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Siderophore Production by *Vibrio vulnificus*

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Previous studies in our laboratory, as well as clinical evidence, have suggested that increased iron levels in the host may be important in infections caused by the halophilic pathogen *Vibrio vulnificus*. To study iron acquisition, we induced siderophore production by growth in a low-iron medium, and biochemical testing indicated the production of both hydroxamate- and phenolate-type siderophores. The siderophores were extracted from growth filtrates with ethyl acetate (for phenolates) and phenol-chloroform-ether (for hydroxamates). These extracts enhanced the growth of *V. vulnificus* when the bacterium was grown in iron-limited medium. The ability of these siderophores to stimulate the growth of *Salmonella typhimurium LT*-2 *enb*-7 (a mutant deficient in the biosynthesis of enterochelin) and *Arthrobacter flavescens* JG-9 (a hydroxamate auxotroph) supported the conclusion that *V. vulnificus* produces both hydroxamate- and phenolate-type siderophores.

Vibrio vulnificus, a halophilic bacterium, has been associated with a number of extraintestinal infections. Unlike clinical symptoms produced by *Vibrio parahaemolyticus*, a closely related species often confused with *V. vulnificus*, those produced by this organism are severe and often result in death (2).

Although several portals of entry have been suggested for *V. vulnificus* (8, 13), a majority of infections by this organism have two distinct clinical presentations—one manifested as a localized infection of wounds exposed to seawater and another as a septicemia probably after the consumption of raw oysters (2). This latter route of infection has been associated with a high incidence (85%) of underlying hepatic diseases (2). Although the overall mortality rate associated with the primary septicemia is 46%, cases involving hepatic disease (especially cirrhosis) have a mortality rate as high as 100%. Another disease most often associated with *V. vulnificus* infections is hemochromatosis. Both the hepatic disease and hemochromatosis are characterized by increased serum iron levels. Iron is essential for bacterial growth, and the ability to acquire iron from the host is a prerequisite for pathogenicity (28). Serum iron in the mammalian system is bound to a globular protein, transferrin, and is generally unavailable for use by microorganisms. The inhibitory effect of human serum reported for a number of organisms has been attributed to this iron deprivation (28). This inhibition can be reversed by the addition of iron-containing compounds (4). The injection of iron-containing compounds has also been shown to decrease the number of microorganisms necessary to produce infection in animal models (11, 14, 25).

Since clinical evidence suggested the correlation of *V. vulnificus* infections with diseases involving increased iron levels, Wright et al. (29) determined the effects of iron on the pathogenicity and the ability of this organism to survive in serum. They showed that, whereas *V. vulnificus* can grow well in rabbit serum (which has transferrin that is 60% saturated with iron), human serum (which is 30% saturated) was bactericidal. This inhibition could be reversed by the addition of iron-containing compounds. They also showed that the injection of ferric ammonium citrate reduced the 50% lethal dose from 6×10^6 to a single cell. Thus, both clinical and experimental evidence suggests that iron may play an important role in the ability of *V. vulnificus* to cause infections.

To obtain iron in environments in which it is not readily available, microorgamisms produce low-molecular-weight chelators, termed siderophores, which bind iron and return it to the cell. These compounds are them transported across the lipid-protein boundary by membrane-bound transport systems. Iron is then removed from the chelator complex for use by the cell (28). Although all siderophores do not fit into these structural categories, two types of siderophores generaly exist: the catechol-like compounds occurring in bacteria and the hydroxamate type found in fungi, yeasts, and bacteria. In a continuation of our previous studies on the role of iron in *V. vulnificus* pathogenesis, the present study was designed to stimulate siderophore production, to determine the class of siderophore pro-

duced, and to examine methods of iron acquisition by this organism when grown under iron-limiting conditions.

MATERIALS AND METHODS

Organisms. *V. vulnificus* strains C7184 and A1402, obtained from the Centers for Disease Control, Atlanta, Ga., were used to study siderophore production. The highly virulent strain C7184 is a human blood isolate, whereas strain A1402 is a weakly virulent isolate from a corneal infection.

