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Mitomycin C-Induced Synthesis of Cloacin DF13 and Lethality in Cloacinogenic *Escherichia coli* Cells

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Treatment of cloacinogenic cultures with increasing concentrations of mitomycin C induced an increasing synthesis of cloacin DF13 accompanied by a decreasing number of colony-forming cells. Cells grown in the presence of glucose required a 10-fold-higher concentration of mitomycin C for optimal induction of cloacin production than did cells grown with lactate. Release of the cloacin was hampered in glucose-grown cells. Experiments with various CloDF13 insertion and deletion mutants revealed that the transcription of CloDF13 deoxyribonucleic acid sequences adjacent to the cloacin structural gene was essential for mitomycin C-induced lethality.

Bacterial cells harboring the nontransmissible plasmid CloDF13 produce a bacteriocin designated cloacin DF13 (9). Cloacin DF13 is characterized by its ability to kill susceptible strains of *Enterobacter* and *Klebsiella* species (8). The native cloacin DF13 is excreted as an equimolar complex of two gene products encoded by the CloDF13 plasmid: the cloacin protein (56,000 molecular weight) and the immunity protein (8,500 molecular weight) (6). Cloacin DF13 inhibits protein synthesis by enzymatic cleavage of 16S rRNA and induces leakage of potassium ions from the susceptible cells (5, 7, 17). The immunity protein functions as an inhibitor of the endoribonucleolytic activity of the cloacin protein, plays a role in the penetration of susceptible cells, and confers immunity to the producing cells (6, 18, 19).

The synthesis of a bacteriocin is repressed in the majority of bacteriocinogenic cells. Treatment of these cells with mitomycin C or UV irradiation induces the synthesis of bacteriocins (9, 12, 20, 24) and is also lethal for cells harboring a bacteriocinogenic plasmid (12, 20). It is still controversial whether the observed cell death is a result of the induced synthesis of the bacteriocin molecule itself. Inselburg (13) observed that cells harboring a ColE1 plasmid mutant that encodes for inactive colicin do not exhibit the lethality associated with colicin induction. This observation suggests that active colicin E1 is required for cell death. On the other hand, Hallewell and Sherratt (11) have shown that cells harboring ColE2 plasmid mutants, carrying nonsense mutations in the colicin structural gene, are still killed by mitomycin C. Apparently the death of these cells is not a result of lesions typical of colicin action (24). Mock and Schwartz (16) have isolated mutants that fail to produce active colicin E3, but treatment with mitomycin C still causes cell death. Using insertion and deletion mutants of plasmid ColE1, Shafferman et al. (21) presented evidence that active colicin E1 is not involved in the death of cells treated with mitomycin C.

To gain more insight into the nature of the mitomycin C-induced lethality, we first studied the relationship between mitomycin C concentration, the extent of cloacin DF13 synthesis, and the death of induced cells grown at different conditions. Furthermore, we examined whether the synthesis of cloacin itself is responsible for the death of induced cells, using various CloDF13 insertion and deletion mutants. The results clearly demonstrate that not the gene for cloacin but the transcription of two other CloDF13 DNA sequences is essential for the lethal events associated with mitomycin C treatment.

MATERIALS AND METHODS

Bacterial strains and plasmids. The *Escherichia coli* K-12 strain used in this study was *E. coli* C600 with the following markers: *thr*, *leu*, *tonA*, *lacY*, *thi*, and *supE*. *E. coli* strains harboring CloDF13 plasmids were constructed by introducing these plasmids by transformation or by conjugation (26). Table 1 lists the various CloDF13 plasmids and some of their properties. The cloacin-susceptible strain *Klebsiella edwardsii* subsp. *edwardsii* was used to determine the killing activity of cloacin DF13 (8) and for lacuna assays.

