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Protein H Encoded by Plasmid CloDF13 Is Involved in Excretion of Cloacin DF13

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Excretion of cloacin DF13 was studied in *Escherichia coli* cells harboring different CloDF13 insertion and deletion mutant plasmids. Insertions of a transposon at position 9.8 or 11.5% of the CloDF13 plasmid blocked the expression of gene H and strongly reduced the specific excretion of cloacin DF13 into the culture medium, but had no effect on the production of cloacin DF13. Insertions in or deletions of regions of the CloDF13 DNA upstream the cloacin operon did not affect the excretion or production of the bacteriocin. Introduction of a CloDF13 plasmid that encodes for the gene H product in cells harboring a CloDF13 plasmid with an insertion in gene H stimulated the excretion of cloacin DF13 significantly in mitomycin C-induced and in noninduced cultures. Cloacin DF13 in cloacinogenic cells that did not produce the gene H protein was found to be about 90% located in the cytoplasm. In cells that did produce the gene H product, about 30% of the cloacin DF13 molecules were found in the cytoplasm, about 18% were found in the periplasm, about 2% were in the membranes, and about 50% were located in the culture supernatant. Cyclic AMP stimulated the production but not the excretion of cloacin DF13 in cells cultivated in the presence of glucose.

The bacteriocin cloacin DF13 is produced by bacterial cells which harbor the CloDF13 plasmid and kills susceptible cells of *Enterobacter* and *Klebsiella* species (10, 11). This bacteriocin is produced as an equimolar complex of two polypeptides, namely, cloacin and immunity protein (8). Cloacin inhibits protein synthesis in susceptible cells by enzymatic cleavage of 16S ribosomal RNA and causes a leakage of potassium ions (7, 9, 18). Immunity protein confers immunity to cloacin-producing cells by blocking the endoribonucleolytic activity of the cloacin and stimulates the penetration of cloacin molecules into the susceptible cells (19, 20).

Van Tiel-Menkveld et al. (29) studied the production and excretion of cloacin DF13. Under conditions where no lysis of cloacinogenic cells could be detected, biologically active cloacin DF13 molecules were first released in the cytoplasm, transported across the cytoplasmic membrane, accumulated in the periplasmic space, and finally excreted into the culture medium. In the presence of fermentable sugars that cause catabolite repression, the production and transport of cloacin DF13 across the cytoplasmic membrane and its excretion into the culture medium are strongly hampered (29). These findings and other published data about the export of colicin E1, E2, and E3 (15, 17, 22) and colicin A (31) support the hypothesis that bacteriocins are transported by a mechanism different from the mechanism of export of most periplasmic and outer membrane proteins (signal hypothesis; 4, 5, 14, 23).

Van Tiel-Menkveld et al. (30) studied the relation between mitomycin C-induced synthesis of cloacin DF13 and lethality (lysis) of cloacinogenic cells. It was found that DNA sequences adjacent to the structural genes for cloacin and immunity protein are essential for the mitomycin C-induced lysis of cloacinogenic cells. Recently, Hakkaart et al. (12, 13) showed that the DNA sequence proximal to the immunity protein gene encodes for a protein, designated as protein H, that is essential for the lethal effect of mitomycin C. Protein H has a molecular mass of about 6,000 and seems to be predominantly located in the cell envelope (12). Hakkaart et al. (13) observed that treatment of cloacinogenic cells with mitomycin C resulted in the induced synthesis of three CloDF13-specified polypeptides: cloacin, the immunity protein, and protein H. The genes for these three polypeptides form one inducible operon with a common promoter in front of the cloacin gene (13, 26). Recently, Van den Elzen et al. showed that mitomycin C treatment results in a derepression of transcrip-

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tion of the cloacin operon and that the chromosomal *lexA* gene product functions as the repressor of the operon (P. J. M. Van den Elzen, H. Walter, J. Maat, E. Veltkamp, and H. J. J. Nijkamp, Nucleic Acids Res., in press).

To obtain more information about the mechanism of export of cloacin DF13 molecules, we studied excretion in strains harboring various mutant CloDF13 plasmids. The excretion of cloacin DF13 into the culture medium was strongly hampered in cells that harbored CloDF13 plasmids with a mutation close to or in gene H.

