The Journal of Microbiology, June 2007, p. 193-198
Vol. 45, No. 3

pVC, a Small Cryptic Plasmid from the Environmental Isolate of *Vibrio cholerae* MP-1

Ruifu Zhang[1], Yanling Wang[1], Pak Chow Leung[2], and Ji-Dong Gu[1,3]*

[1]*Laboratory of Environmental Microbiology and Toxicology, Department of Ecology & Biodiversity, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, P. R. China*
[2]*Biomolecular Interaction Laboratory, Department of Zoology, The University of Hong Kong, Pokfulam Road, Hong Kong SAR, P. R. China*
[3] *The Swire Institute of Marine Science, The University of Hong Kong, Shek O, Cape d'Aguilar, Hong Kong SAR, P. R. China*

(Received August 26, 2006 / Accepted April 14, 2007)

A marine bacterium was isolated from Mai Po Nature Reserve of Hong Kong and identified as *Vibrio cholerae* MP-1. It contains a small plasmid designated as pVC of 3.8 kb. Four open reading frames (ORFs) are identified on the plasmid, but none of them shows homology to any known protein. Database search indicated that a 440 bp fragment is 96% identical to a fragment found in a small plasmid of another *V. cholerae*. Further experiments demonstrated that a 2.3 kb *Eco*RI fragment containing the complete ORF1, partial ORF4 and their intergenic region could self-replicate. Additional analyses revealed that sequence upstream of ORF1 showed the features characteristic of theta type replicons. Protein encoded by ORF1 has two characteristic motifs existed in most replication initiator proteins (Rep): the leucine zipper (LZ) motif located at the N-terminal region and the alpha helix-turn-alpha helix motif (HTH) located at the C-terminal end. The results suggest that pVC replicates via the theta type mechanism and is likely a novel type of theta replicon.

Keywords: *Vibrio cholerae*, plasmid, horizontal gene transfer, replicon

Vibrio cholerae, the causative agent of disease cholera, is one of the most notorious species in the genus of *Vibrio*. Since its first identification in 1854, extensive studies have been conducted on the ecology, pathogenesis, antibiotic resistance and CTX-phage of *V. cholerae* (Islam *et al.*, 1996; Said and Drasar, 1996; Faruque *et al.*, 1998; Salyers *et al.*, 2002). Plasmids, like phages, have often been found to encode virulence factors that play critical roles in the evolution of pathogens. In *V. cholerae*, studies have suggested that the presence of the conjugative P plasmid, which was not widely disseminated among clinical isolates, significantly attenuated pathogenicity due to plasmid-induced loss of virulence (Sinha and Srivastava, 1978). Cook *et al.* (1984) investigated plasmid profiles of many clinical isolates of classical biotype *V. cholerae* strains and found that classical strains possessed two plasmids (the smaller one was about 4.7 kb). Bartowsky and Manning(1988) found three plasmids in the *V. cholerae* classical strain V58, the P plasmid, a large cryptic plasmid (lcp, 34 kb), and a small cryptic plasmid (scp, 4.7 kb). However, little is known about these cryptic plasmids. Rubin *et al.* (1998) sequenced a 4.7 kb toxin-linked plasmid, which was identical to the small plasmid in the above mentioned two studies. This plasmid was designated as pTLC (toxin-linked cryptic). pTLC can exist as both extra- chromosomal double-stranded circular plasmid and tandemly duplicated DNA, inserted on the chromosome at a position only 842 bp upstream from the CTX prophage. This plasmid may play a crucial role in the acquisition and replication of CTX prophage.

Though much of the research is primarily carried out on *V. cholerae* of clinical sources, environmental isolates may serve as an important reservoir for the wide spreading of antibiotic resistance or virulence genes due to horizontal gene transfer and gene cassette capture (Faruque *et al.*, 1998; Chiang and Mekalanos, 1999). Plasmids and other mobile genetic elements (MGEs) such as prophages, integrons, genomic islands and transposons play vital and essential roles in the gene transfer. The high incidence of plasmid isolated from marine bacterial community in sediment suggests that marine environment is an important source for discovery of novel naturally occurring plasmids (Sobecky, 1999; Sobecky, 2002; Zhang *et al.*, 2006). To our knowledge, very limited information is available on the naturally occurring plasmid from the environmental isolates of *V. cholerae*. In this study, we sequenced and characterized a small cryptic plasmid, which was stably maintained in the *V. cholerae* MP-1 strain isolated from water sample of Mai Po Nature Reserve in Hong Kong (Wang *et al.*, 2004; Wang and Gu, 2005; Wang *et al.*, 2006).

