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Use of Transferrin-Iron-Enterobactin Complexes as the Source of Iron by Serum-Exposed Bacteria

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Two smooth and six rough strains of *Salmonella typhimurium* with progressively smaller amounts of sugar and protein in their outer membrane were tested for degree of virulence in normal and iron-injected mice and for ability to acquire iron in mammalian sera. The rate of mortality showed that bacterial virulence for mice was lowered with progressive decrease of outer-membrane sugar and protein. Iron injections increased the rate of mortality in mice infected either with smooth strains or with superficially rough strains but were without effect in mice infected with deep rough strains. In in vitro experiments, iron promoted with equal effectiveness the growth of all serum-exposed bacterial strains, whereas enterobactin (E) was much more effective in promoting the growth of smooth and superficial rough than in promoting that of deep rough strains. Various experiments showed that deep rough strains cannot grow in E-supplemented serum because they are not able to use the transferrin-iron-E complexes that E forms with transferrin-iron. This failure to use transferrin-iron-E complexes by deep rough strains was found to be due to the inability of these strains to adsorb iron-containing complexes to their outer membrane. Adsorption studies with chemically treated bacteria showed that the receptor of transferrin-iron-E or E-iron complexes is a protein of the outer membrane of bacterial cells.

Studies in several laboratories have shown that abilities of bacteria to acquire iron in mammalian sera are related to their degrees of virulence (6). Findings in our laboratory have demonstrated that avirulent bacteria cannot obtain iron from transferrin (Tr), and without this essential metal they die rather rapidly; only in serum supplemented with exogenous iron or with iron-binding bacterial products, siderophores, can avirulent bacteria grow without any hindrance (8). Virulent bacteria can overcome serum-imposed iron starvation by the activity of iron-providing systems associated with their walls. Various experiments have shown that the multiplication of tubercle bacilli in serum is facilitated by the cell wall-associated mycobactin (3) and that the multiplication of *Escherichia coli* is facilitated by the outer-membrane-associated enterobactin (E) (8). These findings led to the conclusion that iron-binding substances of bacterial walls permit the initiation, whereas extracellular siderophores help in the promotion, of bacterial growth in animals or in their sera.

The aim of the present investigation was to indentify mechanisms that help virulent bacteria to obtain iron from Tr and, thus, permit bacterial growth in animal tissues. This study was facilitated by using bacteria that have been made defective in outer-membrane lipopolysaccharides (LPS) and proteins by mutations or chemical treatments. In vitro and in vivo experiments with these outer-membrane-defective bacteria showed that bacterial survival in animals and in their sera is dependent upon the ability of bacterial cells to adsorb Tr-iron-E complexes and to use these complexes as the source of iron.

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MATERIALS AND METHODS

Bacterial strains. Two smooth strains (SL1515 and SL3770) and five rough strains (SL3749, SL3750, SL3748, SL3769, and SL3789) were derived from *Salmonella typhimurium* LT-2 by transduction of mutations in the *rfa* gene cluster with bacteriophage ES18 (9). These mutants were found to possess progressively smaller amounts of sugar in the LPS core of their outer membranes. A sixth rough strain, SL1102, a rough mutant of smooth strain SL1027, contained no sugar. Subsequently, it has been found that loss of LPS sugar lowers amounts of outer-membrane proteins (1); for convenience, in this report these strains are called either rich or poor in LPS sugar. In addition to these strains of *S. typhimurium*, two previously described strains of *E. coli* were used in certain experiments: strain A has been found to be avirulent, and strain C has been found to be virulent (8).

Bacterial strains were maintained with monthly transfers on nutrient agar slants. Before use in experiments, they were cultured in Trypticase soy broth for 18 h at 37°C. After this growth period, bacteria were collected by centrifugation, bacterial pellets were resuspended in physiological saline, and bacterial suspensions were adjusted to a desired turbidity by using a Klett colorimeter.

