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Sensitivity of *Escherichia coli* to Cloacin DF13 Involves the Major Outer Membrane Protein OmpF

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Fourteen spontaneous cloacin DF13-insensitive mutants of an *Escherichia coli* strain expressing the aerobactin-cloacin DF13 receptor protein IutA were isolated. The mutants fell into three classes on the basis of outer membrane profiles analyzed by electrophoresis in denaturing polyacrylamide gels. The most frequent class lacked the IutA protein and was unable to bind cloacin DF13 or aerobactin. A second class of mutants had lost protein species corresponding in size to the porin proteins OmpF and OmpC. To determine which porin was required for the bactericidal activity of cloacin DF13, defined strains with mutations at the *ompB* (*ompR envZ*) locus were transformed with a recombinant plasmid carrying the *iutA* gene and screened for cloacin DF13 sensitivity. OmpF$^-$ strains, whether OmpC$^+$ or OmpC$^-$, were insensitive to cloacin DF13, indicating involvement of the OmpF protein in cloacin DF13 killing. An OmpC$^-$ OmpF$^+$ strain, on the other hand, was more sensitive than the wild-type parent strain, probably because of compensatory overexpression of OmpF. The third class of cloacin DF13-insensitive mutant had lost an outer membrane protein of approximately 31 kDa. The nature and function of this protein are not yet known, but it is not the protease OmpT. Mutants of classes 2 and 3 bound cloacin DF13 and aerobactin as effectively as the cloacin DF13-sensitive parental strain, indicating that they remained IutA$^+$. We propose that these mutants (more accurately described as cloacin DF13 tolerant) are defective in translocation of the active portion of cloacin DF13 across the bacterial membranes.

Cloacin DF13 is a bacteriocin produced by strains of *Enterobacter cloacae* that harbor plasmid CloDF13 (21). Killing of sensitive enteric bacteria by cloacin DF13 involves three distinct stages (13, 25): binding to a specific surface receptor protein, transport of an active fragment of the bacteriocin molecule through the cell envelope, and endoribonucleolytic cleavage of 16S rRNA, resulting in defective protein synthesis. The cloacin DF13 receptor in several enterobacterial species also acts as a receptor for the hydroxamate siderophore aerobactin (2, 14, 25). Thus, strains of *Escherichia coli* that utilize aerobactin as a source of iron, a category that includes many pathogenic isolates (3, 26), are sensitive to cloacin DF13 because they express the 74-kDa outer membrane aerobactin receptor protein IutA (2). Killing of *E. coli* by cloacin DF13, unlike many other bacteriocins, is not dependent on the cytoplasmic membrane protein TonB (20); the aerobactin receptor is therefore the only envelope-associated protein in *E. coli* reported to date to be required for cloacin DF13 activity. In this paper we show that the major outer membrane protein OmpF, and possibly an outer membrane protein of 31 kDa, are also essential for the lethal action of cloacin DF13.

MATERIALS AND METHODS

Bacteria and culture conditions. The bacterial strains used in this study are described in Table 1. Plasmid pLG141 comprises a 6.5-kb *Bam*HI fragment of plasmid ColV-K30 that includes the aerobactin receptor gene *iutA* (4), cloned in the vector plasmid pACYC184 (5); *iutA* in pLG141 is expressed constitutively (4). Plasmid DNA was introduced as required into bacterial strains by transformation (11). Bacteria were routinely grown with aeration at 37°C in nutrient broth (Oxoid No. 2) supplemented where necessary with 20 μg of chloramphenicol (Sigma) ml^{-1} to select for the presence of pLG141. Solid media contained 1.5% agar (Difco).

Outer membrane protein preparation and analysis. Outer membranes were prepared as described previously (19). Briefly, cells were harvested from overnight cultures by centrifugation, resuspended in 0.1 volume of 100 mM Tris-HCl (pH 7.8) containing 10 mM $MgCl_2$, and disrupted by sonication. After removal of cell debris by low-speed centrifugation, supernatant fractions were subjected to ultracentrifugation at 100,000 × *g* for 10 min. Pellets containing total membranes were suspended in the same volume of Tris-$MgCl_2$ buffer containing 2% Triton X-100 to dissolve inner membrane proteins and were again subjected to ultracentrifugation. Pellets containing outer membranes were resuspended as before, incubated at room temperature for 30 min, recovered by ultracentrifugation, and resuspended in the same buffer. Outer membrane proteins were separated by electrophoresis in 11% polyacrylamide gels containing 0.1% sodium dodecyl sulfate (SDS) by the method of Laemmli (15), or 9% gels containing 8 M urea as described by Pugsley and Schnaitman (18). Gels were stained with Coomassie brilliant blue.

