

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

Authors: Nguyen Dong Tu^{1,2}, Ngo Tuan Cuong¹, Tran Huy Hoang¹, Nguyen Thi Phuong Lan³,
Nguyen Binh Minh¹, Kouichi Morita² & Masahiko Ehara^{4*},

Addresses:

¹ Department of Bacteriology, National Institute of Hygiene and
Epidemiology, 1 Yersin St., Hanoi 10000, Vietnam.

² Department of Virology, Institute of Tropical medicine, nagasaki
University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.

³ Department of Microbiology and Immunology, Pasteur Institute HCMC
167 Pasteur St., District 3 Ho Chi Minh, Vietnam.

⁴ Department of Bacteriology, Institute of Tropical medicine, nagasaki
University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.

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*Correspondence: Masahiko Ehara, Department of Bacteriology, Institute of Tropical Medicine,
Nagasaki University, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.

Tel: +81 95 819 7832; fax: +81 95 819 7805; e-mail: ehara@nagasaki-u.ac.jp

Abstract: Auto-agglutinable strains of *Vibrio cholerae* O1 (7 non-fimbriate strains and 1 fimbriate strain) were transformed to resistance to ampicillin. Two distinct mechanisms were found in these strains. One was operating in non-fimbriate 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 *Vibrio cholerae* O1 is controlled by a two component signal transduction system.

Introduction: Dalsgaard et al.(1999) characterized *V. 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 SXT element. Among outbreak strains isolated in Vietnam in 1995, there were 6 rough strains which showed a rugose phenotype with 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, wrinkled colony morphology, increased biofilm formation and increased survival under specific conditions (Rashid, M. H., *et al.*, 2003, 2004).

In *Vibrio cholerae* O1, there is another type of rough strain composed of fimbriated bacteria with type IV fimbriae. Fimbriated vibrios show extremely hydrophobic phenotype different from rugose phenotype. This type of vibrios are easily auto-agglutinated in normal saline and also form biofilm (pellicle). Type IV fimbriae are filamentous appendages expressed by many pathogenic bacteria, including *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae*. In *Vibrio cholerae* O1 and O139, type IV fimbriae consist of a homologous polypeptide formed from fimbrillin (pilin), a 17-kDa protein encoded by the *fimA* (*mshA*) gene (Ehara, M. *et al.*, 1994, Jonson, G., *et al*, 1994). Some type IV fimbriae serve as receptors for filamentous phages (Ehara, M, *et al.*, 1997, Shimodori, S., *et al.*, 1997, Jouravleva, E.T., *et al.*, 1998) and are thought to play a role in bacterial adherence to epithelial cells and mucosal surfaces. Type IV fimbriae play also important roles in twitching motility or social gliding by the retraction and extension of fimbriae (Shi, W. and Sun, H., 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 antibiotics resistance that can be up to 1,000-fold greater than planktonic bacteria (Mah, T-F., *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 mechanism 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 *Vibrio 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, M., *et al.*, 1991) were selected and studied for any protein expression levels. These strains are rough phenotypes with rugose morphology except for 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 aliquote 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 of ampicillin and incubated overnight at 37°C. Resistant colonies were stored in AT broth containing 33% glycerol at -80°C.

Antibiotic susceptibility. MICs of ampicillin of wild and mutant strains were determined by broth microdilution method as described by Andrews, J. M. (2001).

Purification of periplasmic proteins. The ampicillin resistant strain of Bgd17 was cultured in 6 conical flasks (for 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 5,000g for 1 h at 4°C, then suspended in Tris-HCl buffer (TB, 20 mM, pH8.0). Whole cells were broken by French Press. The cell-lysate was centrifuged at 1,600g for 15 min to remove unbroken cells. The resultant supernatant was further centrifuged at 100,000g for 1h 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 0-0.5M NaCl in TB after washing with TB. Each fraction was monitored by a sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) using 15% gel. Fractions which contain dominant proteins were combined and further fractionated by a gel filtration column chromatography using PROTEIN PAK 300(PREP) in Waters 650E Advanced Protein Purification System.

