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Iron-Vibriobactin Transport System is Not Required for Virulence of *Vibrio cholerae*

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The possible requirement of a functional siderophore (vibriobactin)-mediated iron transport system in the pathogenicity of *Vibrio cholerae* was determined. Two mutants of *V. cholerae* defective in the iron-vibriobactin transport system were examined for their ability to multiply and elicit diarrhea in infant mice. One mutant, 40130, was unable to synthesize vibriobactin. The second mutant, 1510, was able to synthesize, but not transport, the siderophore. Both mutants retained the ability to multiply and produce disease in the infant mouse, and virulence was indistinguishable from the parent strains. This indicates that a functional iron-vibriobactin transport system is not essential for cholera pathogenesis. These mutants, like the wild-type strains, were found to have a ferric citrate iron uptake system and could utilize citrate or asparagine for growth in low-iron medium. Compounds of this type may increase the availability of iron to *V. cholerae* in the host.

Iron acquisition is a crucial process for many microorganisms. Iron is an essential component of many enzymes and other proteins and is required for growth of virtually all bacteria (9). However, iron is often difficult to obtain since it is highly insoluble in aerobic environments at neutral or alkaline pH (11). Microbial pathogens are further taxed because most of the iron in animal hosts is sequestered in cells or bound to high-affinity iron-binding proteins, such as transferrin in blood and lactoferrin in secretions (5, 22). Many pathogenic bacteria have efficient iron-scavenging systems which allow them to compete with the host for iron (22).

One iron acquisition system found in some pathogens consists of a microbially produced iron-binding compound, or siderophore, and proteins associated with the transport and utilization of chelated iron (11). Siderophores are synthesized by *Escherichia coli* (4, 13), *Shigella* species (10), and *Salmonella typhimurium* (16, 23) among others. *Vibrio cholerae*, the etiological agent of cholera, produces a phenolate-type siderophore, vibriobactin, when grown under iron-limiting conditions (8, 15). In addition, a number of proteins are found in the outer membranes of iron-starved cells of *V. cholerae* which are not detected when the cells are grown with sufficient iron (20).

Possession of a siderophore-mediated iron transport system is related to increased virulence of some bacterial pathogens (22). Iron acquisition appears to be especially important for invasive pathogens (14), but its role in virulence of surface pathogens is less clear. In this study, the role of the siderophore-mediated iron transport system was determined for *V. cholerae*, a noninvasive pathogen (12, 17). In contrast to invasive pathogens, *V. cholerae* appears to cause disease independent of a high-affinity iron transport system. Iron from other sources, such as ferric citrate, may supply sufficient iron to *V. cholerae* in vivo.

MATERIALS AND METHODS

Bacterial strains. Wild-type *V. cholerae* strains used in this study were CA401, a classical strain, and Lou15, an ElTor biotype (3, 19, 20). CA416, a strain with rough lipopolysaccharide, was used as a negative control for virulence assays (19). These strains were stored frozen in 20% glycerol at −70°C and were cultured on meat extract agar (Difco Laboratories) as described previously (20). Isolation and characterization of the iron transport mutants 40130 and 1510 have been described previously (8).

Media. Brain heart infusion (Difco) was used as an iron-sufficient broth. The Tris-buffered medium of Simon and Tessman (21) with various amounts of added iron was used to induce iron limitation. The use of this medium for determining the effects of iron limitation has been described previously (20). This medium was also used to assay utilization of nonsiderophore iron chelates. Compounds such as sodium citrate and asparagine were added to Tris medium in various amounts. Growth was measured spectrophotometrically, and siderophore production was assayed by the method of Arnow (1) as described previously (20).

Determination of virulence. Groups of 10 infant CFW mice (weight, 3.0 ± 0.5 g) were challenged per os with ~5×10^6 cells per mouse, and fluid accumulation (FA) ratios were determined as described by Baselski et al. (2). At 16 to 18 h postchallenge, the animals were sacrificed and weighed, and the individual intestines were removed and weighed. Secretion of fluid into the gut increases the FA ratio, which is defined as gut weight/(body weight − gut weight). The total number of viable vibrios present in the gut was also determined. The entire intestine was homogenized in 1 ml of saline in a Potter-Elvehjem tissue grinder with a Teflon pestle (Wheaton), diluted, and plated on meat extract agar.