Determinations of virulence. The ability of each bacterial strain of *S. typhimurium* to kill mice was tested by intraperitoneal injection of 10^8 bacterial cells into 20 or more Swiss-Webster mice. To assess the stimulating effect of iron on the growth of bacteria in animals, certain groups of mice at the time of infection were injected intraperitoneally with a solution of ferric ammonium citrate containing 0.1 mg of iron. The mortality rates in infected and iron-treated and untreated mice were recorded during a 5-day period.

Iron starvation of bacteria. The study of the effects of iron unavailability on bacterial strains was performed in media in which iron was bound to Tr or conalbumin. In most cases, the deficiency of iron was achieved by mixing 1 part of bovine serum containing 65% iron-free Tr with 3 parts of iron-poor agar medium, which contained the following ingredients in 1 liter of double-distilled water: asparagine, 2.0 g; N-Z amine, 1.5 g; KH_2PO_4, 1.0 g; Na_2HPO_4, 2.5 g; $MgSO_4$, 10 mg; $CaCl_2$, 0.5 mg; $ZnSO_4$, 0.1 mg; $CuSO_4$, 0.1 mg; dextrose, 5.0 g; and agar, 15.0 g. The content of contaminating iron in this medium was 0.1 μg/ml. The autoclaved, pH 7.5-adjusted medium was mixed thoroughly with serum at 56°C, and the mixture was incubated for 30 min to allow time for binding of all iron to Tr. This serum-agar medium was used in the previously described agar plate diffusion test (7). This test determined not only the effects of iron starvation on bacteria but also permitted study of the serum-neutralizing effects of iron or iron-providing siderophores. The width of bacterial growth on the microbiostatic serum-agar medium around wells charged with iron or E was used as a measure of the effectiveness of the iron-acquiring property of the outer membrane of serum-exposed bacteria.

E. Siderophores of *E. coli* and *S. typhimurium* are chemically identical and serve as iron carriers for both species (15). In this study, the serum microbiostasis was neutralized by enterobactin extracted from spent medium of *E. coli*. Spent medium was prepared by growing strain C of *E. coli* in iron-poor liquid medium, which contained the same ingredients as iron-poor agar medium but no agar. Iron in this medium was depleted to undetectable amounts by treatment with magnesium carbonate. This iron-deficient medium was prepared by adding 25.0 g of magnesium carbonate to 1 liter of the medium and removing the salt by centrifugation after a 5-min incubation at room temperature. One liter of the medium was inoculated with 2.0 ml of bacterial suspension adjusted to a Klett reading of 20. After 24 h of incubation at 37°C, the bacteria were removed from the spent medium by centrifugation, and E was extracted by the Rogers method with ethyl acetate (14). Since E in solution is very unstable, the extracted E was immediately lyophilized, and, when needed, a known amount was solubilized immediately before the use in an experiment.

Adsorption of Tr-iron-E complexes. When results of this study had shown that E does not remove iron from Tr but combines with it, forming Tr-iron-E complexes, the mechanism by which these strains obtain iron in serum became the main subject of our investigation. As the first step, we tested whether heat-killed avirulent and virulent bacteria can adsorb Tr-iron-E complexes. A saline solution containing 2.5 mg of 75% iron-saturated Tr per ml was combined with 25 μg of E, and, after 2 h of incubation at 37°C, it was dialyzed for 14 h against saline at 4°C to remove unbound E. After dialysis, the Tr-iron-E-containing solution was adsorbed with virulent and avirulent bacteria by adding 80 mg (wet weight) of cells per ml of solution. Before use in adsorption experiments, bacteria were killed by exposure to 75°C for 1 h and depleted of outer-membrane-associated E by repeated washings in saline. After a 2-h adsorption period at 37°C, bacteria were removed by centrifugation, and adsorbed and unadsorbed Tr-iron-E solutions were tested for the neutralization of serum microbiostasis by the agar plate diffusion test.

