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Iron Uptake in *Salmonella typhimurium*: Utilization of Exogenous Siderochromes as Iron Carriers

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Aerobic microorganisms have evolved a variety of siderochromes, special ligands which can dissolve insoluble ferric iron and facilitate its transport into the cell. We have found that *enb* mutants of *Salmonella typhimurium* blocked in the biosynthesis of enterobactin (its natural iron carrier) are able to utilize siderochromes of different types made by other microorganisms as iron carriers. The antibiotic albomycin δ_2 was used to select mutants defective in ferrichrome-mediated iron uptake. Twelve classes of albomycin-resistant mutants, named *sid*, were defined on the basis of their growth responses to other siderochromes. Most of these classes have genetic lesions in loci that are cotransduced with *panC* (represented at 9 min on the genetic map). The locus designated *sidJ* is cotransduced with *enb*, whereas *sidK* and *sidL* are linked with neither *panC* nor *enb*. Genetic and physiological data indicate that *S. typhimurium* has several transport systems of high specificity for a variety of siderochromes produced by other microorganisms.

In recent years a number of iron-transporting agents have been isolated from the culture supernatant fluids of a variety of microbial species. The formation of these iron chelators is derepressed at low levels of iron. These substances in general fall into two classes, catechols and hydroxamates, collectively termed siderochromes (7, 8).

Salmonella typhimurium, grown on low-iron medium, secretes enterobactin, a member of the catechol series (11). Enterobactin [enterochelin in the terminology of the Australian workers (9)], is a cyclic trimer of 2,3-dihydroxybenzoyl-serine (DHB-serine) (11). Mutants of *S. typhimurium* were isolated which are blocked in the biosynthesis of enterobactin; they are named *enb* mutants. Their mutations are grouped at about 20 min on the *Salmonella* map (10). The presence of citrate in the medium inhibits the growth of these mutants by decreasing the rate of iron uptake (10). The addition of enterobactin to citrate-containing media restores normal growth of these mutants since enterobactin serves to promote iron uptake by *S. typhimurium*. When ferrichrome, a hydroxamate-type siderochrome commonly produced by fungi, is added to the medium at very low concentrations (10^{-7} M), it also allows normal growth of the *enb* mutants.

The ability of low concentrations of ferrichrome to support the growth of *enb* mutants suggests that *S. typhimurium* possesses a very efficient system for utilizing the iron in ferrichrome. If such a system involves specific uptake of ferrichrome at the cell surface, it could also account for the transport of the structurally similar antibiotic albomycin δ_2 into the cell. We have already described, in a preliminary report, the inability of an albomycin-resistant strain of *S. typhimurium* to take up tritiated ferrichrome (J. R. Pollack, B. N. Ames, and J. B. Neilando, Fed. Proc., p. 801, 1970). In the present investigation we have isolated a number of albomycin-resistant mutants, designated *sid*, and tested their ability to use as growth factors 15 siderochromes of the hydroxamate class. The results indicate that *S. typhimurium* has several specific uptake systems that enable it to obtain iron from exogenous iron chelators.

MATERIALS AND METHODS

Chemicals. Ferrichrome was crystallized from

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cultures of *Ustilago sphaerogena*, and schizokinen was isolated from *Bacillus megaterium*. Rhodotorulic acid and ferrichrome C were gifts of C. L. Atkin, and albomycin δ_2 was generously supplied by J. Turková. Desferal (deferriferrioxamine B mesylate) was obtained from CIBA Pharmaceutical Co., and Nocardamin (deferriferrioxamine E) from Sandoz Co. Ferrichrysin, ferricrocin, ferrirubin, and ferrirhodin were donated by W. Keller-Schierlein, and coprogen B and dimerum acid were provided by H. Diekmann. Saké-Colorant A and danomycin were gifts of S. Sato and H. Kawaguchi, respectively. ^{3}H-acetic anhydride was purchased from the New England Nuclear Corp. ICR 372 was donated by H. J. Creech.

