Iron-Regulated Hemolysin Production and Utilization of Heme and Hemoglobin by *Vibrio cholerae*

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El Tor and non-O1 strains of *Vibrio cholerae* were analyzed to determine whether synthesis of secreted hemolysin was influenced by the concentration of iron in the medium. Synthesis of hemolysin was found to be iron regulated in both El Tor and non-O1 isolates. Increased levels of hemolytic activity were detected in supernatants of iron-starved cells. Spontaneous hemolysin-deficient mutants of one non-O1 strain were found to occur at high frequency. These variants also failed to synthesize vibriobactin, the iron transport compound utilized by *V. cholerae*. Another non-O1 strain was found to synthesize both hemolysin and vibriobactin constitutively. When the cloned *Escherichia coli* fur gene, encoded on the plasmid pABN203, was introduced into this constitutive strain, normal iron regulation of both hemolysin and vibriobactin was reestablished. The ability of *V. cholerae* to utilize mammalian iron compounds was determined, and it was found that both hemin and hemoglobin could serve as sole sources of iron.

Many El Tor and non-O1 strains of *Vibrio cholerae*, the causative agent of human cholera, produce a soluble hemolysin (8, 12, 17, 20). This protein is found both in the periplasmic space and in the extracellular medium in hemolysin-producing (Hly*) V. cholerae strains and appears to be actively excreted (8, 13). The El Tor and non-O1 vibrio hemolysins are biologically and immunologically indistinguishable (29, 30), while a second hemolysin, distinct from that of El Tor strains, has been shown recently to be produced by classical V. cholerae strains (18). Hemolytic variation is often seen within pure cultures of El Tor vibrios, and the possible existence of a unique genetic regulatory element, such as a transposable or invertible element, has been suggested as a means of regulating the expression of the *V. cholerae* hemolysin (4, 13).

*V. cholerae* also secretes a high-affinity iron-binding compound, vibriobactin (6, 16). This catechol-type siderophore is produced by *V. cholerae* in response to an environment containing little free iron. In addition to the siderophore, at least five new outer membrane proteins are detected when *V. cholerae* isolates are grown in low-iron medium (23). One of these iron-regulated proteins may serve as the vibriobactin receptor for iron transport in *V. cholerae*.

Hemolysins and siderophores have been implicated as virulence factors in several model systems. They appear to function by increasing the availability of the essential element iron to pathogenic species. Free iron in the host is extremely limited (3, 14). Almost all iron in the body is intracellular, and the small amount of extracellular iron is bound by transferrin or lactoferrin (3, 28). Establishment of bacterial infections involves competition with the host for acquisition of this nutrient (15, 28). Siderophores can directly remove iron from transferrin (9), while hemolysins can increase the level of available iron in the host via lysis of erythrocytes and subsequent release of hemoglobin. Waalwijk and co-workers (27) have shown that hemolysin enhances the virulence of nephropathogenic *Escherichia coli* by providing iron in vivo. Linggood and Ingram reported similar findings for hemolytic *E. coli* strains (11). Siderophore production is regulated by the concentration of iron in the growth medium (14), and in some *E. coli* strains the synthesis of hemolysin is also repressed by high concentrations of iron (10). Recently Gruenig and co-workers demonstrated that the extracellular hemolytic activity encoded on certain *E. coli* hemolysin plasmids is regulated not only by the environmental iron concentration, but also by the chromosomally encoded *E. coli* fur gene product (7).

