IN VITRO COMPETITION BETWEEN FERRICHROME AND PHAGE FOR THE OUTER MEMBRANE T5 RECEPTOR COMPLEX OF *ESCHERICHIA COLI*

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Summary

Ferrichrome is shown to compete *in vitro* for the partially purified outer membrane phage T5 receptor complex of *Escherichia coli* K-12. Among siderochromes tested the competition is confined to ferrichrome and its analogs. Thus reversal of receptor inhibition of plaque formation is achieved by ferrichrome, ferricrocin, and the aluminum and chromium(III) derivatives of deferriferrichrome but not by rhodotorulic acid, Desferal, or ferrichrome A.

Introduction

Enteric bacteria have evolved a variety of systems for acquiring iron from their environment, including the ability to utilize low molecular weight iron carriers (siderochromes) produced either indigenously or by other microbial species. *Salmonella typhimurium* mutants (termed *sid*) defective in the uptake of hydroxamate-type siderochromes were obtained by selection for resistance to albomycin, a close structural analog of the specific siderochrome ferrichrome (1). Most of the *sid* mutations were found to cotransduce with *panC*, a genetic locus that is close to the *tonA* allele in *Escherichia coli*. The findings that both *tonA* (resistant to phages T1, T5, and Φ80) and *tonB* (T1- and Φ80-resistant) mutants of *E. coli* are albomycin-resistant and that ferrichrome protects sensitive cells from phage Φ80 by preventing phage adsorption led to the suggestion that a component of the Φ80 receptor in the outer membrane of *E. coli* is essential for the uptake of ferrichrome-type compounds (2, 3). The effect of *tonA* and *tonB* alterations on ferrichrome uptake was then confirmed by Hantke

and Braun, who showed that ferrichrome-stimulated ^{55}Fe transport is absent in mutants bearing either of the *ton* phenotypes (4).

Ferrichrome also protects cells from Φ80h, a mutant of Φ80 carrying the host range of T5 (3) and from colicin M (3, 4). The T5 receptor, as extracted from the outer membrane of *E. coli* B, has been shown to bind T5 and colicin M (5). In this report we demonstrate that ferrichrome competes *in vitro* with T5 for the partially purified receptor and we show that the effect is restricted to ferrichrome and its close analogs.

Experimental Procedures

Ferrichrome, ferrichrome A, rhodotorulic acid, Desferal, albomycin, and chromium-deferriferrichrome were obtained as described previously (3). Additionally, ferricrocin was a gift from W. Keller-Schierlein and H. Zahner, and aluminum-deferriferrichrome was obtained from Miguel Llinás (6). Ferrichrome A was esterified in methanolic-HCl and the 2^-, 1^-, and uncharged methyl esters were separated by paper electrophoresis.

E. coli JC6724 was obtained from Alex Karu and grown in M9 medium or in L broth containing 10 gm Bacto tryptone, 5 gm Bacto yeast extract, 5 gm sodium chloride per liter, adjusted to pH 7.0 with sodium hydroxide. Phage T5 was provided by Mirium Golomb and stored in T2 buffer (7).

Partially purified receptor was prepared by mild alkaline extraction of JC6724 grown to late-log phase in L broth, followed by differential centrifugation and chromatography on Bio-gel A-50 (5). Repeated freezing and thawing was found to destroy activity, so the receptor was stored at 4°C in 0.01 M sodium phosphate, pH 7.0, with 0.1% sodium azide added as preservative.

Phage binding activity of the receptor was assayed as described by Braun et al (5), with siderochrome competition determined by inclusion of the compound to be tested with phage and receptor in the incubation mixture. The azide buffer was found to have a negligible effect on the phage assay and the various siderochromes tested, with the exception of enterobactin, did not reduce plaque formation when incubated in the absence of receptor.

Results and Discussion

Receptor was isolated from JC6724 grown to late-log phase in L broth. Comparison of cells grown in M9 minimal medium with those grown in L broth showed negligible difference in the number of plaque-forming units (PFU) obtained, indicating that presence of receptor is not repressed by rich medium. In both media, however, PFU decreased when cells entered stationary phase, falling to *ca.* 50% of the original number after two hr in stationary phase, as shown in Table I.

Receptor was partially purified as described in Experimental Procedures. Phage binding activity of the complex thus obtained was routinely assayed with 100 µl of 4 x 10^3/ml T5. Aliquots of the receptor complex containing *ca.* 100 µg total protein were usually required to completely inhibit this amount of phage. When the receptor preparation is fresh, this phage binding was completely reversed by 10 µM ferrichrome. After three months' storage at 4°C the receptor functions efficiently in inhibiting plaque formation but is only about one-third reversed by 10 µM ferrichrome. Thus while the results obtained with

Table I

Plaque-Forming Ability of Phage T5
on Cells of *Escherichia coli* JC6724 Grown in L Broth and M9 Medium

	L Broth		M9 Medium	
	OD 650	PFU/Plate	OD 650	PFU/Plate
Log	1.02	412	0.8	455
Stationary	1.8	233	1.5	269

At varying points on the growth curve, 0.1 ml cells were incubated 20 min with 100 µl of 4 x 10^3/ml T5 at 37°C, and plated with 2 ml L top agar (0.7%) on fresh L plates.

a given preparation of receptor are fairly reproducible, the percent reversal by ferrichrome may vary considerably and the numbers reported below are given as representative samples.

