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Comparison of *Vibrio cholerae* O139 with *V. cholerae* O1 Classical and El Tor Biotypes

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***Vibrio cholerae* O139 is a recently identified non-O1 *V. cholerae* strain responsible for outbreaks of epidemic cholera in India, Bangladesh, and Thailand in the past 2 years. Other workers have demonstrated the presence of the cholera toxin genetic element in *V. cholerae* O139, unlike the situation for other non-O1 *V. cholerae* strains. We sought to compare further this strain with strains of *V. cholerae* O1, classical and El Tor biotypes, by classic microbiologic methods, Southern blot analysis for restriction fragment length polymorphisms with probes for iron-regulated genes of *V. cholerae* O1, and comparisons of outer membrane protein profiles. Our results were similar for *V. cholerae* O139 and the El Tor biotype of *V. cholerae* O1, with the exception of the constitutive expression in *V. cholerae* O139 of OmpS, an outer membrane protein that was maltose inducible in comparison strains of *V. cholerae* O1.**

In the past, epidemic cholera has been uniquely associated with infection by *Vibrio cholerae* of the O1 lipopolysaccharide serotype. Strains of *V. cholerae* O1 are further differentiated biochemically into two biotypes, classical and El Tor. The present extensive pandemic of cholera, ongoing since 1961, is due to the El Tor biotype. *V. cholerae* strains of other O serotypes (non-O1 *V. cholerae*) generally cause diarrheal syndromes and extraintestinal infections but not classical cholera. In October 1992, however, an outbreak of epidemic cholera caused by a non-O1 strain of *V. cholerae* began in Madras and subsequently spread throughout India, Thailand, and Bangladesh. The causative organism of this outbreak has been designated *V. cholerae* O139 (or *V. cholerae* Bengal), as it does not agglutinate with O1 antisera or with any of the 137 previously described non-O1 antisera (18). *V. cholerae* O139 is distinguished by a high attack rate in young adults, indicating that there is little immunologic protection against this organism in patients previously exposed to *V. cholerae* O1 (3, 17). The origin of *V. cholerae* O139 and its relationship to *V. cholerae* O1 are unknown.

Previous preliminary investigations showed that *V. cholerae* O139 contains virulence genes of *V. cholerae* O1 not normally found in non-O1 strains, including the genes for cholera toxin, zonula occludens toxin, and the toxin-coregulated pilus (2, 7, 17). In addition, previous preliminary biochemical characterizations suggested that *V. cholerae* O139 is similar to the El Tor biotype of *V. cholerae* O1 and possibly arose from the latter (1, 7). We wished to characterize *V. cholerae* O139 in more detail by using biochemical tests, hemolysis, susceptibility to antibiotics, restriction fragment length polymorphisms (RFLPs) in three previously characterized iron-regulated genes of *V. cholerae* O1, and examination of outer membrane protein profiles to obtain further hints regarding its relationship to *V. cholerae* O1.

The two *V. cholerae* O1 isolates used in this study were 0395 (classical biotype, Ogawa serotype) and C6709 (El Tor biotype, Inaba serotype, isolated from a patient with cholera in Peru). *V. cholerae* O139 isolate MO10, from the Madras, India, outbreak, was provided by R. Bradley Sack via John J. Mekalanos. Classical and El Tor biotypes of *V. cholerae* O1 are most characteristically differentiated by the Voges-Proskauer reaction, hemolysis of sheep blood, and susceptibility to polymyxin B. When tested with an API 20E system strip (bioMerieux Vitek, Inc., Hazelwood, Mo.), *V. cholerae* 0395 repeatedly tested Voges-Proskauer negative, whereas both C6709 and MO10 were Voges-Proskauer positive. Hemolysis by the strains was tested on Trypticase soy agar containing 5% sheep erythrocytes (Becton Dickinson, Cockeysville, Md.); isolate 0395 was nonhemolytic, while both isolates C6709 and MO10 were hemolytic at 24 h. Hemolysis by MO10 was more marked than that by C6709, with hemolysis even around single colonies at the edge of the streak. The susceptibility of each isolate to polymyxin B was tested on Mueller-Hinton agar with antibiotic disks containing 300 IU of polymyxin B (Becton Dickinson) (16). While 0395 was susceptible to polymyxin B (15-mm zone of inhibition), both C6709 and MO10 were fully resistant.

