JOURNAL OF BACTERIOLOGY, Nov. 1986, p. 648–654
0021-9193/86/110648-07$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 168, No. 2

Expression of the Cloned ColE1 *kil* Gene in Normal and Kil^r *Escherichia coli*

MARIO ALTIERI,† JOAN L. SUIT,* M.-L. J. FAN, AND S. E. LURIA

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Received 24 March 1986/Accepted 5 August 1986

The *kil* gene of the ColE1 plasmid was cloned under control of the *lac* promoter. Its expression under this promoter gave rise to the same pattern of bacterial cell damage and lethality as that which accompanies induction of the *kil* gene in the colicin operon by mitomycin C. This confirms that cell damage after induction is solely due to expression of *kil* and is independent of the *cea* or *imm* gene products. *Escherichia coli* derivatives resistant to the lethal effects of *kil* gene expression under either the normal or the *lac* promoter were isolated and found to fall into several classes, some of which were altered in sensitivity to agents that affect the bacterial envelope.

Several colicin-encoding plasmids also contain a lysis or lethality gene that is apparently responsible for the colicin release and cell death that usually accompany induced synthesis of colicin. Such a gene, called gene *H*, was first described in pCloDF13 (11); similar genes are *kil* in pColE1 (27), *celB* in pColE2 (22), or *lys* (12) or *hic* (30) in pColE3. Each of these genes, which are very similar in nucleotide sequence (5, 30), is located in the same operon as the colicin (*cea*) gene, positioned downstream from the latter and separated from it by an immunity (*imm*) gene (5, 10–12, 22, 27, 33). In pColE1 the immunity gene is transcribed in the reverse direction from the other two genes (2, 18).

The mechanism and even the primary effect(s) of the Kil function are not known. We have previously described the pattern of physiological cell damage caused by induction of the *cea-kil* operon of pColE1 (27, 28). This pattern includes the inhibitions of active transport and of protein synthesis and development of abnormal permeability. All of these effects as well as cell death were ascribed to the action of the presumed *kil* gene product, since they were present whether colicin E1 was produced or eliminated by certain mutations or deletions and since the E1 immunity gene is not part of the same operon. To analyze further the expression of the *kil* gene we have cloned it under control of the *Escherichia coli* *lac* promoter. We have determined that its expression leads to the same set of physiological effects irrespective of the promoter under which it is expressed, confirming that these effects occur irrespective of the presence of the colicin or immunity genes. As another approach to the study of the *kil* gene action in the bacterial host, we have isolated a series of *E. coli* derivatives that are resistant to killing after induction of the *kil* gene either in its original plasmid or after cloning. The findings are reported in the present paper.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacteriophages. The strains of *E. coli* K-12, plasmids, and bacteriophages used in this study are listed in Table 1.

Media and growth conditions. The media used were LB broth (17), λ broth (27), and Ozeki medium base (OM) (14) supplemented with thiamine (1 μg/ml), glucose or glycerol (0.4%), and Casamino Acids (0.2%; Difco Laboratories). When appropriate, either ampicillin (10 to 200 μg/ml) or tetracycline (10 μg/ml), each from Sigma Chemical Co. was added. Plates of each of the media contained 1.5 to 2% agar. In experiments in which protein synthesis or proline transport was measured, the Casamino Acids were replaced by an amino acid pool (19 amino acids, each 25 μg/ml) without proline. Cultures were incubated with shaking in water baths at the desired temperatures; the turbidity of cultures was followed by readings in a Klett-Summerson photoelectric colorimeter.

Colicin assay. Colicin was assayed by spot tests as described by Suit et al. (28).

Assay of active transport. The uptake of [^{3}H]proline and [α-^{14}C]methylglucoside, both from New England Nuclear Corp., was measured as described by Plate (21).

Measurement of β-galactosidase. Isopropyl-β-D-thiogalactopyranoside (IPTG; Sigma) (10^{-2} M) was used to induce the *lac* promoter. β-Galactosidase was assayed by measuring the rate of hydrolysis of *o*-nitrophenyl-β-D-galactoside in cells treated with $CHCl_3$ and sodium dodecyl sulfate (16). In cells growing on LB plates, β-galactosidase activity was screened for with the indicator 5-bromo-4-chloro-3-indolyl-β-*d*-galactopyranoside (Bachem, Inc.) present at 80 μg/ml.

Transformation procedure. Transformation of bacterial strains with plasmid DNA was carried out as described by Cohen et al. (4).

Isolation and enzymatic treatment of plasmid DNA. pColE2 DNA was isolated by ethidium bromide-cesium chloride centrifugation of cleared Triton X-100 lysates from 1- to 2-liter LB broth cultures of *E. coli* A153(pColE2). All other plasmids used in this study have the ColE1 replicon, so strains containing these plasmids were grown at 30 or 37°C to the late log phase in LB broth or minimal medium (10 to 100 ml) with the appropriate antibiotic and then incubated overnight at the same temperature in the presence of chloramphenicol (175 μg/ml) to amplify plasmid DNA (3). Plasmid DNA was extracted by alkaline lysis (1) and whenever required was purified further by ethidium bromide-CsCl centrifugation. Restriction endonuclease reactions were carried out under conditions specified by the supplier (New England BioLabs, Inc.) as were those with bacterial alkaline

* Corresponding author.
† Present address: Hagedorn Research Laboratory, Copenhagen, Denmark.

TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Relevant genotype or phenotype	Source
Strains		
RR1ΔM15	*lacZ* M15/F′ *lacI*^{q} ZΔM15	Guido DeVos, M.I.T.[a]
W3110I^q	*lacI*^{q}*Z*$^+$ *Y*$^+$	Guido DeVos, M.I.T.[a]
A153	*lacIZ*$^+$ *Y*$^+$	This laboratory
Ymel	*supE supF*	Ethan Signer, M.I.T.
Plasmids		
pColE1	*cea*$^+$ *kil*$^+$ *imm*$^+$	This laboratory
pColE2	*ceaB*$^+$ *celB*$^+$ *ceiB*$^+$	This laboratory
pDMS630	pColE1::Tn*3* (Ap^r)	J. Inselburg, Dartmouth Medical School
pJFS3	pDMS630 *cea*::Tn*5* Kil$^-$	This laboratory
pBR322	pColE1 *ori* region, Ap^r Tc^r	Boehringer Mannheim Biochemicals
pBSE2	pBR322-pColE2 hybrid containing pColE2 *ceaB*$^+$ *celB*$^+$ *ceiB*$^+$ operon, Tc^r	This laboratory
pUR222	Cloning vector, Ap^r	Boehringer Mannheim Biochemicals
Bacteriophage		
λ *c*I857 *S*$^+$ *R*$^+$		Ethan Signer, M.I.T.
λ *c*I857 *S*am7 *R*$^+$		Ethan Signer, M.I.T.
λ *c*I857 *S*am7 *R*am5		This laboratory

[a] M.I.T., Massachusetts Institute of Technology.

phosphatase (Bethesda Research Laboratories, Inc.). Reactions with T4 ligase (New England BioLabs) were performed at 4°C overnight in 50 mM Tris hydrochloride (pH 7.5)–20 mM dithiothreitol–1 mM ATP–10 mM $MgCl_2$ for sticky end ligation. Oligonucleotide synthetic linkers (New England BioLabs) were ligated in the same buffer but at room temperature for 2 h.

Gel electrophoresis and isolation of DNA from gels. Restriction digests were analyzed by gel electrophoresis after gels were stained with ethidium bromide (0.5 μg/ml). DNA bands were visualized under UV light. DNA fragments longer than 1,000 base pairs (bp) were isolated from 1% low-melting-point agarose gels. The bands were cut from the gel and placed in tubes in a water bath at 70°C. The melted solution was diluted with 2 volumes of elution buffer (0.5 mM EDTA, 50 mM Tris hydrochloride [pH 7.5], 500 mM NaCl). The solution was then extracted twice with phenol and concentrated four times by extraction with sec-butanol, and DNA was then ethanol precipitated. DNA fragments of smaller size were isolated from 5% polyacrylamide gels. The DNA bands were cut from the gels, and each acrylamide slice was smashed in a siliconized Corex glass tube with a Teflon pestle and then suspended in 1 ml of 10 mM Tris hydrochloride [pH 8]–10 mM EDTA–500 mM NaCl and 1 ml of phenol. The suspension was shaken overnight at 30°C and then centrifuged at 12,000 × *g* for 10 min. The supernatant was filtered through a Millipore 0.45-μm filter and concentrated to 500 μl with sec-butanol extraction, and the DNA was precipitated with ethanol.

Curing cells of resident plasmids. Cells containing ampicillin-resistant (Ap^r) pColE1 derivatives were transformed with pBR322 (tetracycline resistant [Tc^r]). Selection on tetracycline agar followed by overnight growth in the presence of tetracycline yielded a majority of colonies that had lost the resident pColE1 derivative and were therefore Ap^s. Further growth for 10 to 20 generations without tetracycline yielded about 1% Ap^s Tc^s colonies; these were considered plasmid free.

RESULTS

Construction of plasmids pBK1, pUK1L, and pSEL5. The construction of plasmids pBK1, pUK1L, and pSEL5 is outlined in Fig. 1. The probable site of the *kil* gene of pColE1 is downstream from the *cea* gene, very near the *imm* gene (27, 33). In this region there is an open reading frame of 138 bp, lying in a 412-bp DNA fragment bracketed by two *Hae*III sites and containing the only *Nru*I site on the ColE1 plasmid (2, 8, 18). DNA fragments approximately 400 bp long produced by *Hae*III digestion of pColE1 were cloned into the unique *Bam*HI site of pBR322. The desired product was identified as a plasmid conferring resistance to ampicillin but not tetracycline and containing two *Nru*I sites and was called pBK1. The inserted fragment presumed to contain the *kil* gene was isolated from a *Bam*HI digest of pBK1 and cloned into the unique *Bam*HI site of the plasmid pUR222, which contains the *lac* promoter and the first part of the *lacZ* gene as well as an Ap^r marker. Recombinant plasmids with DNA inserted into the cloning site gave rise to Ap^r transformants of *E. coli* RRIΔM15 that were white on LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside as an indicator (26). Plasmids were isolated from such transformed cells, and those that released a 300-bp fragment upon double digestion with *Nru*I and *Eco*RI were considered to be pUR222 derivatives in which the *kil* gene DNA fragment had been inserted in the direction of transcription of the *lac* promoter. One such plasmid was then called pUK1L.

Plasmid pSEL5 was constructed by inserting a synthetic *Eco*RI linker into the *Nru*I site in the *kil* gene and was identified as an Ap^r plasmid that was nonlethal to strain A153 (see below). Restriction enzyme tests showed it to have two *Eco*RI sites 300 bp apart and no *Nru*I site.

