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Iron Transport in *Salmonella typhimurium*: Mutants Blocked in the Biosynthesis of Enterobactin

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A number of mutants of *Salmonella typhimurium* were isolated which are blocked in the biosynthesis of enterobactin, an iron chelator that is secreted by the wild-type bacteria when they are grown on low iron media. One class of these *enb* mutants accumulates the enterobactin precursor 2,3-dihydroxybenzoic acid, and another class does not accumulate any detectable catechol precursor. The *enb* mutants grow very well on a glucose-mineral salts medium. Addition of citrate, itself an iron chelator, to the medium drastically inhibits growth unless the medium is supplemented with enterobactin or iron salts. Citrate inhibits iron uptake from the medium by *enb* mutants; enterobactin counteracts this inhibition and also, by itself, increases iron uptake. Thus, the apparent function of enterobactin is to promote the absorption of iron from the medium by the bacteria. Transduction experiments showed that the genes for enterobactin biosynthesis are closely linked on the *S. typhimurium* chromosome. It is suggested that they form an operon which is repressed by the presence of iron. *S. typhimurium* can utilize the iron chelate ferrichrome. (Deferriferrichrome is a cyclic hexapeptide that is produced by some fungi but not by *S. typhimurium*.) The *enb* mutants use ferrichrome as an effective growth factor.

In recent years, iron-sequestering agents consisting of 2,3-dihydroxybenzoic acid (DHBA) conjugated with the amino group(s) of a specific amino acid have been isolated from several bacteria: 2,3-dihydroxybenzoyl (DHB)-glycine from *Bacillus subtilis* (4); DHB-serine from *Escherichia coli* (1, 7), *Aerobacter aerogenes* (7), and *Salmonella typhimurium* (16); and di-DHB-lysine from *Azotobacter vinelandii* (3). *S. typhimurium* also produces enterobactin (10), a cyclic trimer of DHB-serine. These compounds are all excreted into the medium under low iron conditions. It has been suggested that these compounds are involved in iron utilization (4, 6), a hypothesis supported by the finding that DHB-glycine and DHB-serine promote the uptake of iron by *B. subtilis* (9) and *E. coli* (15), respectively. In the present communication, a series of mutants of *S. typhimurium* blocked at various stages in the biosynthesis of enterobactin is described.

MATERIALS AND METHODS

Chemicals. DHB-serine was a generous gift of N. Brot. Ferrichrome was isolated from cultures of *Ustilago sphaerogena* (5) and enterobactin was obtained from *S. typhimurium* (10). Iron-55 was purchased from New England Nuclear Corp. All other chemicals were from commercial sources.

Bacterial strains. All mutants were derived from *S. typhimurium* LT2. The ascorbate-requiring mutant *asc-1* was provided by K. E. Sanderson.

Media. Medium A contained the following (per liter of distilled water): K_2HPO_4, 7 g; KH_2PO_4, 4 g; $(NH_4)_2SO_4$, 2 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; and glucose, 5 g. Medium E was the glucose-citrate-mineral salts medium of Vogel and Bonner (14). The glucose was autoclaved separately for both media.

Culture conditions. Inocula (1%) from overnight cultures were used in all experiments. The cultures were grown at 37 C with shaking. Bacterial growth was followed by measuring the turbidity of the cultures at 650 nm with a spectrophotometer (Gilford-Beckman, model DU).

Chromatography. Paper chromatography was performed by the ascending method on Whatman no. 1 paper. Thin-layer chromatography was carried out on Silica Gel G plates. Catechols were detected by spraying with 0.5% (w/v) $FeCl_3$.

Isolation of catechols. The cell-free supernatant from a 12-hr medium A culture was acidified to *p*H 2 with 12 N HCl and then extracted with an equal volume of ether. The ether extract was taken to dryness in vacuo. The catechols in the residue were separated by paper chromatography or assayed quantitatively at 515 nm with the Arnow reagent (3).

