JOURNAL OF BACTERIOLOGY, Apr. 1983, p. 170–176
0021-9193/83/040170-07$02.00/0

Vol. 154, No. 1

Escherichia coli Mutants with an Altered Sensitivity to Cecropin D

INGA SIDÉN AND HANS G. BOMAN*

Department of Microbiology, University of Stockholm, S-106 91 Stockholm, Sweden

Received 19 October 1982/Accepted 6 January 1983

Cecropins are a family of small, basic antibacterial polypeptides which can be isolated from pupae of immunized *Lepidoptera*. They are active against both gram-negative and gram-positive bacteria. We studied a mutant of *Escherichia coli*, strain SB1004, which is more sensitive to cecropin D than is the parental strain. The mutant was selected as resistant to a host range mutant of a *Serratia marcescens* phage. When the protein composition of the outer membrane was examined, strain SB1004 and some other phage-resistant mutants were found to be deficient in the OmpC protein. It was concluded that the OmpC protein is the receptor of the phage. Strain SB1004 was found to differ from other *ompC* mutants in being especially sensitive to hydrophobic antibiotics and to cecropin D. Furthermore, strain SB1004 has a tendency for spontaneous autolysis. A genetic analysis showed the mutations in strain SB1004 and a suppressor mutant to map in the *ompC* region. The activity of cecropin D against different strains of *E. coli* was specifically enhanced when divalent cations were absent. No such effect was found with cecropins A and B, which are less hydrophobic than the D form.

Cell-free immunity in the *Cecropia* moth and several other higher insects is due mainly to the antibacterial action of a new class of small, basic polypeptides, the cecropins. Cecropins A and B are made up of 37 amino acid residues; the sequences show them to be very polar molecules with seven or eight lysine residues in the N-terminal part, whereas the C-terminal is quite hydrophobic (22). Cecropin D contains only 36 amino acid residues and is more hydrophobic than the A and B forms. All three cecropins show a potent lytic activity toward *Escherichia coli*; cecropins A and B also show such activity against other gram-negative as well gram-positive bacteria (15).

It has not been possible to isolate mutants resistant to immune hemolymph, presumably because of the presence of several bactericidal factors with different modes of action. Shortage of material has so far made it impossible to isolate mutants resistant to a single isolated factor, such as one of the cecropins. However, earlier studies of the sensitivity of *Serratia marcescens* Db11 (isolated as a *Drosophila* pathogen) showed that mutants with an increased sensitivity to immune hemolyph of *Cecropia* could be isolated if mutants resistant to phage φJ were selected (12). We have now isolated a host range mutant of φJ which can grow on *E. coli*. By using this phage, φJE, we have isolated phage-resistant mutants of *E. coli* with an altered response to insect immunity, especially to cecropin D. This paper contains a genetic and biochemical characterization of some of these mutant strains and a study of some factors which influence the activity of cecropin D.

MATERIALS AND METHODS

Bacteria, phage, and media. Strain SB100 is believed to be a derivative of a prototrophic *E. coli* K-12 strain, HfrH. It was obtained from D. Pfeifer, Institute for Genetics, Cologne, Germany, in 1980. When tested, its origin and direction of transfer did not agree with the published data on HfrH (2). Instead, the transfer of strain SB100 goes clockwise with the origin of transfer at about 10 min. Thus, if our SB strains are related to HfrH, they must represent mutants in which the F factor has moved and changed orientation. A summary of the properties of the bacteria and phage used is given in Tables 1 and 2.

Bacteria were normally grown in LB broth (18) at 37°C on a rotary shaker. LEG medium (16) was used for the isolation of one revertant. LA plates contained LB medium with 1% agar. Phage stocks were prepared on LA plates by a method described previously (18). Soft agar contained 0.6% agar and 1.3% nutrient broth. Dilution of phages was done in 0.1% nutrient broth containing 0.5% NaCl.

Independent phage-resistant mutants were isolated on LA plates by mixing in soft agar 4×10^7 PFU of the respective phages and 3×10^8 cells from different clones of the parental strain (0.1 ml of a fresh overnight culture). After incubation overnight at 37°C, colonies were picked and purified three times.

