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Expression of a Gene in a 400-Base-Pair Fragment of Colicin Plasmid ColE2-P9 Is Sufficient to Cause Host Cell Lysis

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The colicin E2 immunity (*ceiB*) and lysis (*celB*) genes of colicin plasmid ColE2-P9 were cloned as a 900-base-pair insert under the control of the *lac* promoter in high-copy-number plasmid pUR222. Hosts carrying this plasmid were immune to colicin E2, produced increased amounts of immunity protein (molecular weight, 9,000) and two smaller proteins (molecular weights, 5,000 and 3,000), and lysed when incubated in medium containing isopropyl-β-D-thiogalactopyranoside (IPTG). A 400-base-pair *lacp*-distal fragment derived from the insert in this plasmid was recloned in the same orientation into pUR222. Although hosts carrying this plasmid also lysed when grown in the presence of IPTG, they were sensitive to colicin E2 and produced increased amounts of the 5,000- and 3,000-molecular-weight proteins (but not the full-length immunity protein) when treated with IPTG. The results were consistent with the idea that expression of *celB* (production of the 5,000- and 3,000-molecular-weight proteins) is sufficient to cause host cell lysis in the absence of colicin production and derepression of the host cell SOS system.

The mechanisms whereby colicins are released from producing cells have been the subject of much recent research. It was originally assumed that colicins were secreted into the growth medium, but subsequent studies with cells carrying different colicin plasmids (A, D, E1 through E7, and K) indicated that they lysed when they were induced to produce large amounts of colicin. Furthermore, the majority of the colicin remained within the cells until they lysed, an observation which led to the conclusion that lysis (or more aptly quasilysis [7] since periplasmic proteins constitute the bulk of the material released [16]) was the sole mechanism for colicin export (7, 10, 15, 22). This view was challenged by van Tiel-Menkveld et al. (21), who suggested that the high levels of mitomycin used to obtain full and rapid induction of colicin synthesis could make the cells more susceptible to lysis. In support of this, they noted that cloacin DF13 was apparently released in the absence of lysis when relatively low doses of mitomycin were used to induce cloacin production (21). We suggested previously that the apparently specific secretion of cloacin under these conditions may be a spurious result caused by the asynchronous nature of colicin production and lysis (16).

We recently identified a gene (*celB*) of pColE2-P9 which is essential for colicin export and for mitomycin-induced lysis of host cells (17). *celB* is the third gene of an operon which also comprises the structural genes for the colicin and immunity proteins (*ceaB* and *ceiB*, respectively) and is probably the structural gene for the 5,000-molecular-weight (5K) protein produced by ColE2$^+$ cells after mitomycin treatment (15, 17). Thus, *celB* expression is normally controlled by the SOS-regulated (LexA-repressed and mitomycin plus RecA-induced) promoter located in front of *ceaB* as depicted in Fig. 1 (17).

The *celB* gene of pColE2-P9 is probably functionally analogous to similarly positioned genes of other Col plasmids (gene H of pCloDF13, *hic*/*celC* of pColE3-CA38, and *kil* of pColE1) (4, 5, 12, 17, 19, 23). It now seemed pertinent to ask whether *celB* expresion alone was sufficient to cause host cell lysis or whether lysis was a multifactorial response dependent on the accumulation of large amounts of colicin (16) or on the induction of various host cell SOS functions (8). Our approach was to clone *celB* under *lac* promoter control, and we show here that *celB* expression is sufficient to cause host cell lysis.

MATERIALS AND METHODS

Plasmids and hosts. Plasmid pUR222 (18) was used for cloning fragments of pColE2-P9 under *lacZp* control. pUR222 contains *lacZop* with a polylinker DNA fragment containing several unique restriction endonuclease sites inserted between the fifth and sixth codons of *lacZ* (18) (Fig. 1). The hosts were PAP105 Δ(*lac-pro*) carrying pAPIP502 which is F′ Tn*10* *lacI*^{Q1}