Much work has been done to elucidate the mechanisms of pathogenesis of *V. cholerae*, but the roles of both vibriobactin production and hemolysin production as virulence factors remain to be determined. Most clinical isolates of El Tor and non-O1 *V. cholerae* are hemolytic. The hemolysin is lethal in mice, increases vascular permeability in rabbits, and lyses erythrocytes of numerous animal species (8, 29). However, identical diseases can be produced by both Hly* and Hly− strains (8; M. M. Levine, J. B. Kaper, J. G. Morris, D. Herrington, and G. Lofonsky, Abstr. 21st Joint Conf. on Cholera, 1985). Similarly, the role of vibriobactin and iron acquisition is unclear. Studies by Sciorotto and Finkelstein (21) demonstrated the expression of iron-regulated outer membrane proteins of *V. cholerae* in vivo, indicating iron limitation in the intestine. However, vibriobactin synthesis or transport mutants of *V. cholerae* have proven to be virulent in the infant mouse model (24). This may indicate that iron is not sufficiently limited to prevent growth and expression of virulence or that mechanisms other than siderophore-mediated iron transport may be used for iron acquisition in vivo. It is possible that either the siderophore or the hemolysin could function in iron acquisition in the absence of the other, or the two may work in concert for efficient iron uptake. Effects of hemolysin, such as cytotoxicity to intestinal epithelial cells, may liberate intracellular iron compounds into the environment to be taken up by the vibrios, or the iron may be scavenged by vibriobactin, thus providing the nutrient to infecting vibrios. If the hemolysin does play a role in iron acquisition, it appears likely that the concentration of iron would influence its expression. In this study, the influence of iron on hemolysin synthesis was investigated. The utilization of both intracellular and extracellular host iron compounds by several *V. cholerae* strains was also determined.

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MATERIALS AND METHODS

Bacterial strains and plasmids. V. cholerae strains Q20523, 123-83, 2690-79, 1182-79, 2076-79, and C4752 (26) were provided by James Oliver, University of North Carolina at Charlotte. Strains Lou15 and CA401 have been described previously (22, 23). EB1 and EB2 are spontaneous hemolysin-deficient (Hly-) mutants of 2076-79 isolated on sheep blood agar. CA4015 is a vibriobactin synthesis mutant (sidA) constructed by Tn5 mutagenesis. All strains were maintained at −80°C in L broth with 20% glycerol. The plasmid pABN203 carrying the E. coli fur gene was provided by J. B. Neilands.

Media and reagents. Strains were grown on L agar or in L broth at 37°C. The iron chelator EDDA (ethylenediaminetri(o-hydroxyphenylacetic acid); Sigma Chemical Co.), deferrated by the method of Rogers (19), was added to L broth or L agar to induce iron limitation. T medium without added iron (25) was used as described previously to assay siderophore production (23). Blood agar used for the detection of hemolysin was 5% washed sheep erythrocytes added to tryptic soy broth (Difco). Hemin, transferrin, lactoferrin, hemoglobin, and ferritin were obtained from Sigma Chemical Co.

Liquid hemolysin assay. Cultures of V. cholerae were grown in 5 ml of L broth or L-EDDA broth to a density of 10⁹/ml. The cells were removed by centrifugation, and 200 μl of neat or diluted supernatant was added to 800 μl of hemolysin assay mixture as described by Mercuro and Manning (13). The assay mixture contained 0.25% washed sheep erythrocytes, 0.02 M K₂HPO₄, 0.06 M NaH₂PO₄ (pH 7.0), and 0.12 M NaCl. The reaction mixtures were incubated at 37°C for 60 min and then centrifuged to remove all unlysed erythrocytes. Absorbance of the supernatant (λ₅₀₀) was measured to determine percent erythrocyte lysis; 100% lysis was defined as A₅₀₀ of 1 ml of assay mix in which all erythrocytes were lysed. Hemolysin assay mix incubated with L broth or L-EDDA broth was used as a negative control.

Hemolysin-deficient mutants. Spontaneous hemolysin-deficient mutants were isolated on blood agar. The frequency of Hly⁺→Hly⁻ mutation and the reversion frequency were determined by suspending single hemolytic or nonhemolytic mutants in 1 ml of saline and diluting and plating directly onto sheep blood agar. The proportion of Hly⁺ and Hly⁻ colonies was determined after 24 h of growth at 37°C. Hly⁻ colonies were passaged by diluting the saline suspensions 1:100 into L broth with or without EDDA. The fully grown cultures were diluted and plated on blood agar, and the proportion of Hly⁺ colonies was determined.

