Effects of Divalent Cations and of Phospholipase A Activity on Excretion of Cloacin DF13 and Lysis of Host Cells

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Induction of cloacin DF13 synthesis in *Escherichia coli* harbouring plasmid CloDF13 results in the release of cloacin DF13, inhibition of growth and ultimately in lysis of the host cells. Expression of the pCloDF13-encoded protein H is essential for both the release of cloacin DF13 and the lysis of the cells. The divalent cations Mg²⁺ and Ca²⁺ interfered with the mitomycin C-induced, protein H-dependent lysis, but hardly affected the release of cloacin DF13. Essentially all of the bacteriocin was released from the cells before a detectable degradation of the peptidoglycan occurred, independent of the presence of mitomycin C. Experiments with phospholipase A mutants revealed that activation of detergent-resistant phospholipase A was essential for the export of cloacin DF13 across the outer membrane and the lysis of induced cells. Transport of cloacin DF13 across the cytoplasmic membrane was mainly dependent on protein H. A revised model for the excretion of cloacin DF13 is presented.

INTRODUCTION

Cells of *Escherichia coli* and *Enterobacter cloacae* harbouring the bacteriocinogenic plasmid CloDF13 synthesize cloacin, immunity protein and protein H (Van den Elzen *et al.*, 1980; Hakkaart *et al.*, 1981*a*; Oudega *et al.*, 1982). The genes encoding these proteins are co-ordinately transcribed from one common, mitomycin C-inducible promoter located proximal to the cloacin gene on pCloDF13 (Van den Elzen *et al.*, 1980; Hakkaart *et al.*, 1981*b*).

Cloacin and immunity protein form an equimolar complex, called cloacin DF13, which is excreted into the extracellular environment (Van Tiel-Menkveld *et al.*, 1979; Oudega *et al.*, 1982). Protein H is a polypeptide of 28 amino acid residues with a *M_r* of 2871 (Oudega *et al.*, 1984*a*). The polypeptide is synthesized as a precursor with an amino-terminal signal sequence of 21 amino acid residues, and has been localized in both the cytoplasmic and outer membrane of *E. coli* minicells (Oudega *et al.*, 1984*b*). The mature protein H is essential for the excretion of cloacin DF13, and for the lethal consequences and apparent lysis of cells induced with high concentrations of mitomycin C (Hakkaart *et al.*, 1981*b*; Van Tiel-Menkveld *et al.*, 1981; Oudega *et al.*, 1982, 1984*a, b*).

Genes functionally comparable to gene H of pCloDF13 have been identified on the colicinogenic plasmids ColE1 (the *kil* gene, Sabik *et al.*, 1983) and ColE2 (the *celB* gene, Pugsley & Schwartz, 1983*a, b*). Also the plasmids ColE3, ColN and ColA seem to encode a protein H-like product (Mock & Schwartz, 1978; Jakes & Model, 1979; Varenne *et al.*, 1981; Pugsley, 1984; Jakes & Zinder, 1984). The primary structures of the *kil* gene product, the *celB* gene

Abbreviation: DAP, ϵ -diaminopimelic acid.

product, and the gene H product show extensive homology (Hakkaart *et al.*, 1981*a*; Stuitje *et al.*, 1981; Pugsley & Schwartz, 1984). The *kil* gene product and the *celB* gene product are also involved in the release of their respective colicins and the lysis or quasi-lysis of fully induced host cells (Mock & Schwartz, 1978; Cavard *et al.*, 1981; Pugsley & Rosenbusch, 1981; Sabik *et al.*, 1983; Jakes & Zinder, 1984).

To obtain information on the mechanism by which protein H provokes (i) the release of cloacin DF13 and (ii) the lysis of host cells, and to study to what extent these events are related, we investigated the effect of protein H on the integrity of the cell envelope (i.e. degradation of peptidoglycan and phospholipids), as well as the effect of membrane-stabilizing divalent cations on the functioning of protein H.

METHODS

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study, and their relevant characteristics, are listed in Table 1. Plasmids were introduced into the appropriate strains by transformation according to the method of Dagert & Ehrlich (1979). Plasmid DNA was isolated as described by Birnboim & Doly (1979). To select for antibiotic resistance the following antibiotics and concentrations were used: ampicillin, 100 µg ml⁻¹; kanamycin, 50 µg ml⁻¹; and chloramphenicol, 30 µg ml⁻¹. *E. coli* W7 was cultured in a minimal medium containing 2.0 g K₂HPO₄, 2.6 g NaH₂PO₄·2H₂O, 0.9 g NH₄Cl, 0.2 g MgSO₄·7H₂O, 5 mg FeCl₂, 20 mg lysine and 10 mg DL-α,ε-diaminopimelic acid (DAP, Sigma) per litre distilled water and 0.5% (w/v) sodium lactate as a carbon source. The pH of the medium was 7.4. All other strains were cultured in Lab-Lemco broth (Oxoid) supplemented with 0.5% (w/v) NaCl and 0.5% (w/v) lactate. Cells harbouring a CloDF13 plasmid derivative with an insertion of transposon Tn901 or Tn5 were cultured in the presence of ampicillin or kanamycin, respectively. Cells carrying pPI232 were cultured in medium with chloramphenicol.