Materials and Methods

Water sampling and isolation of plasmid-containing Vibrio species

Water samples were collected from the Mai Po Nature Reserve (22°29′N to 22°31′N and 113°59′E to 114°03′E) of Hong Kong. Surface water was taken in 1 L plastic bottles when the tidal level was at approximately 1.5 m. The samples used in this investigation were collected in May 2004 and were transferred to the laboratory immediately after sam-

* To whom correspondence should be addressed.
(Tel) 852-2299-0605; (Fax) 852-2517-6082
(E-mail) jdgu@hkucc.hku.hk

pling for processing. Water quality at Mai Po was 5.12 mg/L for dissolved oxygen; 7.23 for pH; 65.3 NTU for turbidity; 153.3 mg/L for suspended solids; 3.90 mg/L for BOD_5; 8.66 mg/L for ammoniacal-N; 0.43 mg/L for nitrate-N; 9.08 mg/L for total Kjeldahl N; 0.71 mg/L for total phosphorus; and 14.12 mg/L for chlorophyll *a* (Laboratory of Environmental Toxicology, 2004; Xu *et al.*, 2005). Water samples (0.1 ml) were directly spread on Thiosulphate Citrate Bile Salts Sucrose (TCBS) agar plates (Difco Lab, USA) and incubated at 30°C for about 12 h. Distinctive yellow colonies on the agar plates were then picked and streaked on new TCBS agar plates several times successively to purify the bacterial isolates till pure cultures were obtained. Isolates were identified using morphological and biochemical techniques as reported (Wang *et al.*, 2004; Wang and Gu, 2005; Wang *et al.*, 2006; Zhang *et al.*, 2006). They were also screened for the presence of plasmids and one *V. cholera* strain designated as MP-1 harboring a small plasmid was chosen for further study.

Bacterial strains, plasmids, media, and reagents

Strains and plasmids used in this study are listed in Table 1. *V. cholerae* was grown at 30°C in Tryptic Soy Broth (TSB) medium (Difco Lab., USA). Commercially available *Escherichia coli* for cloning work was grown at 37°C in Luria-Bertani medium (Difco Lab.,USA). When necessary, ampicillin (100 μg/ml) or/and tetracycline (10 μg/ml) was added to the culture medium. Enzymes and reagents for gene manipulation were purchased from New England Biolabs (USA), Promega (USA) and Sigma-Aldrich (USA). Primers for amplification of DNA were synthesized by Invitrogen Hong Kong Ltd. PCR products and plasmid purification kits were from Qiagen Inc (USA).

Identification of the plasmid-bearing Vibrio species

Biochemical identification of bacterial isolates was carried out using API 20NE System (bioMerieux, France) according to the manufacturer's instructions for preliminary identification. Total genomic DNA of strain MP-1 was extracted using DNeasy tissue kit (Qiagen Inc., USA) as recommended by the manufacturer. 16S rRNA gene was amplified using the universal primers pA; 5'-AGAGTTTGATCCTGGCTCA G-3', *E. coli* bases 8 to 27, and reverse primer PC5B; 5'-TA CCTTGTTACGACTT-3', *E. coli* bases 1507 to 1492 (Wilson *et al.*, 1990). Amplification reaction mixtures contained 5 μl deoxynucleoside triphosphate mixture (20 mM), 5 μl 10× Taq DNA polymerase buffer, 6 μl $MgCl_2$ (25 mM), 2 μl of each primer (25 pmol/μl), 3 μl of DNA template (20 ng/μl), 0.5 μl Taq polymerase (5 U/μl) in a final reaction volume of 50 μl. PCR was conducted in a PTC-200 Peltier Thermal Cycler (MJ Research Inc., USA) as follows: 2 min of denaturation at 94°C, followed by 30 cycles of 30 s at 94°C (denaturation), 30 s at 50°C (annealing), and 60 s at 72°C (extension), with a final 5 min 72°C extension step after cycling was complete.

