Molecular analysis of VcfQ protein involved in *Vibrio cholerae* type IV pilus biogenesis

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The nucleotide sequence of an ORF (*vcfQ*) within the type IV pilus gene cluster of *Vibrio cholerae* O34 strain NAGV14 was determined, thereby completing the sequence analysis of the structural operon. The *vcfQ* gene showed homology to the *mshQ* gene of the mannose-sensitive haemagglutinin pilus gene cluster. The *vcfQ* was 651 bp larger than *mshQ*, and the G+C content of the extra 651 bp portion (35.6 mol%) was lower than that of the overall *vcfQ* gene (42.5 mol%). Except for the first 270 aa residues, the deduced amino acid sequence of VcfQ showed high homology to the MshQ protein. There was immunological cross-reaction between VcfQ and MshQ by Western blotting. Cell fractionation studies showed that VcfQ is located in both the inner and the outer membranes. Mutational analysis showed that *vcfQ*-deficient mutant expressed detectable levels of major pilin (VcfA), but failed to assemble them into pili, indicating that VcfQ is essential for pilus assembly. Colony-blotting analyses showed that the N-terminal region of *vcfQ* is variable in *V. cholerae* strains.

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INTRODUCTION

Vibrio cholerae is known to be the causative agent of the acute gastrointestinal disease cholera. Strains of just two of the approximately 200 currently known O-antigen serogroups, O1 and O139, produce cholera toxin and cause cholera disease symptoms (Faruque *et al.*, 1998; Yamai *et al.*, 1997). However, sporadic cases of gastroenteritis including cholera-like diarrhoea due to organisms with serogroups that are non-O1/non-O139 have been reported (Bhattacharya *et al.*, 1998; Dalsgaard *et al.*, 1999; Sharma *et al.*, 1998). In the process of infection, the initial events are attachment of *V. cholerae* to the intestinal epithelium and colonization of the intestine by the organisms. Therefore, in studies of pathogenic mechanisms and in the efforts to develop vaccines, much attention has been focused on these events.

Type IV pili are flexible appendages which are found in a number of important pathogenic bacteria that affect humans and animals. Expression of type IV pili is associated with

colonization of epithelial cells, twitching motility of the organisms and biofilm formation on a variety of surfaces (Strom & Lory, 1993). Several kinds of type IV pili of *V. cholerae* have been isolated, purified and characterized. The toxin-coregulated pilus (TCP) (Taylor *et al.*, 1987) has been recently identified as a coat protein of a filamentous bacteriophage (Karaolis *et al.*, 1999). Expression of a mannose-sensitive haemagglutinin (MSHA) pilus that promotes adherence to zooplankton and biofilm formation appears to be important for survival in an aquatic environment (Chiavelli *et al.*, 2001; Watnick & Kolter, 1999). The MSHA pilus is encoded in a locus that contains 16 genes organized in secretory and structural operons (Marsh & Taylor, 1999). In the structural operon, five genes encode prepilin-like proteins and two encode putative outer-membrane (OM) proteins (Marsh & Taylor, 1999).

In our previous studies, several kinds of pili of *V. cholerae* were purified and characterized (Iwanaga *et al.*, 1989; Nakasone & Iwanaga, 1990; Yamashiro *et al.*, 1993, 1996). Among them, only the pili isolated from strain NAGV14 adhered to rabbit intestinal epithelium (Yamashiro *et al.*, 1996). The major pilin structural gene of the NAGV14 pilus (*vcfA*) was highly homologous to the MSHA pilin gene in the N-terminal region, but there was no homology in the C-terminal region (Kuroki *et al.*, 2001). A subsequent study demonstrated that NAGV14 pilus gene cluster contained five

Abbreviations: IM, inner membrane; MSHA, mannose-sensitive haemagglutinin; OM, outer membrane.

The GenBank/EMBL/DBJ accession number for the *vcfQ* sequence of *V. cholerae* strain NAGV14 is AB064660.