Induction, production, excretion and subcellular localization of cloacin DF13. Cloacinogenic cells were cultured and induced as described by Van Tiel-Menkveld *et al.* (1979, 1981). For sub-optimal induction of cloacin DF13 and protein H, concentrations of 25 or 50 ng mitomycin C (ml culture)⁻¹ were used. Full induction was established with 250 ng (ml culture)⁻¹. The total production of cloacin DF13, the amount of cloacin DF13 excreted into the culture medium, and the amount of cloacin DF13 in various subcellular fractions were determined with *Klebsiella edwardsii* strain S15 as indicator strain for cloacin DF13 activity (*in vivo* killing assay), or immunologically with an enzyme-linked immunosorbent assay (ELISA). Both assays have been described previously (Van Tiel-Menkveld *et al.*, 1979; Oudega *et al.*, 1982). The isolation of subcellular fractions of *E. coli*, the assays of β-galactosidase and β-lactamase activity, and all other basic procedures were done as described previously (Van Tiel-Menkveld *et al.*, 1979, 1981; Oudega *et al.*, 1982).

Measurement of peptidoglycan degradation. The peptidoglycan of cells of *E. coli* W7 harbouring pJN73 was labelled with [³H]DAP by incubation of a minimal medium culture in the presence of 10 mM-[³H]DAP [specific activity 60 mCi mmol⁻¹ (2.22 GBq mmol⁻¹) CEA, France] for 18 h at 37 °C. Subsequently, the cells were collected by centrifugation (12000 g, 10 min), resuspended to an optical density at 660 nm of 0.1 in minimal medium containing lysine, DAP and 0.5% (w/v) lactate, and incubated aerobically at 37 °C. One part of this culture was induced with the indicated concentrations of mitomycin C at an optical density at 660 nm of 0.2. At intervals the loss of peptidoglycan labelled with [³H]DAP was measured essentially as described by Leduc *et al.* (1980, 1982). Samples (0.5 ml) were mixed with 0.5 ml portions of 10% (w/v) TCA, and incubated for 1 h at 0 °C. The suspensions were then filtered on membrane filters (pore size 0.2 µm; Millipore). The filters were washed three times with 5 ml 5% (w/v) TCA (0 °C) and the radioactivity retained on the filters was counted.

Labelling and analysis of lipids. Cells of the *E. coli* strains PC1602, F417 and S17, each without any plasmid, or with pJN73 or pVC3, were labelled overnight in Lab-Lemco broth supplemented with [¹⁴C]acetate [10 µCi ml⁻¹ (370 kBq ml⁻¹); 58 mCi mmol⁻¹ (2.15 GBq mmol⁻¹); Amersham]. The cells were collected by centrifugation (12000 g, 10 min), and were washed and resuspended in fresh broth to an optical density at 660 nm of 0.05. The cultures were further incubated at 37 °C and at an optical density of 0.25 they were induced with mitomycin C. Samples of 0.5 ml were taken at various times and were analysed for lipids and free fatty acids. These were extracted from the samples by a modification of the method of Bligh & Dyer as described by Duckworth *et al.* (1974). Lipids were separated by TLC on silica gel plates (0.75 mm, 60 H, Merck) in chloroform/methanol/water/acetic acid (65:25:4:1, by vol.). Lipids were detected with iodine vapour by comparison with commercially available standards. Spots containing phosphatidylethanolamine, lysophosphatidylethanolamine and free fatty acids were scraped off into scintillation vials and counted. The radioactivity incorporated in these three compounds accounted for about 80–90% of that incorporated into extractable lipid and added to the TLC plate.