Iron uptake by bacteria. The cells from a culture in late exponential phase were collected by centrifugation at 20 C and were resuspended in one-fifth the original volume of 0.1 M $K \cdot PO_4$ (*p*H 6.8)-1 mM $MgSO_4$. At zero time, one volume of the cell suspension was added to four volumes of a solution composed of 0.1

M $K \cdot PO_4$ (*p*H 6.8), 1 mM $MgSO_4$, 5 g of glucose per ml, 0.1 μCi of ^{55}Fe per ml, and appropriate additions; all cells were incubated at 37 C. At given times, the bacteria from 1-ml portions of incubation mixture were collected on filters of (Millipore Corp., 450-nm pore size) and washed with 2 ml of 0.1 M $K \cdot PO_4$ (*p*H 6.8) at room temperature. The radioactivity of each filter was determined with 10 ml of Bray's scintillation fluid by using a Nuclear-Chicago Uni-Lux II scintillation counter.

Transduction. Two drops of phage P22 L4 (13), grown on the donor strain (10^{10} phage per ml), and two drops of fresh Nutrient Broth (Difco) culture of the recipient strain were spread on medium E agar plates supplemented with 10 mg of DHBA per liter. Appropriate controls were run to detect either bacterial contamination of the phage preparation or reversion in the recipient.

RESULTS

Isolation of enterobactin-requiring mutants. Attention was first drawn to a group of mutants that were isolated as able to grow on Nutrient Broth (Difco) but not on minimal medium E by the observation that they did not respond to any of the usual growth factors and that an unusually large halo of bacteria would grow up around each colony of revertants to wild type (Fig. 1). These strains were designated *enb* mutants, since they are blocked in the biosynthesis of enterobactin, the iron chelator secreted by wild-type *S. typhimurium*. The characteristic halo surrounding each colony of revertants to wild type is due to their secretion of enterobactin which feeds the surrounding *enb* mutants.

Iron salts and certain naturally occurring and synthetic chelating agents support the growth of *enb* mutants on medium E (Table 1). It should be noted that enterobactin is 100 times as effective as DHB-serine on a molar basis. The *enb* mutants are divided into two classes according to whether they can grow (class II) or cannot grow (class I) on medium E supplemented with DHBA.

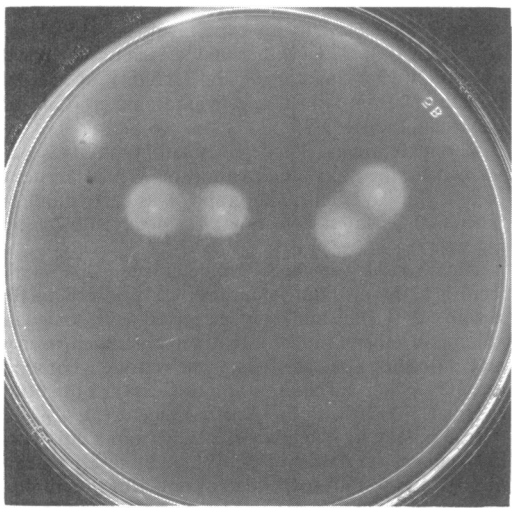

FIG. 1. *Growth of S. typhimurium enb-21 around colonies of revertants to wild type.*

Catechol production by wild-type and mutants. Class II *enb* mutants produce only 5 to 9% of the amount of catechols secreted by wild-type LT2, whereas with class I *enb* mutants the catechol production is as high or higher than wild type (Table 2).

The ether-soluble catechols were extracted as described above and were chromatographed on paper by using 5% (w/v) ammonium formate-0.5% (v/v) formic acid as the solvent. Wild-type LT2 gave four iron-positive spots with R_F = 0.16 (enterobactin), 0.49, 0.63, and 0.70. The identity of the three fastest moving components has not been determined, although work with *E. coli* and *A. aerogenes* (8) suggests that they may be degradation products of enterobactin. Mutants *enb-1* and *enb-13* from class I each gave one spot with R_F = 0.62 or 0.63, respectively, which was shown to be DHBA as follows. The catechol in the ether extract from *enb-1* was purified further by electrophoresis for 2 hr at 1,000 v on Whatman 3MM paper with pyridine-acetic acid-water (42:30:3,000, v/v) as the buffer and eluted from the paper with methanol. The catechol ran similarly to DHBA on paper chromatography with two solvents, *n*-butyl alcohol-acetic acid-water (60:15:25, v/v) and methanol-pyridine-water (80:4:20, v/v), and on thin-layer chromatography with two solvents, benzene-

TABLE 1. *Growth factors for enb mutants on medium E*

Additions[a]	*enb-1* (class I)		*enb-7* (class II)	
	Minimal optimal concn	Generation time	Minimal optimal concn	Generation time
	μM	min	μM	min
$FeSO_4$	50	50	50	50
Enterobactin	0.5	46	0.5	41
DHB-serine	50	54	50	56
DHBA	Not effective		5	46
Ferrichrome	0.1	50	0.1	50
EDTA	2	70	2	57
Ascorbate	100	73	100	63

[a] Abbreviations: DHB, 2,3-dihydroxybenzoyl; DHBA, 2,3-dihydroxybenzoic acid; EDTA, ethylenediaminetetraacetic acid.