Reversibility of the inactivation of receptor by ferrichrome was determined by removal of ferrichrome by dialysis after both short (1 hr, 37°C) and long (22 days, 4°C) incubations of the receptor preparation in 10 μM ferrichrome. Phage binding activity was 80-90% restored and was again competed by ferrichrome.

The ability of ferrichrome to reverse receptor inhibition of plaque formation is shown to be quite specific by the failure of other siderochromes, rhodotorulic acid, Desferal, and ferrichrome A, to have activity when tested in concentrations up to 100 μM. This is illustrated in the two experiments shown in Table II. Enterobactin also showed little or no ability to reverse

Table II

Specificity of Reversal
of Inhibition of Plaque Formation by Receptor

Incubation Mixture	PFU/Plate	
	Receptor Preparation I	Receptor Preparation II
T5 in T2 buffer	358	147
T5 + receptor	6	17
T5 + receptor + ferrichrome	100	72
T5 + receptor + ferrichrome A	11	NT
T5 + receptor + Desferal	NT	18
T5 + receptor + rhodotorulic acid	NT	11

A 1-hr incubation at 37°C of 100 μl T5 phage in T2 buffer with 100 μl receptor (*ca.* 1 mg/ml protein) and 100 μM siderochrome, where indicated, was followed by addition of 0.1 ml exponentially growing JC6724, incubation 20 min at 37°C and plating with L top agar on fresh L plates. NT = not tested.

inhibition of plaque formation in concentrations up to 50 μM, but levels of enterobactin of 50 μM and above were found to reduce PFU in the control incubation mixtures (lacking receptor).

On the other hand, ferricrocin, which differs from ferrichrome only in the substitution of a seryl for a glycyl residue in the cyclohexapeptide, is able to reverse inhibition of plaque formation by receptor, giving 50% activity at 25 μM. Furthermore, the di- and trimethyl esters of ferrichrome A are quite effective in reversing phage binding, while the monomethyl ester has an intermediate level of activity. Thus the lack of activity of ferrichrome A may be attributable to its 3^- charge.

Metal substituted ferrichromes also proved to be active. A 5-μM concentration of aluminum-deferriferrichrome or chromium-deferriferrichrome gives 50% activity in reversing inhibition of plaque formation by receptor.

Ferrichrome has been observed *in vivo* to protect *E. coli* from phages Φ80 and Φ80h, and from colicins M, B, I, and V, and to protect *S. typhimurium* from phage ES18 (2, 3, 4, & unpublished observations in this laboratory). The specific reversible *in vitro* inhibition by ferrichrome-type compounds of T5 binding to a partially-purified T5 receptor complex, as reported here, suggests that the protection of cells *in vivo* by ferrichrome does not require a response of the intact cell but is a property of an outer membrane receptor complex.

The *in vitro* activity is limited to ferrichrome and its close analogs. Changes at the metal binding center do not reduce the activity of ferrichrome, and both kinetically-labile (Al) and stable (Cr) ions give fully active compounds. Minor modifications in the peptide ring, as in ferricrocin, are tolerated; a bulky, charged side chain, as in ferrichrome A, is not. Progressive removal of the negative charges on ferrichrome A by esterification restores activity; a similar effect has been reported for competition with albomycin in the Bonifas test in *Bacillus subtilis*, in which the trimethyl-ferrichrome A gave activity while the free acid did not (8).

The presence of receptor activity in cells grown in rich medium shows

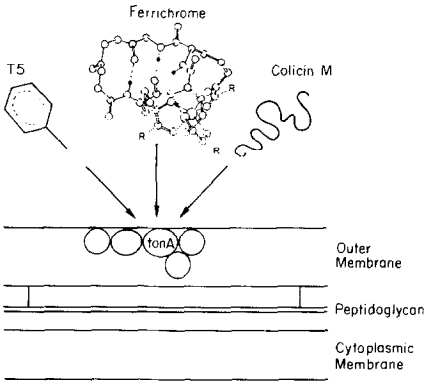

Fig. 1. A schematic diagram of the proposed model for the outer membrane receptor complex in which ferrichrome competes with phage and colicin for the receptor. R = CH_3 for ferrichrome. R = $\overset{CH_3}{\underset{CH_2COOH}{\diagup\!\!=\!\!\diagdown}}$ for ferrichrome A.

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that synthesis of the *tonA* protein is not repressed by iron; likewise cells grown in rich medium are fully sensitive to albomycin inhibition.

These results support the model wherein the outer membrane protein that forms the T5-colicin M receptor functions in the intact cell for ferrichrome uptake and the presence of ferrichrome at this site renders the receptor unavailable for phage (or colicin) attachment (see Fig. 1). The demonstration of loss of ferrichrome uptake in *tonA* and *tonB* mutants also supports this model (4). However, since there is now evidence that the T5 receptor protein exists in an ordered complex of proteins on the surface of the intact outer membrane (9), it is possible that the ferrichrome competition with T5 observed with the partially-purified receptor may involve more than one protein. It remains to be determined whether the *tonA* protein itself is capable of directly binding ferrichrome.

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