Adsorption experiments were used to identify the nature of the receptor of Tr-iron-E complexes on the outer membrane of bacterial cell. In these experiments, ethylenediaminetetraacetate (EDTA)- or trypsin-treated bacteria of strain SL3770 were used to adsorb Tr-iron-E complexes. Treatments with EDTA and trypsin were performed in 0.01 M phosphate buffer at pH 7.1, which, in 1 ml, contained 0.21 mg of EDTA or 10 mg of trypsin and 200 mg of bacteria. After 30 min of EDTA treatment and 6 h of trypsin treatment at 37°C, bacteria were collected by centrifugation and washed twice with saline. Solutions of Tr-iron-E, Tr-iron, and E-iron complexes were adsorbed with treated and untreated bacteria by a previously described method.

RESULTS

Virulence-potentiating effect of iron. The mortality of untreated and iron-injected mice infected with various strains of *S. typhimurium* showed that the lethality of bacterial strains varied with the amount of LPS sugar (Table 1). Smooth strains (SL1515 and SL3770) and strains with superficial lesions in their LPS core (SL3749 and SL3750) killed mice, whereas strains with deep core lesions were avirulent. Injection of infected mice with iron increased the virulence of smooth and superficially rough strains but not that of deep rough strains. Even daily treatment with iron failed to promote lethality in mice infected with strains that possessed little or no LPS sugar.

Neutralization of serum microbiostasis. The ability of serum-exposed LPS-defective strains to use iron and E was tested by the agar plate diffusion test. Bacterial growth on uniformly inoculated serum-agar medium was promoted around wells charged with 5 μg of iron or 2 μg of E. Examination of plates after a 15-h incubation period showed that iron promoted

TABLE 1. *Mortality of saline- or iron-injected mice infected intraperitoneally with smooth (SL1515 and SL3770) and rough strains of S. typhimurium*

Treatment	Mortality[a] of mice infected with strain:							
	SL1515	SL3770	SL3749	SL3750	SL3748	SL3769	SL3789	SL1102
Saline	12/20	12/20	11/20	9/20	1/20	0/20	0/20	0/20
Iron[b]	15/20	15/20	19/20	17/20	14/20	1/20	0/20	0/20
Daily iron[c]	ND	ND	ND	ND	ND	1/5	0/5	0/5

[a] Number of dead mice at day 5/number originally infected with 10^8 bacteria. ND, Not determined.

[b] Mice received 0.1 mg of iron added to the infection dose in the form of ferric ammonium citrate; this quantity of iron in killed-bacilli-injected mice caused no mortality. The mortality in infected mice treated with ammonium citrate was the same as that in the saline-treated group.

[c] Daily injection with 0.1 mg of iron during a 5-day experimental period.

the growth of smooth and rough strains with equal effectiveness (Fig. 1). However, widths of bacterial growth aroung E-charged wells narrowed as the amount of LPS sugar in bacterial strains became progressively smaller. Finally, E failed to promote the growth of serum-exposed strain SL1102, which is void of LPS sugar.

Titration of the growth-promoting effect of E for serum-inhibited bacteria showed that the effectiveness of each quantity of E to promote growth decreased as amounts of LPS sugar became smaller (Table 2). Irrespective of quantity, E failed to promote growth of strain SL1102.

Formation of Tr-iron-E complexes. The inability of strain SL1102 to grow around E-charged wells made in serum-agar medium sugested that this strain is unable to use E-iron complexes. If so, then E should inhibit the growth of strain SL1102 in growth-supporting medium by binding iron. However, the addition of as much as 40 μg of E to wells made in iron-poor agar medium failed to inhibit the growth of these bacteria. This finding demonstrated that strain SL1102 cells can use E-iron complexes in the absence but not in the presence of serum. The possibility that E does not remove iron from Tr but forms with it Tr-iron-E complexes, which cannot be used by strain SL1102 bacteria, became rather obvious.

