Filamentous vibriophage fs2 encoding the rstC gene integrates into the same chromosomal region as cholera toxin X

Dong Tu Nguyen1, Binh Minh Nguyen1, Huy Hoang Tran1, Tuan Cuong Ngo1, Thanh Huong Le1, Hoai Thu Nguyen1, M. John Albert2, Mamoru Iwami3 & Masahiko Ehara3

1Department of Bacteriology, National Institute of Hygiene and Epidemiology, Hanoi, Vietnam; 2Department of Microbiology, Faculty of Medicine, Kuwait University, Safat, Kuwait; and 3Department of Bacteriology, Institute of Tropical Medicine, Nagasaki University, Japan

Correspondence: Masahiko Ehara,
Department of Bacteriology, Institute of Tropical Medicine, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan. Tel.: +81 95 849 7832; fax: +81 95 849 7805; e-mail: ehara@net.nagasaki-u.ac.jp

Received 19 December 2007; accepted 15 April 2008.
First published online 23 May 2008.
DOI:10.1111/j.1574-6968.2008.01200.x

Editor: Wolfgang Schumann

Abstract
The genome of the filamentous phage of Vibrio cholerae fs2 was found to contain rstC and rstB1 (truncated) genes downstream of ORF500. att-fs2-dir and att-fs2-rev sequences homologous to that of att-CTXφ were found between orf500 and rstC of the fs2 genome. This prompted us to search for the integration site of fs2 in the genomes of V. cholerae O1 and O139. The genome of fs2 was found to integrate downstream of attRS of the CTXφ phage, which integrated into chromosome I of V. cholerae O1 and O139. When infected with fs2, a fimbriate strain of V. cholerae O1 appeared to reduce fimbrial production in an adult rabbit ileal loop assay.

Introduction
Cholera is a watery diarrheal disease caused by Vibrio cholerae O1 and O139. These two serotypes of V. cholerae are susceptible to infection with filamentous phages fs1 and fs2. The receptors for fs1 and fs2 are type IV fimbriae (Ehara et al., 1997; Shimodori et al., 1997). Type IV fimbriae are known to cause twitching motility for cell-surface translocation (Shi & Sun, 2002). A V. cholerae-specific filamentous phage, VGJφ was shown to cointegrate with the filamentous phage CTXφ at the same attB chromosomal site (Campos et al., 2003). Different filamentous phages seem to integrate into the bacterial dif locus by a general mechanism which requires the host recombinases XerC and XerD (Huber & Waldor, 2002). In toxigenic El Tor and O139 strains of V. cholerae, CTXφ is integrated at the dif site in the bacterial genome with the satellite phage RS1. The genome of RS1 contains genes encoding proteins RstR, RstA, RstB and RstC. However, the classical strains of V. cholerae O1 contain nonfunctional CTXφ prophages lacking the rstC gene (Davis et al., 2000). The RstC protein was shown to increase production of cholera toxin (CT) and phage CTXφ by forming aggregations with RstR (Davis et al., 2002). When we analyzed the DNA sequence of the fs2 genome, we found that att-fs2-dir and att-fs2-rev were similar to att-CTXφ.

Materials and methods
Phage, bacterial strains and media
Filamentous phage fs2 was isolated from a V. cholerae O139, strain MDO14 isolated in India, and fs1 was isolated from a V. cholerae O139, strain AI1855 (Ehara et al., 1997). Bacterial strains used were: V. cholerae O139 strain AI 4450 and strain AI 1845; V. cholerae O1, El Tor biotype strain UDT 109 ctx (−); and V. cholerae O1, classical biotype strain Bgd 17.
Strains of *V. cholerae* O1 and O139 were cultured in Luria–Bertani medium (LB) at 37 °C overnight with shaking, unless indicated otherwise.

**Infection assay (spot assay)**

Strain MDO 14 (fs2 donor) was grown overnight in alkaline tryptone broth (1% Bacto tryptone, 0.5% NaCl, 0.3% yeast extract, 0.2% sodium bicarbonate) with shaking. One milliliter of the culture supernatant was filtered through a 0.22-μm pore-size filter (Sartorius). A small aliquot (0.3 mL) of the receptor strain, which was cultured overnight in LB, was mixed with 3 mL of soft agar (0.8%) and the mixture was overlaid onto a nutrient agar (Pearlcore, Nutrient Agar, Eiken, Tokyo) plate (10 cm in diameter) and kept at room temperature for 15 min until the soft agar solidified. One drop of cell-free fs2 solution (20–25 μL) was spotted onto the soft agar plate and incubated overnight at 37 °C. Turbid plaques contained receptor strains infected with fs2.

