BACTERIAL STRUCTURE AND PATHOGENICITY

Screening of *TnphoA* mutants of *Vibrio cholerae* O139 for identification of antigens involved in colonisation

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A new serogroup of *Vibrio cholerae* non-O1, designated as O139, has emerged causing cholera-like disease among adults. Laboratory and field studies clearly show that there is no cross-protection between O1 and O139 pathogenic strains. Since colonisation of the intestine is a most important step in the pathogenesis of cholera caused by O1 strains and colonising antigens are known to be protective, investigation of the colonising antigens of O139 strain was initiated. By *TnphoA* mutagenesis, mutants were generated with insertions in the genome encoding membrane spanning or secretory proteins. Screening of the mutants for adherence to rabbit intestinal surface and colonisation in 5-day-old mice resulted in the identification of mutant clones, which were less adhesive than was the wild-type parent strain and which could not efficiently colonise the gut. Such non-colonising strains were attenuated in virulence. Analysis of the proteins by SDS-PAGE revealed that the non-colonising mutants did not express a 40-kDa outer-membrane protein.

Introduction

In late 1992, a cholera-like disease caused by a new non-O1 organism designated as *Vibrio cholerae* O139 spread across India, Bangladesh [1, 2] and Thailand [3]. The disease caused by O139 strains was reported to be indistinguishable from cholera in clinical features and response to treatment [4]. As most of the affected patients were adults who were not primed previously with the O139 serogroup and prior exposure to *V. cholerae* O1 gave no cross-protection, it may be argued that the O139 strains differ from O1 strains in critical antigens involved in protection and the vaccine being developed against *V. cholerae* O1 is not likely to be effective against *V. cholerae* O139. Hence, the virulence factors of serogroup O139 will have to be investigated before formulating strategies to develop vaccine for the control of cholera caused by O139 strains.

Studies on the pathogenesis of *V. cholerae* O1 suggest that adherence of vibrios to the mucosal cell surface is an important step leading to the colonisation and subsequent production of the cholera toxin that causes

diarrhoea [5, 6]. It is also known that the antigens involved in colonisation induced protection in experimental cholera [7–9]. Therefore, the colonising antigens of the serogroup O139 were investigated. A panel of mutants was generated by *TnphoA* mutagenesis and screened to identify the adhesive and colonising antigens of *V. cholerae* O139.

Materials and methods

Bacteria and media

Bacteria, plasmids and *TnphoA* mutants are described in Table 1. The rifampicin-resistant spontaneous mutant, VO15-rif^r, was isolated by plating 2×10^8 cells of strain VO15 on L-agar plates containing rifampicin 100 µg/ml. Nutrient broth and L-broth (Difco) were routinely used for growing the bacteria and were prepared according to the manufacturer's instructions. Plates were prepared by adding Difco agar 1.2%. L-broth, brain heart infusion broth (BHI) and casamino yeast extract broth (CYE) have been described previously [10]. Bacterial dilutions were made in phosphate-buffered saline (PBS), pH 7.3 [11]. Membrane-filtered solutions of tetracycline (12 mg/L), kanamycin (45 mg/L), gentamicin (30 mg/L), rifampicin (50 mg/L) and 5-bromo,4-chloro,3-indolylphosphate (BCIP, 40 mg/L) were added to the sterilised medium.

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Construction of *TnphoA* mutants

Random insertion of *TnphoA* into the VO15 chromosome was accomplished by plasmid pRT291, a derivative of pRK290, that carries a copy of *TnphoA* [12, 13]. The plasmid was transferred by conjugation to mutant VO15-*rif*^r and transposition was allowed to occur at 37°C for 18 h. Chromosomal inserts of *TnphoA* were obtained by conjugal transfer of incompatible plasmid pPH1JI and simultaneous selection for kanamycin and gentamicin resistance, which allowed selection of pPH1JI, retention of *TnphoA* and elimination of pRT291. As *TnphoA* was transposed from pRT291 to the chromosome, all colonies were rifampicin-, kanamycin- and gentamicin-resistant, and susceptible to tetracycline. VO15 isolates carrying *TnphoA* inserts were screened for by alkaline phosphatase activity on L-agar containing glucose 0.2% and BCIP, at 37°C. Transposon insertions that resulted in the production of fusion proteins directed by signal sequences were detected as blue colonies.

