JOURNAL OF BACTERIOLOGY, Jan. 1979, p. 653-657
0021-9193/79/01-0653/05$02.00/0

Vol. 137, No. 1

Genetic and Physiological Studies on the Relationship Between Colicin B Resistance and Ferrienterochelin Uptake in *Escherichia coli* K-12

MARK A. McINTOSH,† SARA S. CHENAULT, AND CHARLES F. EARHART*

Department of Microbiology, University of Texas at Austin, Austin, Texas 78712

Received for publication 6 October 1978

The *Escherichia coli* gene for the ferrienterochelin uptake and colicins B and D receptor protein is located at approximately 13 min, adjacent to or among genes for enterochelin biosynthetic enzymes. The two receptor functions (colicin and siderophore) are separable by mutation.

The *Escherichia coli* outer membrane protein believed to function as the receptor for ferrienterochelin (20) has a molecular weight of 81,000 and is designated 81K (2). The 81K protein also serves as the receptor for colicins B and D (21), and mutations affecting 81K can be isolated on the basis of colicin B resistance. Mutations in genes designated *feuB* (3) and *cbr* (20) result in the absence of 81K and cause defective ferrienterochelin uptake. Both *feuB* and *cbr* mutations map at approximately 13 min (4, 18, 20), as do mutations in the recently described *fepA* gene (22); *feuB*, *cbr*, and *fepA* all probably refer to the same locus. This report provides evidence that the structural gene for the 81K polypeptide is located near 13 min.

Colicin B-insensitive mutants were isolated (15) from strain AB1515 (F^- *thi proC leu trpE purE rpsL tonA*) and from RW193, a $purE^+$ *entA* $tonA^+$ derivative of AB1515 (12). Sixty-five spontaneous colicin B-insensitive mutants derived from RW193 (UT100-UT6500) and 24 from AB1515 (UT6600-UT8900) were tested for several properties, including sensitivity to other group B colicins and to bacteriophages ϕ80 and T5 and receptor activity for colicins B and D; total membrane preparations from 53 of the mutants were analyzed by polyacrylamide gel electrophoresis to test for the presence of 81K. A minimum of 28 independent mutants, isolated from a total of 13 overnight cultures, were identified. Five of these (UT200, UT1100, UT6700, UT7100, and UT7600) had gained colicin and phage resistance characteristics typical of *tonB* mutants (5) and were not studied further.

Adsorption of colicin B by the insensitive mutants was investigated by two techniques (15). Two classes of insensitive mutants were distinguished: tolerant mutants were able to adsorb colicin B, indicating the presence of its receptor, but were not killed by its action; resistant (receptor) mutants were unable to adsorb the colicin. Seven mutants isolated from RW193 were classified as tolerant by both procedures, and one from AB1515 showed this phenotype. The two adsorption assays were also used to screen each of the insensitive mutants for its ability to adsorb colicin D; adsorption results for colicin D were identical to those obtained with colicin B. However, the colicin D-producing strain (col D-CA23), provided by E. M. Lederberg, is reported to also carry colicin X, so these latter results are not definitive.

Typical results obtained when total membrane preparations from representative mutants were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis are shown in Fig. 1. (J. B. Neilands kindly supplied strain RWB18, a colicin B-resistant mutant of RW193 which has been recently characterized [6]; it is included here for comparative purposes.) A direct correspondence was noted between the ability of a mutant to adsorb colicin B and the presence of 81K. That is, tolerant mutants (UT100, UT300, UT2200, UT2400, UT2500, and UT6400) all had a protein band corresponding to 81K, whereas the resistant mutants, with the exception noted immediately below, lacked this polypeptide. An unusual resistant mutant was detected by gel electrophoresis; UT500 possesses a protein corresponding to 81K in that it is regulated by the iron content of the medium (data not shown), but the relevant protein of UT500 has a faster migration rate in these gels. The mutation resulted in the loss of colicin receptor activity even though the protein had a decrease in apparent molecular weight of only 2,000 to 3,000.

