JOURNAL OF BACTERIOLOGY, July 1985, p. 174–179
0021-9193/85/070174-06$02.00/0
Copyright © 1985, American Society for Microbiology
Vol. 163, No. 1

Mitomycin-Induced Lethality of *Escherichia coli* Cells Containing the ColE1 Plasmid: Involvement of the *kil* Gene

SHIPING ZHANG, ALBERT FARO, AND GEOFFREY ZUBAY*

Fairchild Center for Biological Sciences, Department of Biological Sciences, Columbia University, New York, New York 10027

Received 5 December 1984/Accepted 15 April 1985

Escherichia coli **cells containing the ColE1 plasmid or related plasmids are killed by considerably lower levels of mitomycin C (MTC) than are plasmid-free cells. Since exposure to MTC induces high levels of synthesis of the plasmid-encoded colicin toxin, it was originally thought that the killing effect was due to the increased levels of colicin. This possibility was discounted when it was shown that deletion mutations in the plasmid lacking most of the colicin (*cea*) gene still sensitized host cells to MTC. Only when the region containing the *cea* gene promoter was deleted did the killing effect disappear. This led to the suggestion that transcription originating from the *cea* gene promoter and not the colicin protein itself was required for killing. Transcription-blocking mutations in the *cea* gene support this suggestion. It was proposed that there is a gene (*kil*) located downstream from the *cea* gene in the same operon that is responsible for MTC killing and colicin transport. The precise location of the *kil* gene in ColE1 can be predicted by piecing together published sequence information. We used available sequence data to construct a number of well-defined plasmid mutants to further examine the relevance of transcription from the *cea* promoter and the *kil* gene to drug-induced killing and colicin transport. The most informative mutant had a small insertion in the *kil* gene. This mutant behaved as predicted; cells containing it had a greatly lowered sensitivity to MTC and were severely inhibited in the transport of colicin.**

Escherichia coli cells containing the colicin E1 (ColE1) plasmid are unusually sensitive to DNA-damaging reagents such as mitomycin C (MTC) (12, 16, 18). As little as 0.4 μg of MTC per ml will kill more than 90% of the cells after 1 to 2 h. The same cells without plasmid survive this treatment.

Some of the effects of MTC are well understood. It is known that exposure to MTC sets off a chain of reactions known as the SOS response (10). First, MTC produces structural damage in the DNA. This damage somehow signals the activation of a highly specific protease activity of the RecA protein. The protease in turn cleaves the LexA protein which is a repressor of several host genes involved in DNA repair; the LexA protein is also a repressor of the plasmid *cea* gene for colicin (5). Destruction of the LexA protein results in derepression of the *cea* gene, and a high level of colicin synthesis ensues.

Colicin is a toxin which kills plasmidless cells at very low concentrations. Cells containing the plasmid are protected from the harmful effects of colicin by products of the plasmid *imm* gene (e.g., see reference 8). At very high levels of colicin even plasmid-containing cells are killed, so it was quite natural to assume that the sensitivity of such cells to MTC was due to the high levels of colicin synthesis that it stimulates. That colicin itself was not responsible for the increased drug sensitivity was first shown by Shafferman et al. (16). They observed that deletion within the *cea* gene that eliminated active colicin formation had no effect on the drug-induced lethality. However, a deletion which also eliminated the *cea* gene promoter greatly lowered drug sensitivity. Tn*1* insertions within the *cea* gene also lowered drug sensitivity to a comparable extent so that plasmid-containing cells were no more sensitive to MTC than were plasmidless cells. Tn*1* insertions usually have a polar effect blocking downstream transcription of a gene from the point of insertion. The characteristics of the insertion and deletion mutants led to the suggestion that transcription originating from the *cea* gene promoter but not from the colicin protein itself was essential for drug-induced lethality. Derepression of the *cea* promoter after exposure to MTC was presumed to result in the expression of a gene in the same operon as the *cea* gene which in turn caused a lethal reaction. This hypothesized gene was named the *kil* gene.

Before the proposal of a *kil* gene it was known that the *imm* gene, responsible for immunity to exogenous colicin, is located immediately downstream of the *cea* gene. Cells harboring cea^+ *imm* plasmids usually grow poorly if at all. Inselburg found that such cells showed improved growth in liquid culture when trypsin was added to the growth medium (8). This observation suggested that the toxic action of colicin was confined to its effect after transport outside the cell since trypsin action could not otherwise be effective. It also demonstrated most clearly that the function of the *imm* gene was to protect the cell from colicin in the extracellular environment.

In view of this function, it seemed unlikely that the *imm* gene in any way could be responsible for drug-induced killing or, to put it another way, that the *imm* gene could be the *kil* gene. Nevertheless, its location immediately downstream of the *cea* gene did complicate the search for the *kil* gene and the selection for *kil* mutants. An insertion mutation in the *imm* gene might eliminate drug sensitivity due to the *kil* gene; at the same time, it might lead to lethality because of the loss of the *imm* gene function. Recognizing this complication, Sabik et al. (14) and Suit et al. (18) attempted to devise assays that would permit discrimination between two forms of lethality, one resulting from insufficient *imm* gene product and the other resulting from high-level expression of the *kil* gene. They found that increased membrane permeability and the associated transport of colicin could be ascribed to one function (presumably Kil) and that immunity to extracellular colicin could be ascribed to another function (presumably Imm). Suit et al. isolated two particularly

* Corresponding author.

