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Isolation of Enterochelin from the Peritoneal Washings of Guinea Pigs Lethally Infected with *Escherichia coli*

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Escherichia coli secretes enterochelin while infecting normal guinea pigs. Since production of enterochelin is a well-characterized response to an iron-restricted environment, this work establishes that host iron-binding proteins do indeed influence the metabolism of the invading organism.

An important factor in any bacterial infection is the ability of the invading organisms to adapt to and multiply in the host. Many body fluids, particularly mucosal secretions, contain the iron-binding proteins transferrin or lactoferrin or both (1, 3, 15, 16). These proteins have an association constant for iron of about 10^{36} and are normally only partially saturated (2). Although there is plenty of iron present in host body fluids, the amount of free iron in equilibrium with the iron-binding proteins is about 10^{-18} M, which is far too low for normal bacterial growth (2). In spite of this, pathogenic organisms successfully multiply under these conditions. Such bacteria must, therefore, possess specific mechanisms for assimilating the protein-bound iron. Organisms in the genera *Salmonella*, *Escherichia*, and *Klebsiella* secrete the iron-chelating compound enterochelin under conditions of iron restriction in vitro (21). This neutral compound is the cyclic trimer of 2,3-dihydroxybenzoyl serine, and both enterochelin and its acidic degradation products are found in culture fluids (19-21). Since enterochelin mediates the transport of iron from iron-binding proteins to the bacterial cell, it has been proposed that its secretion is essential for the growth of these organisms in vivo (2, 19, 20, 22). Indeed, it has been suggested that one of the mechanisms behind the adaptive, or beneficial, role of fever is the inhibition of the synthesis of enterochelin and other iron-transporting compounds in bacteria at elevated temperatures (4, 5, 12-14). This, it is proposed, reduces the ability of pathogenic organisms to grow in the presence of iron-binding proteins.

In any work on infection, it is essential to show that biochemical mechanisms studied in vitro do apply in vivo. It has already been shown that injected enterochelin and other iron-transporting compounds enhance the virulence of *E. coli* and *S. typhimurium* in infected animals (11, 19). Furthermore, Griffiths et al. (6, 7) have shown that *E. coli* O111 recovered from the peritoneal cavities of lethally infected guinea pigs and rabbits contain transfer ribonucleic acids with the same chromatographic properties as those found in the bacteria growing in vitro in the presence of iron-binding proteins and which are distinct from those found in normal broth grown organisms. The evidence, therefore, strongly suggests that host iron-binding proteins can affect the metabolism of invading bacteria. The present work augments and consolidates previous work by showing that *E. coli* O111 secretes enterochelin while infecting normal guinea pigs.

Groups of 10 to 14 guinea pigs were lethally infected by intraperitoneal injection of about 10^8 *E. coli* O111 K58 H2 in 0.15 M NaCl as described previously (7). Injected bacteria multiplied rapidly in the peritoneal cavities, and the animals were either very ill or were dead within 24 h. A similar number of control animals were injected with 0.15 M NaCl alone or with 10^8 heat-killed *E. coli* O111, neither of which had any apparent deleterious effect. Between 14 and 15 h after injection, surviving guinea pigs were killed with CO_2, and peritoneal washings were obtained as before (7). Peritoneal washings from lethally infected animals contained about 10^9 extracellular viable bacteria per ml. Bacterial and tissue cells were removed from the pooled washings of each group of animals by centrifugation at 5°C, and after lowering the pH to 2.5 with N HCl, the washings were extracted with ethyl acetate (19). Catechols which form iron complexes were separated from contaminating material by adding solid $NaHCO_3$ to the concentrated ethyl acetate extract, followed by 1 mM ferric nitrilotriacetate (20 to 30 ml). Iron complexes of the catechols are insoluble in ethyl acetate and move into the acqueous phase. The characteristic brownish-pink color of Fe^{3+} enterochelin and its degradation products was sometimes seen at this stage in extracts from lethally infected animals. Undissolved $NaHCO_3$ was removed, the pH was adjusted to 2.5, and the aqueous phase was again