The amplified products were purified and sequenced with an ABI Prism model 377XL DNA sequencer (Perkin-Elmer Applied Biosysems, USA) using universal primers pA. Sequence was determined at Department of Zoology, The University of Hong Kong.

Sequences were initially analyzed using BLAST (National Center for Biotechnology Information) to determine the closest available database sequences. Alignment of the 16S rRNA gene sequences and its closest sequences was accomplished by using CLUSTAL X (Thompson *et al.*, 1997). Parsimony tree was constructed by using the DNAPAR program of the PHYLIP package (version 3.6; distributed by J. Felsenstein, University of Washington, USA). Bootstrap analysis was performed with 100 re-sampled data sets.

Molecular genetic techniques and plasmid sequence analysis

Screen of plasmids was carried out using alkaline lysis as described by Sambrook *et al.* (1989) and small-scale purifi-

Table 1. Bacteria strains and plasmids used in this study

Strains and plasmids	Characteristics	Sources
Strains		
Vibrio cholerae MP-1		This study
E. coli JM109	*recA supE*44 *endA*1 *hsdR*17 *gyrA*96*relA*1 *thiΔ*(*lac-proAB*) *F`*[*traD*36 *proAB*$^+$ *lacI*^{q}*lacZΔM*15]	Promega
E. coli XL1-Blue MRF'	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$*Δ*(*mcrA*) 183*Δ*(*mcrCB-hsd SMR-mrr*)173 *F`* [*proAB*$^+$ *lacI*^{q}*lacZΔM*15 *Tn*10 (*Tc*^{r})]	Stratagene
Plasmids		
pVC	3.8 kb cryptic plasmid from *V. cholerae* MP-1	This study
pBR322	4.36 kb, Ap^R, Tc^R source of Tc^R gene	Fermentas
pBluescirpt II KS	3.0 kb, Ap^R, cloning vector	Stratagene
pBluescirpt-F1	pBluescirpt II KS::0.8 kb *EcoR*I pVC, 3.8 kb, Ap^R	This study
pBluescirpt-F2	pBluescirpt II KS::1.5 kb *EcoR*I pVC, 4.5 kb, Ap^R	This study
pBluescirpt-F3	pBluescirpt II KS::1.5 kb *EcoR*I pVC, 4.5 kb, Ap^R	This study
pGEM-T	3.0 kb, Ap^R , PCR products cloning	Promega
pGEM-Tc	pGEM-T::1.5 kb Tc^R gene, 4.5 kb, Ap^R, Tc^R	This study
pVC01	Tc^R::2.3 kb fragment from pVC, 3.8 kb, Tc^R	This study

cation of plasmid DNA from *V. cholerae* MP-1 was extracted with QiaPrep spin miniprep kit (Valencia, USA). The plasmid (approximately 3.8 kb), designated as pVC has three *Eco*RI sites, which were used for cloning into *Eco*RI treated pBluescirpt II KS phage vector (Stratagene, USA). Recombinant plasmids carrying the three fragments (0.8 kb, 1.5 kb, and 1.5 kb) from *Eco*RI digested pVC were constructed and designated pBluescirpt-F1, pBluescirpt-F2, and pBluescirpt-F3, respectively (Table 1). They were electroporated into *E. coli* XL1-Blue MRF' (Stratagene, USA); transformant cells were screened by both blue-white color selection and PCR amplification.

DNA sequence was obtained with an ABI Prism model 377XL DNA sequencer (Perkin-Elmer Applied Biosysems, USA), initially by using standard T3 and T7 primers, then the internal primers derived from the sequence information through the previous run of sequencing by primer walking. Sequencing was conducted at Department of Zoology, The University of Hong Kong.

DNA analysis, GC content calculation and plasmid map construction were performed using Bioedit (Hall, 1999). Database searches were carried out through NCBI using BLASTn, BLASTp (Altschul *et al.*, 1997) and ORF finder. Promoter prediction was conducted with "neural network promoter prediction" (http://www.fruitfly.org/seq_tools/promoter.html) while molecular weight and pI prediction was done with an on-line server "compute Mw/pI tool" (http://us.expasy.org/tools/pi_tool.html). Protein secondary-structure prediction was performed using the neural network algorithm PHD (Rost and Sander, 1993).