Expression of the *kil* gene in pUK1L. The DNA fragment from ColE1 that had been placed downstream from the *lac* promoter to generate pUK1L did in fact contain a functional *kil* gene under control of the *lac* promoter (Fig. 2). Within about 30 min after the addition of IPTG as an inducer to a culture of W3110I^q containing pUK1L, typical signs of *kil* gene action appeared: turbidity began to decrease, over 95% of cells lost colony-forming ability, protein synthesis slowed down, and drastic reduction appeared in the ability to accumulate proline or α-methylglucoside (Fig. 2). In parallel experiments, cells of W3110I^q containing pSEL5 exhibited none of the above changes, confirming the expectation that insertion of an 8-bp oligonucleotide into the *Nru*I site of the presumed *kil* gene sequence would inactivate its product. The physiological effects observed after induction of pUK1L, which contains the *kil* gene under control of the *lac* promoter, are the same as the effects attributed to mitomycin induction of *kil* in the colicin operon (28). We conclude that these effects are solely due to expression of the *kil* gene.

Microscopic examination of *kil*-induced cells plated on the surface of supplemented OM agar showed that most cells failed to undergo even one cell division over several hours, in agreement with the results of colony counts; but cell shape persisted. The solid medium may have prevented lysis.

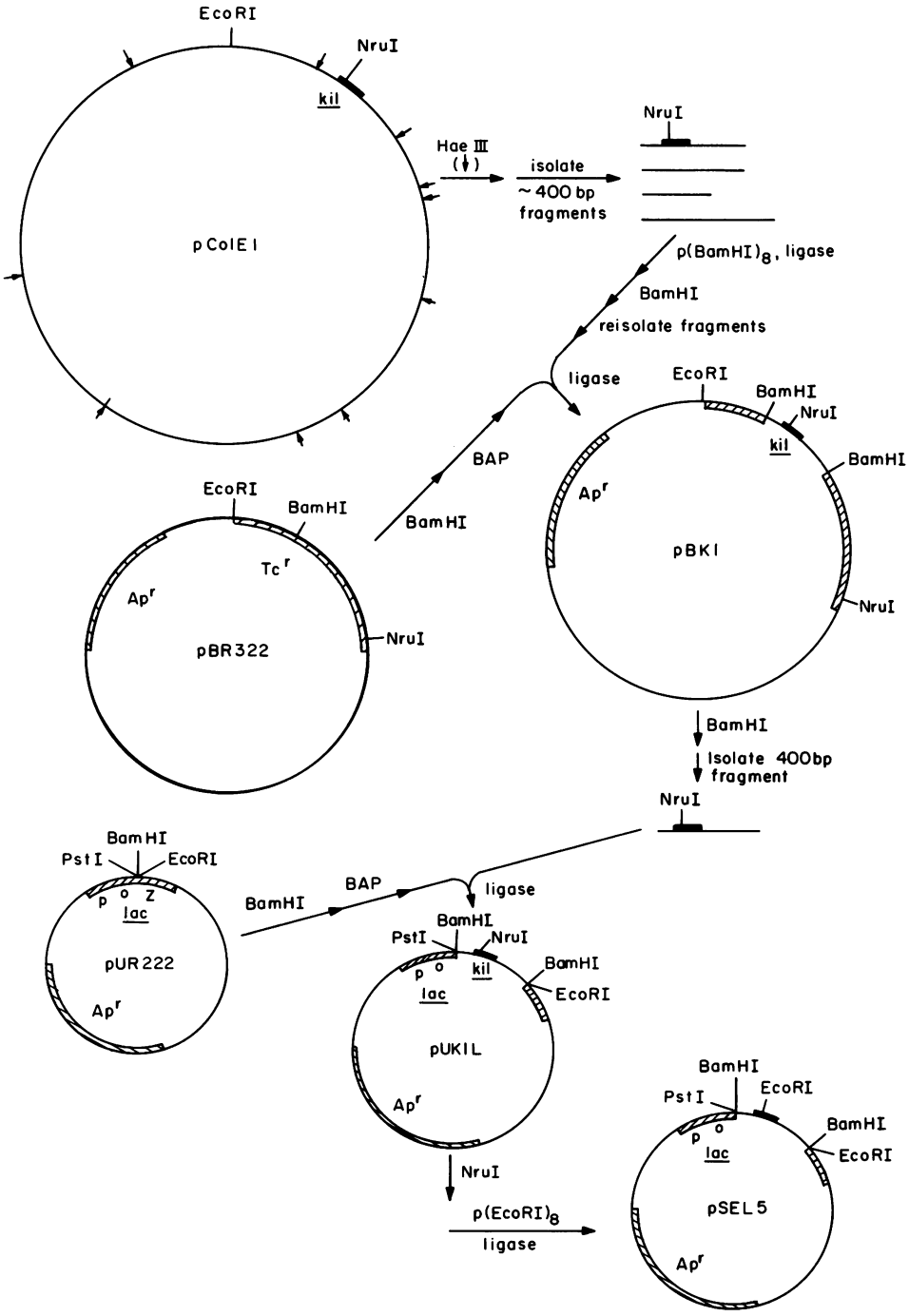

FIG. 1. Construction of plasmids pBK1, pUK1L, and pSEL5. Abbreviations and symbols: (↓) recognition site for *Hae*III restriction endonuclease; p(BamHI)$_8$ and p(EcoRI)$_8$, 8-bp linkers containing the *Bam*HI and *Eco*RI recognition sequences, respectively; ligase, T4 DNA ligase; BAP, bacterial alkaline phosphatase; *kil*, an open reading frame of 138 bp on pColE1 containing a unique *Nru*I recognition sequence; Ap^r and Tc^r, genes coding for resistance to ampicillin and tetracycline, respectively; *lac*, the proximal part of the lactose operon including promoter (p) and operator (o) regions, plus a 30-bp insert containing the indicated restriction endonuclease recognition sequences and the first few base pairs of the gene coding for β-galactosidase (*Z*). See the text for details.

