Purification and Characterization of Cloacin DF13 Receptor from *Enterobacter cloacae* and Its Interaction with Cloacin DF13 In Vitro

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Extraction of the crude cell envelope fraction of cloacin DF13-susceptible *Enterobacter cloacae* strain 02 with Triton X-100 and ethylenediaminetetraacetate solubilized an outer membrane fraction which neutralized the lethal activity of cloacin DF13. A similar fraction could not be isolated from strains known to be lacking functional cloacin DF13 receptors. On this basis the isolated outer membrane fraction was assumed to contain the specific cloacin DF13 receptor. The receptor was purified to homogeneity by acetone precipitation and affinity chromatography, using cloacin DF13 as a ligand. The purified receptor was identified as a protein which consisted of a single polypeptide chain with an apparent molecular weight of 90,000 and a preponderance of acidic amino acids (pI = 5.0). The interaction of equimolar amounts of purified receptor and cloacin DF13 in vitro resulted in a complete, irreversible neutralization of the lethal activity of the bacteriocin. This interaction showed a temperature optimum at 43°C but was only slightly affected by variation of the pH beween 5.0 and 8.5 or by increasing the ionic strength of the incubation buffer. The receptor had no neutralizing activity towards other bacteriocins, such as colicin E1 or colicin E3.

Cloacin DF13 is a bacteriocin produced by bacteriocinogenic strains of *Enterobacter cloacae*(CloDF13) (16, 47). It is characterized by its ability to kill cells of susceptible strains of *Enterobacter* and *Klebsiella* species (16). The bacteriocin is excreted as an equimolar complex of two CloDF13-determined gene products, designated cloacin and immunity protein (13). The cloacin inactivates bacterial ribosomes by endoribonucleolytic cleavage of 16S rRNA and induces a leakage of potassium ions from the susceptible cells (12, 14, 15). The immunity protein serves as an inhibitor of the endoribonucleolytic activity of the cloacin through a direct and specific stoichiometric interaction with the cloacin molecules (34).

The initial event in the killing of susceptible bacteria by cloacin DF13 involves recognition of and binding to specific receptors localized on the surface of the cells (16, 35). Recently, it has been reported that whole cells of cloacin DF13-susceptible *E. cloacae* strain 02 grown in brain heart infusion each adsorb about 425 molecules of cloacin DF13 (35). This adsorption has a temperature optimum between 35 and 40°C, but it is not affected by variation in pH between 5.0 and 8.0 or by extracellular potassium chloride

up to a concentration of 0.5 M. However, little is known about the nature of the interaction of this bacteriocin and its receptor or about the sequence of events that, after adsorption, leads to the biochemical effects observed.

All receptors for bacteriocins affecting gram-negative bateria studied so far have been localized in the outer membrane fraction of the cell envelope (6, 7, 30, 39, 45, 50). Furthermore, it has been shown that some of these receptors also serve as the attachment sites for certain bacteriophages and that they facilitate the passage of low-molecular-weight substrates across the outer membrane (5–7, 18, 23, 24, 32). Sabet and Schnaitman (40) purified the receptor for the colicins of type E, using Triton X-100 and EDTA. It was shown to be a protein with a molecular weight of 60,000, containing little covalently bound carbohydrate. The colicin M receptor has been purified by Braun and co-workers (7, 8). This receptor is a protein which consists of a single polypeptide chain with a molecular weight of 78,000. Manning and Reeves (33) purified the receptor for colicin K and showed it to be a protein with a molecular weight of 26,000. The receptors for colicins Ia and Ib (30–32) and for colicins B and D (23, 36) have also been

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identified as outer membrane proteins with molecular weights of 74,000 (Ia and Ib) and 81,000 (B and D).

To elucidate the nature of the cloacin DF13 receptor and its interaction with the bacteriocin, efforts were made to purify the receptor.

This paper describes the localization of the cloacin DF13 receptor in the outer membranes of susceptible cells, its solubilization from crude envelope fractions with the use of Triton X-100 and EDTA, and its purification by affinity chromatography. Furthermore, the characterization of the purified receptor as well as its interaction with cloacin DF13 in vitro is described.

MATERIALS AND METHODS

Bacterial strains and isolations. The complex of cloacin and its immunity protein, designated as cloacin DF13, was isolated and purified from the cloacinogenic strain *E. cloacae*(CloDF13) after induction with mitomycin C as previously described (13).

E. cloacae 02 was used for the isolation of the outer membrane receptors for cloacin DF13. Resistant mutants of this strain, lacking the outer membrane receptors, were used as control strains (35).

Klebsiella edwardsii subsp. *edwardsii* was used for determinations of the specific killing activities of the cloacin and its immunity protein, designated as cloacin DF13, was isolated and purified from the cloacinogenic strain *E. cloacae*(CloDF13) after induction with mitomycin C as previously described (13).