RESULTS

The role of a high-affinity iron transport system in pathogenicity of *V. cholerae* was determined by comparing the virulence of iron transport mutants with that of the wild-type parent strain. The isolation and characterization of mutants defective in either synthesis or transport of vibriobactin, the *V. cholerae* siderophore, have been described previously (8). Strain 40130 is unable to synthesize vibriobactin, whereas 1510 synthesizes but fails to transport this compound. Infant mice were challenged per os with these mutants and their virulent parent strains, and FA ratios were determined (Table 1). Doses of ~5×10^6 CFU were used to distinguish between strains that could colonize successfully and those that could not colonize but might elicit diarrhea if adminis-

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TABLE 1. FA ratios in infant mice challenged with 5×10^6 CFU of *V. cholerae* per mouse

Strain	FA ratio[a] (±SEM)
CA401	0.089 ± 0.003
40130[b]	0.085 ± 0.004
Lou15	0.079 ± 0.002
1510[c]	0.079 ± 0.002
CA416[d]	0.057 ± 0.001
None (BHI[e] alone)	0.057 ± 0.002

[a] A value of ≥0.070 indicates positive, and a value of ≤0.064 indicates negative.
[b] Vibriobactin synthesis mutant of CA401.
[c] Vibriobactin transport mutant of Lou15.
[d] Avirulent, rough lipopolysaccharide.
[e] BHI, Brain heart infusion.

tered in much higher doses (10^8 CFU) (2, 19). Additional mice were mock infected (brain heart infusion alone) or challenged with an avirulent strain, CA416, as negative controls. Strain 40130 was fully virulent and elicted diarrhea to the same extent as its parent strain, CA401. The transport mutant, 1510, was also as virulent as its parent. Virulence of the mutants was not due to reversion in vivo. Diluted intestinal homogenates were plated on meat extract agar and on Luria agar containing 75 μg of EDDA [ethylenediamine di-(*O*-hydroxyphenyl acetic acid)] per ml, an iron chelator which permits growth of the wild-type but not the iron transport mutants. No colonies were found on EDDA plates.

To determine whether the mutants were actually multiplying in vivo, viability counts were also performed at the same time the FA ratios were determined (Table 2). These numbers are an underestimate of the total number of vibrios since some cells were lost in the diarrhea fluid. Both mutants appeared to be multiplying normally, and the numbers of cells recovered were similar to those obtained with the wild-type parent strains. This indicates that the mutants are able to multiply in the host in the absence of a virbriobactin-iron transport system.

The virulence of the iron transport mutants suggested that sufficient iron is available in the gut. Alternatively, the vibrios may have mechanisms in addition to their siderophores for acquiring iron in vivo. To investigate the latter possibility, the wild-type strains of *V. cholerae* and the iron transport mutants were examined to determine whether other iron transport systems were present. No evidence was found for any functional high-affinity iron transport system in the mutants. Chemical assays for catechol and hydroxamate siderophores and biological assays for siderophores were negative for strain 40130, and both mutants failed to grow in low-iron Tris medium from small inocula. The ability to use compounds other than siderophores for iron acquisition was also investigated. Since an iron-citrate transport system is present in some gram-negative bacteria (7), citrate was tested for its ability to stimulate growth of both wild-type and mutant vibrios in low-iron medium.

TABLE 2. Multiplication of wild-type and iron transport mutants of *V. cholerae* in infant mouse intestine

Strain	Inoculum size (CFU)	CFU at 15 h
Lou15	7.0×10^6	9.1×10^7
1510	8.0×10^6	3.0×10^8
CA401	6.0×10^6	5.4×10^7
40130	5.0×10^6	3.0×10^7

The classical and ElTor vibrios were found to utilize citrate for iron acquisition. The presence of citrate in amounts up to an optimal 10 mM was found to increase the cell density of wild-type and mutant strains in low-iron medium (Table 3). In addition, the presence of citrate led to an increase in the levels of vibriobactin produced by wild-type *V. cholerae* (Table 3). This was an increase both in the total amount of vibriobactin and in the amount of vibriobactin produced per cell. Citrate was not being utilized as a carbon source, and no growth occurred unless an additional carbon source, such as sucrose or succinate, was present in the medium.

