JOURNAL OF BACTERIOLOGY, July 1975, p. 96-101

Vol. 123, No. 1
Printed in U.S.A.

Genetics of Resistance to Colicins in *Escherichia coli* K-12: Cross-Resistance Among Colicins of Group B

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Received for publication 9 October 1974

This paper describes the isolation of mutants resistant to colicins of group B (i.e., colicins B, D, G, H, Ia, Ib, M, Q, S1, and V). The 145 mutants studied in detail can be divided into nine phenotypic classes, based on their colicin-resistance patterns. They include the previously isolated *tonA*, *tonB exbB* and *cir* mutants. Each of the different phenotypic classes of mutants has been partially characterized, and some approximately mapped. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis has revealed substantial changes in the composition of the outer membrane proteins of *exbB* and *tonB* mutants.

Colicins are antibacterial proteins produced by some strains of *Escherichia coli*. We recently described a collection of mutants containing representative strains selected as resistant to each of the known colicins (5). On the basis of the cross-resistance patterns of these mutants it was possible to divide the colicins into two groups, with none of the mutants isolated being resistant to colicins from both groups.

The colicins within a group have interrelated modes of action, as many of the mutants affect more than one colicin of the group. The characterization of the mutants resistant to the group A colicins (colicins A, E1, E2, E3, K, L, N, S4, and X) has been described in the accompanying paper.

This paper describes some of the properties of mutants resistant to colicins of group B (colicins B, D, G, H, Ia, Ib, M, Q, S1, and V). The 145 mutants studied in detail have been placed in nine phenotypic classes and representative mutants have been partially characterized and some approximately mapped. The mutants differ from those resistant to colicins of group A in that they never lack major outer membrane proteins, or show altered sensitivity to detergents or antibiotics.

These different properties of mutants resistant to colicins of the two groups supports the suggestion that the colicins of the two groups themselves have fundamentally different modes of action.

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used have all been described previously (5), with the exception of *E. coli* K-12, strain AG097 (Col B-K260), which produces colicin B alone (B. Stocker, personal communication) and JC3272 *tsx str his lys trp lac gal mal* λ^R. The media used were described in the same paper.

Triple-layer plate tests and electrophoresis of colicins. These techniques have all been described elsewhere (5).

Mapping techniques. Methods used for bacterial crosses and P1 transduction were as described previously (5).

Plate test for strains excreting colicin inhibitors. The plate test used by Guterman and Luria (13) was used to test for strains exhibiting the Exb phenotype.

Inhibition of colicins by enterochelin. The ability of enterochelin to interfere with the action of a particular colicin was tested by streaking the relevant colicinogenic strain across a nutrient agar plate and growing overnight at 37 C. The colicinogenic strain was killed by exposure to chloroform and the plate was overlayed with nutrient agar. Varying dilutions of enterochelin were streaked across the plate at right angles to the original colicinogenic streak and allowed to dry in. The plate was then overlayed with soft agar seeded with approximately 10^7 cells/ml of AB1133, the indicator strain. The ability of the indicator strain to grow across the colicin inhibition zone, at the spot where the enterochelin was cross-streaked, was interpreted as meaning that enterochelin interfered with the action of the particular colicin. We are grateful to I. G. Young for his gift of some enterochelin.

Membrane preparations. Whole cell envelope was prepared from cells grown in nutrient broth to late log phase (1-litre batches, 1.5×10^9 cells/ml). The cells were harvested by centrifugation at $5,000 \times g$ for 20 min, washed in 500 ml of 0.01 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 7.8, and centrifuged again. The pellet was transferred to a plastic bag, frozen by immersion in an alcohol-dry ice bath, and broken in an X-press (LKB Biotec) cooled to −25 C.