Media and buffer solutions. For the isolation of cell envelopes from *E. cloacae* 02, cells were grown at 37°C in a medium containing 4.5 g of KH_2PO_4, 7.5 g of K_2HPO_4, 2.0 g of NH_4Cl, 0.05 g of $MgSO_4 \cdot 7H_2O$, 5 mg of $FeSO_4$, 0.1% yeast extract (Oxoid Ltd., London, England), 0.5% Casamino Acids (Difco Laboratories, Detroit, Mich.), and 0.5% glucose per liter of water under constant aeration and stirring in a New Brunswick fermentor. For killing or receptor activity assays, cells were grown in broth (Difco).

T buffer contained 0.05 M Tris-hydrochloride (pH 7.8). TE buffer was T buffer supplemented with 5 mM EDTA. TTE buffer was TE buffer plus 0.1% or 1% Triton X-100.

Chemicals. DNase I, RNase, lipase or triacylglycerol acyl-hydrolase phospholipases C and D, trypsin inhibitor, lysozyme (chicken egg), α- and β-glucosidase, α- and β-galactosidase, α- and β-mannosidase, and the molecular weight markers RNA nucleotidyl-transferase, phosphorylase *b*, bovine serum albumin, chicken egg albumin, and chymotrypsinogen A were purchased from C. F. Boehringer & Soehne GmbH, Mannheim, West Germany. Trypsin (bovine pancreas) was purchased from Sigma Chemical Co., St. Louis, Mo. Endoglycosidase H was from Seikagaku Fine Biochemicals, Tokyo, Japan. Cyanogen bromide-activated Sepharose 4B was obtained from Pharmacia Fine Chemicals AB, Uppsala, Sweden. Bio-Beads SM-2 were purchased from Bio-Rad Laboratories, Richmond, Calif. All other chemicals used were obtained from E. Merck A. G., Darmstadt, West Germany.

Cell envelope preparations. Cells of *E. cloacae*

02 were grown to a culture density of 5×10^9 cells per ml, collected by centrifugation at $23,000 \times g$ at 4°C, washed with 0.9% NaCl, and suspended in TE buffer (100 g [wet weight]/250 ml of buffer). The cells were broken by two passages through a French pressure cell at 20,000 lb/in^2 in the presence of DNase (20 µg/ml) and RNase (15 µg/ml). The broken-cell suspension was centrifuged for 10 min at $23,000 \times g$ to remove intact cells and supplemented with $MgSO_4$ to a final concentration of 6 mM. The crude cell envelopes were then collected by centrifugation for 3 h at $140,000 \times g$ (4°C).

Assay of killing activity of cloacin DF13. The killing activity of cloacin DF13 was determined with a susceptible strain of *K. edwardsii* subsp. *edwardsii* as described previously (16). The specific activity was expressed as units per milligram of protein; 1 U represented the amount of bacteriocin required to kill 50% of 5×10^8 cells under the assay conditions.

Assay of receptor activity. Tests for receptor activity were performed by incubating cloacin DF13 (10 ng/ml) with or without (control) the receptor material to be tested in broth (Difco) buffered with 10 mM phosphate buffer (pH 7.0) for 60 min at 37°C, unless otherwise indicated. Serial twofold dilutions of the mixtures in broth were then supplemented with susceptible cells of *K. edwardsii* subsp. *edwardsii* to a final concentration of 10^8 cells per ml. Total volume of the assay suspension was 5 ml. After incubation for 2 h at 37°C, the turbidity at 660 nm was measured and plotted as a function of the concentration of cloacin DF13 in each dilution. The decrease in the specific killing activity of the bacteriocin in the sample with regard to the control (containing no receptor material) was taken as a measure for the receptor activity. The specific receptor activity was expressed as units of bacteriocin neutralized per milligram of receptor material. One unit represented the amount of receptor material required to neutralize the lethal action of 1 U of cloacin DF13.

Polyacrylamide gel electrophoresis. Analytical discontinuous gel electrophoresis was performed according to Davis (11) on 7% acrylamide gels. The pH of the separation gel was 8.9, and the pH of the electrophoresis buffer was 8.3.

Sodium dodecyl sulfate electrophoresis was performed on 10% polyacrylamide gels containing 0.1% sodium dodecyl sulfate, essentially as described by Weber and Osborn (49). Samples were preincubated for 5 min at 100°C in the presence of 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. Electrophoresis was performed for 6 h at 7 mA per gel.

The gels were fixed and stained for protein in a solution of 0.4% amido black in 7% acetic acid for 1 h at room temperature. Destaining was performed by diffusion in 7% acetic acid.

The presence of carbohydrates in gels without sodium dodecyl sulfate was determined according to Zacharias et al. (51). In sodium dodecyl sulfate gels the method of Glossmann and Neville (21) was used.