Media and buffers. For cultivation of bacteria, Lab Lemco (LL) broth (Oxoid Ltd., London) was used, supplemented with lactate or glucose at a concentra-

TABLE 1. *Bacterial plasmids*

Plasmid	Parental plasmid	Site of Tn*901* insertion[a]	CloDF13 DNA deleted[a]	Origin or reference
CloDF13				14
CloDF13 *cop-3*	CloDF13			14
pJN60	CloDF13 *cop-3*	11.5		26
pJN61	CloDF13 *cop-3*	93.5		26
pJN63	CloDF13 *cop-3*	43		22
pJN66	CloDF13 *cop-3*	37		22
pJN67	CloDF13 *cop-3*	11.5		26
pJN73	CloDF13 *cop-3*	83		26
pJN77	CloDF13 *cop-3*	27.5		26
pJN78	CloDF13 *cop-3*	19		26
pJN81	CloDF13 *cop-3*	15.5		26
pEV2	pJN60	11.5	15–29	22
pEV32	pJN63	43	15–43	22
pEV202	pJN61	93.5	15–29, 45–93.5	E. Veltkamp

[a] The site of the Tn*901* insertion and deleted regions are presented as percentages of the CloDF13 physical map.

tion of 0.5% (wt/vol). LL agar and LL soft agar were composed of LL broth with 12 and 6 g of agar per liter, respectively. Phosphate-buffered saline was 0.01 M phosphate buffer (pH 7.2) plus 0.15 M NaCl.

Induction of cloacin synthesis. An overnight culture was harvested by centrifugation, and the pellet was suspended in prewarmed medium to an optical density at 660 nm of about 0.10. Portions of this suspension were then incubated at 37°C with aeration and induced with various concentrations of mitomycin C (Kyowa, Hakko Ltd., Tokyo) after about 1 hour (optical density at 660 nm of approximately 0.25).

Assay for cloacin DF13. The killing activity of cloacin DF13 was determined essentially as described by De Graaf et al. (8). Serial twofold dilutions of samples containing cloacin DF13 and 4 ml of LL broth buffered with 10 mM phosphate (pH 7.0) were supplemented with 5×10^8 cells of the indicator strain. The mixtures were incubated with vigorous aeration for 2 h at 37°C, and the optical density at 660 nm was measured and plotted versus the dilution of the samples. The killing activity of the bacteriocin in the sample was calculated from the plots and expressed in units per milliliter of sample. One unit represents the amount of cloacin required to inhibit the growth of the added cells by 50%. In this assay, concentrations of cloacin DF13 as low as 1 ng/ml could be determined with good reproducibility. Usually less than 10% variation per test was found. Extracellular cloacin was measured in the culture medium after the cells were removed by centrifugation. Intracellular cloacin was measured in preparations of disrupted cells, obtained by sonication of cell suspensions in phosphate-buffered saline as described previously (27). The total amount of cloacin produced was the sum of the extra- and intracellular cloacin.

Assays of lacuna-forming cells. The number of lacuna-forming cells was determined by a modification of the lacuna technique of Ozeki et al. (20), essentially as described by Kool and Nijkamp (14). Dilutions of cloacin-producing cultures were made in cold phosphate-buffered saline. Samples (0.1 ml) of the appropriate dilutions were mixed with 2.5 ml of LL soft agar with or without 10^7 susceptible cells. The mixtures were poured onto 10-ml LL agar plates. After overnight incubation at 37°C, lacunae and colonies were counted.

RESULTS

Relationship between mitomycin C concentration, cloacin synthesis, and viability of cloacinogenic cells. Addition of mitomycin C to a culture of cloacinogenic cells results in an induction of cloacin production concomitant with a decrease in the number of viable cells (27). The lethality of mitomycin C induction is evident from the observation that induced cells lose their colony-forming ability and are only detectable as lacuna-forming cells in a layer of susceptible bacteria.

To get more information about the relationship among mitomycin C concentration, cloacin synthesis, and loss of colony-forming ability, we measured the amount of cloacin synthesized at 180 min after the addition of various concentrations of mitomycin C together with the number of lacuna- and colony-forming cells. An incubation period of 3 h was chosen since within that time period the extent of cloacin synthesis in the various cultures has reached its maximum (27). An increasing concentration of mitomycin C induced an increasing synthesis of cloacin accompanied by a decreasing number of colony-forming cells (Fig. 1A). Cloacin production was optimal with mitomycin C concentrations of 50 ng/ml or higher. The number of lacuna-forming cells was proportional to the total amount of cloacin produced with mitomycin C concentrations of up to 25 ng/ml. At higher concentrations, a reduced number of lacuna-forming cells was detected after the 3 h incubation period. The explanation for this reduction is given by