Revertants of the mutant strain SB1004 were isolat-

TABLE 1. Main strains of *E. coli* K-12, genetic markers, and references

Strain	Genotype	Source or reference
AB1157	*thi thr leu his proA argE lac mtl xyl ara gal rpsL tsx sup-37$_{UGA}$*	(1)
SB100	Prototrophic Hfr[a]	D. Pfeifer
SB101	*gyrA*	Derivative of SB100
SB1001	*ompC1*	φJE-resistant derivative of SB100
SB1004	*ompC4*	φJE-resistant derivative of SB100 Supersensitive to immune hemolymph
SB1011	*ompF*	TuIa-resistant derivative of SB100
SB1023	*ompC6*	TuIb-resistant derivative of SB100
SB1032	*ompA*	TuII*-resistant derivative of SB100
SB1101	*ompC1 gyrA*	P1(SB101) → SB1001
SB1104	*ompC4 gyrA*	P1(SB101) → SB1004
SB1106	*ompC6 gyrA*	P1(SB101) → SB1023
SB1110	*ompC4 sup$_{ompC4}$*	"Revertant" of SB1004[b]
SB1115	*ompC4 gyrA*	P1(SB1104) → SB100
D21	*proA trp his lac rpsL ampA1*	(5)
D21e7	*proA trp his lac rpsL ampA1 lpsA1*	(5)
D22	*proA trp his lac rpsL ampA1 envA1*	(14)

[a] See text.
[b] Strains SB1111 and SB1112 are similar to SB1110 but are isolated in different ways.

ed in three different ways. (i) Clones were selected which grew on LA plates containing 1.25% immune hemolymph from *Hyalophora cecropia*. The parental strain SB100 gave confluent growth on these plates, whereas only two colonies were obtained with a fresh stationary-phase culture of mutant SB1004. One of the colonies was purified and designated SB1110. (ii) When strain SB1004 was streaked on LA plates and incubated for 3 days at 37°C, it produced a few small thick colonies on a background of confluent, transparent growth. Of these small colonies, 26 were picked, restreaked on LA plates, incubated overnight at 37°C, and then left in the cold for 1 additional day (after which the transparent appearance of mutant SB1004 becomes more pronounced and thereby more easily distinguished from the revertant). One of the small thick colonies was further purified by restreaking for single colonies (strain SB1111). (iii) One colony from an LA plate was used to inoculate 1 ml of LEG medium, which then was incubated overnight. From this culture, 0.1 ml was transferred to 1 ml of the same medium and again incubated overnight. The procedure was repeated twice. From the last culture, a sample was streaked on an LA plate. The colonies obtained were all thick and yellow, as opposed to the thinner and more transparent appearance of mutant SB1004. One of the colonies was used for further work (strain SB1112). All revertants were tested by growth on minimal agar and sensitivity to phages φJE, Tula, Tulb, TuII* and P1.

Preparation of outer membrane proteins. The essential steps in the protocol were taken from Wolf-Watz and Masters (24). Each strain was grown overnight in 50 ml of LB supplemented with 0.2% glucose. The bacteria were harvested by centrifugation at 12,000 × *g* for 10 min. The pellet was suspended in 10 mM Tris-hydrochloride–1 mM EDTA (pH 8.0) and sonicated four times for 15 s. Afterward, debris and remaining intact cells were removed by centrifugation at 3,000 × *g* for 20 min. The supernatant fluid was centrifuged for 1 h at 40,000 rpm in a Sorvall ultracentrifuge OTD2 with rotor T865. The pellet, containing undissolved outer and cytoplasmic membranes, was dissolved in 0.5% Sarkosyl and left in the cold overnight. Sarkosyl selectively dissolves the cytoplasmic membrane, and the purified outer membrane can therefore be isolated by an additional ultracentrifugation as described above (11). Finally, the otuer membrane was dissolved in 50 μl of 40% sucrose–0.05% bromphenol blue–2% sodium dodecyl sulfate (SDS) in 0.125 M Tris-hydrochloride (pH 6.8).

SDS-polyacrylamide gel electrophoresis. The following modification of the method of Laemmli was used (H. Wolf-Watz, personal communication). The gel was 19 by 21 by 0.2 cm and contained 16% acrylamide, 0.09% *N,N'*-methylenebisacrylamide, and 0.1% SDS in 0.375 M Tris-hydrochloride (pH 8.8). It was polymerized by the addition of 50 mg of ammonium peroxodisulfate and 50 μl of *N,N,N',N'*-tetramethylethylenediamine (TEMED) per 100 ml of acrylamide solution. The stacking gel contained 3% acrylamide, 0.085% *N,N'*-methylenebisacrylamide, and 1% SDS in 0.032 M Tris-hydrochloride (pH 6.8). The electrophoresis buffer contained, in 1 liter, 3 g of Tris, 14.4 g of glycine, and 1 g of SDS. Before application, all samples (15 μl) were boiled for 5 min. Electrophoresis was carried out at 60 V until the samples had entered the running gel, at which time the