Electroporation of V. cholerae. Cells were grown in L broth to an A₅₅₀ of 0.5. The cells were washed in sucrose electroporation buffer (272 mM sucrose, 7 mM sodium phosphate buffer, pH 7.4, 1 mM MgCl₂) and then suspended in ice-cold sucrose buffer at 1/20 the original volume. Five micromolars of plasmid DNA was added to the cell suspension to give a final volume of 0.8 ml, which was placed in a Gene Pulser (BioRad) cuvette and mixed with the tip of a pipette. Cells plus DNA were allowed to sit on ice for 30 min. Electroporation conditions were 2,000 V at 25-μF capacitance, producing a time constant of 5.2 ms. The cells were returned to ice for 30 min, diluted in 8 volumes of L broth, and incubated at 37°C for 1.5 h. Samples were plated on appropriate antibiotic media and grown at 37°C for 24 to 48 h.

Siderophore assays. Vibriobactin was measured in low-iron T medium supernatants by the Arnow assay for catechols (1) as described previously (23). A bioassay specific for vibriobactin was also used to detect secretion or utilization of the siderophore. L-EDDA agar (250 μg of EDDA per ml) was seeded with 10⁴ indicator organisms per ml and allowed to solidify. Samples (10 μl) of fully grown bacterial cultures were spotted on plate surfaces to test for vibriobactin production and utilization. Plates were incubated at 37°C for 18 h, and producer strains were checked for zones of stimulation of indicator organisms. No growth of the indicator strain was detected in the absence of iron or usable siderophores. To determine the effect of Fur protein on siderophore production, both strains 1182-79 and 1182-79(pABN203) were passaged twice in T medium with no or 10 μM added iron and then analyzed for the production of siderophore. Tetracycline (6 μg/ml) was added to 1182-79(pABN203) cultures to stably maintain the plasmid. After growth for 24 h in second passage, the A₅₅₀ of the cultures was determined and supernatants were assayed for production of vibriobactin by the method of Arnow (1).

Host iron compound utilization assays. Strains were grown to an A₅₅₀ of 0.7, diluted, and inoculated into 20 ml of L-EDDA agar (250 μg/ml) at 10⁴ organisms per ml. Wells were punched in agar plates and filled with 50 μl of the iron compound tested at the concentrations indicated in Table 4. Lactoferrin and transferrin were 100% saturated with FeCl₃·6H₂O. Plates were incubated at 37°C and were examined at 24 and 48 h for growth.

RESULTS

Iron-regulated synthesis of hemolysin. Several El Tor, non-O1, and classical V. cholerae isolates were initially plated on blood agar for detection of hemolysin (Table 1). The classical strain CA401 only produced hemolysin when grown at 30 or 37°C and subsequently shifted to 42°C for several hours. Because V. cholerae hemolysin is excreted externally into the growth medium, El Tor and non-O1 cell-free culture supernatants were also examined for their ability to lyse sheep erythrocytes in a more sensitive liquid assay. Strains which were Hly⁺ on blood agar also lysed sheep erythrocytes in the liquid assay, whereas the classical strains did not (Table 1).

These strains were also analyzed for vibriobactin production, using the Arnow assay for catechols (1). The catechol

<table>
<thead>
<tr>
<th>Strain</th>
<th>Type</th>
<th>Hemolysin synthesis</th>
<th>Vibriobactin synthesis a</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA401</td>
<td>Classical</td>
<td>-(++)</td>
<td>0.225 16</td>
</tr>
<tr>
<td>CA4015</td>
<td>Classical, sidA b</td>
<td>-(++)</td>
<td>0.001 0</td>
</tr>
<tr>
<td>Lou15</td>
<td>El Tor</td>
<td>+</td>
<td>0.435 21</td>
</tr>
<tr>
<td>123-83</td>
<td>El Tor</td>
<td>+</td>
<td>0.389 20</td>
</tr>
<tr>
<td>2690-79</td>
<td>Non-O1</td>
<td>+</td>
<td>0.467 25</td>
</tr>
<tr>
<td>2076-79</td>
<td>Non-O1</td>
<td>+</td>
<td>0.497 24</td>
</tr>
<tr>
<td>Q20523</td>
<td>El Tor</td>
<td>–</td>
<td>0.087 9</td>
</tr>
<tr>
<td>1182-79</td>
<td>Non-O1</td>
<td>+</td>
<td>0.483 26</td>
</tr>
<tr>
<td>C4752</td>
<td>Non-O1</td>
<td>–</td>
<td>0.053 6</td>
</tr>
</tbody>
</table>

a Hemolysin was determined on blood agar and in liquid assay.
b Vibriobactin synthesis was determined chemically by the method of Arnow (2) and by bioassay.