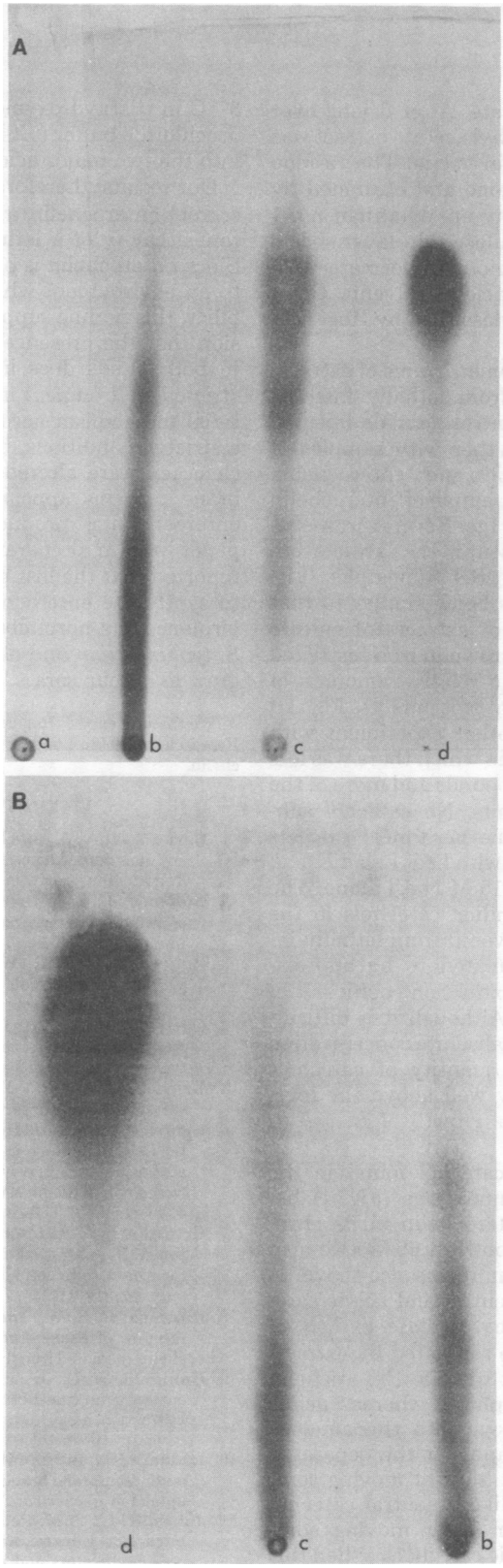

FIG. 1. *Paper chromatograms showing the presence of iron-binding catechols in the ethyl acetate extracts of peritoneal washings from guinea pigs infected with E. coli O111, together with controls. A and B show the results from two separate experiments. Solvent, 6% acetic acid. (a) Extract from guinea pigs injected with heat-killed E. coli O111; (b) enterochelin; (c) extract from guinea pigs injected with live E. coli O111; (d) 2,3-dihydroxybenzoic acid.*

extracted with ethyl acetate. After drying over anhydrous Na_2SO_4, the ethyl acetate extract was concentrated to dryness in vacuo. The residue was dissolved in *n*-butanone and examined by ascending chromatography on Whatman no. 1 or 3MM paper or on cellulose thin-layer sheets by using either 5% ammonium formate–0.5% formic acid or 6% acetic acid as solvents. Compounds were detected as described by Rogers et al. (20).

Figure 1 shows the chromatograms of extracts of peritoneal washings from lethally infected guinea pigs and from control animals injected with killed bacteria, together with samples of 2,3-dihydroxybenzoic acid and enterochelin. Some degradation of this sample of enterochelin had occurred during storage. Several iron-chelating catechols were present in the extract from one group of lethally infected guinea pigs (Fig. 1A), the pattern of spots being similar to that seen in chromatograms of extracts of culture fluids from *E. coli* O111 grown in iron restricted media (20). However, the relative amounts of each spot varied between experiments. Fig. 1B shows the results of another experiment with lethally infected animals in which there was less of the faster moving compounds and more of the slower moving components. No catechol substances were found in the peritoneal extracts from guinea pigs injected with heat killed *E. coli* O111 (Fig. 1A) or with 0.15 M NaCl alone. The concentration of iron-binding catechols in the peritoneal washings recovered from lethally infected animals was estimated to be approximately 1 μM, using the extinction coefficient of ferric enterochelin (20). Although it is difficult to relate this figure to the absolute concentration in the peritoneum, the quantity of catechols formed in vivo is clearly well above the level required to stimulate the growth of *E. coli* in body fluids (19, 20).

The slowest running catechol found in the extract from infected guinea pigs (Fig. 1) behaved like authentic enterochelin in its chromatographic properties both in 6% acetic acid and in the formate system. It was also shown to be a neutral catechol. Neutral and acidic catechols present in the ethyl acetate extract of peritoneal washings were separated by extracting the organic phase at pH 6.5. Enterochelin remains in the organic phase, whereas acidic degradation products move into the aqueous phase (20). Chromatography of the separated products showed that the slowest moving compound (Fig. 1) behaved like a neutral catechol and that the two major faster moving compounds behaved like acidic catechols. When this neutral catechol was incubated overnight at 37°C in tris(hydroxymethyl)aminomethane-hydrochloride buffer (0.1 N; pH 8), it was degraded into the two major acidic catechols.

Our results, therefore, show that *E. coli* O111 secrete enterochelin when growing in the peritoneal cavity of a lethally infected guinea pig. Since enterochelin is produced only in response to an environment which is low in iron availability, this finding supports our previous conclusion that the presence of iron-binding proteins in body fluids does indeed create an iron-restricted environment in vivo and will affect bacterial metabolism accordingly (7). Under iron-restricted conditions, bacterial transfer ribonucleic acids are altered (6, 7), new outer-membrane proteins appear (8, 9, 10, 17, 18), and enterochelin is produced (19, 21). While this paper was in preparation, Yancey et al. (23) reported that the loss by mutation of the ability to synthesize enterochelin greatly reduced the virulence of a normally highly virulent strain of *S. typhimurium* and also inhibited its ability to grow in human sera.

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