Siderophore Synthesis in *Klebsiella pneumoniae* and *Shigella sonnei* During Iron Deficiency†

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*Klebsiella pneumoniae* 298/53 and *Shigella sonnei* 43-GG9 exhibited restricted growth and enterochelin synthesis only under iron-deficient conditions. *S. sonnei* also produced an unidentified secondary hydroxamate siderophore.

When ionic iron is present in relatively high concentrations, a low-affinity membrane-bound iron transport system in *Escherichia coli* is capable of satisfying its iron requirements. At lower iron concentrations, this system is unable to accumulate sufficient iron to meet microbial needs (5, 18); under these conditions many microorganisms synthesize and secrete small organic compounds (siderophores) which solubilize exogenous iron, making it available for transport into the cells (9). Two classes of compounds, phenolics (2,3-dihydroxybenzoate amino acid conjugates) and secondary hydroxamates, have been shown to be effective siderophores. The most thoroughly studied phenolate siderophore, enterochelin (or enterobactin, a cyclic trimer of 2,3-dihydroxybenzoylserine) is produced by iron-deficient cultures of *E. coli*, *Enterobacter aerogenes*, *Enterobacter cloacae*, and *Salmonella typhimurium* (11, 16, 20). Only a few bacterial species secrete hydroxamate siderophores during iron deprivation: *Bacillus megaterium* (schizokinen), *E. aerogenes* strain 62-1 (aerobactin), *Arthrobacter pascens* (arthrobactin or terregens factor), and *Pseudomonas fluorescens* (ferribactin) (3, 6, 9). In this study, we report on the synthesis of enterochelin by both *Klebsiella pneumoniae* 298/53 and *Shigella sonnei* 43-GG9 and the additional synthesis of a secondary hydroxamate siderophore by the *S. sonnei* strain.

The iron-starved cells of *K. pneumoniae* secreted phenolate compounds and failed to attain the level of growth exhibited by the iron-sufficient cells (Fig. 1). Growth of the iron-deficient culture was reduced by 38%. In the iron-deficient culture, the secretion of extracellular phenolate compounds paralleled growth. Secretion of phenolate compounds by iron-sufficient cells was below detectable levels (less than 1 μg of 2,3-dihydroxybenzoic acid equivalents per ml).

Hydroxamate compounds were not observed in supernatant fluids of either culture (less than 10 μg of schizokinen equivalents per ml).

When compared with iron-sufficient *S. sonnei* cells, iron-restricted cells exhibited an increased generation time and a reduced growth yield (Fig. 1). Iron restriction caused a 38% reduction in growth and nearly a twofold increase in generation time (1.3 h:0.75 h). Iron-starved cells synthesized both phenolate and hydroxamate compounds; secretion of these compounds paralleled growth.

Enterochelin, its degradation products, and 2,3-dihydroxybenzoic acid have previously been separated on paper chromatograms run in 5.0% (wt/vol) ammonium formate in 0.5% (vol/vol) aqueous formic acid (11, 15, 17). In this study, purified phenolate isolates from each organism were separated into five iron-reactive spots [i.e., enterochelin, linear (2,3-dihydroxybenzoylserine)_3, (2,3-dihydroxybenzoylserine)_2, 2,3-dihydroxybenzoylserine, and 2,3-dihydroxybenzoic acid] by the formate solvent (Table 1). Although the formate solvent system has previously been reported to separate phenolate isolates from *E. coli* into six (17) and four (15) compounds, differences in growth conditions and isolation procedures may account for these discrepancies. The experimental *R*~f~ values for phenolate compounds from *E. coli* K-12 (Table 1) correlate reasonably well with previously published values of analogous compounds (15, 17). Phenolate compounds from *E. coli* yielded spots with *R*~f~ values which were nearly identical with analogous spots from phenolate isolates of *K. pneumoniae* and *S. sonnei* (Table 1). The slowest moving compounds from all three organisms turned a shade of mauve when sprayed with 0.5% ferric chloride; these results are in agreement with those previously reported by O'Brien et al. (11), who identified this compound as enterochelin.

Thus, under iron-deficient conditions, both *K. pneumoniae* 298/53 and *S. sonnei* 43-GG9 syn-
Fig. 1. Growth and siderophore synthesis by K. pneumoniae and S. sonnei at 37°C. (A) Viability (in colony-forming units per milliliter) and (B) optical density (620 nm) of cultures: iron-sufficient cultures of K. pneumoniae (○) and S. sonnei (□); iron-deficient cultures of K. pneumoniae (●) and S. sonnei (■). Optical density values (540 nm) of the Czakó assay (4) for the secondary hydroxamate in the iron-deficient culture of S. sonnei (■, A). No detectable hydroxamate production occurred in the iron-sufficient cultures. (B) Equivalent μg of 2,3-dihydroxybenzoic acid (DHB) produced per ml by the iron-deficient cultures of K. pneumoniae (○) and S. sonnei (■). No detectable production of phenolate compounds occurred in the iron-sufficient cultures. Growth curves were performed in a chemically defined medium as previously described (13). Iron was extracted from this medium by the method of Waring and Werkman (18); iron-deficient medium contained 0.1 to 0.3 μM iron as determined by a modified bathophenanthroline iron assay (14). Iron-deficient medium was prepared by supplementing the iron-deficient medium with ferric chloride to a final concentration of 20 μM. Phenolate compounds were detected by the method of Arnow (1).

