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# Synthesis and Utilization of Siderophores by *Shigella flexneri*

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Strains of *Shigella flexneri* secrete a hydroxamate-type siderophore when grown in low-iron media. This hydroxamate appears to be identical with aerobactin, a siderophore synthesized by *Aerobacter aerogenes*. In contrast to other enteric bacteria, *S. flexneri* did not produce detectable phenolate siderophores, although it could utilize an exogenously supplied phenolate.

Iron is a relatively abundant element, but its availability for microbial growth is limited by its tendency to undergo hydrolysis and polymerization at neutral pH. To meet their requirement for this essential element, many aerobic and facultative anaerobic microorganisms possess high-affinity systems to solubilize and transport iron into the cell (10). These consist of a low-molecular-weight ferric iron-chelating compound, or siderophore, and its specific membrane receptors. Most of the microbial siderophores are one of two chemical types, the hydroxamic acids and the phenolates or catechols. Hydroxamates are very common among fungi but are relatively rare among bacteria. Examples of hydroxamate siderophores include ferrichrome, produced by *Ustilago* (16); schizokinen, which is secreted by *Bacillus megaterium* and the cyanobacterium *Anabaena* sp. (4, 6, 26); aerobactin, a product of *Aerobacter* (*Enterobacter*) *aerogenes* (7); and terregens factor (ferric arthrobactin), which is synthesized by *Arthrobacter pascens* (12). Enterochelin (also known as enterobactin), a phenolate-type siderophore, has been isolated from cultures of *Salmonella typhimurium* (22, 28), *Escherichia coli* (3), and *Shigella sonnei* (20). Related compounds are produced by *A. aerogenes* (17), *Klebsiella* (9), *Bacillus subtilis* (8), *Vibrio cholerae* (19), *Agrobacterium tumefaciens* (18), and *Paracoccus denitrificans* (27) among others.

Membrane receptors are required for the transport of the iron-siderophore complex into the cell, and some bacteria possess specific transport systems not only for their own siderophores but also for siderophores produced by other microorganisms. Thus *S. typhimurium* can utilize ferrichrome, schizokinen, or other hydroxamates for uptake of iron, although it does not synthesize any of these compounds (13).

In this study, the ability of *Shigella flexneri* to produce and utilize siderophores was determined. Unlike other enteric bacteria that have been studied, *S. flexneri* apparently synthesizes only a hydroxamate-type siderophore, although other exogenously supplied siderophores could be utilized for growth.

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## MATERIALS AND METHODS

**Bacterial strains.** *S. flexneri* 2457 was obtained from S. B. Formal, Walter Reed Army Institute of Research. *S. flexneri* strains NTCC 4839 and UP 4204 were provided by I. Ketyi, University Medical School, Pecs, Hungary. Other bacterial strains used were *E. coli* B, *E. coli* K-12, and *A. aerogenes* 62-1 (7). Two mutants defective in the synthesis of enterochelin, *E. coli* K-12 strain RW 193 (11) and *S. typhimurium enb-1* (21), were used in the siderophore utilization assay. All strains were stored frozen at −70°C in Trypticase soy broth (BBL Microbiology Systems) containing 20% glycerol.

**Growth of cultures.** The Tris-buffered medium of Simon and Tessman (25) without added iron was supplemented with 0.4% glucose. The high pH (7.4) and relatively low levels of iron contamination (<0.5 μM Fe) make Tris medium an iron-poor medium suitable for production of siderophores without further deferration. Nicotinic acid was added for growth of *S. flexneri*. Iron in the form of ferric chloride was added to a final concentration of 10 μM where indicated. Cultures were grown at 37°C with shaking, and growth was measured turbidimetrically at 650 nm in a Zeiss spectrophotometer.

**Estimation of siderophores.** The production of hydroxamic acids was estimated by adding 1 ml of the culture supernatant to 1 ml of a solution of 5 mM ferric perchlorate in 0.14 M perchloric acid and measuring the absorbance of the ferric hydroxamate at 500 nm (2). The method of Arnow (1) was used for detection of catechols.

**Chemicals.** Aerobactin was the gift of B. Rowe Byers, University of Mississippi Medical School. Ferrichrome, rhodotorulic acid, enterochelin, and desferrioxamine B (Desferal) were generously provided by J. B. Neilands, University of California at Berkeley. Ethylenediamine di-(*o*-hydroxyphenylacetic acid) (EDDA) was obtained from Sigma and deferrated by the method of Rogers (24).