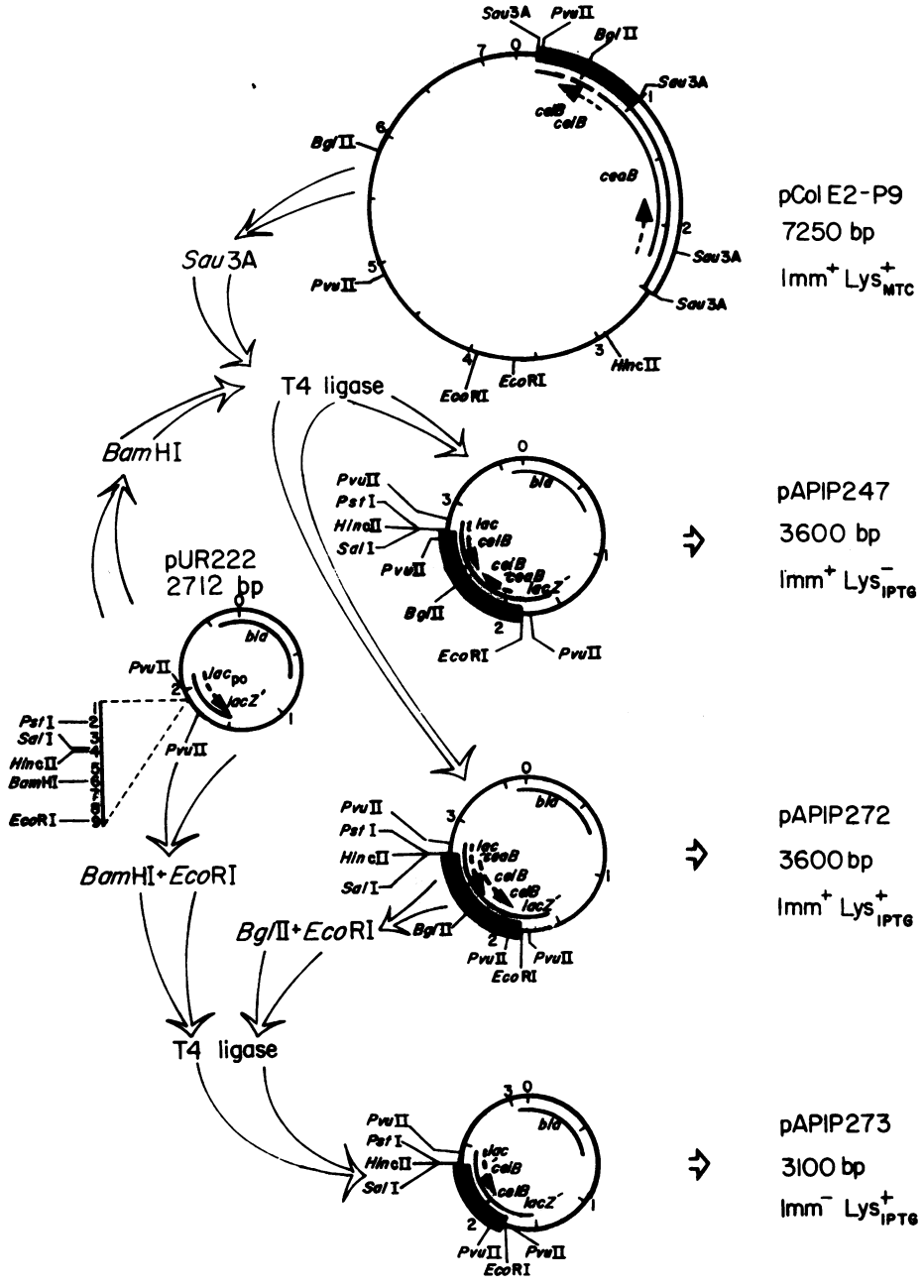

FIG. 1. Physical maps of pColE2-P9 and pUR222 and strategy for cloning fragments of pColE2-P9. Previous experiments (17) indicated that the entire colicin E2 operon is contained within the boxed region of pColE2-P9. The approximate positions of the *ceaB*, *ceiB*, and *celB* genes (thin bars inside the circles) and the directions of transcription from *ceaBp* and the putative *ceiBp* (arrows on inside of circle) were determined by subcloning and transposon mutagenesis (17). The black boxed region of pColE2-P9 contained *ceiB*, *celB*, and part of *ceaB*. This region was cloned into pUR222 to give pAPIP247 and pAPIP272. Part of the insert in pAPIP272 was recloned into pUR222 as shown. pColE2-P9 has several *Sau*3A sites in addition to the *Sau*3A and *Bgl*II sites noted. The polylinker region of pUR222 is shown in exploded view; the numbers 1 to 9 refer to codons in the polylinker. Position 0 would be codon 5 of *lacZ*.