Southern analysis

A 2.65-kb *Bgl*II fragment containing the kanamycin resistance gene of *TnphoA* served as the DNA probe for hybridisation. The probe was labelled with digoxigenin (Boehringer Mannheim Biochemicals) according to the manufacturer's protocol. Briefly, total DNA was extracted from VO15 and *TnphoA* mutants by SDS lysis, phenol-chloroform extraction and ethanol precipitation. DNA was digested overnight with restriction endonuclease *Eco*RV, which does not recognise sequences present within *TnphoA*, and separated by electrophoresis in agarose 0.8%. Southern hybridisation was performed as described previously [10].

Adherence

Adherence of VO15 and *TnphoA* mutants to freshly isolated rabbit intestinal discs was measured as described previously [9]. The adherence index is the number of adherent vibrios expressed as a percentage of the total number of vibrios to which the disc of intact rabbit intestinal mucosa was exposed.

Colonisation

Intestinal colonisation of the vibrios was measured in 5-day-old mice by oral infection with a single strain [9, 11]. Infant mice were fed with (5×10^5)– 10^6 vibrios in 0.1 ml of PBS containing Evan's blue dye. Viable counts of vibrios in the intestine were made after 48 and 72 h. The intestine was dissected out and homogenised in PBS. After suitable dilutions, samples were plated on nutrient agar plates containing rifampicin for VO15 and kanamycin for *TnphoA* mutants. To measure the effect of antibodies, vibrios were opsonised with a sub-agglutinating concentration of antibodies, given orally, and the survival of opsonised vibrios in intestine was measured after 24 h as described previously [9].

Virulence

The virulence of VO15 and *TnphoA* mutant strains was measured in 5-day-old mice. Bacterial dilutions containing 10^7 cells were inoculated orally and mortality was observed up to 72 h.

SDS-PAGE

Cells from a fresh culture were collected and suspended in Tris-Cl buffer (10 mM, pH 8.0). Total protein was estimated with the Folin phenol reagent [14] and analysed on polyacrylamide 4% v/v stacking and 12% v/v separation gels. The sample buffer contained SDS 1%. Proteins to be analysed were mixed with sample buffer [9, 15] and kept in a boiling water bath for 5 min before being loaded on to the gel. Gels were stained with Coomassie Blue 0.1% and destained in acetic acid 10% v/v and methanol 20% v/v.

Immunisation and protection

Swiss albino rabbits weighing 1 kg were immunised subcutaneously with two injections (50 µg each dose) of 40-kDa protein and challenged with 10^4 cells of VO15 in the ileal loop model as described previously [9]. The 40-kDa antigen was obtained by separating the total proteins of VO15 by SDS-PAGE. The 40-kDa protein band was cut out and electroeluted [15]. After dialysis with several changes of Tris-Cl buffer at 4°C, the protein was ready to be used as an immunising agent.

Results

TnphoA mutants

Several epidemic strains of *V. cholerae* O139 isolated from different parts of India were analysed and found to be identical in adherence and colonisation characteristics. The total protein profile in SDS-PAGE was also identical. Hence, one of the strains, VO15, was selected for investigation. *TnphoA* mutagenesis of strain VO15 was designed to effect mutations in secretory or membrane spanning proteins; mutants that had in-frame insertions of *TnphoA* and produced fusion proteins were detected as blue colonies on BCIP plates. About 2% of colonies carrying *TnphoA* inserts were blue. Sixty-three blue colonies were picked and purified by streaking on BCIP plates. Southern analysis of total DNA of several randomly chosen blue colonies digested with *Eco*RV revealed that the mutants described in Table 1 contained a single *TnphoA* insertion in the genome (Southern blot not shown).