Cloacin sensitivity assay and inhibition by membranes. Cloacin DF13 was prepared by mitomycin induction of a bacteriocinogenic strain essentially as described by de Graaf et al. (7). Cloacin DF13 inhibits growth of cultures of sensitive bacteria, but as cloacin preparations are diluted inhibitory activity is lost (19). Sensitivity of bacterial strains to cloacin DF13 was assayed by adding 0.5 ml of cell suspensions at an optical density at 620 nm (OD_{620}) of 0.5 to serial doubling dilutions of a cloacin DF13 preparation in 2 ml of nutrient broth, incubating with shaking at 37°C for 4 h, and again determining OD_{620}. To quantify cloacin DF13 binding to membranes, cloacin dilution series were preincu-

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TABLE 1. Bacterial strains

Strain	Characteristics	Source or reference
E. coli K-12		
CC118	*araD139* Δ(*ara leu*)*7697* Δ*lacX74 phoA*Δ*20 galE galK thi rpsE rpoB argE*(Am) *recA1* OmpC$^+$ OmpF$^+$	16
LG1700	CC118 carrying pLG141 (Cm^r IutA$^+$)	This study
LG1701	LG1700, cloacin DF13 resistant (*iutA*)	This study
LG1702	LG1700, cloacin DF13 tolerant (OmpC$^-$ OmpF$^-$)	This study
LG1703	LG1700, cloacin DF13 tolerant (lacking a 31-kDa outer membrane protein)	This study
MC4100	*araD139 lacU169 rpsL relA thiA fibB* (OmpC$^+$ OmpF$^+$)	23
MH760	MC4100, *ompR472* (OmpC$^-$ OmpF$^+$)	23
MH1160	MC4100, *ompR101* (OmpC$^-$ OmpF$^-$)	23
MH1460	MC4100, *envZ11* (OmpC$^+$ OmpF$^-$)	23
UT4400	*ompT*	9
K. pneumoniae M5a1	Cloacin DF13 sensitive	6

bated with outer membrane preparations before titration against the cloacin DF13-sensitive *Klebsiella pneumoniae* indicator strain M5a1 (6, 19).

Aerobactin uptake and inhibition by cloacin DF13. ^{14}C-labeled aerobactin was prepared from bacterial cultures incubated with precursor [^{14}C]lysine by the method of Ford et al. (8). The ability of mutants to take up exogenously supplied [^{14}C]aerobactin and inhibition of uptake by preincubation of cells with cloacin DF13 were tested as described previously (19).

RESULTS

Isolation of mutants. Spontaneous cloacin DF13-insensitive mutants of strain LG1700 were isolated by spotting partially purified cloacin DF13 (7) onto bacterial lawns (approximately 10^7 cells per plate) on nutrient agar containing chloramphenicol. Fourteen independent colonies were picked from zones of inhibition after overnight incubation at 37°C. SDS-polyacrylamide gel electrophoresis of outer membrane protein preparations of these isolates revealed three classes of mutant (Fig. 1). The most numerous class (8 of the 14 mutants, represented by strain LG1701, lane B) were true cloacin DF13-resistant mutants which, compared with protein profiles of the parent strain LG1700 (lane A), had lost the 74-kDa IutA protein. Plasmid DNA isolated from representative strains of this class was introduced by transformation into virgin CC118 cells; all transformants were cloacin DF13 resistant, confirming that the relevant mutations were plasmid borne (i.e., *iutA* mutants).

Mutants of class 2 (5 isolates, represented by strain LG1702, lane C) lacked proteins corresponding to the major outer membrane proteins species OmpF and OmpC (apparent molecular weight, 36,000). Class 3, represented by the single mutant LG1703 (lane D), had lost a protein of approximately 31 kDa; in addition, the 74-kDa IutA band was replaced by a novel protein of 71 kDa and there were differences in intensity of several other bands compared with those of the wild type. Transformation of strain CC118 with plasmid DNA isolated from mutants of classes 2 or 3 yielded transformants that were sensitive to cloacin DF13, indicating that the plasmids in these mutants were still *iutA*$^+$ and that the mutations causing cloacin insensitivity were chromosomal.