c CA401 produces no hemolysin in liquid assay. Hemolysin was detected on blood agar when isolates were grown at 30 or 37°C and subsequently shifted to 42°C for several hours.
d sidA, Vibriobactin synthesis mutation.
produced by each strain was confirmed to be vibriobactin by a specific bioassay. Both El Tor and non-O1 Hly⁺ V. cholerae strains produced vibriobactin (Table 1). The El Tor strain Q20523 and the non-O1 strain C4752 produced low levels of both vibriobactin and hemolysin as compared to the other strains. This suggested a correlation between the synthesis of hemolysin and vibriobactin. To determine whether hemolysin and vibriobactin might be similarly regulated, cells were grown in L broth with various amounts of EDDA to analyze progressively more iron-starved cells for hemolysin production, using the liquid assay (Fig. 1). Hly⁺ strains showed a marked increase in the amount of hemolysin they produced as the environment in which the cells were growing became more iron limited. One strain, 2076-79, produced greater amounts of hemolysin than the other strains, and supernatants of this strain were diluted 10-fold to measure relative amounts of hemolysin at different EDDA concentrations. Those strains which produced no hemolysin on blood agar also failed to produce hemolysin even at an EDDA concentration of 125 μg/ml (Fig. 1). Surprisingly, strain Lou15 failed to produce detectable hemolysin in L broth with EDDA. When this strain was grown in low-iron minimal medium, however, hemolysin was detected and its synthesis was repressed by iron (Fig. 2). The quantity of vibriobactin synthesized by Lou15 under these same conditions was estimated by the method of Arnow (1) (Fig. 2). Vibriobactin synthesis was also repressed by iron and could not be detected at iron concentrations of 5 μM or higher. It is not clear whether vibriobactin synthesis is repressed at a lower iron concentration than is hemolysin or whether this simply reflects the greater sensitivity of the hemolysin assay.

**Effect of the E. coli fur gene on hemolysin and vibriobactin production in V. cholerae.** To more clearly define the regulation of hemolysin and vibriobactin production in V. cholerae, one wild-type non-O1 strain, 1182-79, was chosen for further analysis. It was observed that 1182-79 constitutively produced hemolysin and vibriobactin at both low (0.2 μM) and high (30.0 μM) iron levels. If hemolysin and vibriobactin production were indeed coordinately regulated by iron in V. cholerae, reestablishing iron-regulated siderophore production would similarly influence hemolysin expression. In E. coli, repression of siderophore synthesis is mediated by the iron-binding protein Fur (2). Because similarities were noted in the activity and expression of the cloned siderophore genes from V. cholerae and those of E. coli (J. Stoebner and S. M. Payne, Abstr. Annu. Meet. Am. Soc. Microbiol. 1988, D182, p. 101), it was anticipated that expression of the Vibrio siderophore would be regulated by a Fur-like repressor.

The E. coli fur gene carried on the plasmid pABN203 (J. B. Neilands) was introduced into strain 1182-79 via electroporation to determine whether Fur might complement the defect in iron regulation of the siderophore system in this strain. The parental 1182-79 produced high levels of siderophore in both iron-depleted and iron-replete conditions, whereas 1182-79(pABN203) produced high levels of siderophore only under conditions of iron starvation (Table 2). Additionally, culture supernatants of cells grown in various concentrations of EDDA were used in bioassays to quantitate amounts of vibriobactin produced. Strain 1182-79 produced similar amounts of vibriobactin at all EDDA concentrations, whereas strain 1182-79(pABN203) produced increasing amounts of vibriobactin as the cells became more iron starved (Fig. 3A). Thus the introduction of the cloned fur gene from E. coli into 1182-79 reestablished normal iron regulation of vibriobactin in this strain.