The physiological effects of *kil* induction illustrated in Fig. 2 (decline in culture turbidity and inhibition of protein synthesis and active transport) were significantly delayed when cells were induced in the early or midlog phase rather than in the late log phase (data not shown). As already reported for the pColE2 lysis gene (24) the physiological effects of *kil* gene induction were largely eliminated when the cells were induced in the presence of 10 or 20 mM Mg^{2+}. Yet the killing rates as determined by colony counts or by failure of microscopically detected cell division remained similar for all conditions. We comment below on the possible significance of the delayed expression of physiological damage.

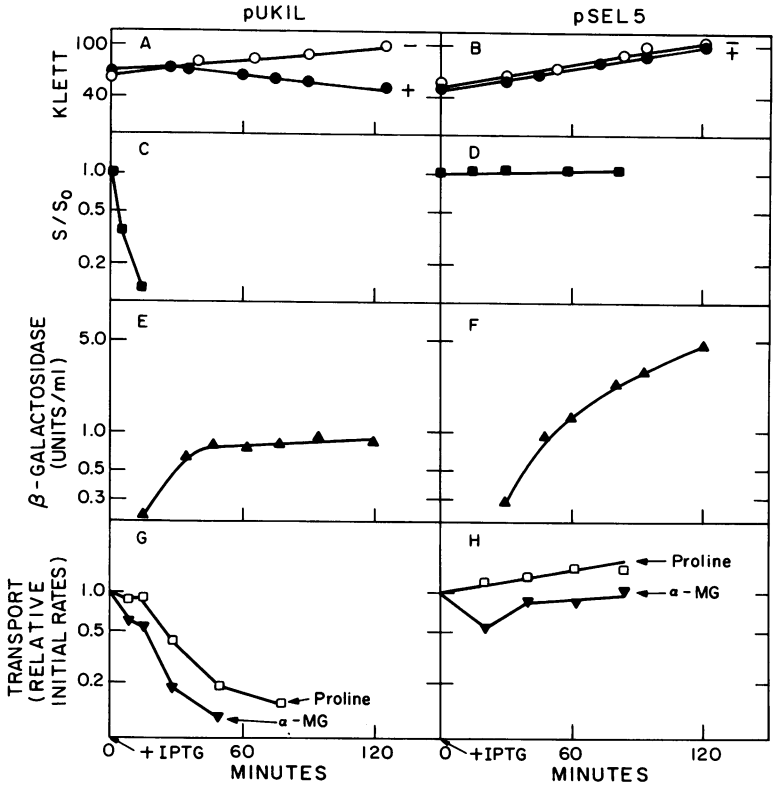

FIG. 2. Response to IPTG induction by W3110I^q cells containing either pUK1L or pSEL5. The cells were grown at 30°C with shaking in OM medium. The supplements were glycerol, thiamine, and an amino acid pool lacking proline. At 0 time IPTG (0.01 M) was added; at intervals the culture turbidity was determined by Klett measurement (A and B), and samples were withdrawn for assay of survival (C and D), β-galactosidase content (E and F), and uptake of proline and of α-methylglucoside (α-MG) (G and H). For uptake assays, 1 ml of culture was withdrawn and mixed with [^{3}H]proline and [α-^{14}C]methylglucoside (final concentrations, 0.5 μCi [0.005 μmol/ml] and 0.25 μCi [0.005 μmol/ml], respectively) and incubated at 30°C. After 1, 2, and 5 min 0.2 ml was removed from the mix, quickly filtered, and washed, and the filters were dried and counted. The initial rates were the amounts of the labeled substances taken up in the first minute. The decline in capacity for uptake of α-methylglucoside during the first 15 to 20 min is probably a transient effect of IPTG addition. Survival was measured on OM plates. In A and B results are presentred for cultures either with (+) or without (−) IPTG. In C through H, only the results for the cultures with IPTG are presented.

The W3110I^q (high *lac* repressor level) strain used for the experiments shown in Fig. 2 was the only strain tested that grew reliably at 30°C when containing pUK1L, so that the Kil function could be induced in a controlled way. At temperatures above 35°C pUK1L was lethal for W3110I^q. In strain RR1ΔM15 pUK1L could not be induced, presumably because of stronger repression. In several *lacI*$^+$ strains it was lethal; repression of the *lac* promoter was probably insufficient to prevent expression of the *kil* gene.

As deduced from the DNA base sequence, the open reading frame for the pColE1 *kil* gene codes for a small protein 45 amino acids long, of which the first 17 probably comprise a leader signal sequence typical for the precursors of exported proteins (19). The presumed peptidase cleavage site resembles that of the outer membrane lipoprotein precursor (32). Since cleavage of the lipoprotein precursor is inhibited by the antibiotic globomycin (29) we tested the effect of this antibiotic on *kil* expression. Globomycin (40 μg/ml) did not prevent the IPTG-induced killing of W3110I^q cells containing pUK1L (data not shown). This may mean either that processing of the *kil* gene product is not required for its action or that it is processed by a globomycin-insensitive peptidase.