Adherence of *TnphoA* mutants

All the 63 mutants were screened for their adherence to freshly isolated rabbit intestinal discs. Three mutants (M-14, M-19 and M-32) were less adhesive than the parent strain VO15 (Table 1). These least adhesive

Table 1. Bacterial strains, plasmids and indices of adherence and colonisation, virulence

Bacteria with relevant markers	Adherence index*	Colonisation index†		Virulence‡
		48 h	72 h	
<i>V. cholerae</i> O1§				
KB207, wild-type El Tor	1.9	10 ²	ND	ND
CD11, non-adhesive mutant	0.02	< 1	ND	ND
<i>V. cholerae</i> O139				
VO15, wild type	5.4	7.0 × 10 ³	ND	6/6
VO15-rif ^r , rifampicin-resistant spontaneous mutant of VO15	5.0	3.3 × 10 ³	ND	6/6
M-14, Tn <i>phoA</i> mutant of VO15-rif ^r	0.95	2.0	3.6	1/6
M-19, Tn <i>phoA</i> mutant of VO15 rif ^r	0.33	1.6	0.6	0/6
M-32, Tn <i>phoA</i> mutant of VO15 rif ^r	0.86	1.4	5.6	1/6
<i>E. coli</i>				
SM10 (pRT291), Tn <i>phoA</i> , Km ^r , Tc ^r				
MM294 (pPH1JI), Gm ^r				

ND, not determined.
*The number of vibrios adherent to the intestinal disc as a percentage of the total number of vibrios to which it was exposed.
†Increase in viable counts of vibrios over viable counts of the inoculum.
‡Number of deaths over the number of mice inoculated.
§Strains described in Jacob *et al.* [9].
||Plasmids described by Singh *et al.* [10].

mutants were selected to examine intestinal colonisation.

Colonisation of the Tn*phoA* mutants

The less adhesive mutants did not colonise the intestine as efficiently as the parent strain. The parent strain VO15 persisted in the gut in high numbers. The viable counts increased 20–100-fold in 24 h. The viable count of the three mutants was several fold less and the colonisation index was very low (Table 1). When strain VO15 was opsonised with rabbit anti-VO15 antibodies, a decrease in viable counts was observed (Table 2), suggesting a role for cell surface antigens in colonisation and its inhibition by antibodies.

Virulence of Tn*phoA* mutants

Virulent strains of *V. cholerae* O1 are lethal in infant mice as a consequence of colonisation of intestine and the ensuing disease. Hence, the virulence of non-colonising mutants was determined in an infant mouse model and deaths were observed up to 72 h and compared with deaths in uninfected control mice and in

mice infected with the parent strain VO15. Whereas all the mice infected with VO15 died, the non-colonising mutants were attenuated in virulence (Table 1).

Analysis of proteins

Strain VO15 and non-colonising mutants were analysed by SDS-PAGE. The total protein profile showed that the mutants do not express a major outer-membrane protein of 40 kDa (Fig. 1). The expression of the 40-kDa protein was examined in different media such as nutrient broth, CYE and BHI, and in different incubation conditions such as growth at 28°C and 37°C with and without aeration. In the parent strain VO15, the 40-kDa protein was enriched in the outer-membrane fraction (data not shown) and was observed irrespective of media composition and growth conditions, whereas the mutants lacked this protein (Fig. 1). This suggested that the integration of Tn*phoA* may have occurred in the structural gene.

Protection

Protection against challenge with strain VO15 in rabbits immunised with 40-kDa protein is shown in Table 3. Control unimmunised rabbits showed no protection. The 40-kDa antigen did not provide good protection but the degree of protection was similar to that given by membrane preparation of VO15.

Discussion

An apparent lack of cross-protection between *V. cholerae* serogroups O1 and O139 [16, 17] means that serogroup O139 has different somatic and other protein antigens that are critical for protection. They need to

Table 2. Inhibition of intestinal colonisation of VO15 by anti-VO15 and anti-40-kDa sera in infant mice

Incubation medium (for 30 min pre-injection)	Viable count in intestine at 24 h (cfu)
PBS	2.4 × 10 ⁸
PBS + normal serum	1.3 × 10 ⁸
PBS + anti-VO15 serum	1.2 × 10 ⁵
PBS + anti-40-kDa serum	1.9 × 10 ⁶

Before oral administration to infant mice, 1.2 × 10⁶ bacteria were incubated at room temperature in PBS or PBS with 10-fold dilutions of the lowest agglutinating titre of the respective serum; 0.1 ml was fed orally to each mouse.

