Two different mechanisms of ampicillin resistance operating in strains of *Vibrio cholerae* O1 independent of resistance genes

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Abstract
Autoagglutinable strains of *Vibrio cholerae* O1 (seven nonfimbriate strains and one fimbriate strain) were transformed to obtain resistance to ampicillin. Two distinct mechanisms were found in these strains. One was operating in nonfimbriate strains by reducing OmpU protein production and the other was operating in a fimbriate strain (Bgd17) by newly overproducing cpxP protein. The twitching motility in the fimbriate Bgd17 strain disappeared depending on the production of cpxP protein, suggesting that fimbriation of *V. cholerae* O1 is controlled by a two-component signal transduction system.

Introduction
Dalsgaard *et al.* (1999) characterized *Vibrio cholerae* O1 isolated in Vietnam from 1979 to 1996 and found that strains isolated after 1990 were resistant to sulfonamide and streptomycin, and harbored class 1 integron containing an *aadA2* gene cassette. In 1996, most strains isolated in this country became resistant to several antibiotics independent of R-plasmid by acquiring an SXT element.

Among the outbreak strains isolated in Vietnam in 1995, there were six rough strains that showed a rugose phenotype with a wrinkled colony morphology. *Vibrio cholerae* O1 can alter its phenotype and reversibly switch from a smooth colony morphology to a rugose colony morphology, which is characterized by an exopolysaccharide matrix, a wrinkled colony morphology, increased biofilm formation and increased survival under specific conditions (Rashid *et al.*, 2003, 2004).

In *V. cholerae* O1, there is another type of rough strain composed of fimbriated bacteria with type IV fimbriae. Fimbriated vibrios show an extremely hydrophobic phenotype different from the rugose phenotype. These types of vibrios are easily autoagglutinated in normal saline and also form biofilms (pellicle). Type IV fimbriae are filamentous appendages expressed by many pathogenic bacteria, including *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*. In *V. cholerae* O1 and O139, type IV fimbriae consist of a homologous polypeptide formed from fimbriillin (pilin), a 17-kDa protein encoded by the *fimA* (*mshA*) gene (Ehara *et al.*, 1994; Jonson *et al.*, 1994). Some type IV fimbriae serve as receptors for filamentous phages (Ehara *et al.*, 1997; Shimodori *et al.*, 1997; Jouravleva *et al.*, 1998) and are thought to play a role in bacterial adherence to epithelial cells and mucosal surfaces. Type IV fimbriae also play important roles in twitching motility or social gliding by the retraction and extension of fimbriae (Shi & Sun, 2002).

Biofilms are surface-attached microbial communities with characteristic architecture and biochemical properties distinct from their free-swimming, planktonic bacteria. One of the best-known of these biofilm-specific properties is the development of antibiotic resistance, which can be up to 1000-fold greater than that of planktonic bacteria (Mah *et al.*, 2003).
For the treatment of cholera, we know clinically that ampicillin is not effective even if strains are susceptible in the laboratory test. However, the mechanisms of ampicillin resistance operating in *V. cholerae* O1 are not well understood. Here, we show two different patterns of resistance to ampicillin in *V. cholerae* O1.

**Materials and methods**

**Bacterial strains**

Six strains (V1–V6) of *V. cholerae* O1 isolated in Vietnam in 1995 and one strain (98-42) isolated in Laos in 1998, together with one fimbriated strain (Bgd17, classical in biotype, Inaba in serotype) (Ehara *et al*., 1991), were selected and studied for any protein expression levels. These strains are rough phenotypes with a rugose morphology, except for the Bgd17 strain, which produces hydrophobic fimbriae.

**Culture conditions**

Vibrio strains were cultured in alkaline tryptone broth (AT broth – 1% Bacto tryptone, 0.5% NaCl, 0.3% yeast extract, 0.2% sodium bicarbonate) at 37°C overnight with shaking, unless otherwise indicated.

**Isolation of strains resistant to ampicillin**

A small aliquot taken from biofilm was transferred to AT broth containing 50 μg mL⁻¹ of ampicillin and cultured overnight at 37°C. Specimens taken from the above culture were streaked on TCBS agar plates containing 100 μg mL⁻¹ of ampicillin and incubated overnight at 37°C. Resistant colonies were stored in AT broth containing 33% glycerol at −80°C.

**Antibiotic susceptibility**

Minimal inhibitory concentrations (MICs) of ampicillin of wild and mutant strains were determined using the broth microdilution method as described by Andrews (2001).