The formation of Tr-iron-E complexes was tested by dialysis experiments. In these experiments, solutions of 20 and 100% iron-saturated Tr were combined with E and tested for neutralization of the antibacterial activity of serum before and after 48 h of dialysis against saline. Results showed that serum-imposed iron starvation of bacteria cannot be alleviated with undialyzed or dialyzed iron-saturated Tr (Table 3). However, after the treatment of Tr-iron with E, the mixture supported bacterial growth before and after dialysis. The retention of serum-neutralizing activity by dialyzed Tr-iron and E mixtures demonstrated that E does not remove iron from Tr but forms with Tr-iron serum-neu-

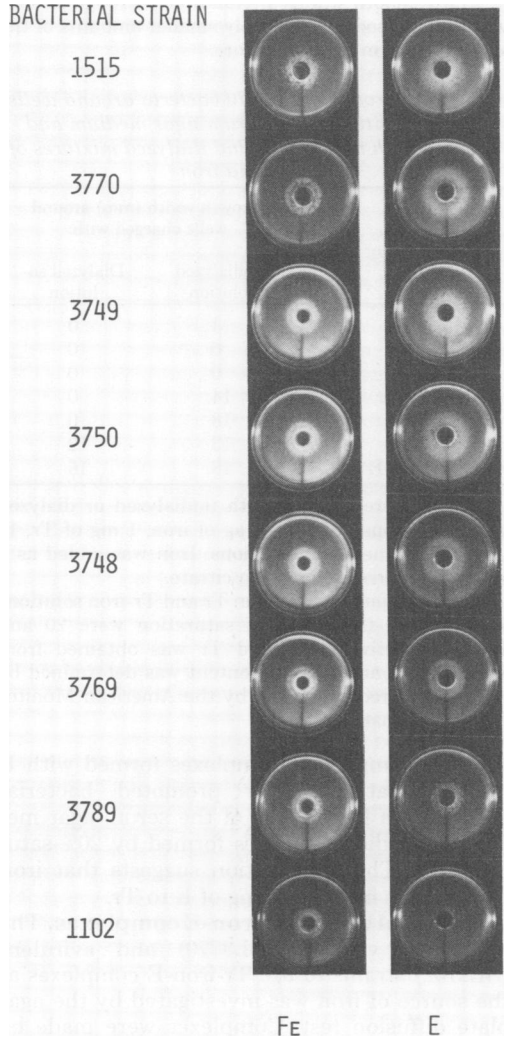

FIG. 1. *Growth of various strains of S. typhimurium on microbiostatic serum-agar medium around wells charged with solutions of ferric ammonium citrate (5 μg of Fe per well) or E (0.5 μg/well).*

TABLE 2. *Growth of various strains of S. typhimurium around E-charged wells made in antibacterial serum-agar medium*

Strain[a]	Growth width (mm) around wells charged with E at (μg/well):				
	8.0	4.0	2.0	1.0	0.5
SL1515	12	10	9	8	5
SL3770	13	12	10	8	7
SL3749	13	13	10	8	7
SL3750	12	12	10	7	4
SL3748	10	9	8	6	3
SL3769	9	8	6	4	0
SL3789	6	5	3	0	0
SL1102	0	0	0	0	0

[a] Two smooth strains are followed by rough strains, which possessed progressively smaller amounts of the sugar moiety in their LPS core.

TABLE 3. *Growth of SL3770 bacteria around wells made in antibacterial serum-agar medium and charged with undialyzed and dialyzed mixtures of Tr, E, and iron*

Solution[a]	Growth width (mm) around wells charged with:	
	Undialyzed solution	Dialyzed solution
Iron	3	0
Tr[b]	0	0
Tr-iron[b]	0	0
E	14	0
E-iron	18	0
Tr-E	13	12
Tr-iron-E	18	16

[a] Wells were charged with undialyzed or dialyzed saline solutions of either 1 μg of iron, 1 mg of Tr, 10 μg of E, or their combinations. Iron was added as a solution of ferric ammonium citrate.

[b] Determinations of iron in Tr and Tr-iron solutions showed that the levels of saturation were 20 and 100%, respectively. Purified Tr was obtained from Pentex, Inc., and its iron content was determined by the method recommended by the American Monitor Corp. (Indianapolis, Ind.).

tralizing complexes. Complexes formed with E by 100%-saturated Tr promoted bacterial growth on a larger area of the serum-agar medium than did complexes formed by 20%-saturated Tr. This observation suggests that iron participates in the binding of E to Tr.