In this paper, we demonstrate that protein H is involved in the process of export of cloacin DF13 both in noninduced and in mitomycin C-induced cultures. Protein H appears to be specifically involved in the translocation of cloacin DF13 molecules across the cytoplasmic membrane.

MATERIALS AND METHODS

Bacterial strains and plasmids. The production and excretion of cloacin DF13 was studied in *Escherichia coli* K-12 strain C600 *thr leu thi lacY tonA supE*. CloDF13 plasmids were introduced by transformation by the method of Dagert and Ehrlich (6). Plasmid DNA was isolated as described by Birnboim and Doly (3).

The relevant characteristics of the various CloDF13 plasmid derivatives used in this study are listed in Table 1. A schematic presentation of the CloDF13 insertion and deletion mutants is shown in Fig. 1. These mutant plasmids were kindly donated by E. Veltkamp, Vrije Universiteit, Amsterdam, The Netherlands. The cloacin DF13-susceptible strain *Klebsiella edwardsii* subsp. *edwardsii* was used for the determination of the killing activity of cloacin DF13 (10).

Media and buffers. Cells were cultured in Lab-Lemco broth (LL broth; Oxoid Ltd., London, England) supplemented with 0.5% (wt/vol) lactate or glucose. Cells harboring a CloDF13 plasmid derivative with an insertion of transposon Tn*901* were grown in medium with ampicillin (50 μg/ml), and cells harboring a CloDF13 plasmid with an insertion of Tn*5* were cultured in medium with kanamycin sulfate (50 μg/ml).

Induction, production, excretion, and localization of cloacin DF13. Cloacinogenic cells were cultured and induced as described by Van Tiel-Menkveld et al. (29, 30). The amount of mitomycin C used for the induction of cloacin DF13 production was 10 or 100 ng/ml of culture. The assay for the total production of cloacin DF13 and the amount excreted into the medium were determined as described elsewhere (29, 30). All other basic procedures were as described previously (29, 30).

RESULTS

Excretion of cloacin DF13 by cells harboring different CloDF13 insertion and deletion mutant plasmids. In the experiments described below, 10 ng of mitomycin C per ml, a suboptimal concentration, was used to minimize any effect on cell growth, viability, and lysis. The rate of excretion of cloacin DF13 is not affected by concentrations of mitomycin C up to 10 ng/ml (30). Under these conditions, cells harboring the wild-type CloDF13 plasmid or the copy mutant CloDF13-*cop3* produced up to about 50% of the maximal amount of cloacin DF13 (30), and about one-half of the bacteriocin was excreted into the culture medium (Table 2). Cells harboring the mutant CloDF13 plasmids pJN60 or pJN67 excreted little cloacin DF13, whereas production was not affected significantly. These mutant

TABLE 1. Characteristics of CloDF13 plasmid derivatives

Plasmid	Transposon integrated	Map position[a]	Orientation of transposon[b]	CloDF13 DNA deleted[c]	Relevant characteristics[d]	Origin or reference
CloDF13					Clo^+ Imm^+ H^+ Lys^+	16
CloDF13-*cop3*					Clo^+ Imm^+ H^+ Lys^+ *cop3*	12, 13, 16
pJN01	Tn*901*	45	ac		Clo^+ Imm^+ H^+ Lys^+	28
pJN60	Tn*901*	11.5	c		Clo^+ Imm^+ H^- Lys^- *cop3*	1, 28, 30
pJN67	Tn*901*	11.5	ac		Clo^+ Imm^+ H^- Lys^- *cop3*	12, 13, 28, 30
pJN73	Tn*901*	83	c		Clo^+ Imm^+ H^+ Lys^+ *cop3*	1, 28, 30
pEV36	Tn*901*	38.5	c	47–90	Clo^+ Imm^+ H^+ Lys^+ *cop3*	E. Veltkamp
pEV41	Tn*901*	11.5	c	47–90	Clo^+ Imm^+ H^- Lys^- *cop3*	E. Veltkamp
pVU215	Tn*901*	37	ac	11.5–29 and 37–100	Clo^- Imm^- H^+ Lys^+	12, 13
pVC3	Tn*5*	9.8	ac		Clo^+ Imm^+ H^- Lys^- *cop3*	12, 13

[a] The site of transposon insertions is shown as a percentage of the CloDF13 physical map (1, 12, 13, 28).