Amino acid analysis. Amino acid composition was determined on a Rank Hilger Chromaspek amino acid analyzer by J. M. Walker, Chester Beatty Research Institute, London, England. Samples were hydrolyzed in 0.4 ml of 6 M HCl for 24 h at 110°C in

evacuated, sealed glass ampoules. Tryptophan was determined spectrophotometrically by the method of Goodwin and Morton (22).

Protein assay. Two different methods were used to determine protein concentration. For protein solutions in non-Triton X-100-containing buffers, the method of Hartree (25) was used. For samples containing Triton X-100, the method of ChandraRajan and Klein (10) was used. Bovine serum albumin was used as a standard in both cases.

RESULTS

Demonstration and localization of the cloacin DF13 receptor in the cell envelope fraction of *E. cloacae* 02. Cells of the cloacin DF13-susceptible strain *E. cloacae* 02 were grown to the late log phase, and a crude envelope fraction was prepared as described in Materials and Methods. Similar cell envelope fractions were prepared from two resistant mutants of this strain known to be lacking functional cloacin DF13 receptors. The presence of a cloacin-neutralizing substance in these envelope fractions was determined (Fig. 1). From the shifting of the activity curves in Fig. 1 it could be concluded that the crude cell envelopes of the susceptible strain were able to neutralize considerable amounts of cloacin DF13, whereas those from the two resistant mutants were not. This result indicated that the bacteriocin-neutralizing ability was presumably due to the presence of spe-
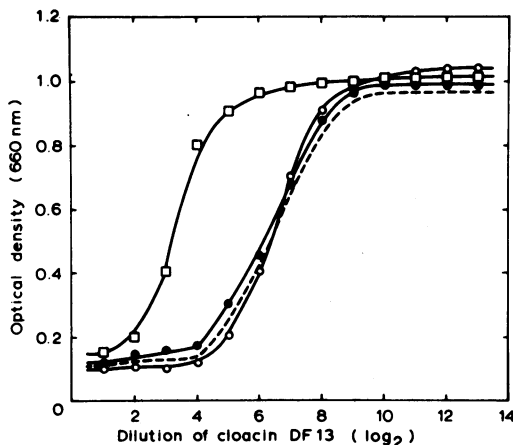
cific cloacin DF13 receptors. Another indication for the functioning of specific receptors as the cloacin DF13-neutralizing substance in the crude envelope fraction came from the observation that about 90% of the bacteriocin (about 10^{12} molecules) was neutralized by 10 μl of envelope fraction, which was obtained from about 4.0 × 10^9 cells of *E. cloacae* 02. Thus, about 250 molecules of cloacin DF13 were neutralized by the envelope fraction of 1 cell, which is known to possess about the same number of specific receptor sites for this bacteriocin in vivo (16, 35).

Like other bacteriocin receptors, receptors for cloacin DF13 are thought to be exposed on the outsides of susceptible cells and to be integral components of the outer membranes. To obtain experimental evidence for this localization, the cell envelope fraction of susceptible cells was subjected to sucrose density gradient centrifugation to separate cytoplasmic and outer membrane fragments (Fig. 2). After this separation, the various fractions were assayed for cloacin DF13-neutralizing activity. The cytoplasmic membrane fragments banded at buoyant densities of 1.18 and 1.20 g/ml, whereas the outer membrane peak appeared at 1.24 g/ml (41). Cloacin DF13-neutralizing activity could only be demonstrated in that part of the outer membrane peak that banded at a buoyant density of more than 1.24 g/ml. This result showed that the cloacin DF13 receptor was an outer membrane component of the susceptible cells.
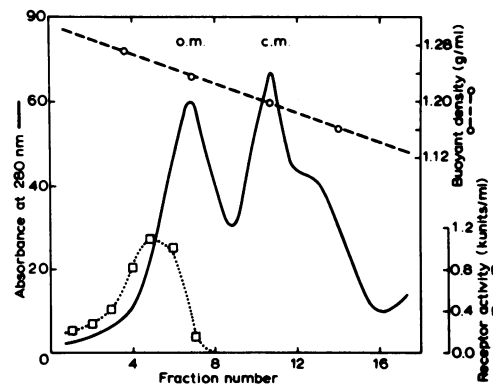

FIG. 1. *Cloacin DF13-neutralizing activity of crude cell envelope fractions. Portions (10 μl) of the crude envelope fractions of susceptible E. cloacae strain 02 and of two resistant mutants of this strain were incubated with 100 ng of cloacin DF13 in 10 ml of broth buffered at pH 7.0 with 10 mM phosphate buffer. After 60 min of incubation at 37°C, the mixtures were diluted and tested for cloacin DF13 activity as described in the text. Symbols: ——, control, no envelope material present; □, crude envelope fraction of E. cloacae 02 (susceptible to cloacin DF13); ○ and ●, crude envelope fractions of resistant mutants.*