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

Development of antiserum. Each band was cut from the gel after SDS-PAGE and homogenized in Tris-HCl buffer (20 mM, pH 7.4) using Teflon-coated homogenizer. Equal volumes of antigen solution and complete Freund's adjuvant (0.5 ml each) were mixed and rabbits immunized by injecting intramuscularly (on the thigh) and subcutaneously (on the back). Two boosting doses were given every

two weeks by substituting incomplete adjuvant for complete adjuvant. Whole blood was obtained from carotid artery two 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 by the techniques of Towbin *et al.*(1979).

Detection of twitching motility (toothpick method). After drying the surface of 1% agarmedium (Bactotryptone 1%, yeast extract 0.3%, NaCl 0.5%, agar 1%, pH 7.4) at 37°C for 10 min, vibrio strains taken from over-night culture on 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 phenotype of 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 otherhand, the fimbriate Bgd 17 strain did not show reversion when kept 37°C for 3 days.

Minimum inhibitory concentration (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 resistant strains. The OmpU protein production was decreased markedly among wild strains (V4, V6, 98-42), when they were transformed to resistant strains to ampicillin (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 over-produced.

Dose-response of OmpU and 16 kDa protein production depending on Amp 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 Amp (Fig. 2a). When the classical Bgd17 strain was transformed to a resistant strain, it produced a new protein with 16 kDa (estimated value) molecular weight at the Amp concentration of 40-60 µg ml⁻¹ (Fig. 2b).

Purification of the 16kDa protein. When whole cells were broken by French-Press, the 16 kDa protein remained in the supernatant after 1 hour ultracentrifugation at 100,000g. The 16 kDa protein was purified by an ion-exchange chromatography and a 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), YGGHGWDKEG (16 kDa). The amino acid residue is expressed in a sigle letter. The 32 kDa protein was found as a dimmer (oxidized form) of 16 kDa protein (periplasmic protein, cpxP). The 22 kDa protein was an antioxidant, AhpC/Tsa family (alkylhydrogenperoxidoreductase). 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 blot. 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 Amp resistance, the production of OmpU protein was decreased in a dose-response manner (Fig.5b). In case of fimbriate Bgd17 strain, the production of 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 Yce I like protein was also detected (Fig 5a,5b and 5c). This Yce I like protein was not detected in a Western blot when anti-oxidized form of cpxP protein antiserum was used (data not shown).

Inhibition of twitching motility by cpxP protein. The twitching motility of fimbriate Bgd17

strain was completely suppressed when the strain was transformed under the stress of ampicillin to produce the cpxP protein (Fig6).

Discussion

Among the isolates of *V. cholerae* O1 in Vietnam in 1995, we found 6 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 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 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 cpxP protein was first demonstrated in *V. cholerae* O1 strain. OmpU and cpxP (reduced form) proteins seemed to form a complex with 18 kDa inner membrane protein (Yce I like protein), because antisera against OmpU and cpxP (reduced form) reacted with Yce I like protein, although the antiserum against cpxP (oxidized form) could not react with Yce I like protein.. This suggests that reduced form of cpxP protein is active to form complex with Yce I like inner membrane protein. Furthermore, these findings suggest that a three proteins complex such as Yce I like (inner membrane protein), cpxP (periplasmic protein) and OmpU (outer membrane protein, porin) contribute to survival of the organism such as a fimbriate strain of *V. cholerae* O1 under oxidative stress environment, because cpxP protein is the oxidative stress-combative protein (Danese PN & Silhavy TJ, 1998)

Expression of 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 DL, *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 early stage of cholera may facilitate colonization of vibrios by releasing the oxidative stress.

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Legend to figures

Fig. 1 SDS-PAGE analysis of whole cells of *Vibrio cholerae* O1, sensitive (S) or resistant (R) to ampicillin.

Each strain was cultured in a test tube containing 2 ml of AT broth with 100 μgml^{-1} of ampicillin or without ampicillin at 37°C overnight. Whole cells were harvested by centrifugation and suspended in 30 μl of TB (20 mM, pH7.4). Each sample was mixed with 6 μl of 6x sample buffer and heated for 7 min at 95°C. Fifteen μl of the mixture was loaded into the well.

Fig. 2a. SDS-PAGE analysis of an El Tor, rough strain (V4) resistant to ampicillin.

Fig. 2b. SDS-PAGE analysis of a fimbriate strain (Bgd17) resistant to ampicillin.

Sample preparation was same as 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 kDa, 18 kDa, 22 kDa, and 32 kDa proteins for the determination of N-terminal amino acid sequence.