Δ(*lacZ*)M15, BZB1019 (*met gal hsdR rpsL*), and PAP88 [BZB1019(pAPIP502)]. The *lacI*^{Q1} mutation is a 15-base-pair deletion in the *lacI* promoter which causes a 50- to 100-fold increase in the level of *lac* repressor (1). pAPIP226 is pColE2-P9 *celB*::Tn*5* (17).

Growth conditions. The rich media used were L broth and L agar prepared by the method of Miller (9). The minimal salts medium was M63 (13) supplemented with 1% glycerol and 30 μg of methionine per ml. The indicator media were MacConkey agar containing 1% lactose and L agar containing 5-bromo-4-indolyl-β-D-galactoside (XG) (9). Ampicillin (200 μg/ml) and tetracycline (16 μg/ml) were included in all media to prevent the outgrowth of clones lacking pUR222 and its derivatives or pAPIP502, respectively. All plate cultures were incubated at 30°C. Broth cultures were established by inoculation with a single colony and incubation with good aeration at 30°C. Mitomycin C was used at 0.5 μg/ml, and colicin E2 was used at concentrations which were approximately 100 times the amount required to prevent colony formation by >95% of 10^8 sensitive cells when added 5 min before plating.

Genetic techniques. Plasmids were purified from cells grown in liquid culture by a scaled-up and slightly modified Holmes and Quigley extraction procedure (6) followed by treatment with DNase-free RNase and agarose gel electrophoresis in the presence of ethidium bromide. The DNA band corresponding to the closed circle plasmid form was eluted from the gel by the method of Tabak and Flavell (20). Restriction endonuclease digest fragments were also purified by elution from agarose gels. Agarose gel electrophoresis, digestion with restriction endonucleases, ligation with T4 DNA ligase, and transformation were all performed as described by Davis et al. (3). The sizes of restriction digest fragments were determined by comparing their mobilities with fragments of known sizes in 0.7 to 1.2% agarose gels. The enzymes used to analyze hybrid plasmids were those for which the sites are indicated in Fig. 1.

Other techniques. Cells were labeled with ^{14}C-amino acids by a modification of the procedure used previously (14). Samples (1 ml) were removed from L broth cultures and centrifuged at 12,000 × *g* for 2 min. The pellets were drained and suspended in 0.5 ml of prewarmed minimal salts medium containing 20 μCi of ^{14}C-amino acids per ml and incubated for 10 min at 37°C. The reaction was stopped with cold 10% trichloroacetic acid, and the acid-precipitated proteins were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using 7 to 16% gradient acrylamide gels with Tris-phosphate buffers (17). In other experiments, proteins in cell-free culture media were precipitated with 66% acetone at −20°C. The precipitates and total cell pellets were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using 10% acrylamide gels and Tris-glycine buffers (15). β-Galactosidase was assayed by the method of Miller (9), except that the cells were disrupted by sonication.

RESULTS

Cloning of *celB* under *lac* promoter control. Fragments of pColE2-P9 generated by partial digestion with restriction endonuclease *Sau*3AI were ligated into the *Bam*HI site in pUR222 (18), and the resulting plasmids were used to transform strain PAP105 with selection for resistance to ampicillin and immunity to colicin E2. The smallest plasmids thus obtained (pAPIP247 and pAPIP272) contained 900 base pairs derived from pColE2-P9 and differed only with respect to the orientation of the insert (Fig. 1). Transformants carrying these plasmids were Lac$^-$ on XG plates at 30°C, indicating that the *lac* reading frame had been interupted and that there were no correctly positioned promoters and open reading frames to permit transcription and translation of *lacZ* beyond the insert. Hosts bearing these plasmids did not produce colicin.