Tritiated ferrichrome was prepared by the following procedure. Exactly 8 mg of ferrichrome was dissolved in a minimal amount of water and treated overnight with 80 mg of 8-hydroxyquinoline in methanol. The solution was evaporated to dryness in vacuo, and the residue was dissolved in a mixture of chloroform and water. The aqueous phase was washed with chloroform until the washings were colorless. Concentrated HCl was added to the aqueous phase to a final concentration of 0.1 N, and the solution was heated in a boiling water bath for 10 min. The solution was rapidly cooled and evaporated to dryness in vacuo. The residue was dissolved in 1 ml of pyridine and acetylated overnight with 10 μliters of ^{3}H-acetic anhydride (10 mCi/mmole). After the addition of 4 ml of 4 N NH_4OH, the mixture was allowed to sit for 3 hr. The mixture was evaporated to dryness in vacuo, the residue was dissolved in 1 ml of water, and 10 mg of $FeCl_3 \cdot 6H_2O$ was added. The ^{3}H-ferrichrome was purified by passage through a column (1 by 45 cm) of Sephadex G-10 equilibrated with water; the ferrichrome was eluted between 15.5 and 20 ml. The ferrichrome-containing fraction was concentrated and subjected to electrophoresis at 1,000 v for 1 hr on Whatman 3 MM paper with pyridine-acetic acid-water (14:10:1,000, v/v) used as buffer. The product, after elution from the paper, consisted of 55 ml of 8.4×10^{-5} M ^{3}H-ferrichrome (5 mCi/mmole).

Bacterial strains. Four parent strains of *S. typhimurium* LT-2 were used in deriving *sid* mutants. The 66 mutants classified were derived from mutants *enb-1*, *enb-7*, and *enb-23*, which are blocked in enterobactin biosynthesis (10). The albomycin-resistant mutants used for conjugation experiments were derived from strain TK223 (Hfr K5 *aroD hisT1529*), provided by T. Klopotowski.

Growth of cultures. The following three media were used. Medium A was a minimal salts medium described previously (10). Medium E contained Vogel-Bonner minimal salts (17) (which contain citrate) supplemented with 5 g of glucose per liter. Difco nutrient broth was used for overnight cultures and storage. Liquid cultures were grown aerobically on a shaker at 37 C. Growth was measured turbidimetrically at 650 nm in a Gilford-Beckman DU spectrophotometer.

Isolation of mutants. Mutations were obtained both spontaneously and by mutagenesis with ICR 372 (1, 13). Albomycin-resistant mutants were selected by spreading the appropriate strain on Difco nutrient agar plates and dropping several small particles of albomycin δ_2 on each plate. Colonies which appeared in the zone of inhibition after 24 hr of incubation were purified and rechecked for albomycin resistance by radial streaking (13).

Genetic crosses. Conjugation experiments (13) were performed with strain Hfr K5 *aroD5 hisT1529 sid21* and the markers *purB12*, *purE54*, *aroA148*, *proAB47*, and *panC3*. Linkage with *panC* was then determined by transduction (13) with the *int-4* mutant (15) of phage P22 grown on *enb sid* cultures with the *panC* strain as recipient on medium E. Linkage with *enb* was determined by transduction with phage grown on a wild-type host (*enb*$^+$*sid*$^+$) with *enb sid* recipients on medium E. Albomycin resistance was tested by radial streaking.

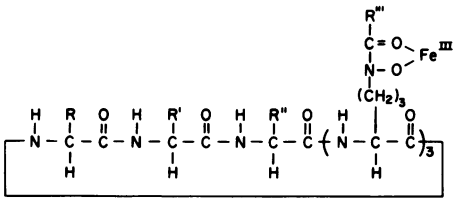
Growth factor assay. The ability of the siderochromes to support growth of mutants on medium E was examined by placing filter-paper discs on pour plates seeded with the mutant as described by Roth (13). Each disc contained 25 μliters of approximately 10^{-4} M siderochrome solution. The radius of visible growth was measured after 18 and 24 hr of incubation at 37 C.