Table 1. *Bacterial strains and plasmids*

Strain or plasmid designation	Relevant characteristics*	Reference
<i>Klebsiella edwardsii</i> subsp. <i>edwardsii</i> S15	Susceptible to cloacin DF13	De Graaf <i>et al.</i> (1969)
<i>Escherichia coli</i> W7	Lys ⁻ DAP ⁻	Leduc & Van Heijenoort (1980); Leduc <i>et al.</i> (1982)
<i>Escherichia coli</i> K12 N3406	Thr ⁻ Leu ⁻ Thi ⁻ LacY ⁻ TonA ⁻ SupE ⁻	Van Tiel-Menkveld <i>et al.</i> (1979); Oudega <i>et al.</i> (1982, 1984a,b)
PC1602	F ⁻ MetE ⁻ ThyA ⁻ Bio ⁻ EndA ⁻ Sup ⁻	De Geus <i>et al.</i> (1983)
F417	Derivative of PC1602; lacking detergent-resistant phospholipase A activity	P. De Geus (personal communication)
S17	F ⁻ Thr ⁻ Leu ⁻ Thi ⁻ ; lacking both detergent-resistant and detergent-sensitive phospholipase A activity	Doi <i>et al.</i> (1972); De Geus <i>et al.</i> (1983)
Plasmids		
pJN73	pCloDF13::Tn901, <i>cop3</i> ; Clo ⁺ Imm ⁺ H ⁺ Amp ^r ; insertion of Tn901 at 83% not affecting expression of the cloacin operon	Van Tiel-Menkveld <i>et al.</i> (1979); Oudega <i>et al.</i> (1982)
pVC3	pCloDF13::Tn901, <i>cop3</i> ; Clo ⁺ Imm ⁺ H ⁻ Amp ^r Km ^r ; insertion of Tn5 in gene H not affecting expression of cloacin and its immunity protein	Oudega <i>et al.</i> (1982)
pPI232	Derivative of pACYC184 with a 11.0 kb DNA fragment of <i>E. coli</i> encoding detergent-resistant phospholipase A; Cm ^r	De Geus <i>et al.</i> (1983)

* Clo⁺, Imm⁺ and H⁺ indicate that cells harbouring pJN73 or pVC3 produce biologically active cloacin, immunity protein and protein H, respectively; *cop3* means that the plasmid originates from the CloDF13 copy mutant CloDF13*cop3*.

RESULTS

Effect of divalent cations on the functioning of protein H

The effect of various concentrations of Mg²⁺-ions on the growth of mitomycin C-induced cultures of *E. coli* N3406 harbouring pJN73 is shown in Fig. 1(a). In the absence of Mg²⁺ the optical density of the culture declined markedly within 2–3 h after induction. The presence of Mg²⁺ ions protected the cells against this apparent lysis, the optimal concentration being 20 mM. At this concentration the increase in optical density of the culture was comparable to the growth of a non-induced culture (not shown). Higher concentrations of Mg²⁺ (50 or 100 mM) did not result in further protection or increase in culture optical density. When cultures were induced with low concentrations of mitomycin C, the decline in optical density was less pronounced, and 20 mM-Mg²⁺ completely prevented the observed growth inhibition (data not shown).

Ca²⁺ ions had a similar protective effect to Mg²⁺ ions. The optimal concentration of Ca²⁺ was 10 mM. The protective effect of Mg²⁺ and Ca²⁺ remained during prolonged incubation of the cells in the presence of high concentrations of mitomycin C. Moreover, addition of Mg²⁺ to a 'lysing' culture protected the cells against further 'lysis' (Fig. 1a).

The effect of Mg²⁺ on the excretion of cloacin DF13 was also determined (Fig. 1b). Mg²⁺ slightly reduced the excretion of bacteriocin produced after induction with a moderate (25 ng ml⁻¹) or high (250 ng ml⁻¹) concentration of mitomycin C, but the cells still excreted more than half of the bacteriocin they produced within 3 h. Prolonged incubation of the cells resulted in a further excretion of bacteriocin (not shown). The total production of cloacin DF13 after sub-optimal and strong induction was about 17 and 28 µg ml⁻¹, respectively. Mg²⁺ had no marked effect on the total production of cloacin DF13.

Similar results were obtained with cells harbouring the wild-type CloDF13 plasmid. The results indicated that divalent cations, as Mg²⁺ and Ca²⁺, reduce the mitomycin C-induced lysis of cloacinogenic cells, but hardly affect the excretion of cloacin DF13.

Fig. 1. Effect of Mg^{2+} ions on (a) the growth of *E. coli* N3406 harbouring pJN73, and (b) excretion of cloacin DF13. (a) —, Optical density (OD_{660}) of cultures in broth supplemented with 0.5% (w/v) lactate and the indicated concentration of Mg^{2+} (250 ng mitomycin C ml^{-1} was added at $t = 0$); ----, OD_{660} of an induced culture to which 20 mM- Mg^{2+} was added 90 min after induction was started. (b) Cells were induced with 25 ng (○, ●), or with 250 ng (□, ■) mitomycin C (ml culture) $^{-1}$, respectively. Mg^{2+} (20 mM) was added at $t = 0$ (●, ■). The amount of cloacin DF13 in the cells and in the culture supernatant fractions was determined with the *in vivo* killing assay. The percentage of cloacin DF13 excreted is indicated to the right of the Figure.