We next explored the possibility of RFLPs in these three isolates by using three previously described iron-regulated genes of *V. cholerae* O1, *irgA* (a virulence gene [6]), *viuA* (the gene for the receptor for the siderophore vibriobactin [4]), and *fur* (an iron regulatory gene [14]). The probes used were each internal to the respective genes and radiolabelled as previously described (4, 6, 14); Southern hybridizations of restriction enzyme-digested chromosomal DNAs were done under high-stringency conditions as previously described (4, 14). Chromosomal DNAs from all three isolates hybridized with the *viuA* and *fur* probes, with no RFLPs seen with three and four different restriction enzymes, respectively (data not shown). Chromosomal DNAs from all three isolates also hybridized with the *irgA* probe (Fig. 1), with RFLPs seen between 0395 and C6709 following digestion with the enzymes *Eco*RV and *Hin*dIII. With both enzymes, isolate MO10 shared identical restriction fragment lengths with the El Tor isolate C6709.

V. cholerae O139 has been reported to be more resistant to

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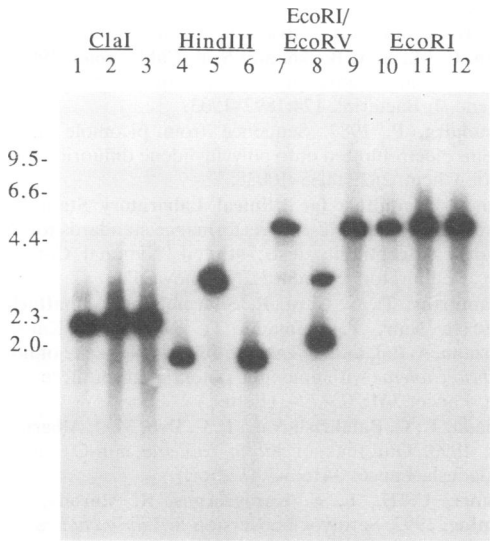

FIG. 1. Southern blot hybridization with a probe internal to *irgA*. Lanes: 1 to 3, chromosomal DNAs from isolates MO10, 0395, and C6709 digested with *Cla*I, respectively; 4 to 6, same isolates digested with *Hin*dIII; 7 to 9, same isolates digested with both *Eco*RI and *Eco*RV; 10 to 12, same isolates digested with *Eco*RI. Numbers to the left of the gel reflect the positions of size markers in kilobase pairs.

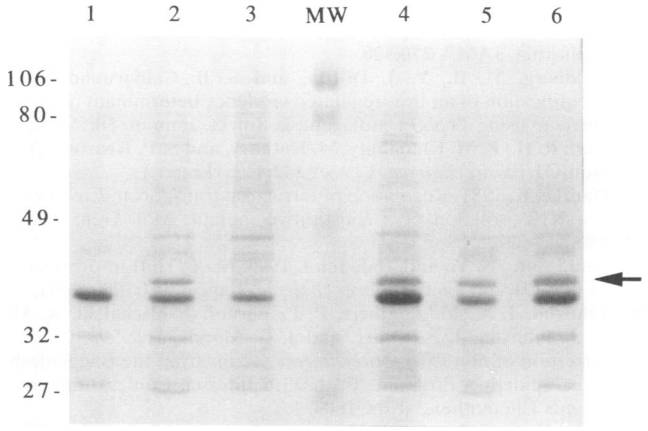

FIG. 2. SDS-PAGE of outer membrane proteins prepared from *V. cholerae* 0395 (lanes 1 and 4), MO10 (lanes 2 and 5), and C6709 (lanes 3 and 6) after growth in media without maltose (lanes 1 to 3) and with 0.4% maltose (lanes 4 to 6). The arrow to the right of the gel identifies OmpS. Numbers to the left of the gel reflect the positions in lane MW of the prestained molecular weight markers, in thousands.