Cloning of the replication region

To locate the pVC replication origin, a selective marker was introduced, the tetracycline resistant (Tc^R) gene amplified from plasmid pBR322 was selected as this marker. The primers used to amplified Tc^R gene were as follows: 5'-CG TCTTCAAGAATTCTCATG-3' and 5'-CGGTGATGAATTC TGCTAAC-3', *Eco*RI sites were contained (underline) in both primers. This pair of primers covered both the structural gene and promoter region of the Tc^R gene (about 1.5 kb) in plasmid pBR322.

The PCR reaction mixtures consisted of 50 ng of pBR322 as template, 50 pmol each of the forward and reverse primers, 1× Taq DNA polymerase reaction buffer, 1.5 mmol/L $MgCl_2$, 0.2 mmol/L dNTP (deoxynucleoside triphosphate), and 2.5 U of Taq DNA polymerase for a final volume of 50 µl. PCR was conducted with a PTC-200 Peltier Thermal Cycler (MJ Research Inc., USA) as follows: 2 min of denaturation at 96°C, followed by 30 cycles of 1 min at 96°C (denaturation), 1 min at 40°C (annealing), and 1 min at 72°C (extension), with a final 5 min 72°C extension step after cycling was complete. The PCR products were loaded onto 0.75% horizontal agarose gels. Gels were run at 5 V/cm, stained with ethidium bromide and photographed on a UV transilluminator.

The PCR product was firstly cloned into pGEM-T vector to confirm its resistance to tetracycline and then treated with *Eco*RI and gel extracted from pGEM-Tc. The mixture of pVC fragments resulted from complete and partial digestion of the plasmid by *Eco*RI were ligated with the Tc^R gene, the ligation products were transformed into *E. coli* JM109 competent cells, transformants showing resistant to tetracycline were obtained.

Plasmid curing experiments

Several attempts to eliminate the plasmid pVC from its original host strain *V. cholerae* MP-1 were carried out to establish the possible relationship between a phenotype and presence of the plasmid. Elevated incubation temperature, ethidium bromide, sodium dodecylsulfate (SDS) and acridine orange were employed to eliminate pVC from the host strain.

Nucleotide sequences accession numbers

The 16S rRNA gene sequence of *V. cholerae* MP-1 and the pVC complete nucleotide sequence have been deposited in the GenBank under accession no. AY911390 and AY 423429, respectively.

Results and Discussion

Identification of plasmid containing strain MP-1

V. cholerae MP-1 containing a small plasmid was chosen for the current study. Its preliminary identification was *V. cholerae* by API 20NE biochemical tests (data not shown) and then by phylogenetic analysis of partial 16S rRNA gene sequence (Fig. 1). Its plasmid band is shown in Fig. 2.

Complete sequence of the plasmid pVC

The whole sequence of the circular plasmid pVC contains

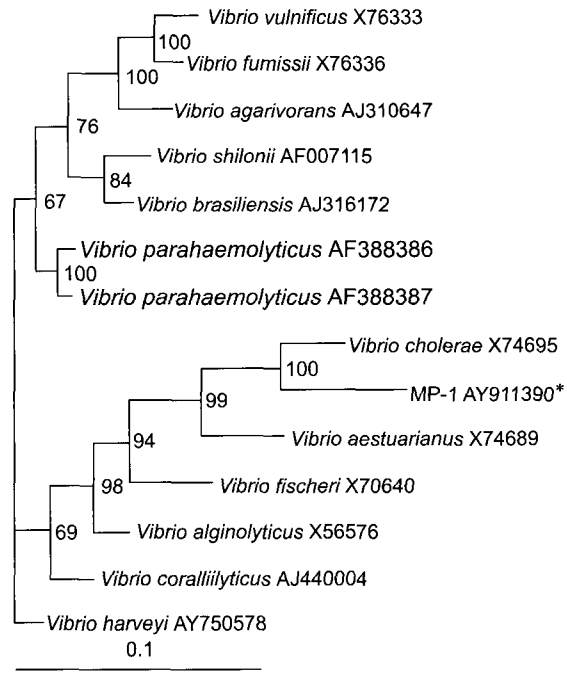

Fig. 1. Phylogenetic relationship of strain MP-1 and its closely relatives. The tree was constructed by the parsimony method, *Vibrio harveyi* AY750578 was ordered as outgroup. Bootstrap analysis was performed with 100 re-sampled data sets and bootstrap values were shown. Asterisks indicate the tested isolates in this study, and others are the references.