Samples separated by ion-exchange and a gel filtration column chromatography were loaded into duplicate wells.

Fig. 5a. Western blot analysis of whole cells of *V. cholerae* O1 sensitive (S) or resistant (R) to ampicillin. SDS-PAGE profile is shown in Fig. 1. Anti-cpxP antiserum was used.

Fig. 5b. 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.

Fig. 5c. Western blot analysis of the fimbriate strain (Bgd17) resistant to ampicillin.

Anti-cpxP antiserum was used. SDS-PAGE profile is shown in Fig. 2b.

Fig. 6. Effect of cpxP protein on the twitching motility of the fimbriate Bgd17 strain.

Fig. 2a.

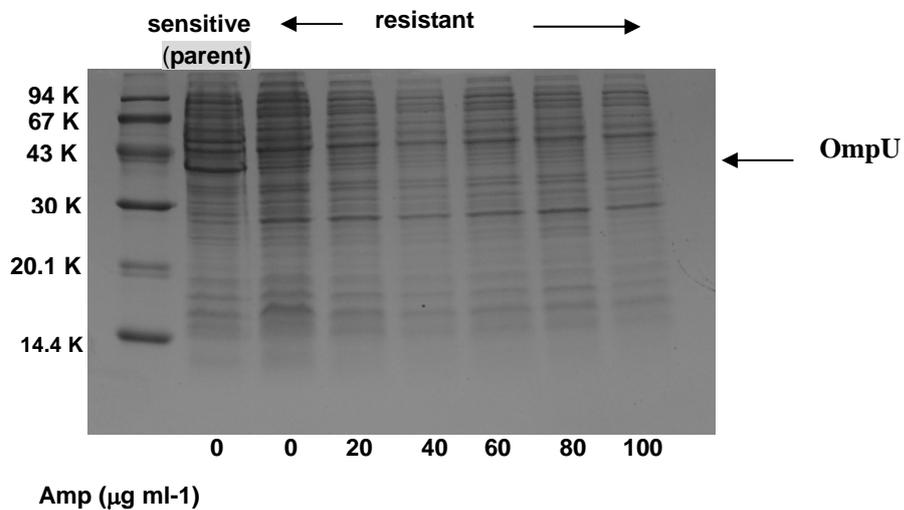


Fig. 2b.

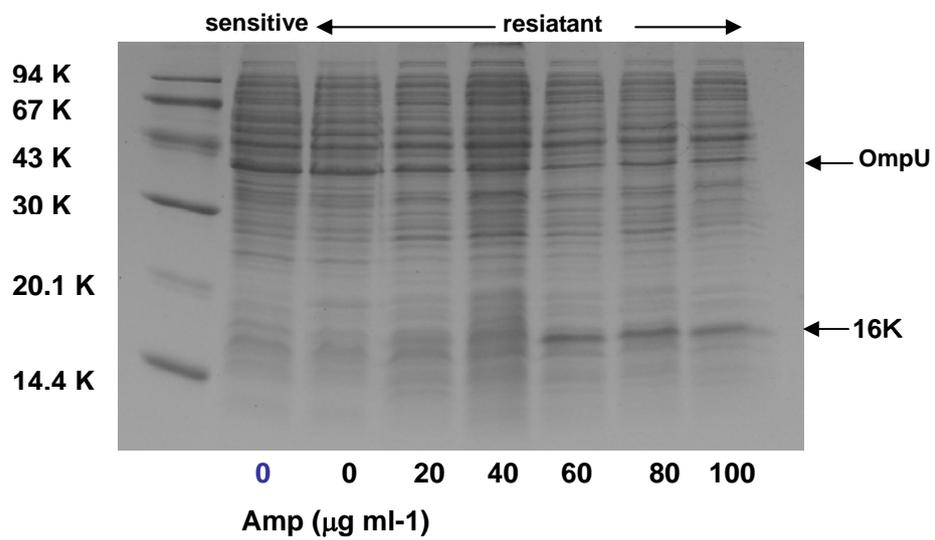


Fig. 3.