**Thin-layer chromatography.** Cellulose (Polygram Cel 300, Macherey-Nagel and Co.) and polyethyleneimine cellulose (PEI-cellulose) (Polygram Cel 300 PEI, Macherey-Nagel and Co.) thin-layer plates

were utilized for chromatography of the purified siderophore. Plates were spotted with 5-μl samples of approximately 10 μM solutions of the siderophores. The following solvent systems were used and the following $R_f$ values were noted for authentic aerobactin: system A, cellulose, aqueous ammonia-ethanol-water (1:16:3 by volume) (7), $R_f$ = 0.15; system B, cellulose, *n*-butanol-acetic acid-water (60:15:25 by volume) (4), $R_f$ = 0.58; system C, PEI-cellulose, 0.4 M LiCl, $R_f$ = 0.29; system D, PEI-cellulose, 0.4 M guanidine hydrochloride, $R_f$ = 0.28; system E, PEI-cellulose, 0.5 M Tris-hydrochloride (pH 8.0), $R_f$ = 0.51; system F, PEI-cellulose, 1.0 M LiCl (pH 4.5), $R_f$ = 0.82; system G, PEI-cellulose, saturated ammonium sulfate (pH 3.5), $R_f$ = 0.39; system H, PEI-cellulose, 1.0 M ammonium acetate (pH 3.5), $R_f$ = 0.01. Dried plates were sprayed with 0.4% $FeCl_3 \cdot 6H_2O$ in 0.04 M HCl to determine the position of iron-binding compounds.

**Absorbance spectra.** Absorbance spectra of the iron-complexed siderophores were determined in a Zeiss spectrophotometer. The pH of the siderophore solution was adjusted by the addition of HCl or KOH.

**Nuclear magnetic resonance spectra.** The nuclear magnetic resonance spectra were recorded on a Nicolet NT200 spectrometer. The sample was dissolved in $D_2O$, and dioxane was added as a reference after the run.

**Siderophore utilization assay.** An assay similar to that described by Miles and Kjimji (15) was used. In this assay, the iron chelator EDDA was added to agar media to bind the iron and make it less accessible for microbial growth. Luria broth agar containing 1 mg of EDDA per ml (EDDA medium) was prepared according to Ong et al. (18) and stored for 48 h before use. The agar was remelted, inoculated with $10^3$ indicator bacteria per ml, and poured into sterile petri dishes. Volumes of 10 μl of the siderophore solutions (approximately 10 to 100 μM) were placed on sterile disks (Sensi-discs, BBL) on the surface of the agar. Plates were examined after 24 h of incubation at 37°C for zones of growth around the disks.

## RESULTS

**Siderophore synthesis.** *S. flexneri* 2457 grown in Tris medium without added iron was analyzed for the production of both hydroxamate- and catechol-type siderophores. Culture supernatants were found to contain a compound which reacted with iron to give a red color, and a test for hydroxamate groups, the Csáky reaction (5), gave a positive result, indicating the presence of hydroxamic acids. Absorbancy measurements (at 526 nm) of supernatant fluids treated by the Csáky method ranged from 0.1 to 0.3, compared with an absorbance of 0.2 for a 0.04 mM solution of aerobactin that was used as a standard. Similar results were obtained with *S. flexneri* strains NTCC 4839 and UP 4204. Supernatant fluids from cultures of all three strains gave consistently negative results when assayed for catechol-type siderophores by the method of Arnow (1). In contrast, both *S. typhimurium* LT2 and *E. coli* K-12 readily produced the catechol enterochelin when grown in Tris medium (absorbancy at 515 nm of supernatant fluids tested by the method of Arnow, 0.4 to 0.9). *A. aerogenes* 62-1 synthesized both aerobactin and enterochelin, and *E. coli* B produced both catechol and hydroxamate siderophores under these same conditions. Thus it seems unlikely that failure to detect a catechol siderophore in *S. flexneri* was the result of improper growth conditions.

The *S. flexneri* hydroxamate siderophore was found to accumulate slowly in the culture supernatant (Fig. 1). As has been noted for other siderophores (10), synthesis of this compound was suppressed when the medium contained added 10 μM iron (Fig. 1).