antibiotics than strains of *V. cholerae* O1 (1, 5, 9). We compared the antibiotic susceptibilities of the three isolates on Mueller-Hinton agar by using disk susceptibility techniques (16). On the basis of interpretative criteria for members of the family *Enterobacteriaceae*, both 0395 and C6709 were susceptible to tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, and ciprofloxacin (data not shown). Isolate 0395 was resistant to streptomycin as the result of a laboratory mutation, while isolate C6709 was susceptible. Isolate MO10 retained susceptibility to tetracycline and ciprofloxacin but was resistant to trimethoprim-sulfamethoxazole and streptomycin and tested intermediate for susceptibility to chloramphenicol. All isolates were ampicillin susceptible.

Extracts of outer membrane proteins were made following growth under high- and low-iron conditions as previously described (6, 8) and compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The outer membrane protein profiles under both growth conditions were highly similar (Fig. 2; data for low-iron conditions are not shown), with the exception of a unique protein band slightly larger than 40 kDa in extracts of MO10 (Fig. 2) that was not iron regulated. This protein band was recovered from the gel for amino-terminal protein sequencing (15), and the first 20 amino acid residues were shown to be identical to the sequence (EMBL accession number X69379) of a previously described 43-kDa, maltose-inducible outer membrane protein of *V. cholerae* O1, designated OmpS (12, 13). The growth of isolates 0395 and C6709 in the presence of 0.4% maltose (Fig. 2) showed the induction of a protein of a size identical to that of the protein in MO10.

The O-antigenic specificity of *V. cholerae* is encoded in the *rfb* gene cluster (19). Recently, other investigators showed that eight O139 isolates failed to hybridize with an O1 antigen-specific oligonucleotide probe derived from *rfbS*. This result suggests that O139 isolates lack the O1 antigen gene cluster (10).

One current hypothesis is that *V. cholerae* O139 arose from an El Tor biotype strain of *V. cholerae* O1 by mutation or loss of the O1 antigen gene cluster (1, 7). Most of our results are consistent with this hypothesis, including biochemical testing, hemolysis, and Southern blot analysis for RFLPs in three iron-regulated genes of *V. cholerae* O1. Isolate MO10, however, did constitutively express OmpS, unlike isolates 0395 and C6709. Occasional isolates of both classical and El Tor biotypes which constitutively express OmpS have been described; in addition, this protein has been shown to be induced during in vivo infection (11). The significance of the constitutive expression of OmpS for the pathogenesis of *V. cholerae* O139 infection is currently being examined.

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REFERENCES

1. **Albert, M. J., M. Ansaruzzaman, P. K. Bardhan, A. S. G. Faruque, S. M. Faruque, M. S. Islam, D. Mahalanabis, R. B. Sack, M. A. Salam, A. K. Siddique, M. D. Yunus, and K. Zaman.** 1993. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. Lancet **342:**387–390.
2. **Albert, M. J., A. K. Siddique, M. S. Islam, A. S. G. Faruque, M. Ansaruzzaman, S. M. Faruque, and R. B. Sack.** 1993. Large outbreak of clinical cholera due to *Vibrio cholerae* non-O1 Bangladesh. Lancet **341:**704. (Letter.)
3. **Bhattacharya, M. K., S. K. Bhattacharya, S. Garg, P. K. Saha, D. Dutta, G. Balakrish Nair, B. C. Deb, and K. P. Das.** 1993. Outbreak of *Vibrio cholerae* non-O1 in India and Bangladesh. Lancet **341:**1346–1347. (Letter.)
4. **Butterton, J. R., J. A. Stoebner, S. M. Payne, and S. B. Calderwood.** 1992. Cloning, sequencing, and transcriptional regulation of *viuA*, the gene encoding the ferric vibriobactin receptor of *Vibrio cholerae*. J. Bacteriol. **174:**3729–3738.