Chemicals and glassware. All glassware was acid washed with 6 N HCl and rinsed 10 times with deionized water before use. Desferal was purchased from Ciba Pharmaceutical Co., Summit, N.J. Enterochelin was the generous gift of J. B. Neilands, University of California, Berkeley. All other chemicals were purchased from Sigma Chemical Co., St. Louis, Mo.

Media and growth of *V. vulnificus*. Brain heart infusion broth (Difco Laboratories, Detroit, Mich.) was used routinely for growth of *V. vulnificus*. For optimum siderophore production, organisms were harvested after growth to the stationary stage in brain heart infusion broth, washed with phosphate-buffered saline, and inoculated into a defined medium (Synbase) to which no iron was added. This low-iron medium was prepared as described by Payne and Finkelstein (20), except sodium succinate replaced sucrose. The cells grown in Synbase were harvested in the stationary stage, washed with phosphate-buffered saline, and inoculated into deferrated medium.

To prepare deferrated medium, we removed contaminating iron from a 10× concentrated solution of Synbase by the addition of conalbumin (10 mg/ml in 5 mM $NaHCO_3$) to a final concentration of 1 mg of conalbumin per ml of medium. After the medium was stirred for 30 min the conalbumin-iron complexes were removed by ultrafiltration through a UM20 filter (Amicon Corp. Lexington, Mass.). The iron content of this medium was 0.6 μM as determined by the ferrozine method of Stookey (24).

To stimulate siderophore production, we placed an inoculum of 10^7 *V. vulnificus* cells into 250 ml of deferrated Synbase. After incubation overnight at 25°C on a rotary shaker, the culture was diluted with 250 ml of fresh deferrated medium and incubated an additional 24 h. This culture was centrifuged (10,000 × *g*, 10 min, 5°C), and the supernatant was filtered through a 0.45-μm (pore size) membrane filter (Nuclepore Corp. Pleasanton, Calif.). This fluid was then concentrated 10-fold by freeze-drying.

Detection and extraction of siderophores. The Arnow phenolic acid assay (1) was used to detect phenolic-type siderophores. For detection of hydroxamate-type siderophores, the Csaky hydroxylamine hydroxamic acid assay (6) was carried out with and without the sulfuric acid digestion step. For extraction of phenolic-type siderophores, 20 ml of concentrated culture filtrate was adjusted to pH 1.5 with HCl and extracted with 10 ml of ethyl acetate (23). The ethyl acetate fraction was taken to dryness and dissolved in 1 ml of either absolute ethanol or phosphate-buffered saline.

For extraction of hydroxamate-type siderophores, a modification of the method described by Mullis et al. (17) was used. The pH of 10 ml of the concentrated filtrate was adjusted to 7.0 with 5 N NaOH and extracted three times with 5-ml volumes of $CHCl_3$-phenol (1:1, wt/wt). The $CHCl_3$-phenol layer was extracted with 50 ml of ethyl ether, and this mixture was extracted with 10 ml volumes of deionized water. The resultant extract was washed with ethyl ether and concentrated by freeze-drying to approximately 5 ml. The pH was adjusted to 7.2 and the extract was filter sterilized.

Paper chromatography. Paper chromatography of the ethyl acetate extract was carried out as described by Rogers (23). The extract dissolved in ethanol was spotted on Whatman no. 1 filter paper and chromatographed for 8 h, with 5% ammonium formate plus 0.5% formic acid used as the solvent. The dried paper was examined under UV light and by spraying with an aqueous solution of 1% ferric ammonium chloride (23) to detect phenolic compounds.

