JOURNAL OF BACTERIOLOGY, June 1976, p. 1052-1062
Vol. 126, No. 3

Iron Uptake in Colicin B-Resistant Mutants of *Escherichia coli* K-12

ANTHONY P. PUGSLEY* AND PETER REEVES

Department of Microbiology, University of Adelaide, Adelaide, S.A. 5001, Australia

Received for publication 20 January 1976

Four classes of colicin B-resistant mutants of *Escherichia coli* K-12 were examined for defects in iron uptake. All four mutant classes (*cbt*, *exbC*, *exbB*, and *tonB*) were defective in the uptake of ferri-enterochelin. The *tonB* mutant was also defective in citrate-, ferrichrome-, and rhodoturulic acid-mediated iron uptake. The defects in iron transport were reflected in increased sensitivity to iron chelators and to chromium and aluminium salts, and in hypersecretion of enterochelin. One of the mutants (*cbt*) was apparently defective in outer membrane ferri-enterochelin receptor activity. *aroE* derivatives (unable to synthesize enterochelin) of the four mutant classes and the parent strain produced increased amounts of two outer membrane polypeptides when grown under iron stress. These polypeptides are implicated in ferri-enterochelin receptor activity.

Several workers have noted that mutants of *Escherichia coli* K-12 that are resistant to certain colicins in group B (Davies and Reeves classification, see reference 5) also hypersecrete enterochelin (5, 8–10, 12). This microbial iron chelator or siderochrome functions in iron uptake in *E. coli* K-12 and is secreted when the cells are grown in the absence of utilizable iron (for a review, see reference 21). Enterochelin (also called enterobactin, a cyclic trimer of 2,3-dihydroxybenzoyl serine (DBS) [20, 21]) has been shown to inhibit the action of certain group B colicins on *E. coli* (5, 9) and to protect the hypersecreting strains from the action of some of these colicins (9; unpublished data). Enterochelin is not the only iron chelator that can be utilized by *E. coli;* a variety of siderochromes produced by a wide range of microorganisms and also citrate have been shown to stimulate the growth of *E. coli* in the absence of freely available iron (7). Specific iron uptake systems have been identified for enterochelin- (4); for ferrichrome- (11, 28), a siderochrome produced by *Ascomycetes, Basidiomycetes,* and Fungi Imperfecti (20); and for citrate-mediated iron uptake (6) in *E. coli* K-12.

Frost and Rosenberg (7) have shown that the group B colicin-resistant mutant *tonB* is unable to utilize iron complexed with enterochelin or with citrate. Hantke and Braun (11) have shown that *tonB* mutants are also defective in ferrichrome-mediated iron uptake. The *tonB* locus may therefore control a step common to all siderochrome uptake systems in the outer membrane of *E. coli* (7).

The defect in ferri-enterochelin uptake in *tonB* mutants results in hypersecretion of this siderochrome when the cells are grown under limiting iron conditions (10). Three additional types of group B colicin-resistant mutants have been found to hypersecrete enterochelin (5; A. P. Pugsley and P. Reeves, Proc. Soc. Gen. Microbiol. III, p. 49; unpublished data), and these are examined in the present paper to determine whether these mutants are also defective in chelator-mediated uptake of iron. The results show that the *cbt* (tolerance to colicins B and D), *exbB* (tolerance to colicins B, D, G, H, Ia, Ib, M, Q, S1, and V), *exbC* (tolerance to colicins B, D, G, H, and M), and *tonB* (tolerance to colicins B, D, G, H, Ia, Ib, M, Q, S1, and V, and resistance to bacteriophage T1 and ϕ80) mutants are all defective in ability to take up ferri-enterochelin. The citrate-, ferrichrome-, and rhodoturulic acid- (a siderochrome produced by certain yeasts [20]) mediated iron uptake systems are fully or partally functional in all mutant classes except *tonB*.

MATERIALS AND METHODS

Bacterial strains. Strains of *E. coli* K-12 used in this study are listed in Table 1. The colicin-resistant mutants used in this study have all been described previously (5). The *aroE* marker was inserted into these strains by cotransduction with spectinomycin resistance (*spc*) from strain AN366, using P1 phage (19).

Culture media and glassware. Nutrient broth, nutrient agar, and soft nutrient agar were as described previously (5). Minimal salts medium was a basic salts solution containing (grams per liter): K_2HPO_4, 10.6; $NaH_2PO_4 2H_2O$, 6.1; $MgCl_2 6H_2O$, 0.17; $(NH_4)_2$, 2; and $Ca(NO_3)_2$, 0.01; adjusted to pH 7.2.

TABLE 1. *Bacterial strains*

Strain no.	Colicin resistance genotype (5)	Growth factor requirements and antibiotic resistances	Immediate source/reference
AB1133		F⁻/*thi, argE, his, proA, thr, leu, str*	A. L. Taylor
P295	*cbt*	As AB1133	Mutants of AB1133 (5)
P535	*exbC*	As AB1133	
P575	*exbB*	As AB1133 but *met*	
P585	*tonB*	As AB1133	
P1552		As AB1133 but *aroE spc*	Derivative of AB1133
P1553	*cbt*	As P1552	Derivative of P295
P1554	*exbC*	As P1552	Derivative of P585
P1555	*exbB*	As P1552 but *met*	Derivative of P575
P1556	*tonB*	As P1552	Derivative of P585
AN366		*aroE, pabA, his, arg, ilv, purE, str, spc*	G. Woodrowe

Trace-salt solutions were added as recommended by Neidhardt et al. (15). Analar-grade reagents were used throughout. The media were supplemented when necessary with amino acids (0.1 g/liter), thiamine (0.001 g/liter), and 4-aminobenzoic acid and 4-hydroxybenzoic acid (0.007 g/liter). The *aroE* strains were grown in the absence of shikimic acid or 2,3-dihydroxybenzoic acid, unless otherwise stated, to suppress enterochelin synthesis. Glucose (5 g/liter) or succinate (0.2 g/liter) was used as a carbon source. Minimal media were solidified where required by the addition of 1.6% Difco agar.

Iron-free media were prepared by extracting 10×-concentrated salts solutions with 100 μg of 8-hydroxyquinoline (BDH) per ml. Iron-hydroxyquinoline and excess hydroxyquinoline were removed by repeated back extraction with chloroform until hydroxyquinoline could not be detected in the chloroform phase by spectrophotometry (244 nm). Deionized, distilled water was used in the preparation of all iron-free media.

Glassware for iron-free media was rinsed with 0.1 N HCl followed by copious quantities of deionized, distilled water before being autoclaved.

