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SIDEROPHORE PRODUCTION BY *SALMONELLA TYPHI*

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This study was initiated to determine the mechanism of iron-uptake in *Salmonella typhi*. When stressed for iron, microorganisms produce siderophores to obtain the necessary nutrient. Generally two types of siderophores exist: the phenolate-type predominantly produced by bacteria and the hydroxamate-type commonly secreted by fungi. Results of this investigation showed that *S. typhi* produced siderophores of the phenolate-type since culture supernatant of the organism grown under iron-deprivation supported the growth of the phenolate-dependent auxotroph. The culture supernatant when extracted for phenolate siderophores, also supported the growth of the phenolate auxotroph but not the hydroxamate auxotroph. Production of phenolate-type siderophores were further confirmed using biochemical assays. These results showed that *S. typhi* utilized the high-affinity iron transport system to obtain the necessary iron.

Iron although abundant in the human host is made unavailable to microorganisms since it is bound to host-iron binding proteins. The ability of microorganisms to acquire iron from the host is a prerequisite for pathogenecity (1, 2). Pathogenic microorganisms such as *Salmonella typhi* presumably must be able to utilize the iron sources available in the human host. An obligate parasite of humans, *S. typhi* is the causative agent of typhoid or Enteric fever which remains as an important public health problem in underdeveloped countries. The organism is capable of invading the blood stream and disseminating into many organs of the body. Transferrin is probably the principal source of iron in the serum and lactoferrin is probably the main source of iron on mucosal surfaces (2). The mechanism by which *S. typhi* obtains iron from these host-iron binding proteins has not been defined.

Microorganisms have devised several mechanisms which can remove iron from host iron-binding proteins (3, 4). One such mechanism is a high-affinity iron acquisition system consisting of iron-chelating agents called siderophores and outer membrane proteins that serve as receptors for the iron-siderophore complexes and aid in their internalization. These iron-

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uptake systems are usually induced when the microorganisms are stressed for iron.

Generally two types of siderophores exist: the hydroxomate-type predominantly produced by fungi and the phenolate-type secreted by bacteria (4). S. typhimurium, a common food-poisoning organism, was reported to secrete phenolate-type siderophores (5).

This study was initiated to aid in defining the mechanism by which S. typhi acquires iron. The results indicate that S. typhi utilized the high-affinity iron-acquisition system by producing siderophores of the phenolate-type to obtain its iron.

MATERIALS AND METHODS

Growth and maintenance of cultures

Clinical isolates of S. typhi (USM 1-10) and 2 clinical isolates of S. paratyphi A (USM 11) and S. paratyphi B (USM 12) were obtained from the stock culture collection, School of Medical Sciences, Universiti Sains Malaysia, Penang. Bacterial strains were maintained on nutrient agar slants kept at 4°C and transferred monthly. For experimental purposes the organisms were grown in nutrient broths at 37°C for 18 hours and were subcultured at least three times before being used.

Siderophore-dependent auxotrophs, Arthrobacter flavescens JG9 and Salmonella typhimurium LT 2 enb - 7, were the generous gifts of J.B. Neilands, Univ. of California, Berkeley. Arthrobacter flavescens was maintained on medium 424 (American Type culture collection, Rockville, MD) while S. typhimurium was maintained on medium E (6) at 37°C.

All glassware were acid washed and rinsed with deionised water. Disposable plasticware were used throughout the study to minimize iron contamination.

Siderophore production

To enrich for siderophore production, strains of S. typhi and paratyphi A and B were grown in a deferrated medium. Deferration of the nutrient broth was achieved by addition of 100 uM 8-Hydroxyquinoline (May and Baker Ltd, Dagenham, England) prior to inoculation. Overnight cultures of S. typhi or paratyphi were added to the deferrated medium to a final concentration of $4 - 8 \times 10^6$ cells/ml and incubated at 37°C for 48 hours. Culture supernatants obtained were either tested immediately for the presence of siderophores or concentrated 10 fold by lyophilization.

Detection of siderophores

The presence of iron-free siderophores in the culture supernatant was tested following the method of Haydon et al (7). The following reagents were added accordingly. Acidified $FeCl_3$ (150 ul) (0.06 M in 0.005 N HCl) was added to samples of the culture supernatant (1350 ul) and allowed to react for about 5 mins. Absorbance of the mixture was read at 480 nm. The presence of the yellow-orange color was indicative of siderophore-iron complex formation. Uninoculated deferrated medium was used as a blank.