Uptake of ^{55}Fe was examined in representatives of each of the classes of colicin-insensitive mutants (Fig. 2A). Iron uptake in all strains possessing 81K, including the tolerant mutant UT2200, was wholly dependent upon the pres-

† Present address: Department of Biochemistry, University of California, Berkeley, CA 94720.

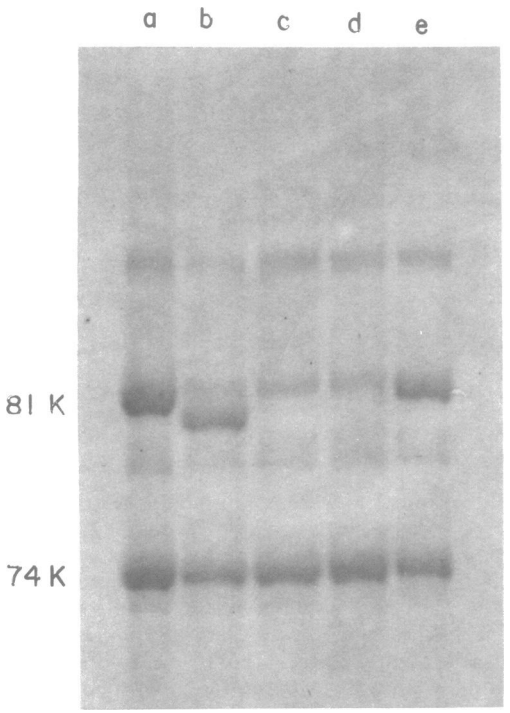

FIG. 1. *Membrane proteins of colicin B-insensitive mutants of RW193. Only proteins with molecular weights ranging from approximately 65,000 to 100,000 are shown. Cells were grown overnight at 37°C in T medium (14) supplemented with 100 μg of Casamino Acids per ml. Total membranes were isolated (9), and samples containing 40 μg of protein were analyzed on a 28-cm 7.5% polyacrylamide slab gel (10) run at 300 V for 6 h. Protein determinations and staining were as before (14). The strains examined were as follows: (a) UT300, tolerant; (b) UT500, altered 81K; (c) UT400, resistant; (d) RWB18, resistant; (e) RW193, parent.*

ence of enterochelin. The receptor mutant UT2300 was unable to transport ^{55}Fe regardless of the presence or absence of enterochelin. Interestingly, the altered-81K mutant UT500 was still capable of transporting ferrienterochelin; this strain thus possesses an outer membrane protein that is defective in colicin B adsorption but which still functions in ferrienterochelin transport.

The ability of outer membrane from resistant mutants UT2300 and UT500 to bind ferrienterochelin was tested directly and compared with that of RW193 (Fig. 2B). Membrane from strain RW193 bound approximately four times more ferrienterochelin than UT2300 and about twice more than UT500 membrane. The reduction in ferrienterochelin binding capacity of UT500 membrane as compared with that of membrane from its parent has been confirmed by microcolumn assay (6) (M. A. McIntosh, unpublished data). Several control experiments indicated that the observed ferrienterochelin-UT2300 membrane binding was nonspecific: (i) cytoplasmic (inner) membrane from RW193 bound the same amount of iron-siderophore complex as did UT2300 outer membrane; (ii) outer membrane from strain RWB18, which does not bind ferrienterochelin when measured by the microcolumn assay (6), behaved identically to UT2300 membrane; and (iii) the binding capacity of RW193 outer membrane was not reduced to that of UT2300 even when isolated from RW193 cells grown in M9 medium supplemented to 10 μM $FeCl_3$.