[b] The orientation of the Tn*901* and Tn*5* insertions is indicated by ac or c; ac means that the left inverted repeat is orientated counterclockwise, whereas c indicates a clockwise orientation.

[c] The DNA region deleted from the plasmid is shown as a percentage of the CloDF13 physical map. (1, 12, 13).

[d] Clo^+, Imm^+, and H^+ indicate that cells harboring this plasmid produce biologically active cloacin, immunity protein and protein H, respectively; Lys^+ indicates that cells harboring this plasmid lyse within 180 min at 37°C when treated with mitomycin C at concentrations of 0.1 to 0.5 μg/ml (13, 30); *cop3* means that the plasmid originates from the CloDF13 copy mutant CloDF13 *cop3*.

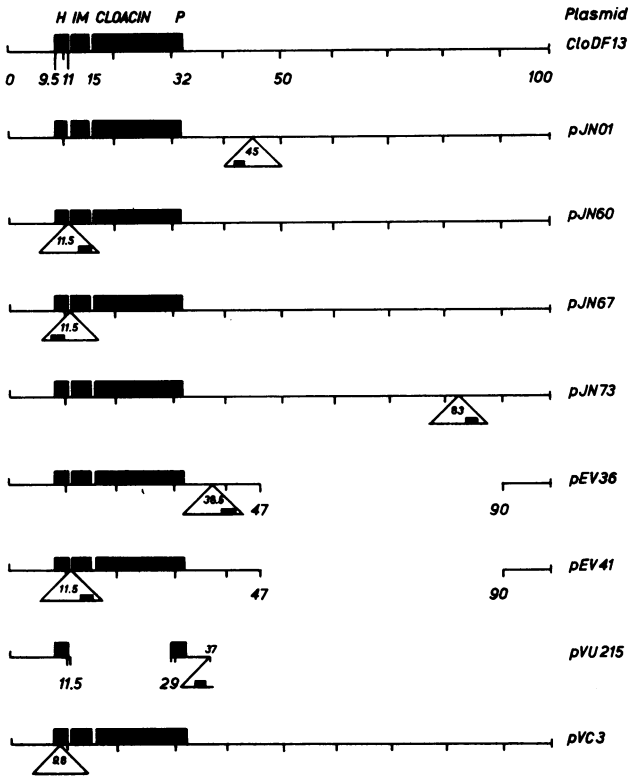

FIG. 1. Schematic presentation of CloDF13 insertion and deletion mutants used in this study. The first line shows the genome of CloDF13 with the genes for cloacin, immunity protein, and protein H. The other lines represent physical maps of different CloDF13 insertion and deletion mutants (see also Table 1). The sites of transposon insertions are indicated with triangles, and the positions are expressed in percentages of the physical map (1, 12, 28). The location of the gene for β-lactamase in Tn*901* is indicated by the boldface bars. The promoter for cloacin, immunity protein, and protein H is indicated by a P.

plasmids have an insertion of transposon Tn*901* at the same position, namely, between the genes for immunity protein and gene H (1, 12, 28). Cells harboring pJN67 do not express protein H (12, 13). In cultures of cells harboring the mutant plasmid pJN73 or pJN01, both of which have an insertion of transposon Tn*901* outside the region of the cloacin operon, production and excretion were not affected significantly (Table 2).