Fig. 1. SDS-PAGE profile of the total proteins of VO15 (lane 1), M-14, M-19 and M-32 (2–4), two *TnphoA* colonising mutants (5, 6); VO15 grown in CYE (7), BHI (8); M-14 grown in CYE (9), BHI (10); M-19 grown in CYE (11), BHI (12); M-32 grown in CYE (13), BHI (14). Pre-stained protein mol.wt markers (Sigma) of 48.5, 36.8 and 26.6 kDa and the 40-kDa protein are indicated.

Table 3. Protection provided by 40-kDa antigen and membrane proteins in rabbit intestinal experiments in which the rabbits were immunised subcutaneously and the challenge strain was VO15

Vaccine	Protection (%)
Control	0
40-kDa antigen	37
Membrane proteins	45

be investigated to develop a vaccine against serogroup O139. Previous experiments with *V. cholerae* O1 suggest clearly that the colonising antigens of O1 strains provide significant protection in experimental cholera [7–10]. Antigens of O139 strains involved in colonisation have not been identified so far. The toxin co-regulated fimbria, an essential colonisation factor of *V. cholerae* O1, has been found in all strains of O139 [18]. Similarly, fimbriae common to O1 and non-O1 strains were found to be present among many strains of O139; but all these studies are rather inconclusive regarding their role in colonisation by O139 strains.

In the past few years, *TnphoA* insertion mutagenesis has been used successfully to characterise genes encoding cell surface and membrane spanning proteins of *V. cholerae* [10, 13, 19]. With the same strategy, a panel of mutants was isolated in which the integration of transposon occurred in-frame in genes encoding cell surface or membrane spanning proteins. When analysed for colonisation, three mutants were identified which did not colonise the gut of the infant mouse as efficiently as did the parent strain VO15. The mutants lacked a protein of *c.* 40-kDa, which is a major outer-membrane protein. Membrane proteins of O139 strains in the same molecular range have been reported previously [20, 21].

That the 40-kDa protein has a role in colonisation and pathogenesis is evident because a single transposon insertion caused loss of expression of this protein associated with reduced colonisation and attenuation of virulence. The purified protein recovered from SDS-PAGE induced protection which was not significant but strikingly identical to the protection given by the membrane protein fraction of strain VO15. Perhaps it might be possible to achieve higher levels of protection by modifications in the dose of the antigen and schedule of immunisation. While the colonising factors of *V. cholerae* O1 play a significant role in protection, the colonising antigen of O139 identified in this work is less effective. Are there other antigens that contribute synergically in colonisation and protection? Unpublished data generated in this laboratory strongly suggest this possibility and that the role of a polysaccharide capsule covering the O139 strains but absent in O1 strains may be important in protection. A good correlation has been found between colonisation and protection in passive immunisation experiments with antibodies to the polysaccharide capsule [22]. Therefore, O139 strains need to be extensively investigated to identify relevant antigens, determine their virulence and to develop strategies for immunoprophylaxis.

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References

1. Shimada T, Nair GB, Deb BC, Albert MJ, Sack RB, Takeda Y. Outbreak of *Vibrio cholerae* non-O1 in India and Bangladesh.