Conjugation experiments indicated that the resistant mutations were localized very near *entA*, probably in or near the *ent-fep* gene cluster, as has been determined for other resistant mutations (20); the mutants were therefore tentatively designated *cbr*. The *cbr* gene was mapped by P1 transduction with *cbr* mutants derived from RW193 (*purE*$^+$ *entA*) as donors and AB1515 (*cbr*$^+$ *purE entA*$^+$) as the recipient or RW193 as the donor and *cbr* mutants from AB1515 as recipients; 200 *purE*$^+$ transductants were selected in each experiment and scored for both the *cbr* and the *entA* markers. The results of some of these experiments are presented in Table 1. The minimal cotransduction frequencies for *entA* and *cbr* with respect to *purE* were 4 and 10%, respectively, which, when analyzed by the equation of Wu (23), correspond to map distances of 1.3 and 1.0 min. All the *purE*$^+$ *entA* cotransductants also carried the appropriate *cbr* marker, indicating that the gene order is *purE-cbr-entA*. In addition, the *cbr* mutation in UT500, which gives rise to an electrophoretically altered 81K and presumably results from a mutation in the structural gene, maps 0.9 min from *purE*. AB1515 *purE*$^+$ *cbr* transductants from this experiment produce the altered 81K polypeptide electrophoretically identical to that present in UT500, and, conversely, all *purE*$^+$ *cbr*$^+$ transductants had a normal 81K protein. These data indicate that the *cbr* locus is the structural gene for 81K.

Many of the mutations mapped had enhanced cotransduction frequencies for both *entA* and *cbr* (e.g., UT1500, UT2300, UT4400, and UT5600). The degree of increase in cotransfer with *purE* was similar for both markers, suggesting that the lesions resulting in the absence of 81K in these mutants were deletions of considerable size located between *cbr* and *purE*. Nutritional studies and reversion data also suggest that these mutations are deletions. The procedure and *Salmonella typhimurium* strains of Luckey et al. (13) were used to determine that