Phenolate-type siderophores in the culture supernatants were determined biochemically using the Arnow assay (8). Hydroxamic acids were determined following the methodology of Emery and Neilands (10, 11). Desferoxamine (a hydroxamate compound, Ciba Pharmaceutical Co., Summit, NJ) and catechol (a phenolate compound, Sigma Chemical Co., St. Louis, MO) in the deferrated medium were used as positive and negative controls.

Extraction of siderophores

Extraction of the phenolate-type siderophores was carried out following the method of Rogers (11). The concentrated culture filtrate

of S. typhi (USM 1) (10) was extracted using ethyl acetate. Hydroxamate-type siderophores were extracted from the concentrated culture supernatant following the method proposed by Simpson and Oliver (12). The extracts obtained were further concentrated by lyophilization and reconstituted to 3 ml with deionized water. The siderophore extracts were adjusted to pH 7.0 and filter-sterilized using a 0.45 um membrane filter.

Siderophore bioassays

Bioassays were performed with A. flavescens, a hydroxamate-dependent auxotroph and S. typhimurium, a phenolate-dependent auxotroph. Samples (50 - 200 ul) of the test extracts were added to the appropriate siderophore-deficient media. Medium 424 without desferoxamine was used to test for the growth of the hydroxamate-dependent auxotroph while medium E without enterochelin (a phenolate siderophore produced by Escherichia coli) was used for the phenolate-dependent auxotroph. Growth of the auxotrophs after 48 hours at 35°C was measured by the increase in turbidity at 600 nm. Auxotrophs grown in the respective siderophore-deficient media with or without the addition of siderophores were used as positive and negative controls.

RESULTS

Production of siderophores

Salmonella strains were grown in a deferrated medium at 37°C in order to enhance siderophore production. The secretion of iron-free siderophores into the culture supernatant was detected via formation of the colored complex upon binding to acidified ferric chloride. Culture supernatants from 10 strains of S. typhi and 1 strain of S. paratyphi A and 1 strain of S. paratyphi B were assayed. The results shown in Table 1 revealed that all strains of S. typhi (USM 1 - 10) gave readings greater than the uninoculated deferrated media (blank) suggesting the presence of siderophores in their supernatants. Siderophores were also detected in the culture supernatants of S. paratyphi A and B.

Determination of siderophore classes

A siderophore bioassay was performed to determine the class of siderophores produced by S. typhi. Concentrated culture supernatant from S. typhi (USM 1) was extracted for hydroxamate and phenolate-type siderophores. The test extracts were then examined for the presence of siderophores using siderophore-dependent auxotrophs. The results showed that S. typhi produced siderophores of the phenolate-type only (Table 2). The culture supernatant as well as the phenolate extract of the concentrated supernatant supported growth of the phenolate auxotroph but not the hydroxamate auxotroph. The hydroxamate extract failed to support growth of A. flavescens JG 9 suggesting the lack of hydroxamate production.

To rule out the possibility that the uninoculated culture media contained small amounts of siderophore activity, phenolate and hydroxamate extracts of the concentrated uninoculated medium was performed. The test extracts did not support growth of either auxotrophs.

Table 1. Siderophore production by Salmonella spp. under iron-limiting conditions [a]

Strains	Absorbance (480 nm) [b]
USM 1	0.095
2	0.110
3	0.110
4	0.068
5	0.120
6	0.150
7	0.100
8	0.140
9	0.113
10	0.100
11 [c]	0.120
12 [d]	0.140

[a] All values are means of duplicate assays.

[b] Iron-binding activity of culture fluids indicating the presence of siderophores in the medium. Formation of the colored iron-siderophore complex was recorded at 480 nm using uninoculated deferrated medium as a blank.

[c] S. paratyphi A

[d] S. paratyphi B

To further confirm that the siderophores secreted were of the phenolate-type, the Arnow assay was done (Table 3). The concentrated as well as unconcentrated supernatant fluids showed positive for phenolate. The Arnow assay also confirmed the presence of phenolate siderophores in the phenolate extract. The hydroxamate extract gave an absorbance equal to that of desferoxamine (the negative control) suggesting the specificity of the Arnow assay for the phenolates. The biochemical assay also confirmed the lack of siderophore activity in the siderophore extracts of the uninoculated deferrated medium.