- Lancet* 1993; **341**: 1346–1347.
2. Cholera Working Group. International Centre for Diarrhoeal Diseases Research, Bangladesh. Large epidemic of cholera-like disease in Bangladesh caused by *Vibrio cholerae* O139 synonym Bengal. *Lancet* 1993; **342**: 387–390.
 3. Chongsang-nguan M, Chaicumpa W, Moolasart P *et al.* *Vibrio cholerae* O139 in Bangkok. *Lancet* 1993; **342**: 430–431.
 4. Bhattacharya SK, Bhattacharya MK, Nair GB *et al.* Clinical profile of acute diarrhea cases infected with the new epidemic strain of *Vibrio cholerae* O139: designation of the disease as cholera. *J Infect* 1993; **27**: 11–15.
 5. Nelson ET, Clements JD, Finkelstein RA. *Vibrio cholerae* adherence and colonization in experimental cholera: electron microscopic studies. *Infect Immun* 1976; **14**: 527–547.
 6. Srivastava R, Sinha VB, Srivastava BS. Events in the pathogenesis of experimental cholera: role of bacterial adherence and multiplication. *J Med Microbiol* 1980; **13**: 1–9.
 7. Sharma DP, Thomas C, Hall RH, Levin MM, Attridge SR. Significance of toxin-coregulated pili as protective antigens of *Vibrio cholerae* in the infant mouse model. *Vaccine* 1989; **7**: 451–456.
 8. Sun D, Mekalanos JJ, Taylor RK. Antibodies directed against the toxin-coregulated pilus isolated from *Vibrio cholerae* provide protection in the infant mouse experimental cholera model. *J Infect Dis* 1990; **161**: 1231–1236.
 9. Jacob A, Sinha VB, Sahib MK, Srivastava R, Kaper JB, Srivastava BS. Identification of a 33 kDa antigen associated with an adhesive and colonizing strain of *Vibrio cholerae* El Tor and its role in protection. *Vaccine* 1993; **11**: 376–382.
 10. Singh SN, Srivastava R, Sinha VB, Srivastava BS. A 53 kDa protein of *Vibrio cholerae* classical strain O395 involved in intestinal colonization. *Microb Pathog* 1994; **17**: 69–78.
 11. Kumar KK, Srivastava R, Sinha VB, Michalski J, Kaper JB, Srivastava BS. *recA* mutations reduce adherence and colonization by classical and El Tor strain of *Vibrio cholerae*. *Microbiology* 1994; **140**: 1217–1222.
 12. Manoil C, Beckwith J. *TnphoA*: a transposon probe for export signals. *Proc Natl Acad Sci USA* 1985; **82**: 8129–8133.
 13. Taylor RK, Miller VL, Furlong DB, Mekalanos JJ. The use of *phoA* gene fusions to identify a pilus colonization factor coordinately regulated with cholera toxin. *Proc Natl Acad Sci USA* 1987; **84**: 2833–2837.
 14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265–275.
 15. Hames BD. An introduction to polyacrylamide gel electrophoresis. In: Hames BD, Rickwood D (eds) *Gel electrophoresis of proteins: a practical approach*. London, IRL Press. 1981: 1–86.
 16. Albert MJ, Alam K, Ansaruzzaman M, Qadri F, Sack RB. Lack of cross-protection against diarrhea due to *Vibrio cholerae* O139 (Bengal strain) after oral immunization of rabbits with *Vibrio cholerae* O1 vaccine strain CVD103-HgR. *J Infect Dis* 1994; **169**: 230–231.
 17. Takeda T, Ramamurthy T, Chowdhury AS *et al.* *Vibrio cholerae* O1 vibriocidal and anti-cholera toxin antibodies in O139 Bengal cholera patients. *J Infect* 1994; **29**: 233–235.
 18. Waldor MK, Mekalanos JJ. ToxR regulates virulence gene expression in non-O1 strains of *Vibrio cholerae* that cause epidemic cholera. *Infect Immun* 1994; **62**: 72–78.
 19. Comstock LE, Maneval D, Panigrahi P *et al.* The capsule and O antigen in *Vibrio cholerae* O139 Bengal are associated with a genetic region not present in *Vibrio cholerae* O1. *Infect Immun* 1995; **63**: 317–323.
 20. Calia KE, Murtagh M, Ferraro MJ, Calderwood SB. Comparison of *Vibrio cholerae* O139 with *V. cholerae* O1 classical and El Tor biotypes. *Infect Immun* 1994; **62**: 1504–1506.
 21. Nandy RK, Sengupta TK, Mukhopadhyay S, Ghose AC. A comparative study of the properties of *Vibrio cholerae* O139, O1 and other non-O1 strains. *J Med Microbiol* 1995; **42**: 251–257.
 22. Sengupta DK, Boesman-Finkelstein M, Finkelstein RA. Antibody against the capsule of *Vibrio cholerae* O139 protects against experimental challenge. *Infect Immun* 1996; **64**: 343–345.