TABLE 2. Phages, host ranges, and receptors[a]

Indicator strain	Plating efficiency				
	TuIa	TuIb	TuII*	φJ	φJE
E. coli SB100	1.0	1.0	1.0	10^{-6}	1.0
E. coli B[b]	ND[c]	0.5	ND	$<10^{-8}$	0.5
E. coli C	ND	ND	ND	$<10^{-8}$	10^{-5}
S. marcescens Db10	$<10^{-8}$	$<10^{-8}$	10^{-6}	1.0	10^{-5}
S. marcescens SM6	ND	ND	ND	$<10^{-8}$	$<10^{-8}$

[a] The Tu phage (8) were obtained from U. Henning. Phage φJ has been described previously (12), and φJE is described in this paper. The receptor proteins are: TuIa, OmpF; TuIb, OmpC; TuII*, OmpA; φJ, unknown; and φJE, OmpC.
[b] Our strain of *E. coli* B is atypical because it can function as host for phage TuIb, known to require the OmpC protein. A similar strain has been reported previously (21).
[c] ND, Not determined.

voltage was raised to 170 V and electrophoresis was continued for 7 to 8 h. All gels were stained with Coomassie blue.

Genetic techniques. Conjugation was performed as described by Miller (18). Transduction with P1 was done essentially as previously described (18), with the following exceptions. Before adsorption, the recipient bacteria were concentrated 10 times. After adsorption of the phage, 0.1 ml of 0.2 M sodium citrate was added to 0.2 ml of the culture, which then was added to soft agar and poured on LA plates. All plates were incubated for 3 h at 37°C, and then 3 ml of soft agar with 1.5 mg of nalidixic acid was added. Incubation was continued for 24 to 36 h at 37°C.

Antibacterial assays. The inhibition zone assay used is essentially the classical Fleming method for antibiotics, as modified by Hultmark et al. (15) to permit calculation of lethal concentrations. Inhibition zones were measured around 3-mm wells, into which the serial dilutions of the antibacterial agent were added. Each thin-layer agar plate (diameter, 9 cm) contained 6 ml of LA medium with about 10^5 bacterial cells and, when indicated, 0.5 mM EDTA.

Killing of buffer-suspended bacteria was done by the method of Boman et al. (6), except that dithiothreitol was omitted from the reaction buffer. About 10^6 exponentially growing bacteria, suspended in buffer solution, were incubated at room temperature with 0.2% immune hemolymph. Samples were withdrawn between 0 and 6 min, and viable count was determined. Immune hemolymph from diapausing pupae of *H. cecropia* was collected 7 to 10 days after immunization (10). Cecropins A, B, and D were purified as described by Hultmark et al. (15).

RESULTS

Properties of phage φJE. The parental phage φJ was isolated as specific for an insect-pathogenic strain, *S. marcescens* Db10. When phage-resistant mutants of strain Db10 were tested for susceptibility to cecropins, they were found to be more sensitive than the parental strain (12). To be able to study the same phenomena in *E. coli*, we first isolated φJE, a host-range mutant of φJ which grew well on *E. coli* SB100 and *E. coli* B (Table 2). Neither phage φJ nor φJE could infect *E. coli* C or *S. marcescens* SM6, and φJE had lost its ability to grow on strain Db10 (Table 2). Phage φJE has a burst size of about 50, an eclipse period of 11 min, and a latent period of 15 min. On all strains, it produced clear plaques with a halo. The receptor is the OmpC protein (see below).

Resistance to φJE as a way of obtaining mutants sensitive to immune hemolymph. Spontaneous φJE-resistant mutants of *E. coli* SB100 were isolated as described above. Of 12 independent mutants, 5 were selected for further studies. For a first characterization of these φJE-resistant mutants, the bacteria were incubated in vitro with immune hemolymph from *H. cecropia* pupae (details in Materials and Methods). Conditions were chosen to give only a slight reduction of viable count of the parental strain SB100 (Fig. 1). All five mutants tested were more sensitive to immune hemolymph than the parental strain. However, the mutants differed in susceptibility, and strain SB1004 was found to be the most sensitive mutant.

