.5 μg of mitomycin C per ml, 3 h, 1 M NaCl wash of W3110(ColE1); (i) 0.5 μg of mitomycin C per ml, 3 h, SDS-lysate, W3110(ColE1); (j) 0.5 μg of mitomycin C per ml, 3 h, 1 M NaCl wash of W3110; (k) 0.5 μg of mitomycin C per ml, 3 h, SDS-lysate, W3110. Markers, lane e, colicin E1, molecular weight 56,000; lane 1, purified EF-G.*

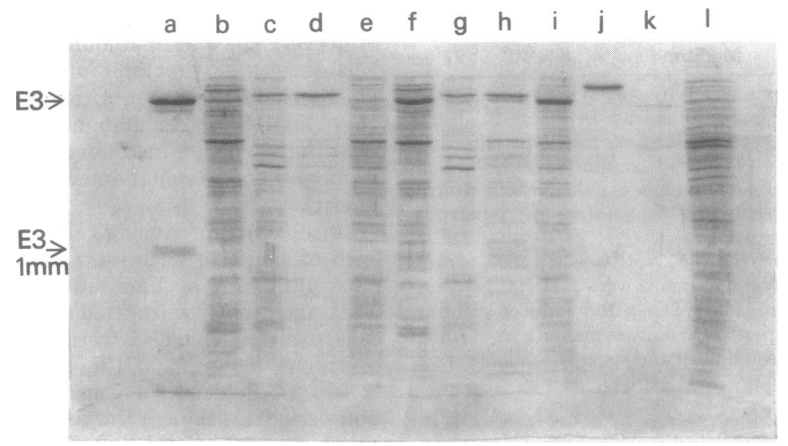

FIG. 3. *Polyacrylamide slab gel electrophoresis of fractions from colicin E3-producing cells and non-colicinogenic control cells, stained with Coomassie blue. Experiment A (lanes b, c, f, and g), all W3110(ColE3): (b) no mitomycin, cytosol; (c) no mitomycin, membranes; (f) 0.5 μg of mitomycin per ml for 1 h, cytosol; (g) 0.5 μg of mitomycin per ml for 1 h, membranes. Experiment B (lanes d, e, h, i, k, and l): (d) no mitomycin, 1 M NaCl wash of W3110(ColE3); (e) no mitomycin, SDS-lysate, W3110(ColE3); (h) 0.5 μg of mitomycin per ml for 1 h, 1 M NaCl wash of W3110(ColE3); (i) 0.5 μg of mitomycin per ml for 1 h, SDS-lysate, W3110(ColE3); (k) 0.5 μg of mitomycin per ml for 1 h, 1 M NaCl wash of W3110; (l) 0.5 μg of mitomycin per ml for 1 h, SDS-lysate, W3110. The samples were from relative amounts of culture volume in the ratio of 1:1:1:0.23, for cytosol, membranes, 1 M NaCl wash, and lysates, respectively. Markers, lane a, purified colicin E3 (molecular weight 60,000), containing immunity protein (molecular weight 10,000); lane j, purified EF-G.*

increase was greatest in the medium, since proportional amounts of wash, lysate, and medium were run on the gel shown in Fig. 5. The differences in the amount of total stained protein from the different cultures shown in Fig. 5 were due to the fact that ColE3 cultures begin to show a sharp decrease in optical density about 1 h after mitomycin C addition, whereas ColE1 cultures continue to grow (Fig. 6). The samples for the gel shown in Fig. 5 were normalized for culture