FIG. 2. *Sucrose density centrifugation of the crude cell envelope fraction of E. cloacae 02. The crude envelope fraction was prepared as described in the text, and 0.5 g was applied on a 25 to 65% (wt/vol) discontinuous sucrose gradient in TTE buffer containing 1% Triton X-100. After centrifugation for 18 h at 4°C and 60,000 × g in a swing-out rotor (MSE 75; 3 by 25 ml), fractions of 1 ml were collected. For the determination of the receptor activity in the various fractions, each fraction was dialyzed overnight against TTE buffer (1% Triton X-100) and used in an activity assay. Abbreviations: c.m., cytoplasmic membrane; o.m., outer membrane.*

To obtain information about a possible association of the cloacin DF13 receptor with the peptidoglycan–lipoprotein layer, cell envelopes were extracted with 2% sodium dodecyl sulfate at 60°C as described by Rosenbusch (38). The isolated complex of peptidoglycan and outer membrane proteins was examined for cloacin DF13 receptor activity and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The result showed that the cloacin DF13 receptor was not a peptidoglycan-associated protein.

Solubilization and purification by affinity chromatography of the cloacin DF13 receptor. Cells of cloacin DF13-susceptible *E. cloacae* strain 02 were grown in 75 liters of medium to the late log phase (about 4.0×10^9 cells), and a crude envelope fraction was prepared from these cells. The crude envelope fraction obtained (250 g of protein) was extracted with Triton X-100 in the presence of EDTA by a modification of the method described by Sabet and Schnaitman (39, 40). First, the envelope fraction was extracted twice by two incubations with 1 liter of 1% Triton X-100 in TE buffer (pH 7.8) for 20 h at 4°C, which solubilizes principally cytoplasmic membrane components (42). After each incubation period, the extraction mixture was centrifuged for 3 h at 140,000 × *g* (4°C), and the Triton-soluble and Triton-insoluble fractions were assayed for receptor activity. During this stage of the extraction procedure, no receptor activity could be demonstrated in the Triton-soluble fractions. Then, the Triton-insoluble pellet fraction was extracted with the same buffer but at 30°C for 20 h followed by centrifugation at 15°C. After this procedure, the supernatant and pellet fractions were again assayed for receptor activity. The extraction at 30°C was repeated several times (usually three to six times) until most of the receptor activity was solubilized. The supernatant fractions containing receptor activity were precipitated by the addition of 2 volumes of cold acetone (−20°C). The precipitate was then collected by centrifugation, suspended in TE buffer (pH 7.8), dialyzed extensively against distilled water, and finally lyophilized. The lyophilized material (8.4 g of protein) contained about 80% of the receptor activity present in the crude envelope fraction.

The same extraction and precipitation procedure was performed with the cell envelope fraction of a cloacin DF13-resistant mutant, and the protein composition of both lyophilized acetone precipitates were examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (see Fig. 4a and b). In addition to the major outer membrane proteins, several polypeptides could

be seen on the gels. The most remarkable difference between both preparations was the absence of a polypeptide in the acetone precipitate prepared from the envelope extracts of the resistant mutant. This missing polypeptide might be an essential component of the specific cloacin DF13 receptor.

To purify the cloacin DF13 receptor, an affinity chromatography procedure was developed in which immobilized cloacin DF13 was used as a ligand for the receptor. Cloacin DF13 was coupled to cyanogen bromide-activated Sepharose 4B by the basic coupling procedure (1). The cloacin DF13–Sepharose conjugate was then used to prepare a column, and the crude acetone precipitate was subjected to affinity chromatography on this column. The receptor material was eluted as a single peak (Fig. 3). The peak fractions were pooled and dialyzed against 0.05 M T buffer (pH 7.0), and the protein content as well as the specific receptor activity were determined.

Finally, Triton X-100 was removed from the receptor solution, using Bio-Beads SM-2 according to the method of Holloway (28). Removal of Triton X-100 did not affect the cloacin DF13-neutralizing activity of the receptor. The receptor solution was then dialyzed extensively against distilled water at 4°C, lyophilized, and stored at −20°C without significant loss in neutralizing activity.
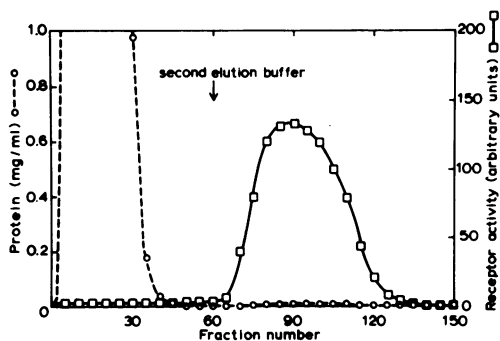