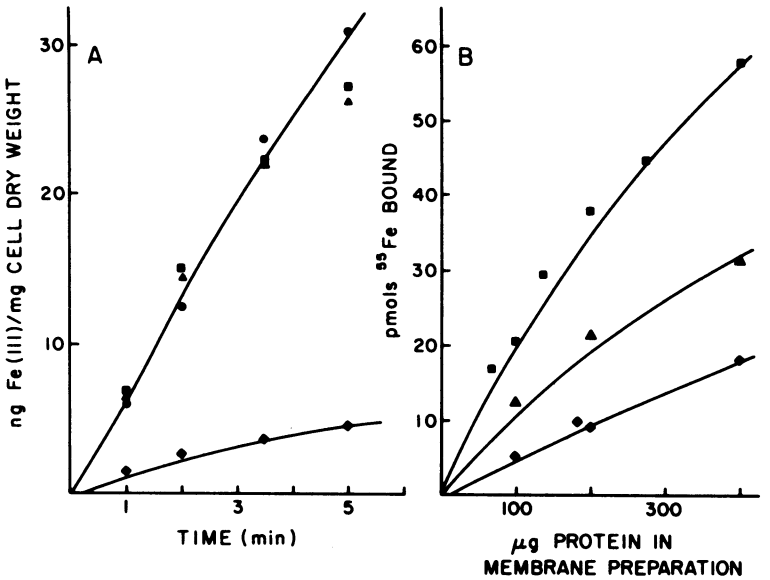

FIG. 2. *Uptake and binding of ^{55}Fe-enterochelin by colicin-resistant mutants of E. coli K-12 strain RW193. (A) Uptake. The procedure used was based on those described previously by Langman et al. (11) and Pugsley and Reeves (19). Cells were prepared as described elsewhere (19), except that appropriately supplemented iron-depleted M9 Medium (16), rather than medium A, was used for growth of overnight cultures and subcultures and for uptake medium. Assays were performed at 37°C in plastic tubes with vigorous shaking. At zero time, 1.5 ml of a mixture containing nitrilotriacetic acid, ^{55}Fe, and enterochelin prepared in iron-depleted M9 was added to 1.5 ml of cells (1.5 × 10^9 to 2 × 10^9) in uptake medium; final concentrations were 100 μM nitrilotriacetic acid, 1 μM ^{55}Fe (780 cpm/pmol), and 2 μM enterochelin. Samples (0.5 ml) were diluted into 3 ml of 10 mM ethylenediaminetetraacetic acid (pH 7) and filtered immediately through Gelman GA-6 membrane filters (pore size, 0.45 μm) which had been presoaked in 10 mM ethylenediaminetetraacetic acid. The filters were washed twice with 10 ml of 0.85% (wt/vol) NaCl, dried, placed in scintillation vials, and counted after the addition of toluene-Permaflour (Packard) scintillation fluid. In the absence of enterochelin, the level of Fe(III) accumulated was less at all time points than that present in zero-time samples containing enterochelin [1 to 2 ng of Fe(III) per mg of cell dry weight]. Strains examined were: ■, RW193; ●, colicin B-tolerant strain UT2200; ▲, altered 81K strain UT500; and ◆, colicin B-resistant strain UT2300. (B) Binding of ferrienterochelin to outer membrane preparations. The procedure of Ichihara and Mizushima (8) was used with two modifications. Outer membrane fractions were isolated by isopycnic sucrose density gradient centrifugation (17), and appropriately supplemented iron-depleted M9 was utilized as the growth medium, iron-binding medium, and washing medium. Symbols for the three strains examined are as in (A).*

TABLE 1. *Transductional analysis of relationships among purE, cbr, and entA genes*

Donor	Recipient	% Cotransduction with *purE*$^+$	
		cbr	*entA*
RW193	UT6600	14.2	4.9
	UT6900	10.0	4.7
UT500	AB1515	16.2	4.9
UT700		14.3	4.4
UT1500		38.5	19.2
UT2300		33.0	12.2
UT4400		54.4	28.3
UT5600		52.2	17.4

UT1500, UT2300, UT4400, UT5600, and UT6100 were unable to produce enterochelin even when provided with 2,3-dihydroxybenzoic acid. This is consistent with the idea that the mutations in these strains have extended into *ent* genes responsible for later steps in enterochelin biosynthesis. In contrast, the tolerant mutant UT2200 and two resistant mutants (RWB18 and UT700) were able to synthesize enterochelin when the *entA* lesion was bypassed by adding 2,3-dihydroxybenzoic acid. No true revertants of any resistant mutant, regardless of its ability to synthesize enterochelin from 2,3-dihydroxybenzoic acid, have been isolated. Twelve resistant mutants, including UT500, have been tested for reversion by plating on iron-deficient medium (15). Colonies appear with a frequency of 10^{-6}; 12 to 16 of these colonies derived from each mutant tested were screened for colicin B sensitivity, and in no case was sensitivity restored. These pseudorevertants have been described elsewhere (15).

Additional transductions were performed by

using UT2300 as the donor and W1485 (*lip-2*) as the recipient. Lip$^+$ transductants were selected; all *lip*$^+$ *cbr* transductants were *entA*, as expected, and the transduction frequencies indicated that *cbr* and *entA* are, respectively, 0.8 and 0.5 min from *lip*. In addition to confirming that *cbr* and *entA* are 0.3 min apart, the results were interesting for several reasons: (i) UT2300 is a large deletion and yet *lip*$^+$ *cbr* transductants were readily attained, and (ii) 10 of 62 *lip*$^+$ *cbr* transductants were able to synthesize enterochelin when provided with 2,3-dihydroxybenzoic acid.

Mutations that resulted in tolerance to colicins B and D were mapped by P1 transduction and also found to cotransduce with *purE* (data not shown). The cotransduction frequencies showed that the mutations in these strains (UT2200, UT2400, UT2500) are located approximately 1.0 min from *purE*, suggesting that they are located in or very near the structural gene for 81K.