Table 1. Rf values for the components of the phenolate isolates and 2,3-dihydroxybenzoic acid separated by descending paper chromatography in an ammonium formate solvent system

<table>
<thead>
<tr>
<th>Phenolates</th>
<th>Rf in 5% (wt/vol) ammonium formate in 0.5% (vol/vol) aqueous formic acid&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>From E. coli</td>
<td>0.19 0.32</td>
</tr>
<tr>
<td>From K. pneumoniae</td>
<td>0.19 0.35</td>
</tr>
<tr>
<td>From S. sonnei</td>
<td>0.20 0.35</td>
</tr>
<tr>
<td>2,3-dihydroxybenzoic acid</td>
<td>0.76</td>
</tr>
</tbody>
</table>

<sup>a</sup> Phenolate compounds were isolated by ethyl acetate extraction as described by O'Brien and Gibson (12). The phenolate isolates were diluted with redistilled ethanol such that each solution contained equivalent quantities of Arnow assay-reactive material.

A, Enterochelin; B, linear (2,3-dihydroxybenzoylserine)<sub>5</sub>; C, (2,3-dihydroxybenzoylserine)<sub>5</sub>; D, 2,3-dihydroxybenzoylserine; E, 2,3-dihydroxybenzoic acid. Chromatograms were developed by spraying with a 0.5% (wt/vol) ferric chloride solution.

The synthesis of siderophores has been reported (7, 8). This compound was either not secreted under the conditions employed in this study or indistinguishable from enterochelin and its degradation products in the formate solvent system.

The hydroxamate compound from S. sonnei has not been completely isolated and purified. This compound gave a positive reaction for the presence of hydroxylamine only after hydrolysis with 6 M sulfuric acid at 100°C, indicating that the compound is a secondary hydroxamate. The compound can be extracted into phenol when fully chelated with iron at pH 4.0, whereas the unchelated compound is not. Attempts to remove the hydroxamate from phenol have been unsuccessful. Ethyl acetate extraction at pH 1.0 did not remove this compound from culture supernatant fluids. Dowex-1 columns (4 by 7 cm) did not remove the hydroxamate from culture supernatant fluids adjusted to pH’s of 7.0 and 9.0. At pH 12.0, the hydroxamate was partially retained on a Dowex-1 column and appeared to be eluted with 0.1 M NH₄Cl (data not shown). The compound was partially purified by passage of culture supernatant fluids at pH 7.0 through a Dowex-1 column. The eluant was adjusted to pH 9.0 with 1 M NaOH and passed through a second Dowex-1 column. The eluant was flash evaporated and applied to a water-equilibrated Sephadex G-10 column which ex-
cluded the hydroxamate compound. Positive fractions were pooled, concentrated by flash evaporation, and analyzed by descending paper chromatography in n-butanol-acetic acid-water (60:15:25, by vol). A chromatogram developed with 0.5% ferric chloride revealed a single red spot with an Rf of 0.63. In the same system, aerobactin, desferrioxamine B, and schizokinen gave reddish-purple spots with respective Rf's of 0.69, 0.86, and 0.73. Development of a second chromatogram with ammoniacal silver nitrate indicated that the hydroxamate sample from S. sonnei contained several other organic compounds as minor contaminants (data not shown).

For growth and inhibition factor studies, S. typhimurium LT-2 enb-7 (a mutant blocked in the synthesis of 2,3-dihydroxybenzoic acid) was grown in iron-sufficient medium before inoculation onto petri plates. Petri plates contained either iron-sufficient solid medium (iron-deficient medium supplemented with 1% [wt/vol] Ionagar no. 2 and 50 μM ferric chloride) or citrate-supplemented solid medium E, which completely inhibits the growth of the S. typhimurium mutant (10, 15). Petri plates were seeded by overlaying with approximately 10⁵ cells in the appropriate solid medium. Volumes of 10 μl of sterile siderophore and iron solutions (1 mM) were spotted onto the seeded plates. All phenolate isolates were diluted with water to 0.15 optical density units by the Arnow assay (1) before spotting 10-μl volumes onto the seeded plates. The same volume of the S. sonnei hydroxamate solution (0.58 optical density units by the Csáky assay [4]) was applied to seeded plates. The hydroxamate solution was the eluant obtained by passing culture supernatant through a Dowex-1 column and was not contaminated with phenolate compounds since these compounds were irreversibly bound by Dowex-1 (Perry, unpublished data).

The growth of S. typhimurium LT2 enb-7 on citrate-supplemented medium E was stimulated by ferric chloride, ferrous chloride, 2,3-dihydroxybenzoic acid, desferrioxamine B, schizokinen, the S. sonnei hydroxamate, and the phenolate isolates from E. coli, K. pneumoniae, and S. sonnei. Aerobactin, hemin, and unincubated iron-deficient medium did not stimulate growth. Only aerobactin inhibited the growth of the S. typhimurium mutant on iron-sufficient solid medium (data not shown).

The secondary hydroxamate produced by S. sonnei is not aerobactin, desferrioxamine B, or schizokinen since its chemical or biological properties or both differ from the properties of these secondary hydroxamate siderophores. The synthesis of phenolate and hydroxamate siderophores by Shigella boydii M44 (Perry, unpublished data) and S. sonnei 43-GG9 suggests that the simultaneous expression of both types of siderophores may be common within this genus. The simultaneous synthesis of both phenolate and hydroxamate compounds has been previously reported only in E. aerogenes 62-1 (enterochelin and aerobactin) and in Bacillus subtilis var. niger (a secondary hydroxamate and 2,3-dihydroxybenzoylglycine) (2, 6, 18). The physiological necessity for synthesizing two separate siderophores is not readily apparent.

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LITERATURE CITED