The region of the CloDF13 DNA located between 0 and 12% is known to be essential for the replication of the plasmid (25) and to contain gene H, which was mapped between 9.3 and 11% on the CloDF13 physical map (12). Therefore, the results described above indicate that this protein H might be involved in the process of export of cloacin DF13. However, transposon insertions might have polar effects on other DNA sequences (2, 27). Therefore we studied the excretion in cells harboring the miniplasmids pEV36 and pEV41, both with a deletion between 47 and 90% of the CloDF13 DNA (Fig. 1) and an insertion of Tn*901*. In pEV36 the insertion is located at 38.5% in front of the cloacin operon that starts at 32%, whereas in pEV41 the insertion is located at 11.5%, as in pJN60 (Fig. 1). As shown in Table 2, cells harboring pEV36 excreted 46.5% of the bacteriocin, whereas cells with pEV41 excreted only 1.0%. The production of cloacin DF13 was about the same in both strains. These results indicated that information on the CloDF13 DNA between positions 47 and 90% is not important for the export of cloacin DF13.

Plasmid pVC3 has an insertion of transposon Tn*5* in gene H at 9.8%. Plasmid pVU215 has deletions between 11.5 to 29% and 37 to 100%. This plasmid still encodes for protein H (12, 13), but not for cloacin and immunity protein (Fig. 1). Cells harboring pVC3 excreted only 2.5% of the bacteriocin synthesized (Table 2). To demonstrate that protein H is able to stimulate the excretion of cloacin DF13, cells harboring pVC3 were transformed with pVU215 DNA.

TABLE 2. Excretion of cloacin DF13 by cells harboring different CloDF13 insertion and deletion mutants

Plasmid	Induced cultures[a]		Noninduced cultures[a]	
	Total production[b] (kU/ml)	Excretion[c] (%)	Total production[b] (kU/ml)	Excretion[c] (%)
CloDF13	26.8	51.3	3.6	49.1
CloDF13-*cop3*	31.7	52.7	5.4	54.5
pJN60	22.4	1.9	2.2	5.7
pJN67	20.6	0.7	4.9	4.1
pJN73	17.4	55.0	4.5	37.8
pJN01	15.0	38.1	3.2	51.6
pEV36	21.3	46.5	4.4	48.9
pEV41	15.6	1.0	2.9	3.4
pVC3	24.9	2.5	3.3	4.6
pVC3 + pVU215	22.1	57.5	1.2	57.2

[a] Cells were cultured in LL broth supplemented with lactate and induced with 10 ng of mitomycin C per ml. At 180 min after induction, samples of the cultures were collected, and the total production of cloacin DF13 and the amount excreted into the culture medium were determined. Noninduced cultures were treated in the same manner except for the addition of mitomycin C.

[b] Total production is the sum of cloacin DF13 activity found in the medium supernatant and associated with the cells.

[c] The excretion of cloacin DF13 in the medium supernatant is expressed as a percentage of the total production. The number given is the mean value of three different experiments.

Transformants resistant to both ampicillin and kanamycin were then isolated. These transformants, which harbored two different plasmids, excreted more than 50% of the cloacin DF13 synthesized. This complementation experiment showed that protein H encoded on plasmid pVU215 was able to stimulate the export of cloacin DF13 encoded on pVC3.

The excretion experiments were all carried out in the presence of 10 ng of mitomycin C per ml to induce cloacin DF13 production. However, in noninduced cultures similar results were obtained (Table 2).

Subcellular localization of cloacin DF13 in cells that do not excrete cloacin DF13. Cells harboring pJN67 were cultured and induced with 10 ng of mitomycin C per ml. The amount of cloacin DF13 in the various subcellular fractions was determined (Table 3). The cells had only excreted 0.6% of the bacteriocin. Cellular cloacin DF13 was mainly located in the cytoplasm. Only minor amounts were found in the membrane fraction and in the periplasm.

The distribution of cloacin DF13 over the various subcellular fractions was also studied in cells harboring pJN73, which excreted one-half of the bacteriocin. Of the half still cell associated, most was found in the cytoplasm and periplasm (Table 3). This distribution of cloacin DF13 in cells containing pJN73 is in good agreement with results described by Van Tiel-Menkveld et al. (29).

As controls, the distribution of β-galactosidase, a cytoplasmic marker enzyme, and β-lactamase, a periplasmic enzyme, was also determined (Table 3). The results showed that the cells did not become leaky during the induction period and that spheroplasts did not leak cytoplasmic proteins. The cytoplasmic location of the bacteriocin in cells harboring pJN67 together with the results presented above showed that protein H is involved in the specific transport of cloacin DF13 molecules across the cytoplasmic membrane.