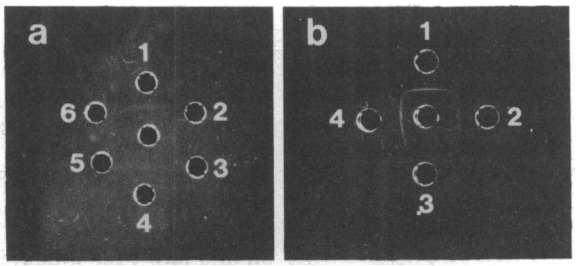

FIG. 4. *Ouchterlony double-diffusion analysis. (a) Concentrated 1 M NaCl washes of mitomycin C-induced and uninduced colicin producers and non-colicinogenic control culture: center well, anti-EF-G IgG, 2 mg/ml; well 1, W3110(ColE3) plus mitomycin for 1 h; well 2, W3110(ColE3), no mitomycin; well 3, W3110 plus mitomycin; well 4, W3110(ColE1) plus mitomycin for 3 h; well 5, W3110(ColE1), no mitomycin; well 6, blank well. (b) Concentrated medium from overnight (16 h) mitomycin C treatment: center well, anti-EF-G IgG, 2 mg/ml; well 1, W3110(ColE3); well 2, W3110(ColE1); well 3, W3110; well 4, purified EF-G, 250 μg/ml.*

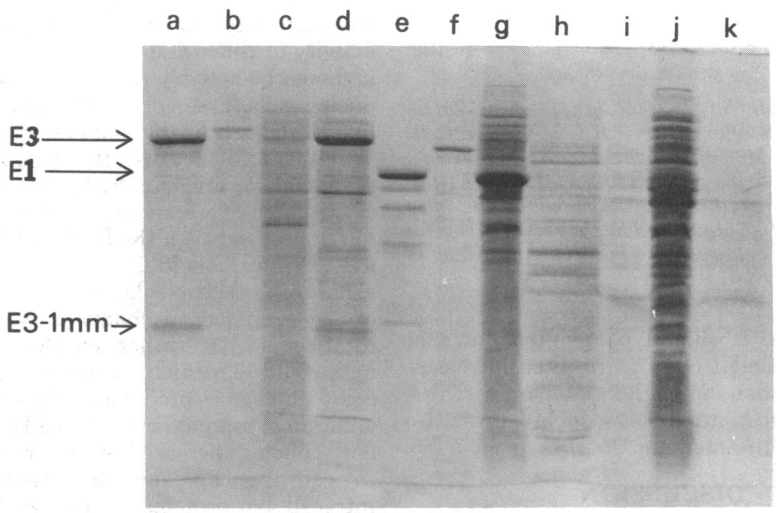

FIG. 5. *Polyacrylamide slab gel electrophoresis of samples from overnight mitomycin C induction of colicinogenic cultures and non-colicinogenic control, stained with Coomassie blue. (a) purified colicin E3, containing immunity protein; (b–d) from W3110(ColE3); (b) 1 M NaCl wash; (c) SDS-lysate of 1 M NaCl-washed cells; (d) culture medium; (e) purified colicin E1; (f–h) from W3110(ColE1); (f) 1 M NaCl wash; (g) SDS-lysate of 1 M NaCl-washed cells; (h) culture medium; (i–k) from W3110 non-colicinogenic control; (i) 1 M NaCl wash; (j) SDS-lysate of 1 M NaCl-washed cells; (k) culture medium. The samples were all from identical volumes of the starting cultures.*

volume, not final cell density. Ouchterlony double-diffusion analysis (Fig. 4b) of media from cultures induced overnight showed significant amounts of EF-G from the two colicin producers, and none from a similarly induced non-colicinogenic strain. We therefore conclude that lysis of induced ColE3 cultures and an increase in permeability of ColE1 cultures does occur after synthesis of substantial amounts of colicin. It should be noted (Fig. 5) that most colicin E1 remains in the lysates even after many hours in mitomycin C, although a significant increase in permeability of the producers occurred (Fig. 5, g and h), and the percentage of colicin in the medium increased almost 30-fold.