FIG. 3. *Affinity chromatography of crude receptor material. Lyophilized receptor material (acetone precipitate) was dissolved in 100 ml of TTE buffer (1% Triton) and applied to a column (0.9 by 12 cm) of cloacin DF13–Sepharose conjugate previously equilibrated with TTE buffer (1% Triton; pH 7.8). A 10-mg amount of cloacin DF13 was used per 1.0 g of Sepharose 4B. The column was then washed with the first elution buffer (TTE buffer [pH 7.8], 0.1% Triton X-100) at 20°C until the effluent was free from protein. Thereafter, the column was eluted with the second elution buffer (0.05 M T buffer [pH 4.0] plus 0.1% Triton X-100, 2 M NaCl, and 6 mM EDTA) at 4°C. Receptor activities and protein contents of the fractions (±3.0 ml) were determined as described in the text.*

The results of the total purification procedure are summarized in Table 1. The specific receptor activity of the purified receptor was about 11,000-fold higher than that determined in the original crude envelope fraction. Forty percent of the total activity was recovered after the final purification step. Ten milligrams of receptor protein was obtained from about 800 g (wet weight) of cells. Assuming a molecular weight of 90,000 for the receptor protein and about 400 receptors per cell, it can be calculated that the total crude envelope fraction isolated contained about 25 mg of receptor protein. Thus, the result of the purification procedure was in good agreement with the theoretical purifiable quantity, taking the yield into account.

Characterization of the purified receptor for cloacin DF13. Information about the nature of the purified receptor was obtained by measuring the effects of various substances and incubation conditions on the bacteriocin-neutralizing activity. The various enzymes, agents, and incubation conditions used and their influences on the receptor activity are summarized in Table 2. Trypsin inactivated the receptor activity, indicating that the receptor required intact protein for its activity.

Analysis of the lyophilized material showed that the receptor contained 80 to 90% protein as assayed by the method of Hartree (25). The carbohydrate content was less than 5% as assayed by the phenol method of Dubois et al. (19) and the orsinol method of Herbert and Phipps (26). Triton X-100 could not be detected (20). The absorption spectrum was characteristic for protein, with a maximum around 280 nm. The absorption coefficient at 280 nm for a 1% solution in 0.1 M NaOH was determined as 14.3 cm^{-1}.

The homogeneity of the purified bacteriocin was tested by polyacrylamide gel electrophoresis. Discontinuous gel electrophoresis at pH 8.3 showed that the receptor ran as a single protein

TABLE 1. *Purification of the cloacin DF13 receptor from E. cloacae 02*

Fraction	Total protein (g)	Receptor activity		
		Total ($\times 10^6$ U)	Specific ($\times 10^3$ U/ mg of protein)	Yield (%)
Crude envelope	250	17.3	0.07	100
Triton X-100 extracts	62	15.6	0.25	90
Acetone precipitate	8.4	13.8	1.64	80
Affinity chromatography	0.01	6.9	690	40

TABLE 2. *Various treatments of the cloacin DF13 receptor and their effects on receptor activity*

Treatment[a]	Special conditions	Effect
DNase (20 µg/ml)	6 mM MgCl$_2$	None
RNase (20 µg/ml)	6 mM MgCl$_2$	None
Triacylglycerol lipase (100 µg/ ml)	6 mM CaCl$_2$	None
Phospholipase C (100 µg/ml)	6 mM CaCl$_2$	None
Phospholipase D (100 µg/ml)	6 mM CaCl$_2$	None
Trypsin (20 µg/ml)[b]	pH 8.0	Complete inactivation
Lysozyme (50 µg/ml)[c]	10 mM EDTA	None
Various exoglycosidases (20 µg/ml)[d]	pH 5.0	None
Endoglycosidase H (5 units)[e]	pH 5.0	None
Deoxycholate (1%)[f]		None
Triton X-100 (2%)[f]		None
Urea (6 M)[f]		None
Heat (60 min at 70°C)		None

[a] Portions of 1.0 µg of purified receptor protein in 0.5 ml of 50 mM T buffer (pH 7.0) were incubated with various substances for 60 min at 37°C or under special conditions as indicated. Then, the treatments were stopped and the receptor activities were assayed as described in the text. Control experiments without receptor material were performed.
[b] The action of trypsin was stopped by the addition of trypsin inhibitor (75 µg/ml) and another incubation of 15 min at 37°C before the receptor test was performed.
[c] The action of lysozyme was stopped by the addition of MgSO$_4$ (final concentration, 20 mM).
[d] The various exoglycosidases used were α- and β-glucosidase, α- and β-galactosidase, and α- and β-mannosidase. In addition, a mixture of these enzymes was used. The incubations were performed in a 0.1 M sodium acetate buffer (pH 5.0).
[e] Endoglycosidase H (endo-β-N-acetylglucosaminidase) was used as described by Koide and Muramatsu (29).
[f] This treatment was followed by dialysis against 0.05 M T buffer (pH 7.0) for 16 h at 4°C.