Siderophore bioassays. Bioassays were performed with two microorganisms that are unable to produce siderophores but have receptors for a variety of those produced by other organisms. *Salmonella typhimurium LT-2 enb-7*, a mutant blocked in the synthesis of phenolates, was obtained from J. B. Neilands. Twelve-hour broth cultures were used in the assay described by Luckey et al. (16). *Arthrobacter flavescens* JG9, a hydroxamate auxotroph, was obtained from Paul Szaniszlo, University of Texas, Austin, and maintained on *Arthrobacter* medium 424 (American Type Culture Collection, Rockville, Md.) with Desferal. Assays were performed with lawns of these cultures on this medium containing no Desferal. Plate assays were performed by inoculating wells with 15 μl of the concentrated extracts and observing growth enhancement of the test organism.

Growth enhancement studies. The extracted siderophores were also tested for their ability to enhance growth of *V. vulnificus* in the deferrated medium with either added iron (80 μg/ml), concentrated filtrate, or extracted siderophore. Cultures were incubated for 30 h at 37°C, and at 2, 4, 8, 20, 24, and 30 h, total viable counts were determined with the marine medium (MSWYE) described by Oliver and Colwell (19). This same assay procedure was used to determine the ability of *V. vulnificus* to transport iron by using Desferal, a hydroxamate-type siderophore produced by *Streptomyces* spp., and enterochelin, a catechol-type siderophore produced by many of the enterics.

RESULTS

Detection of siderophore production. For stimulation of siderophore production, *V. vulnificus* cells were added to Synbase that had been deferrated with conalbumin. After concentration by freeze-drying, the cell-free supernatants of strains C7184 and A1402 were examined for the presence of siderophores by biochemical testing. The concentrated filtrate from *V. vulnificus* C7184 gave a positive result in the Arnow test, indicating the presence of a phenolate-type siderophore. The filtrate from the weakly virulent strain A1402, however, gave negative results. Both strains gave positive results in the Csaky test for hydroxamates. The uninoculated medium gave negative results in the Arnow and Csaky tests.

To determine whether a filtrate could act to reverse the effects of iron limitation, 0.3 ml of the concentrate from the C7184 culture was added to 2.4 ml of deferrated Synbase and inoculated with 7 × 10^5 cells of *V. vulnificus* C7184 per ml. The culture was incubated at 37°C and plate counts were determined at time intervals. The filtrate resulted in an increase in growth as compared with growth in the deferrated medium alone (Fig. 1). By 24 h, the number of cells was equivalent to that of the culture grown in the medium to which iron had been added.

Since the Arnow and Csaky tests indicated that *V. vulnificus* C7184 produced both phenolate- and hydroxamate-type siderophores, the filtrate from the culture of this organism was treated with organic solvents to extract the siderophores.

Ethyl acetate was used to extract the catechol-type siderophore. There was an increase in cell numbers from 3 × 10^5 per ml in the deferrated medium alone to 5 × 10^8 per ml in the medium containing the ethyl acetate extract (Fig. 2).

When the ethyl acetate extract of *V. vulnificus* C7184 was analyzed by paper chromatography, a catechol was detected with R_fs of 0.9 and 0.48. A small amount of iron-binding compounds were also detected in the *V. vulnificus* extract with R_fs of 0.33 and 0.67. In this system, enterochelin migrated with R_fs of 0.43 and 0.62. When the ethyl acetate extract of *V. vulnificus* C7184 was added to the *S. typhimurium* mutant *LT-2-enb-7*, growth enhancement of this mutant was observed.