**Preparation of bacterial genomic DNA and total RNA**

Total DNA of host bacteria and lysogenic bacteria was extracted by using phenol/chloroform/isoamyl alcohol after digestion with proteinase K, then precipitated with cold ethanol (99.5%) and washed with 70% ethanol. Bacterial DNA thus obtained was suspended in TE (Tris-EDTA) buffer and stored at −20 °C until use. Total bacterial RNA was extracted by using RNA protect™ Bacteria Reagent and RNasy® Mini Kit (QIAGEN Sciences). Reverse transcriptase (RT)-PCR was performed according to the manufacturer’s instructions (Marigen Biosciences, First-strand cDNA Synthesis for Quantitative RT-PCR). For RT-PCR, RstCF and RstCR (see Table 1) were used for detecting *rstC* mRNA.

**PCR analysis and sequencing**

PCR analysis was used to detect the junction between the chromosomal DNA of the host and the integrated phage genome. The primers used in this study are listed in Table 1.

**Table 1.** Sequences of primers used

<table>
<thead>
<tr>
<th>Primer used</th>
<th>Description</th>
<th>Sources or references</th>
</tr>
</thead>
<tbody>
<tr>
<td>RstCF</td>
<td>5′-TTACAGTGATGCTCAGTCA-3′</td>
<td>This study</td>
</tr>
<tr>
<td>RstCR</td>
<td>5′-ATGAGTTGGAAACCTACAC-3′</td>
<td>This study</td>
</tr>
<tr>
<td>TLCF1i</td>
<td>5′-ACCTAGAAGACAAATGTTTCCT-3′</td>
<td>This study</td>
</tr>
<tr>
<td>Rst Cri</td>
<td>5′-CAACTCAAGGCATTGAAGTA-3′</td>
<td>This study</td>
</tr>
<tr>
<td>ORFS500Fii</td>
<td>5′-TGCACATTTGCATGACGC-3′</td>
<td>This study</td>
</tr>
<tr>
<td>RstR-Revi</td>
<td>5′-ACAGTTAGATGAGAAGAGAGCA-3′</td>
<td>This study</td>
</tr>
</tbody>
</table>

PCR analysis with TaKaRa Taq DNA polymerase was performed in 100 μL reaction mixture under the following conditions: initial denaturation at 94 °C for 90 s (one cycle); denaturation at 94 °C for 1 min, annealing at 58 °C for 1 min and extension at 72 °C for 1 min (30 cycles); final extension at 72 °C for 5 min; and then holding at 4 °C. PCR mixtures were analyzed by 3% agarose gel electrophoresis, stained with ethidium bromide and photographed under UV illumination. Each PCR amplicon was gel purified by using QIAEX® II (Qiagen). DNA sequencing of all PCR products was carried out with the *Taq* dye-terminator sequencing kit (Perkin-Elmer) and an automated 373A DNA sequencer (Applied Biosystems).

**Rabbit ileal loop assay**

A rabbit ileal loop assay was conducted as described by De & Chatterjee (1953). A New Zealand White male rabbit (2.5 kg) was fasted for 48 h before surgery and fed only water *ad libitum*. The animal was anesthetized by intramuscular injection of ketamine (35 mg kg⁻¹ of body weight) and xylazine (5 mg kg⁻¹). A laparotomy was performed, and the ileum was washed and ligated with a double ligature. Each loop was 10 cm long and was inoculated with 10⁸ CFU of the challenge strain in phosphate-buffered saline (PBS). The challenge strain was fimbriate Bgd 17 with or without infection with fs2. As a reference, *V. cholerae* O139 strain AI 1845 (nonfimbriate wild-type strain) infected with the filamentous phage fs1 was used. Purified CT (10 μg) and PBS were used as positive and negative controls, respectively. The intestine was returned to the peritoneum, the abdomen was sutured and the animal was returned to the cage. After 8 h, the rabbit was sacrificed by intravenous injection of pentobarbital (100 mg kg⁻¹), and loops were excised. Fluid volume and loop length were measured, and the fluid accumulation ratio was recorded as milliliters per centimeter loop.

**Electron microscopy**

For sample preparation for scanning electron microscopy (SEM), a part of the washed loop was immediately placed in cold 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4), and held overnight at 4 °C. The specimen was postfixed with 2% osmium tetroxide for 1 h, and was then dehydrated through a series of ethanol baths, critical-point dried (Polaron E3000), trimmed, mounted on copper stubs and coated with gold–palladium. The specimen was examined with a JSM 840A SEM (JEOL) operated at 10 kV.