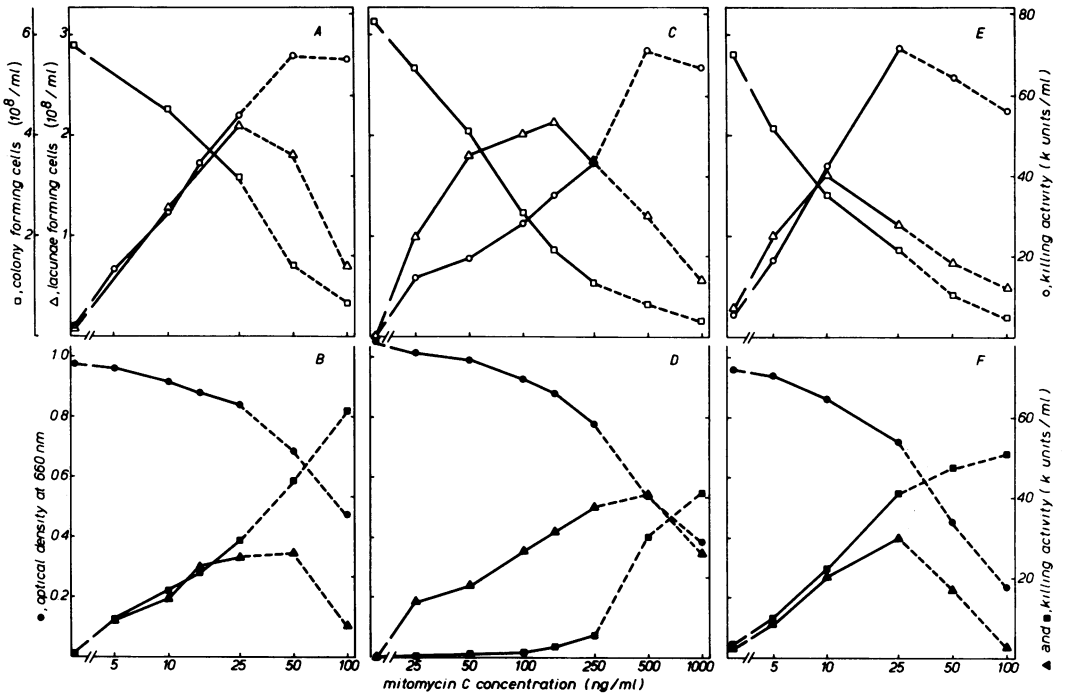

FIG. 1. *Effect of mitomycin C on cloacin production and colony-forming ability of cloacinogenic cells grown in different media. E. coli C600 (CloDF13) was cultured in LL broth supplemented with lactate (A, B) or glucose (C, D). Strain C600 (CloDF13 cop-3) was grown with lactate (E, F). At 180 min after the addition of mitomycin C, the numbers of lacuna-forming* (Δ) *and colony-forming* (□) *cells and the total amount of cloacin produced* (○) *were determined (A, C, and E). Furthermore, we measured the distribution of cloacin in the culture medium* (■) *and cells* (▲) *(B, D, and F). The optical density of the cultures was followed, and the value measured at 180 min was plotted* (●). *The broken lines connect values obtained with cultures that underwent lysis. A culture was considered to have lysed when a significant decrease of the optical density was observed (see also reference 27 and Fig. 2).*

the previously described effects of mitomycin C on the growth of cloacinogenic cells (27). Measurements of the optical density of the cultures during 3 h of incubation showed that a slight inhibition of growth occurred after the addition of mitomycin C in concentrations lower than 25 ng/ml. At concentrations higher than 25 ng/ml, an increasing portion of the cells lysed during the incubation period, which resulted in a decreasing number of lacuna-forming cells. This lysis was reflected in the proportion of the bacteriocin present in the cells and in the culture supernatant (Fig. 1B). The number of lacuna-forming cells paralleled the amount of intracellular cloacin. Therefore, the lack of a continued constant relationship between cloacin production and number of lacuna-forming cells at relatively high mitomycin C concentrations is very likely due to the loss of lacuna-forming cells caused by lysis before plating.