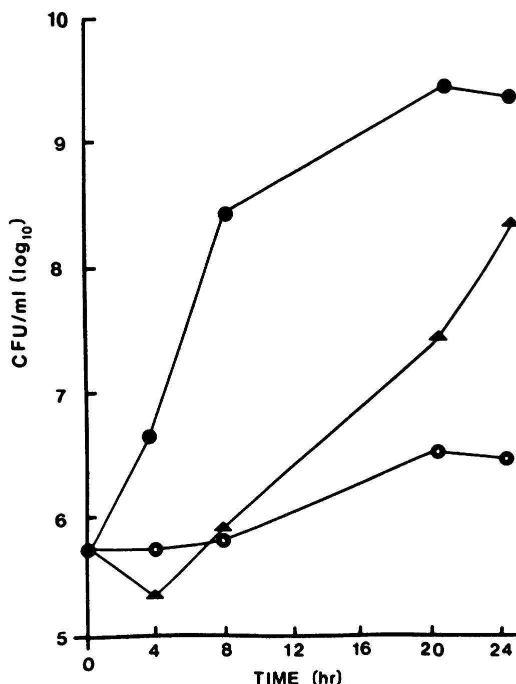

FIG. 2. Growth enhancement of *V. vulnificus* with addition of ethyl acetate extract containing the phenolate siderophore. ○, Deferrated medium; ●, deferrated medium plus ferric ammonium citrate; ▲, deferrated medium plus ethyl acetate extract.

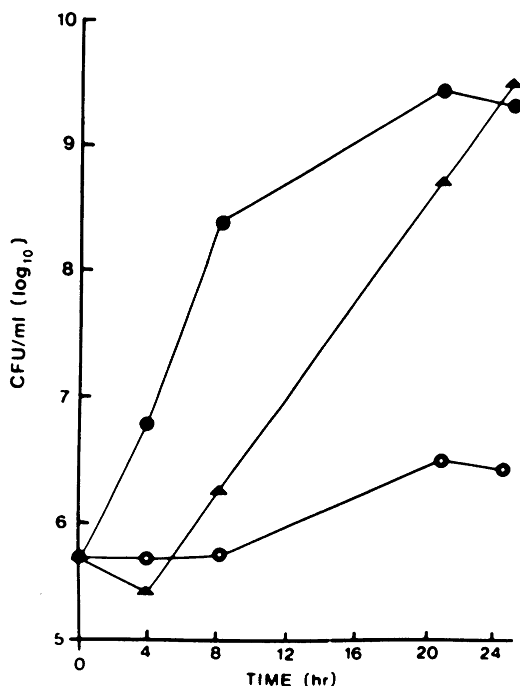

FIG. 1. Growth enhancement of *V. vulnificus* with addition of concentrated culture filtrate. ○, Deferrated medium; ●, deferrated medium plus ferric ammonium citrate; ▲, deferrated medium plus culture filtrate.

The hydroxamate siderophore extracted from *V. vulnificus* C7184 was also tested for its ability to enhance growth of *V. vulnificus* C7184 in the deferrated medium. The addition of this extract increased the growth of *V. vulnificus* over the 24-h test period from 9.5 × 10^6 to 7 × 10^8 cell per ml (Fig. 3). Furthermore, evidence of hydroxamate production was obtained with the hydroxamate auxotroph *A. flavescens* JG9. Growth of this mutant was stimulated around the well to which the *V. vulnificus* strain C7184 filtrate was added.

Effects of exogenous siderophores. Since previous studies (29) have suggested that *V. vulnificus* may be able to use Desferal to transport iron, the ability of this siderophore to reverse iron limitation was studied. The addition of 0.1 mg of Desferal per ml was found to increase the growth of *V. vulnificus* in the deferrated medium from 9.5 × 10^6 to 9.1 × 10^9 cells per ml in 24 h

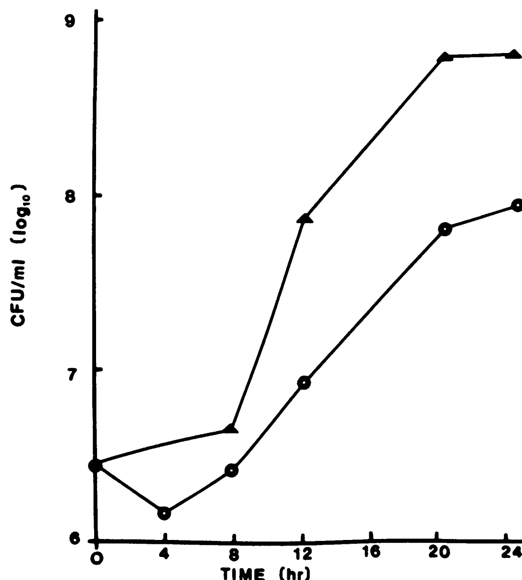

FIG. 3. Growth enhancement of *V. vulnificus* with addition of ether extract containing the hydroxymate siderophore. O, Deferrated medium; ▲, deferrated medium plus ether extract.