Degradation of peptidoglycan

A close correlation exists between autolysis of *E. coli* cells and peptidoglycan degradation (Leduc & Van Heijenoort, 1980; Leduc *et al.*, 1982). We have investigated whether induction of cloacin DF13 and protein H, and the subsequent decline in optical density of the culture, also resulted in a degradation of peptidoglycan. Cells of *E. coli* W7 harbouring pJN73 were labelled with [3H]DAP. After induction with two different concentrations of mitomycin C, the optical density of the culture, the production and excretion of cloacin DF13, and the loss of peptidoglycan labelled with [3H]DAP were determined simultaneously (Fig. 2). Induction with a high concentration of mitomycin C (250 ng ml^{-1}) resulted in a decline in optical density of the culture after 3 h, followed by release of [3H]DAP after 6 h. Under these conditions, excretion of cloacin DF13 started 2 h after induction and reached a maximum value of about 90% within 4–6 h. Essentially all of the bacteriocin was excreted before release of [3H]DAP was observed. The presence of Mg^{2+} ions prevented the decrease of optical density and the release of [3H]DAP, but hardly affected the excretion of cloacin DF13 or its total production (about 50 μg ml^{-1}).

Induction with a suboptimal amount of mitomycin C (25 ng ml^{-1}) resulted in a slight decline in optical density after 8 h, but no release of [3H]DAP was observed (data not shown). About 2 h after induction the cells started to excrete cloacin DF13 and about half of the bacteriocin was released by the cells within 4 h. After that time the total amount of cloacin DF13 produced (about 16 μg ml^{-1}) and the percentage of cloacin DF13 excreted remained constant. Comparable results were obtained in the presence of 20 mM- Mg^{2+} , except that no decline in optical density was observed (data not shown). Induction of *E. coli* W7 harbouring pVC3 with a transposon insertion in gene H did not result in cloacin excretion, apparent lysis, or in a detectable degradation of labelled peptidoglycan. The results indicated that the initial decrease in the optical density of the culture and the excretion of cloacin DF13 after induction are not correlated with a detectable loss of peptidoglycan labelled with [3H]DAP.

Preliminary morphological investigations with the electron microscope revealed that induction of cloacinogenic cells resulted in local protuberances of the cell envelope during the time-period in which the initial decline in optical density of the culture and excretion of cloacin DF13 are observed. These protuberances were not observed when the cells were induced in the presence of Mg^{2+} or Ca^{2+} . It is possible that the initial decline in optical density is caused by an altered scattering of light as a result of these protuberances.

Fig. 2. Effect of mitomycin C and Mg^{2+} on the release of $[^3H]DAP$, the excretion of cloacin DF13, and the OD_{660} of cultures of *E. coli* W7 harbouring pJN73 and precultured in the presence of $[^3H]DAP$. The cells were incubated in minimal medium in the absence ($\circ$, $\triangle$, $\square$), or presence ($\bullet$, $\blacktriangle$, $\blacksquare$) of 20 mM- Mg^{2+} ; they were induced at $t = 0$ with 250 ng mitomycin C ml^{-1} . $\circ$, $\bullet$, OD_{660} of the cultures; $\triangle$, $\blacktriangle$, excretion of cloacin DF13; $\square$, $\blacksquare$, acid-precipitable $[^3H]DAP$ -labelled peptidoglycan. The amount of cloacin DF13 in the cells and in the culture supernatant fractions was determined using ELISA. The total production of bacteriocin was about $50 \mu g\ ml^{-1}$, both in the presence and absence of Mg^{2+} . Excretion is expressed as a percentage of the total production. The data on the release of $[^3H]DAP$ are the mean values of several experiments, each done in duplicate (variance 5–10%).

Fig. 3. Effect of mitomycin C on the growth of various *E. coli* phospholipase A mutants. (a) Strain PC1602 with pJN73; (b) strain F417 with pJN73; (c) strain PC1602 with pVC3 (similar growth curves were obtained with strain F417 harbouring pVC3 and with strain S17 harbouring pVC3); (d) strain F417 harbouring pJN73 and pPI232. The cultures were grown without mitomycin C ($\circ$), or with 50 ng ($\square$) or 250 ng ($\triangle$) mitomycin C ml^{-1} .