Measurement of ferrichrome uptake. Medium A supplemented with 8.4×10^{-7} M ^{3}H-ferrichrome (5 mCi/mmole) was inoculated with the desired strain and incubated at 37 C with shaking. At given times the bacteria from 1-ml samples of culture were collected on membrane filters (Millipore) and washed with 2 ml of 0.1 M $K \cdot PO_4$, pH 6.8. The radioactivity on each filter was determined in Bray's scintillation fluid with a Nuclear-Chicago Uni-Lux II counter.

RESULTS

Growth factors for enb sid mutants. The ability of 15 siderochromes (structures shown in Fig. 1) to support the growth of the mutants on medium E agar plates was examined (Table 1). The *enb sid* mutants fall into 12 phenotypic classes on the basis of their response to these compounds. They range from Sid A mutants, which are unable to use any of the siderochromes as growth factors, to Sid E mutants which can use all of them. Intermediate classes are able to grow on some but not all of the siderochromes. Parent *enb sid*$^+$ strains utilize all of the siderochromes for growth, but are inhibited by albomycin. Growth of the *enb sid* mutants, like the parent strains, is promoted by iron salts, enterobactin, DHB-serine, ascorbate, and ethylenediaminetetraacetic acid.

The first *sid* mutants isolated contained spontaneous mutations, but, since about 20% of spontaneous mutations are deletions (5), many of which go through several genes, mutants were also induced with the acridine-like agent ICR 372, which causes frameshift mutations (1); these ICR mutations are point mutations

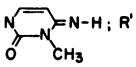


Ferrichrome: R = R' = R'' = H; R''' = CH_3

Ferrichrome C: R = R'' = H; R' = R''' = CH_3

Ferricrocin: R = R'' = H; R' = $-CH_2OH$; R''' = CH_3

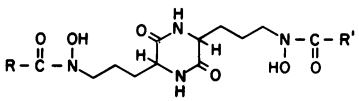
Saké Colorant A: R = $-CH_2OH$, R' = R''' = CH_3; R'' = H

Albomycin δ_2: R = $-CH_2-O-SO_2-$  $-N-H$; R' = R'' = $-CH_2OH$; R''' = CH_3

Ferrichrysin: R = R' = $-CH_2OH$; R'' = H; R''' = CH_3

Ferrirhodin: R = R' = $-CH_2OH$; R'' = H; R''' = $-CH=C(CH_3)-CH_2-CO_2H$ (cis)

Ferrirubin: R = R' = $-CH_2OH$; R'' = H; R''' = $-CH=C(CH_3)-CH_2-CO_2H$ (trans)



Rhodotorulic Acid: R = R' = CH_3

Dimerum Acid: R = R' = $-CH=C(CH_3)-CH_2-CH_2OH$ (trans)

Coprogen B: R = $-CH=C(CH_3)-CH_2-CH_2OH$;

R' = $-CH=C(CH_3)-CH_2-CH_2O-CO-CH(NH_2)-(CH_2)_3-N(OH)-CO-CH=C(CH_3)-CH_2-CH_2OH$

Desferal (Deferriferrioxamine B mesylate):

$$CH_3SO_3H\cdot H-\left[NH-(CH_2)_5-N(OH)-CO-(CH_2)_2-CO-\right]_2NH-(CH_2)_5-N(OH)-\overset{O}{\overset{\|}{C}}-CH_3$$

Nocardamin (Deferriferrioxamine E):

$$\text{Cyclo}\ \left[NH-(CH_2)_5-N(OH)-CO(CH_2)_2-CO-\right]_3$$

Danomycin:

Structure unknown, but believed to be closely related to ferrimycin (H. Kawaguchi, personal communication)(7)