Iron assays. The concentration of iron in media was determined by the Merkotest system (Merck and Co., Inc.). Assays were performed on 10×-concentrated salts solutions and on 100×-concentrated amino acid solutions. The test system was found to give reproducible results for iron concentrations over 3 μM. Media were considered to be iron free when the final calculated iron concentration was less than 0.5 μM.

Preparation of enterochelin. Enterochelin was purified from culture supernatants of strain P585 (*tonB*). Minimal medium containing 100 μM 2,2'-dipyridyl (DP) (BDH) and 80 μM $FeSO_4$ was inoculated with washed cells and incubated with good aeration for 36 h at 37 C. Additional $FeSO_4$ was added during incubation to maintain the concentration of $FeSO_4$ at the same molarity as the enterochelin in the medium. Cells were removed by centrifugation at 6,000 × *g* for 20 min, and the supernatant was loaded directly onto a DE52 cellulose column (40 by 3 cm, equilibrated with 10 mM phosphate buffer [pH 7.1] at 4 C). The column was washed with 250 ml of the phosphate buffer followed by 500 ml of 1 M NH_4Cl (pH 7.1). Enterochelin was eluted with 2 M NH_4Cl (pH 7.1). Fractions containing the red ferri-enterochelin were extracted with ethyl acetate and crystallized by rotary evaporation. The dried enterochelin was dissolved in fresh ethyl acetate, washed successively with equal volumes of phosphate buffer (100 mM, pH 7.1) and distilled water, and concentrated to a final volume of 20 ml by rotary evaporation. Benzene (analar grade) was added to the ethyl acetate-enterochelin until faint turbidity persisted, and the enterochelin was crystallized by further rotary evaporation. The enterochelin was further dried over phosphorous pentoxide and stored at −20 C. Purity of the enterochelin was confirmed by dissolving it in fresh ethyl acetate and analyzing by thin-layer chromatography (18). Ferri-enterochelin was filtered through cellulose acetate membrane filters before use. Enterochelin concentrations were determined from the extinction coefficient (17).

Preparation of purified colicin D. Purified colicin D was prepared from sonically oscillated cells of *E. coli* K-12.CA23 induced with mitomycin C (0.4 μg/ml of culture). The colicin was precipitated in a 30 to 50% saturated ammonium sulfate fraction and purified by gel filtration on a Sephadex G200 column followed by column chromatography on DE52 cellulose (Whatman), using procedures similar to those described by Timmis (26). The colicin was purified further by repeating the gel filtration and DE52 chromatography stages. The resulting colicin was similar to the purified colicin D described by Timmis (26).

Effects of iron, iron chelators, chromium salts, and aluminum salts on growth. Cells from overnight minimal medium cultures (10 μM iron) were washed twice in iron-free minimal medium and subcultured into appropriate media to give an initial optical density at 660 nm (OD_{660}) of 0.01. Cultures were grown with vigorous shaking in 20-ml volumes in 150-ml side-arm flasks or in 400-ml volumes in 2-liter flasks, and the cell density was estimated from the OD_{660}. Doubling times (OD_{660}) in the middle of

the logarithmic growth phase and yields (OD_{660}; 24 h) were used as parameters of growth. Samples were removed from the 400-ml cultures and assayed for DBS-containing compounds by extraction with ethyl acetate and measurement of OD_{315} (17).

Iron uptake studies. Iron uptake experiments using $^{55}Fe(III)$ were performed as described by Langman et al. (13) with the following minor modifications. *aroE* strains were used throughout to eliminate problems caused by secretion of enterochelin during the assay. Bacteria were grown overnight in minimal medium (5 μM Fe), washed twice in iron-free medium, and subcultured into iron-free medium to an OD_{660} of 0.01. For experiments on inducible iron transport systems (ferrichrome, rhodoturulic acid, and citrate), bacteria were grown overnight in minimal medium containing the appropriate chelating agent before subculture. Subcultured cells were grown to an OD_{660} of approximately 0.2, harvested by centrifugation (4,000 × *g* for 20 min), washed twice in iron-free medium, and finally resuspended in iron-free medium to an OD_{660} of 0.8 to 1.0. Cells were then chilled to 4 C. Chelators were used in the desferri form throughout. Inhibitors were added where indicated to the resuspension medium. The amount of $^{55}Fe(III)$ taken up by the cells was determined by removing samples from the reaction mixture and filtering through a Gelman GA-6 membrane filter (0.45-μm pore diameter). After rinsing (13), the filter was counted in a Packard 3003 liquid scintillator counter. The scintillator fluid used in our experiments was 2,5-diphenyloxazole (Packard, 0.4%) plus 1,4-bis-[2]-(4-methyl-5-phenyloxazolyl)-benzene (Packard, 0.05%) in toluene.

Preparation of outer membranes. Triton-insoluble walls (outer membranes) were prepared by the method of Schnaitman (23) with minor modifications. Cells were grown to mid-log phase in well-aerated minimal salts medium cultures, harvested by centrifugation (5,000 × *g* for 20 min at 4 C), and washed once in 10 mM HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4). The packed cells were flash frozen (−20 C) and cracked in an LKB X-press. HEPES buffer (10 mM) was then added to the cracked cells (4 ml of buffer per ml of cells) and mixed together with 5 μg each of deoxyribonuclease and ribonuclease (Sigma Chemical Co.) per ml for 30 s in an MSE blender. Cell debris was removed by centrifugation (5,000 × *g*, 5 min). The crude cell envelope was then pelleted by centrifugation of the supernatant (40,000 × *g*, 60 min), suspended in 10 mM HEPES buffer containing 2% Triton X-100 (BDH), and allowed to stand at room temperature for 10 min. The Triton-insoluble material was then pelleted by centrifugation (40,000 × *g*, 60 min), the supernatant was discarded, and the Triton extraction was repeated. The final pellet was suspended in distilled water. Protein concentrations were determined by the method of Schacterle and Pollack (22).

PAGE. Triton-insoluble walls were solubilized for polyacrylamide gel electrophoresis (PAGE) by method II of Schnaitman (24). The samples were run on 7.5% polyacrylamide disc-gels, using the alkaline Bragg-Hou system (1). Gels were stained with Coomassie brilliant blue (16, 25) and, after destaining, were scanned in a Quick Scan densitometer (Helena Labs, Beaumont, Tex.).