Identification of φJE receptor. Phage φJE appears to be a virulent phage, and it does not adsorb to the resistant mutants isolated. Thus, the φJE-resistant bacteria were assumed to be affected in the receptor of the phage. The receptors of phages TuIa, TuIb, and TuII*, respectively (Table 2), are the major outer membrane proteins OmpF, OmpC, and OmpA (8). We therefore isolated additional mutants of strain SB100 which were resistant to these phages. The cross-resistance pattern indicates that phages TuIb and φJE could share a receptor, the OmpC protein. To test this directly, outer membranes were prepared from strains SB1001 and SB1004 (φJE resistant), SB1011 (TuIa resistant), SB1023 (TuIb resistant), and SB1032 (TuII* resistant). All bacterial strains were grown in LB medium supplemented with glucose, and the main porin protein was therefore the OmpC protein (3). The outer membrane proteins were then separated by SDS-polyacrylamide gel electrophoresis. The protein patterns obtained showed that the OmpC protein was missing in strains SB1001, SB1004, and SB1023 (Fig. 2). We have not been able to identify the OmpF protein, which, under our

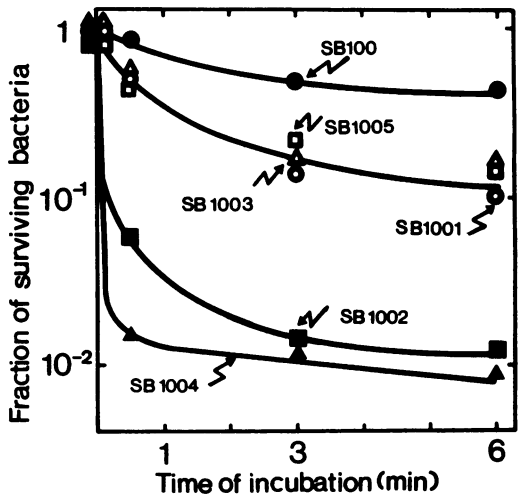

FIG. 1. Susceptibility of different φJE-resistant mutants of SB100 to immune hemolymph from *H. cecropia* pupae. Growing bacteria were incubated at room temperature with 0.2% immune hemolymph. Samples were withdrawn at different intervals and plated for viable count. SB100 (●), the parental strain, and strains SB1001 (○), SB1002 (■), SB1003 (△), SB1004 (▲), and SB1005 (□) are all φJE-resistant mutants.

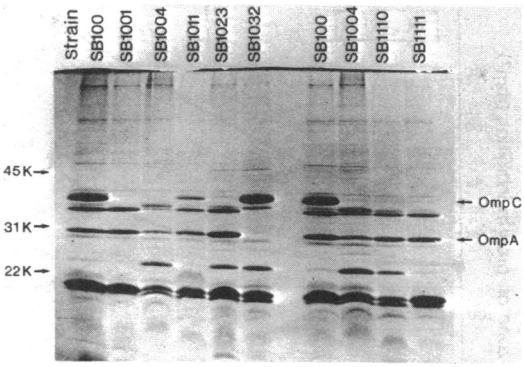

FIG. 2. SDS-polyacrylamide gel electrophoresis of the outer membrane proteins of the strains indicated above the gel. On the left side of the gel, SB100 is the parental strain, SB1001 and SB1004 are φJE resistant, SB1011 is Tula resistant, SB1023 is TuIb resistant, and SB1032 is TuII* resistant. The right side of the gel includes preparations from strains SB1110 and SB1111 containing suppressor mutations. The arrows on the left indicate the positions and sizes in kilodaltons of three molecular weight markers.

growth conditions, is a minor component. However, the presence of the OmpF protein was monitored as sensitivity to phage TuIa. Only strain SB1011 was resistant to this phage. No new protein bands could be seen in the preparations from strain SB1004, although the relative amounts of some of the proteins, especially one with a size around 25,000, had increased (Fig. 2).

Correlation between OmpC deficiency, sensitivity to cecropin D, and autolytic character. The parental strain SB100 and most OmpC-deficient mutants grew normally. However, the growth behavior of the φJE-resistant mutant SB1004 was different. When the cells entered stationary phase, they started to aggregate. A fraction of the cells then lysed, thereby giving a lower growth yield. On agar plates, colonies of SB1004 were whiter and thinner than those of the parental strain. The strain was also supersensitive to immune hemolymph (Fig. 1).

We isolated revertants of SB1004 by the three methods described in detail above. Three different revertants were selected which grew normally and did not show any tendency to lyse spontaneously. They were all found to be φJE and TuIb resistant but to retain their sensitivity to the phages TuIa, TuII*, and P1. As shown in Fig. 2, these strains were not true revertants with restored synthesis of the OmpC protein but rather suppressor mutant strains. Two of these strains, SB1110 and SB1111, still lacked the OmpC protein. The third strain, SB1112, did not differ from SB1111 (results not shown). None of the strains showed any new proteins in their outer membranes.