The cell envelope preparations were resuspended in

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30 ml of tris(hydroxymethyl)aminomethane-hydrochloride buffer, a speck (approximately 100 μg) of deoxyribonuclease (Sigma Chemical Co.) was added, and the preparations were left on ice for 30 min. Cell debris was removed by centrifugation at 5,000 × *g* for 20 min. $MgCl_2$ was added to 2 mM and the supernatant fluid was centrifuged (78,000 × *g*, 60 min) in a Spinco 30 rotor. The resultant pellet was resuspended in tris(hydroxymethyl)aminomethane-hydrochloride buffer, $MgCl_2$ was added to 2 mM, and the pellet centrifuged again at 78,000 × *g* for 60 min. The pellet was resuspended in distilled water, and the protein concentration was estimated by the method of Schacterle and Pollack (15).

Outer membrane preparations were prepared from the whole envelope preparation by using Triton X-100 to solubilize the cytoplasmic membrane. The method used was basically that of Schnaitman (16). *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid buffer was added to the whole envelope preparation to 0.01 M, an equal volume of 4% (vol/vol) Triton X-100 in 0.01 M *N*-2-hydroxyethyl-piperazine-*N'*-2'-ethanesulfonic acid buffer was added, and the sample was held for 15 min at 23 C. The preparation was centrifuged at 4 C in either a Spinco 40 (68,000 × *g*, 120 min) or Spinco 65 (161,000 × *g*, 60 min) rotor, the pellet was resuspended in distilled water, and the centrifugation was repeated. The resultant pellet was resuspended again in distilled water.

Three methods were used for solubilization of the above preparations in sodium dodecyl sulfate (SDS) before polyacrylamide gel electrophoresis. Method 1 was essentially that of Neville (14). Na_2CO_3 was added to 0.05 M and exposed to 8 mg of SDS per mg of protein at room temperature for 1 min before the addition of 10% by volume β-mercaptoethanol. The preparations were then dialyzed against upper gel buffer (14) overnight. Methods 2 and 3 are basically those described by Schnaitman (17). The sample, suspended at a concentration of about 10 mg of protein/ml in distilled water, was added to 2 volumes of a solution containing 3% (wt/vol) SDS, 0.15% (vol/vol) β-mercaptoethanol, and 7.5 mM ethylenediaminetetraacetic acid in 0.1 M sodium phosphate buffer, pH 7.2. The sample was then incubated for 2 h at 37 C under nitrogen, dialyzed overnight against a solution containing 48% (wt/vol) urea, 0.1% (wt/vol) SDS, 0.1% (vol/vol) β-mercaptoethanol and 0.5 mM ethylenediaminetetraacetic acid in 0.1 M sodium phosphate buffer, pH 7.2, and then heated for 5 min at 100 C (method 2). For method 3 preparations, the final dialysis and heating were omitted. All samples were stored frozen until used.

Polyacrylamide gel electrophoresis. Membrane samples prepared by method 1 were run on gels containing 11.1% total acrylamide (wt/vol) (0.1% [wt/vol] *N,N'*-methylenebisacrylamide), using a 3.2% (wt/vol) polyacrylamide (0.2% [wt/vol] bisacrylamide) stacking gel and buffers described by Neville (14).

Samples prepared by methods 2 and 3 were run on gels containing 7.5% (wt/vol) acrylamide, 0.2% (wt/vol) bisacrylamide and 0.5 M urea, in 0.1% (wt/vol) SDS in 0.1 M sodium phosphate buffer, pH 7.2, as described by Schnaitman (17).

Gels were stained overnight in Coomassie brilliant blue (0.025%) (14, 19) in fixative (methanol 45%, acetic acid 10%, water 45%) and destained in an aqueous solution of methanol (25%) and acetic acid (7.5%). Stained bands were recorded with a densitometer (Quick Scan Jr., Helena Laboratories Corp.).

RESULTS

Isolation of colicin-resistant mutants. Mutants have been selected as resistant to each of the colicins of group B, and checked for cross-resistance to all other colicins available. None of the mutants showed cross-resistance to any colicin of group A, confirming our previous finding (5).