(Fig. 4), confirming the ability of this organism to use Desferal to reverse iron limitation.

A study was also made to determine whether enterochelin, a catechol siderophore produced by many enterics, could reverse iron limitation. A 0.1 mM solution of this siderophore did not enhance growth of this organism in the deferrated medium.

DISCUSSION

Siderophores are produced by virtually every aerobic and facultatively anaerobic organism examined. The production of these iron chelators is markedly affected by the amount of iron in the medium. When these siderophores are added to a medium containing low levels of iron, they have been shown to alleviate growth inhibition (18). When stressed for iron, *V. vulnificus* produced a substance which, when added to iron-depleted medium, reversed growth inhibition.

Since biochemical testing of the culture supernatant suggested that the highly virulent *V. vulnificus* C7184 produced both hydroxamate- and catechol-type siderophores, an attempt was made to extract them and to determine whether both were active in iron transport.

To confirm the results of the Arnow test for phenolate-type siderophores, the supernatant was extracted with ethyl acetate, and this extract, when introduced into cultures of the organism in deferrated medium, alleviated growth inhibition within 24 h. Further evidence for the production of a phenolate-type siderophore was shown by the growth stimulation of the mutant *S. typhimurium LT-2-enb-7*. This mutant has been studied extensively by Leong and Neilands (15) and, although the mutant does not produce a phenolate siderophore, it can rapidly take up iron supplied by ferric-catechol complexes.

An analysis of the extract of paper chromatography indicated that the siderophore is distinct from enterochelin, the siderophore produced by many of the enterics. The catechol siderophores of *V. cholerae* (20) and *Bacillus subtilis* (12) also differ from enterochelin in their amino acid composition. Thus, it is possible that the catechol produced by *V. vulnificus* varies in a similar manner.

The results of the Csaky test, suggesting the production of a hydroxamate siderophore, were confirmed by the growth enhancement of the hydroxamate auxotroph *A. flavescens* JG9. This organism has been used extensively to detect hydroxamate siderophores because of its high degree of specificity for these specific iron-binding compounds (7). A chloroform-phenol extract of the culture supernatant also enhanced the growth of *V. vulnificus* in iron-limited medium, further suggesting the presence of a hydroxamate-type siderophore.

The simultaneous production of both pheno-

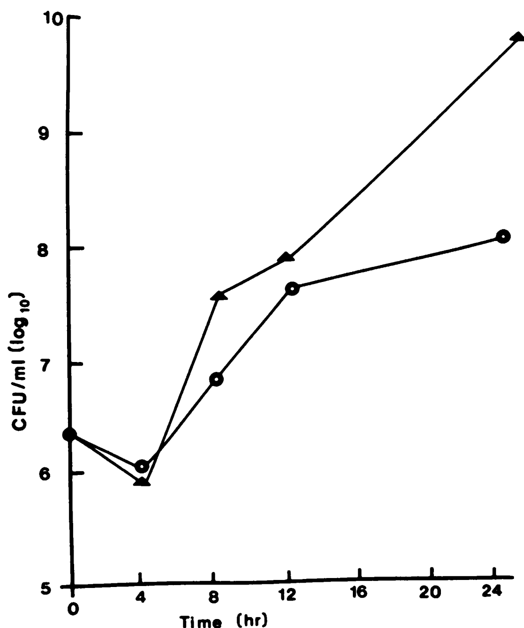

FIG. 4. Reversal of growth inhibition of *V. vulnificus* by addition of Desferal. O, Deferrated medium; ▲, deferrated medium plus Desferal.

