JOURNAL OF BACTERIOLOGY, Feb. 1975, p. 497-503

Vol. 121, No. 2
Printed in U.S.A.

Evidence for Common Binding Sites for Ferrichrome Compounds and Bacteriophage φ80 in the Cell Envelope of *Escherichia coli*

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Received for publication 28 October 1974

Mutants *tonA* and *tonB* of *Escherichia coli* K-12, known to be resistant to bacteriophage φ80, were found to be insensitive as well to albomycin, an analogue of the specific siderochrome ferrichrome. Ferrichrome at micromolar concentrations strongly inhibited plaque production by φ80. Preincubation with ferrichrome did not inactivate the phage. At a concentration at which ferrichrome allowed 90% inhibition of plaque formation, the chromium analogue of ferrichrome showed no detectable activity. Similarly, ethylenediaminetetraacetic acid, ferrichrome A, and certain siderochromes structurally distinct from ferrichrome, such as ferrioxamine B, schizokinen, citrate, and enterobactin, did not show detectable inhibitory activity. However, rhodotorulic acid showed moderate activity. A host range mutant of φ80, φ80h, was also inhibited by ferrichrome, as was a hybrid of phage λ possessing the host range of φ80. However, phage λcI⁻ and a hybrid of φ80 possessing the host range of λ were not affected by ferrichrome. Finally, ferrichrome and chromic deferriferrichrome were shown to inhibit adsorption of φ80 to sensitive cells, ferrichrome giving 50% inhibition of adsorption at a minimal concentration of 8 nM. It is suggested that a component of the ferrichrome uptake system may reside in the outer membrane of *E. coli* K-12 and may also function as a component of the receptor site for bacteriophage φ80, and that ferrichrome inhibition of the phage represents a competition for this common site.

A number of enteric bacterial strains possess iron transport systems that utilize low-molecular-weight, ferric ion-specific carrier molecules termed siderochromes, structures for which have been published elsewhere (16, 19). Luckey et al. (16) used the antibiotic albomycin to select mutants of *Salmonella typhimurium* defective in the utilization of a number of specific siderochromes, including ferrichrome. Most of these mutants had genetic lesions in loci that co-transduced with *panC*. In examining the susceptibility of *Escherichia coli* to albomycin, we found that two well-known mutant classes of this organism, *tonA* and *tonB*, the former mapping near *panC* (21), were resistant to the antibiotic. Since both of these mutations are known to impart resistance to T1 and φ80, a possible relationship was suggested between the ferrichrome uptake system and the receptors for these phages.

In this paper we report that ferrichrome specifically inhibits φ80 infection, and we present evidence that this inhibition is due to a competition between ferrichrome and the phage for a common site in the cell envelope, a site which we believe functions physiologically as a component of the ferrichrome uptake system.

MATERIALS AND METHODS

Chemicals. Ferrichrome and ferrichrome A (10) were crystallized from cultures of *Ustilago sphaerogena* by M. Luckey. Chromic deferriferrichrome was prepared from deferriferrichrome (15) by John Leong. Rhodotorulic acid (1) was isolated and recrystallized four times from supernatant liquids of *Rhodotorula pilimanae* by H. Akers. Crystalline schizokinen was a gift of B. R. Byers. Ferrioxamine B was obtained by addition of ferric ion to Desferal (deferriferrioxamine B mesylate) and was purified further by ion exchange and filtration on Bio Gel P2. Desferal was a gift of CIBA Pharmaceutical Company. Crystalline 2,3-dihydroxybenzoic acid was obtained from Aldrich Chemical Company. Albomycin δ_2 was a gift of J. Turková.

Enterobactin was obtained from culture supernatant fluids of *Aerobacter aerogenes* 62-1 grown in low-iron medium with vigorous aeration for 10 h in a 37 C incubator-shaker. Cells were removed by centrifugation from 1 liter of culture, and the supernatant liquid was adjusted to pH 2 with concentrated HCl.

The acidified supernatant fraction was extracted with ethyl acetate until negligible catechol remained, and the extract was washed with 0.5 volume of 0.1 M sodium citrate buffer, pH 5.5. The catechol concentration was determined with the aid of the Arnow reagent (4), and the washed organic phase was then evaporated over the stoichiometric amount of 1 mM $FeSO_4$ in 0.1 M sodium phosphate buffer (pH 7), yielding about 50 μmol of ferric enterobactin per liter of original culture supernatant fluid. This crude ferric enterobactin contained oxidized degradation products and other impurities and was therefore purified further by passage through a column (1.5 by 80 cm) of Sephadex LH-20 (Pharmacia) in sodium phosphate buffer, pH 7. Ferric enterobactin eluted as a slow-moving, compact, red-purple band following a rapidly moving, broad, blue-purple band of unknown composition and preceding a barely motile, purple-black band of oxidized impurity.