Figures showing the results of Western blotting analyses are available as supplementary data in JMM Online (<http://jmm.sgmjournals.org>).

ORFs that encoded prepilin-like proteins and corresponded to *mshB*, *A*, *C*, *D*, *O* (Toma *et al.*, 2002). An ORF that encoded a putative OM protein and corresponded to *mshP* (Marsh & Taylor, 1999; Toma *et al.*, 2002) was also demonstrated. Considering this similarity of the both gene cluster organization in the two organisms, an orthologous protein (same functional protein) (Alm *et al.*, 2000) of MshQ might exist in the NAGV14 pilus structural operon. In the present study, the *vcfQ* gene encoding the putative MshQ-orthologous protein was investigated to clarify the gene function and to complete the nucleotide sequence of the NAGV14 pilus structural operon. The distribution of the gene in *V. cholerae* isolates was also studied.

METHODS

Bacterial strains, vector and media. The strains and plasmids used in this study are listed in Table 1. Other strains stocked in our laboratory were also used in colony blotting. The organisms were cultured in Luria–Bertani (LB) broth [1% tryptone (Difco), 0.5% yeast extract (Difco), 0.5% NaCl (nacalai tesque)] or on LB agar plates that were supplemented, as necessary, with 100 µg ampicillin ml⁻¹, 5 µg kanamycin ml⁻¹ and/or 5 or 25 µg chloramphenicol ml⁻¹.

DNA manipulation. Chromosomal DNA was isolated by using QIAGEN Genomic-tip (Qiagen). Plasmid DNA was isolated according to the method described by Birnboim & Doly (1979) or by using Qiagen resin columns. Restriction enzyme digestion, ligation, gel electrophoresis, and transformation of DNA were carried out as described by Bagdasarian & Bagdasarian (1994) or electroporation in a 0.1 cm cuvette, using the Gene Pulser II Electroporation System (Bio-Rad) set at 0.75 kV. PCR products were purified on a GFX column (Amersham).

Sequencing and cloning of *vcfQ*. For sequencing and cloning of *vcfQ*, construction of the primers and PCR were performed as shown in Fig. 1. First, primers *mshQ3* and *mshQ4* were designed on the basis of the *mshQ* sequence previously reported by Marsh & Taylor (1999), and the PCR product was named fragment-1. The nucleotide sequence of this fragment was determined, and new primers, *mshQ10* and *LA3* were constructed. For amplification of fragment-2, fragment-3 and fragment-4, primers DSV11, *mreB*, DSV13 and DSV8 were designed on the basis of previously reported sequences (Marsh & Taylor, 1999; Toma *et al.*, 2001). Fragment-5, which contains the complete *vcfQ* gene, was amplified by using primers DSV13 and *mshQ6* with KOD plus DNA polymerase (TOYOBO). Fragment-5 was cloned into pCR 2.1 vector to obtain plasmid pCTQ20 after addition of 3' A-overhangs which were necessary for TA cloning with *Taq* DNA polymerase (TOYOBO). All primers used in this study are presented in Table 2. Nucleotide sequencing was performed on both strands with Big Dye Terminator Cycle Sequencing FS Ready Reaction kits (Applied Biosystems) and analysed with an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). The program BLAST 1.4.9 was used to search for homologous sequences in the database.

Cloning of *mshQ*. The same protocol used for cloning of *vcfQ* was used to clone the *mshQ* gene from *V. cholerae* O1 strain 86B3. The complete *mshQ* gene was amplified by using primers QCE1 and *mshQ6*. The PCR product was cloned into pCR 2.1 vector and the resulting plasmid (pQ28-4) was used to transform *Escherichia coli* XL-1 Blue. *E. coli* XL-1 Blue harbouring pQ28-4 was used for immunological detection of MshQ.

Cloning the C-terminal region of *vcfQ* into the expression vector. The C-terminal region (*VcfQT*) of *vcfQ* was amplified by PCR using primers *nagQ15* and *nagQ18*. The PCR product was digested with *Bam*HI and *Sma*I and cloned into the corresponding sites of pQE30 vector. The recombinant plasmid (pTM2) was transformed into *E. coli* M15.