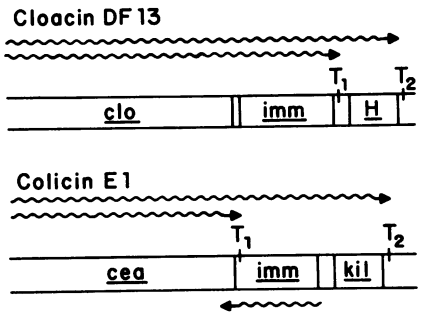

FIG. 1. Genetic regions in cloacin DF13 and colicin E1 that contain the bacteriocin genes and other nearby genes in the same operon. Transcripts are indicated by wavy lines with termination points at the head of the arrow. In cloacin DF13, transcription of all three genes *clo*, *imm*, and *H* is initiated from a promoter on the left (not shown). This transcript terminates at T1 (T_1) which is located between the *imm* and the *H* gene or at T2 (T_2) which is located after the *H* gene. In colicin E1, transcription also initiates from a promoter on the left (not shown). This transcript terminates about 50 bp downstream from the *cea* gene at T1 or about 500 bp further downstream after the *kil* gene at T2. In this case transcription of the *imm* gene initiates from an *imm*-specific promoter in the opposite direction. The ColE1 plasmid contains 6,646 bp. The transcription and translation start and stop sites for the three genes are given. *cea* gene: transcription start (4, 13), 5065; transcription stops (4), ~115 (T1) and ~613 (T2); translation start (20), 5140; translation stop (20), 60. *kil* gene: transcription start (4), same as *cea* gene; transcription stop (4), same as T2; translation start (inferred by comparison of references 7 and 11), 448; translation stop (inferred by comparisons of references 7 and 11), 583. *imm* gene: transcription start (13), 409; transcription stop, unknown; translation start (11), 400; translation stop (11), 61.

interesting mutant plasmids containing Tn*5* insertions in the *imm* gene. One of these mutant plasmids gave the Cea^+ Kil^- Imm^+ phenotype, as evidenced by the fact that cells carrying this plasmid were not killed by low levels of MTC even though they made large amounts of colicin when exposed to the drug. The colicin made in such cells was severely inhibited in transport. Even though the insert in this plasmid was in the *imm* gene, it did not appear to affect *imm* gene function. This is possible since it is known that the C-terminal portion of the immunity protein is not required for its function (see reference 11). The second plasmid mutant gave a Cea^+ Kil^- Imm^- phenotype. Cells containing it were killed by low levels of MTC. Unlike cells carrying the wild-type plasmid, however, their membranes do not become highly permeable after MTC treatment and they very poorly transport the colicin they make after induction. The mutant studies of Suit et al. were helpful in suggesting certain properties associated with the *kil* gene. They were confusing in that they did not establish the location of the *kil* gene. This is because Tn*5* insertions like Tn*1* insertions (discussed above) tend to be polar, disrupting the gene in which they are located but also inhibiting transcription of downstream genes in the same operon. Thus, in any particular mutant the *kil* gene could be located either at the site of the Tn*5* insertion that gives a Kil^- phenotype or further downstream from the site of the insertion.

Evidence suggesting the location of the *kil* gene in ColE1 comes from studies on a related plasmid CloDF13 and from sequence comparisons between the two plasmids. The CloDF13 plasmid contains three clustered genes, *clo*, *imm*, and *H*, in the same operon (Fig. 1; 7). These genes are contranscribed from a promoter adjacent to the *clo* gene. The *clo* and *imm* genes of CloDF13 are completely different in structure from the *cea* and *imm* genes of ColE1, although they serve comparable functions. In each case the first gene, *clo* or *cea*, encodes a protein toxin (bacteriocin), whereas the *imm* gene encodes an immunity factor that protects plasmid-harboring cells from the toxin. The *H* gene of CloDF13 encodes a 6-kilodalton (kDa) protein called protein H (6, 7). A high concentration of protein H, due either to the drug-induced expression of the *H* gene or to a gene dosage effect, results in the killing of bacterial cells. The gene for protein H is located immediately downstream of the *imm* gene in CloDF13 (Fig. 1). Determination of the precise location of the gene involved: (i) sequence analysis of the plasmid in that region, (ii) comparison of reading frames in that region which could encode a 6-kDa protein with the actual amino acid composition of protein H, and (iii) finally showing that deletion mutations involving part of the presumptive gene result in a loss of the ability to make protein H and the absence of cell lysis associated with exposure to MTC.