Other compounds tested for stimulation of growth of *V. cholerae* in low-iron medium included aspartic acid, glutamic acid, asparagine, glutamine, and succinate. Asparagine stimulated growth of the wild-type and mutant vibrios (Table 3). Growth and siderophore production of the vibriobactin transport mutant, 1510, was the same with 20 mM asparagine as with 10 mM citrate. Asparagine also stimulated growth of the parent strain Lou15 but had less effect on the production of siderophore by this strain. Similar results were obtained with the classical strain CA401. With the exception of succinate, the other compounds stimulated growth of the wild-type strains in low-iron medium. None of these compounds, however, stimulated growth of the mutants to the levels obtained with asparagine or iron.

DISCUSSION

The ability to efficiently acquire iron from the host appears to be crucial for many pathogens. This is particularly true for invasive pathogens (14), but the role of high-affinity iron acquisition systems in virulence of surface pathogens has been less clear. *V. cholerae* infection of infant mice (2) was chosen as a model system to determine the role of iron transport in the pathogenesis of a noninvasive bacterium, since this model mimics the natural disease in humans. In addition, the high-affinity iron transport system of *V. chol-*

TABLE 3. Growth and siderophore production by *V. cholerae* in T medium with supplements

Strain	Supplement	Growth (A_{625})[a]	Siderophore (A_{515})[b]	Siderophore/growth ratio
Lou15	None	0.27	0.015	0.05
	10 μM iron	1.03	0.005	0.005
	1 mM citrate	0.41	0.04	0.10
	5 mM citrate	0.75	0.11	0.15
	10 mM citrate	1.24	0.19	0.15
	20 mM asparagine	0.73	0.04	0.06
	20 mM aspartic acid	0.64	0.05	0.08
	20 mM glutamine	0.56	0.06	0.11
	20 mM glutamic acid	0.58	0.06	0.10
	20 mM succinate	0.26	0.02	0.07
1510	None	0.14	0.01	0.05
	10 μM iron	1.10	0.01	0.01
	1 mM citrate	0.28	0.04	0.14
	5 mM citrate	0.52	0.07	0.14
	10 mM citrate	0.75	0.09	0.13
	20 mM asparagine	0.76	0.11	0.15

[a] A_{625} of cells in second-passage T medium plus sucrose plus supplement at 24 h.
[b] A_{515} of Arnow assay of supernatant.

erae has been characterized. The siderophore, vibriobactin, has been isolated, and its structure has been previously determined (8). Mutants blocked in either the synthesis or transport of this compound have been previously isolated and characterized (8). These mutants fail to grow in low-iron medium, indicating that other high-affinity iron transport systems are not present in these strains.

Mutants defective in either synthesis or transport of vibriobactin, the *V. cholerae* siderophore, were compared with their parent strains in the infant mouse model. Both mutants multiplied in the mouse and elicited diarrhea to the same extent as the parent. These data indicate that a high-affinity iron transport system is not essential to colonization, multiplication in the host, or production of disease by *V. cholerae*. Previous studies have shown that siderophore production is greatest among environmental isolates of *V. cholerae* (20). It is possible that the vibriobactin-mediated iron transport system plays a greater role in survival of the vibrios outside the host than in vivo.

It is not clear from these data whether the iron transport system is unnecessary because sufficient iron is available in the host or whether alternative methods of iron acquisition are being utilized in vivo. It is possible that compounds such as citrate or asparagine may supply the necessary iron. However, it seems unlikely that compounds of this type could efficiently compete with the high-affinity iron-binding protein lactoferrin found on the mucosal surface of the intestine. It appears more likely that sufficient iron is available to the vibrios at the surface of the intestine. Studies by Sciortino and Finkelstein (18) have shown that iron-regulated outer membrane proteins of *V. cholerae* are synthesized by vibrios in the gut fluids of infected infant rabbits. This would suggest that iron is limiting in vivo. The authors indicate that the in vivo-grown vibrios represent a mixed population of bacteria from the lumen and from the epithelial surfaces, and iron may not be uniformly distributed in these environments. Our preliminary evidence also indicates that vibrios from infected infant mice can be divided into two fractions. Vibrios found in the gut fluid appear to express some iron-regulated proteins, whereas those associated with the mucosal surface do not (unpublished data). Freter et al. (6) have shown that intestinal fluids are not an optimal growth environment. It is possible that the fluids are deficient in iron, and the mucin at the gut surface may provide a sufficient supply of this element. Thus, the ability to synthesize and transport vibriobactin might enhance multiplication within the lumen but would not be required for growth at the epithelial surface and production of disease.