Table 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics*	Source or reference
Strains		
<i>Vibrio cholerae</i>		
NAGV14	Serotype O34; NAGV14 pili ⁺ ; environmental isolate	Yamashiro & Iwanaga (1996)
86B3	Serotype O1 Ogawa; Biotype Classical	Nakasone & Iwanaga (1998)
88UDT119	Serotype O34; NAGV14 pili ⁺ ; clinical isolate	Kuroki <i>et al.</i> (2001)
WM4	88UDT119 <i>vcfQ</i> ::pQM14; Cm ^r	This work
50-2	WM4 strain harbouring pCTQ20; Cm ^r ; Ap ^r	This work
<i>Escherichia coli</i>		
XL-1 Blue	<i>recA endA gyrA thi-1 hsdR supE relA lac</i> [F' <i>proAB lacIqZ</i> .M15 Tn10 (Tet ^r)]	Stratagene
M15	<i>lacI</i> Kan ^r on pRPE4; F ⁻ <i>recA</i> ⁺ <i>uvr</i> ⁺ <i>lon</i> ⁺ <i>lac</i>	Qiagen
SM10λpir	<i>thi thr leu tonA lacY supE recA</i> [RP4-2-Tc::Mu] λpirR6K Km ^r	Simon <i>et al.</i> (1983)
Plasmids		
pCR2.1	Cloning vector; Ap ^r ; Km ^r	Invitrogen
pCTQ20	pCR2.1 carrying <i>vcfQ</i> gene	This work
pQ28-4	pCR2.1 carrying <i>mshQ</i> gene	This work
pQE30	Expression vector; Ap ^r	Qiagen
pTM2	pQE30 containing 730 bp <i>Bam</i> HI– <i>Sma</i> I fragment of <i>vcfQ</i>	This work
pKY719	R6k– <i>ori</i> suicide vector; Cm ^r	Whayeb <i>et al.</i> (1996)
pQM14	pKY719 containing 1.5 kb fragment of <i>vcfQ</i> at <i>Sma</i> I site	This work

*Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance; Km^r, kanamycin resistance.

Fig. 1. Sequencing strategy for *vcfQ* gene (black box). Arrows and open boxes below the ORF represent the primers and the fragments (F) amplified by PCR, respectively. Horizontally striped box shows the 3' portion of ORF5 (Toma *et al.*, 2002) and the vertically striped box shows the 5' portion of *mreB*. The two probes used for colony blotting in this study are indicated.

Table 2. Oligonucleotide primers used in PCR, protein expression or probes

All primers were designed in this study. Underlined letters indicate restriction endonuclease sites. F, Forward primer; R, reverse primer.

Primer or probe	Sequence (5'→3')	Positions or locus	Use
mshQ3(F)	TCC <u>GAGCTC</u> GGTGGCTCTCTAGGTTATGC	2510–2529*	Sequencing
mshQ4(R)	CGG <u>GGTACC</u> GGCTGCACCAGAGTGTGTTG	3995–4014*	Sequencing
mshQ10(R)	GACTTGACTGGCATTACGGG	1826–1845	Sequencing
LA3(F)	AGTGATGGTCAATGCCCTGC	2818–2837	Sequencing
DSV11(F)	ATTGAGTCCACGCTTGTTGGC	2294–2313†	Sequencing, probe 1
mreB(R)	GGCGGTACCTAAATCGATCG	5369–5388*	Sequencing
DSV13(F)	CTGGGGGCTTTAGATAAT	2001–2020†	Sequencing
DSV8(R)	GTGTCTAGTTAGCTTGTAAG	1–20	Sequencing
mshQ6(R)	TTAATTCGATTTCGCGCCACC	5142–5161*	Sequencing, probe 2
QCE1(F)	GAGATTGGAGCTTACAACGC	1361–1380*	Cloning
nagQ15(F)	CCG <u>GGATCC</u> GATTCTCTTTGGTCGCCCC	3100–3119	Expression
nagQ18(R)	TCC <u>CCCGGG</u> GTAAGCCTGATATCGCAGG	3817–3836	Expression
PUCF(F)	GAATTCGAGCTCGGTACCC	pKY719 <i>EcoRI</i> site	Mutant confirmation
nagQ7(R)	CAAG <u>GATCC</u> CAACGCCATTCTGTGGAGG	1228–1247	Mutant confirmation
nagQ10(R)	CGTCACTGGATTATTCGCTG	284–303	Probe 1
nagQ11(F)	TTCCACCAATTGATGGAGC	3658–3677	Probe 2