Genetic characterization of φJE resistance. Since strain SB1004 was super sensitive to immune hemolymph, deficient in the OmpC protein, and showed aberrations in growth, it was considered necessary to map its mutation. The mutations in two other φJE-resistant mutants, SB1001 and SB1023, as well as the suppressors, were also mapped. For all strains, the respective mutations were first transferred to strain AB1157 by conjugational crosses. Selection was made for markers in three regions of the chromosome, *argE* at 89 min, *his* at 44 min, and *leu* at 2 min. Recombinants obtained from strains SB1001, SB1004, and SB1023 showed that φJE resistance was linked to *his* (data not shown), indicating that the mutations could reside in the *ompC* gene at 47 min on the *E. coli* chromosome map (2).

We next mapped the mutations by cotransduction with *gyrA* (Table 3). Moreover, all suppressor mutations in strain SB1110, SB1111, and SB1112, which counteract the autolytic character of SB1004 without restoring the synthesis of OmpC protein, were also found to map close to *gyrA*. These results agree well with data previously published for *ompC* mutations (3).

Sensitivity to antibiotics and melittin. As we had reason to believe that strain SB1004 had a changed permeability barrier, we tested resistance to various antibiotics. Earlier studies have indicated that a change in the permeability barrier, notably alterations in lipopolysaccharide (LPS), leads to increased sensitivity to various hydrophobic compounds, whereas sensitivity to hydrophilic agents is unaffected (7, 19). However, the results in Table 4 do not support such a simple relationship. Strain SB1004 showed an increased sensitivity to some hydrophobic antibiotics, such as erythromycin, nalidixic acid, chloramphenicol, and novobiocin. However, no change in resistance to rifampin and actinomy-

TABLE 3. Cotransduction between *gyrA* and alleles of *ompC*

Donor[a]	Recipient	Cotransduction frequency (%)
SB101 *gyrA ompC*$^+$	SB1001 *gyrA*$^+$ *ompC1*	35
SB101 *gyrA ompC*$^+$	SB1004 *gyrA*$^+$ *ompC4*	57
SB101 *gyrA ompC*$^+$	SB1023 *gyrA*$^+$ *ompC6*	45
SB1101 *gyrA ompC1*	SB100 *gyrA*$^+$ *ompC*$^+$	27
SB1104 *gyrA ompC4*	SB100 *gyrA*$^+$ *ompC*$^+$	29
SB1106 *gyrA ompC6*	SB100 *gyrA*$^+$ *ompC*$^+$	43

[a] Transduction was done with phage P1, and *gyrA* was the selected marker. A total of 300 transductants in each experiment were scored for *ompC* by cross-streaking with phage φJE.

TABLE 4. Resistance of strain SB100 and two of its *ompC* mutants to antibiotics

Antibiotic	Concentration[a] (μg/disk)	Partition coefficient[b]	Inhibition zone diameter (mm)		
			SB100	SB1004	SB1110
Rifampin	30	65	15	15	13
Actinomycin D	12.5	20	<6	<6	<6
Novobiocin	50	20	7	13	12
Chloramphenicol	30	12.4	18	25	18
Nalidixic acid	30		17	24	20
Erythromycin	15		10	24	9
Bacitracin	30	0.12	<6	<6	<6
Tetracycline	30	0.07	18	18	18
Penicillin G	10	0.02	9	10	10
Ampicillin	10	0.01	20	20	20

[a] The diameter of the disks was 6 mm.

[b] All values of partition coefficients were taken from Nikaido (19), except for rifampin, which was from Grundström et al. (14). Note that Grundström et al. determined the partition coefficient in a different system from that used by Nikaido. A higher value means that the substance is more hydrophobic. A substance is considered to be hydrophilic if its partition coefficient is less than 0.02.

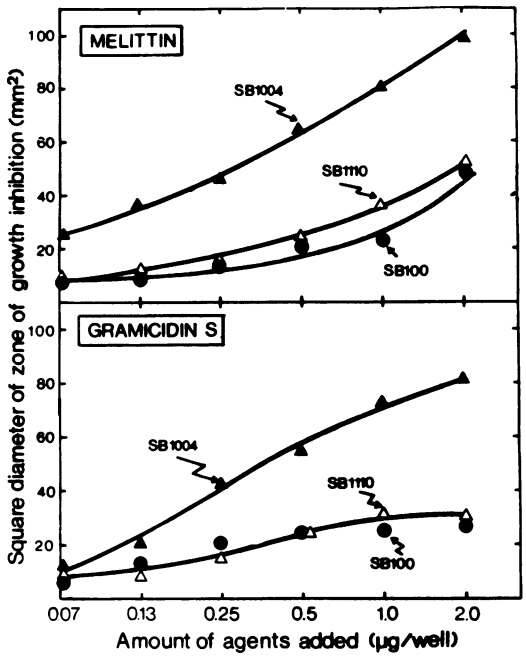

FIG. 3. Susceptibility of different bacterial strains to melittin and gramicidin S.