Since it has been observed that fermentable sugars like glucose repress both cloacin production and excretion (27), a comparable experiment was carried out with cultures grown in the presence of 0.5% glucose (Fig. 1C, D). In this medium, the concentration of mitomycin C required to induce optimal cloacin synthesis was about 10-fold higher than that needed with cultures grown in the absence of glucose. Also, in this experiment the number of lacunae decreased at high mitomycin C concentrations because an increasing portion of the cells were already lysed before plating. The ultimate amount of cloacin produced in both media (7×10^4 U/ml), however, was the same. This observation indicated, in contrast to earlier statements (27), that the production of cloacin was not repressed by glucose, provided a sufficiently high concentration of mitomycin C was used for induction. Comparison of the data in Fig. 1C and D indicated that the number of lacuna-forming cells paralleled the amount of intracellular cloacin in the culture. Remarkably, almost no cloacin was detected in the culture supernatant before the cultures started to lyse during incubation with higher mitomycin C concentrations.

Apparently, glucose prevented release of the bacteriocin.

The observation that cloacinogenic cells grown in the absence or presence of glucose produced a comparable amount of cloacin when almost all of the cells in the population were induced suggested that under these conditions the total protein-synthesizing capacity of the cells was shifted toward the synthesis of plasmid-specified gene products. In that case, the maximal amount of cloacin that can be produced by an induced cell should be independent of an increase in the number of plasmid copies per cell. To investigate this possibility, a third experiment was carried out with a strain possessing seven times more plasmid copies (CloDF13 *cop-3*) (14, 28). With these cells a concentration of 25 ng of mitomycin C per ml was sufficient to induce optimal cloacin synthesis (Fig. 1E, F). The ultimate amount of cloacin produced was about 7×10^4 U/ml. The number of lacuna-forming cells was proportional to the amount of intracellular cloacin up to a mitomycin C concentration of 10 ng/ml. This experiment demonstrated that, compared with the strain harboring the wild-type CloDF13 plasmid, the copy mutant had an increased sensitivity to mitomycin C. Furthermore, the production of cloacin under conditions of optimal induction was not increased when more plasmid copies were present in the cell.

CloDF13 DNA sequences involved in the mitomycin C-induced lethality. Although the death of mitomycin C-induced cloacinogenic cells appeared to be correlated with cloacin synthesis, the question remained whether the death of induced cells resulted from lesions typical of the cloacin itself or from other mitomycin C-induced events. We first investigated the effect of mitomycin C treatment on the viability of plasmidless cells as well as on various mutants containing an insertion of transposon Tn*901* at different locations on the CloDF13 plasmid. As a measure of mitomycin C-induced lethality, one can determine either the decrease in colony-forming cells or the effect on bacterial growth as illustrated in Fig. 1. We chose the simple and rapid determination of mitomycin C activity on bacterial growth of the various strains by following the optical density of cultures incubated with different concentrations of mitomycin C during several hours. Using plasmidless cells, only a slight growth inhibition was observed after the addition of a relatively high concentration of mitomycin C (Fig. 2A). Cells harboring the plasmid copy mutant CloDF13 *cop-3*, however, were already inhibited in their growth at low mitomycin C concentrations and lysed at higher concentrations (Fig. 2B). This observation clearly showed that commitment to death was dependent on the presence of the CloDF13 plasmid. The mitomycin C-induced lethality in various CloDF13 insertion mutants is illustrated in Fig. 3, where the final optical density of the cultures, measured at 180 min, is plotted versus mitomycin C concentration. A strain with an insertion of the transposon Tn*901* within the cloacin structural gene (pJN78) showed a response to mitomycin C treatment similar to that observed with plasmidless cells, but insertion of the transposon into other CloDF13 genes (pJN63 and -73) did not prevent cell lysis, and these strains showed a sensitivity to mitomycin C similar to that observed with the copy mutant from which the insertion mutants were derived. These results indicated that the transcription of the CloDF13 DNA region comprising the cloacin structural gene was involved in the events leading to lethality after mitomycin C treatment.