**Purification of periplasmic proteins**

The ampicillin-resistant strain of Bgd17 was cultured in six conical flasks of 5 L, each containing 1 L of AT broth in the presence of 50 μg mL⁻¹ of ampicillin. Whole cells were harvested by centrifugation at 5000 g for 1 h at 4°C and then suspended in Tris–HCl buffer (TB – 20 mM, pH 8.0). Whole cells were broken using a French Press. The cell lysate was centrifuged at 1600 g for 15 min to remove unbroken cells. The resultant supernatant was further centrifuged at 100 000 g for 1 h at 4°C, then treated with 20–60% ammonium sulfate following dialysis in TB. The dialysate was centrifuged briefly to remove insoluble materials. The clarified supernatant was loaded onto an ion-exchange column chromatography using PROTEIN PAK G-DEAE (PREP) (Waters, Nihon Waters Ltd, Japan). Elution was started with a linear gradient of 0–0.5 M NaCl in TB after washing with TB. Each fraction was monitored by a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using 15% gel. Fractions that contained dominant proteins were combined and further fractionated by gel filtration column chromatography using PROTEIN PAK 300(PREP) in the Waters 650E Advanced Protein Purification System.

**N-terminal amino acid determination**

For the determination of the N-terminal amino acid sequence, purified proteins were transblotted onto immobilon membranes (Millipore) after SDS-PAGE. The amino-terminal amino acid sequences were determined using an Applied Biosystems model 470A automated sequencer. Homology search was carried out using BLAST 2 program (NCBI).

**Development of antiserum**

Each band was cut from the gel after SDS-PAGE and homogenized in TB (20 mM, pH 7.4) using a Teflon-coated homogenizer. Equal volumes of antigen solution and complete Freund’s adjuvant (0.5 mL each) were mixed, and rabbits were immunized by injecting intramuscularly (on the thigh) and subcutaneously (on the back). Two boosting doses were given every 2 weeks by substituting an incomplete adjuvant for a complete adjuvant. Whole blood was obtained from the carotid artery 2 weeks after the last injection.

**SDS-PAGE and Western blot**

SDS-PAGE was performed according to the system of Laemmli (1970), while Western blotting was performed using the techniques of Towbin *et al.* (1979).

**Detection of twitching motility (the toothpick method)**

After drying the surface of 1% agar medium (Bacto tryptone 1%, yeast extract 0.3%, NaCl 0.5%, agar 1%, pH 7.4) at 37°C for 10 min, vibrio strains taken from an overnight culture on a BTB agar plate were inoculated vertically with a toothpick to the bottom of the plate. The twitching motility was observed between the bottom of the plate and agar.
Results

Characterization of rugose phenotype

All the rugose phenotypes of the strains tested showed reversion on the edges of colonies when cultured for 3 days at 37°C (data not shown). These rugose strains formed visible aggregates when liquid cultures were kept without shaking at room temperature. On the other hand, the fimbriate Bgd17 strain did not show reversion when kept at 37°C for 3 days.

MIC

MIC of ampicillin of each parent strain tested was 4 μg mL⁻¹, and after transformation MIC of each strain showed over 65 μg mL⁻¹.

SDS-PAGE analysis of the resistant strains

The OmpU protein production was decreased markedly among wild strains (V4, V6, 98-42), when they were transformed to ampicillin-resistant strains (Fig. 1). On the other hand, when the Bgd17 fimbriate strain was transformed, the production of OmpU protein was not decreased markedly; a new protein with a molecular weight of 16 kDa was overproduced.

Dose response of OmpU and the 16-kDa protein production depending on the ampicillin concentration

Once an El Tor wild strain (V4) was transformed into a resistant strain, the production of OmpU protein was decreased independent of the presence or absence of ampicillin (Fig. 2a). When the classical Bgd17 strain was transformed to a resistant strain, it produced a new protein of 16 kDa (estimated value) molecular weight at the ampicillin concentration of 40–60 μg mL⁻¹ (Fig. 2b).

Purification of the 16-kDa protein

When whole cells were broken using a French Press, the 16-kDa protein remained in the supernatant after 1 h of ultracentrifugation at 100 000 g. The 16-kDa protein was purified by ion-exchange chromatography and gel-filtration chromatography (Fig. 3).