The *celB* and *ceiB* genes were in the same orientation with respect to *lacp* in pAPIP272 as they were with respect to the *ceaBp* in pColE2-P9 (Fig. 1) (17) and should therefore constitute a hybrid operon Φ(*lac-ceiB-celB*). We next attempted to divide the cloned fragment by using the *Bgl*II site within *ceiB* (17) and the *Sal*I and *Eco*RI sites in the flankng vector DNA (Fig. 1). Transformants carrying hybrid plasmid pAPIP273 with the 400-base-pair *Bgl*II-*Eco*RI fragment from pAPIP272 cloned into pUR222 after *Bgl*II-*Eco*RI digestion were Lac$^-$ on indicator plates and sensitive to colicin E2. Ampicillin-resistant transformants were not obtained when strain PAP105 was transformed with plasmids formed by ligating the 500-base-pair *Sal*I-*Bgl*II fragment of pAPIP272 into *Sal*I-*Bam*HI-digested pUR222. This is probably because these hybrid plasmids contain a portion of *ceaB* which encodes the catalytic domain of colicin E2 (11); hosts carrying these plasmids could therefore produce a catalytic colicin peptide in the absence of immunity protein, a potentially suicidal situation.

Effects of *ceiB* and *celB* expression on host cells. Strain PAP88 (*lac*$^+$ *lacI*^{Q1}) was transformed with pUR222 or with the pColE2-P9-pUR222 hybrid plasmids to compare their effects on host cells. Transformants carrying pAPIP272 or pAPIP273 produced dark blue colonies on XG plates, with a halo of hydrolyzed XG surrounding the colony. This indicated that the chromosomal *lac* genes were being expressed at high levels without inducer and that cells in the colonies were lysing to release either hydrolyzed XG or β-galactosidase. These strains were unable to grow on rich medium at 42°C or on minimal media at any temperature. A short burst of increased β-galactosidase synthesis was observed when cultures in L broth at 30°C were transferred either to L broth at 42°C or to minimal medium at any temperature. Thus, a probable explanation for the inability of these strains to grow at elevated temperature or in minimal medium may be that the *lacI*^{Q1}-encoded

repressor is being titrated by an increased number of *lacZo* sites as the plasmids continue to replicate during a period of reduced protein synthesis. Strains which carried pAPIP272 or pAPIP273 in the absence of the *lacI*^{Q1} allele could only be grown on L agar plates at 30°C.

The turbidity of cultures of strains carrying pAPIP272 or pAPIP273 began to decline within 60 to 120 min of the additon of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) (Fig. 2). The use of decreasing amounts of IPTG caused progressively increased delays in lysis onset (data not shown). Delayed lysis was also observed in untreated cultures (Fig. 2). The onset of lysis under these conditions was always preceded by an increase in the specific activity of chromosomal *lacZ*-encoded β-galactosidase, indicating that lysis was probably caused by derepression of *lacop*-regulated *celB* because of an absence of sufficient Lac repressor to bind to all *laco* sites.

In contrast to these results, neither IPTG nor mitomycin caused lysis of hosts carrying pUR222 or pAPIP247 (data not shown). Strain PAP105 carrying these plasmids formed dark blue colonies on XG plates and produced substantially increased amounts of β-galactosidase when grown to the late-exponential phase in L broth. Thus, the increased expression of chromosomal and plasmid *lac* operons in the absence of inducer was not because of the presence of *celB* or *ceiB*.

Proteins released by lysis. The onset of lysis occurred earlier in IPTG-treated cultures of strains carrying pAPIP272 or pAPIP273 than in mitomycin-treated cultures of strains carrying pColE2-P9 (Fig. 1). We noted previously that in the latter case, the lysing cells release mainly periplasmic proteins (16). The results shown in Fig. 3 indicate that the proteins released after IPTG-mitomycin treatment of hosts carrying pAPIP273 and pAPIP226 (pColE2-P9 *celB*::Tn*5* [17]) were similar, although not identical, to the proteins released by pColE2^{+} cells which had been induced to lyse with mitomycin. The qualitative and quantitative differences in protein content between the two samples may be variously caused by the presence of peptides derived from proteolysis of released colicin (15), by the difference in incubation temperature (which causes differences in the relative abundance of a number of periplasmic proteins), and by differences in the extent of lysis. It is important to note that in both cases, the β-galactosidase was released more slowly than the colicin, with only 10 to 20% of the total activity being present in the medium 3 h after the onset of lysis.