cin D, which are also hydrophobic antibiotics, was found. None of the strains was altered in its response to the hydrophilic antibiotics tested. With the inhibition zone assay, we tested our mutants for sensitivity to the bee venom toxin melittin and gramicidin S (Fig. 3), two peptides believed to disrupt lipid bilayer membranes (9, 13). The mutant SB1004 was significantly more sensitive to melittin and gramicidin S than both the parental strain SB100 and the suppressor mutant SB1110.

Cecropin sensitivity of different outer membrane mutants. For strains SB100 and three of its *ompC* mutants, we determined the lethal concentrations for cecropins A, B, and D with the inhibition zone assay and calculations by the method of Hultmark et al. (15). We also included the LPS mutant D21e7, the *envA* mutant D22, and their common ancestor, strain D21. The test bacteria were grown in LA medium in either the presence or absence of EDTA (Table 5). The two *ompC4* strains SB1004 and SB1115 were specifically sensitive to cecropin D, whereas all SB strains showed the same susceptibility to cecropins A and B. In the suppressor mutant SB1110, the specific sensitivity toward cecropin D was lost. Strains D21e7 and D22 were also more sensitive to cecropin D than their parental strain, D21. All three strains that were insensitive to cecropin D (SB100, SB1110, and D21) became susceptible when EDTA was added to

TABLE 5. Sensitivity of strains SB100 and D21 and some of their derivatives to purified cecropins

Strain	Relevant genotype	Lethal concentration[a] (μM)					
		Cecropin A		Cecopin B		Cecopin D	
		NA	+EDTA	NA	+EDTA	NA	+EDTA
SB100	Wild type	0.32	0.27	0.32	0.24	5.7	0.45
SB1004	*ompC4*	0.30	0.21	0.23	0.21	0.51	0.20
SB1110	*ompC4* sup_{ompC4}	0.30	NT	0.25	NT	5.6	0.19
SB1115	*ompC4*	0.21	NT	0.21	NT	0.62	0.25
D21	Wild type	0.35	0.24	0.37	0.37	2.8	0.19
D21e7	*lpsA1*	0.23	NT	0.26	NT	0.47	0.22
D22	*envA*	0.25	NG	0.25	NG	0.56	NG

[a] Lethal concentrations were calculated by the method of Hultmark et al. (15). For parallel determinations, using standardized conditions for dilutions, the accuracy is believed to be 10 to 20% for values <1.0 μM; for higher values (with small zones), the errors may be around 50 to 100%. NA, No addition; NT, not tested; NG, no growth.

the growth medium. The addition of either $MgSO_4$ or $CaCl_2$ in a concentration exceeding that of EDTA by 1 mM fully canceled the effects of EDTA. Other *ompC* mutations were tested and found to be less sensitive to cecropin D than strains SB1004 and SB1115. There was no uniform pattern because strain SB1023 was just as resistant as the parental strain SB100, whereas strain SB1001 had an intermediate level of sensitivity (data not shown).

DISCUSSION

What is the nature of the *ompC4* mutation? Strain SB1004 grows normally until the onset of stationary phase, at which time the cells begin to aggregate and eventually to lyse. The *ompC4* mutation in strain SB1004 is pleiotropic because it affects growth behavior, sensitivity to cecropin D and melittin, and the response to some hydrophobic drugs (Fig. 3 and Tables 4 and 5). The suppressor mutant SB1110 has lost the pleiotropic phenotype, but it does not produce the OmpC protein. The respective mutations in strains SB1004 and SB1110 both map in the *ompC* region (Table 3). The *ompC4* allele can therefore be explained in two different ways. First, the mutation could lie in the *ompC* gene itself. If so, it could either change the OmpC protein in such a way that it cannot be properly translocated to the outer membrane, or it could affect some unknown regulatory function of the OmpC protein. Second, the mutation could affect a nearby gene involved in sensitivity to cecropin D. The suppressor mutation would then abolish the deleterious effect of the *ompC4* mutation without restoring the synthesis of the gene product. One could argue that the pleiotropic effects of the *ompC4* mutation are due to an independent mutation outside the *ompC* locus. However, the mutation occurred spontaneously, and P1 transduction experiments have never given rise to any transductant being φJE resistant and lacking the *ompC4* phenotype.