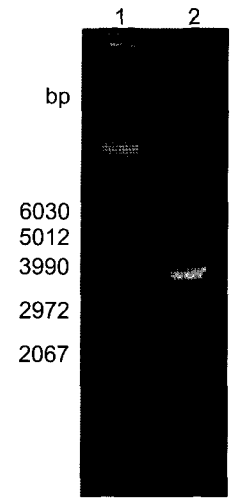

Fig. 2. Plasmid pattern of strain *V. cholerae* MP-1. Lane 1, Supercoil DNA marker; Lane 2, plasmid band of MP-1.

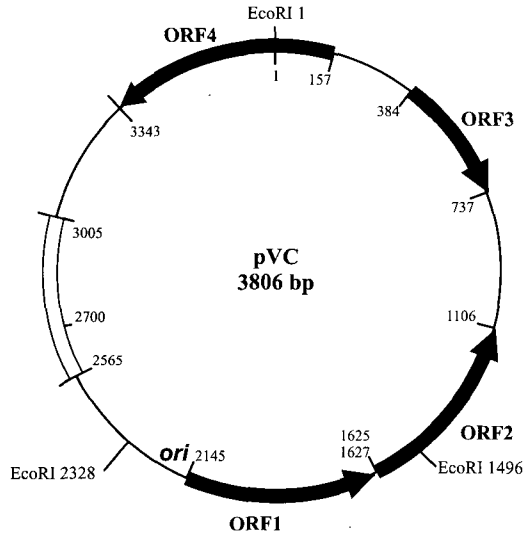

Fig. 3. Physical map of the plasmid pVC from *V. cholerae* MP-1. Gene position and direction of transcription are indicated by solid arrows. The fragment with high similarity to *V. cholerae* strain SIO plasmid pSIO1 is indicated by an open box.

3,806 bp and the calculated molecular weight is 2,309,334 Dalton (double strands) and its G+C content is 43.25%. This G+C content value is close to the reported range (approximately 45 to 48%) of the chromosome in the marine *Vibrionaceae* group (Farmer, 1992). G+C content of *V. cholerae* chromosomal DNA is 47.19% (Heidelberg *et al.*, 2000), suggesting that the plasmid either is indigenous to the *Vibrionaceae* or has resided in the host strain for a considerable period of time (Lawrence and Roth, 1998).

With no demonstrated replication origin, position 1 of the sequence is assigned to nucleotide of *Eco*RI site, which is within ORF4. Database searches reveal a fragment (from position 2565 to 3005) of 440 bp (Fig. 3) is 96% identical to a fragment in the 4.9 kb plasmid pSIO1 (AY876057) of *V. cholerae* strain SIO and also a section of this fragment (position 2632-2766) is 88% identical to a small section of the 13.5 kb plasmid (Z10rZ9, AF009904) in *V. shiloi*. Computer analyses predict four ORFs with >100 amino acids, identified as putative coding regions (Fig. 4) on the basis of putative ribosome-binding site (RBS, also called Shine-Dalgarno sequence), promoter sequence and transcription terminator sequence (Holtwick *et al.*, 2001). Other smaller size ORFs (<50 amino acid residues) are neglected due to no homology to known proteins and additionally no putative ribosome-binding sites proceeded.

ORF1, ORF2, and ORF4 are transcribed in the same orientation. The predicted protein from ORF1 has 173 amino acids with a predicted molecular weight (MW) of 19.9 kD. Similarly, the putative product of ORF2 contains 171 amino acids with a predicted MW of 18.6 kD. Transcription of ORF3 is in opposite orientation with the other 3 ORFs and it encodes a smaller protein with 117 amino acids and MW of 13.3 kD. ORF4 encodes the largest protein in this plasmid, the putative protein has 206 amino acids with a predicted MW of 22.9 kD. Searches using BLASTp (Altschul *et al.*, 1997) show that none of them have homology to any known proteins.