Ouchterlony double diffusion analysis of the reaction of varying amounts of purified EF-G with anti-EF-G IgG was done to titrate approximately the amounts of EF-G that were required to give precipitin bands such as those from the reactions shown in Fig. 4 (titration not shown). It was calculated that the EF-G in the medium of 16-h cultures was about 60 times more than that in the 1-h ColE3 wash and about 30 times more than that in the 3-h ColE1 wash. The colicin titers of those fractions differed by about 13-fold for both colicins. Thus the increases in EF-G and colicin in the various fractions correlate within a factor of five, a reasonable figure, considering the inherent inaccuracies of some of

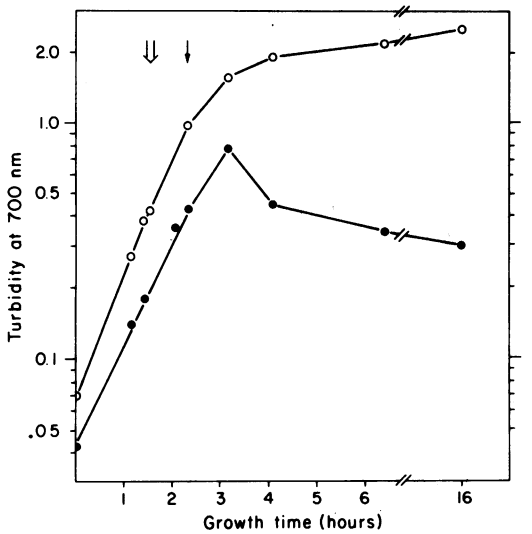

FIG. 6. *Growth of W3110(ColE1) and W3110-(ColE3) cultures after mitomycin C induction. Cells were grown in M9 supplemented with yeast extract and Casamino Acids. Symbols:* ○, *W3110(ColE1);* ●, *W3110(ColE3). At the time indicated by the open arrow for W3110(ColE1), and by the closed arrow for W3110(ColE3), cultures were induced with 0.5 μg of mitomycin C per ml.*

the estimates. It should be pointed out that induction of ColE1 cultures resulted in release of less EF-G than did ColE3 (compare Fig. 4b-1 with 4b-2). Substantially less colicin E1 was also released into the medium than was colicin E3.

DISCUSSION

We have shown here that colicin E1, colicin E3, and colicin E3 immunity protein, all of which can be found outside of producing cells at some point after induction with mitomycin C, are not synthesized with amino-terminal extensions as their means of exit from the cells. Instead, these proteins remain in the soluble cytosol of the producing cells until long after their synthesis. Exit of these proteins is accompanied by extensive increases in permeability, or actual lysis, of the producing cells, since the rise in extracellular colicin is accompanied by a corresponding rise in extracellular elongation factor G, an intracellular protein.

If there were two mechanisms for the export of colicin, (i) a specific one to account for the colicin which can be washed off of producing cells with 1 M salt at relatively early times after mitomycin C induction, and (ii) lysis, to account for the ultimate appearance of large amounts of colicin in the culture medium long after induction, then it would be difficult to explain the presence of EF-G in the early washes. The fact that EF-G is found in 1 M salt washes of induced colicin producers, along with some colicin, indicates that probably both the colicin and the EF-G are actually being washed out of permeable cells.

In addition to the colicin, significant amounts of colicin E3 immunity protein appear in salt washes of induced ColE3-containing cells (Fig. 3h and 5h). Free immunity protein, in addition to that found in a 1:1 complex with the colicin E3 (13), has been purified from such washes (12). Colicin E3 immunity protein, a ColE3-specified protein (28), most likely protects the ribosomes of cells making it from cleavage by the colicin E3 they are also making (12, 27; K. S. Jakes and D. Fischhoff, unpublished data). Since excess free immunity protein does not affect the in vivo activity of colicin E3 (12), it is hard to see why it should be actively transported out of the producing cells. Free colicin E3 immunity protein, like EF-G, is probably found outside of induced W3110(ColE3) because the cells have become permeable or are actually lysing.