Liquid hemolysin assays were also performed on the culture supernatants of strains 1182-79 and 1182-79 (pABN203) used in the vibriobactin bioassays. The parental 1182-79 produced similar amounts of hemolysin at all EDDA concentrations tested, whereas 1182-79(pABN203) produced increasing amounts of hemolysin as the cells became more iron starved (Fig. 3B). The expression of fur in 1182-79, therefore, reestablished the iron-regulated expression of both vibriobactin and hemolysin in this strain.

**Isolation of hemolysin-deficient mutants.** Hemolytic variation within cultures of some El Tor V. cholerae has been described (4). Single hemolytic colonies of strain 2076-79 were suspended in saline and plated on blood agar, and approximately 10% of the colonies were found to be nonhemolytic. Two of these spontaneous Hly⁻ mutants, designated EB1 and EB2, were further characterized. Both produced a faint zone of partial alpha-hemolysis on blood agar, but failed to produce the clear beta-hemolysis of the parent strain. The absence of vibriobactin production by the two mutants was observed in bioassays in which the mutants and the parental strain were tested as producer strains (Table 3), suggesting a common regulatory mechanism for the synthe-
sis of both hemolysin and vibriobactin. These mutants were found to revert to Hly<sup>+</sup> at a high frequency. After a single passage in L broth, approximately 1% of the EB1 cells were Hly<sup>+</sup>, while passage in low-iron EDDA broth yielded 2 and 20% Hly<sup>+</sup> colonies on first and second passage, respectively. The frequency of reversion without passage in broth medium was less than 0.1%. The revertants produced wild-type levels of vibriobactin (Table 3), presumably allowing the greater accumulation of revertants in the EDDA broth. The high frequency of mutation and reversion supports the suggestions of other authors (4, 5, 13) of the existence of DNA rearrangements or an invertible element within or controlling the V. cholerae hemolysin genes.

**Utilization of host iron sources.** Hemolytic cytotoxicity to intestinal epithelial cells may play a role in the pathogenesis of V. cholerae by liberating intracellular iron stores for use by the organisms during growth. We therefore tested several V. cholerae strains for their ability to utilize different host iron sources, including intracellular and extracellular iron compounds (Table 4). Most of the strains tested were unable to utilize transferrin or lactoferrin directly as a sole iron source, although growth of the non-O1 strain 2076-79 was stimulated by iron-saturated transferrin at the highest concentration tested (30 μM). Utilization of ferritin appears to be dependent upon siderophore production, since Vib<sup>b</sup>- strains failed to utilize ferritin as a sole iron source.

All strains tested were able to use hemin and hemoglobin as sole iron sources. Furthermore, the utilization of both of these compounds appears to be independent of vibriobactin production. CA4015, a sidA (vibriobactin synthesis) mutant of CA401, utilizes hemin and hemoglobin as well as the wild-type strain. This suggests that in addition to the vibriobactin iron transport system, an alternative mechanism for iron acquisition exists in V. cholerae.

**DISCUSSION**

The data presented show that V. cholerae hemolysin production is regulated by the concentration of iron in the environment. Reducing the availability of iron by addition of the iron chelator EDDA resulted in increased synthesis of hemolysin. Similarly, production of hemolysin in low-iron T medium was repressed by the addition of iron. Hemolysin synthesis has also been reported to be regulated by iron in an E. coli strain (27). However, concentrations of iron greater than 100 μM were required to repress hemolysin synthesis in E. coli, while the V. cholerae hemolysin was repressed at iron levels which also repressed siderophore production (<10 μM).

Hemolysin synthesis may be coordinately regulated with synthesis of the siderophore of V. cholerae, vibriobactin. The production of both vibriobactin and hemolysin was repressed by the addition of iron. Strains Q20523 and C4752, which were deficient in hemolysin synthesis, were also deficient in the production of vibriobactin. In addition, a reversible mutation has been found in strain 2076-79 which controls hemolysin and vibriobactin production. Because interconversion between Hly<sup>+</sup> Vib<sup>b</sup> and Hly<sup>-</sup> Vib<sup>−</sup> phenotypes occurs at a high frequency, a single mutation or switch most likely influences both. This is suggestive of an invertible element acting on these iron-regulated genes.