Isolation of Kil-resistant bacterial mutants. The observation that induction of the *kil* gene has similar effects whether the gene is placed under the *cea* or *lac* promoter is strong evidence that it is a translated gene and that its expression is through a specific peptide. We were unsuccessful in detecting on sodium dodecyl sulfate-urea-polyacrylamide gels any peptide attributable to the *kil* gene, that is, a peptide of the expected size whose presence was correlated with induction of cells containing pUK1L. Protein bands thought to represent the products of the genes analogous to *kil* in other Col plasmids have been revealed by comparable techniques (5, 10, 12, 20, 23).

We decided to seek information on Kil action by isolating bacterial mutants with an abnormal response to the expression of the *kil* gene. Putative Kil^r mutants of strain A153 were isolated in the following manner. Cells of A153 were transformed with plasmid DNA, that of either pDMS630 or *cea*$^-$ deletion derivatives of it, and 22 individual Ap^r transformants were subjected to several successive cycles of treatment with mitomycin C. For each cycle of treatment, an overnight culture was diluted 1/50 and incubated at 37°C with shaking for about 3 h and then with mitomycin C (2 μg/ml) for 2 h, at which time survival was 0.01 to 0.1%. The culture was then diluted 10^{-5} and incubated overnight. After three to five such cycles, cultures were streaked out for isolated colonies on LB-ampicillin plates. Five colonies were picked from each, grown up, treated for 2 h with mitomycin C, and

tested by scoring for the time of appearance of visible growth. Cultures that showed greater survival than the parental control were retained and rechecked by measuring culture turbidity at intervals after the addition of mitomycin C. Those that did not show the loss of turbidity typical for mitomycin C-induced cells with *kil*$^+$ plasmids were the putative Kil-resistant (Kil^r) mutants. Each putative Kil^r isolate was put through the usual plasmid DNA amplification and extraction procedures. When plasmid DNA was found, it was used to transform A153 cells to Ap^r, and the transformants were checked for normal *kil*$^+$ expression upon mitomycin C induction. Of 37 putative resistant mutants tested in this way, 34 were found to contain a *kil*$^+$ plasmid.

Twelve mutants were screened for sensitivity or resistance to the following agents thought likely to detect changed membrane properties: methylene blue, acriflavine, EDTA, neomycin, and deoxycholate. The screen used LB plates into which the chemicals had been incorporated at various concentrations, and the plates were scored for growth after incubation at 30°C. The mutants fell into three classes: class I (one mutant), sensitive to EDTA, neomycin, and deoxycholate (no growth at concentrations of 2 mM, 1 μg/ml, and 1%, respectively); class II (eight mutants) resembling the control strain (growth at concentrations of 5 mM, 2 μg/ml, and 3%, respectively); and class III (three mutants) resistant to 6 μg of neomycin per ml. The response to methylene blue or to acriflavine was normal in all isolates.

One representative of each of these classes was cured of its resident plasmid. The cured strains were designated I-1, II-1, and III-1, respectively, and were studied further. Each was found to display the same response to EDTA, neomycin, and deoxycholate as before being cured. The growth of mutant II-1 was found to be slow at temperatures above 35°C, so all incubations were carried out at that or lower temperatures. Mutant III-1 grew somewhat more slowly than the parent and the other mutants at all temperatures.

Response of putative Kil^r mutants to plasmid pUK1L. The mutants were derived from the *lacI*$^-$ strain A153 and retained the same high level of β-galactosidase (2×10^4 to 5×10^4 U/mg of protein) with or without 10^{-2} M IPTG. In this strain the Kil function of plasmid pUK1L, in which the *kil* gene is under *lac* promoter control, should be fully expressed; only cells bearing a specific resistance to the Kil function should survive introduction of pUK1L. We tested the presumed resistant strains I-1, II-1, and III-1 by transformation with pUK1L as well as with pSEL5 and pDMS630 as controls (Table 2). Each of the Kil-resistant mutants yielded many transformants with pUK1L. The pUK1L-containing mutants II-1 and III-1 were completely stable, retaining the plasmid during repeated assays and at 37°C as well as at 30°C without any observed deleterious effects. They are considered fully Kil^r. Transformants from I-1 were not stable; when picked and streaked out most did not grow. This mutant may be resistant to Kil action only when the Kil product is present at low concentrations, such as may exist early after transformation when the copy number is probably low. We consider I-1 to be partially Kil resistant. As expected, no transformants of the parent strain A153 by pUK1L were found; the few colonies that appeared on the LB ampicillin plates proved to be Ap^s, presumably coming from untransformed cells that grew into colonies because of being protected by penicillinase released by neighboring cells.

TABLE 2. Ability of A153 *lacI*$^-$ Kil^r derivatives to harbor pUK1L[a]

Expt	Plasmid	No. of Ap^r transformants/competent cell (×10^6)			
		A153	Kil^r I-1	Kil^r II-1	Kil^r III-1
1	pUK1L	0	44.1	7.1	21.3
	pDMS630	1.8	8.8	2.5	1.4
2	pUK1L	0	0.5	7.0	0.6
	pSEL5	4.4	0.3	4.1	0.6

[a] After 2 h of recovery in 5 ml of LB at 30°C, 0.1-ml samples were plated in duplicate on LB plates containing ampicillin (20 μg/ml). The plates were scored for colonies after overnight incubation at 30°C, and 20 to 500 colonies per plate were observed in all cases except that of A153(pUK1L). The viable count of cells rendered competent by the standard $CaCl_2$ treatment (4) varied among the organisms and between the experiments. The range was 0.5×10^9 to 2×10^9 cells per transformation mix. Approximately 0.5 μg of each plasmid DNA per transformation mix was used.