Studies on the transcription of the CloDF13 plasmid in minicells of *E. coli* (25) have shown that three mRNA species are synthesized in relatively large amounts. These three mRNA's were found to be transcripts from the CloDF13 DNA region from 0 to 40% on the CloDF13 physical map, a region containing the cloacin and immunity genes as well as the genetic information involved in plasmid DNA replication (Fig. 4). The cloacin and immunity genes were coordinately transcribed into mRNA's of about 2,200 and 2,400 nucleotides, which differ in length at their 3′ termini (25). Transcription of these genes initiated at a promoter located around 32% on the CloDF13 physical map. The third 100-nucleotide mRNA was encoded by the CloDF13 region between 7.7 and 8.8%, and its synthesis proceeded in a direction opposite to that of transcription of the cloacin and immunity genes. These data indicated the necessity to investigate the expression of the entire DNA region encoding these mRNA's to explain the lethality of mitomycin C treatment. Therefore, a larger set of plasmid mutants containing Tn*901* insertions within the 0 to 40% DNA region was investigated for sensitivity to mitomycin C (Fig. 3). All strains harboring plasmids with an insertion at different locations within the cloacin structural gene (pJN77, -78, and -81) appeared to respond to mitomycin C as observed with plasmidless cells. These strains could produce cloacin fragments as described previously (2). Insertions upstream of the cloacin promoter (pJN63 and -66) did not affect cloacin synthesis, and the host cells remained as sensitive to mitomycin C as the parent strain. Surprisingly, however, an insertion located at 11.5% on the

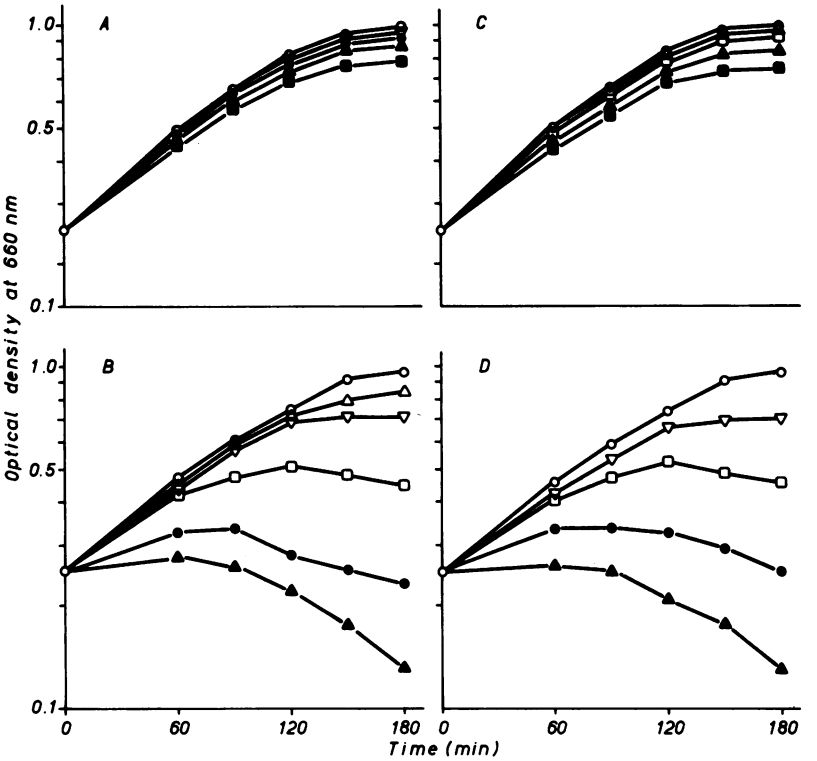

FIG. 2. *Effect of mitomycin C on bacterial growth. E. coli C600 was grown in LL broth with lactate and induced with various mitomycin C concentrations as described in Material and Methods. (A) Plasmidless cells; (B) cells harboring CloDF13 cop-3; (C) cells harboring pJN67; (D) cells harboring pEV202. The mitomycin C concentrations used were: ○, none; △, 10 ng/ml; ▽, 25 ng/ml; □, 50 ng/ml; ●, 100 ng/ml; ▲, 250 ng/ml; and ■, 1,000 ng/ml.*