Ferri-enterochelin binding to membrane fractions. Outer membranes were suspended in minimal salts solution containing 100 μM nitrilotriacetic acid (NTA; Sigma Chemical Co.). Equal volumes of NTA (100 μM), enterochelin (20 μM), and $^{55}Fe(III)$ (10 μM) were then added to 0.1-ml volumes of the outer membrane suspensions and incubated for 5 min at 37 C. The reaction mixture was then filtered through a cellulose nitrate filter (Sartorius 11307, 0.2-μm pore diameter) and washed with two aliquots of 20 ml of 0.9% saline, and the $^{55}Fe(III)$ retained by the filter was counted as described above. According to Lever (14), protein is quantitatively adsorbed by cellulose nitrate filters, thus facilitating measurements of binding of small molecules to proteins. Preliminary experiments with horse transferrin (Koch-Light) demonstrated that siderochrome-mediated iron binding could be detected by this method (1) and that 100 μg of outer membrane protein gave reproducible results in ferri-enterochelin binding assays.

RESULTS

Growth responses and enterochelin production in minimal media. The four colicin B-resistant mutants and their *aroE* derivatives were subcultured into minimal media containing: various concentrations of $FeCl_3$; chelating agents citrate, DP, and NTA; and siderochromes desferrioxamine B (Desferal, Ciba), desferri-enterochelin, and 2,3-dihydroxybenzoate (DHB). Desferrioxamine is a siderochrome produced by *Micromonospora, Nocardia,* and *Streptomyces* (20), and DHB is a precursor of enterochelin (21). The parent strain (AB1133) and its *aroE* derivative (P1552) were used as controls. Mid-logarithmic-phase doubling times are shown in Table 2. Cell yields were reduced in all cases in which growth rates were retarded. Strain P585 *(tonB)* was clearly the most sensitive of all the *aro*$^+$ strains to incubation in the absence of available iron (low-iron medium or a medium containing desferrioxamine B, NTA, or DP, which chelate iron and cannot be taken up by *E. coli* K-12). All strains carrying the *aroE* marker grew less well under iron stress conditions than the corresponding *aro*$^+$ strains. Growth with succinate as the sole carbon source also increased the inhibitory effects of low iron concentrations on the mutants. Citrate was apparently able to function as an iron transport compound in all *aroE* strains other than P1556 *(tonB). aroE* strains grown in the presence of DHB behaved like *aro*$^+$ cells. Enterochelin strongly inhibited the growth of strains P1553 *(cbt, aroE)* and P1556 *(tonB, aroE)* and reduced the growth of strains P1554 *(exbC, aroE)* and P1555 *(exbB, aroE).*

Growth of the *aro*$^+$ colicin B-resistant mu-

TABLE 2. *Growth rates of the colicin B-resistant mutants under varying conditions of iron stress*

Iron and iron chelators	Carbon source	Mid-log doubling time (min) measured as OD_{660}									
		AB1133	P1552 (*aroE*)	P295 (*cbt*)	P1553 (*aroE, cbt*)	P535 (*exbC*)	P1554 (*aroE, exbC*)	P575 (*exbB*)	P1555 (*aroE, exbB*)	P585 (*tonB*)	P1556 (*aroE, tonB*)
3 μM Fe	0.5% glucose	56	63	55	58	60	61	58	58	59	103
<1 μM Fe	0.5% glucose	66	86	63	96	66	126	65	116	71	174
3 μM Fe, 100 μM NTA	0.5% glucose	53	114	53	156	63	118	61	104	108	280
3 μM Fe, 100 μM DP	0.5% glucose	51	>500	63	>500	68	>500	73	>500	175	>500
3 μM Fe, 100 μM desferrioxamine B	0.5% glucose	53		64		75		78		188	
3 μM Fe, 10 mM citrate, 100 μM NTA	0.5% glucose	57	62	53	53	66	58	66	60	66	250
3 μM Fe	0.2% succinate	75	136	85	160	83	166	85	185	86	176
1 mM DHB, 100 μM NTA, 3 μM Fe	0.2% succinate		63		73		69		79		114
10 μM enterochelin, 100 μM NTA, 3 μM Fe	0.2% succinate		70		186		103		120		>500

tants under the various conditions of iron stress resulted in the appearance of high concentrations (100 to 300 μM) of DBS-containing compounds in the culture supernatants. To compare DBS secretion by the four mutants under identical growth conditions, the strains were cultured in minimal medium containing 3 μM iron. This level of iron was just sufficient to maintain normal growth of strain P585 (*tonB*). All four mutants secreted more DBS than the parent strain (Fig. 1). When the level of iron was reduced further, either by using extracted medium or by adding strong iron-chelating agents, all four mutants secreted up to 10 times more DBS-containing compounds than the parent strain.

Examination of 12 independently isolated representatives of each of the *cbt*, *exbB*, and *tonB* mutant classes demonstrated that yields of DBS-containing compounds (OD_{315}) varied from strain to strain. The *tonB* mutants consistently secreted more DBS-containing compounds than the *cbt* and *exbB* mutants, which in turn secreted more of these compounds than the single *exbC* strain tested (we have so far been unable to isolate additional representatives of this class of mutants).

Chromium and aluminium sensitivity. Strains of *E. coli* that carry a mutation in the *tonB* locus are known to have increased sensitivity to chromium salts (9, 27). We were previously unable to demonstrate increased chromium sensitivity of the *tonB* mutant studied here but suggested that this may have been due to the presence of iron in the growth medium (5). The results in Table 3 show the effect of chromium on growth of the four colicin B-resistant mutant classes in minimal media containing various concentrations of iron. Chromium inhibited the growth of strains P295 (*cbt*) and P585 (*tonB*). The inhibitory effect of the chromium was eliminated by adding iron to the growth medium. Results similar to those shown in Table 2 were obtained with aluminium salts, but we were unable to demonstrate increased sensitivity of any of the mutants to cobalt, copper, or nickel salts. All strains carrying the *aroE* marker were more sensitive to chromium (100 μM) than the corresponding *aro*$^+$ strains. The toxic effect of chromium on *aroE* strains was again eliminated by adding iron (10 μM) to the growth medium.