Effect of cyclic AMP on production and excretion of cloacin DF13. Van Tiel-Menkveld et al. (29) showed that the production and excretion of cloacin are lowered in cultures grown on glucose as carbon source or with other fermentable sugars that cause catabolite repression. We studied the effect of cyclic AMP (21) on production and excretion of cloacin DF13 by cells cultured with glucose as carbon source (Table 4). Although cyclic AMP caused a limited stimulation of total production of cloacin DF13 in cells harboring pJN01 and about a twofold increase in production of cells harboring pJN73, the excretion of cloacin DF13 by these cells was not stimulated by the addition of cyclic AMP.

DISCUSSION

Van Tiel-Menkveld et al. (30) showed that expression of CloDF13 DNA adjacent to the gene for immunity protein is important for the lysis of cells when induced with relatively high concentrations of mitomycin C. It was assumed that the DNA region from 9.5 to 11.5% encoded for a toxic product and that the expression of its gene (H) required the cloacin promoter located

TABLE 3. Localization of cloacin DF13, β-galactosidase, and β-lactamase in the various subcellular fractions[a]

Plasmid	Total activity[b]	Activity in following fraction (%)[c]:				
		Spheroplasts	Cytoplasm	Membranes	Periplasm	Medium supernatant
pJN73						
Cloacin DF13	34.5	31.8	30.2	1.6	18.3	49.9
β-Galactosidase	20.9	96.0	nd	nd	2.4	1.6
β-Lactamase	29.4	2.0	nd	nd	91.9	5.1
pJN67						
Cloacin DF13	37.6	94.0	89.3	4.7	5.4	0.6
β-Galactosidase	14.6	97.5	nd	nd	1.4	1.1
β-Lactamase	31.7	1.5	nd	nd	92.8	5.7

[a] The cells were grown in LL broth supplemented with lactate. The activities of cloacin DF13, β-galactosidase, and β-lactamase were assayed 180 min after the addition of mitomycin C (10 ng/ml). Preparation of the various subcellular fractions and determination of activities were as described in the text.

[b] Total activity is the sum of the activities determined in the various fractions. The total activity of cloacin DF13 is presented in kilounits per milliliter; the activities of β-galactosidase and β-lactamase are given in arbitrary units per milliliter.

[c] The activities in the various fractions are expressed as a percentage of the total activity determined; nd, not determined.

at 32%. Under noninduced conditions, the expression of gene H was assumed to be repressed. Recent studies of Hakkaart et al. (12, 13) confirm and extend these hypotheses. Treatment of cells that are able to express gene H with relatively high concentrations of mitomycin C leads to the induced synthesis of protein H and subsequent lysis of the cells within 180 min. Protein H is a relatively small polypeptide (6,000 dalton) and is located predominantly in the cell envelope of glucose-grown cloacinogenic cells (12). The mechanism by which this protein induces lysis of the cells remains unclear.

The results presented in this paper concern the function of protein H in noninduced cells and in cells induced with a relatively low concentration of mitomycin C. The CloDF13 region between positions 0 and 11.5% is essential for the export of cloacin DF13 molecules. Mutants that are blocked in the expression of gene H and mitomycin C-induced lysis are hampered in the export of cloacin DF13 molecules. In these mutants, most of the bacteriocin molecules remain located in the cytoplasm. The following mechanism for the release of cloacin DF13 molecules and for the function of protein H in this process can be proposed. Induction of cultures with low concentrations of mitomycin C (lower than 10 to 25 ng/ml) leads to the synthesis of relatively low amounts of cloacin, immunity protein, and protein H. Equimolar complexes of cloacin and immunity protein are formed and released into the cytoplasm, whereas the synthesized protein H molecules associate with the cytoplasmic membrane and possibly also with the outer membrane. The bacteriocin molecules are then translocated across the cytoplasmic membrane in cooperation with membrane-bound protein H molecules and released into the periplasm, without a concomitant release of cytoplasmic proteins. Finally, the bacteriocin molecules are translocated across the outer membrane and excreted into the medium. This excretion process is specific since periplasmic proteins are not excreted. In cultures induced