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LITERATURE CITED

1. **Arnow, L. E.** 1937. Colorimetric determination of the components of 3,4-dihydroxyphenylalanine-tyrosine mixtures. J. Biol. Chem. **228**:531–537.
2. **Baselski, V., R. Briggs, and C. Parker.** 1977. Intestinal fluid accumulation induced by oral challenge with *Vibrio cholerae* or cholera toxin in infant mice. Infect. Immun. **15**:704–712.
3. **Blake, P. A., D. T. Allegra, J. D. Snyder, T. J. Barrett, L. McFarland, C. T. Caraway, J. C. Feeley, J. P. Craig, J. V. Lee, N. D. Puhr, and R. A. Feldman.** 1980. Cholera—a possible endemic focus in the United States. New Engl. J. Med. **302**:305–309.
4. **Brot, N., J. Goodwin, and H. Fales.** 1966. In vivo and in vitro formation of 2,3-dihydroxybenzoylserine by *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **25**:454–461.
5. **Bullen, J. J.** 1982. The significance of iron in infection. Rev. Infect. Dis. **3**:1127–1138.
6. **Freter, R., H. L. Smith, Jr., F. J. Sweeney, Jr.** 1961. An evaluation of intestinal fluids in the pathogenesis of cholera. J. Infect. Dis. **109**:35–42.
7. **Frost, G. E., and H. Rosenberg.** 1973. The inducible citrate-dependent iron transport system in *Escherichia coli* K12. Biochim. Biophys. Acta **330**:90–101.
8. **Griffiths, G. L., S. P. Sigel, S. M. Payne, and J. B. Neilands.** 1984. Vibriobactin, a siderophore from *Vibrio cholerae*. J. Biol. Chem. **259**:383–385.
9. **Lankford, C. E.** 1973. Bacterial assimilation of iron. Crit. Rev. Microbiol. **2**:273–331.
10. **Lawlor, K. M., and S. M. Payne.** 1984. Aerobactin genes in *Shigella* spp. J. Bacteriol. **160**:266–272.
11. **Neilands, J. B.** 1981. Iron absorption and transport in microorganisms. Annu. Rev. Nutr. **1**:27–46.
12. **Nelson, E. T., J. D. Clements, and R. A. Finkelstein.** 1976. *Vibrio cholerae* adherence and colonization in experimental cholera: electron microscopic studies. Infect. Immun. **14**:527–547.
13. **O'Brien, I. G., and F. Gibson.** 1970. The structure of enterochelin and related 2,3-dihydroxy-*N*-benzoylserine conjugates from *Escherichia coli*. Biochim. Biophys. Acta **215**:393–402.
14. **Payne, S. M., and R. A. Finkelstein.** 1978. The critical role of iron in host-bacterial interactions. J. Clin. Invest. **61**:1428–1440.
15. **Payne, S. M., and R. A. Finkelstein.** 1978. Siderophore production by *Vibrio cholerae*. Infect. Immun. **20**:310–311.
16. **Pollack, J. R., and J. B. Neilands.** 1970. Enterochelin, an iron transport compound from *Salmonella typhimurium*. Biochem. Biophys. Res. Commun. **38**:989–992.
17. **Schrank, G. D., and W. F. Verwey.** 1976. Distribution of cholera organisms in experimental *Vibrio cholerae* infections: proposed mechanisms of pathogenesis and antibacterial immunity. Infect. Immun. **13**:195–203.
18. **Sciortino, C. V., and R. A. Finkelstein.** 1983. *Vibrio cholerae* expresses iron-regulated outer membrane proteins in vivo. Infect. Immun. **42**:990–996.
19. **Sigel, S. P., S. Lanier, V. S. Baselski, and C. D. Parker.** 1980. In vivo evaluation of pathogenicity of clinical and environmental isolates of *Vibrio cholerae*. Infect. Immun. **28**:681–687.
20. **Sigel, S. P., and S. M. Payne.** 1982. Effect of iron limitation on growth, siderophore production, and expression of outer membrane proteins of *Vibrio cholerae*. J. Bacteriol. **150**:148–155.
21. **Simon, E. H., and I. Tessman.** 1963. Thymidine-requiring mutants of phage T4. Proc. Natl. Acad. Sci. U.S.A. **50**:526–532.
22. **Weinberg, E. D.** 1978. Iron and infection. Microbiol. Rev. **42**:45–66.
23. **Wilkins, T. D., and C. E. Lankford.** 1970. Production by *Salmonella typhimurium* of 2,3-dihydroxybenzoylserine and its stimulation of growth in human serum. J. Infect. Dis. **121**:129–136.