Iron uptake. The results in Fig. 2 show iron uptake in the *aroE* derivatives of the colicin B-resistant mutants and in strain P1552 in the presence of enterochelin, citrate, ferrichrome, and rhodotorulic acid. Enterochelin-mediated iron uptake was completely blocked in the *tonB* and *cbt* mutants, although a low level of iron was consistently bound by the cells. Ferri-en-

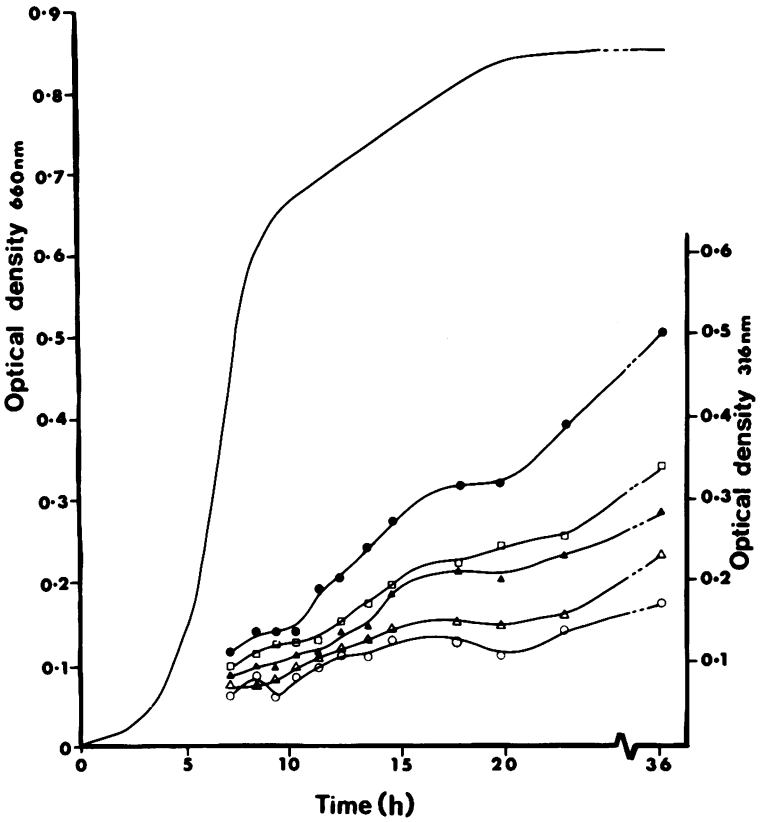

FIG. 1. *Growth (——) and appearance of DBS-containing compounds in minimal medium culture supernatants of strains AB1133 (○), P295 (cbt; ▲), P535 (exbC; △), P575 (exbB; □), and P585 (tonB; ●). Growth was measured as an increase in* OD_{660}, *and DBS was assayed by extraction of 10-ml volumes of supernatant with ethyl acetate and measurement of* OD_{315}. *Growth curves for all strains were identical to that shown. The medium contained 3 μM iron.*

TABLE 3. *Effect of iron on chromium inhibition of colicin B-resistant mutants*

$CrCl_3$ (μM)	$FeCl_3$ (μM)	Mean mid-log doubling time (min)				
		AB1133	P295 *(cbt)*	P535 *(exbC)*	P575 *(exbB)*	P585 *(tonB)*
100	2	63	114	65	58	155
100	3	59	78	64	58	90
100	4	57	64	59	62	75
100	5	58	60	57	61	60
50	2	55	81	62	65	101
0	2	57	67	63	62	70

terochelin uptake was reduced in the *exbB* and *exbC* mutants. The *tonB* strain was also blocked in the uptake of iron complexed with citrate, rhodotorulic acid, and ferrichrome. Ferrirhodotorulic acid uptake was apparently only partially functional in the *exbB* and *exbC* mutants, although the level of ferrirhodotorulic acid taken up was consistently very low, even in strain P1552 *(aroE)*.

Iron uptake in the *aroE* strains in the absence of siderochrome (the so-called low-affinity iron uptake system [7, 21]) and in the presence of NTA alone or NTA plus ferrioxamine B was also studied. The results for all five strains were identical, and Fig. 3 shows the results obtained with strain P1552 *(aroE)*. The low-affinity iron uptake system appears to be quite efficient in our derivatives of *E. coli* K-12, but little or no iron was taken up in the presence of NTA or NTA plus ferrioxamine B.

Ferri-enterochelin binding by whole cells. No enterochelin-mediated iron uptake could be detected after 1 min in iron-starved cells of strain P1552 *(aroE)* treated with 2,4-dinitrophenol (DNP; Fig. 4). We conclude that DNP prevents active uptake of ferri-enterochelin and that the initial rise in the level of iron bound by the DNP-treated cells is due to an energy-independent absorption stage in the uptake of ferri-enterochelin. The surface binding of ferri-enterochelin was therefore measured after incubation of cells with labeled ferri-enterochelin in the presence of DNP. The level of ferri-entero-