Cloacin DF13 binding. Figure 2 shows a representative experiment in which approximately 10 doubling dilutions of a cloacin DF13 preparation (incubated with membranes prepared from the IutA$^-$ strain CC118) were required for 50% inhibition of growth of the indicator strain *K. pneumoniae* M5a1. Addition of membranes prepared from the IutA$^+$ strain LG1700, however, significantly increased the level of cloacin DF13 required for 50% inhibition of M5a1 (to approximately 5 to 6 doubling dilutions) by binding, and so inactivating, bacteriocin molecules at each dilution. Interestingly, outer membranes prepared from the cloacin DF13 insensitive mutants LG1702 and LG1703 also titrated cloacin DF13 activity in the dilution series with the same efficiency. As would be expected of an *iutA* mutant, however, membranes of mutant strain LG1701 did not bind cloacin DF13 and therefore did not alter the effective bactericidal concentration of the cloacin preparation used (Fig. 2).

Aerobactin uptake. Similarly, strain LG1701 (*iutA*) did not bind exogenously supplied [^{14}C]aerobactin (Table 2), while mutant strains LG1702 and LG1703 (IutA$^+$) took up aero-

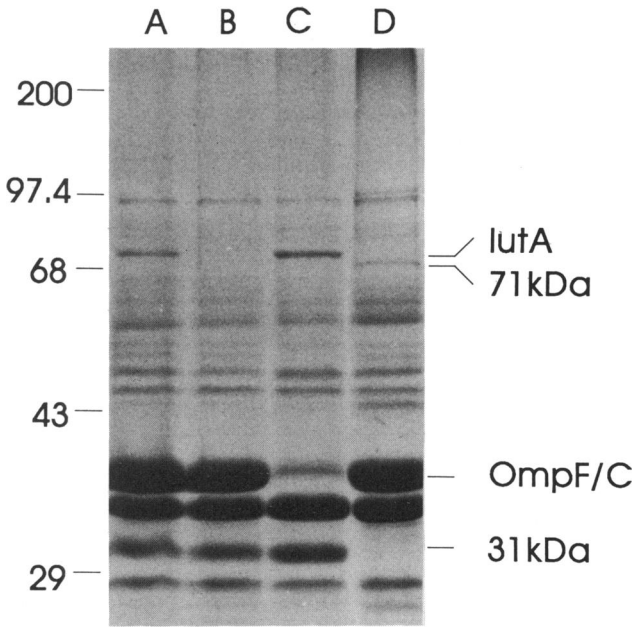

FIG. 1. SDS-polyacrylamide gel electrophoresis of outer membrane proteins of strain LG1700 (lane A) and representative cloacin DF13-insensitive mutants LG1701 (lane B), LG1702 (lane C), and LG1703 (lane D). Molecular weight standards were myosin heavy chain (200,000), phosphorylase *b* (97,400), bovine serum albumin (68,000), ovalbumin (43,000), and carbonic anhydrase (29,000).

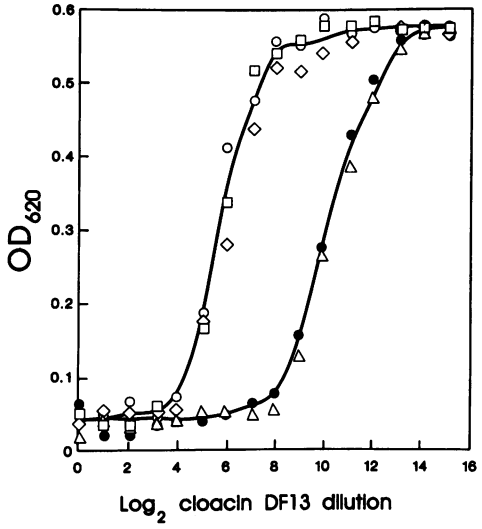

FIG. 2. Cloacin binding to membranes of cloacin DF13-insensitive derivatives of strain LG1700. Growth of the indicator strain M5a1 was monitored (culture OD_{620}) after addition to the indicated dilutions of a cloacin DF13 preparation treated with membranes prepared from strains LG1700 ($IutA^+$, ○) and CC118 ($IutA^-$, ●) and from cloacin DF13-insensitive mutants LG1701 (△), LG1702 (□), and LG1703 (◇).