Each of the 145 mutants studied in detail can be placed into one of seven phenotypic classes on the basis of their colicin resistance pattern. These include mutants with the colicin resistances reported for the well known TonA and TonB mutants (4, 9, 12). TonA mutants are known to be resistant to bacteriophages φ80, T1, and T5, and TonB mutants resistant to bacteriophages φ80 and T1 (4, 9). Therefore, all the mutants were checked for their resistance to bacteriophages φ80, T1, and T5 by cross-streaking. This allowed the differentiation of a further two phenotypic classes of mutants and the colicin and bacteriophage resistance patterns for the classes are shown in Table 1. One mutant of each class was selected as the type mutant strain and these are also listed in Table 1. The Cir and Ivt mutants are differentiated by the triple-layer plate test, as Cir mutants are receptor mutants for colicins Ia, Ib, and S1, whereas Ivt mutants are tolerant to these three colicins. Both classes of mutants are tolerant to colicins Q and V.

Excretion of colicin inhibitors. Guterman et al. (10, 12, 13) have shown that *tonB* (*exbA*) and *exbB* mutants excrete enterochelin, an iron chelator that has the ability to interfere with the action of colicins B and I (11).

By using a plate test, we tested the type mutant strains and several other strains from each of the mutant classes for the excretion of colicin inhibitors. None of them produced a substance that could be seen to inhibit colicins G, H, M, S1, Q, or V. Only occasionally could we detect inhibition of colicins Ia and Ib, and the results were not consistent. Several of the strains, however, excreted inhibitors of colicins B and D. The results are summarized in Table 2. TonB mutants excreted inhibitors of both colicins B and D, as did what are presumably the ExbB mutants, previously isolated by Guterman (12). The ExbC mutant, P535, comprises a new group. It produces a substance that interfered with colicin B action, but did not inhibit colicin D action. The Cbt mutants did

TABLE 1. *Mutants resistant to colicins of group B*

Phenotypic class	Mutant example	No. studied	Colicin resistance pattern[a]									
TonA	P1205	5					M					(φ80, T1, T5)
TonB	P585	17	B	D	G	H	M	I	S1	Q	V	(φ80, T1)
ExbB	P575	70	B	D	G	H	M	I	S1	Q	V	
ExbC	P535	1	B	D	G	H	M					
Cbt[b]	P295	16	B	D								
Cmt	P1209	4					M					
Cir	P625	10						I^R	$S1^R$	Q	V	
Ivt	P645	12						I	S1	Q	V	
Cvt	P1235	10								Q	V	

[a] The colicins listed are those to which the particular mutants are resistant. They have been tested against, and are sensitive to, all other colicins used. Cir mutants lack receptor for colicins I and S1. All other resistance is due to tolerance.

[b] New designations are derived as follows: Cbt (colicin B tolerant), Cmt (colicin M tolerant), Cir (colicin I resistance), Ivt (colicin I and V tolerance), Cvt (colicin V tolerant).

TABLE 2. *Ability of mutants to excrete colicin inhibitors*

Phenotypic class	Strain	Colicin inhibitory excretion[a]	
		Exb[b]	Exd[c]
TonA	P1205	S	S
TonB	P585	+	+
ExbB	P575	+	+
ExbC	P535	+	−
Cbt	P295	−	−
Cmt	P1209	S	S
Cir	P625	S	S
Ivt	P645	S	S
Cvt	P1235	S	S

[a] S, sensitive to colicin; +, colicin resistant, inhibitor excreted; −, colicin resistant, inhibitor not excreted.

[b] Exb, excretes an inhibitor of colicin B.

[c] Exd, excretes an inhibitor of colicin D.

not appear to produce any colicin inhibitory substances. The remaining mutants were all sensitive to colicins B and D, and did not produce any detectable inhibitor. Some ExbB mutants have been reported to require methionine (12) and our type ExbB strain, P575, was also found to require methionine. The other type strains had only the parental strain requirements.

Inhibition of colicin action by enterochelin. To check that enterochelin itself would inhibit these colicins, we checked the ability of enterochelin to interfere with their action in a plate test. Both colicins B and D were inhibited, and both at approximately the same level of enterochelin (100 to 1,000 μg/ml).