Crude colicin B + V lysate was a gift of D. Elseviers.

Bacterial strains. *A. aerogenes* 62-1, a strain auxotrophic for the aromatic amino acids, was obtained from E. Wawszkiewics and was from the collection of F. Gibson. *E. coli* K-12 strain CGSC 856 (F^- *serA*$^-$, *serB*$^-$, λ^-) was obtained from S. Guterman and was originally from the collection of S. Luria.

Isolation of mutants. *tonA* and *tonB* mutants were selected as spontaneously occurring resistant colonies on plates of strain CGSC 856 confluently lysed with ϕ80vir. The *tonA* phenotype was confirmed by resistance to T5 infection. Mutants displaying the *tonB* phenotype could be distinguished by their small colony size. The *tonB* phenotype was confirmed by sensitivity to T5 infection and by resistance to crude lysates of colicin B + V as determined by streaking against an absorbed spot of colicin solution on a nutrient agar plate. Albomycin resistance was tested by radial streaking.

Media. All bacteria used in this study were grown in Difco nutrient broth for overnight cultures and storage. *A. aerogenes* 62-1 was grown in the low-iron minimal salts medium of Pollack and Neilands (18), supplemented with the following (per liter): $CaCl_2$, 10 mg; thiamine-hydrochloride, 0.1 mg; DL-methionine, 40 mg; DL-tryptophan, 30 mg; L-tyrosine, 20 mg; and DL-phenylalanine, 15 mg. The following additional media were used: tryptone broth, plates, and top agar (7) for work with all phage but T5, and LC broth, plates, and top agar (tryptone medium plus 0.5% yeast extract plus 5 mM $CaCl_2$) for T5 work. LC plates contain 1.5% agar. LC top agar contains 0.6% agar.

Phage. ϕ80vir was obtained from D. Elseviers, ϕ80h and T5 were from M. Chamberlin, and λcI was from U. Kühnlein. The hybrids $h^{+80}att^{80}imm^{\lambda}$ and $h^{+\lambda}att^{80}imm^{80}$ were obtained from N. Franklin.

Assay of phage titer. All phage were assayed with strain CGSC 856 grown to mid-log phase in the appropriate medium. For infection with phage other than T5, the bacteria were washed once with 1 mM $MgSO_4$ and resuspended in this solution for preadsorption. T5 was preadsorbed without this wash. Exactly 0.1 ml of a suitable dilution of phage was mixed with 0.1 ml of strain CGSC 856 and allowed to adsorb for 20 min (30 min was allowed for phages with λ immunity). The appropriate top agar (2 ml) was then added, and the mixture was poured onto plates and incubated overnight at 37 C.

Assay of inhibitory activity. A 0.1-ml volume of the solution to be tested, or 0.1 ml of distilled water, was added to the bacteria prior to addition of phage. Plaque production was then assayed by the usual procedure on plates of constant volume.

Adsorption assay. For measurement of adsorption of ϕ80, strain CGSC 856 was prepared in the same manner as for assay of ϕ80 titer. To 0.1 ml of phage was added 0.8 ml bacteria plus 0.1 ml of siderochrome or distilled water. After 15 min at 37 C, cells were removed by centrifugation for 12 min. The supernatant liquid was diluted into cold 1 mM $MgSO_4$ to give 10^3 to 4×10^3 phage per ml. This suspension was assayed for phage titer by the usual procedure, and the plaque count was compared to controls prepared with 0.8 ml of $MgSO_4$ solution substituted for bacteria during the adsorption period.

RESULTS

Mutant response to albomycin. A total of 20 spontaneously occurring ϕ80vir-resistant mutants of *E. coli* CSCG 856 (an albomycin-susceptible strain) were purified and streaked to test their susceptibility to albomycin. Of these, 19 proved to be resistant to the antibiotic, 18 displaying a classical *tonA* phenotype (resistance to phage T5 and ϕ80vir) and one displaying the *tonB* phenotype (sensitivity to T5 with resistance to colicins B and V, and resistance to ϕ80vir). An additional 10 *tonB*-type mutants of CGSC 856 were independently selected and all were found resistant to albomycin.