Although there are rather large quantitative differences between the final localization of colicin E3 and colicin E1, the difference in the export of these two colicins seems to be only one of degree, not of mechanism. Only 8.5% of the total colicin E1 synthesized ultimately appears in the culture medium, whereas almost all of the colicin E3 leaves producing cells within 3 h after addition of mitomycin C (Table 1). However, for both colicins, increased release from the producing cells is accompanied by increased release of intracellular proteins. Thus, we feel that the mechanism of export—a nonspecific increase in permeability of colicin-producing cells—is basically the same for both colicins. The differences in the extent of the increase in permeability are very likely due to the many differences between the two colicins. The genes for the two colicins are carried on plasmids which differ in size and possibly in their mechanism of replication (1, 3; Jakes, unpublished data), and the proteins themselves are only somewhat physically related (26). Since both the Col factors and the colicins are different, it is not possible to decide from our results whether the increase in permeability is due to a function that is inherent in the colicin molecules themselves, or to some other protein encoded by the Col factors, which is also induced by mitomycin C. All that can be said is that the kinetics of colicin synthesis and of the increased permeability are related for both colicins. The bulk of the colicin E3 is made sooner after mitomycin C addition than is colicin E1, and colicin E3 can be found extracellularly sooner than

colicin E1 (Table 1) (Jakes, unpublished data). There may be some critical intracellular colicin concentration required for the increase in permeability to occur.

After this work was completed, Mock and Schwartz (19) reported on the mechanism of export of colicin E3. They also concluded that the ultimate means by which colicin E3 leaves producing cells is probably lysis. Although they reported that, at 1 h after mitomycin C induction, up to 50% of the total colicin E3 activity could be released from the bacteria by washing with 1 M NaCl, examination of their figures suggests that a considerably smaller proportion of the protein is actually in those washes, when they are analyzed by slab gel electrophoresis. Thus, their slab gel data are in fairly good agreement with our results, for colicin E3, since we never find more than about 7% of the activity in 1 M NaCl washes. Mock and Schwartz also pointed out that at the same time (1 h after mitomycin C addition) they found 30 to 50% of the colicin activity in 1 M NaCl washes, only 5% of that activity was membrane associated, while the rest was in the cytosol, a result that we have confirmed in this work (Fig. 3 and Table 1). This suggests that the colicin E3 activity they extracted with 1 M NaCl was not at the cell surface, but rather was coming out by some mechanism which increased the permeability of the producing cells. In addition, with the use of an immunofluorescence assay, Mock and Schwartz showed that at 1 h after induction colicin E3 becomes accessible to antiserum at the outer surface of induced cells. This colicin may correspond to that which they (and we) find can be released by 1 M NaCl, or to the small fraction which both groups have found to be membrane associated. Either a specific export process, or a generalized but gradual increase in permeability might be expected to give rise to such immunofluorescence.

The present work, together with that of Mock and Schwartz (19), suggests that the export of colicin E1 and colicin E3 is accompanied by a generalized increase in the permeability of the producing cells, and provide no support for a specific export mechanism. Although nothing we have done excludes specific transport, the simplest hypothesis consistent with the observed data is that colicin leaves producing cells by a process of lysis or quasi-lysis.