TABLE 2. *Catechol production by S. typhimurium strains*

Strain	Relative amt excreted
LT2 (wild-type)	100
enb-1 (class I)	83
enb-7 (class II)	9
enb-10 (class II)	9
enb-11 (class II)	5
enb-13 (class I)	292

TABLE 3. *Effect of citrate on growth of enb mutants*

Additions to medium A	Generation time	
	enb 1	*enb 7*
	min	*min*
None	56	58
1 mM citrate	255	221
1 μM enterobactin	53	50
1 mM citrate and 1 μM enterobactin	43	54

methanol-acetic acid (90:16:8, v/v) and benzene-dioxane-acetic acid (90:25:4, v/v). Thus, it appears that class I mutants, unable to produce enterobactin, excrete DHBA instead. The three mutants from class II which we examined showed no detectable iron-positive spots on paper chromatography.

Effect of citrate on enb mutants. The *enb* mutants grow very well on the unsupplemented glucose-mineral salts of medium A, but growth of both classes of mutants is inhibited by the addition of citrate to the medium. This inhibition was reversed by the addition of enterobactin (Table 3).

Citrate (1 mM) inhibits the initial rate of uptake of iron by a suspension of *enb-1* by about 70%. The addition of enterobactin to the incubation mixture containing citrate restores the rate of iron uptake to about 70% of the original level (Fig. 2). Enterobactin by itself significantly increases the initial rate of iron uptake. Thus, it appears that the inhibition of growth by citrate is due to its interference with the uptake of iron from the medium.

Trace metals and growth of mutants. Mn^{2+} and Co^{2+} inhibit the growth of all *enb* mutants on medium A at concentrations that do not inhibit the wild-type LT2 (Table 4). Zn^{2+} and Cr^{3+} showed no such differential effect on growth.

Linkage of enb mutants. Mutations in class I and class II are cotransducible at high frequency (Table 5). This implies that the genes are very close together on the chromosome. The *enb-13*, which was previously isolated by Sanderson as an ascorbate-requiring auxotroph, *asc-1*, is located at 20 min on the *S. typhimurium* chromosomal map (12).

DISCUSSION

We conclude that *enb* mutants are blocked in the enterobactin biosynthetic pathway. Since chorismate is the point at which the pathway to DHBA diverges from the main aromatic sequence in *E. coli* and *A. aerogenes* (17), the class II mutants are probably blocked at the steps between chorismate and DHBA, whereas the class I mutants are blocked between DHBA and enterobactin (Fig. 3). The evidence for this is based on the production of enterobactin by wild type, the accumulation of the precursor DHBA in the medium instead by class I, and the greatly reduced catechol excretion by class II. Also, class II, but not class I, can use DHBA as a growth factor on medium E.

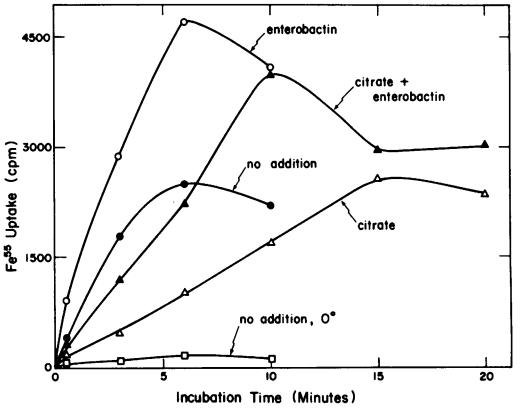

FIG. 2. *Iron uptake by S. typhimurium enb-1. Standard assay conditions were used except where indicated. Symbols: ●, no addition; △, assay mixture supplemented with 1 mM citrate; ○, assay mixture supplemented with 1 μM enterobactin; ▲, assay mixture supplemented with 1 mM citrate and 1 μM enterobactin; □, assay mixture incubated at 0 C.*