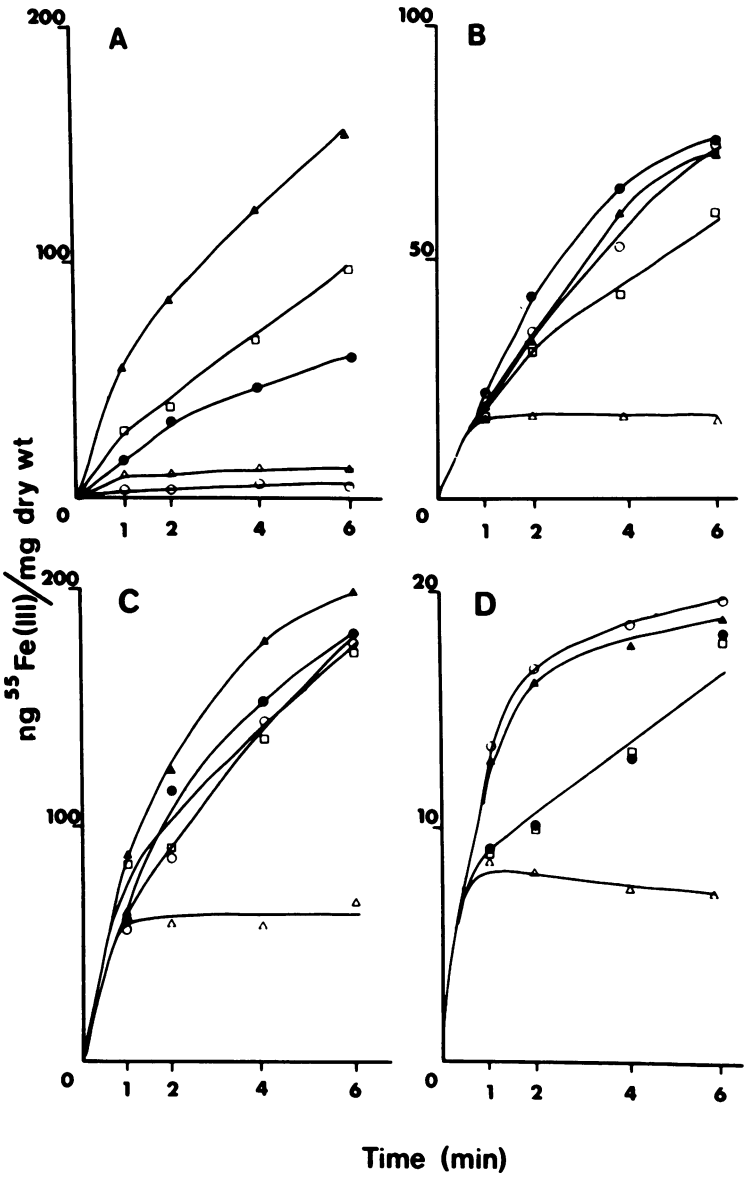

FIG. 2. *Uptake of iron by cells of strains P1552 (▲), P1553 (cbt; ○), P1554 (exbC; □), P1555 (exbB; ●), and P1556 (tonB; △) in the presence of: 2.5 μM enterochelin, 2.5 μM ^{55}Fe(III), and 100 μM NTA (A); 1 mM trisodium citrate, 2.5 μM ^{55}Fe(III), and 100 μM NTA (B); 5 μM ferrichrome, 5 μM ^{55}Fe(III), and 100 μM NTA (C); or 5 μM rhodoturulic acid, 5 μM ^{55}Fe(III), and 100 μM NTA (D).*

chelin bound by cells of strain P1552 *(aroE)* was increased by growing the cells in iron-free medium and further increased by growth in the presence of NTA. The level of ferri-enterochelin bound was reduced, although not completely abolished, by growing cells in the presence of 100 μM $FeCl_3$ (Table 4). The amount of ferri-enterochelin bound, therefore, appears to be inversely correlated with the level of iron available to the cells.

The *aroE* colicin B-resistant mutants were also examined for their ability to bind ferri-enterochelin in the presence of DNP. All four strains bound as much ferri-enterochelin after growth in medium containing 100 μM $FeCl_3$ as did strain P1552. Ferri-enterochelin binding could not, however, be increased by growing any of the *aroE* colicin B-resistant mutants in the absence of available iron.

Examination of outer membranes of *aroE* strains by PAGE. Outer membranes were prepared from the parent strain (AB1133) and

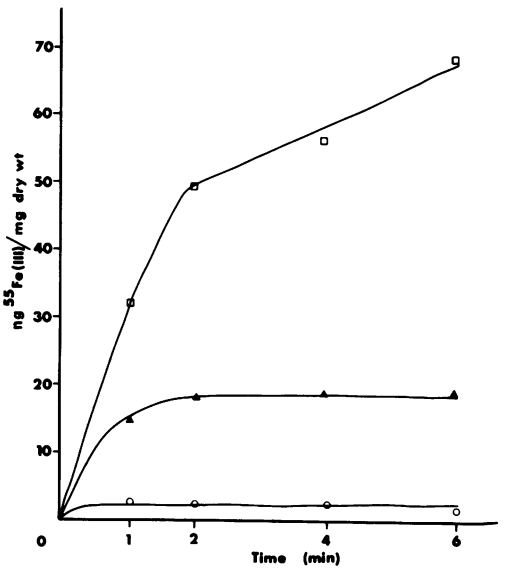

FIG. 3. *Uptake of iron by strain P1552 in the presence of: 100 μM NTA, 5 μM ferrioxamine B, and 5 μM ^{55}Fe(III) (○); 100 μM NTA and 5 μM ^{55}Fe(III) (▲); and 2.5 μM ^{55}Fe(III) (□).*

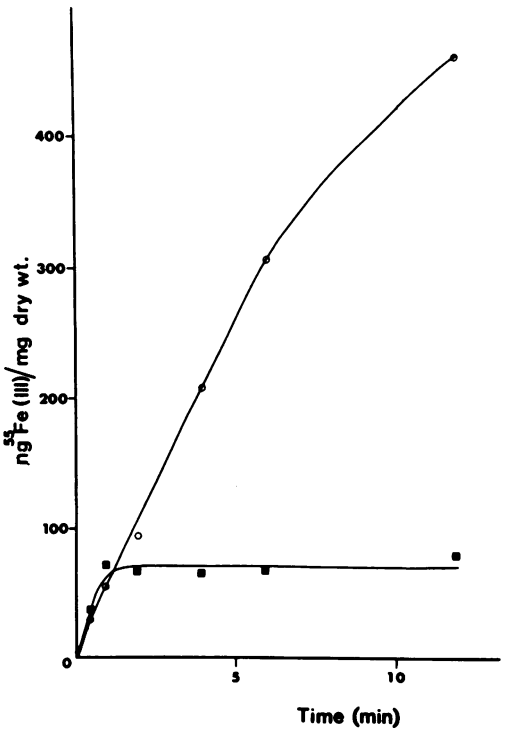

FIG. 4. *Comparison of ferri-enterochelin uptake by cells of strain P1552 in the presence (■) or absence (○) of 2 mM DNP. Cells were grown in iron-free medium containing 100 μM NTA to enhance iron requirement. Uptake medium contained 5 μM ^{55}Fe(III), 5 μM enterochelin, and 100 μM NTA.*

TABLE 4. *Comparison of levels of ferri-enterochelin bound in 5 min by DNP-treated cells of the aroE strains grown under varying conditions of iron stress*

Strain no.	Relevant genotype	Growth medium		^{55}Fe(III)-enterochelin bound[a] (ng/mg of cell dry wt)
		Fe (μM)	NTA (μM)	
P1552	*aroE*	100		10
		<1		40
		<1	100	75
P1553	*cbt, aroE*	100		10
		<1		10
		<1	100	15
P1554	*exbC, aroE*	100		10
		<1		12
		<1	100	21
P1555	*exbB, aroE*	100		12
		<1		12
		<1	100	18
P1556	*tonB, aroE*	100		11
		<1		10
		<1		15

[a] Mean of several determinations.

aroE derivatives of both the parent strain and the colicin B-resistant mutants as described above. The preparation of outer membranes from iron-deprived *aroE* strains presented some difficulties since the strains do not grow well under these conditions. This problem was overcome by growing the cells to early logarithmic phase (OD_{660} = 0.25) in minimal medium containing 5 μM iron and then adding 100 μM DP. Cells were then harvested in the late log phase of growth in the normal way (OD_{660} = 0.9 to 1.1). Outer membranes prepared from cells grown under these conditions were then compared with similar preparations from cells grown in medium containing 100 μM $FeCl_3$. PAGE of the outer membrane preparations demonstrated the presence of three major polypeptide peaks in outer membranes from all iron-deprived *aroE* strains and their absence from strain AB1133 (Fig. 5). The two high-molecular-weight polypeptides are identical to those found previously in iron-deprived *aro*$^+$ colicin B-resistant strains (unpublished data) and do not appear in outer membranes prepared from *aroE* strains grown in the presence of 100 μM iron. The new lower-molecular-weight polypeptide was present in outer membranes prepared from all *aroE* strains irrespective of whether or not the cells were cultured in the absence of available iron.