TABLE 3. Resistance of Kil^r mutants to mitomycin C-induced pColE1 *kil*[a] or pColE2 *celB*[b] lethality

Organism	Survival after 2-h mitomycin C treatment[c]
A153	0.16
A153 (pEl *kil*$^+$)	0.0004
A153 (pE2 *celB*$^+$)	0.00014
Kil^r I-1	0.67
Kil^r I-1 (pE1 *kil*$^+$)	0.72
Kil^r I-1 (E2 *celB*$^+$)	0.81
Kil^r II-1	0.2
Kil^r II-1 (pE1 *kil*$^+$)	0.25
Kil^r II-1 (pE2 *celB*$^+$)	0.22
Kil^r III-1	0.13
Kil^r III-1 (pE1 *kil*$^+$)	0.65
Kil^r III-1 (pE2 *celB*$^+$)	0.33

[a] pDMS630.
[b] pBSE2.
[c] Cells were grown in LB at 35°C to a density of approximately 6×10^8 cells per ml. Mitomycin C was added to give a final concentration of 3 μg/ml.

Response of Kil^r mutants to mitomycin C induction of *kil*$^+$ plasmids. As noted above several Col plasmids are known to bear genes similar in structure and function to the ColE1 *kil* gene and similarly induced by mitomycin C. Bacterial mutants resistant to pColE1 Kil action might be resistant to the Kil or lysis action of other Col plasmids. We introduced into A153 and each of the Kil-resistant mutants either the plasmid pDMS630 (ColE1 *kil*$^+$) or plasmid pBSE2 (which bears gene *celB*$^+$, the analog of *kil* in pColE2 [5]) and tested the plasmid-containing cells for mitomycin C-induced lethality. All Kil^r mutants are resistant to the Kil function of pColE2 as well as to that of pColE1 (Table 3). Loss of turbidity such as is observed in mitomycin C-treated cultures of plasmid-containing A153 cells was not seen in any of the resistant mutants (data not shown). Normal amounts of each colicin were produced in the induced plasmid-containing cells of mutants I-1 and II-1, and only low amounts were produced in those of mutant III-1.

Plasmid-containing colonies of mutant III-1 showed markedly reduced colicin halos (colicin released after spontaneous induction) of either E1 or E2 compared to the parent strain. Mutants I-1 and II-1 each showed considerably reduced halos of E1 but not of E2. This may indicate that the Kil resistance trait in mutants I-1 and II-1 is less complete than in III-1 and allows release of some colicin. This may be sufficient to produce an almost normal halo of colicin E2, which for unknown reasons always produces much larger halos than does E1 at comparable levels of induction.

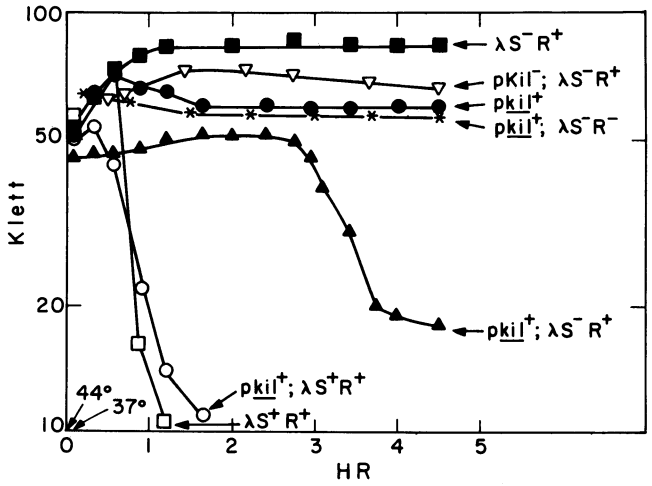

FIG. 3. Lysis of cells containing λ *S*am7 phage upon induction of pColE1 *kil* gene expression. Cells of strain A153 carrying the indicated plasmids or λ *c*I857 bacteriophage (or both) with *S* and *R* genes in the indicated configurations were grown with shaking at 35°C in LB. When the cultures attained a Klett reading of about 90, mitomycin C (1 μg/ml) was added, and incubation was continued at 30°C for 25 min to induce expression of the *cea-kil* operon if present. (With this induction regimen 50 to 70% of the cells gave rise to lucunae when exposed to chloroform and plated with indicator cells.) At 0 time each culture was diluted 1:2 in LB prewarmed to 44°C and held at 44°C for 5 min to destroy the λ *c*I857 temperature-sensitive repression and initiate λ expression and replication. Thereafter, the cultures were incubated with shaking at 37°C, and turbidity was measured at intervals. After 4.5 h samples were removed from some of the cultures and assayed for bacteriophage titer before and after exposure to $CHCl_3$. PFU were detected on indicator strain Ymel in soft λ agar poured on λ agar base plates. The titers with and without $CHCl_3$ exposure, respectively, were 19×10^8 and 0.16×10^8 PFU/ml in the culture of cells carrying λ S^- only; 4.9×10^8 and 4.9×10^8 PFU/ml in the culture of cells carrying p*kil*$^+$ and λ S^-; and 7.3×10^8 and 7.2×10^8 PFU/ml in that of cells carrying p*kil*$^+$ and λ S^+. The pColE1 *kil*$^+$ and *kil*$^-$ plasmids were pDMS630 and pJFS3, respectively; λ S^- was λ *S*am7, and λ R^- was λ *R*am5.