CloDF13 physical map (pJN67), close to the immunity gene and within the DNA region encoding the 2,400-nucleotide mRNA, completely abolished the mitomycin C-induced lethality, although this strain produced as much active cloacin and immunity protein as the parent strain (Fig. 2C and 4). These observations clearly indicated that the synthesis of cloacin or a cloacin fragment was not involved in mitomycin C-induced lethality. Next, several Tn*901* insertion mutants were investigated that had deletions in the cloacin structural gene (pEV202, -32, and -2) (Fig. 4). Cells harboring plasmid pEV202, with a deletion of almost the entire cloacin gene, appeared to be as sensitive to mitomycin C as the parent strain (Fig. 2D). This observation confirmed that synthesis of cloacin or cloacin fragments did not commit the cells to death. Extension of the deletion into the DNA region upstream of the cloacin promoter (pEV32) rescued the cells from mitomycin C-induced lethality (Fig. 4). The same result was obtained with a strain harboring pEV2 which did contain the cloacin promoter but carried the Tn*901* insertion at the same location as pJN67. Both strains harboring pEV32 and pEV2 responded to mitomycin C as shown for the insertion mutant pJN67 in Fig. 2C. These results clearly demonstrated that not the synthesis of the cloacin but the transcription of the cloacin promoter region as well as the DNA region adjacent to the immunity protein gene is essential for mitomycin C-induced lethality.

DISCUSSION

The bacteriocinogenic plasmid CloDF13 is a small, nonconjugative plasmid (molecular weight of about 5.75×10^6) which encodes for at least eight polypeptides, including cloacin and immunity protein (2). In a noninduced culture, only a small fraction of the population spontaneously produces a measurable amount of cloacin DF13. If, however, bacteriocinogenic cells are treated with mitomycin C, the majority of the cells produce cloacin DF13. Apparently mitomycin C induces the transcription of the cloa-

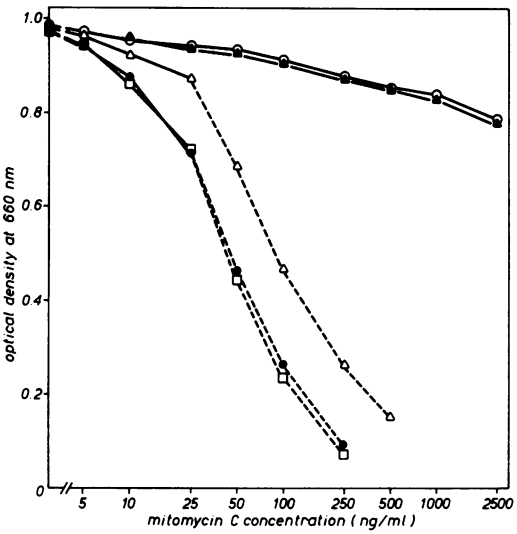

FIG. 3. *Sensitivity of various CloDF13 insertion mutants to mitomycin C. E. coli C600 cells harboring different CloDF13 plasmids or plasmidless cells were grown in LL broth supplemented with lactate. Induction with mitomycin C was as described in Materials and Methods. The optical density of the cultures was followed at 660 nm, and the value measured at 180 min after the addition of mitomycin C was plotted versus mitomycin C concentration. Symbols: ○, no plasmid; △, CloDF13; □, CloDF13 cop-3; ●, pJN63, -66, and -73; ▲, pJN 67, -77, -78, and -81. Values obtained with cultures that underwent lysis during incubation are connected by broken lines (see also Fig. 2).*

cin operon, resulting in a coordinated synthesis of cloacin and immunity protein; the synthesis of other plasmid-coded gene products seems not be enhanced (10). The mechanism of mitomycin C induction and the reason why only the expression of certain plasmid genes is induced are still unknown. Experiments on the relationship between mitomycin C concentration and cloacin synthesis by cells grown in the presence or absence of glucose or by cells containing a different number of plasmid copies have shown that the efficiency of mitomycin C-induced cloacin synthesis is strongly reduced when the cloacin operon is under catabolite repression and enhanced when more plasmid copies are present in the cell. These results suggest a direct interaction of mitomycin C with the promoter region for the cloacin operon on the CloDF13 DNA. The inhibiting effect of glucose on cloacin synthesis can be abolished by treating the cells with relatively high concentrations of mitomycin C. However, the major part of the cloacin synthesized at these conditions was not released. Probably other effects of glucose, for instance, on cell envelope composition (15), interfere with the translocation of the cloacin through the cell membranes.