Nucleotide sequences of the junctions *attfs2/Rs1, attfs2/CTX*, *attRs1/fs2* and *attCTX/fs2* have been deposited in the DNA Data Bank of Japan (DDBJ) under accession numbers AB299797, AB299798, AB299799 and AB299800, respectively.
Results and discussion

Sequence re-analysis of fs2 genomic DNA

The genome of fs2 (AB002632) was found to contain \( \text{rstC} \) (8023 nt) and also revealed the presence of two sites homologous to \( \text{att} \) sequences known to function in integrative filamentous phages, such as CTX\( \Phi \) (Huber & Waldor, 2002). The \( \text{att} \)-like sites in fs2 were found to overlap in opposite directions (Fig. 1a and b). The \( \text{att} \)-site in the viral plus strand was designated \( \text{att-}\text{fs2-dir} \), and the opposite site was designated \( \text{att-}\text{fs2-rev} \) as per Campos et al. (2003). Both sites mapped in the intergenic region between ORF500 and RstC different from that of VGJ\( \Phi \). In the case of VGJ\( \Phi \) both sites mapped inside ORF154 (Campos et al., 2003).

Toxigenic strains of \( V. \) cholerae O1, biotype El Tor and \( V. \) cholerae O139 have an adjacent prophage RS1, which contains the \( \text{rstC} \) gene. Although classical strains lack the \( \text{rstC} \) gene, filamentous phage fs2 seems to transfer the \( \text{rstC} \) gene horizontally to the classical vibrios.

RT-PCR to detect the \( \text{rstC} \) gene in a classical strain after infection with fs2

Even the El Tor UDT109 strain lacking the ctx gene and the classical Bgd17 strain were found to have the mRNA for the \( \text{rstC} \) gene when infected with fs2 (Fig. 2). This finding
suggests that the filamentous phage fs2 transferred the rstC gene to a classical strain as well as to an El Tor strain lacking the ctx gene. This finding also suggests that CT production in vivo may far greater than expected, if Vibrio strains were infected with fs2 owing to the presence of rstC on the genome of fs2. The significance of rstC transfer by fs2 indicates an increased ability for CT production by inactivating the RstR repressor protein.

Fig. 4. DNA sequence of the left and right junction regions. (a) The left junction region of strains of AI4450, UDT109 and Bgd17. (b, c) The right junction region of strains of AI4450 and Bgd17, respectively.
Detection of the right and left junction region of integrated fs2 by PCR

Surrounding the att sequences for the toxin-linked cryptic (TLC) element, RS1, CTXφ and rtxA were the target regions to be examined. Each primer was prepared for the region of each junction and the resulting amplicons were compared between fs2 infected cells and fs2 noninfected cells.

Amplicons produced by primers TLCF1i and RstCRi (Fig. 3a) and by ORF500Fii and RstR-Revi (El Tor) or RstR-Ri (classical) primers (Fig. 3b) were sequenced and shown in Fig. 4. The left junction of fs2 locates just downstream of attL of CTXφ.

The boxed region (Fig. 3) shows the integration site of fs2 in El Tor or O139 vibrios.

In the case of a classical strain, fs2 integrates between TLC and CTXφ. Sequencing data of attP and attB for classical, El Tor and O139 strains and of attL and attR for classical, El Tor, O139 strains are shown in Fig. 5. The overlap region (TAAGGCCG) where the cutting and rejoining occur was shifted downstream and was different from that of VGJφ (ATTATGT). This difference suggests an ancestral phage, as suggested by phylogenetic relationships (Faruque et al., 2005).

Effect of fs2 infection on the fimbriate strain of V. cholerae O1 (Bgd17)

Lateral transfer of rstC is one of the major roles of fs2 infection in strains of V. cholerae O1 and O139. Another important role was suggested by the fact that filamentous phages (fs1, fs2, VGJ and KSF) require type IV fimbriae as their receptor.

Therefore, after infection with any of these filamentous phages, Vibrio cells may stop producing type IV fimbriae. Wild-type strains are never isolated as fimbriate phase. We developed the fimbriate Bgd17 strain in 1991 (Ehara et al., 1991). When inoculated in the ileal loop of a rabbit, the surfaces of fimbriate Vibrio cells were shown as irregularly shaped solid masses following critical-point drying. However, when infected with fs2, surfaces of Vibrio cells were relatively smooth (Fig. 6). This finding is consistent with our unpublished data that the hemagglutination activity of fimbriate cells decreased following infection with fs1 or fs2. The reduction in fimbriae associated with fs2 in vivo indicates a reduced colonizing ability on to epithelial cells. We suspect that infection with any of the filamentous phages may cause detachment of colonized vibrios from the surface of the small intestine in vivo. Thus, filamentous phage infection may play a role in phase variation. The fluid accumulation ratio of strains of Bgd17 and Bgd17 infected with fs2 were 1.5 and 1.33, respectively. These values were higher than that (0.85) of V. cholerae O139 strain AI1845 infected with fs1.

Acknowledgements

This study was supported by the Core University Program of Japan Society for the Promotion of Science and by the 21st
Century COE Program of Ministry of Education, Culture, Sports, Science and Technology of Japan.

References