All mutants without plasmids were as fully sensitive to colicins E1, E2, E3, and A as the parent, A153. When harboring the appropriate plasmid, they were normally immune to colicins E1 and E2 (other immunities have not been tested).

Capacity of mutants to grow and release bacteriophage. Several bacteriophages have lysis genes (gene *S* of λ, gene *t* of T4) whose products are needed for cell lysis, presumably to allow an endogenous phage lysozyme (coded for by another gene) to reach the peptidoglycan layer and induce lysis (13, 25). Some of the reported effects of λ *S* gene action (9, 31) are very similar to those of *kil* gene action. The *S* gene of λ has no detectable nucleotide sequence homology with the *kil* gene of pColE1 (6, 7). We found that expression of the *kil* gene in pColE1 allows release of λ *S*am7 phage from induced lysogens (Fig. 3). Cells carrying λ *S*am7 replicated the phage but did not lyse or release phage. If these cells contained also a ColE1 *kil*$^+$ plasmid inducible by mitomycin C, the addition of mitomycin caused cell lysis and phage release after some delay. The lysis was apparently brought about by the phage lysozyme because it did not occur with λ *S*am7 *R*am5 (lysozyme negative).

The three Kil^r mutants were tested for their response to phage λ lysis; each replicated and released the phage normally. This means that the mutants are not resistant to the product of the λ *S* gene. Presumably, the helping action of *kil* for phage release in the absence of the *S* function reflected a nonspecific damage of the cell membrane by the Kil product. The three Kil^r mutants were normal also for replication and release of bacteriophage T4.

DISCUSSION

The experiments here described provide evidence that the effects earlier ascribed to the *kil* gene of pColE1 (27, 28) are dependent solely on the expression of this gene. This has been established also for the lysis gene of pColE2 (23) and is probably true for its analogs in other Col plasmids (10–12). Although the release of colicin provides the assay by which these genes are normally identified, neither colicin nor the immunity protein need be present for the *kil* gene product to act. The mechanism by which the *kil* gene product or its analogs lead to cell death and release of colicin and other proteins (24, 33) remains unknown. It is presumed to act on the cytoplasmic membrane; a possible role of a phospholipase has been suggested (24). Experiments are underway to devise a satisfactory subcellular assay to isolate and purify the Kil protein either as a 28-amino-acid peptide or as an unprocessed precursor.

The presumed action of the Kil peptide on the cytoplasmic membrane leading to cell damage and protein release may be direct or indirect. One clue comes from the rate of loss of viability of the *kil*-induced bacteria as a function of time after induction. With the *kil* gene under the *lac* promoter, we can extrapolate from the known kinetics of induction of β-D-galactosidase in *E. coli* and assume that, by 10 min after the addition of inducer, induction has occured in all cells; yet over 10% of the cells are still able to form colonies when removed from the inducer. Longer exposure to an inducer results in progressively fewer survivors. This suggests that recovery is possible after some Kil peptide has been present, or that a relatively large amount of it must be present to kill. Accumulation of a sufficient amount might be delayed if, for example, the gene transcripts were translated slowly because of some sequence constraint or if some of the peptide were inactivated or improperly folded within the cell. Another possibility is that each bacterial cell becomes sensitive to irreversible damage caused by the Kil protein only at a certain period in the growth cycle.

The fact that rapidly growing cells and cells in the presence of Mg^{2+} continue to be physiologically active for longer times, although already destined to die, would suggest a progressive membrane damage influenced by metabolic rate and not directly interfering with energy metabolism. Note the contrast with colicin E1 itself, which like several other colicins immediately abolishes the membrane potential in the bacterial cell (15). Preliminary experiments (in collaboration with Michael Weiss) have confirmed that early-log-phase colicin-sensitive cells in which a pUK1L plasmid has been induced maintain their membrane potential for at least 90 min and are promptly deenergized by the addition of colicin E1.

The Kil-resistant bacterial mutants described in this report may help identify some of the steps involved in *kil* gene action. Mapping of the gene or genes affected is currently underway using indirect selection methods, since direct selection for the resistant trait is inefficient. Examination of partial diploids should help clarify the nature of the resistance process. The fate of colicin produced in these mutants after the nonlethal mitomycin C induction as well as that of colicin produced under other nonlethal conditions (28, 33) remains to be investigated.

Our observations on the physiological effects of the Kil function suggest that it damages the cytoplasmic membrane, so that cell resistance to the damage may be expected at the membrane level. The preliminary observations on sensitivity of some of the mutants to various external agents suggests that resistance may also affect, directly or indirectly, the outer bacterial membrane. Profiles of membrane proteins of the three mutants in sodium dodecyl sulfate-polyacrylamide gels showed only small changes in minor bands compared with the parental strain. Further characterization of mutant cell membranes is underway.

ACKNOWLEDGMENTS

The work was supported by National Science Foundation grant CM-8108866 and by Public Health Service grant 5-R01-AI03038 from the National Institutes of Health. We thank H. Wu and M. Arai for generous gifts of globomycin and G. DeVos for many helpful discussions.