Characterization of mutants. Mutants resistant to colicins of group A often show changed sensitivity to antibiotics, detergents, and surfactants (5). The mutants listed in Table 1 were therefore tested with Multodisks for altered susceptibility to the same range of antibiotics used previously (5). None of them showed any change in sensitivity. In addition, none of them showed super-sensitivity to Triton X-100, SDS, ethylenediaminetetraacetic acid, phenethyl alcohol, or sodium deoxycholate, in contrast to the mutants selected as resistant to colicins of group A (5).

The TonB mutant, P585, did not appear to have the greatly increased sensitivity to $CrCl_3$ in nutrient broth reported previously (12), when compared with AB1133, its parent strain. The discrepancy may be due to the use of different strains or differences in the nutrient broth used. The viable count of both the TonB mutant, P585, and its parent strain, AB1133, falls dramatically on the addition of 400 μM $CrCl_3$. Although P585 does appear to be slightly more sensitive to $CrCl_3$, the difference in percentage of survival is not great (0.012% for AB1133, compared with 0.004% for P585, after 1 h).

Map location of colicin resistance loci. Although we have not confirmed its map position, the TonA mutant, P1205, shows the characteristics reported previously (1, 2, 6) of those mutants mapping at 3 min on the genetic map,

resistance to colicin M and bacteriophages T1 and T5. The TonB mutant, P585, has a colicin resistance locus co-transducible with *trp*, as expected (9). We find *tonB* to be 62% co-transducible with *trp*.

The colicin resistance locus of the Cbt mutant, P295, was transferred by both HfrH and HfrC, and appears from an examination of the recombinant classes to be between *lac* (9 min) and the HfrC origin (12 to 14.5 min). It is co-transducible with *lip* (14.6 min) at a frequency of 2.6%, which means that it maps at either 13.2 or 16 min on the genetic map (20). Since a locus mapping at 16 min would not be transferred by HfrC, *cbt* must be located near 13.2 min.

The type strains of the ExbB, ExbC, Cir, and Ivt mutant classes (P575, P535, P625, and P645) all have colicin resistance loci that are transferred by HfrH, when *his*$^+$ recombinants are selected. The loci in P625 and P645 are linked to *his*, with 88 and 85% *his*$^+$ recombinants, respectively, being colicin sensitive.

The resistance loci in the Cmt mutant, P1209, and the Cvt mutant, P1235, are not transferred by HfrH when selecting for *thr*$^+$ or *his*$^+$ recombinants, or by HfrC when selecting for *pro*A$^+$ or *arg*E$^+$ recombinants. The *cmt* and *cvt* loci therefore presumably map somewhere between *his* (38.5 min) and *argE* (78.5 min).

SDS-polyacrylamide gel electrophoresis. Whole cell envelope samples (methods 1, 2 and 3) and outer membrane samples (methods 2 and 3) from each of the type mutants were run on SDS-polyacrylamide gels as described in Materials and Methods.

When cell envelope preparations, prepared by method 3, of the TonB mutant, P585, and ExbB mutant, P575, were compared with those of the parent strain, AB1133, it was immediately apparent that a new high-molecular-weight peak had appeared on scans of the gels loaded with membrane preparations from the two mutants. It has been suggested that proteins in membrane preparations prepared by method 2 run in a position that is more indicative of their true molecular weight, since they are fully unfolded or denatured (17). Whole membrane preparations of P585 and P575 prepared by method 2 showed two distinct additional peaks running in approximately the same position as the single peak seen in samples prepared by method 3. Scans of gels loaded with whole cell envelope preparations from P585 and P575, solubilized by method 1, also showed the appearance of two additional peaks.

These protein species do not appear to be part of the cytoplasmic membrane, since they are not solubilized by Triton X-100 (16). The scans of gels loaded with outer membrane preparations, prepared by methods 2 and 3, from AB1133 and P575, are shown in Fig. 1. The results for P585, the TonB mutant, were identical to those of P575.

None of the other mutants isolated showed any major difference in the protein composition of either the whole or outer membrane preparations.