Bacterial use of Tr-iron-E complexes. The ability of virulent (SL3770) and avirulent (SL1102) strains to use Tr-iron-E complexes as the source of iron was investigated by the agar plate diffusion test. Complexes were made by mixing solutions containing 70% iron-saturated Tr with E in the proportion of 1 mg of Tr to 1 μg of E and incubating the mixture for 2 h at 37°C. After the incubation, unbound E was removed by 24 h of dialysis against saline at 4°C. Wells in serum-agar medium were charged with 1 mg of E-saturated Tr, and the medium was inoculated with bacterial suspensions containing 15,000 bacterial units. Examination of plates after 24 h of incubation at 37°C showed that Tr-iron-E complexes supported the growth of LPS sugar-rich strains on a 13-mm-wide area but did not support that of the LPS sugar-void strain.

Adsorption of Tr-iron-E complexes. Adsorption of Tr-iron-E complexes to outer membranes of virulent bacteria was tested as the first step in the mechanism of iron acquisition by serum-exposed bacteria. Solutions of Tr-iron-E were adsorbed with heat-killed virulent and avirulent bacteria (SL3770 and SL3789 of *S. typhimurium* and strains C and A of *E. coli*). Wells made in serum-agar medium were charged with adsorbed solutions, and the medium was inoculated with strain SL3770. Examination of plates after 24 h of incubation at 37°C showed that Tr-iron-E solutions adsorbed with virulent strain SL3770 or strain C lost, whereas those adsorbed with avirulent strain SL3789 or strain A retained, their growth-supporting activity for serum-exposed bacteria (Fig. 2). The ability of virulent and the failure of avirulent bacteria to adsorb Tr-iron-E complexes indicate that better survival of virulent bacteria in serum can be attributed to the possession of receptors for the complexes.

Receptors of Tr-iron-E complexes. The chemical nature of the outer-membrane receptor of Tr-iron-E complexes was investigated by adsorption experiments. Solutions of Tr-iron-E, E-iron, and Tr-iron were adsorbed with untreated and EDTA- or trypsin-treated cells of *E. coli* C or *S. typhimurium* SL3770. Unadsorbed and adsorbed solutions of iron-containing complexes were tested for the neutralization of serum microbiostasis by the agar plate diffusion test. Results showed that the microbiostasis-neutralizing activities of Tr-iron-E and E-iron solutions were lost after adsorption with untreated bacteria (Table 4). Adsorption of these solutions with EDTA- or trypsin-treated bacteria failed to remove their neutralizing effects for serum bacteriostasis; in comparison with activities of unadsorbed solutions of Tr-iron-E or E-iron, solutions adsorbed with trypsin-treated cells did not lose any, and those adsorbed with EDTA-treated cells lost about 50%, of the microbiostasis-neutralizing activity. Unadsorbed and adsorbed Tr-iron solutions exerted no growth-promoting activity for serum-exposed bacteria.

DISCUSSION

The demonstration that avirulent bacteria succumb to iron starvation in spent mammalian

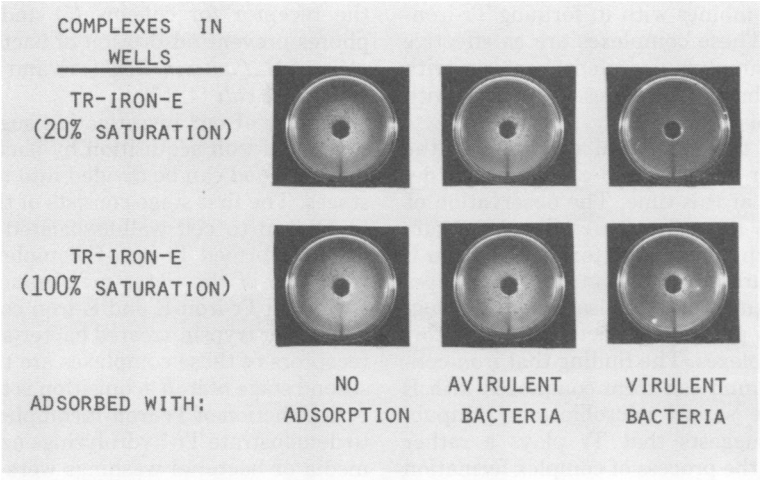

FIG. 2. *Growth of E. coli strain A on microbiostatic serum-agar medium around wells charged with solutions of Tr-iron-E complexes untreated or adsorbed with avirulent (strain A) or virulent (strain C) bacteria. Similar results were obtained after adsorption with avirulent and virulent strains of S. typhimurium.*