Involvement of phospholipase A activity

Recently, Pugsley & Schwartz (1984) showed that the release of colicin E2 might be due, at least in part, to the activation of the detergent-resistant phospholipase A by the pColE2-encoded lysis protein. To study whether phospholipase A activities are also involved in the functioning of protein H, plasmid pJN73 was transferred to the *E. coli* strains PC1602, F417 and S17. Strain PC1602 has 'normal' phospholipase A activities and induction of cultures of this strain harbouring pJN73 resulted in 'normal' growth-inhibition and decrease in optical density of the culture (Fig. 3a). Strain F417 is defective in detergent-resistant phospholipase A activity in its

outer membrane, whereas strain S17 lacks both detergent-sensitive phospholipase A in its cytoplasmic membrane and detergent-resistant phospholipase A activity in the outer membrane (Doi *et al.*, 1972; Ohki *et al.*, 1972; De Geus *et al.*, 1983). These mutations strongly affected the mitomycin C-induced lysis of cells harbouring pJN73. Induction of cultures of strain F417 harbouring pJN73 resulted in less lysis (Fig. 3*b*). Induction of cultures of strain S17 harbouring pJN73 had an even smaller effect on cell growth (not shown). A comparable small effect of induction, as found with the latter strain, was observed with cultures of strains PC1602, F417 and S17 harbouring pVC3 (Fig. 3*c*) or harbouring no plasmid at all (not shown).

The absence of significant lysis in strain F417 harbouring pJN73 after induction could be completely complemented by the introduction of plasmid pPI232, which encodes detergent-resistant phospholipase A activity (Fig. 3*d*). Similarly, the introduction of plasmid pPI232 into strain S17 harbouring pJN73 also resulted in a decline in optical density of the culture after strong induction (data not shown). Obviously, phospholipase A activity, especially the outer membrane-associated detergent-resistant phospholipase A activity, is required for the lysis of mitomycin C-induced cloacinogenic cells.

Further evidence for the involvement of detergent-resistant phospholipase A activity came from experiments in which the degradation of phospholipids, especially of phosphatidylethanolamine, the major phospholipid in the outer membrane, was determined (Fig. 4). Induction of cultures of strain PC1602 harbouring pJN73 with a sub-optimal amount of mitomycin C resulted in a decrease in phosphatidylethanolamine and an increase in free fatty acids. These effects were also found in induced cultures of strain F417 harbouring both pJN73 and pPI232 but not in non-induced cultures; comparable results were obtained in the presence of divalent cations (Ca^{2+} , Mg^{2+}) (data not shown). Control experiments revealed that induction of cultures of strain F417 or strain S17 harbouring either pJN73 or pVC3 did not result in a loss of labelled phosphatidylethanolamine or in an increase in labelled free fatty acid (data not shown). The experiments indicated that induction of cells harbouring a plasmid encoding protein H results in an activation of detergent-resistant phospholipase A at an early stage, concomitant with or even prior to the induced decline in optical density of the culture (see also Fig. 3).

Excretion and subcellular localization of cloacin DF13 in phospholipase A mutants.

Production and excretion of cloacin DF13 was determined in the phospholipase A mutants harbouring pJN73. Cultures were induced with a sub-optimal (50 ng ml^{-1}) or high (250 ng ml^{-1}) concentration of mitomycin C and the excretion of cloacin DF13 was determined at various times (Fig. 5). Under both induction conditions, the amount of bacteriocin excreted by the mutant strains was low as compared to the excretion by strain PC1602 harbouring pJN73. Cells of strain S17 excreted only a very low percentage of the bacteriocin during the induction period, independent of the mitomycin C concentration. Cells of strain F417 released more bacteriocin, especially after strong induction and longer periods. The total production of cloacin DF13 was about the same in all strains (Fig. 5), and comparable to the total production in *E. coli* N3406 harbouring pJN73 (Fig. 1*b*; Oudega *et al.*, 1982). Cells harbouring pVC3 did not excrete significant amounts of cloacin DF13 (data not shown).

The subcellular localization of cloacin DF13 in the phospholipase A mutants was also determined (Table 2). In cells of strain F417 harbouring pJN73 more than half of the cell-associated bacteriocin was found in the periplasm, whereas only one-third could be detected in the spheroplast (cytoplasmic) fraction. In cells of strain S17 harbouring pJN73 about half of the bacteriocin was present in the spheroplasts and a smaller amount was detected in the periplasm. Only a small amount was excreted. The location of cloacin DF13 was also determined in cells of strains PC1602, F417 and S17 harbouring pVC3, and in cells of strains F417 and S17 harbouring both pJN73 and pPI232. In the absence of protein H practically all of the bacteriocin was found in the spheroplast (cytoplasmic) fraction, which is in agreement with previous experiments done with *E. coli* N3406 harbouring pVC3 (Oudega *et al.*, 1982). Subcellular localization and excretion of cloacin DF13 by cells of strain F417 harbouring both pJN73 and pPI232 were comparable to those of strain PC1602 with pJN73. Cells of strain S17 harbouring both pJN73

Fig. 4

Fig. 5

Fig. 4. Degradation of phospholipid. Cells of strain PC1602 harbouring pJN73 were labelled with [14 C]acetate and further cultured with 50 ng mitomycin C ml $^{-1}$. At the indicated times samples were removed and analysed for lipids and free fatty acids, and the 14 C-radioactivity present in phosphatidylethanolamine (O), lysophosphatidylethanolamine (□) and in free fatty acids (▲) was determined.