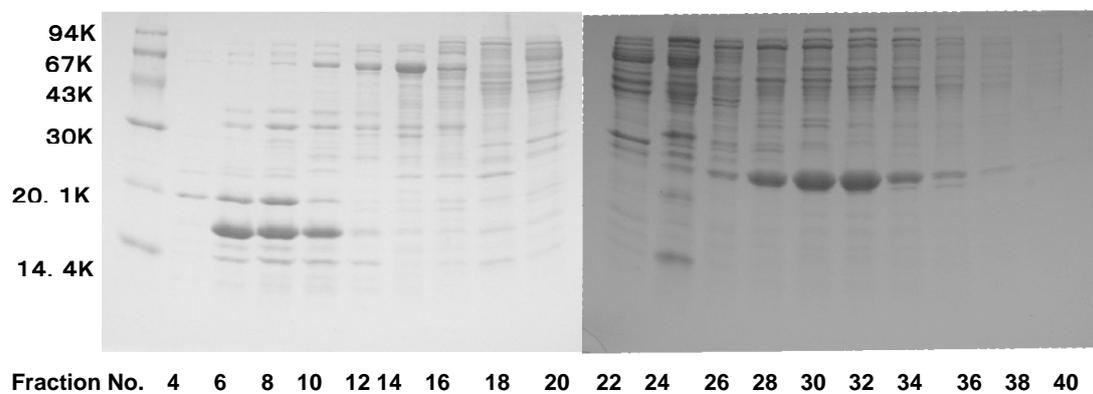


Fig.4.

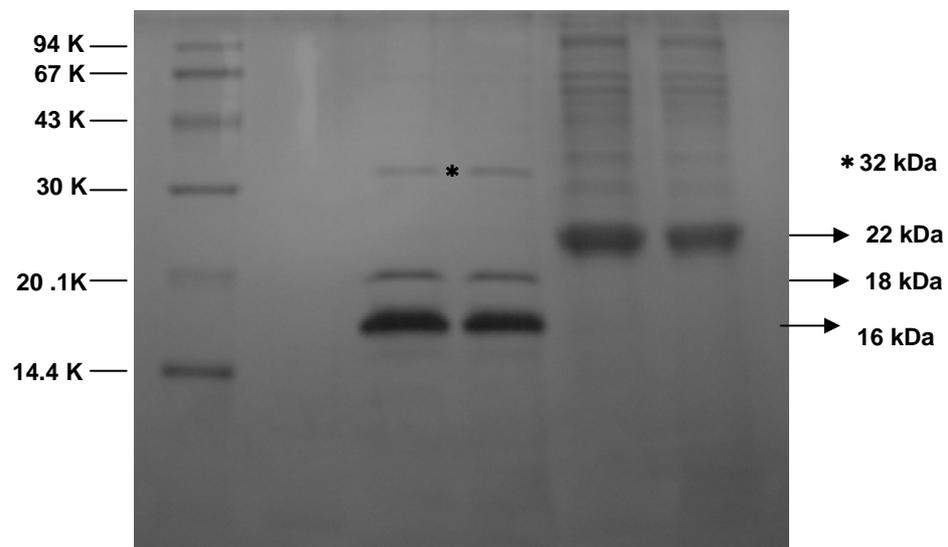


Fig. 5a.

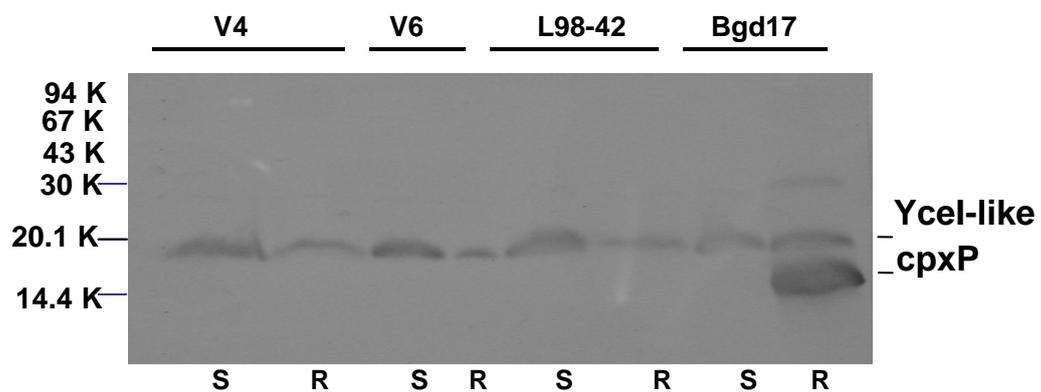


Fig. 5b.