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LITERATURE CITED

1. **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.
2. **Blobel, G.** 1977. Synthesis and segregation of secretory proteins: the signal hypothesis, p. 318–325. *In* B. R. Brinkley and K. R. Porter (ed.), International cell biology 1976–1977. The Rockefeller University Press, New York, N.Y.
3. **Chang, C. N., G. Blobel, and P. Model.** 1978. Detection of prokaryotic signal peptidase in an *Escherichia coli* membrane fraction: endoproteolytic cleavage of nascent f1 pre-coat protein. Proc. Natl. Acad. Sci. U.S.A. **75**: 361–365.
4. **Clewell, D. B.** 1972. Nature of Col E1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **110**:667–676.
5. **Devillers-Thiery, A., T. Kindt, G. Scheele, and G. Blobel.** 1975. Homology in amino-terminal sequence of precursors to pancreatic secretory proteins. Proc. Natl. Acad. Sci. U.S.A. **72**:5012–5016.
6. **Fredericq, P.** 1958. Colicins and colicinogenic factors. Symp. Soc. Exp. Biol. **12**:104–122.
7. **Herschman, H. R., and D. R. Helinski.** 1967. Purification and characterization of colicin E2 and colicin E3. J. Biol. Chem. **242**:5360–5368.
8. **Holland, I. B.** 1977. Colicin E3 and related bacteriocins: penetration of the bacterial surface and mechanism of ribosomal inactivation, p. 100–127. *In* P. Cuatrecasas (ed.), The specificity and action of animal, bacterial and plant toxins. Chapman and Hall, London.
9. **Horiuchi, K., G. F. Vovis, V. Enea, and N. D. Zinder.** 1975. Cleavage map of bacteriophage f1: location of the *Escherichia coli* B-specific modification sites. J. Mol. Biol. **95**:147–165.
10. **Inouye, H., and J. Beckwith.** 1977. Synthesis and processing of an *Escherichia coli* alkaline phosphatase precursor *in vitro*. Proc. Natl. Acad. Sci. U.S.A. **74**:1440–1444.
11. **Inouye, S., S. Wang, J. Sekizawa, S. Halegoua, and M. Inouye.** 1977. Amino acid sequence for the peptide extension on the prolipoprotein of the *Escherichia coli* outer membrane. Proc. Natl. Acad. Sci. U.S.A. **74**:1004–1008.
12. **Jakes, K., N. D. Zinder, and T. Boon.** 1974. Purification and properties of colicin E3 immunity protein. J. Biol. Chem. **249**:438–444.
13. **Jakes, K. S., and N. D. Zinder.** 1974. Highly purified colicin E3 contains immunity protein. Proc. Natl. Acad. Sci. U.S.A. **71**:3380–3384.
14. **Kennedy, C. K.** 1971. Induction of colicin production by high temperature or inhibition of protein synthesis. J. Bacteriol. **108**:10–19.
15. **Kessler, S. W.** 1976. Cell membrane antigen isolation with the *Staphylococcus* protein-A antibody adsorbant. J. Immunol. **117**:1482–1490.
16. **Lingappa, V. R., A. Devillers-Thiery, and G. Blobel.** 1977. Nascent prehormones are intermediates in the biosynthesis of authentic bovine pituitary growth hormone and prolactin. Proc. Natl. Acad. Sci. U.S.A. **74**: 2432–2436.
17. **Lotz, W.** 1978. Effect of guanosine tetraphosphate on in vitro protein synthesis directed by E1 and E3 colicinogenic factors. J. Bacteriol. **135**:707–712.
18. **Luria, S. E.** 1973. Colicins, p. 293–320. *In* L. Leive (ed.), Bacterial membranes and walls. Marcel Dekker, New York.
19. **Mock, M., and M. Schwartz.** 1978. Mechanism of colicin

E3 production in strains harboring wild-type or mutant plasmids. J. Bacteriol. **136**:700–707.
20. **Model, P., and N. D. Zinder.** 1974. *In vitro* synthesis of bacteriophage f1 proteins. J. Mol. Biol. **83**:231–251.
21. **Osborn, M. J., and R. Munson.** 1974. Separation of the inner (cytoplasmic) and outer membranes of gram-negative bacteria. Methods Enzymol. **31**:642–653.
22. **Randall, L., S. Hardy, and L.-G. Josefsson.** 1978. Precursors of three exported proteins in *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **75**:1209–1212.
23. **Rice, R. H., and G. E. Means.** 1971. Radioactive labeling of proteins *in vitro*. J. Biol. Chem. **246**:831–832.
24. **Sander, G.** 1977. Mechanism of action of colicin E3. Effect on ribosomal elongation-factor-dependent reactions. Eur. J. Biochem. **75**:523–531.
25. **Schaller, K., and M. Nomura.** 1976. Colicin E2 is a DNA endonuclease. Proc. Natl. Acad. Sci. U.S.A. **73**:3989–3993.
26. **Schwartz, S. A., and D. R. Helinski.** 1971. Purification and characterization of colicin E1. J. Biol. Chem. **246**:6318–6327.
27. **Sidikaro, J., and M. Nomura.** 1974. E3 immunity substance: a protein from E3-colicinogenic cells that accounts for their immunity to colicin E3. J. Biol. Chem. **249**:445–453.
28. **Sidikaro, J., and M. Nomura.** 1975. *In vitro* synthesis of the E3 immunity protein directed by Col E3 plasmid deoxyribonucleic acid. J. Biol. Chem. **250**:1123–1131.