Treatment of cells with mitomycin C appeared to be a lethal event for a cloacinogenic cell. It is quite clear from studies on other bacteriocins that lethality parallels bacteriocin production, but it has not been shown unambiguously that lethality is a direct consequence of the production of the bacteriocin itself (11, 12, 16, 20, 21, 23, 24). This study was an attempt to get more insight into lethality that occurs upon mitomycin C-induced synthesis.

The experiments presented demonstrate that the presence of the CloDF13 plasmid is a prerequisite for mitomycin C-induced lethality. The question arises of how the plasmid is involved. Induced cloacinogenic cells did not show any depletion of ATP or ADP, indicating that maintenance of the plasmid and the strongly increased synthesis of cloacin and immunity protein did not exhaust the cellular energy pool (data not shown). It seemed more likely that plasmid-encoded gene products are involved in mitomycin C-induced lethality of cloacinogenic cells. A possible candidate for such a gene product is the cloacin itself, because it inactivates the ribosomes of the susceptible cells by cleavage of the 16S rRNA and induces of leakage of potassium ions (5, 7). However, no such effects of intracellular cloacin could be detected (data not shown). Furthermore, the synthesis or release of cloacin did not result in alterations of the protein composition of the cell membranes or in drastic alterations of the permeability of the membranes for cytoplasmic and periplasmic proteins (27; G. J. van Tiel-Menkveld, unpublished data).

To gain more evidence that cloacin is not involved in mitomycin C-induced lethality of cells, we made use of different CloDF13 plasmid mutants obtained by insertion of transposon Tn*901* or deletion of particular DNA fragments with restriction endonucleases (22, 26). We observed that insertions within the cloacin structural gene abolished the lethal effect of mitomycin C on the host, although these cells are capable to produce cloacin fragments (2). Since it is known that insertion of a transposon may have polar effects on adjacent genes, we also used CloDF13 mutants with insertions outside the cloacin gene and mutants that contain deletions within the cloacin gene. From these studies it was evident that the presence of the two DNA regions adjacent to these genes and not the structural genes for cloacin and immunity protein are essential for mitomycin C-induced lethality. A comparable situation has been observed with the ColE1 plasmid. Shafferman et al. (21) have reported that mitomycin C-induced lethality is also not due to colicin E1 itself, but