Fig. 5. Effect of mitomycin C on excretion of cloacin DF13 by *E. coli* phospholipase A mutants harbouring pJN73. Cultures of strain PC1602 harbouring pJN73 (○, ●), strain F417 harbouring pJN73 (□, ■), and of strain S17 harbouring pJN73 (△, ▲) were induced with 50 ng (○, □, △), or with 250 ng (●, ■, ▲) mitomycin C ml $^{-1}$. The percentage of cloacin DF13 excreted into the culture supernatant fractions was determined using ELISA. The total production of cloacin DF13 (the sum of the amounts in the cell and culture supernatant fractions) after 6 h is given in brackets (μg ml $^{-1}$).

Table 2. Localization of cloacin DF13 in subcellular fractions of phospholipase A mutants

Cells were induced by the addition of 25 ng mitomycin C (ml culture) $^{-1}$; after 4 h supernatant fractions were collected. The cells were resuspended and periplasmic fractions and spheroplast fractions were prepared. The spheroplasts were not separated in a cytoplasmic and a membrane fraction, because no significant amounts of cloacin DF13 are present in the membranes (Van Tiel-Menkveld *et al.*, 1979). The amount of cloacin DF13 in each fraction and the total production in the culture were determined immunologically (ELISA). The amount in each fraction was expressed as percentage of the total production. As controls, the percentages of β -galactosidase (cytoplasmic marker) and of β -lactamase (periplasmic marker) in the various fractions were determined and used for correction of the bacteriocin data due to experimental artefacts (corrections usually less than 6%; see also Oudega *et al.* 1982). The bacteriocin data given are the mean values of several experiments, the variance being about 10%.

Strain and plasmid(s)	Amount of cloacin DF13			
	Spheroplasts (%)	Periplasm (%)	Medium fraction (%)	Total production (μg ml $^{-1}$)
PC1602 + pJN73	21	18	61	25
PC1602 + pVC3	95	3	2	19
F417 + pJN73	30	53	17	24
F417 + pVC3	95	4	1	21
F417 + pJN73 + pPI232	18	19	63	22
S17 + pJN73	49	43	8	19
S17 + pVC3	94	4	2	24
S17 + pJN73 + pPI232	33	16	51	22

and pPI232 excreted slightly less cloacin DF13. About half of the bacteriocin they produced was excreted, whereas cell-associated cloacin DF13 was located mostly in the spheroplasts. The total production of cloacin DF13 was about the same in all of the strains used.

DISCUSSION

The mechanism by which protein H molecules provoke the excretion of cloacin DF13 and the lysis of fully induced cells is unknown. Cloacin and immunity protein are both synthesized without an amino-terminal signal sequence that could trigger their export (Oudega *et al.*, 1982, 1984*a, b*). In non-induced conditions, or after moderate induction with mitomycin C, cloacin DF13 molecules first accumulate in the cytoplasm of producing cells, and are subsequently released into the culture medium during the late exponential phase of growth (Van Tiel-Menkveld *et al.*, 1979). This release occurs without a detectable decline in optical density of the culture, or apparent lysis, and appears to be rather specific since soluble proteins such as β -galactosidase and β -lactamase are not or hardly released (Van Tiel-Menkveld *et al.*, 1979; Oudega *et al.*, 1982). Under fully induced conditions, a strong reduction in culture optical density and a maximal release of cloacin DF13 are observed in the exponential growth phase (Van Tiel-Menkveld *et al.*, 1979, 1981). Mature protein H molecules were found in both the cytoplasmic and outer membrane of *E. coli* minicells (Oudega *et al.*, 1984*b*). It has been proposed that protein H might form some kind of pores in both membranes through which cloacin DF13 molecules traverse the cell envelope. A large number of these 'pores' might induce a decline in culture optical density and lysis.

The experiments described in this paper show that the two phenomena observed after induction of protein H synthesis, namely the decline in optical density of the culture and the excretion of cloacin DF13, are separate events. Divalent cations such as Mg^{2+} and Ca^{2+} strongly inhibited the mitomycin C-induced, protein H-dependent decline in culture optical density, possibly by stabilizing the outer membrane or preventing autolysis, but did not affect the excretion of cloacin DF13 significantly. Furthermore, the experiments with *E. coli* W7 demonstrated that essentially all of the cloacin DF13 was excreted before a release of [3H]DAP from labelled peptidoglycan could be detected.