Effect of ferrichrome on plaque production by ϕ80vir. Since the resistance to albomycin observed in the *tonA* and *tonB* mutants suggested that the phage resistance in these strains might be due to a defect in the normal ferrichrome uptake system, it was of interest to determine whether the infection of a susceptible strain by a phage such as ϕ80vir could be inhibited by ferrichrome. Therefore, ϕ80vir was plated on strain CGSC 856 in the presence or absence of micromolar concentrations of ferrichrome. When about 100 plaque-forming units were employed per plate, inhibition of plaque production by ϕ80vir began to appear at a final ferrichrome concentration of about 0.15 μM, and was 90% complete at 3 μM ferrichrome. Inhibition of phage growth by ferrichrome was manifested both in decreased plaque count and in smaller plaque size (Fig. 1), so that the percent inhibition of plaque count was inversely related to the final plaque size attained by the control. Since the latter parameter

was difficult to standardize, the data yielded by the plate inhibition assay should be regarded as only roughly quantitative.

Preincubation of phage with ferrichrome. To investigate the possibility that ferrichrome might inactivate φ80vir directly, a portion of phage was preincubated with 100 μM ferrichrome for 15 h, then diluted (10^4) and plated, and the plaque count was compared to that of a control portion which had not been treated with ferrichrome. When water was added during preincubation 4.2×10^8 plaque-forming units per ml resulted, and when 0.1 mM ferrichrome was added during preincubation 4.1×10^8 plaque-forming units per ml resulted (average of three determinations). Clearly, prolonged exposure to ferrichrome at a concentration sufficient to completely inhibit plaque production when present in a plate had no substantial irreversible effect on φ80vir itself.

Response of φ80vir to other siderochromes and chelating agents. Chromium[III] deferriferrichrome and seven additional siderochromes at micromolar concentrations were tested for their effect on plaque production by φ80vir to assess the specificity of the response to ferrichrome (Table 1). Substitution of chromium[III] for iron resulted in a reduction of the inhibitory activity below the level detectable by the plate assay at the concentration tested. Ferrichrome A, an analogue of ferrichrome which at best shows slight growth enhancement toward *enb*⁻ mutants of *S. typhimurium* (unpublished observation) likewise did not detectably inhibit plaque production by φ80vir. Furthermore, most of the siderochromes structurally distinct from ferrichrome showed no inhibitory activity at the concentration tested, and the synthetic general chelating agent, ethylenediaminetetraacetic acid was similarly inactive. However, the dihydroxamate, rhodotorulic acid, showed substantial inhibitory activity.

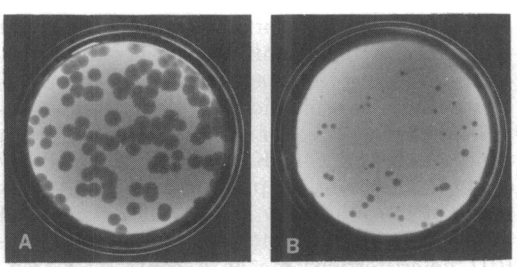

FIG. 1. *Plaques of φ80vir on a lawn of E. coli K-12 strain CGSC 856: (A) No siderochrome added; (B) 50 nmol of ferrichrome added during preadsorption of phage to cells prior to plating.*

TABLE 1. *Siderochrome and chelate inhibition of plaque formation by φ80vir*

Addition[a]	Inhibition[b] (%)
Chromium [III] deferriferrichrome	<10
Ferrichrome A	<5
Ferrioxamine B	<10
Rhodotorulic acid	20–40
Schizokinen	<10
Ferric citrate	<10
Enterobactin	<10
2,3-Dihydroxybenzoic acid	<10
Ethylenediaminetetraacetic acid	<10

[a] Each compound tested was added to a final concentration of 0.1 μmol/30-ml plate. With the exception of chromium deferriferrichrome and ethylenediaminetetraacetic acid, all compounds were added as the ferric chelates.

[b] Average of three determinations.