zone migrating towards the anode (Fig. 4d). Carbohydrate could not be detected on a Schiff-stained duplicate gel. To demonstrate the presence of receptor activity in this single protein zone, an unstained duplicate gel was cut into slices of 2 mm. The slices were homogenized and incubated for 16 h at 37°C in 1 ml of T buffer (pH 7.8). These incubation mixtures were then tested for cloacin DF13-neutralizing activity. The result showed that the protein zone possessed full receptor activity. No activity could be detected in other parts of the gel.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis showed a single protein zone (Fig. 4c). Again, no free or protein-bound carbohydrate could be detected after Schiff staining of duplicate gels. Omission of 2-mercaptoethanol or treatment of the sample at 37 instead of 100°C did not affect the mobility of the purified receptor on sodium dodecyl sulfate–polyacrylamide gels. The molecular weight of the polypeptide, estimated by comparison with a set of standard calibration proteins, was about 90,000. The data
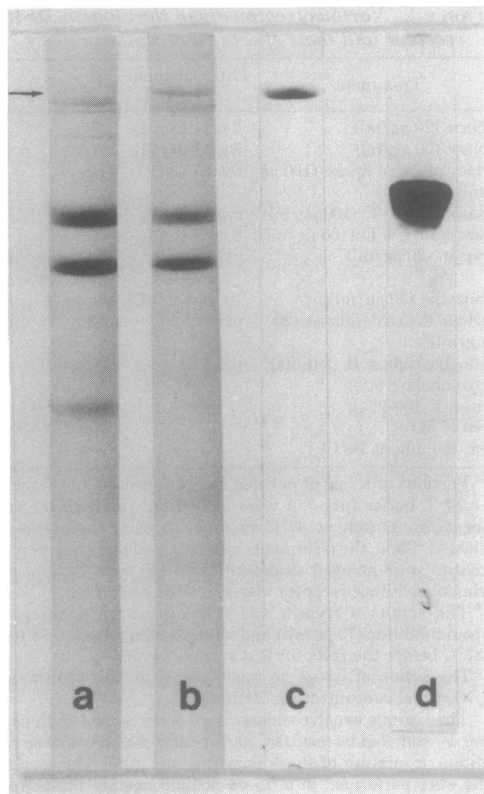
FIG. 4. *Polyacrylamide gel electrophoresis of crude receptor material and purified cloacin DF13 receptor. Gels a through c were performed on sodium dodecyl sulfate–polyacrylamide (see text). Gel a represents an analysis of the acetone precipitate of Triton extracts prepared from a cloacin DF13-resistant mutant of E. cloacae 02; gel b represents an analysis of the acetone precipitate of Triton extracts prepared from cloacin DF13-susceptible E. cloacae strain 02; gel c represents an analysis of the receptor purified by affinity chromatography. The arrow indicates the position of the cloacin DF13 receptor protein in gels a through c. Gel d represents an analysis of the purified receptor on polyacrylamide without sodium dodecyl sulfate (see text).*

TABLE 3. *Amino acid composition of the cloacin DF13 receptor protein*

Amino acid	Amt	
	mol of amino acid/mol of protein	No. of residues/receptor molecule[a]
Aspartate + asparagine	43.0	109
Threonine	21.1	53
Serine	25.6	65
Glutamate + glutamine	30.2	76
Proline	10.5	27
Glycine	44.8	113
Alanine	25.9	66
Cysteine	—[b]	—
Valine	19.6	50
Methionine	4.3	11
Isoleucine	12.3	31
Leucine	19.8	50
Tyrosine	14.9	38
Phenylalanine	11.5	29
Histidine	4.6	12
Lysine	18.4	47
Arginine	9.1	23
Tryptophan	8.1	20

[a] Probable number, calculated on the basis of a molecular weight of 90,000.

[b] —, Not detectable.

indicated that the receptor was a protein consisting of a single polypeptide with an apparent molecular weight of about 90,000 that did not contain or need any carbohydrate for its activity.

The amino acid composition of the purified receptor protein is presented in Table 3. The molecule appeared to have a relatively high content of acidic amino acids. The isoelectric point of the receptor protein was found to be 5.0 as determined by isoelectric focusing on polyacrylamide gels according to Vesterberg (48).

Characterization of the interaction of cloacin DF13 and its receptor protein in

vitro. The specificity of the purified receptor was investigated by testing its neutralizing activity on other bacteriocins. The receptor, however, did not neutralize the lethal activity of colicin E3, a bacteriocin comparable to cloacin DF13 with respect to its ultimate biochemical effects (2–4), or that of colicin E1. Most probably, the receptor had the same specificity for cloacin DF13 in vitro as in the intact cells (35).