A strikingly similar sequence to the H protein gene is located on the other side of the *imm* gene in ColE1 (for relevant sequence data, see references 11 and 20). This gene, believed to be the *kil* gene, is oriented so that transcription initiated from the *cea* gene promoter and extending through the *kil* gene should make a productive messenger for the *kil* gene protein. Related to this, Ebina and Nakazawa (4) showed that transcripts initiated from the *cea* gene promoter are of two types, some terminate at T1, located about 60 base pairs (bp) downstream from the *cea* gene, whereas others proceed an additional 500 bp downstream to T2 (Fig. 1). The latter transcript includes the presumptive coding sequences for the Kil protein.

In this paper we describe observations made on various plasmid mutants designed to examine the significance of transcription originating from the *cea* promoter and the *kil* gene on cell viability and colicin transport.

MATERIALS AND METHODS

E. coli K-12 514 was used in most of the studies reported here (2). This strain contains wild-type $recA^+$ and $lexA^+$ genes. *E. coli* K-12 GW1000 contains a *tif* mutation of the *recA* gene (19). The recA protein in this mutant has a hyperactive protease, especially at elevated temperatures. Other properties of the strain are described elsewhere (9). All of the restriction enzymes used were obtained from International Biotechnologies, Inc.

Assay for MTC-induced lethality. Cells were grown in TS broth (2% tryptose and 0.5% NaCl at 37°C). In mid-exponential phase, 0.4 μg of MTC per ml was added to the cells, and growth was continued for an additional period of 2 to 6 h. Viable cells were determined by plating suitable samples of MTC-free cells on 0.5% agar plates containing TS broth. Colonies arising after overnight growth were taken as an estimate of the number of viable cells in the original culture and the MTC-treated culture.

Preparation of plasmid mutants. All plasmid mutants were made from ColE1 or pDS1107, a ColE1 derivative containing a Tn*1* insertion (Fig. 2; 3). Three plasmids with deletions pAF1 (deletion of bp 5280 to 6549), pAF2 (deletion of bp 1652 to 3432), and pSZ1 (deletion of bp 1682 to 4627) were obtained by exhaustive digestion with a single restriction endonuclease, *Pst*I, *Pvu*II, or *Xmn*I, respectively. The resulting DNA fragments containing the replication origins were recircularized with T4 DNA ligase. Three plasmids

with deletions pSZ2 (deletions of bp 4072 to 6646), pSZ3 (deletion of bp 5145 to 6646), and pSZ4 (deletion of bp 5280 to 6646) were obtained by exhaustive digestion with combinations of two different restriction enzymes, *Aha*III-*Eco*RI, *Sac*II-*Eco*RI, or *Pst*I-*Eco*RI, respectively. After digestion these fragments were blunt ended by treatment with T4 DNA polymerase in the presence of the four deoxynucleoside triphosphates. This enzyme degrades ends containing 3′ overhangs and fills in ends containing 5′ overhangs. The blunt-ended linear fragments then were circularized by treatment with T4 DNA ligase. After ligation the plasmids were transfected into strain 514. Transfected cells were selected by the immunity property which permits survival on agar plates containing colicin. Plasmids were reisolated from the transfected cells, and their structures were confirmed by restriction enzyme analysis.

Two plasmid mutants were made from pDS1107 which contains a Tn*1* insertion around base pair 4100 (Fig. 2). This plasmid was convenient to use because it contains an ampicillin resistance marker that facilitates selection of mutant variants of the plasmid constructed in vitro.

A *kil* mutant (pSZ5) was constructed from pDS1107 by first treating pDS1107 exhaustively with *Nru*I endonuclease. This endonuclease produces a single blunt end cleavage between bp 478 and 479 which is 40 bases from the start

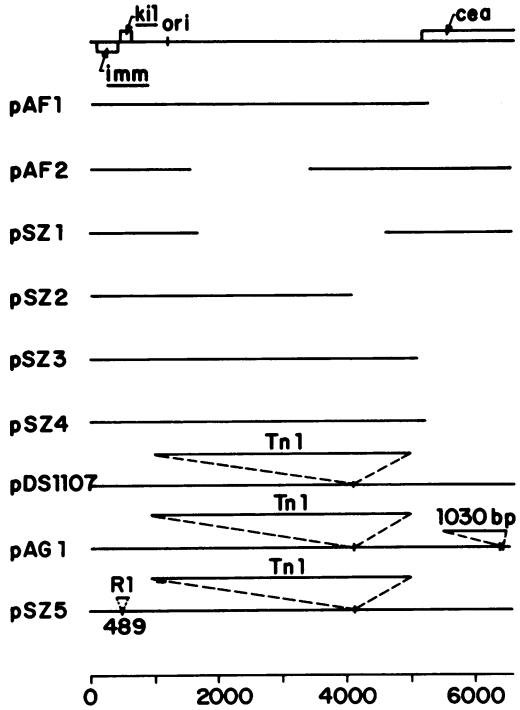

FIG. 2. Genetic map of ColE1. The chromosome is shown in the linear form, with the numbering of bases (1 to 6646) starting at the unique *Eco*RI cleavage site. The locations of the origin of replication (ori) and of the *cea*, *imm*, and *col* genes are shown. pAF1, pAF2, pSZ1, pSZ2, pSZ3, and pSZ4 are mutant plasmids containing deletions as shown. pDS1107, pAG1, and pSZ5 are mutant plasmids containing inserts. All three of these plasmids contain a Tn*1* insert around bp 4100. pAG1 contains an additional 1,030-bp tandem repeat which is shown. Greater detail on the structure of this insert is given in Fig. 3. pSZ5 contains an 8-bp insert with an *Eco*RI restriction site at bp 479. The preparation of the mutant plasmids and the precise location of the deletion and the insertion are described in the text.