Ferri-enterochelin binding to outer mem-

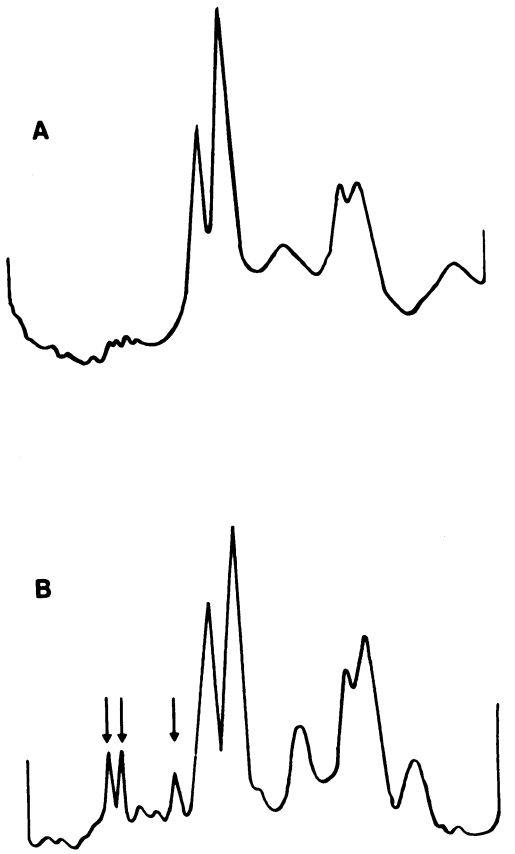

FIG. 5. *Comparison by densitometry of stained bands of outer membrane polypeptides prepared from strains AB1133 (A) and P1552 (aroE; B) grown under iron stress conditions and examined by PAGE as described in the text. Outer membrane profiles similar to that shown in B were obtained with strains P1553, P1554, P1555, and P1556 when these strains were also grown under iron stress. The direction of electrophoresis is from right to left.*

brane preparations. Outer membranes were analyzed to determine whether the presence of the new polypeptides was correlated with increased ferri-enterochelin binding activity. The results (Table 5) show that outer membranes prepared from all *aroE* strains adsorbed approximately the same level of ferri-enterochelin when the cells were grown in the presence of iron. Bovine serum albumin, which was used as a negative control in binding assays, consistently adsorbed relatively high levels of ferri-enterochelin, indicating that nonspecific adsorption of ferri-enterochelin to proteins may account for some of the ferri-enterochelin bound to these membrane preparations. The ferri-enterochelin binding capacity of outer membranes prepared from cells of strains P1552 *(aroE)*, P1554 *(aroE, exbC)*, P1555 *(aroE, exbB)*, and P1556 *(aroE, tonB)* was, however, increased by growing the cells used to prepare the membranes under iron stress, although we were consistently unable to demonstrate increased binding of ferri-enterochelin to outer membranes of strain P1553 *(aroE, cbt)* under these conditions (Table 4). The level of ferri-enterochelin binding to outer membranes detectable in this assay was only 10% of that predicted from the results with whole cells (Table 4). This may be due to incomplete retention of the outer membrane protein by the membrane filters.

Inhibition of ferri-enterochelin binding to outer membranes by preincubation with purified colicin D. Volumes (0.1 ml) of purified colicin D diluted in minimal salts medium containing 100 μM NTA were incubated for 30 min at 37 C with an equal volume of outer membrane suspension (100 μg of protein) prepared from iron-stressed cells of strain P1552 *(aroE)*. The mixture was then assayed for ferri-enterochelin binding activity as described above. The results (Fig. 6) show that low levels of colicin D substantially reduced the amount of ferri-enterochelin bound by the membrane preparation. At higher levels of colicin D, the level of ferri-enterochelin bound appeared to increase slightly. This increase was due to binding of ferri-enterochelin to the colicin, and the data shown in Fig. 6 are therefore corrected for this nonspecific binding by subtracting the amount of ferri-enterochelin bound by colicin D under the same conditions but in the absence of outer membrane.

TABLE 5. *Enterochelin-mediated binding of iron to outer membranes prepared from aroE strains grown in the presence or absence of available iron*

Strain no.	Relevant genotype	Iron in culture medium (μM)	DP added to culture medium	^{55}Fe(III)-enterochelin bound (ng/mg of outer membrane protein)
P1552	*aroE*	100	–	13
		5	+	36
P1553	*cbt, aroE*	100	–	14
		5	+	14
P1554	*exbC, aroE*	100	–	14
		5	+	56
P1555	*exbB, aroE*	100	–	13
		5	+	39
P1556	*tonB, aroE*	100	–	8
		5	+	54

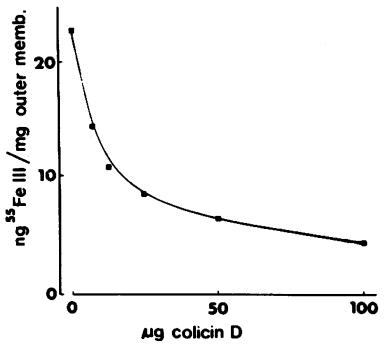

FIG. 6. *Neutralization of ferrienterochelin binding activity of outer membranes prepared from iron-deprived cells of strain P1552 (aroE) by preincubation of the membrane preparation with purified colicin D. Outer membrane and colicin concentrations are expressed as milligrams and micrograms of protein, respectively.*

DISCUSSION

At least two genes are known to control the uptake of ferri-enterochelin in *E. coli* (*fep* and *tonB* [21]). The latter gene is also involved in the uptake of iron complexed with ferrichrome, rhodotorulic acid, and citrate (Fig. 2) (7, 11). The data presented in this paper indicate that the *cbt*, *exbB*, and *exbC* genes may also be involved in the enterochelin-mediated uptake of iron in *E. coli* K-12. Another gene (*tonA*) is known to control the uptake of ferrichrome by *E. coli* K-12 (11, 28).