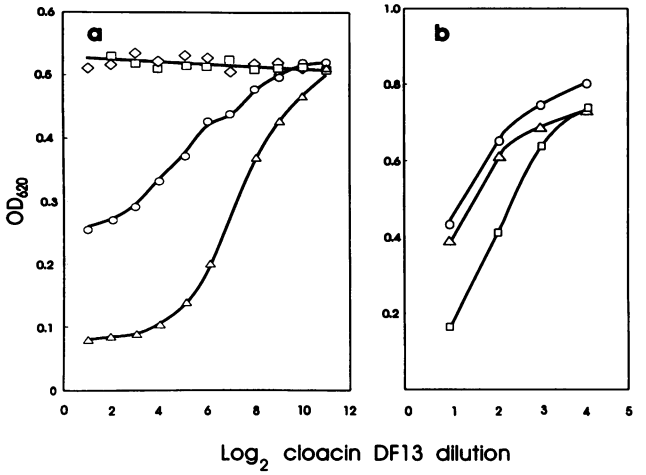

FIG. 3. Sensitivity to cloacin DF13 of *E. coli* strains altered in the activity of major outer membrane proteins. Bacterial growth was monitored (culture OD_{620}) after addition to the indicated dilutions of a cloacin DF13 preparation. (a) Strains tested were MC4100 (pLG141) ($OmpC^+$ $OmpF^+$, ○), MH760(pLG141) ($OmpC^-$ $OmpF^+$, △), MH1160(pLG141) ($OmpC^-$ $OmpF^-$, □), and MH1460(pLG141) ($OmpC^+$ $OmpF^-$, ◇). (b) Sensitivity of strain MC4100(pLG141) was assayed in the presence (○) and absence (△) of $ZnCl_2$ to inhibit OmpT protease activity, and compared with that of strain UT4400(pLG141) (*ompT*, □).

bactin at levels comparable to that of the cloacin DF13-sensitive parent strain LG1700. Moreover, preincubation of LG1702 and LG1703 cells with partially purified cloacin DF13 caused greater than 80% inhibition of aerobactin uptake, similar to the level of inhibition observed with the parental strain (Table 2). Mutants of classes 2 and 3 have normal receptor activity (despite the fact that the latter appears to express a slightly smaller protein than wild-type IutA [Fig. 1]) but are presumably defective in uptake of active bacteriocin; they are therefore more accurately termed cloacin DF13 tolerant.

Involvement of OmpF in cloacin DF13 sensitivity. To determine whether the apparent loss of porin proteins from the outer membranes of class 2 mutants was responsible for cloacin DF13 insensitivity, we examined strains carrying well-defined mutations at the *ompB* locus resulting in particular OmpC and OmpF phenotypes. Strains were transformed with plasmid pLG141 and tested for cloacin DF13 sensitivity essentially as described previously (19). As shown in Fig. 3a, strains MH1460 ($OmpC^+$ $OmpF^-$) and MH1160 ($OmpC^-$ $OmpF^-$) were insensitive to cloacin DF13; on the other hand, strain MH760 ($OmpC^-$ $OmpF^+$) showed susceptibility to cloacin DF13 markedly greater even than that of the parent strain MC4100. By using a gel system that resolves OmpF and OmpC proteins (18), it was observed that strain MH760 expresses significantly higher levels of OmpF (in the absence of OmpC) than the wild type (Fig. 4).

OmpT as a candidate for the 31-kDa protein absent from outer membranes of mutant class 3. The mature form of the outer membrane protease OmpT has approximately the same molecular mass, calculated from nucleotide sequence data, as the protein band absent from the class 3 mutant (9). The possibility arose, therefore, that OmpT may be responsible for proteolytic cleavage of cloacin DF13 as it passes through the membranes of susceptible *E. coli* cells. To test this, we determined the cloacin sensitivity of MC4100 (pLG141) in the presence and absence of 0.5 mM $ZnCl_2$, which completely inhibits OmpT activity in vitro (22) and in

TABLE 2. Uptake of radioactive aerobactin by cloacin DF13-insensitive mutants of strain LG1700

Strain	Aerobactin (cpm) uptake[a] by:	
	Untreated cells	Cloacin DF13-treated[b] cells
LG1700	1,219	194
LG1701	82	71
LG1702	1,315	205
LG1703	1,054	75
CC118	77	—[c]

[a] [^{14}C]aerobactin associated with cells after incubation at 37°C for 90 min.
[b] Cells were incubated with cloacin DF13 for 30 min at 37°C before the addition of [^{14}C]aerobactin. Untreated samples contained equivalent concentrations of bovine serum albumin.
[c] —, Not done.