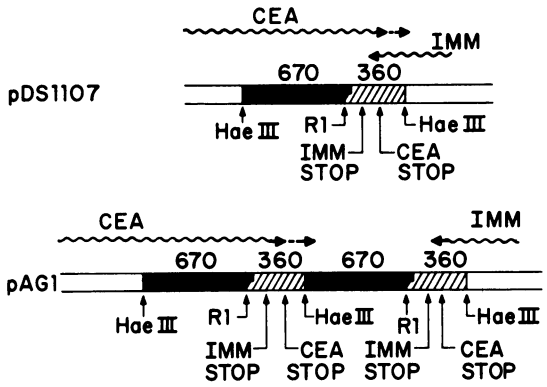

FIG. 3. Structure of *cea-imm* boundary in pDS1107 and in pAG1 which contains a 1,030-bp tandem duplication of the boundary region. pDS1107 has the same structure in this region as does wild-type ColE1. Construction of pAG1 is described in the text. Transcripts as in Fig. 1 are indicated by wavy lines with arrowheads. The *cea* stop that is indicated corresponds to the ρ-dependent termination signal T1. The alternative T2 termination signal is not shown for this transcript, but further transcription beyond T1 is suggested by a dashed arrow. The actual transcription termination signal for the *imm* gene is not known. The locus designated as the *imm* stop should be labeled presumptive *imm* stop. The DNA sequence in this region shows the dyad symmetry characteristic of a stop signal. It can be seen that leftward transcription ending at the T1 stop signal ordinarily overlaps, with rightward transcription terminating at the presumptive *imm* stop signal. This overlap is eliminated in the pAG1 plasmid.

of the *kil* gene reading frame (bp 448 to 583). The resulting linear fragment was reacted with a 20-fold excess of an 8-base blunt end linker containing an *Eco*RI site (GGAATTCC) in the presence of T4 DNA ligase. This product was redigested with *Nru*I to remove plasmid that recircularized without incorporating linker. After transfection the construction of the reisolated plasmid was verified by restriction enzyme digestion and gel electrophoresis. This analysis showed that the plasmid had lost its *Nru*I site and gained a second *Eco*RI site (normally pDS1107, like ColE1, contains only one *Eco*RI recognition site).

Plasmid pAG1 contains a 1,030-bp duplication (bp 5979 to 364) in the region bounding the *cea* and the *imm* genes (Fig. 3). The following procedure was used to construct the plasmid. ColE1 was digested with *Eco*RI and *Hae*III. This treatment led to the production of 12 blunt-ended *Hae*III fragments and 2 fragments of 360 and 670 bp, which contain one *Hae*III blunt end and one *Eco*RI 5′ overhang. pDS1107 was digested with *Eco*RI and calf alkaline phosphatase. Treatment with calf alkaline phosphatase prevented self-ligation in the subsequent step. The fragments from the *Eco*RI-*Hae*III digestion were mixed with the linearized pDS1107 and treated with T4 ligase, first under conditions favoring staggered end ligation and then under conditions favoring blunt end ligation. The reaction mixture should contain a variety of products. First, transfectants were selected for ampicillin resistance. Then they were selected for colicin-producing cells. Finally, plasmids were isolated from different amp^+ cea^+ clones and analyzed by restriction enzyme treatment and gel electrophoresis to find one that carried a tandem duplication of the *Hae*III B segment of ColE1.

RESULTS

ColE1-containing cells in liquid culture were treated with 0.4 μg of MTC per ml in mid-exponential phase. At specified