Schizokinen:

$$HO_2C-C(OH)\left[-CH_2-CO-NH-(CH_2)_3-N(OH)-CO-CH_3\right]_2$$

FIG. 1. *Siderochromes tested as growth factors for enb sid mutants. [According to Maehr and Pitcher (6) albomycin δ_2 contains only one serine residue instead of the three serine residues shown here, which is the structure originally proposed by Turková et al. (16).]*

TABLE 1. *Classification of enb sid mutants*[a]

Mutant class	Ferri-chrome	Ferri-chrome C	Ferri-crocin	Sake-Colorant A	Albo-mycin	Ferri-chrysin	Ferri-rhodin	Ferri-rubin	Rhodo-torulic acid	Dimerum acid	Copro-gen B	Desferal	Nocar-damin	Dano-mycin	Schi-zokinen	Genetic linkage
enb parent	++	++	++	++	0	++	++	++	++	++	++	++	++	++	++	
Sid A (25)	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	*pan*
Sid B (7)	±	++	0	0	0	0	0	0	0	0	±	0	±	0	0	*pan*
Sid C (13)	±	+	+	0	+	±	+	+	++	++	++	++	++	++	++	*pan*
Sid D (3)	++	++	++	++	+	++	++	++	++	++	++	++	++	0	++	*pan*
Sid E (4)	+	+	+	±	+	+	+	±	+	+	+	+	+	+	±	*pan*
Sid F (2)	±	±	±	0	0	0	±	+	++	++	+	0	++	0	+	*pan*
Sid G (1)	++	++	++	++	±	++	+	++	±	0	+	++	++	±	+	*pan*
Sid H (1)	+	++	+	±	+	+	±	±	0	0	±	+	++	±	++	u
Sid J (6)	+	+	+	+	0	+	+	++	+	++	+	+	+	+	+	*enb*
Sid K (1)	±	±	±	±	0	±	±	±	±	+	+	++	++	+	+	n
Sid L (1)	++	++	+	++	±	+	±	+	0	0	+	+	+	0	++	n
Sid M (2)	++	++	++	++	0	++	+	++	+	+	+	0	++	0	++	*pan*

[a] Classification is based on growth response to the siderochromes shown in Fig. 1. Each mutant was tested on medium E pour plates to which filter-paper discs containing 25 μliters of siderochrome solution were applied. The solutions ranged in concentration from 1.6×10^{-4} to 6.4×10^{-4} M. The radius of visible growth was measured after 18 and 24 hr of incubation at 37 C. The symbols used indicate growth relative to the *enb sid*$^+$ parent strains, for which ++ designates ca. 20- to 30-mm radii of growth from the disc on all compounds except schizokinen and desferal, which supported growth to ca. 15 mm. The number of mutants in each class is indicated in parentheses. ++, Good growth, equivalent to parent; +, fair growth; ±, poor growth; 0, no visible growth; n, neither; u, undetermined.

that usually result in complete loss of function of the protein product. ICR-derived mutations fell into classes Sid A, Sid B, Sid C, Sid E, Sid J, Sid K and Sid M, indicating that mutations in each of these classes result from the mutation of a single gene.

Uptake of ferrichrome by enb and enb sid mutants. To determine whether the decreased ability of most *enb sid* mutants to utilize siderochromes as growth factors was due to defective siderochrome uptake, we examined their ability to take up tritiated ferrichrome from the medium. The first attempt, using a resting cell suspension of the *enb-1 sid*$^+$ strain, failed to demonstrate ferrichrome uptake. However, ferrichrome was taken up by this strain during mid-exponential growth on either medium A or medium E (Fig. 2). A Sid D mutant (strain TA2745) which is able to use ferrichrome as a growth factor as efficiently as the *enb-1 sid*$^+$ strain also showed normal uptake. A Sid A mutant (strain TA2700) which is unable to use ferrichrome as a growth factor did not accumulate ferrichrome intracellularly during growth (Fig. 3). A strain of type Sid C (TA2733) similarly failed to accumulate ferrichrome during growth (data not shown).