Sensitivity to lipopolysaccharide-specific bacteriophage. Several of the mutants resistant to colicins of group A showed a changed pattern of sensitivity to the two lipopolysaccharide-specific bacteriophages C21 and U3 (5). None of the mutants resistant to colicins of group B showed any change in the pattern of sensitivity, suggesting an absence of drastic changes to the lipopolysaccharide.

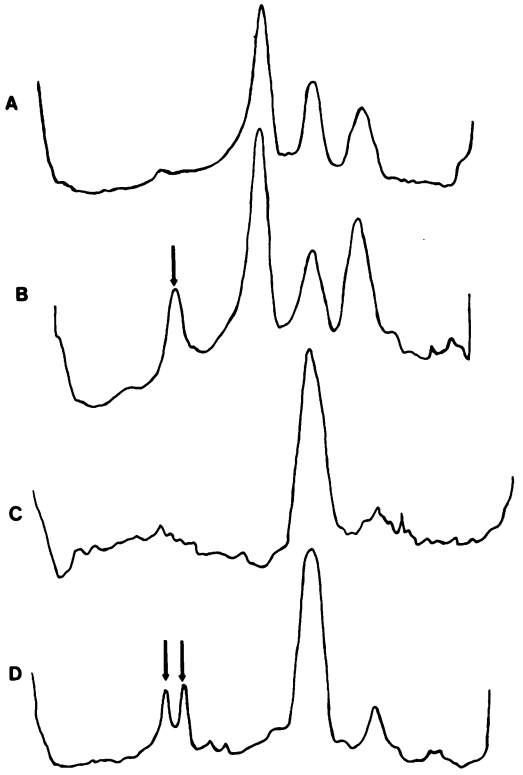

FIG. 1. *Comparison, by densitometry, of stained bands of outer membrane proteins, run using the SDS-polyacrylamide gel system described by Schnaitman (17). Figures 1A and B show the outer membrane proteins of AB1133 and P575 (ExbB), respectively, solubilized by method 2. Figures 1C and D show the outer membrane proteins of AB1133 and P575, respectively, solubilized by method 3. The membrane preparations and gel systems used are described in the text.*

DISCUSSION

Mutants resistant to combinations of colicins of group B have been described before, but only partially characterized. Gratia (9) described the isolation of mutants partially resistant to colicin B, and resistant to bacteriophages T1 and T5, that mapped at or near *tonA*. The apparent partial resistance to colicin B may have been due to resistance to colicin M, as *E. coli* CA18, the strain used here as a producer of colicin B, also produces colicin M (5, 18). In addition, he described a series of mutants mapping hear *trp* on the genetic map, and still another set, mapping between *his* and *str*, some of which had a requirement for methionine. The two groups included mutants resistant to colicins B, I, and V, and phage T1; or to colicins B, I, and V; B and I; I and V; or to B alone, I alone and V alone.

Guterman (12) has described the isolation of mutants resistant to colicins B, I, and V; B and I; I and V; B and V; B alone; I alone and V alone. Some of these had a requirement for methionine, and some excreted enterochelin.

The methionine requirement of some of the mutants isolated by Gratia (9) suggest that they may in fact be the same as the methionine-requiring ExbB mutants described by Guterman (12), mapping near *serA*, and our ExbB mutant also requires methionine.

More recently (3) two mutants, one (*tolI*) tolerant to colicins Ia and Ib, and the other (*cir*) a receptor mutant for colicin I, have been described. Both mutants were sensitive to colicin B.

The mutants isolated here include classes with all the colicin resistance patterns previously observed, except for those classes described as resistant to colicins B and I (9, 12), B and V (10), and I alone (9, 12), although in several cases we have extended the known phenotypes by studying more colicins. We have differentiated two classes of mutants resistant to colicin B, but sensitive to colicins I and V (Cbt and ExbC), and two different classes of mutants resistant to colicins I and V, but sensitive to colicin B (Cir and Ivt). The Cir mutants described here are almost certainly the same as those described by Cardelli and Konisky (3). The Cbt, ExbC, and Ivt classes have not been properly defined or characterized previously. In addition, we have failed to isolate the *tolI* mutant described in the same paper, as our only mutants that are tolerant to colicin I and sensitive to colicin B (Ivt) map near *his*, and not near 89—1 min as reported for *tolI*. The Cmt mutants may have been isolated before by Gratia (9) and described as partially tolerant to colicin B, for the reasons described above.