LITERATURE CITED

1. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
2. **Chan, P. T., H. Ohmori, J. Tomizawa, and J. Lebowitz.** 1985. Nucleotide sequence and gene organization of ColE1 DNA. J. Biol. Chem. **260**:8925–8935.
3. **Clewell, D. B.** 1972. Nature of ColE1 replication in *E. coli* in the presence of chloramphenicol. J. Bacteriol. **11**:667–676.
4. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *E. coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
5. **Cole, S. T., B. Saint-Joanis, and A. P. Pugsley.** 1985. Molecular characterization of the colicin E2 operon and identification of its products. Mol. Gen. Genet. **198**:465–472.
6. **Daniels, D. L., J. L. Schroeder, W. Szybalski, F. Sanger, and F. R. Blattner.** 1983. Appendix I: a molecular map of coliphage lambda, p. 469–517. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
7. **Daniels, D. L., J. L. Schroeder, W. Szybalski, F. Sanger, A. R. Coulson, G. F. Hong, D. F. Hill, G. B. Peterson, and F. R. Blattner.** 1983. Appendix II: complete annotated lambda sequence, p. 519–676. *In* R. W. Hendrix, J. W. Roberts, F. W. Stahl, and R. A. Weisberg (ed.), Lambda II. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Dougan, G., M. Saul, G. Warren, and D. Sherrat.** 1978. A functional map of plasmid ColE1. Mol. Gen. Genet. **158**: 325–327.
9. **Garret, J. M., and R. Young.** 1982. Lethal action of bacteriophage λ S gene. J. Virol. **44**:886–892.
10. **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. I. Localization of the gene and identification and subcellular localization of the gene H product. Mol. Gen. Genet. **183**:318–325.
11. **Hakkaart, M. J. J., E. Veltkamp, and J. 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.
12. **Jakes, K. S., and N. D. Zinder.** 1984. Plasmid ColE3 specifies a lysis protein. J. Bacteriol. **157**:582–590.
13. **Josslin, R.** 1971. Physiological studies on the *t* gene defect in T4-infected *Escherichia coli*. Virology **44**:101–107.
14. **Luria, S. E., M. J. Adams, and R. C. Ting.** 1960. Transduction of lactose utilizing ability among strains of *E. coli* and *S. dysenteriae*. Virology **12**:348–390.
15. **Luria, S. E., and J. L. Suit.** 1982. Transmembrane-channels produced by colicin molecules, p. 279–284. *In* A. Martonosi (ed.), Membranes and transport, vol. 2. Plenum Publishing Corp., New York.
16. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Nagel de Zwaig, R., and S. E. Luria.** 1967. Genetics and physiology of colicin-tolerant mutants of *Escherichia coli*. J. Bacteriol. **94**:1112–1123.
18. **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.
19. **Osborn, M. J., and H. C. P. Wu.** 1980. Proteins of the outer membrane of Gram-negative bacteria. Annu. Rev. Microbiol. **34**:369–422.
20. **Oudega, B., A. Ykema, F. Stegehuis, and F. K. de Graaf.** 1984. Detection and subcellular localization of mature protein H, involved in excretion of cloacin DF13. FEMS Microbiol. Lett. **22**:101–108.
21. **Plate, C. A.** 1976. Mutants of *Escherichia coli* defective in response to colicin K and in active transport. J. Bacteriol. **125**:467–474.
22. **Pugsley, A. P., and M. Schwartz.** 1983. A genetic approach to the study of mitomycin induced lysis of *Escherichia coli* K-12 strains which produce colicin E2. Mol. Gen. Genet. **190**: 366–372.
23. **Pugsley, A. P., and M. Schwartz.** 1983. Expression of a gene in a 400-base-pair fragment of colicin plasmid ColE2-P9 is sufficient to cause host cell lysis. J. Bacteriol. **156**:109–114.
24. **Pugsley, A. P., and M. Schwartz.** 1984. Colicin E2 release: lysis, leakage or secretion? Possible role of a phospholipase. EMBO J. **3**:2393–3297.
25. **Reader, R. W., and L. Siminovitch.** 1971. Lysis defective mutants of bacteriophage lambda: genetics and physiology of S cistron mutants. Virology **43**:607–622.
26. **Ruther, U., M. Koenen, K. Otto, and B. Muller-Hill.** 1981. pUR222, a vector for cloning and rapid chemical sequencing of DNA. Nucleic Acids Res. **9**:4087–4098.
27. **Sabik, J. F., J. L. Suit, and S. E. Luria.** 1983. *cea-kil* operon of the ColE1 plasmid. J. Bacteriol. **153**:1479–1485.
28. **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. USA **80**:579–583.
29. **Tokunaga, M., H. Tokunaga, and H. C. Wu.** 1982. Posttranslational modification and processing of *Escherichia coli* prolipoprotein *in vitro*. Proc. Natl. Acad. Sci. USA **79**:2255–2259.
30. **Watson, R. J., P. C. K. Lau, T. Vernet, and L. P. Visentin.** 1984. Characterization and nucleotide sequence of a colicin-release gene in the *hic* region of plasmid ColE3-CA38. Gene **29**:175–184.
31. **Wilson, D. B.** 1982. Effect of the lambda S gene product on properties of the *Escherichia coli* inner membrane. J. Bacteriol. **151**:1403–1410.
32. **Wu, H. C., M. Tokunaga, H. Tokunaga, S. Hayashi, and C.-Z. Giam.** 1983. Posttranslational modification and processing of membrane lipoproteins in bacteria. J. Cell Biochem. **22**: 161–171.
33. **Zhang, S., A. Faro, and G. Zubay.** 1985. Mitomycin-induced lethality in *Escherichia coli* cells containing the ColE1 plasmid: involvement of the *kil* gene. J. Bacteriol. **163**:174–179.