Hantke and Braun (12) recently described several mutants (*feu*, ferri-enterochelin uptake) that are both resistant to colicins in group B and are defective in ferri-enterochelin uptake. However, none of the *feu* mutants described by Hantke and Braun is identical with respect to colicin resistance to those described in this paper. Differences between the parent strains may account for some of the differences in colicin resistance of the two groups of mutants, but the two sets of data undoubtedly indicate that several distinct mutational events may block both the action of group B colicins and the uptake of ferri-enterochelin in *E. coli*. Although it cannot be assumed that all of the mutants described control an outer membrane function (12), it is important to decide which, if any, of the mutants carry a specific block in the transport of ferri-enterochelin across the outer membrane.

Enterochelin-mediated accumulation of iron was inhibited in the presence of DNP. This enabled us to distinguish between the binding and active uptake stages of ferri-enterochelin transport. The sensitivity of ferri-enterochelin transport to DNP, and to sodium azide (data not presented), may indicate that energy for ferri-enterochelin transport is furnished through oxidative phosphorylation (2), although further studies will be needed to confirm this.

Secretion of enterochelin by *E. coli* K-12 is increased by growth in the absence of utilizable iron (8, 21). One might therefore predict that an increase in ferri-enterochelin uptake ability would result from growing *E. coli* K-12 under iron stress conditions. Indeed, when the cells are grown in the presence of iron before assaying, little or no active uptake of ferri-enterochelin could be detected (data not presented). Derepression of ferri-enterochelin uptake appears to be independent of the presence of enterochelin since it occurred in *aroE* cells grown under iron stress in the absence of enterochelin precursors.

In the ferrichrome-mediated iron transport system in *E. coli*, increase in ferrichrome uptake is associated with induction of ferrichrome receptor (11), and this also appears to be the case with the derepression of the ferri-enterochelin uptake system. Whole cells and outer membranes prepared from cells grown under iron stress (derepressed) bind more ferri-enterochelin than similar preparations from cells grown in the presence of available iron (repressed) (Tables 4 and 5). This increase in ferri-enterochelin binding and uptake activities coincides with the appearance in the outer membrane of two new polypeptides (Fig. 5). The 3- to 7-fold increase in ferrienterochelin binding is less than the 10- to 20-fold increase in the sizes of the two polypeptide peaks. However, the basal level of ferri-enterochelin binding to the surface of repressed cells may, in part, be nonspecific. This hypothesis is supported by the observation (unpublished data) that ferri-enterochelin binds to bovine serum albumin. This nonspecific binding may be due to the highly anionic nature of ferri-enterochelin, as indicated by its high affinity for the anion-exchanger DE52 cellulose. Hence, the increase in ferri-enterochelin binding by outer membranes of derepressed cells may be greater than is indicated by the results in Tables 4 and 5.

We have also noted that the presence of these two outer membrane polypeptides is correlated with a marked increased binding activity for colicins B and D but not for other colicins in group B (unpublished data). These observations, together with those reported here, suggest that ferri-enterochelin and colicins B and D share a common cell surface receptor component and that one or both of the polypeptides may comprise the whole or part of the receptors. The inhibition of ferri-enterochelin bind-

ing by purified colicin D (Fig. 6) and the protection afforded by ferri-enterochelin against killing by colicins B and D (5) support the hypothesis that both receptors share a common component.

The *aroE cbt* strain P1553 is very similar to the *aroB fep* mutant described by Cox et al. (4, 21). Both *cbt* and *feb* mutations block the active uptake of ferri-enterochelin but do not affect ferrichrome-, rhodotorulic acid-, or citrate-mediated iron uptake. Furthermore, the *cbt* mutation maps close to the *ent-fep* cluster of genes on the *E. coli* K-12 chromosome (5). The *cbt* mutation may therefore produce a specific block in ferri-enterochelin transport similar or identical to that produced by *fep* mutations but also blocking a stage in colicin B and D action. The *cbt* mutant is apparently defective in ferri-enterochelin binding activity since this activity is not increased by growing the cells under iron stress conditions. This is so both for whole cells treated with DNP (Table 4) and for outer membrane preparations (Table 5). The level of ferri-enterochelin bound by derepressed cells or outer membranes prepared from these cells was the same for the *cbt* mutant (P1553) as for wild-type cells (P1552). However, as suggested above, some of this binding may be nonspecific. It is therefore not possible to say at this stage whether the *cbt* mutant shows any specific binding of ferri-enterochelin. The simplest hypothesis is that receptor functions in the *cbt* mutant are altered such that ferri-enterochelin is not bound specifically at all and, hence, not actively taken up. However, receptor function for colicins B and D is normal in the *cbt* mutant (unpublished data), and the two additional polypeptides appear normally in iron-stressed *cbt* cells. If colicins B and D and ferri-enterochelin do indeed share a common receptor, it may be altered in the *cbt* mutant so as to affect ferri-enterochelin binding and colicin action but not colicin binding.

The ability of the *cbt* mutant P295 to grow more quickly under iron stress conditions than the *cbt aroE* and *cbt*$^+$ *aroE* strains (P1553 and P1552, respectively; Table 2) indicates that the *cbt* mutant can utilize an aromatic compound for iron uptake. Since the defect in strain P1553 *(cbt, aroE)* is eliminated by growth in the presence of DHB but not enterochelin (Table 2), DHB may be able to function as a siderochrome, albeit inefficiently. To do this, the DHB must form a complex with iron and be transported across both the outer and inner membranes. Alternatively, enterochelin located in the periplasmic space may form a complex with the iron after the ferri-DHB has crossed the outer membrane and then be transported across the inner membrane (7). This would require a functional inner membrane transport system for ferri-enterochelin (i.e., *fep*$^+$), again indicating that the *cbt* and *fep* mutations are not identical.

Outer membranes prepared from iron-stressed cells of strains P1554 *(exbC, aroE)*, P1555 *(exbB, aroE)*, and P1556 *(tonB, aroE)* all have increased ferri-enterochelin binding activity (Table 5). Whole cells of these strains do not, however, have increased ferri-enterochelin receptor activity when grown under iron stress (Table 4). As was the case with the *cbt* mutant, it is not possible to say whether the basal level of ferri-enterochelin in repressed cells of these strains is specific. However, the results with iron-stressed cells suggest that the outer membrane ferri-enterochelin receptor is normal in these strains but is blocked in some way in whole cells. Binding of colicins B and D to both whole cells and to outer membranes is normal in these strains (unpublished data), and it is difficult to explain these observations if colicins B and D share a common receptor. Whatever the nature of the block in *exbB* and *exbC* cells, some active ferri-enterochelin uptake activity is retained.