Phage specificity of ferrichrome inhibition. The possibility remained that ferrichrome was acting as a general inhibitor of phage growth or infection, i.e., that its effects would not be limited to φ80. Therefore, several phage strains, related and unrelated to φ80, were assayed for plaque production in the presence of ferrichrome. When ferrichrome was added during preadsorption to give 0.1 μmol/30-ml plate, there was 100, <5, <5, and 100% inhibition (average of three determinations) with φ80h, λcI, $h^{+\lambda}att^{80}imm^{80}$, and $h^{+80}att^{80}imm^{\lambda}$, respectively. Infection by phage φ80h, a host range mutant of φ80 which will form plaques on *tonB* strains but not on *tonA* strains of *E. coli* K-12 (7), was found to be highly susceptible to ferrichrome inhibition. When this response was investigated further, it was discovered that plaque production by φ80h was inhibited by as much as 60% when 1 nm of ferrichrome was added per plate, an amount nearly 50 times below that required to give a similar inhibition of φ80vir infection in the plate assay.

Adsorption of φ80 in the presence of ferrichrome. Since the structure of the siderochrome and the host range of the phage appeared to be the decisive parameters involved in the inhibition of φ80, the interaction of ferrichrome with the phage at the adsorption step in infection was examined (Table 2). Phage were allowed to adsorb to cells of strain CGSC 856 in the presence and absence of ferrichrome for 15 min. Cells were then removed by centrifugation, the remaining unadsorbed phage were plated, and plaque count was compared to phage controls that had not been allowed to adsorb. Ferrichrome was found to inhibit φ80 adsorp-

tion, giving 50% inhibition at a minimal concentration of 8 nM. By contrast, rhodotorulic acid did not detectably inhibit adsorption at 1 μM. Chromium [III] deferriferrichrome and aluminum deferriferrichrome were also tested and shown to inhibit adsorption, the former by 50% and the latter by 30% at 1 μM in a representative experiment. It should be noted that the percent inhibition of adsorption for a given ferrichrome concentration showed considerable variation from one experiment to the next, and therefore the data presented in Table 5 represent the maximal values obtained for each concentration tested.

DISCUSSION

Mutants of *E. coli* resistant to phage φ80vir by virtue of a receptor defect fall into two classes, *tonA* and *tonB* (17). The former mutation also imparts resistance to phages T1, T5, and φ80h (7), and to colicin M (8), whereas mutations in the *tonB* region can provide simultaneous or separate resistance to phage T1 (but not T5 or φ80h) (7) and colicins B, M, V, and I (8, 21). We have now found that spontaneously occurring *tonA* and *tonB* mutants of *E. coli* K-12 strain CGSC 856 also display resistance to albomycin, whereas the parent *ton*$^+$ strain shows distinct susceptibility to this ferrichrome-type antibiotic.

Resistance to albomycin may be due either to a defect in the specific system that transports the antibiotic into the cell or to cellular changes unrelated to permeation. Mutants in the *tonB* region are known to show reduced citrate-dependent iron uptake (22-24), and they seem unable to transport ferric enterobactin (20) (also called enterochelin), the siderochrome secreted by *E. coli* strains under iron stress. It would not be entirely surprising, therefore, to find that *tonB* mutants are also unable to take up albomycin and are resistant to the drug by virtue of this defect. The *tonA* mutation, on the other hand, has not been associated previously with any defect in cell transport or metabolism outside of the loss of phage receptor activity. For this reason, we sought to investigate the possibility that one or more of the phage receptors inactivated by the *tonA* mutation might function physiologically in the uptake of ferrichrome compounds and albomycin by *E. coli* cells.

TABLE 2. *Siderochrome inhibition of φ80vir adsorption to E. coli strain CGSC 856*

Addition	Concn (nM)	Inhibition[a] (%)
Ferrichrome	1	<5
	4	15
	6	30
	7	35
	8	50
	10	80
	15	>95
Chromium [III] deferriferrichrome	1,000	50
Aluminum deferriferrichrome	1,000	30

[a] Average of three determinations of final phage titer.

Our initial approach to this problem has been the indirect one of looking for a competition between phage and ferrichrome at the postulated common receptor site. Phage φ80vir was first selected for this purpose because the receptor for this phage is apparently inactivated by both the *tonA* and the *tonB* mutation. Additionally, φ80 can be handled and assayed more conveniently than phage T1 or colicin M, agents with host range identical to φ80vir.

Our investigation immediately disclosed that ferrichrome was a potent inhibitor of plaque formation by φ80vir. Fewer and smaller plaques appeared in the presence of ferrichrome added to pour plates in final concentrations ranging from about 0.1 to 3 μm. Above about 3 μM ferrichrome completely prevented plaque production.