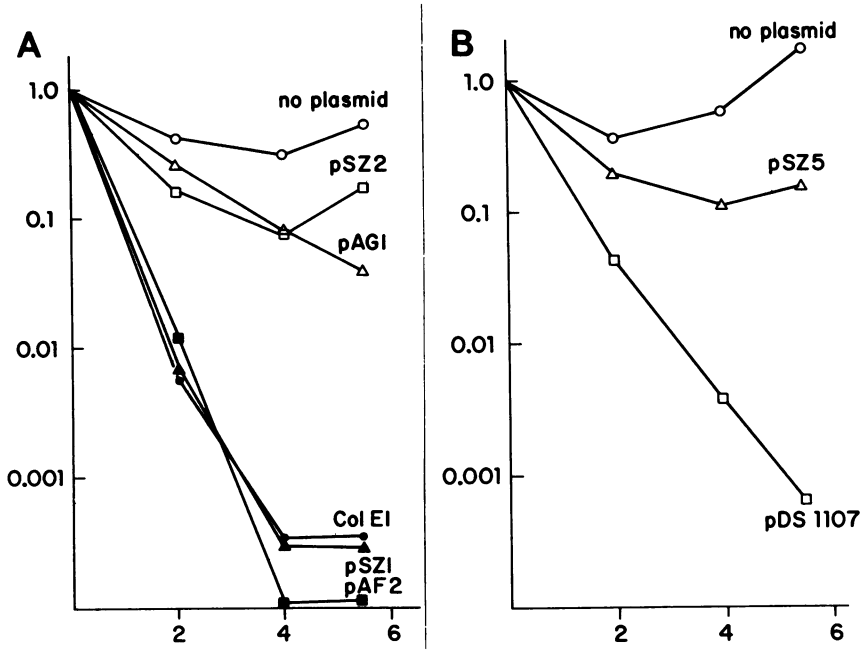

FIG. 4. Survival of cells in liquid broth containing 0.4 μg of MTC. On the ordinate the logarithmic scale indicates the ratio of viable cells at time zero to viable cells after exposure to MTC. The time of exposure to MTC is indicated on the abscissa in hours. The viable cell number is determined by spinning cells down from liquid broth and plating a suitable sample on nutrient agar in the absence of drug. The number of colonies after overnight incubation at 37°C is taken as an indication of the number of viable cells in the sample. Treated cells either contained no plasmid or plasmid as indicated. The structure of different plasmids is indicated in Fig. 2.

time intervals samples were removed from the culture, sedimented, suspended, and plated to assay for the number of viable cells. About 90% of the cells containing the ColE1 plasmid were killed within 1 h. The effect was progressive so that after 6 h of exposure to the drug less than 0.1% of the cells survived (Fig. 4). Comparable plasmidless cells showed better than 50% survival after a 6-h exposure to the drug, and the effect of prolonged exposure was not progressive. Indeed, the population usually seemed to recover after the first 2 h of exposure, as evidenced by an increased number of survivors.

Nine mutants of ColE1 were examined for their effect on MTC killing. The structures of these plasmids are indicated in Fig. 2 (more data on the precise location of the mutations is given above). In the absence of drug these plasmids did not influence the cell growth rate, and their copy number (the number of plasmids per cell) was comparable, except for plasmid pAF2 which tends to be present in slightly higher numbers. The relative copy number was visually estimated on an ethidium bromide-stained gel after electrophoresis of a plasmid preparation. Whereas more refined methods exist for plasmid copy number estimation, this procedure was considered adequate for our purpose.

Two of these mutant plasmids, pAF2 and pSZ1, contained deletions upstream of the *cea* gene (Fig. 2). Cells with these plasmids showed the usual high sensitivity to MTC (Fig. 4A). Drug hypersensitivity was also observed for cells carrying the pDS1107 plasmid which contains a Tn*1* insertion about 100 bp upstream from the *cea* gene (Fig. 4B). Several mutant plasmids, pAF1, pSZ3, and pSZ4, carried deletions within the *cea* gene. These mutants also made their host cells hypersensitive to MTC (results not shown). Finally, three mutants, pSZ2, pAG1, and pSZ5, were found to be greatly reduced in their ability to increase drug sensitivity of harboring cells (Fig. 4A and B). The drug sensitivity of such cells was just slightly greater than that observed for plasmidless cells. Plasmid pSZ2 contains a large deletion which removes most of the *cea* gene including the promoter and some sequences upstream from the promoter. Plasmid pAG1 contains a tandem duplication (1,030 bp) of a region bordering the *cea* and *imm* genes (see Fig. 3 for details). Plasmid pSZ5 contains a mutation situated exclusively in the *kil* gene.

Plasmids pSZ5 and pDS1107 were examined in more detail in a *tif* strain of *E. coli* (GW1000) in which colicin synthesis is induced by a temperature upshift from 30 to 41°C in the absence of MTC. Under these conditions of induction, cell death is not apparent (17). The effect of a temperature upshift was examined 6 and 18 h after a temperature upshift. Intracellular colicin levels were examined by acrylamide gel electrophoresis of whole-cell lysates (Fig. 5). After 6 h, both cells contained high levels of colicin, as evidenced by the prominence of a 56-kDa band characteristic of colicin (Fig. 5, lanes 2 and 5). AFter 18 h at 41°C, the cells carrying the pSZ5 *kil* plasmid gave a similar gel profile (Fig. 5, lane 6). In marked contrast, the cells carrying the pDS1107 *kil*$^+$ plasmid lost almost all of their colicin (Fig. 5, lane 3). With neither culture was there any evidence for cell death or lysis. Thus, after 18 h at 41°C, both cultures contained about the same number of cells, as measured by turbidimetry or by plating. In addition, examination of six clones arising from both 18-h cultures showed that unmodified plasmid was present in the cells of both cultures. The growth medium after 18 h of exposure also was examined by gel analysis. For this purpose, the protein present in the cell-free growth medium was precipitated by the addition of a 20-fold excess of acetone. The precipitate was collected by centrifugation, and the acetone was removed by air drying. The sample derived from the pSZ5-containing cells showed almost no protein (results not shown). The sample derived from the pDS1107-

containing cells after 18 h at 41°C showed a substantial concentration of protein (Fig. 5, lane 8) dominated by a prominent band with an estimated molecular weight of about 27 kDa but also containing some 56-kDa protein characteristic of colicin.