Recently, Pugsley & Schwartz (1984) described the effects of Mg^{2+} , Triton X-100, and of detergent-resistant phospholipase A activity in the outer membrane, on the growth of *E. coli* cells harbouring pColE2 and the release of colicin E2. They concluded that colicin E2 release is a consequence of a semi-specific leakage resulting from an alteration of the permeability properties of the cell envelope, and that this alteration might be due, at least in part, to the activation of the detergent-resistant phospholipase A by the pColE2-encoded 'lysis' protein. This activation might result in the formation of lysophosphatidylethanolamine, a membrane perturbant which could alter the permeability properties of the cell envelope. The assumption that such an activity is important for the release of bacteriocins is supported by the observation that the non-ionic detergent Triton X-100 enhances the release of colicin E2 in mitomycin C-induced wild-type, as well as phospholipase A-deficient strains (Pugsley & Schwartz, 1984). Our experiments with *E. coli* mutants defective in detergent-resistant and/or detergent-sensitive phospholipase A confirmed and extended these findings. Cells defective in detergent-resistant phospholipase A excreted only a low amount of cloacin DF13 into the culture medium, and more than half of the bacteriocin accumulated in the periplasm. Cells defective in both phospholipase A activities excreted less cloacin DF13 into the culture medium, and about half of the bacteriocin was found in the cytoplasm. However, about 40% of the cloacin DF13 produced was still found in the periplasm. Complementation of the mutation affecting detergent-resistant phospholipase A, by introduction of a plasmid encoding this enzyme, completely restored bacteriocin excretion and induced lysis in cells defective in detergent-resistant phospholipase A. Complementation of cells defective in both phospholipase A activities resulted in a significant release of cloacin DF13 and lysis of cells after strong induction, but complementation did not restore these effects completely. This indicates that detergent-sensitive phospholipase A activity might play a minor role in the translocation of cloacin DF13 molecules across the cell envelope.

Based on the data presented in this paper we propose a modified model for the export of cloacin DF13 molecules. In the cytoplasmic membrane protein H molecules form specific pores through which molecules of cloacin DF13 can pass. Activation of detergent-sensitive phospholipase A in the cytoplasmic membrane possibly somewhat enhances the permeability of this membrane for cloacin DF13, but is not essential. Translocation of cloacin DF13 across the outer membrane and release into the culture medium is largely dependent on the activation of detergent-resistant phospholipase A in the outer membrane. Protein H molecules might directly activate phospholipase A, or the activation could be the result of the interaction of protein H with the membrane. The combined activities of protein H and detergent-resistant phospholipase A might result in local permeability zones which rather selectively facilitate the release of cloacin DF13. Strong induction of cloacinogenic cells, resulting in large amounts of protein H, causes a strong induction of both phospholipase A activities and the formation of numerous permeability zones. This process leads to severe damage to the membranes and ultimately to cell lysis, possibly by induction of the autolytic system of *E. coli* (Leduc *et al.*, 1982). Ca^{2+} and Mg^{2+} ions interfere with this autolytic process and thus inhibit the decline in culture optical density and cell lysis.