We sought to determine whether this observed inhibition of plaque formation by ferrichrome could indeed be interpreted as representing a competition between the siderochrome and the phage for a site involved both in the absorption of the phage and in the uptake of ferrichrome compounds. It was shown, first of all, that ferrichrome did not inactivate the phage directly: lengthy preincubation of φ80vir with the siderochrome at a concentration two orders of magnitude higher than that required to give 90% inhibition of plaque formation in the plate assay had no significant irreversible effect on the ability of the phage to infect *E. coli* K-12. However, this result did not rule out a nonspecific protective effect of iron or siderochromes in general for *E. coli* K-12 when infected by φ80vir. Therefore, a preliminary survey was made of the inhibitory activity of a number of additional siderochromes, including chromium-[III] deferriferrichrome as a control for iron. The latter compound did not detectably inhibit plaque formation at the concentration tested. However, as was shown later, the chromium complex did retain a significant, if re-

duced, ability to inhibit adsorption of the phage to cells. This residual activity of the chromium complex was not due to contaminating ferrichrome, since the chromium does not exchange with iron at pH 7 and since the chromium complex was prepared from apoferrichrome containing less than 1% iron and recrystallized from methanol after chromatography by cation exchange and gel filtration (John Leong, personal communication). Thus, it appears that iron potentiates the inhibitory effect of the siderochrome, but the capacity of the siderochrome to provide iron to cells, which is lacking for the chromium chelate, is not required for phage inhibition.

Capacity to inhibit φ80 plaque production at the concentration tested by the plate assay was not found to be a general characteristic of siderochromes or chelating agents, since enterobactin, ferric citrate, schizokinen, 2,3-dihydroxybenzoic acid, and ethylenediaminetetraacetic acid did not detectably reduce plaque count or plaque size. Although we had previously reported a marked effect of ferrioxamine B on plaque formation by φ80vir when the siderochrome preparation obtained by adding iron to commercially available deferrioxamine B mesylate was employed (R. Wayne and J. B. Neilands, Pacific Slope Biochemistry Conference, Abstracts, p. 55, 1974), we have now found that after purification by ion-exchange chromatography and gel filtration this siderochrome preparation is without inhibitory activity. In any event, it is clear from the responses to the siderochromes tested that φ80vir infection is especially susceptible to inhibition by ferrichrome. Additionally, it appears that a ferrichrome-type structure is not in itself sufficient for activity detectable by the plate assay. Thus, ferrichrome A, a biologically "inactive" analogue of ferrichrome which is able only poorly to support the growth of siderochrome-requiring *enb*$^-$ mutants of *S. typhimurium*, did not inhibit φ80vir infection at a concentration allowing 90% depression of plaque count by ferrichrome. Presumably the siderochrome must be specifically "recognized" at a binding site for phage inhibition to occur.

In this latter regard, it is of interest that the dihydroxamate, rhodotorulic acid, was detectably active in the plate inhibition assay, indicating that a ferrichrome-type structure of the siderochrome is not required for inhibitory activity toward φ80vir. However, unlike ferrichrome, rhodotorulic acid at a concentration of 1 μM did not inhibit adsorption of φ80 to cells. Thus, it is not likely that the inhibitory activity of this compound can be ascribed to a specific competition with phage for a cell envelope site required for phage attachment. Of the siderochromes tested, only ferrichrome seems of interest in this respect.

Further investigation showed that the inhibitory activity of ferrichrome behaves as though it depends heavily on the receptor specificity of the phage. Phage λcI$^-$, whose receptor is unrelated to that of φ80, was unaffected by ferrichrome. Similarly a λ/φ80 hybrid carrying the λ host range with φ80 immunity and attachment genes was oblivious to the siderochrome. However, ferrichrome effectively inhibited a λ/φ80 hybrid carrying the φ80 host range and attachment genes with λ immunity. Thus, the φ80 host range appears to be sufficient to impart sensitivity of phage plaque formation to ferrichrome, although a role of the attachment genes cannot be excluded with the hybrids used. However, the heightened sensitivity of φ80h infection to ferrichrome supports the contention that the host range of the phage determines its response to the siderochrome, as would be expected if a competition is occurring between siderochrome and phage for a specific site on the cell surface.

Finally, solutions of ferrichrome, chromium-[III] deferriferrichrome, and aluminum deferriferrichrome were all shown to prevent adsorption of φ80vir to sensitive cells of *E. coli* K-12. Under conditions allowing 75% total adsorption of added phage to the control cells, ferrichrome reduced adsorption by half when present at a concentration of 8 nM in the adsorption mixture. Chromium and aluminum deferriferrichrome, however, proved to be on the order of 100 to 200 times less potent than ferrichrome in preventing adsorption. This may indicate that the *E. coli* cell is able to "recognize" the metal ion upon binding of the siderochrome to the cell envelope, in this way avoiding the accumulation of the metabolically inert metal complexes in favor of the more useful iron chelate.