The mutants resistant to colicins of group B show none of the changed sensitivity patterns to antibiotics, detergents, and surfactants that often accompany mutation to resistance of colicins of group A (5). Apart from the *tonA* and *tonB* mutants, the mutants resistant to group B colicins show a complete lack of cross-resistance to a wide range of bacteriophage (R. E. W. Hancock, J. K. Davies, and P. Reeves, manuscript in preparation), unlike the mutants resistant to group A colicins, supporting the division of colicins into these two basic groups.

We have been unable to differentiate, either on the colicin resistance patterns of the mutants isolated, or on zone morphology and electrophoretic mobility (unpublished data), between several of the colicins. Colicins Q and V are almost indistinguishable, having only a very slight difference in electrophoretic mobility, as reported previously (18). It was not possible to differentiate colicins Ia and Ib from S1 with the techniques used and colicins G and H also appear to be very similar. This last point has been commented on before by Fredericq (7), who reclassified them both as a single colicin, called P. We have attempted to cross-check that the strains used here are the ones originally described, and have obtained several stocks of the same strain, or several strains producing the one colicin, from different sources (5). In addition, we have been unable to identify the second group B colicin produced by *E. coli* CA7, in addition to colicin V (5). Fredericq has shown that mutants of CA7 resistant to phages T1 and T5 produce colicin M (6). However, the second colicin which we find CA7 to produce is indistinguishable from colicin V on the colicin resistance pattern of the mutants isolated. It does differ from colicin V in its electrophoretic mobility and the morphology of its zone of inhibition (5). In addition, *tonA* mutants, which are resistant to colicin M (1, 2, 6), are sensitive to this unidentified colicin, which is not produced by any other colicin V producer (5).

The similarity between colicins B and D has not been commented on before. They are reported to have different modes of action. Colicin D inhibits protein synthesis (21), while colicin B is said to affect energy metabolism, as simultaneous inhibition of deoxyribonucleic acid and ribonucleic acid protein synthesis is observed (11). However, the colicin B preparations used in that study were contaminated with colicin M, and it is possible that colicins B and D are very similar. Colicin D has also been reported to have a very wide activity spectrum, and colicin

D-resistant mutants are reported to be very rare (7). Both of these observations may have been due to the use of *E. coli* CA23, which we have shown to produce colicin X, in addition to colicin D (5). Colicins B and D are differentiated, however, by the ExbC strain, P535, which inhibits colicin B but not colicin D under our test conditions.

This raises the interesting possibility that P535, and perhaps also the TonB and ExbB strains, produces a colicin inhibitor other than, or in addition to, enterochelin, as enterochelin inhibits the action of colicins B and D at approximately the same concentration. However, the studies we report all relate to inhibition on agar plates, and need to be confirmed by using purified colicins and inhibitors.

The different activity spectrum of the inhibition produced by the ExbC strain, P535, correlates well with the observed changes in protein composition of the outer membranes of these mutants. Of the three classes of mutants that excrete inhibitors, only the ExbC mutant showed no substantial alterations. It would seem, therefore, that the observed changes are characteristic of mutants that excrete enterochelin in large amounts.

It has been suggested that *tonB* mutants cannot accumulate enough iron to repress enterochelin synthesis and are therefore acting as constitutive (12). If this were due to the observed changes in the composition of the outer membrane proteins, one would expect a similar situation to occur in ExbB mutants, which show identical changes. Yet ExbB strains can grow on media in the absence of additional iron (12). It is hard to understand why identical changes in the outer membranes of these two mutants should lead to different pleiotropic properties in the two strains.

Of interest also is the fact that the *cbt* locus appears to map very close to the *ent* cluster of loci, which are involved in enterochelin synthesis. The elucidation of the gene products of the *tonB*, *exbB*, *exbC*, and *cbt* loci would help considerably in our understanding of this complex phenomenon.

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