Mapping of sid mutants. Conjugation using strain Hfr K5 *aroD5 hisT1529 sid-21* showed that *sid-21* is closely linked to *panC*. Transduction using this strain as donor and strain

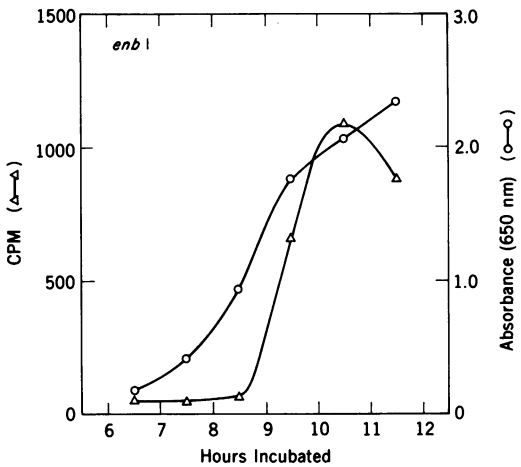


FIG. 2. *Ferrichrome uptake by the enb-1 sid*$^+$ *strain when grown on medium A supplemented with 8.4 × 10*$^{-7}$ *M* ^{3}*H-ferrichrome (5 mCi/mmole). At given times the bacteria from 1-ml samples of culture were collected with membrane filters and washed with 2 ml of 0.1 M K·PO*$_4$*, pH 6.8. The radioactivity on each filter was determined in Bray's scintillation fluid by using a Nuclear-Chicago Uni-Lux II counter.*

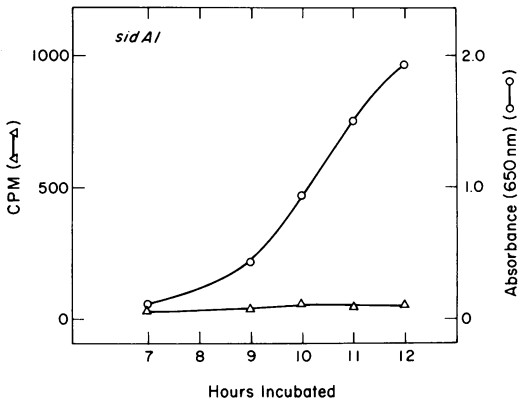


FIG. 3. *Ferrichrome uptake by the sidA1 mutant (strain 2700) when grown on medium A supplemented with 8.4 × 10*$^{-7}$ *M* ^{3}*H-ferrichrome (5 mCi/mmole). Radioactivity taken up by the cells was determined as in Fig. 2.*

panC3 as recipient indicated that *panC* and *sid-21* are cotransducible, since 28 of the 79 *pan*$^+$ transductants examined (35%) were albomycin resistant. The *panC* gene is represented at 9 min on the *S. typhimurium* chromosome map (14). The lesions in several other *sid* mutants derived from strain TK223 were tested for cotransducibility with *panC*. Three of the nine mutations were cotransducible with *panC* at frequencies ranging from 30 to 50%.

The mutants derived from *enb* strains were therefore tested for transduction with *panC*. Twenty-two *sid* mutations from *enb sid* strains were shown to be cotransduced with *panC* at frequencies of 20 to 60% (Table 2). These strains represented phenotypic classes Sid A, Sid B, Sid C, Sid D, Sid E, Sid F, Sid G, and Sid M, and it seems likely that each of these classes represents the inactivation of one of a cluster of genes near the *panC* locus. This conclusion is supported by the observation that all of the mutants within each class have the same unique phenotype and by the isolation of frameshift mutants in all of the major classes. (We have used gene designations for all of the major phenotypic classes, but we wish to do further genetic studies before designating genotypes for the minor classes.)

Transductions performed with LT-2 wild type as donor and *enb sid* mutants as recipients showed that *sidJ* mutations were cotransduced with *enb* at very high frequencies (Table 3).