TABLE 4. *Bacterial growth on microbiostatic serum-agar medium around wells charged with unadsorbed and adsorbed solutions of Tr-iron-E complexes*

Adsorbing bacteria	Treatment of bacteria	Growth[a] width (mm) around wells charged with:		
		Tr-iron-E	E-iron	Tr-iron[b]
None	None	15	13	0
SL3770 or *E. coli* C	None	0	0	0
	EDTA	10	9	0
	Trypsin	15	12	0

[a] Growth of *S. typhimurium* SL3748 or *E. coli* A around wells charged with unadsorbed iron-containing complexes.

[b] Determinations of the amounts of iron and Tr remaining after the adsorption of Tr-iron solution with untreated bacteria showed that bacteria removed 60% of Tr-iron. Iron determinations were done according to the procedure recommended by the American Monitor Corp. (Indianapolis, Ind.), and amounts of Tr were determined by total iron-binding capacity tests.

sera of virulent bacteria showed that the ability of virulent bacteria to grow in serum cannot be attributed to the production of extracellular siderophores but to factors associated with bacterial walls (8). Since results in several laboratories indicated that the ability to acquire iron in animals is a significant factor in bacterial virulence (2, 3, 8, 13, 14), we investigated the mechanisms of iron acquisition by virulent and avirulent bacteria. As the first step in this investigation, we determined the fate of rfa mutants of *S. typhimurium* in untreated and in iron-injected mice. We hoped that the use of these mutants, whose virulence has been associated with the amount of LPS sugar (12), would help in identification of factors that permit virulent cells to acquire iron in animals and their sera. We found that the virulence of rfa mutants for mice decreased as the amount of the sugar moiety in the LPS became smaller. The injection of infected mice with iron increased the virulence of LPS sugar-rich but not of LPS sugar-poor or -void strains. Platings of liver and spleen homogenates of untreated and iron-injected mice infected with strains that contained little or no LPS sugar showed that these LPS-defective bacteria died very rapidly.

The finding that iron increases virulence of bacteria with superficial but not with deep lesions in LPS core does not permit the conclusion that only LPS sugar facilitates the acquisition of iron by bacteria in animals. Also, this activity can be attributed to outer-membrane proteins, which have been shown to be depressed in mutants with short sugar chains in their LPS (1). In either case, the different potentiating effects of iron for virulence of rfa mutants suggested that study of iron acquisition by these bacteria may reveal factors that determine bacterial virulence.

Determinations of the fate of serum-exposed rfa mutants revealed a principal difference between LPS sugar-rich and -poor strains. Although the growth-supporting effect of exogenous iron was equally beneficial for all serum-exposed strains, the effect of E was much more beneficial for LPS sugar-rich than it was for LPS sugar-poor bacteria; finally, E failed to support the growth of serum-exposed LPS sugar-void strain SL1102. The observation that strain SL1102 can use E-iron in the absence but not in the presence of serum led to the discovery of Tr-iron-E complexes. Dialysis and adsorption studies showed that E does not remove iron

from Tr but combines with it, forming Tr-iron-E complexes. These complexes are as effective as E-iron in supplying virulent bacteria with iron; avirulent bacteria use these complexes with difficulty or not at all.

The role of the individual reactants in the formation of Tr-iron-E complexes cannot be defined precisely at this time. The observation of more effective neutralizing activity for serum microbiostasis by complexes formed between E and 100%-saturated Tr than for complexes formed between E and 20%-saturated Tr suggests that iron participates actively in the formation of complexes. The finding that iron-contaminated albumin can form complexes with E that neutralize serum microbiostasis (unpublished data) suggests that Tr plays a rather passive role in the process of complex formation with E.