We note with interest that the data in Table 5 give an S-shaped curve for ferrichrome concentration versus inhibition of phage adsorption. However, further experimentation will be necessary to confirm the sigmoidal nature of this relationship, and it is the aim of this paper merely to provide evidence of a competition between ferrichrome and φ80 for a common receptor site.

The findings reported here bear a striking resemblance to those reported by DiMasi et al. (6) and by Kadner and Liggins (14) detailing the interaction of colicins E1 and E3 with another coordination compound, vitamin B_{12}, at an outer membrane site involved in adsorption of the colicins and binding for uptake of the vitamin. Additionally, Guterman has described

the inhibition of colicin B by the siderochrome enterobactin (11–13). This inhibition may also represent a competition for a site on the outer membrane of the cell that functions physiologically in the binding for uptake of the siderochrome, since enterobactin was shown to inhibit adsorption of colicin B (12) and since *tonB* mutants are simultaneously insensitive to the colicin and unable to take up ferric enterobactin.

E. coli K-12 has been shown to possess an inducible transport system for ferric citrate (5, 9), and recently Frost and Rosenberg (G. E. Frost and H. Rosenberg, Proc. Aust. Biochem. Soc. 6, p. 49, 1973) have reported the existence of a separate system that transports the ferric chelate of rhodotorulic acid. The cellular organization of these transport systems has not been elucidated, but the findings of the present paper should focus interest on the possibility that these systems, together with those for ferrichrome and enterobactin, may involve outer membrane-binding sites.

The participation of such outer membrane-binding sites in the transport of specific siderochromes into the bacterial cell would distinguish the siderochrome uptake systems from those for sugars and amino acids, which apparently do not require the involvement of outer membrane components. An explanation for a novel cellular organization of the siderochrome systems might lie in the permeability properties of the outer membrane, as recent research on *S. typhimurium* and *E. coli* has defined an "exclusion limit" of molecular weight 800 to 900 for the outer cell envelope of these strains (G. Decad, T. Nakae, and H. Nikaido, Fed. Proc., p. 1240, 1974). Monosaccharides and amino acids would have no difficulty passing through a protective barrier of this porosity. However, since most siderochromes have molecular weights approaching the exclusion limit, it could be expected that special systems would have been retained by the bacterial cell to ensure efficient penetration of these iron carriers through the outer membrane. A location of the siderochrome-binding components at the site of adhesions between cell wall and cytoplasmic membrane, adhesions to which a number of bacteriophage including T1 and T5 appear to adsorb (2), would also facilitate the transfer of the siderochromes across the *E. coli* cell envelope into the cytoplasm. That certain of these binding components may have been retained in spite of their utilization as phage and colicin receptors would attest to the selective advantage conferred by their participation in the maintenance of an adequate cellular supply of iron.

A final confirmation of the identity of one or more of the phage receptors in the outer membrane of *E. coli* with a binding component of the ferrichrome uptake system will require the isolation of a given phage receptor and demonstration of its ability to specifically bind ferrichrome. The phage T5-colicin M receptor originally isolated by Weidel et al. (25) appears particularly promising in this regard, since ferrichrome protects cells of *E. coli* from colicin M (unpublished observation, this laboratory), and we have recently found that ferrichrome will specifically inhibit binding of coliphage T5 to crude, cell-free preparations of the T5 receptor protein when the siderochrome is present at a concentration of 1 μM (M. Luckey, R. Wayne, and J. B. Neilands, manuscript in preparation). Thus, it is a reasonable hypothesis that the outer membrane protein which forms the phage T5-colicin M receptor (3), and which presumably also forms part of the ϕ80 receptor, functions in the intact cell in the specific binding for uptake of ferrichrome.

ACKNOWLEDGMENTS

We thank Naomi Franklin for invaluable technical advice and for phage strains, Mary Luckey for her assistance and helpful discussions, and the several people who contributed phage, chemicals, and bacterial strains.

This research was supported by Public Health Service grants AI 04156 and AM 17146 from the National Institute of Allergy and Infectious Diseases and the National Institute of Arthritis, Metabolism, and Digestive Diseases, respectively.

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