Chromium and aluminium killing occurs when iron is not readily available to the cells. Thus, all *aroE* strains are sensitive to these metal salts unless iron is present in the growth medium. However, *aro*$^+$ cells that are able to take up ferri-enterochelin were not sensitive to chromium and aluminium (wild type, *exbC*, and *exbB* strains, Table 2), presumably because the enterochelin enables the cells to maintain a high internal level of iron. In contrast, *cbt* and *tonB* mutants do not take up ferri-enterochelin at all (Fig. 2) and are sensitive to chromium and aluminium (Table 3). These results suggest that chromium and aluminium are not taken up by the cells as enterochelin complexes but rather by some other means, as was also suggested by Wang and Newton (27).

The studies reported here have further defined the interaction between colicins B and D and iron uptake. However, the nature of the lesions in the four classes of mutants studied still remains to be elucidated.

ACKNOWLEDGMENTS

We are grateful to P. Wookey for introducing us to the techniques used in iron uptake studies and for providing ferrichrome and rhodotorulic acid. We also thank G. Frost and H. Rosenberg for allowing us to see their manuscripts prior to publication, and A. Richardson and A. Beizaitis for technical assistance.

LITERATURE CITED

1. **Bates, G. W., and J. Wernicke.** 1971. The kinetics and mechanism of iron (III) exchange between chelators

and transferrin. IV. The reaction of transferrin with iron (III) nitrilotriacetate. J. Biol. Chem. 246:3679–3685.
2. **Berger, E. A., and L. A. Heppel.** 1974. Different mechanisms of energy coupling for the shock-sensitive and shock-resistant amino acid permeases in *Escherichia coli*. J. Biol. Chem. 249:7747–7755.
3. **Bragg, P. D., and C. Hou.** 1972. Organization of proteins in the natural and reformed outer membranes of *Escherichia coli*. Biochim. Biophys. Acta 274:478–488.
4. **Cox, G. B., F. Gibson, R. K. J. Luke, N. A. Newton, I. G. O'Brien, and H. Rosenberg.** 1970. Mutations affecting iron transport in *Escherichia coli*. J. Bacteriol. 104:219–226.
5. **Davies, J. K., and P. Reeves.** 1975. Genetics of resistance to colicins in *Escherichia coli* K-12: cross-resistance among colicins of group B. J. Bacteriol. 123:96–101.
6. **Frost, G. E., and H. Rosenberg.** 1973. The inducible citrate-dependent iron transport system in *Escherichia coli* K-12. Biochim. Biophys. Acta 330:90–101.
7. **Frost, G. E., and H. Rosenberg.** 1975. Relationship between the *tonB* locus and iron transport in *Escherichia coli*. J. Bacteriol. 124:704–712.
8. **Guterman, S. K.** 1971. Inhibition of colicin B by enterochelin. Biochem. Biophys. Res. Commun. 44:1149–1155.
9. **Guterman, S. K.** 1973. Colicin B: mode of action and inhibition by enterochelin. J. Bacteriol. 114:1217–1224.
10. **Guterman, S. K., and L. A. Dann.** 1973. Excretion of enterochelin by *exbA* and *exbB* mutants of *Escherichia coli*. J. Bacteriol. 114:1225–1230.
11. **Hantke, K., and V. Braun.** 1975. Membrane receptor dependent iron transport in *Escherichia coli*. FEBS Lett. 49:301–305.
12. **Hantke, K., and V. Braun.** 1975. A function common to iron-enterochelin transport and action of colicins B, I, V in *Escherichia coli*. FEBS Lett. 59:277–281.
13. **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.
14. **Lever, J. K.** 1972. Quantitative assay of the binding of small molecules to protein. Comparison of dialysis and membrane filter assays. Anal. Biochem. 50:73–83.
15. **Neidhardt, F. C., P. L. Block, and D. F. Smith.** 1974. Culture medium for *Enterobacteriaceae*. J. Bacteriol. 119:736–747.
16. **Neville, D. M., Jr.** 1971. Molecular weight determination of protein-dodecyl sulphate complexes by gel electrophoresis in a discontinuous buffer system. J. Biol. Chem. 246:6328–6334.
17. **O'Brien, I. G., G. B. Cox, and F. Gibson.** 1971. Enterochelin hydrolysis and iron metabolism in *Escherichia coli*. Biochim. Biophys. Acta 237:537–549.
18. **O'Brien, I. G., and F. Gibson.** 1970. The structure of enterochelin and related 2,3-dihydroxy-N-benzoylserine conjugates from *Escherichia coli*. Biochim. Biophys. Acta 215:393–402.
19. **Pittard, J.** 1965. Effect of integrated sex factor on transduction of chromosomal genes in *Escherichia coli*. J. Bacteriol. 89:680–686.
20. **Rogers, G. D., and J. B. Neilands.** 1974. Microbial iron transport compounds, p. 823–830. *In* A. L. Laskin and H. A. Lechevalier (ed.), Handbook of microbiology, vol. 2. Microbial composition. C.R.C. Press, Cleveland.
21. **Rosenberg, H., and I. G. Young.** 1974. Iron transport in the enteric bacteria, p. 67–82. *In* J. B. Neilands (ed.), Microbial iron metabolism. Academic Press Inc., New York.
22. **Schacterle, G. E., and R. L. Pollack.** 1973. A simplified method for quantitative assay of small amounts of protein in biologic material. Anal. Biochem. 51:654–655.
23. **Schnaitman, C. A.** 1971. Solubilization of the cytoplasmic membrane of *Escherichia coli* by Triton X-100. J. Bacteriol. 108:545–552.
24. **Schnaitman, C. A.** 1973. Outer membrane proteins of *Escherichia coli*. I. Effect of preparative conditions on the migration of proteins on polyacrylamide gels. Arch. Biochem. Biophys. 157:541–555.
25. **Swank, T. T., and K. D. Munkres.** 1971. Molecular weight of oligopeptides by electrophoresis in polyacrylamide gels with sodium dodecyl sulfate. Anal. Biochem. 39:462.
26. **Timmis, K.** 1972. Purification and characterization of colicin D. J. Bacteriol. 109:12–20.
27. **Wang, C. C., and A. Newton.** 1969. Iron transport in *Escherichia coli*: relationship between chromium sensitivity and high iron requirement of *Escherichia coli*. J. Bacteriol. 98:1135–1141.
28. **Wayne, R., and J. B. Neilands.** 1975. Evidence for common binding sites for ferrichrome compounds and bacteriophage ϕ80 in the cell envelopes of *Escherichia coli*. J. Bacteriol. 121:497–503.