The extracellular fluid from the two cultures was also tested for the presence of colicin by the kill unit bioassay. This assay measures the killing effect of colicin on normal plasmidless *E. coli* cells which are sensitive to colicin, as described elsewhere (17). By this assay the extracellular fluid from the GW1000(pDS1107 *kil*$^+$) cells was always 100 to 1,000 times more potent than that from the GW1000 (pSZ5 *kil*) cells.

Parallel gel analyses were done on cell extracts and on the extracellular fluid from strain 514 cells containing either pSZ5 or pDS1107 (results not shown). The cells were examined at 4 h after exposure to 0.4 μg of MTC per ml, as described in the legend to Fig. 4. Cells carrying the pDS1107 (*kil*$^+$) plasmid showed no accumulation of colicin. All of the colicin was in the extracellular fluid, along with a spectrum of other proteins very similar to that found in a whole-cell lysate. This suggests that all of the colicin had been transported and that a substantial percentage of the cells (~30%) had lysed. Cells carrying the pSZ5 (*kil*) plasmid accumulated a significant amount of colicin (about one-quarter of that observed in strain GW1000 after 6 h [Fig. 5, lane 2]). A very small amount of colicin, but only minute traces of other proteins, was also observed in the extracellular fluid. This suggests that even though colicin transport is strongly inhibited there is some indication that it is selectively transported.

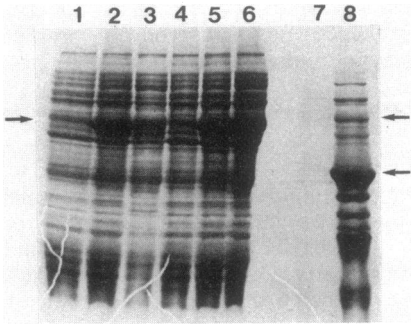

FIG. 5. Polyacrylamide gel electrophoresis patterns of proteins isolated from plasmid-containing cells induced to make colicin. Strain GW1000 cells were used. These either contained the *kil*$^+$ pDS1107 plasmid (lanes 1 to 3) or the *kil* pSZ5 plasmid (lanes 4 to 6). Cells grown in L broth at 30°C were transferred in mid-log phase to a 41°C water bath at time zero. Crude whole-cell lysates were prepared from these cells shortly thereafter (lanes 1 and 4) and at 6 h (lanes 2 and 5) and 18 h (lanes 3 and 6) thereafter. The extracts were electrophoresed on a 5 to 20% polyacrylamide gradient gel and treated with the general protein stain Coomassie blue. In lanes 7 and 8 are shown the extracellular protein prepared from the cell-free growth media of the GW1000(pDS1107) cells 6 and 18 h after the temperature upshift. Lanes 7 and 8 should be compared with lanes 2 and 3, respectively, except that the extracellular protein was prepared from a twofold larger sample of the liquid culture (see the text for further details). Comparable gel patterns are not shown for the extracellular extracts of the culture containing the *kil* plasmid because they were very faint. The arrow adjacent to lane 1 indicates the position of the 56-kDa colicin protein. The upper arrow adjacent to lane 8 indicates the position of colicin, and the lower arrow indicates the location of the main band with an estimated molecular weight of 27 kDa.

DISCUSSION

The currently favored model for MTC-induced lethality in ColE1-containing cells is that lethality results from expression of the *kil* gene (e.g., see reference 18). This model is supported by findings in this study.

Lethality is usually measured by plating efficiency of cells previously exposed to MTC in liquid culture. High levels of MTC (2 μg/ml or greater) will kill plasmidless cells, but only plasmid-containing cells are extensively killed by low levels (0.4 μg/ml) of the drug (compare no plasmid and ColE1 in Fig. 4A). Deletions in the plasmid outside of the *cea-imm-kil* gene cluster do not lower the killing effect (see pSZ1 and pAF2 in Fig. 4A). Deletions within the *cea* gene do not lower the killing effect unless they remove the *cea* gene promoter. Thus, plasmids pAF1, pSZ3, and pSZ4 which contain extensive deletions of the coding region of the *cea* gene (Fig. 2) do not lower the killing effect of the plasmid. But pSZ2, which also removes the *cea* promoter (Fig. 2), greatly lowers drug-induced killing (Fig. 4A). These findings are in agreement with those of Shafferman et al. (16) and Suit et al. (18).