TABLE 4. *Effect of Co^{2+} and Mn^{2+} on growth of S. typhimurium strains*

Additions to medium A	Generation time	
	LT2	*enb 1*
	min	*min*
None	51	56
10 μM $MnSO_4$	52	95
100 μM $MnSO_4$	52	>300
10 μM cobaltous acetate	56	124

TABLE 5. *Transduction of enb mutants*[a]

Phage derived from	Recipient	
	enb-1	*enb-13*
	%	%
enb-7	89	56
enb-10	88	60
enb-11	94	85

[a] Transductions were performed on medium E agar plates supplemented with 10 mg of 2,3-dihydroxybenzoic acid (DHBA) per liter with phage P22 L4 derived from class II mutants and class I mutants as recipients. Results expressed as percentage of transductants requiring DHBA for growth on medium E (about 20 transductants per cross tested). The variation in linkage is probably not significant and may be due to the small number of transductants analyzed.

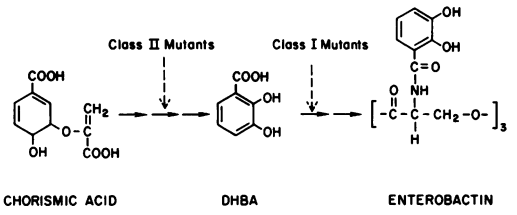

FIG. 3. *Blockage points of S. typhimurium enb mutants.*

Investigations with *E. coli* and *A. aerogenes* (2, 17) showed that the production of the enzymes in the pathway from chorismate to DHBA and "DHB-serine synthetase" is repressed by growing the bacteria in media containing high levels of added iron salts. Adding modest amounts of iron salts to the medium drastically reduces the amount of enterobactin secreted by *Salmonella* (J. R. Pollack and J. B. Neilands, *unpublished data*). In view of this and the observed cotransducibility of the *enb* mutations, the genes for enterobactin biosynthesis in *S. typhimurium* may form an operon with iron as the corepressor.

The function of enterobactin, which chelates Fe^{3+} extremely strongly, in *S. typhimurium* appears to be the promotion of iron uptake from the medium by the bacteria. Transfer of iron from enterobactin to an unknown receptor could occur at the cell surface or inside the bacterium if ferric enterobactin is transported across the cell membrane. The iron could be released from the complex with Fe^{3+} by reduction to Fe^{2+} since ligands with only oxygen atoms in the coordination sphere display a relatively low affinity for ferrous iron.

The ability of *enb* mutants to grow well on medium A suggests that enterobactin is only required to maintain an adequate iron uptake when *S. typhimurium* is growing in certain environments. Media containing citrate is one such environment. Citrate is an effective chelating agent, and the iron bound to it is apparently unavailable for use by the bacteria. Enterobactin would partly remove the iron from the citrate complex, forming ferric enterobactin which the bacteria can use as an iron source. Human serum is probably another such environment. DHB-serine eliminated the bacteriostatic property of human serum for *S. typhimurium* (16). The bacteria appear unable to use the iron bound to transferrin in the serum.

The mechanism by which manganous and cobaltous ions inhibit the growth of *enb* mutants remains unknown. Wang and Newton (15) have isolated a mutant of *E. coli* K-12 that lacks DHB-serine synthetase activity and thus would be equivalent to a class I *enb* mutant except that it is chromium sensitive and its growth is stimulated by citrate; chromium was shown to inhibit iron uptake from the medium. This suggested to us that the inhibitory effect of Mn^{2+} and Co^{2+} might be due to an inhibition of iron uptake by the *S. typhimurium enb* mutants. We were not able to demonstrate such an effect, however.

The ability of ferrichrome, a cyclic hexapeptide containing ferric iron complexed to three hydroxamate groups (11) produced by smut fungi, to act as a growth factor at a concentration of 0.1 μmole/liter implies that *S. typhimurium* possesses a very efficient system for utilizing the iron contained in ferrichrome. Preliminary experiments (J. R. Pollack, B. N. Ames, and J. B. Neilands, Fed. Proc. **29:** 801) have indicated that *S. typhimurium* has a specific transport system for ferrichrome and other siderochromes which are excreted by a wide variety of microorganisms in response to iron starvation. It is presumably of survival value in its natural habitats for *S. typhimurium* to have an uptake system for these compounds.

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