5. **Centers for Disease Control.** 1993. Imported cholera associated with a newly described toxigenic *Vibrio cholerae* O139 strain—California. JAMA **270:**428.
6. **Goldberg, M. B., V. J. DiRita, and S. B. Calderwood.** 1990. Identification of an iron-regulated virulence determinant in *Vibrio cholerae* using Tn*phoA* mutagenesis. Infect. Immun. **58:**55–60.
7. **Hall, R. H., F. M. Khambaty, M. Kothary, and S. P. Keasler.** 1993. Non-O1 *Vibrio cholerae*. Lancet **342:**430. (Letter.)
8. **Hantke, K.** 1981. Regulation of ferric iron transport in *Escherichia coli* K12: isolation of a constitutive mutant. Mol. Gen. Genet. **182:**288–292.
9. **Jesudason, M. V., and T. J. John.** 1993. Major shift in prevalence of non-O1 and El Tor *Vibrio cholerae*. Lancet **341:**1090–1091.
10. **Johnson, J. A., M. J. Albert, P. Panigrahi, J. Michalski, A. Ali, R. J. Johnson, J. B. Kaper, and J. G. Morris, Jr.** 1993. Characterization of non-O1 *Vibrio cholerae* strains from the Bangladesh-India epidemic. Program Abstr. 33rd Intersci. Conf. Antimicrob. Agents Chemother., abstr. 1604.
11. **Lang, H. A., G. Jonson, A. M. Svennerholm, and E. T. Palva.** 1988. The maltose-inducible 43 kDa major outer membrane protein in *Vibrio cholerae* is immunogenic and common to different isolates. Microb. Pathog. **5:**169–175.
12. **Lang, H. A., and E. T. Palva.** 1987. A major outer membrane protein in *Vibrio cholerae* is maltose-inducible. Microb. Pathog. **3:**143–147.
13. **Lang, H. A., and E. T. Palva.** Unpublished data.
14. **Litwin, C. M., S. A. Boyko, and S. B. Calderwood.** 1992. Cloning, sequencing, and transcriptional regulation of the *Vibrio cholerae fur* gene. J. Bacteriol. **174:**1897–1903.
15. **Matsudaira, P.** 1987. Sequence from picomole quantities of proteins electroblotted onto polyvinylidene difluoride membranes. J. Biol. Chem. **262:**10035–10038.
16. **National Committee for Clinical Laboratory Standards.** 1990. Approved standard M2-A4. Performance standards for antimicrobial disk susceptibility tests, 4th ed. National Committee for Clinical Laboratory Standards, Villanova, Pa.
17. **Ramamurthy, T., S. Garg, R. Sharma, S. K. Bhattacharya, G. Balakrish Nair, T. Shimada, T. Takeda, T. Karasawa, H. Kurazano, A. Pal, and Y. Takeda.** 1993. Emergence of novel strain of *Vibrio cholerae* with epidemic potential in southern and eastern India. Lancet **341:**703–704. (Letter.)
18. **Shimada, T., G. Balakrish Nair, B. C. Deb, M. J. Albert, and R. B. Sack.** 1993. Outbreak of *Vibrio cholerae* non-O1 in India and Bangladesh. Lancet **341:**1347 (Letter.)
19. **Stoehner, U. H., L. E. Karageorgos, R. Morona, and P. A. Manning.** 1992. Serotype conversion in *Vibrio cholerae* O1. Proc. Natl. Acad. Sci. USA **89:**2566–2570.