The structural gene for 81K is located between *purE* and *entA*; it is 0.3 min from *entA* and is therefore situated at approximately 13 min on the *E. coli* map (1). Assignment of a structural gene role for this locus was possible from studies of UT500, a resistant mutant which synthesized an 81K polypeptide of altered mobility in sodium dodecyl sulfate-polyacrylamide gels. Evidence that the relevant polypeptide in UT500 is related to 81K is as follows: its abundance is regulated by iron, normal 81K and altered 81K were never present together in outer membrane, outer membrane from UT500 bound ferrienterochelin but to a lesser degree than membrane containing normal 81K, and the ferrienterochelin uptake rate of UT500 cells was equal to that of its parent. UT500 failed to bind colicin B, but there is a precedent for separation of 81K receptor functions (see below). Also, the fact that all mutants isolated by us and others which specifically confer colicin B resistance or tolerance bear mutations mapping near 13 min is circumstantial evidence that this chromosomal region contains the 81K structural gene. It is likely that the locus studied is identical to one or all of the genes designated *cbr*, *feuB*, and *fepA*, but definitive proof will require complementation studies.

Most, if not all, of the resistant mutants isolated in this study may contain deletions. Transductional analyses indicate that many of the mutants have lost DNA, and there was a direct correspondence between the amount of DNA apparently lost and the ability to synthesize enterochelin from 2,3-dihydroxybenzoic acid. Also, it was impossible to isolate true revertants of any resistant mutant; however, the sensitivity of these tests was reduced by the high frequency at which suppressor mutations occurred. The *E. coli* genome contains an IS3 insertion sequence between *purE* and *fep* (7); this IS3 or an additional, thus far undetected, insertion sequence may (i) be the site of the end points of the deletions and (ii) account for the prevalence of deletions in this region. Genetic analyses indicate that some of the deletions are large (0.3 to 0.7 min; UT1500, UT2300, UT4400, and UT6600). The existence of these mutants argues against the presence of any essential genes in the chromosomal region extending from approximately 12.3 to 13 min of the map, a region which at present is known to contain only the *supQ* and *stsB* genes in addition to *cbr* and possibly some genes for enterochelin biosynthesis. If UT500 is a small deletion, the carboxyl terminus of 81K is essential for colicin B receptor activity but not for ferrienterochelin uptake.

This work is consistent with previous studies regarding the receptor properties of the 81K polypeptide. We here demonstrated that the two receptor functions of 81K are separable by mutation (UT500). This finding is in accord with the report (6) that ferrienterochelin binding activity and colicin B binding activity differ in sensitivity to thermal inactivation. Colicin D as well as colicin B use 81K as a receptor, and both colicins appear to recognize the same region on the receptor (21). This conclusion is supported by our results that, with the caveat mentioned above, all of the insensitive mutants, including UT500, behaved identically with respect to colicin B and colicin D binding.

This research was supported by grant BMS75-07693 from the National Science Foundation. The bulk of the data were collected while M.M. was a Public Health Service predoctoral trainee (1 T32 GM-07126) from the National Institute of General Medical Sciences.

We are indebted to J. R. Walker for his interest and advice regarding the mapping studies and this manuscript, to J. R. Pierce for performing the binding studies, and to D. G. Moore for enterochelin.