Previous findings in our laboratory showed that cell wall-associated siderophores are much more important to the initiation of bacterial growth in animals than are siderophores produced extracellularly. We have found that mycobactin is bound more firmly to lipid-rich, virulent than to lipid-poor, avirulent tubercle bacilli, and, therefore, virulent bacteria acquire iron much more effectively in animals or their sera (3). Studies with *E. coli* showed that virulent bacteria are richer in cell wall-associated E (8). Results of the present study show that cell wall-associated siderophores bind Tr-iron to bacterial cells and indicate that the production of utilizable iron for serum-exposed bacteria occurs on bacterial cells. These results suggest that the initiation of bacterial growth in animals depends upon bacterial ability to bind and to use Tr-iron or Tr-iron-E complexes, whereas the production of extracellular siderophores secures unrestricted bacterial growth.

Adsorption of Tr-iron-E complexes with cells of various bacterial strains showed that virulent cells are much more effective in binding these complexes than are avirulent cells. This property enables virulent cells to derive iron from extracellularly formed Tr-iron-siderophore complexes in animals. Adsorption experiments with EDTA- and trypsin-treated bacteria showed that bacteria have receptors in their outer membrane that bind Tr-iron-E complexes. It has been reported that treatments of *E. coli* with EDTA removes from bacteria not only LPS but also surface proteins (10) and that trypsin cleaves off a part of a protein 7 that is exposed to the outside of the cell (5). These chemical treatments suggest that the receptor for Tr-iron-E is an outer-membrane protein. This conclusion is supported by observations showing that the protein 7 provides the receptor for colicins (5) and that siderophores prevent adsorption of bacteriophages to cells of *S. typhimurium* (11) and of colicins to cells of *E. coli* (4, 16).

Results of our investigation suggest that the process of iron acquisition by bacteria in mammalian blood can be divided into three separate stages. The first stage consists of the adsorption of Tr-iron to cell wall-associated siderophores or of preformed Tr-iron-E complexes to protein receptors of the outer membrane. The failure to adsorb Tr-iron-E and E-iron complexes with EDTA- or trypsin-treated bacteria suggests that receptors of these complexes are the same. The second stage of iron acquisition is the separation or destruction of Tr-iron-E complexes. Attempts to demonstrate Tr-hydrolyzing enzymes in spent media or bacterial washings were unsuccessful. There is some evidence to suggest that the inability of LPS sugar-void strain SL1102 to use Tr-iron-E as the source of iron may be attributed not only to the weak adsorbing activity of these cells for Tr-iron-E complexes but also to the lack of enzymes that liberate E-iron from the association with Tr. The last stage of iron acquisition consists of the transport of E-iron into bacterial cells. The neutralization of serum bacteriostasis for *S. typhimurium* by various siderophores (such as ferrichrome, rhodotorulic acid, and desferrioxamine B) suggests that the adsorption of Tr-iron-siderophore complexes to bacterial cells and the passage of siderophore-iron into bacterial cells are not specific. Preliminary results in our laboratory suggest that LPS may play a role in the acquisition of iron from various siderophores. More work is necessary to define the role of various iron-binding substances of the outer membrane in the acquisition of iron by bacteria.

The failure of iron to promote either virulence or the survival of LPS sugar-poor or -void bacteria became understandable after the discovery of Tr-iron-E complex formation. In iron-injected mice, the elevated serum iron level lasts only until 6 h, when bacterial chances to bind Tr-iron to cell wall-associated E are increased. However, the LPS sugar-deficient bacteria have no ability to use Tr-iron-E complexes as the source of iron, and without iron they die very rapidly (8). Preliminary tests of bacterial abilities to use Tr-iron-siderophore complexes indicate that bacterial virulence for mice can be predicted on the basis of bacterial efficiency to use the complexes. The present study indicates that the efficiency of serum-exposed bacteria to obtain iron is determined by at least three factors: the amount of cell wall-associated siderophores, the presence of Tr-iron-E-splitting enzymes, and the

number of protein receptors for siderophore-iron in the outer membrane of bacterial cells.

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