Proteins whose production was stimulated by IPTG treatment. IPTG-treated cultures of PAP88 carrying pAPIP272 produced three low-molecular-weight proteins which were not detected in untreated cultures (Fig. 4). These proteins comigrated with the immunity protein (9K) and the 5K and 3K proteins whose production

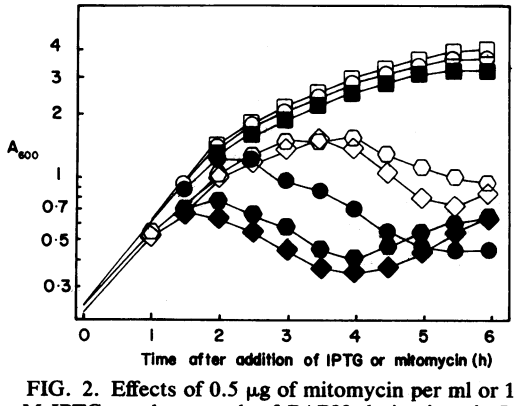

FIG. 2. Effects of 0.5 μg of mitomycin per ml or 1 mM IPTG on the growth of PAP88 derivatives in L broth at 37°C. Open symbols indicate untreated cultures, and closed symbols indicate treated cultures: ● and ○, pColE2-P9 with and without mitomycin, respectively; ■ and □, pAPIP226 with and without mitomycin, respectively; ● and ○, pAPIP272 with and without IPTG, respectively; and , pAPIP273 with and without IPTG, respectively.

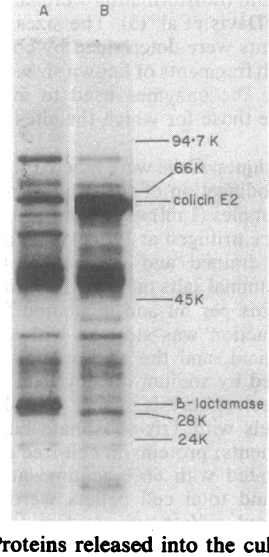

FIG. 3. Proteins released into the culture medium by IPTG-induced lysis of PAP88(pAPIP226 plus pAPIP273) (A) or by mitomycin-induced lysis of PAP88(pColE2-P9) (B). Samples loaded were equivalent to 0.2 ml of medium concentrated by precipitation with acetone. Sample A was taken 150 min after the addition of IPTG to the culture at 30°C; sample B was taken 240 min after the addition of mitomycin at 37°C. The positions of molecular weight standards are shown at the right of the gel.

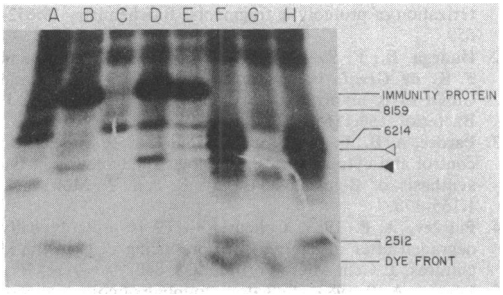

FIG. 4. Proteins whose production was stimulated by treatment with 0.5-μg/ml mitomycin (strains carrying pColE2-P9) or 1 mM IPTG (strains carrying pAPIP272 or pAPIP273). Cells were grown in L broth at 37°C to an absorbance at 600 nm (A_{600}) of 0.2 before the addition of IPTG or mitomycin. Samples were withdrawn for labeling with ^{14}C-amino acids at time intervals thereafter. Only the lower part of the gel is shown, and the positions of molecular weight markers (cyanogen bromide fragments of myoglobin) are shown at the right. Lanes A and B, pColE2-P9$^+$ after incubation for 80 min in the absence (A) or presence (B) of mitomycin; lanes C and D, pAPIP272$^+$ after 15 min of incubation in the absence (C) or presence (D) of IPTG; lanes F and G, pAPIP273 after 15 min of incubation in the absence (G) or presence (F) of IPTG; lanes E and H, pAPIP272$^+$ or pAPIP273$^+$, respectively, after 120 min of incubation in the absence of IPTG. The positions of the 5K protein (symbol ◀ in lanes B, D, E, F, and H), immunity protein (lanes B, D, and E), and the putative β-galactosidase–immunity fusion protein (symbol ◁ in lanes F and H) are as indicated.