The nature of the interaction of cloacin DF13 and its receptor in vitro was investigated by incubating equimolar amounts of cloacin DF13 and receptor protein at various temperatures, pH values, and increasing concentrations of potassium chloride in 0.05 M T buffer. After 30 min of incubation, the residual activity of the bacteriocin in each mixture was determined and used as a measure for the extent of neutralization. The experiments showed that the neutralization of cloacin DF13 by its receptor had a temperature optimum between 40 and 45°C but that it was not affected by variation in pH between 5.0 and 8.5 or relatively high ionic strength (potassium chloride up to a concentration of 0.5 M). These data are similar to the adsorption characteristics of this bacteriocin in vivo (35) and suggest that the recognition and binding of cloacin DF13 to its purified receptor protein does not depend only on electrochemical interaction.

The kinetics and the stoichiometry of the

neutralization of cloacin DF13 activity by its receptor were investigated as described in the legends of Fig. 5 and 6, respectively. The rate of "inactivation" of cloacin DF13 by its receptor protein was dependant on the temperature (Fig. 5A) and on the concentration of receptor protein used (Fig. 5B). When equimolar amounts (15 mM) of both preparations were incubated at 37°C in 0.05 M T buffer (pH 7.0), the half-time of the inactivation reaction was 380 s. A certain amount of receptor protein neutralized under the conditions used the lethal activity of a commensurate amount of cloacin DF13 (Fig. 6). This result indicated that the neutralization of the lethal activity of cloacin DF13 by its purified receptor protein was the result of a stoichiometric and irreversible interaction.

FIG. 6. Stoichiometry of neutralization of bactericidal activity of cloacin DF13 by its purified receptor protein. Various amounts of purified receptor were incubated with a constant amount of cloacin DF13 (60 ng) for 3 h at 37°C in 6 ml of broth buffered at pH 7.0 with 10 mM phosphate buffer. After these incubations, the residual activity of bacteriocin was determined for each concentration of receptor material used. Concentrations of receptor and cloacin DF13 were calculated on the basis of milligrams (dry weight) of material per milliliter and molecular weights of 90,000 and 66,000, respectively (13).

DISCUSSION

This paper describes the isolation and purification to homogeneity of a substance from the outer membrane fraction of cloacin DF13-susceptible cells of E. cloacae that possesses the ability to neutralize the lethal activity of cloacin DF13. Mainly based on the fact that a similar outer membrane component could not be isolated from cells of two resistant mutants of the susceptible strain, we concluded that this substance is the cloacin DF13 receptor or a cloacin DF13-binding component of the receptor. Moreover, the other data presented strongly indicate that this conclusion is permissible: (i) the amount of cloacin DF13-neutralizing activity demonstrated in the crude cell envelope fraction is in good agreement with the amount of receptor activity demonstrated in a commensurate amount of whole cells, and (ii) the specificity and characteristics of the interaction of the purified receptor protein and cloacin DF13 in vitro are comparable to those of the interaction in vivo.

The cloacin DF13 receptor has been identified as a non-heat-modifiable outer membrane protein which is not associated with the peptidoglycan and which consists of a single polypeptide (molecular weight, 90,000). The amino acid composition of the cloacin DF13 receptor protein has been determined and resembles the amino
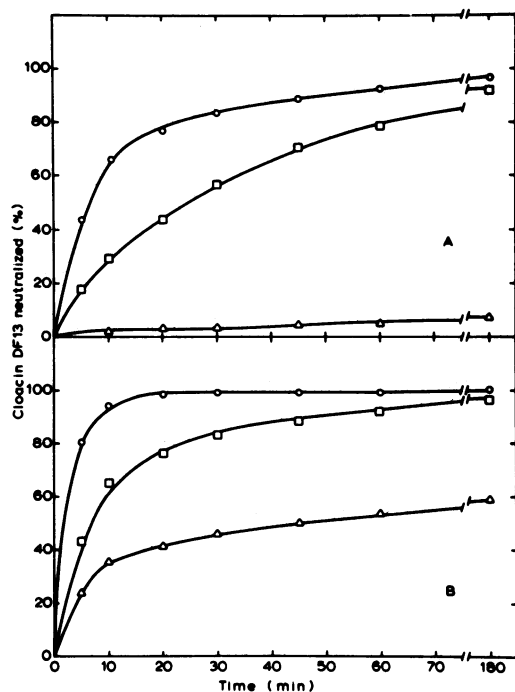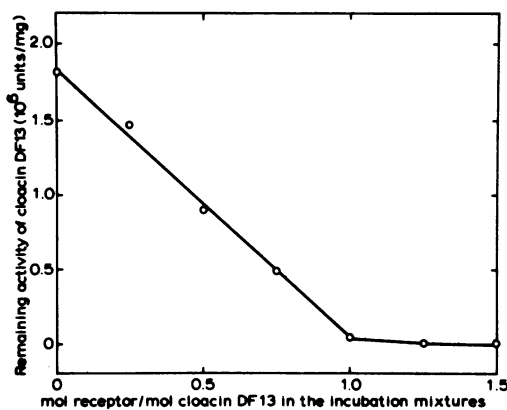