*GenBank accession no. AF079234.

†GenBank accession no. AB049152.

Expression and purification of recombinant His-tagged protein.

VcfQT was purified from *E. coli* M15 harbouring plasmid pTM2 as a (His)₆-tagged recombinant protein. Overnight culture of the recombinant organism was diluted 1:50 in LB medium (containing ampicillin and kanamycin) and induced with 1 mM IPTG. The harvested cells were dissolved in start buffer (8 M urea, 0.1 M NaH₂PO₄, 0.01 M Tris, 10 mM imidazole, pH 8.0). The soluble fraction was collected and purified using a His Trap nickel column (Amersham) using 500 mM imidazole phosphate buffer as the elution buffer.

Antiserum preparation. A rabbit was immunized with 87.5 µg purified VcfQT protein in Freund's complete adjuvant. The animal was boosted three times with the same antigen in Freund's incomplete adjuvant at 2-week intervals.

SDS-PAGE and Western blotting analysis. SDS-PAGE and

Western blotting were carried out according to the methods of Laemmli (1970) and Towbin *et al.* (1979), respectively. Pre-stained molecular markers (New England Biolabs) were used as size standards.

Cellular fractionation. Cellular fractionation was accomplished by a modification of the method of Ramer *et al.* (2002). *V. cholerae* 88UDT119 was cultured in 500 ml LB broth at 37 °C overnight with shaking. The harvested cells were suspended in 8 ml 1 M sucrose in 30 mM Tris/HCl (pH 8.0), to which 80 µl 0.5 M EDTA and 80 µl of a 20 mg ml⁻¹ lysozyme solution were added. The suspension was then incubated on ice for 40 min. MgCl₂ was added to a final concentration of 75 mM. The cells were centrifuged at 15 000 *g* for 30 min and the resulting supernatant was collected as the periplasmic fraction. The cell pellet was resuspended in 8 ml 1 M sucrose-30 mM Tris/HCl (pH 8.0)-75 mM MgCl₂. The resulting suspension was sonicated four times (1 min each) and then subjected to two rounds of freezing and thawing.

The unbroken cells were removed by centrifugation at 5000 *g* for 10 min and supernatant was subjected to further centrifugation at 113 000 *g* for 1 h. The resulting supernatant was termed the cytoplasmic fraction. The pellet was resuspended in 1 ml 10 mM PBS, and then 100 µl 10% Sarkosyl was added. The suspension was treated with a glass homogenizer, and after centrifugation at 113 000 *g* for 1 h, the Sarkosyl-soluble fraction was termed the inner-membrane (IM) fraction. The pellet resuspended in 500 µl PBS was termed the OM fraction.

Construction of *vcfQ* insertion mutant. To confirm that VcfQ is required for type IV pilus biogenesis, the *vcfQ* gene was disrupted by insertion of a suicide plasmid. A 1.5 kb central fragment amplified by PCR with primer *mshQ*3 and *mshQ*4 (fragment-1, Fig. 1) was cloned in the suicide plasmid pKY719 (Whayeb *et al.*, 1996) to obtain plasmid pQM14. Bacterial conjugation was accomplished by mating *E. coli* SM10λ*pir* bearing pQM14 as a donor and *V. cholerae* 88UDT119 as recipient. The *vcfQ* exconjugants were selected on TCBS agar containing 5 µg chloramphenicol ml⁻¹. The *vcfQ* plasmid insertion mutant, WM4, was identified by PCR using primer pUCF, which is specific for the multiple cloning site of pKY719, and primer *nagQ*7, which is specific for sequences upstream of primer *mshQ*3. To complement the *vcfQ* mutation in WM4, pCTQ20, containing *vcfQ*, was electroporated into WM4 to obtain strain 50-2 (Table 1).