was substantially increased after mitomycin treatment of cells carrying pColE2 (Fig. 4) (17). Cells carrying pAPIP273 produced the 5K and 3K proteins, but not the 9K protein, when treated with IPTG (Fig. 4). These cells also produced a 6K protein when grown in the presence of IPTG; we suspect that this protein is the product of a fused gene formed from the first five codons of *lacZ* and the promoter-distal part of *ceiB*. Production of the 3K, 5K, and immunity (6K) proteins was also detected in untreated cultures shortly before the onset of spontaneous lysis (Fig. 4). This supports the above suggestion that cells carrying pAPIP272 or pAPIP273 undergo spontaneous induction of the *lac* system after prolonged incubation. IPTG treatment of hosts carrying pAPIP247 or pUR222 did not elicit increased production of any of these proteins.

Possible host cell involvement in lysis. Cultures of strains carrying pAPIP272 contained very few survivors (ca. 1/10^9 cells) when grown for 80 min in L broth containing ampicillin, colicin E2, and IPTG. These survivors were either Lac$^-$ (indicating inactivation of the chromosomal *lac* operon) or plasmid negative (ampicillin sensitive) and resistant to other BtuB group colicins in addition to E2 or both. Mutations inactivating the *lacY* gene encoding Lac permease are unlikely to reduce IPTG uptake sufficiently to account for the IPTG resistance of any of the Lac$^-$ clones, but mutations affecting the Lac repressor gene *lacI* might affect the ability of IPTG to induce the *lac* system. No mutations affecting genes other than *lac* were obtained by this selection procedure; there was thus no evidence for the involvement of dispensable host cell functions in lysis. We also failed to obtain lysis-negative plasmid mutations by using the above selection procedure, but this may not be surprising because the high copy number of the hybrid plasmid almost precludes the possibility that a plasmid carrying a mutation affecting *celB* could segregate to give an IPTG-resistant clone during the time course of the experiment.

DISCUSSION

In this report, we describe two hybrid plasmids in which expression of parts of the colicin E2 operon are regulated by a *lac* promoter. In the first (pAPIP272), the immunity and lysis genes (*ceiB* and *celB*) are under *lac* control; hosts carrying this plasmid produced substantially increased amounts of immunity protein and 5K and 3K proteins and lysed when treated with IPTG. Hosts carrying a similar plasmid with the same fragment cloned in reverse orientation with respect to *lacp* (pAPIP247) did not exhibit these features, although they were fully immune to colicin E2. Plasmid pAPIP273 was derived from pAPIP272 and effectively carried a 500-base-pair deletion starting from a site in the *lacZ* polylinker to the *Bgl*II site in *ceiB*. IPTG treatment of hosts carrying this plasmid resulted in increased production of the 5K and 3K proteins as well as a putative LacZ-CeiB fusion protein. The 5K and 3K proteins which appeared in increased amounts after IPTG induction comigrated with proteins whose production was increased in mitomycin-treated pColE2$^+$ cells (Fig. 4). Both proteins were absent from mitomycin-treated cells which carried pColE2 with Tn*5* inserted in *celB* (17). There was probably insufficient cloned DNA in pAPIP273 to encode three different proteins, which makes it more likely that the 3K protein was derived from the 5K protein as discussed previously (17).

The mode of action of the *celB*-encoded lysis protein remains to be elucidated. It is tempting to speculate that the lysis protein may form a channel to allow export of cytoplasmic colicin to the periplasm, but it is difficult to imagine how the formation of such pores would lead to cell lysis. Lysis may result from the leakage of other cytoplasmic proteins into the periplasm or, more

likely, from the general perturbation of inner and outer membrane structure. It is interesting that genes with apparently similar functions exist not only on other Col plasmids (4, 5, 7, 10, 12, 15–17, 19, 22, 23) but also on certain bacteriophages (2, 19, 24, 25).

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