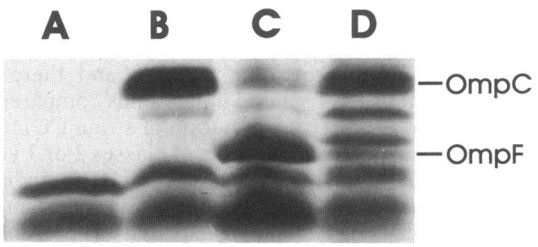

FIG. 4. Urea polyacrylamide gel electrophoresis to resolve major outer membrane proteins OmpF and OmpC. Strains tested were MH1160 ($OmpC^-$ $OmpF^-$, lane A), MH1460 ($OmpC^+$ $OmpF^-$, lane B), MH760 ($OmpC^-$ $OmpF^+$, lane C), and MC4100 ($OmpC^+$ $OmpF^+$, lane D).

vivo (1). Cloacin susceptibility was unaffected by the presence of $ZnCl_2$ (Fig. 3b), suggesting that OmpT is not involved in the lethal action of cloacin DF13. This was confirmed by the observation that the defined *ompT* mutant UT4400 (9) containing plasmid pLG141 was sensitive to killing by cloacin DF13 (Fig. 3b). Therefore the nature of cloacin insensitivity in the class 3 mutant remains unresolved.

DISCUSSION

The observation that cloacin DF13-resistant mutants of susceptible strains of *E. coli* were also unable to take up aerobactin provided the first evidence that aerobactin and cloacin DF13 share a common receptor (2). Among 14 spontaneous cloacin DF13-insensitive mutants examined in this study, the *iutA* type of mutation was indeed the most common, in all cases resulting in total absence of IutA protein from the outer membrane. Two additional classes of mutants were also identified on the basis of outer membrane protein profiles. Binding of both aerobactin and cloacin DF13 were unaffected in these two classes (which are therefore most accurately designated cloacin DF13-tolerant mutants), emphasizing that although the two very different ligands share a common receptor, their subsequent translocation across the outer membrane occurs by different processes. It has been known for some time that uptake of aerobactin (27), but not of cloacin DF13 (20), is dependent on the inner membrane protein TonB, which is thought to provide energy for translocation of the siderophore across the outer membrane (12).

The evidence provided here suggests roles for OmpF and for a 31-kDa outer membrane protein in the lethal action of cloacin DF13 for susceptible strains of *E. coli*. These proteins are not involved in the initial binding of cloacin DF13, nor presumably in its enzymic activity. Rather, they appear to be involved in the fragmentation and/or translocation of active cloacin DF13 across the cell envelope and into the cytoplasm of sensitive cells. The 31-kDa protein absent from mutant LG1703 has not yet been identified, but it is not the protease OmpT. Indeed, other alterations in the protein profile of LG1703, including conversion of the IutA protein from 74 kDa to about 71 kDa, might indicate increased protease activity in this mutant. Moreover, we cannot rule out the possibility that the 71-kDa form of the receptor may have lost translocation functions while retaining binding activity.

Class 2 mutants, represented by strain LG1702, have simultaneously lost the ability to express the two similar-sized porin proteins OmpF and OmpC. Synthesis of these proteins in *E. coli* is regulated by the *ompB* (*ompR envZ*) locus, which encodes the cytoplasmic protein OmpR and the inner membrane-associated EnvZ protein. EnvZ is thought to act as an environmental sensor that responds to changes in osmolarity of the surrounding milieu by switching OmpR between two functionally active states which separately act as inducers of the *ompC* and *ompF* gene promoters (10). Thus mutants of class 2 appear to be like strain MH1160 (*ompR101*), lacking active OmpR protein so that neither *ompC* nor *ompF* is expressed (23). Mutants of the MH1460 type (*envZ11*), in which the OmpR protein acts only on the *ompC* promoter (23), might be predicted, as indeed would mutants harboring mutations within the OmpF structural gene, but such mutants were not observed in this study.

Membranes of wild-type *ompB*$^+$ (*ompR*$^+$ *envZ*$^+$) *E. coli* contain both OmpC and OmpF; in circumstances where one of these proteins is synthesized at submaximal levels, however, there may be compensatory expression of the other to maintain the total porin content of the outer membrane (10). For example, expression of OmpC is preferred over that of OmpF in conditions of high osmolarity (24), such as in brain-heart infusion broth. Therefore the observation that cells grown in brain-heart infusion broth were less sensitive to cloacin DF13 than cells grown in nutrient broth, despite the fact that they both expressed the same number of receptor molecules per cell (13), may be explained in terms of the relative lack of OmpF in the membranes of the former. In addition, we have shown here that the OmpC$^-$ strain MH760(pLG141) is significantly more sensitive than the wild type to killing by cloacin DF13. MH760(pLG141) expresses compensatory high levels of OmpF, as has previously been demonstrated for this class of mutants (10). Thus we have demonstrated that sensitivity to cloacin DF13, like that previously reported for the closely related E colicins (17) correlates with expression of OmpF, but how the porin is involved in the process of cloacin killing is not yet known.

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