Crude type IV pili preparation. Crude NAGV14 pili were prepared as described previously (Nakasone & Iwanaga, 1990).

Colony blotting. A single colony was picked and placed onto a nylon membrane Hybond-N⁺ (Amersham). Alkaline lysis of the cells was performed and then probed with peroxidase-labelled PCR-amplified DNA fragments (Fig. 1). Hybridization was performed overnight at 42 °C, and high-stringency washes were performed before detection with the ECL direct nucleic acid labelling and detection system (Amersham).

RESULTS AND DISCUSSION

Nucleotide sequence analysis

The nucleotide sequences of fragments-1, 2, 3 and 4 (Fig. 1) were determined. To confirm the sequence, fragment-5 (Fig. 1) was cloned and sequenced. The sequence of fragment-5 contained a complete ORF (*vcfQ*) of 4410 bp. The deduced amino acid sequence revealed that *vcfQ* encoded a 1470 aa residue with a calculated molecular mass of 157.9 kDa and an isoelectric point of 4.77. VcfQ is predicted by PSORT analysis (Nakai & Kanehisa, 1991) to be an OM protein with a potential signal sequence of 19 aa.

Homology searches comparing the nucleotide sequence of *vcfQ* with other sequences indicated that portions of *vcfQ* showed homology to those of *mshQ*; however, *vcfQ* was 651 bp larger than *mshQ*. The size difference between *vcfQ* and *mshQ* might be explained by the difference of G+C

content of the extra 651 bp region. The G+C content of *vcfQ* (42.5 mol%) and *mshQ* (45 mol%) are similar; however, the G+C content was lower (35.6 mol%) in the extra region. This may indicate that the region was introduced into the NAGV14 strain from some other organism since different G+C content is a reliable indicator of horizontal transfer (Lawrence & Ochman, 1998). As in *V. cholerae* O1, a portion of the *mreB* gene (Marsh & Taylor, 1999) was also found downstream of *vcfQ*.

BLASTP analysis of the deduced amino acid sequence revealed that the first 270 residues of VcfQ had no similarity with any known protein. However, high homology was detected between the VcfQ (residues 271–1470) and MshQ (residues 43–1253) (61 % identity, 70 % similarity) (Fig. 2). This homology was supported by the immunological cross-reaction found in Western blotting using VcfQ specific antiserum (anti-VcfQ antiserum) and whole-cell lysates of *E. coli* harbouring pCTQ20 or pQ28-4 (see gel in the supplementary data system in JMM Online; <http://jmm.sgmjournals.org>). By determining the *vcfQ* gene nucleotide sequence, we completed the genetic sequence analysis of the NAGV14 pilus structural operon. The *vcfQ* gene was identified as the last gene of the pilus operon.

Localization of VcfQ

Many Gram-negative bacteria express type IV pili on their surface; however, the mechanism of type IV pili biogenesis is poorly understood. The genes required for type IV pili biogenesis encode major pilin, minor pilin(s) and IM or OM proteins (Marsh & Taylor, 1999). The predicted amino acid sequence of *vcfQ* showed that VcfQ might be an OM protein. To investigate this possibility, cell fractions prepared from *V. cholerae* 88UDT119 were analysed by Western blotting with anti-VcfQ antiserum. As shown in the gel in the supplementary data system in JMM Online (<http://jmm.sgmjournals.org>), anti-VcfQ antiserum recognized a 157 kDa VcfQ protein in OM fraction of 88UDT119. The result shows that this protein is OM-associated. However, a detectable level of VcfQ was also found in the IM. Therefore, VcfQ might coalesce into an assembly complex by anchoring to IM and thus may articulate between the proteins in the IM and OM components. The presence of VcfQ in the IM and OM could be explained by disruption of this complex during cell fractionation. In *E. coli* type IV pilus (bundle-forming pilus, BFP), the assembly complex has been reported to consist of IM, OM and periplasmic components. One of