All attempts to cure pVC using different chemical agents at a range of concentration including ethidium bromide, SDS, acridine orange and elevated temperature of culturing conditions were unsuccessful, suggesting a phenomenon that is most likely due to the relatively high copy number and stability of this plasmid within its natural bacterial host.

Cloning and characterization of the replication origin

Surprisingly, none of the proteins encoded by plasmid pVC show similarity to the known replication initiator protein (Rep) needed by most of the plasmids. To locate the pVC replication origin, the tetracycline resistant (Tc^R) gene amplified from plasmid pBR322 was selected as a selection marker, ligated with the *Eco*RI digest of pVC, and transformed into *E. coli* JM109 competent cells. Recombinant plasmid from the transformants was 3.8 kb in length, and it is designated pVC01 (Table 1). Analysis of pVC01 reveals that it was consisted of a 2.3 kb fragment (position 1496-3806, containing the 0.8 kb and 1.5 kb *Eco*RI fragments) of plasmid pVC and the 1.5 kb Tc^R gene.

Further analysis of the 2.3 kb fragment indicated that it contains partial ORF4, the complete ORF1 and an intergenic region between ORF4 and ORF1. Detailed analyses of the sequence upstream of ORF1 show that it possesses characteristics of plasmid replication origins (Helinski *et al.*, 1996; Del Solar *et al.*, 1998) and several features typical of iteron-containing theta-type replicons (Fig. 4). They are specifically (i) an AT rich region containing sequence repeats where opening of the strands and assembly of host initiation factors occur, (ii) four potential direct repeats (called iterons) spaced approximately one DNA helical turn apart (11-13 bp), which are the binding sites of Rep proteins. Generally the sequences of the iterons are not identical for a particular origin, but a consensus motif is identified (Del Solar *et al.*, 1998). In this plasmid, the consensus sequence is ATAAA, (iii) two *dnaA* boxes where the host DnaA initiator protein binds (Fuller *et al.*, 1984), (iv) three Dam methylation sites,