Some mutants resistant to phage TuIb were also found to be sensitive to cecropin D, but none of these mutants was more sensitive than SB1004. Altogether, 25 independently isolated *ompC* mutants (isolated as resistant to phage φJE or TuIb) were tested for sensitivity to cecropin D; only 12 were found to be more sensitive than the parent. Thus, sensitivity to cecropin D and certain mutations in the *ompC* region must in some way be coupled phenomena. However, the reverse is not always true.

What makes a bacterium cecropin D sensitive? Cecropin D differs from the A and B forms in two important respects: it is less basic than the two other forms (a net difference in four or five positive charges), and it has a stretch of 19 amino acid residues without a single charge (15, 22). Both of these differences make the D form more hydrophobic. When tested for bactericidal activity against a number of different bacteria, cecropin D was significantly less active than the A and B forms (15). Table 5 shows that the susceptibility of *E. coli* to cecropin D was increased by several different mutations as well as by the removal of Ca^{2+} and Mg^{2+} by EDTA. Our earlier assays (15) included medium E (22), which contains enough citrate to produce the same increase in sensitivity as we obtained here with EDTA. Therefore, one must not compare lethal concentrations for cecropin D (and perhaps for other hydrophobic agents) without considering the concentration of divalent cations.

The major outer membrane protein OmpC was lost in strain SB1004 (Fig. 2). The mutation in strain SB1004 also decreased the lethal concentration of cecropin D by a factor of 10, without altering the sensitivity to the other two cecropins (Table 5). In addition, the mutation makes the bacteria more sensitive to several hydrophobic antibiotics (Table 4) and to gramicidin S and melittin (Fig. 3), two agents known to act on membranes (9, 13). These results, together with the observation of spontaneous autolysis, suggest that the mutation in strain SB1004 leads to a weakening of the outer membrane. It would be easiest to assume that this alteration could be fully explained by the sole loss of the OmpC protein. However, two findings would then be difficult to explain: (i) not all mutants lacking the OmpC protein have the properties of strain SB1004, and (ii) the suppressor mutant SB1110, despite the fact that it still lacks the OmpC protein, has regained the parental levels of sensitivity to cecropin D and some hydrophobic antibiotics and has lost the tendency for spontaneous autolysis.

We also included in Table 5 two other outer membrane mutations which lead to an increased sensitivity to cecropin D. The *lpsA1* mutation in strain D21e7 leads to the loss of phosphates bound to heptose and ethanolamine (20), alterations which make the LPS and the cell surface considerably more hydrophobic. Moreover, the *envA1* mutation in strain D22 makes the bacteria more hydrophobic, presumably by decreasing the amount of LPS present in the outer membrane (14). Also, divalent cations are necessary for the organization of the outer membrane of *E. coli*, and low concentrations of EDTA lead to the release of LPS (4) and increased permeability (17). This in turn can explain the effects of EDTA on the activity of cecropin D (Table 5).

Taken together, the information presented here on the activity of cecropin D and the properties of strains SB1004, D21e7, and D22 can be interpreted as follows. (i) The mutation in strain SB1004 has increased the hydrophobicity

of the cell surface. (ii) In comparison to the A and B forms, cecropin D is restricted in its penetration through the outer membrane. (iii) Genetic information for sensitivity to cecropin D is linked to the *ompC* locus. (iv) The penetration of cecropin D through the outer membrane is facilitated by alterations which make this surface layer more hydrophobic. A more refined genetic analysis, direct measurements of the hydrophobicity of the bacterial surfaces, and binding studies with labeled cecropins are needed to substantiate these tentative conclusions.

ACKNOWLEDGMENTS

We thank Hans Wolf-Watz for helpful suggestions.
This work was supported by grant BU2453-110 from the Swedish Natural Science Research Council.