**Purification.** The purification procedure was similar to that described by Gibson and Magrath for aerobactin (7). An overnight culture of *S. flexneri* 2457 in Tris medium was diluted 100-fold into 3 liters of fresh Tris medium and grown to stationary phase at 37°C with shaking. Cells were removed by centrifugation, and the supernatant was evaporated to dryness in a rotary evaporator. This material was taken up in a small volume of water (approximately 6 ml) and applied to the top of a column (1.5 by 40 cm) of Bio-Gel P10 (BioRad) equilibrated with water. The column was eluted with water, and 2-ml

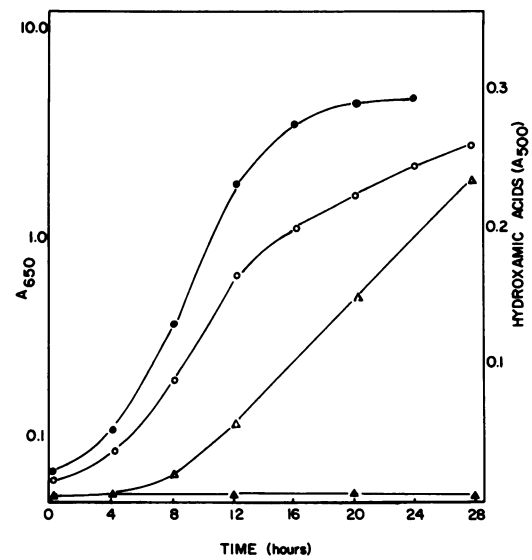


FIG. 1. *Effect of iron concentration on growth and hydroxamic acid production by S. flexneri. Growth of cells in medium with* (●) *or without* (○) *added 10 μM iron was determined by measuring turbidity (absorbancy at 650 nm [$A_{650}$]). Culture supernatants were assayed for hydroxamic acid production during growth with* (▲) *and without* (△) *added iron as described in the text.*

fractions were collected. Small samples of each fraction were added to a solution of 5 mM ferric perchlorate in 0.14 M perchloric acid (2). Those fractions giving a red or purple color with the iron solution were pooled and evaporated to dryness. This residue was dissolved in water and applied to a column (3 by 10 cm) of Dowex 1 (chloride form). The column was washed with 0.4 M $NH_4Cl$, and the siderophore was eluted with 0.5 M $NH_4Cl$. The solution was evaporated to dryness, taken up in 1 ml of water, and chromatographed on Sephadex G-100 (column, 1.8 by 100 cm) to remove the $NH_4Cl$. This column was eluted with water, and the fractions containing iron-reactive material were pooled and dried. This material was dissolved in water, applied to the top of a column (2 by 28 cm) of Dowex 50W-X8 ($H^+$ form), and eluted with water. Iron-reactive fractions were pooled and lyophilized, yielding 40 mg of siderophore.

**Siderophore identification.** Preliminary chromatographic studies indicated that the *Shigella* hydroxamate might be aerobactin or a closely related compound. The purified *Shigella* hydroxamate was compared with aerobactin in a number of chromatographic systems based on solubility (systems A and B) and on solubility and charge (systems C to H) (Materials and Methods). $R_f$ values were essentially identical in each system (data not shown). Both compounds produced relatively broad spots after chromatography, and accurate $R_f$ values were occasionally difficult to ascertain.

Absorbance spectra were determined for the iron complex of the *Shigella* siderophore and aerobactin at several pH values. The variation of $\lambda_{max}$ with pH was found to be approximately the same for the two siderophores (Table 1) and was similar to that reported by Gibson and Magrath for aerobactin (7). The pH-dependent spectral shifts were compatible with a dihydroxamate structure.

Nuclear magnetic resonance spectra confirmed that the *S. flexneri* hydroxamate was identical with aerobactin. The nuclear magnetic resonance pattern was entirely consistent with the structure of aerobactin and was essentially the same as that shown by Gibson and Magrath (7).

**Siderophore utilization.** Media containing EDDA to bind iron inhibited the growth from small inocula of all the bacterial strains tested. Only usable siderophores or high concentrations of iron relieved this inhibition (Table 2). Growth of *S. flexneri* in EDDA medium was stimulated by its own hydroxamate, by aerobactin from *A. aerogenes*, and by ferrichrome. The other hydroxamates tested, desferrioxamine B and rhodotorulic acid, were ineffective in stimulating the growth of *S. flexneri*. Enterochelin was somewhat less effective than aerobactin in reversing the inhibition of *S. flexneri* by EDDA. Only a small zone of growth was observed around disks containing this phenolate.