late and hydroxamate siderophores has been reported for *Enterobacter aerogenes* (9), *Shigella sonnei* (22), and *Escherichia coli* strains harboring the ColV plasmid (3). In a study of siderophore production by *Enterobacter cloacae* (27), enterochelin was observed to be synthesized at maximal concentrations when cells were placed under mild iron limitation. The production of the hydroxamate aerobactin, however, was highest in medium without iron. This finding is in contrast to a report by Harris et al. (10) that enteric bacteria secrete aerobactin under mild iron stress and produce and excrete enterochelin under extreme stress. Since both strains (C7184 and A1402) of *V. vulnificus* were grown in media with the same iron concentration, neither of these theories explain the lack of production of a phenolate by strain A1402. One possible explanation for the lack of phenolate production by this strain could be that this less virulent strain may have a block in the aromatic pathway that controls the synthesis of catechols. Another explanation could be that the less virulent strain may have more cell-associated iron, which might make it less stressed for iron. This would support the report of Harris et al. (10) that phenolate siderophores are produced only under extreme stress.

Although the Csaky assay for hydroxamates is not strictly quantitative (5), the results indicating that both strains C7184 and A1402 produce hydroxamate-type siderophore suggests that both have an effective means of transporting iron. This possibility would also agree with our observation (unpublished data) which showed that the virulence of A1402 is not enhanced by the injection of iron-containing compounds. Thus, the difference in virulence between the two strains does not appear to involve iron transport.

Of all the vibrios studied to date, only *V. vulnificus* produces both siderophore types. *Vibrio cholerae* produces only a phenolate compound (2). *V. parahaemolyticus* appears to have the capacity to produce both types, but no siderophore is observed unless the catechol pathway is blocked (C. R. Andrus and S. M. Payne, Abstr. Annu. Meet. Am. Soc. Microbiol. 1982, CC I, II, 11, p. 89). Under these conditions, a hydroxamate siderophore is produced.

Some organisms have the capacity to transport not only their own siderophores but also those produced by other organisms. For example, *V. parahaemolyticus* can utilize the phenolate siderophore produced by *V. cholerae* and the hydroxamate siderophore aerobactin (Andrus and Payne, Annu. Meet. ASM 1982, CC I, II, 11). The results of the present study illustrate that, although *V. vulnificus* cannot use enterochelin, the siderophore produced by many of the enterics, it can use at least one other siderophore, Desferal, to alleviate iron limitation. This finding agrees with our previous report (29) showing that the addition of Desferal reversed the bactericidal effects of human serum and decreased the 50% lethal dose of *V. vulnificus* for mice from 6×10^6 to a single cell.

The exact role played by the siderophores produced by the highly virulent strain (C7184) of *V. vulnificus* in the pathogenesis of the disease caused by this organism remains to be determined. Since at least one siderophore has been detected in the less virulent strain, it does not appear that the inability to bind iron can be the sole determinant of virulence in this organism. The inability of the organism to grow in normal serum (29) suggests that the siderophores cannot successfully compete with transferrin when it is not saturated with iron, in contrast to organisms such as *S. paratyphi* B, which can mobilize transferrin-bound iron even when the protein is only partially saturated with iron (26). Payne and Finkelstein (21) have suggested that gram-negative pathogens can be classified according to how they respond to iron. One class they describe includes highly virulent microorganisms such as the invasive strains of *Neisseria gonorrhoea*, *Haemophilus influenzae*, and *Escherichia coli* K1, which can compete for low levels of iron such as those found in normal serum. They describe another class of pathogens whose virulence is proportional to iron availability. The virulence of these organisms is typically inhibited by iron-binding proteins. The amount of siderophore produced appears to be less in these organisms. Clinical and experimental observations suggest that *V. vulnificus* belongs to the latter group, and elevated iron levels may be necessary for this organism to compete with transferrin for iron and thus produce systemic infections.

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