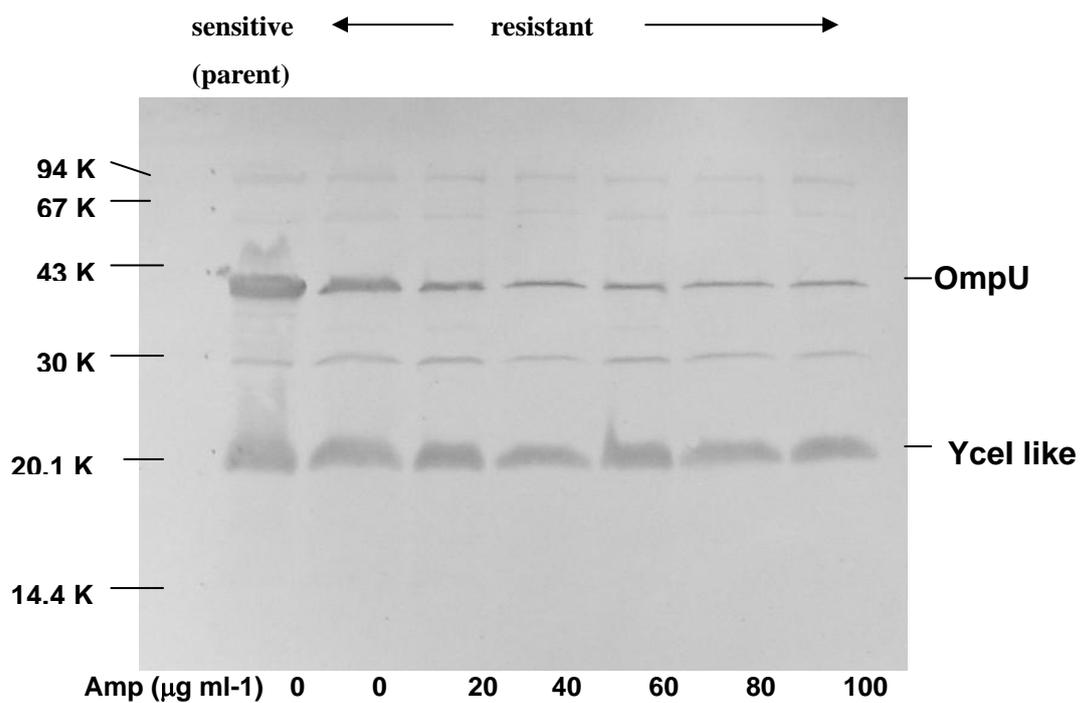


Fig. 5c.

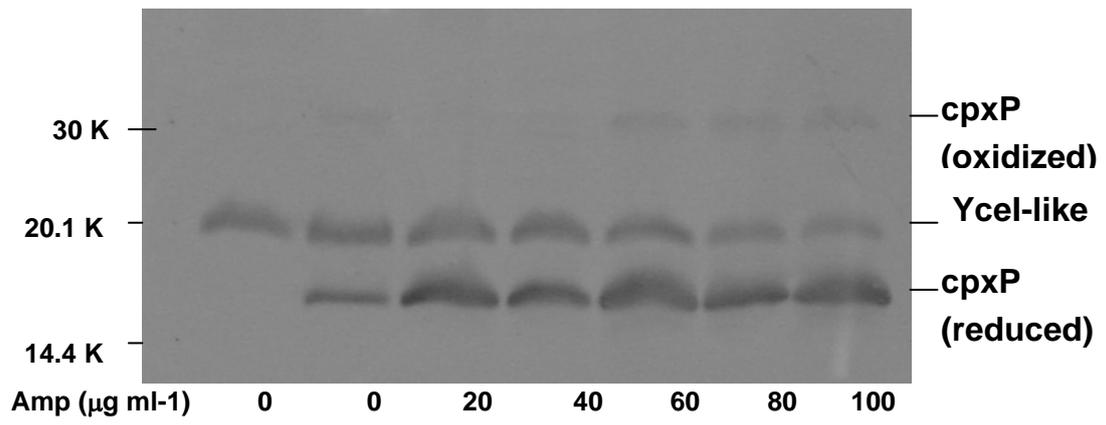


Fig. 6.