To determine if other strains of S. typhi produced siderophores of the phenolate-type, 9 clinical strains of S. typhi and 2 strains of S. paratyphi

Table 2. Bioassay with siderophore-dependent auxotrophs to determine the class of siderophore produced by S. typhi (USM 1)

Samples	Growth of auxotrophs	
	Phenolate-dependent S. typhimurium	Hydroxamate-dependent A. flavescens
Siderophore-deficient medium [a]	-	-
Culture supernatant (200 ul)	0.10 [b]	0.04
Phenolate extract (50 ul)	0.07	-
Hydroxamate extract (50 ul)	0.03	0.02
Controls		
Enterochelin (50 ul)	0.08	-
Desferoxamine (50 ul)	-	0.115
Deferrated medium (50 ul) (phenolate extract)	0.005	-
Deferrated medium (50 ul) (hydroxamate extract)	0.02	-

[a] Auxotroph grown in the siderophore-deficient medium (Medium E or Medium 424) was used as a blank. Absorbance was recorded at 600 nm.

[b] Growth was considered positive at absorbance >0.05.

A and B were screened using the Arnow assay. All the strains tested gave a positive Arnow assay (data not shown).

To confirm that S. typhi did not produce siderophores of the hydroxamate-type, the methodology of Emery and Neilands was followed. If hydroxamic acids were present, addition of the periodic acid would result in the formation of nitrosodimers, that absorbed maximally at 270 nm. Desferoxamine was used as the positive control while catechol was used as a negative control. All 10 strains of S. typhi and two strains of S. paratyphi did not give a positive hydroxamate assay (data not shown).

DISCUSSION

The ability to efficiently acquire iron from the host appears to be crucial for many pathogens. This is particularly true for most invasive

Table 3. Biochemical assay to detect phenolate-type siderophores of S. typhi (USM 1)

Samples	Absorbance (510 nm) [a]
Culture supernatant	0.63
Concentrated supernatant	0.180
Phenolate extract	0.200
Hydroxamate extract	0.020
Deferrated medium (phenolate extract)	0.010
Deferrated medium (hydroxamate extract)	0.010
Controls	
Catechol (174 um)	0.370
Desferoxamine (174 um)	0.020

[a] Phenolate compounds absorb maximally at 510 um. An absorbance > 0.040 (2 x O.D of negative control) was considered positive.

pathogens (13). In this study, biochemical as well as biological assays were used to elucidate the mechanism of iron-acquisition by S. typhi.

When grown under iron-limitation, siderophores were detected in the culture supernatants of clinical isolates of S. typhi and S. paratyphi A and B. In order to determine the class of siderophores produced, siderophore dependent auxotrophs were used. The supernatant fluids of S. typhi supported the growth of the phenolate-dependent auxotroph but not the hydroxamate auxotroph. Extraction of the concentrated supernatant for phenolate siderophore was performed to assess for its presence. The phenolate extract supported growth of the phenolate auxotroph suggesting that S. typhi produced siderophores of the phenolate-type. S. typhi did not produce hydroxamic acids since the hydroxamate extract of the concentrated supernatant did not support the growth of A. flavescens JG 9. Results of the bioassay were confirmed using the biochemical assay. This investigation revealed that the organisms involved in enteric fever possessed a high-affinity iron-transport system to sequester the necessary iron.

Possession of a siderophore-mediated iron transport system is related to increased virulence of some bacterial pathogens (2). Siderophore production has been shown to increase the virulence of E. coli (14), Vibrio anguillarum (15) and Pseudomonas aeruginosa (13). Previous work on S. typhimurium showed that the organism failed to cause mouse typhoid due to its inability to synthesize enterochelin (a phenolate siderophore) (16). If siderophore production is necessary for the virulence of the organism, then conditions which either promote or suppress its synthesis must be examined in order to control the course of the disease. The effect of febrile temperature on siderophore production by S. typhi is currently being examined.

Although data indicating the importance of siderophores in the pathogenesis of most bacteria are overwhelming, several authors have published reports that are on the countrary. Reports on S. typhimurium (17) and V. cholerae (18) have suggested that production of a functional siderophore-mediated iron-transport system did not contribute to the virulence of non-invasive as well as intracellular pathogens.

Due to the conflicting reports in the literature, a comprehensive investigation is necessary to determine if the high-affinity iron-transport system plays a vital role in the pathogenesis of S. typhi.

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