TABLE 1. *Comparison of absorption maxima of ferric ion complex forms of aerobactin and the S. flexneri hydroxamate*

| pH | Absorption maximum (nm) | |
|---|---|---|
| | *S. flexneri* ferric hydroxamate | Ferric aerobactin |
| 1.1 | 485 | 485 |
| 1.8 | 473 | 473 |
| 2.5 | 463 | 463 |
| 3.5 | 450 | 455 |
| 6.0 | 430 | 430 |

The hydroxamate produced by *Shigella* and the authentic aerobactin exhibited the same spectrum of growth-promoting activity (Table 2). *S. flexneri*, *A. aerogenes*, and *E. coli* B were stimulated by aerobactin, whereas RW 193, an *E. coli* K-12 derivative, and *S. typhimurium enb-1* were apparently unable to utilize this compound for iron transport.

Supernatants of the three *S. flexneri* strains grown in Tris medium were also tested for growth-promoting activity to determine whether siderophores other than aerobactin might be produced by *S. flexneri*. The supernatants had the same type of stimulatory activity as the purified siderophore. Only those bacteria that utilized aerobactin grew around disks containing the supernatants. The inability of *S. typhimurium* or *E. coli* K-12 to utilize these supernatants suggests that little or no enterochelin is secreted by *S. flexneri*. In a similar assay, the *S. flexneri* strains were inoculated directly onto the surface of seeded indicator plates to test for secreted siderophores. These inocula stimulated only the growth of those bacteria which were stimulated by aerobactin.

## DISCUSSION

Although *S. flexneri* is closely related to *E. coli* and *S. typhimurium*, it differs distinctly in its ability to synthesize and utilize iron transport compounds. Unlike these and other enteric bacteria, the *S. flexneri* strains tested do not produce detectable levels of phenolate-type siderophores, and they apparently rely upon synthesis of a hydroxamate for the acquisition of iron in low-iron media.

Despite its lack of phenolate production, *S. flexneri* can utilize the phenolate enterochelin for growth in iron-poor EDDA medium. This

TABLE 2. *Utilization of siderophores for growth in medium containing EDDA*

| Test bacteria | Growth around siderophore or iron solution[a] | | | | | | |
|---|---|---|---|---|---|---|---|
| | *S. flexneri* hydroxamate | Aerobactin | Ferrichrome | Rhodotorulic acid | Desferrioxamine B | Enterochelin | Fe |
| *S. flexneri* 2457 | ++ | ++ | ++ | − | − | + | + |
| *S. typhimurium enb-1* | − | − | ++ | + | + | ++ | ++ |
| *E. coli* RW 193 | − | − | ++ | − | − | ++ | ++ |
| *E. coli* B | ++ | ++ | ++ | + | − | ++ | ++ |
| *A. aerogenes* 62-1 | ++ | ++ | ++ | − | ++ | ++ | ++ |

[a] ++, Diameter of zone of growth around disk was ≥12 mm after 24 h of incubation; +, zone < 12 mm; −, no detectable growth. Disks contained 100 to 1,000 pmol of siderophore or 100 nmol of iron.

suggests that *S. flexneri* has membrane receptors for enterochelin. In *E. coli*, receptor synthesis is coordinately regulated with enterochelin synthesis, and after iron starvation increased levels of several membrane proteins can be detected by polyacrylamide gel electrophoresis (14). Increased levels of membrane proteins were not observed when *S. flexneri* 2457 was starved for iron, although basal levels of phenolate receptors may be present (polyacrylamide gel electrophoresis; data not shown). Thus *S. flexneri* may have some or all of the genetic information required for a phenolate-mediated iron transport system but may be unable to derepress this system during iron starvation.

The hydroxamate synthesized by *S. flexneri* is identical with aerobactin, the siderophore secreted by some strains of *A. aerogenes*. Solubilities and charge, as determined by chromatography, were the same for the two compounds, as were their absorbance and nuclear magnetic resonance spectra. Additionally, the biological activities were identical. Both compounds stimulated growth of *S. flexneri*, *A. aerogenes*, and *E. coli* B but not *S. typhimurium enb-1* or *E. coli* K-12.

Another *Shigella* species, *S. sonnei*, has recently been shown to synthesize both a phenolate and a hydroxamate siderophore (20). The hydroxamate has not been identified, but its chemical and biological properties differ from those of the *S. flexneri* hydroxamate. It is interesting that the iron transport system of *S. flexneri* should differ not only from *E. coli* and *S. typhimurium* but also from the closely related species *S. sonnei*. These differences in ability to synthesize and utilize various siderophores may be important factors in bacterial ecology and pathogenicity.

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