Thus far, we have considered examples of plasmids which are *cea*$^+$ *imm*$^+$ *kil* or *cea*$^+$ *imm kil* but none which are *cea*$^+$ *imm kil*$^+$. We mentioned above a *cea*$^+$ *imm* plasmid isolated by Inselburg that grew best in liquid culture when trypsin was present. Since the mutation in this plasmid was due to a Tn*3* insertion into the *imm* gene, it is very likely that a polar effect would result, making the strains effectively *kil*. This was never tested, but it might help account for the survival of cells containing this plasmid since they would be expected to transport colicin poorly. It would be informative to have plasmid mutants that are altered in immunity expression without being altered in *kil* gene expression. In fact, plasmid pSZ4 appears to be such a plasmid. Measured by resistance to exogenous colicin, this plasmid shows a much higher level of immunity than does plasmid pAF1 or ColE1 (15; unpublished data). Plasmid pSZ4 has 100 bp less than pAF1 in the promoter distal region of the *cea* gene (Fig. 2). We do not know the reasons for the large effect of this gene segment on immunity expression. The most important point here is that plasmid mutations do exist which have a huge effect on immunity without significantly affecting *kil* expression.

The *cea* and the *imm* reading frames abut one another to the extent that their stop codons share 2 bp (see the legend to Fig. 1). A mutant containing a duplication of this region was constructed (Fig. 3) to determine the effect that eliminating the overlap would have on immunity and drug-induced killing. No effect on immunity was seen (results not shown), but drug-induced killing was greatly reduced (see pAG1 in Fig. 4A). Since this duplication introduces an additional T1 transcription termination site between the *cea* gene and the *kil* gene, it is possible that this is the cause of the reduced killing. This interpretation is consistent with the findings of Shafferman et al. (16) and Suit et al. (18) that transposons inserted in the *cea* gene usually block killing.

The most important mutant plasmid generated for our studies was pSZ5 which contains a small DNA insertion in the middle of the *kil* gene. This is the only mutation that is solely located within the *kil* gene. The properties of this mutant plasmid confirm and extend expectations from previous work. Thus, MTC sensitivity is largely eliminated (Fig. 4A), and colicin is synthesized but cannot be found in the extracellular fluid. The striking absence of all but a trace of extracellular colicin in induced cells containing the pSZ5 plasmid indicates that the *kil* gene is needed for efficient transport of colicin.

Whereas it is clear that extracellular colicin is drastically reduced in the absence of the Kil protein, this by itself does not explain how the Kil protein functions in colicin release. The first question to resolve is whether the Kil protein stimulates colicin release by a discreet secretion process, or whether lysis is required. Our results favor the conclusion that colicin release does not require lysis. Thus when strain GW1000 containing either plasmid is shifted from 30 to 41°C, this results in a high level of colicin synthesis (Fig. 5). The turbidity of both cultures rises steadily until it reaches a plateau. Plating of samples from either culture yields approximately the same viable cell count. Indeed, the only difference to be found between the two cultures is that the *kil*$^+$ plasmid-containing cells lose most of their colicin in addition to minor amounts of some other proteins. Since there is no evidence for cell lysis, we are inclined to the view that this loss of protein involves a Kil protein-dependent transport process. This does not outrule the participation of other specific protein factors as suggested by Yamada et al. (21).

The spectrum of proteins released upon prolonged incubation of *kil*$^+$ plasmid-containing cells is complex (Fig. 5, lane 8). Some of these proteins are clearly plasmid encoded, but some may also be host encoded. Comparison of lanes 2 and 3 (Fig. 5) shows that prolonged incubation results in the selective loss or reduction of several intracellular proteins in addition to the 56-kDa colicin. Colicin is unstable in the extracellular state (e.g., see reference 1), and this may account for the low intensity of the 56-kDa band in the extracellular fluid. From the large amount of the 27-kDa protein found in the extracellular fluid (lower arrow in Fig. 5, lane 8), it is tempting to suggest that this band represents a major degradation product of the colicin. In this connection it may be relevant that a polypeptide of this size is the second most abundant species synthesized in the ColE1 DNA-directed system for protein synthesis (2). It was shown that this polypeptide is encoded by the promoter-proximal region of the *cea* gene. Other minor proteins or peptide fragments found in the extracellular fluid may be host encoded or plasmid encoded.

The most puzzling aspect of cell death associated with colicin production is related to the fact that the conditions of induction are critical. On the one hand, when induction is caused by DNA-damaging reagents such as UV irradiation or MTC, massive cell death often accompanied by cell lysis results when the wild-type plasmid is present. On the other hand, when induction is caused by host mutants that result in a deficiency of the LexA repressor, cell death does not necessarily follow. The critical factor may be the Kil protein concentration which in turn could be related to the extent to which transcripts stop at the T1 and the T2 terminators (Fig. 1). Only the latter transcripts would lead to Kil protein synthesis. Possibly the effectiveness of termination at T1 is influenced by metabolic conditions. With the help of the pSZ5 mutant plasmid, it should now be possible to systematically investigate the effect of different modes of colicin induction on the types of transcripts that are synthesized. Attempts are also being made to assay for the Kil protein directly.