DISCUSSION

Mutants resistant to antibiotics fall into two general classes: those that have lost the system

TABLE 2. *Linkage of sid to panC by transduction*

Strain[a]	Mutation	No. of *pan*$^+$ transductants tested for albomycin resistance	Linkage between *sid* and *pan* (%)
TA2700	*sidA1*	60	52
TA2701	*sidA2*	100	33
TA2703	*sidA4*	40	45
TA2714	*sidA15*	100	30
TA2706	*sidA7*	60	35
TA2765	*sidA66*	60	33
TA2725	*sidB26*	60	53
TA2729	*sidB30*	40	65
TA2730	*sidB31*	120	21
TA2727	*sidB28*	40	25
TA2732	*sidC33*	110	49
TA2736	*sidC37*	100	41
TA2738	*sidC39*	40	50
TA2744	*sidC45*	60	30
TA2745	*sid-46* (D type)	60	26
TA2746	*sid-47* (D type)	60	30
TA2747	*sid-48* (D type)	60	31
TA2748	*sidE49*	60	55
TA2750	*sidE51*	120	25
TA2752	*sid-53* (F type)	60	50
TA2753	*sid-54* (F type)	55	41
TA2754	*sid-55* (G type)	100	50
TA2756	*sidJ57*	40	<2
TA2757	*sidJ58*	40	<2
TA2759	*sidJ60*	40	<2
TA2760	*sidJ61*	100	<2
TA2761	*sidK62*	60	<2
TA2762	*sidL63*	100	<2
TA2764	*sidM65*	60	50
TA2763	*sidJ64*	60	<2

[a] Strain numbers have been assigned to strains carrying the double mutations *enb sid*.

TABLE 3. *Linkage of sid to enb by transduction*

Strain[a]	Mutation	No. of *enb*$^+$ transductants tested for albomycin resistance	Linkage between *sid* and *enb* (%)
TA2756	*sidJ57*	60	100
TA2757	*sidJ58*	60	100
TA2759	*sidJ60*	80	100
TA2760	*sidJ61*	80	100
TA2761	*sidK62*	120	<2
TA2762	*sidL63*	60	<2
TA2763	*sidJ64*	100	94

[a] Strain numbers have been assigned to strains carrying the double mutations *enb sid*.

transporting the antibiotic into the cell and those which are no longer susceptible to its toxic effects. The coupling of albomycin resistance with impaired ability to use siderochromes as iron donors suggests that a *sid* mutation usually results in a defect in the uptake system that is normally utilized by both albomycin and other iron chelators. It is possible that some *sid* mutations affect different elements of the system; for example, the lesion in Sid J mutants still enables them to utilize all siderochromes tested and may be causing some other defect.

Three experimental approaches to the characterization of *sid* mutations indicate that a number of specific uptake systems exist in *S. typhimurium*. Thus, (i) the considerable variation observed in phenotypes of *sid* mutants shows rather intricate discriminatory abilities for transport of different siderochromes. (ii) The *sid* mutations are represented in at least three areas on the *Salmonella* chromosome map. (iii) The ability of some, but not all, *sid* mutants to take up tritiated ferrichrome has been lost.

From the complex set of responses to the siderochrome growth factor assay (Table 1) a few observations on the nature of the *sid* mutations may be made. Mutants of the Sid A type, having lost the ability to utilize all of the siderochromes tested, may have a defect in either a regulatory mechanism for the transport systems or an essential activity such as the iron release mechanism. The latter may correspond to the reductase activity described for *Mycobacterium smegmatis* by Ratledge (12), since ferrous iron is only weakly bound by hydroxamic acids.

Phenotypes of other *sid* mutants indicate specific recognition of the siderochrome does occur. For example, Sid B mutants discriminate between ferrichrome and its several analogues ferrichrysin, ferricrocin, ferrirhodin, and ferrirubin. Strains of the class Sid C utilize all of the ferrichromes and other siderochromes tested except Saké-Colorant A. Sid G, Sid H, and Sid L mutants do not grow on one or both of the dihydroxamates, rhodotorulic acid and dimerum acid. These chelators carry a positive charge on their ferric complexes. Thus specificity may be based on structural detail and electronic properties.