The establishment of normal iron-regulated siderophore and hemolysin production when the E. coli fur gene was

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**TABLE 3.** Vibriobactin production in V. cholerae 2076-79

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth in low-iron medium</th>
<th>Vibriobactin&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Hemolysin production on blood agar&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Utilization</td>
<td>Synthesis</td>
</tr>
<tr>
<td>2076-79</td>
<td>+</td>
<td>25</td>
<td>26</td>
</tr>
<tr>
<td>EB1</td>
<td>−</td>
<td>26</td>
<td>−</td>
</tr>
<tr>
<td>EB2</td>
<td>−</td>
<td>24</td>
<td>−</td>
</tr>
<tr>
<td>EB1 Hly&lt;sup&gt;+&lt;/sup&gt;</td>
<td>+</td>
<td>26</td>
<td>28</td>
</tr>
</tbody>
</table>

<sup>a</sup> Determined by bioassay; millimeters of zone diameter.

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**TABLE 4.** Utilization of host iron sources by V. cholerae<sup>a</sup>

<table>
<thead>
<tr>
<th>Zone of stimulation (mm) with:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>2076-79</td>
</tr>
<tr>
<td>EB1</td>
</tr>
<tr>
<td>EB2</td>
</tr>
<tr>
<td>Lou15</td>
</tr>
<tr>
<td>Lou1510</td>
</tr>
<tr>
<td>CA401</td>
</tr>
<tr>
<td>CA4015 (sidA)</td>
</tr>
</tbody>
</table>

<sup>a</sup> Diameter of zone stimulation around wells containing iron sources (50 μl) at the following concentrations: FeCl<sub>3</sub> (2.0 μM), ferritin (30 μM), hæmin (8 μM), and iron-saturated transferrin or lactoferrin (30 μM).
introduced into strain 1182-79, a constitutive hemolysin and vibriobactin overproducer, provides further evidence that hemolysin and vibriobactin are coregulated by iron in V. cholerae. The regulation of hemolysin and vibriobactin synthesis in V. cholerae was found to be similar to iron-regulated systems in E. coli, since the E. coli fur gene product controlled the expression of both of these systems in V. cholerae. This suggests that regulation by Fur of various microbial systems which require iron as a corepressor is highly conserved. Additional genetic evidence will be needed, however, to confirm the V. cholerae regulatory mechanisms.

Our results indicate that V. cholerae possesses at least two mechanisms for iron acquisition, either of which could function in vivo. These bacteria possess an efficient siderophore-mediated vibriobactin iron transport system. Additionally, hemin and hemoglobin can be used as iron sources in a vibriobactin-independent manner. Any means of liberating these intracellular heme compounds, therefore, would be advantageous to a growing organism. The cytotoxic hemolysin of V. cholerae could effect release of intracellular heme or ferritin from damaged epithelial cells in the intestine. The bloody diarrhea produced by many non-O1 V. cholerae strains also indicates the presence of hemoglobin which could be utilized directly as an iron source.

It is of interest that the only strain which could utilize transferrin was the non-O1 strain 2076-79. This strain produces large amounts of vibriobactin and is also invasive. Vibriobactin may play a beneficial role in the direct removal of iron from transferrin for non-O1 V. cholerae utilization.

Intestinal pathogens such as V. cholerae must overcome numerous host defenses to establish infection. Withholding of the essential element iron by the host may serve as one of these defense mechanisms. The requirement for iron is low, but it is absolutely necessary for growth and proliferation of V. cholerae. To rely upon only one method of iron acquisition without a “backup” system would therefore seem to put the organism at a major disadvantage in an iron-limited environment such as in the human host. Both hemolysin and vibriobactin production may be beneficial to V. cholerae during establishment of infection by providing cooperative or alternative mechanisms for obtaining iron from the host.

ACKNOWLEDGMENTS

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LITERATURE CITED