LITERATURE CITED

1. **Bachmann, B. J.** 1972. Pedigrees of some mutant strains of *Escherichia coli* K-12. Bacteriol. Rev. **36**:525–557.
2. **Bachmann, B. J., and K. B. Low.** 1980. Linkage map of *Escherichia coli* K-12, edition 6. Microbiol. Rev. **44**:1–56.
3. **Bassford, P. J., Jr., D. L. Diedrich, C. L. Schnaitman, and P. Reeves.** 1977. Outer membrane proteins of *Escherichia coli*. VI. Protein alteration in bacteriophage-resistant mutants. J. Bacteriol. **131**:608–622.
4. **Bayer, M. E., and L. Leive.** 1977. Effect of ethylenediaminetetraacetate upon the surface of *Escherichia coli*. J. Bacteriol. **130**:1364–1381.
5. **Boman, H. G., and D. A. Monner.** 1975. Characterization of lipopolysaccharides from *Escherichia coli* K-12 mutants. J. Bacteriol. **121**:455–464.
6. **Boman, H. G., I. Nilsson-Faye, K. Paul, and T. Rasmuson, Jr.** 1974. Insect immunity. I. Characteristics of an inducible cell-free antibacterial reaction in hemolymph of *Samia cynthia* pupae. Infect. Immun. **10**:136–145.
7. **Coleman, W. G., Jr., and L. Leive.** 1979. Two mutations which affect the barrier function of the *Escherichia coli* K-12 outer membrane. J. Bacteriol. **139**:899–910.
8. **Datta, D. B., B. Arden, and U. Henning.** 1977. Major proteins of the *Escherichia coli* outer cell envelope as bacteriophage receptors. J. Bacteriol. **131**:821–829.
9. **Dawson, C. R., A. F. Drake, J. Helliwell, and R. C. Hider.** 1978. The interaction of bee melittin with lipid bilayer membranes. Biochim. Biophys. Acta **510**:75–86.
10. **Faye, I., A. Pye, T. Rasmuson, H. G. Boman, and A. Boman.** 1975. Insect immunity. II. Simultaneous induction of antibacterial activity and selective synthesis of some hemolymph proteins in diapausing pupae of *Hyalophora cecropia* and *Samia cynthia*. Infect. Immun. **12**:1426–1438.
11. **Filip, C., G. Fletcher, J. L. Wulff, and C. F. Earhart.** 1973. Solubilization of the cytoplasmic membrane of *Escherichia coli* by the ionic detergent sodium-lauryl sarcosinate. J. Bacteriol. **115**:717–722.
12. **Flyg, C., K. Kenne, and H. G. Boman.** 1980. Insect pathogenic properties of *Serratia marcescens*: phage-resistant mutants with a decreased resistance to *Cecropia* immunity and a decreased virulence to *Drosophila*. J. Gen. Microbiol. **120**:173–181.
13. **Gale, E. F., E. Cundliffe, P. E. Reynolds, M. H. Richmond, and M. J. Waring.** 1981. The molecular basis of antibiotic action, 2nd ed., p. 187–188. John Wiley & Sons, London.
14. **Grundström, T., S. Normark, and K.-E. Magnusson.** 1980. Overproduction of outer membrane protein suppresses *envA*-induced hyperpermeability. J. Bacteriol. **144**:884–890.
15. **Hultmark, D., A. Engström, H. Bennich, R. Kapur, and H. G. Boman.** 1982. Insect immunity: isolation and structure of cecropin D and four minor antibacterial components from Cecropia pupae. Eur. J. Biochem. **127**:207–217. (Correction of formula 9, Eur. J. Biochem., in press.)
16. **Landén, R., A. Heierson, and H. G. Boman.** 1981. A phage for generalized transduction in *Bacillus thuringiensis* and mapping of four genes for antibiotic resistance. J. Gen. Microbiol. **123**:49–59.
17. **Leive, L.** 1968. Studies on the permeability change produced in coliform bacteria by ethylenediaminetetraacetate. J. Biol. Chem. **243**:2373–2380.
18. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
19. **Nikaido, H.** 1976. Outer membrane of *Salmonella typhimurium* transmembrane diffusion of some hydrophobic substances. Biochim. Biophys. Acta. **433**:118–132.
20. **Prehm, P., S. Stirm, B. Jann, K. Jann, and H. G. Boman.** 1976. Cell-wall lipopolysaccharides of ampicillin-resistant mutants of *Escherichia coli* K12. Eur. J. Biochem. **66**:369–377.
21. **Schmitges, C. J., and U. Henning.** 1976. The major proteins of *Escherichia coli* outer cell envelope membrane. Heterogeneity of protein I. Eur. J. Biochem. **63**:47–52.
22. **Steiner, H., D. Hultmark, A. Engström, H. Bennich, and H. G. Boman.** 1981. Sequence and specificity of two antibacterial proteins involved in insect immunity. Nature (London) **292**:246–248.
23. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218**:97–106.
24. **Wolf-Watz, H., and M. Masters.** 1979. Deoxyribonucleic acid and outer membrane: strains diploid for the *oriC* region show elevated levels of deoxyribonucleic acid-binding protein and evidence for specific binding of the *oriC* region to outer membrane. J. Bacteriol. **140**:50–58.