Strikingly different growth responses to albomycin have been observed, as five classes of mutants utilize it for iron uptake with varying efficiency. Sid C mutants only grow very close to the disc containing the antibiotic, whereas Sid E mutants grow in a halo around a zone of

inhibition. Although Sid D mutants grow on albomycin, they will not grow where albomycin and a second active siderochrome are present (unpublished observation by Rush Wayne). Although all of the mutants were selected for albomycin resistance, the above classes are able to utilize it as an iron carrier. The ability of these albomycin-resistant strains to obtain iron from albomycin implies that albomycin transport into the cell may consist of at least two steps, a step subsequent to iron release being blocked in this case. It should be noted, however, that the albomycin used was a clinical preparation which certainly contained degradation products of albomycin δ_2, the active form. Although the degradation products have been observed to have little or no antibiotic activity (2), they could function as iron chelators and could be recognized by the altered transport system of these *sid* mutants.

Some classes of mutants, Sid F, Sid K, Sid L, and Sid M, do not grow on albomycin or danomycin (which is similar to ferrimycin and may also have bacteriostatic action). Although these mutants are resistant to both compounds on nutrient medium (determined by radial streaking), they may acquire sensitivity to these antibiotics if the *sid* uptake system is derepressed on low iron medium.

The plural genetic loci for *sid* mutations suggest that a variety of genes are involved in siderochrome transport. Preliminary experiments indicate the different genes are coordinately regulated in response to iron starvation conditions.

Ferrichrome uptake studies illustrate the different siderochrome transport abilities of *sid* mutants. With a counting efficiency of 10% for intracellular tritiated ferrichrome, it can be calculated that the *enb1 sid*$^+$ strain accumulated intracellularly almost all of the ferrichrome added to the medium. The ferrichrome, which was present at a concentration of about 1 μM, would furnish iron greatly in excess of the amount required by the bacteria.

The evidence presented above indicates that *S. typhimurium* has developed a scavenger ability to utilize siderochromes that are excreted into the environment by other microorganisms. [We have not found any ability of *Salmonella* to make hydroxamate-type siderochromes. A determination of hydroxylamine by the method of Csaky (3) indicates that the level of hydroxylamine compounds in the cells and supernatant fluid of a wild-type culture growing on medium A with and without added iron is below the detectable level of 10^{-5} M.]

Siderochrome transport systems appear to exist in *Staphylococcus aureus* as well (18). It has been shown that antibiotic A22,765, a siderochrome, is taken up from the medium against a concentration gradient by *S. aureus*. Bacteria resistant to A22,765 have a greatly reduced ability to take up the antibiotic. Ferrioxamine B (desferal is its iron-free mesylate) is taken up by *S. aureus* only in minute quantities, but nevertheless it antagonizes permeation by A22,765. The investigators have proposed that the observed reversal by other siderochromes of bacteriostasis due to siderochrome antibiotics is caused by competition for a siderochrome transport system, which prevents toxic levels of the antibiotic from accumulating inside the bacteria.

It may be that prokaryotic cells once possessed the ability to form both hydroxamate- and catechol-type siderochromes and the corresponding permeases. Thus *Aerobacter aerogenes* forms aerobactin (4), a dihydroxamic acid, as well as the catechol, enterobactin. Other enteric bacteria, such as *Escherichia coli* and *S. typhimurium*, synthesize only enterobactin. However, these species may have retained the hydroxamate permeases as a survival mechanism that can be invoked under conditions of iron stress. The ability of albomycin and other siderochrome antibiotics to antagonize the growth of a wide variety of bacteria suggests that siderochrome transport systems are common among microorganisms and are present in those that do not apparently produce siderochromes.

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