N-terminal amino acid determination

The N-terminal amino acid sequences of the four proteins (as shown in Fig. 4) were YGGHGWDKEG (32 kDa), MVLVGRKAPD (22 kDa), ADYVIDTKGA (18 kDa) and YGGHGWDKEG (16 kDa). The amino acid residue is expressed in a single letter. The 32-kDa protein was found as a dimer (oxidized form) of the 16-kDa protein (periplasmic protein, cpxP). The 22-kDa protein was an antioxidant, AhpC/Tsa family (alkylhydroperoxidoreductase). The 18-kDa protein was a conserved hypothetical protein (inner membrane protein, YceI like).

Western blot analysis of ampicillin-resistant strains

Three wild strains and one fimbriate strain were analyzed by Western blotting. When reacted with anti-cpxP antiserum, only the resistant strain of Bgd17 (fimbriate) was shown to produce cpxP protein (Fig. 5a). When wild strains were transformed to ampicillin-resistant ones, the production of the OmpU protein was decreased in a dose–response manner (Fig. 5b). In the case of the fimbriate Bgd17 strain, the production of the cpxP protein was initiated when transformed to resistance even in the absence of ampicillin, and increased in a dose–response manner (Fig. 5c).

When reacted with each antiserum, the YceI-like protein was also detected (Fig. 5a–c). This YceI-like protein was not detected in a Western blot when the antioxidized form of cpxP protein antiserum was used (data not shown).
Inhibition of twitching motility by the cpxP protein

The twitching motility of the fimbriate Bgd17 strain was completely suppressed when the strain was transformed under the stress of ampicillin to produce the cpxP protein (Fig. 6).

Discussion

Among the isolates of V. cholerae O1 in Vietnam in 1995, we found six rough strains exhibiting rugose colonies. A fimbriate Bgd17 strain was developed earlier. These two types of V. cholerae O1 are known to easily become resistant to antibiotics. We observed natural reversion of rugose 

Fig. 2. (a) SDS-PAGE analysis of an El Tor, rough strain (V4) resistant to ampicillin. (b) SDS-PAGE analysis of a fimbriate strain (Bgd17) resistant to ampicillin. Sample preparation was the same as in Fig. 1. Vibrio strains were cultured in the presence of ampicillin at the indicated concentration.

Fig. 3. SDS-PAGE analysis of the fractions eluted by PROTEIN PAK G-DEAE (ion-exchange chromatography).
Fig. 4. SDS-PAGE profile showing 16, 18, 22 and 32 kDa proteins for the determination of the N-terminal amino acid sequence. Samples separated by ion-exchange and gel filtration column chromatography were loaded into duplicate wells.

Fig. 5. (a) Western blot analysis of whole cells of *Vibrio cholerae* O1 sensitive (S) or resistant (R) to ampicillin. SDS-PAGE profile is shown in Fig. 1. Anti-cpxP antiserum was used. (b) Western blot analysis of an El Tor, rough strain (V4) resistant to ampicillin. Anti-OmpU antiserum was used. SDS-PAGE profile is shown in Fig. 2a. (c) Western blot analysis of the fimbriate strain (Bgd17) resistant to ampicillin. Anti-cpxP antiserum was used. SDS-PAGE profile is shown in Fig. 2b.
colonies to smooth ones. Here we showed two distinct mechanisms operating in V. cholerae O1 strains. One is shown in El Tor strains, which exhibit rugose colonies. The production of OmpU protein (outer membrane protein, porin) was markedly suppressed when transformed to be resistant to ampicillin. The other one is shown in the fimbriate Bgd17 strain. When the fimbriate strain was transformed under the stress of ampicillin, a new protein (cpxP) was produced even in the absence of ampicillin. This transformed under the stress of ampicillin, a new protein fimbriate Bgd17 strain. When the fimbriate strain was resistant to ampicillin. The other one is shown in the porin) was markedly suppressed when transformed to be production of OmpU protein (outer membrane protein, shown in El Tor strains, which exhibit rugose colonies. The V. cholerae mechanisms operating in organism such as a fimbriate strain of V. cholerae O1 under an oxidative stress environment, because the cpxP protein is the oxidative stress-combative protein (Danese & Silhavy, 1998).

Expression of the cpxP protein in V. cholerae O1 strain led to the suppression of twitching motility. The Cpx two-component signal transduction system controls a stress response and is activated by misfolded proteins in the periplasm. Cpx also controls genes necessary for pilus biogenesis (pap pili) (Hung et al., 2001).

Under the oxidative stress condition, strains of V. cholerae cannot colonize onto the upper small intestine due to diminished twitching motility. Thus, vomiting at the early stage of cholera may facilitate colonization of vibrios by releasing the oxidative stress.

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References