TABLE 4. Effect of cyclic AMP on production and excretion of cloacin DF13[a]

Plasmid	Carbon source	Cyclic AMP (mM)	Activity of cloacin DF13[b]	
			Total production (kU/ml)	Excretion (%)
pJN01	Lactate	0	15.0	35.1
	Glucose	0	14.5	1.0
	Glucose	5	18.2	0.7
pJN73	Lactate	0	17.4	55.0
	Glucose	0	15.6	2.0
	Glucose	5	30.1	2.1

[a] Cells were grown in LL broth with lactate or glucose. The activity of cloacin DF13 was determined 180 min after the addition of mitomycin C. Cells grown with lactate were induced with 10 ng of mitomycin C per ml. Cells grown with glucose were induced with 100 ng of mitomycin C per ml.

[b] Activity of cloacin DF13 was determined as described in the text. Total production was determined as the sum of the activities in the medium and in the cell pellet. Excretion is expressed as a percentage of the total production.

with higher concentrations of mitomycin C (100 to 500 ng/ml), relatively high amounts of cloacin, immunity protein, and protein H are synthesized per cell (12, 13, 29, 30). A high concentration of protein H might induce structural alterations in the cell envelope and subsequent lysis of cells. As a result of this lysis, a maximal amount of cloacin DF13 molecules are released into the environment together with other cellular proteins. In noninduced cultures, the same mechanism of export as in cells induced with relatively low concentrations of mitomycin C might occur, but only a few cells are spontaneously induced to produce the bacteriocin.

Recent reports on the synthesis and export of other bacteriocins (colicins E1, E2, E3, and A) are in agreement with the proposed model for the mechanism of excretion (15, 17, 22, 31). For all of these bacteriocins, indications were obtained that the molecules were synthesized without an N-terminal extension or signal peptide and that the mechanism of excretion was different from the mechanism of export of periplasmic and outer membrane proteins. The excretion of these bacteriocin molecules (except colicin A) into the culture medium, however, appeared to be the result of a rather nonspecific release because of an increased permeability of the cells (15, 17, 22). However, these results were obtained with cells induced with relatively high concentrations of mitomycin C. It is not yet known whether cells producing colicin E1, E2, E3, or A also synthesize a polypeptide similar to protein H. However, as discussed by Hakkaart et al. (12), comparison of the nucleotide sequence of the replication region of the plasmids CloDF13 and ColE1, in particular the region containing gene H, showed that the corresponding region of ColE1 contains an open reading frame potentially coding for a protein of about 6,000 daltons. The amino acid sequence of this protein would be almost identical to that of protein H encoded by CloDF13 (12).

The inhibiting effect of glucose on the excretion of cloacin DF13 molecules remains intriguing. Cyclic AMP does not reverse this effect. It remains to be investigated whether glucose inhibits somehow the expression of gene H in the absence or presence of cyclic AMP; furthermore, the functioning of protein H may be hampered by glucose or glucose-induced changes in the cell envelope.

The molecular mechanism by which protein H is able to transport cloacin DF13 molecules across the cytoplasmic membrane and possibly also across the outer membrane is unknown. Hakkaart et al. (12) showed that this protein is located in the cell envelope of glucose-grown cloacinogenic cells. The C-terminal part of protein H contains a large hydrophobic stretch of 17 amino acids (12, 24). Another observation which can explain the localization of protein H is that this protein might have an N-terminal signal peptide (4, 14, 23) of 21 amino acids (12). Membrane-associated protein H molecules might form specific patches through which cloacin DF13 molecules can traverse the membranes. Molecules of cloacin DF13 (complexes of cloacin and immunity protein) that have been excreted into the culture medium were not found to be associated with molecules of protein H (unpublished data). Further studies of the subcellular localization of protein H, the possible N-terminal signal peptide, and the interaction of protein H molecules with the cytoplasmic and outer membranes might give more information on the mechanism of export of cloacin DF13 and the protein H-induced lysis of cells.

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