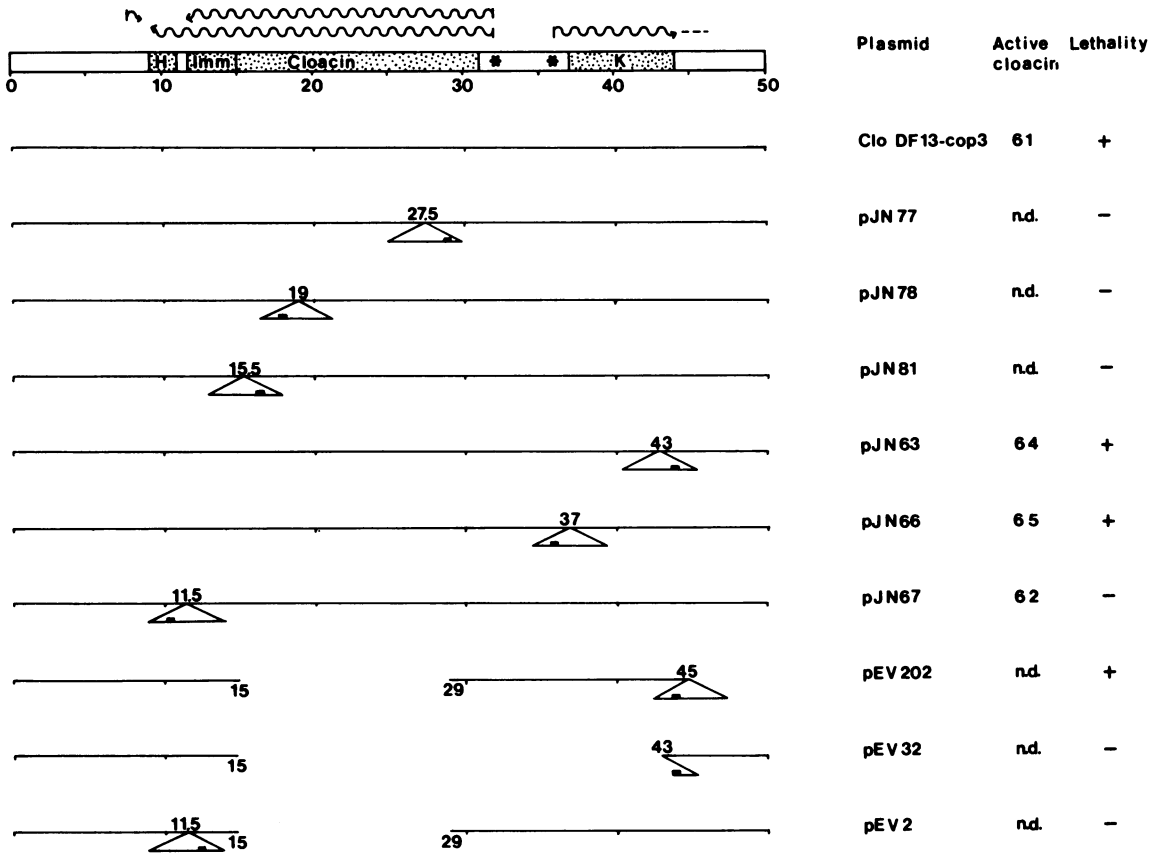

FIG. 4. *Physical maps of the plasmid mutants used, their ability to produce active cloacin DF13, and their effect on mitomycin C-induced lethality. The upper line represents a part (0 to 50%) of the genetic map of the CloDF13 plasmid according to Van den Elzen et al. (25). The locations of gene H, gene K, and the structural genes coding for immunity protein and cloacin, and the position of the promoters (*), are indicated. RNA transcripts are indicated by curved lines. △, Location of transposon Tn901; ■, β-lactamase structural gene. Active cloacin production was determined as described in Materials and Methods. The total amount of cloacin produced by the various strains in LL broth supplemented with lactate after induction with 25 ng of mitomycin C per ml is given in units × 10^3 per milliliter. n.d., No detectable cloacin production. The mitomycin C-induced lethality was determined by measuring the optical density at 660 nm of the cultures in the presence of various concentrations of mitomycin C as shown in Fig. 2 and 3. —, Strain responded to mitomycin C as observed with plasmidless cells; +, strain responded to mitomycin C as observed with the strain harboring CloDF13 cop-3.*

to the presence of ColE1 DNA regions located adjacent to the colicin gene.

In view of the DNA regions involved and since it is known from studies on the transcription of the CloDF13 plasmid that a large mRNA molecule is transcribed from the DNA region from 32 to 9% (25), mitomycin C-induced lethality might be explained by assuming that the DNA region from 9 to 11.5% encodes for a gene product that is toxic for the cell and that the expression of this gene requires the promoter located at 32%. This hypothesis suggests that under noninduced conditions the expression of the gene is repressed. Recently, it was observed that integration of transposon Tn*5* between 9 and 11.5% resulted in the disappearance of a CloDF13-specified gene product (protein H; molecular weight of 6,500) (M. J. J. Hakkaart, unpublished data). It might be that this protein is translated from the 200-terminal nucleotides of the 2,400-nucleotide mRNA. In that case, treatment of cells with mitomycin C will induce the expression of the cloacin and immunity genes and the expression of the proposed "kill" gene. Plasmid mutants have been isolated that are able to kill the host cells. For instance, Andreoli et al. (1) have reported the isolation of a temperature-sensitive CloDF13 mutant, CloDF13 *cop-1*(Ts), that kills host cells at 42°C. Recently it was found that the *cop-1*(Ts) muta-

tion resides within the CloDF13 DNA region of 0 to 15% (A. R. Stuitje and E. Veltkamp, Plasmid, in press). These observations support the hypothesis that CloDF13 contains a gene, normally repressed, that is involved in the killing of the host. Whether other bacteriocinogenic plasmids contain such a gene is not known at the moment. The intriguing possibility exists that the function of this gene product resembles the 75-amino acid polypeptide translated from the RNA of certain phages which appears to be essential for cell lysis (3, 4).

To solve the problem of how the CloDF13 plasmid is involved in the mitomycin C-induced lethality of host cells, further research will be focused on the isolation and identification of the kill gene product.

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