Fig. 2. Comparison of the deduced amino acid sequences of VcfQ and MshQ. Black boxes represent homologous regions of VcfQ and MshQ. White boxes represent variable regions. Numbers show amino acid position relative to the methionine of VcfQ and MshQ taken as 1. Asterisks show signal sequence. The location of recombinant protein VcfQT is indicated.

these components, BfpL, was also shown to be associated with the IM and OM fractions and essential for BFP biogenesis (Ramer *et al.*, 2002).

Phenotype of *vcfQ*-deficient mutant

To assess the role of *vcfQ* in type IV pilus biogenesis, we constructed a *V. cholerae* 88UDT119 strain containing insertion mutation in the *vcfQ* gene (WM4). Western blotting analysis using whole-cell lysates of WM4 with an anti-VcfA antiserum (Toma *et al.*, 2002) revealed that the WM4 did produce normal levels of *vcfA* (see gel in the supplementary data system in JMM Online; <http://jmm.sgmjournals.org>). Multiple bands that appeared at 20, 21 and 22 kDa were likely due to the post-translational modification of pili as previously described by Kuroki *et al.* (2001). The type IV pilus of *Pseudomonas aeruginosa* and *Neisseria meningitidis* have been reported to be post-translationally modified by glycans (Castric, 1995; Power *et al.*, 2000). Despite the presence of pilin, no pilus was detected in the crude pili fraction of WM4. Therefore, WM4 failed to assemble VcfA into pili. The complemented strain, 50-2, produced normal levels of pilin and extracellular pili. These results indicate that VcfQ is essential for pilus assembly but not for expression of VcfA.

Colony-blotting analysis

To investigate the conservation of *vcfQ* and *mshQ* in various strains, 93 strains of *V. cholerae* (Table 3) were tested by colony blotting using probe 1 and probe 2 (Table 2, Fig. 1). The results presented in Table 3 show that only one of the 25 *V. cholerae* O1 strains tested and none of the 18 *V. cholerae* O139 strains tested gave a positive signal with probe 1, which anneals to the N-terminal region of *vcfQ*. For *V. cholerae* non-O1/non-O139, 20 % (10 of 50) of the strains tested gave positive signals with probe 1. In contrast, 92 % (23 of 25) of *V. cholerae* O1 strains, 100 % (18 of 18) of *V. cholerae* O139 strains and 98 % (49 of 50) of *V. cholerae* non-O1/non-O139 strains tested produced strong signals with probe 2, which anneals to the C-terminal region of *vcfQ* and *mshQ*. We previously demonstrated that the NAGV14 pilus and MSHA pilus gene clusters are different in the C-terminal region of the gene (*vcfA*) encoding the major pilin subunit (Kuroki *et al.*, 2001). In this study we elucidated that the N-terminal region of the gene (*vcfQ*) encoding the OM-associated protein is also variable.

Table 3. Conservation of *vcfQ* and *mshQ*

Serogroup	No. strains	No. (%) strains positive with probe:	
		1	2
O1	25	1 (4)	23 (92)
O139	18	0 (0)	18 (100)
Others	50	10 (20)	49 (98)

One possible way for *V. cholerae* to cause epidemic disease is adhesion to the zooplankton using type IV pili and survival in the environment (Chiavelli *et al.*, 2001). *V. cholerae* has to assemble the pili under many environmental conditions. Thus, the molecular variation detected here in the gene encoding an OM protein included in the type IV pilus gene cluster might be required for type IV pilus biogenesis.

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