FIG. 5. Kinetics of neutralization of bactericidal activity of cloacin DF13 by its purified receptor protein, as a function of temperature (A) and concentration of receptor protein (B). (A) Equimolar amounts of cloacin DF13 and purified receptor were incubated in 0.05 M T buffer (pH 7.0) for various time intervals at 37°C (O), 20°C (□), and 4°C (△). (B) Cloacin (15 nM) DF13 was incubated with 30 (O), 15 (□), and 7.5 (△) nM receptor in 0.05 M T buffer (pH 7.0) for various time intervals at 37°C. After these incubation periods the lethal activities of cloacin DF13 in the mixtures were determined as described in the text and expressed as percentages of the lethal activities of control incubation mixtures without receptor.

acid composition of the colicin K receptor protein isolated by Manning and Reeves (33), although minor differences can be observed.

Some investigators (8, 30, 40, 50) obtained indications for the involvement of carbohydrate in the activities of the receptors for colicins E2/E3, K, and A. However, these indications were mainly based on the inactivation of receptor activity upon periodate treatment, for which independent verification is required, since oxidation by periodate can also lead to protein modification (46). Our experiments also showed the presence of very small amounts of carbohydrate in the purified receptor material. Attempts to demonstrate the direct and essential involvement of carbohydrates in the functioning of the cloacin DF13 receptor were not successful, since (i) various endo- and exoglycosidases did not destroy its activity and (ii) no protein-bound carbohydrate could be detected on polyacrylamide gels, whereas full receptor activity could be demonstrated. Based on these data, we concluded that the cloacin DF13 receptor neither contains nor requires any carbohydrate for its activity observed in vitro.

The recognition of and initial interaction of cloacin DF13 molecules with the specific receptor sites in vivo has been characterized as a temperature-sensitive and probably reversible process (35). The initial interaction proceeds within 2 min and is most probably followed by a relatively slow and irreversible adsorption of the bacteriocin molecules (16, 35). Comparable phenomena have been observed with colicin K (9) and colicin E2 (37, 44). The recognition and adsorption process in vivo has a temperature optimum between 35 and 40°C, but it is not affected by variation in pH or ionic strength (35). Subsequent to the adsorption process, a sequence of unknown events results in the penetration of a few cloacin DF13 molecules or of a few cloacin fragments possessing endoribonucleolytic activity and finally in cell death (16, 17, 35). The data presented in this paper show that the interaction of cloacin DF13 molecules and receptor protein in vitro results in a neutralization (inactivation) of the bactericidal activity of the bacteriocin molecules. This neutralizing interaction has been characterized as a relatively slow, temperature-sensitive, stoichiometric, and irreversible process, suggesting that the interaction determined in vitro is comparable to the irreversible adsorption process observed in vivo. Its temperature optimum lies between 40 and 45°C, which is slightly different from the temperature optimum of the interaction in vivo. Comparable to the situation in vivo, variation in pH or ionic strength does not affect the neutral-

izing interaction in vitro. These observations indicate that ionic bonds and electrostatic attractions do not play an important role in the interaction of cloacin DF13 molecules and their receptor protein or that ionic considerations are not important in maintaining cloacin DF13 and receptor protein in their active conformation. In contradiction to this situation, Konisky and Liu (31) reported that the interaction of colicin I molecules with their receptors is very much dependent on ionic conditions in vivo as well as in vitro, since various chloride salts inhibit the binding of colicin I to its receptor. Recently Cavard (9) also reported that the rate of reversible colicin K adsorption is progressively inhibited with increasing ionic strength of the medium. Moreover, a reversible interaction between phage lambda and its receptor in vitro has also been shown to be dependent on the ionic strength of the medium (43).

The nature of the process by which the bactericidal activity of the cloacin DF13 molecules is neutralized by their receptor protein in vitro is not known at the moment. This neutralization might be the result of an irreversible binding of both proteins, but, in addition, changes in the active conformation or even a fragmentation of the bacteriocin molecules might occur upon binding to the receptor protein. The elucidation of this adsorption process in vitro can provide important information on the process of uptake of bacteriocin by susceptible cells and will be a subject for further investigations.

Several bacteriocin receptors are known to function in the uptake of nutrients or the attachment of bacteriophages. The receptor for colicin E3, a bacteriocin which is comparable to cloacin DF13 with respect to its mode of action, functions in the uptake of vitamin B_{12} and the attachment of phage BF23 (5, 18). At the moment, nothing is known about such a function for the cloacin DF13 receptor protein.

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