ACKNOWLEDGMENTS

We are very grateful to Graham Walker for advice and help in obtaining some of the bacterial strains, to David Sherratt for plasmid pDS1107, and to Alexandra Gampel for constructing the pAG1 plasmid used in this study. P. Chan and J. Lebowitz supplied us with total sequence data of ColE1 in advance of publication. This was most helpful in some of the plasmid constructions. Eva Dworkin advised us on procedures to use in plasmid construction.

This work was supported by grant PCM-80-21625 from the National Science Foundation, grant 5 RO1 GM 29217 from the National Institutes of Health, and a grant from Boehringer Ingelheim.

LITERATURE CITED

1. **Brey, R. N.** 1982. Fragmentation of colicins A and E1 by cell surface proteins. J. Bacteriol. **149**:306–315.
2. **Chen, H.-Z., and G. Zubay.** 1983. Analysis of ColE1 expression in vitro after chromosome fragmentation. J. Bacteriol. **154**:650–655.
3. **Dougan, G., and D. Sherratt.** 1977. The transposon Tn1 as a probe for studying ColE1 structure and function. Mol. Gen. Genet. **151**:151–160.
4. **Ebina, Y., and A. Nakazawa.** 1983. Cyclic AMP-dependent initiation and ρ-independent termination of colicin E1 gene transcription. J. Biol. Chem. **256**:7072–7078.
5. **Ebina, Y., Y. Takahara, F. Kishi, A. Nakazawa, and R. Brent.** 1983. LexA protein is a repressor of the colicin E1 gene. J. Biol. Chem. **258**:13258–13264.
6. **Hakkaart, M. J. J., E. Veltkamp, and H. J. J. Nijkamp.** 1981. Protein H encoded by plasmid CloDF13 involved in lysis of the bacterial host. II. Functions and regulation of synthesis of the gene H product. Mol. Gen. Genet. **183**:326–332.
7. **Hakkaart, M. J. J., E. Veltkamp, and J. J. Nijkamp.** 1981. Protein H encoded by plasmid CloDF13 involved in lysis of the bacterial host. I. Localization of the gene and identification and subcellular localization of the gene H product. Mol. Gen. Genet. **183**:318–325.
8. **Inselburg, J.** 1979. Studies of colicin induction with an *imm*$^-$ *col*$^+$ mutant of the plasmid. Mol. Gen. Genet. **173**:333–338.
9. **Krueger, J. H., S. J. Elledge, and G. C. Walker.** 1983. Isolation and characterization of Tn*5* insertion mutations in the *lexA* gene of *Escherichia coli*. J. Bacteriol. **153**:1368–1378.
10. **Little, J. W., and D. W. Mount.** 1982. The SOS regulatory system of *Escherichia coli*. Cell **29**:11–22.
11. **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 repliction and colicin E1 immunity. Mol. Gen. Genet. **171**:151–159.
12. **Ozeki, H., B. A. D. Stocker, and H. deMargerie.** 1959. Production of colicine by single bacteria. Nature (London) **184**:337–339.
13. **Patient, R. K.** 1979. Characterization of in vitro transcription initiation and termination sites in ColE1. Nucleic Acids Res. **6**:2647–2665.
14. **Sabik, J. F., J. L. Suit, and S. E. Luria.** 1983. cea-kil operon of the ColE1 plasmid. J. Bacteriol. **153**:1479–1485.
15. **Shafferman, A., S. Cohen, and Y. Flashner.** 1978. A DNA segment within the colicin E1 structural gene on ColE1 affecting immunity to colicin. Mol. Gen. Genet. **164**:259–264.
16. **Shafferman, A., Y. Flashner, and S. Cohen.** 1979. ColE1 DNA sequences interacting in *cis*, essential for mitomycin-C induced lethality. Mol. Gen. Genet. **176**:139–146.
17. **Spangler, R., S. Zhang, J. Krueger, and G. Zubay.** 1985. Colicin synthesis and cell death. J. Bacteriol. **163**:167–173.
18. **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 ColE1 plasmids. Proc. Natl. Acad. Sci. U.S.A. **80**:579–583.
19. **Tessman, E. S., and P. K. Peterson.** 1980. tif-dependent induction of colicin E1, prophage lambda and filamentation in *Escherichia coli* K12. J. Bacteriol. **143**:1307–1317.
20. **Yamada, M., Y. Ebina, T. Mujata, T. Nakazawa, and A. Nakazawa.** 1982. Nucleotide sequence of the structural gene for colicin E1 and predicted structure of the protein. Proc. Natl. Acad. Sci. U.S.A. **79**:2827–2831.
21. **Yamada, M., T. Miki, and A. Nakazawa.** 1982. Translocation of colicin E1 through the cytoplasmic membrane of *Escherichia coli*. 1982. FEBS Lett. **150**:465–468.