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REFERENCES

- BIRNBIOM, H. C. & DOLY, J. (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Research* **7**, 1513–1523.
- CAVARD, D., BERNADAC, A. & LAZDUNSKI, C. (1981). Exclusive localization of colicin A in cell cytoplasm of producing bacteria. *European Journal of Biochemistry* **119**, 125–131.
- DAGERT, M. & EHRLICH, S. D. (1979). Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. *Gene* **6**, 23–28.
- DE GEUS, P., VAN DIE, I., BERGMANS, H., TOMMASSEN, J. & DE HAAS, G. (1983). Molecular cloning of *pldA*, the structural gene for outer membrane phospholipase of *E. coli* K12. *Molecular and General Genetics* **190**, 150–155.
- DE GRAAFF, F. K., SPANJAERDT-SPECKMAN, E. A. & STOUTHAMER, A. H. (1969). Mode of action of a bacteriocin produced by *Enterobacter cloacae* DF13. *Antonie van Leeuwenhoek* **35**, 287–306.
- DOI, O., OHKI, M. & NOJIMA, S. (1972). Two kinds of phospholipase A and lysophospholipase in *Escherichia coli*. *Biochimica et biophysica acta* **260**, 244–258.
- DUCKWORTH, D. H., BEVERS, E. M., VERKLEY, A. J., OP DEN KAMP, J. A. F. & VAN DEENEN, L. L. M. (1974). Action of phospholipase A₂ and phospholipase C on *Escherichia coli*. *Archives of Biochemistry and Biophysics* **165**, 379–387.
- HAKKAART, M. J. J., VELTKAMP, E. & NIJKAMP, H. J. J. (1981a). Protein H encoded by plasmid CloDF13 involved in lysis of the bacterial host. I. Location of the gene and identification and subcellular location of the gene H product. *Molecular and General Genetics* **183**, 318–325.
- HAKKAART, M. J. J., VELTKAMP, E. & NIJKAMP, H. J. J. (1981b). Protein H encoded by plasmid CloDF13 involved in lysis of the bacterial host. II. Functions and regulation of synthesis of the gene H product. *Molecular and General Genetics* **183**, 326–332.
- JAKES, K. S. & MODEL, P. (1979). Mechanism of export of colicin E1 and colicin E3. *Journal of Bacteriology* **138**, 770–778.
- JAKES, K. & ZINDER, N. D. (1984). Plasmid ColE3 specifies a lysis protein. *Journal of Bacteriology* **157**, 582–590.
- LEDUC, M. & VAN HEIJENOORT, J. (1980). Autolysis of *Escherichia coli*. *Journal of Bacteriology* **124**, 52–59.
- LEDUC, M., KASRA, R. & VAN HEIJENOORT, J. (1982). Induction and control of the autolytic system of *Escherichia coli*. *Journal of Bacteriology* **152**, 26–45.
- MOCK, M. & SCHWARTZ, M. (1978). Mechanism of colicin E3 production in strains harboring wild-type or mutant plasmids. *Journal of Bacteriology* **136**, 700–707.
- OHKI, H., OSAMU, D. & NOJIMA, S. (1972). Mutant of *Escherichia coli* K-12 deficient for detergent-resistant phospholipase A. *Journal of Bacteriology* **110**, 864–869.
- OUDEGA, B., STEGEHUIS, F., VAN TIEL-MENKVELD, G. J. & DE GRAAF, F. K. (1982). Protein H encoded by plasmid CloDF13 is involved in excretion of cloacin DF13. *Journal of Bacteriology* **150**, 1115–1121.
- OUDEGA, B., STEGEHUIS, F. & DE GRAAF, F. K. (1984a). Effect of glucose fermentation on the functioning of protein H in the excretion of cloacin DF13 by *Escherichia coli*. *FEMS Microbiology Letters* **21**, 125–131.
- OUDEGA, B., YKEMA, A., STEGEHUIS, F. & DE GRAAF, F. K. (1984b). Detection and subcellular localization of mature protein H involved in excretion of cloacin DF13. *FEMS Microbiology Letters* **22**, 101–108.
- PUGSLEY, A. P. (1984). Genetic analysis of ColN plasmid determinants for colicin production, release, and immunity. *Journal of Bacteriology* **158**, 523–529.
- PUGSLEY, A. P. & ROSENBUSCH, J. P. (1981). Release of colicin E2 from *Escherichia coli*. *Journal of Bacteriology* **147**, 186–192.

- PUGSLEY, A. P. & SCHWARTZ, M. (1983*a*). A genetic approach to the study of mitomycin-induced lysis of *Escherichia coli* K-12 strains which produce colicin E2. *Molecular and General Genetics* **190**, 366–372.
- PUGSLEY, A. P. & SCHWARTZ, M. (1983*b*). Expression of a gene in a 400-base-pair fragment of colicin plasmid ColE2-P9 is sufficient to cause host cell lysis. *Journal of Bacteriology* **156**, 109–114.
- PUGSLEY, A. P. & SCHWARTZ, M. (1984). Colicin E2 release: lysis, leakage or secretion? Possible role of phospholipase. *EMBO Journal* **3**, 2393–2397.
- SABIK, J. F., SUIT, J. L. & LURIA, S. E. (1983). *Cea-kil* operon of the ColE1 plasmid. *Journal of Bacteriology* **153**, 1479–1485.
- STUITJE, A. R., SPELT, C. E., VELTKAMP, E. & NIJKAMP, H. J. J. (1981). Identification of mutations affecting the replication control of plasmid CloDF13. *Nature, London* **290**, 264–267.
- VAN DEN ELZEN, P. J. M., KONINGS, R. N. H., VELTKAMP, E. & NIJKAMP, H. J. J. (1980). Transcription of bacteriocinogenic plasmid CloDF13 *in vivo* and *in vitro*: structure of the cloacin-immunity operon. *Journal of Bacteriology* **144**, 579–591.
- VAN TIEL-MENKVELD, G. J., REZEE, A. & DE GRAAF, F. K. (1979). Production and excretion of cloacin DF13 by *Escherichia coli* harboring plasmid CloDF13. *Journal of Bacteriology* **140**, 415–423.
- VAN TIEL-MENKVELD, G. J., VELTKAMP, E. & DE GRAAF, F. K. (1981). Mitomycin C-induced synthesis of cloacin DF13 and lethality in cloacinogenic *Escherichia coli* cells. *Journal of Bacteriology* **146**, 41–48.
- VARENNE, S., CAVARD, D. & LAZDUNSKI, C. (1981). Biosynthesis and export of colicin A in *Citrobacter freundii* CA31. *European Journal of Biochemistry* **116**, 615–620.