LITERATURE CITED

1. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116–167.
2. **Braun, V., R. E. W. Hancock, K. Hantke, and A. Hartman.** 1976. Functional organization of the outer membrane of *Escherichia coli*: phage and colicin receptors as components of iron uptake systems. J. Supramol. Struct. **5**:37–58.
3. **Hancock, R. E. W., K. Hantke, and V. Braun.** 1976. Iron transport in *Escherichia coli* K-12: involvement of the colicin B receptor and of a citrate-inducible protein. J. Bacteriol. **127**:1370–1375.
4. **Hancock, R. E. W., K. Hantke, and V. Braun.** 1977. Iron transport in *Escherichia coli* K-12: 2,3-dihydroxybenzoate-promoted iron uptake. Arch. Microbiol. **114**: 231–239.
5. **Hantke, K., and V. Braun.** 1975. Membrane receptor dependent iron transport system in *Escherichia coli*. FEBS Lett. **49**:301–305.
6. **Hollifield, W. C., Jr., and J. B. Neilands.** 1978. Ferric

enterobactin transport system in *Escherichia coli* K-12. Extraction, assay, and specificity of the outer membrane receptor. Biochemistry **17**:1922–1928.

7. **Hu, S., E. Ohtsubo, and N. Davidson.** 1975. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*: structure of F13 and related F-primes. J. Bacteriol. **122**:749–763.
8. **Ichihara, S., and S. Mizushima.** 1978. Identification of an outer membrane protein responsible for the binding of the Fe-enterochelin complex to *Escherichia coli* cells. J. Biochem. **83**:137–140.
9. **Inouye, M., and J. P. Guthrie.** 1969. A mutant which changes a membrane protein of *E. coli*. Proc. Natl. Acad. Sci. U.S.A. **64**:957–961.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Langman, L., I. G. Young, G. E. Frost, H. Rosenberg, and F. Gibson.** 1972. Enterochelin system of iron transport in *Escherichia coli*: mutations affecting ferric-enterochelin esterase. J. Bacteriol. **112**:1142–1149.
12. **Leong, J., and J. B. Neilands.** 1976. Mechanisms of siderophore iron transport in enteric bacteria. J. Bacteriol. **126**:823–830.
13. **Luckey, M., J. R. Pollack, R. Wayne, B. N. Ames, and J. B. Neilands.** 1972. Iron uptake in *Salmonella typhimurium*: utilization of exogenous siderochromes as iron carriers. J. Bacteriol. **111**:731–738.
14. **McIntosh, M. A., and C. F. Earhart.** 1976. Effect of iron on the relative abundance of two large polypeptides of the *Escherichia coli* outer membrane. Biochem. Biophys. Res. Commun. **70**:315–322.
15. **McIntosh, M. A., C. L. Pickett, S. S. Chenault, and C. F. Earhart.** 1978. Suppression of iron uptake deficiency in *Escherichia coli* K-12 by loss of two major outer membrane proteins. Biochem. Biophys. Res. Commun. **81**:1106–1112.
16. **Miller, J. H.** 1972. Experiments in molecular genetics, p. 431. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
17. **Osborn, M. J., J. E. Gander, E. Parisi, and J. Carson.** 1972. Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. Isolation and characterization of cytoplasmic and outer membrane. J. Biol. Chem. **247**:3962–3972.
18. **Pugsley, A. P.** 1977. Map location of the *cbr* gene coding for production of the outer membrane receptor for ferrienterochelin and colicins B and D in *Escherichia coli* K-12. FEMS Lett. **2**:275–277.
19. **Pugsley, A. P., and P. Reeves.** 1976. Iron uptake in colicin B-resistant mutants of *Escherichia coli* K-12. J. Bacteriol. **126**:1052–1062.
20. **Pugsley, A. P., and P. Reeves.** 1977. The role of colicin receptors in the uptake of ferrienterochelin by *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **74**: 903–911.
21. **Pugsley, A. P., and P. Reeves.** 1977. Comparison of colicins B-K260 and D-CA23: purification and characterization of the colicins and examination of colicin immunity in the producing strains. Antimicrob. Agents Chemother. **11**:345–358.
22. **Wookey, P., and H. Rosenberg.** 1978. Involvement of inner and outer membrane components in the transport of iron and in colicin B action in *Escherichia coli*. J. Bacteriol. **133**:661–666.
23. **Wu, T. T.** 1966. A model for three-point analysis of random general transduction. Genetics **54**:405–410.