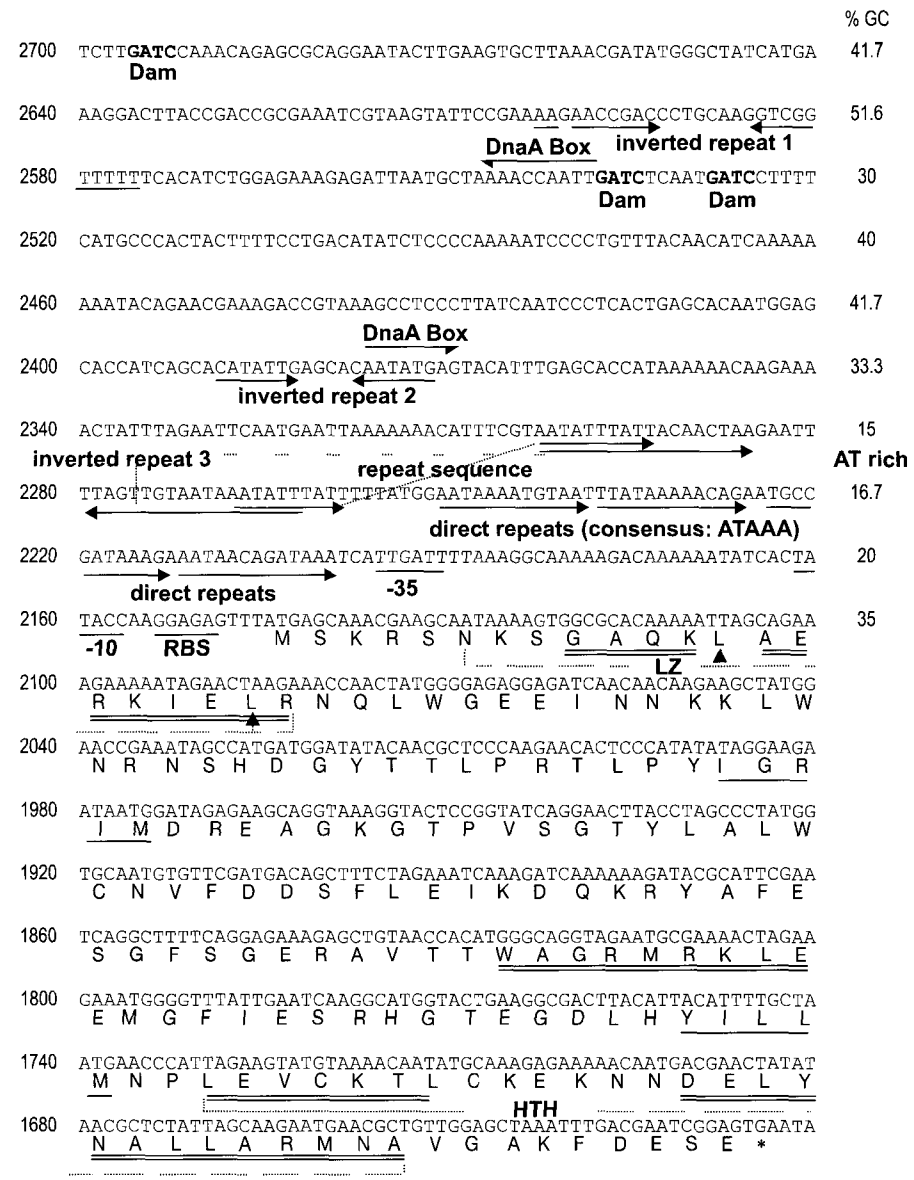

Fig. 4. Sequence analysis of pVC origin of replication. The 5'-3' DNA sequence of this origin and corresponding amino acid sequence of ORF1 are shown. The %GC content of each line (60 bp) is shown at the right. Boldface letters indicate putative Dam methylation sites. Arrows on top of the sequences indicate putative DnaA boxes, -35, -10 promoter region and ribosomal binding site (RBS) of ORF1 are underlined and indicated. Direct repeats and inverted repeats are indicated by arrows, mismatches in the imperfect inverted repeat by discontinuous arrows. Double lines in the amino acid sequence corresponding to ORF1 mean alpha helical structure and single lines mean beta sheets. The leucine zipper (LZ) and helix-turn-helix (HTH) motifs are indicated.

and (v) inverted repeats. Therefore, we postulate that the plasmid pVC replication origin may be located most likely in the region upstream of ORF1.

Most of the plasmids have a self-encoded Rep protein. We propose that ORF1 of pVC may encode this protein even though it has no similarity with the currently known Rep proteins. ORF1 is smaller than the common reported Rep proteins in theta plasmid replicons, but secondary structure prediction by the neural network algorithm PHD (Rost and Sander, 1993) indicates that two characteristic motifs exist in most Rep proteins (Del Solar *et al.*, 1998), the leucine zipper (LZ) motif located at the N-terminal region of the Rep protein (Fig. 4) which is involved in protein-protein interaction (Del Solar *et al.*, 1998), and the alpha helix-turn-alpha helix motif (HTH) located at the C-terminal end of the protein, involved in the protein-DNA binding. However, further direct evidences are needed to support this postulation.

The small cryptic plasmid, pVC, might replicate via theta replication mode and this replicon is likely a new class of theta-type replicon, suggesting the potential replicon diversity in marine microbial community.

Acknowledgements

This research was financially supported by 863 Project (No. 2002AA601160) supplement fund of The University of Hong Kong and RGC Central Allocation CA04/05.SC01. We would also like to thank Miss Jessie Lai for laboratory assistance.

References

Altschul, S.F., T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, and D.J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25, 3389-3402.

Bartowsky, E.J. and P.A. Manning. 1988. Molecular cloning of the plasmids of *Vibrio cholerae* O1 and the incidence of related plasmids in clinical isolates and other *Vibrio* species. *FEMS Microbiol. Lett.* 50, 183-190.

Chiang, S.L. and J.J. Mekalanos. 1999. Horizontal gene transfer in the emergence of virulent *Vibrio cholerae*, p. 156-169. *In* E. Rosenberg (ed.), *Microbial Ecology and Infectious Disease*. American Society for Microbiology Press, Washington, D.C., USA.

Cook, W.L., K. Wachsmuth, S.R. Johnson, K.A. Birkness, and A.R. Samadi. 1984. Persistence of plasmids, cholera toxin genes, and prophage DNA in classical *Vibrio cholerae* O1. *Infect. Immun.* 45, 222-226.

Del Solar, G., R. Giraldo, M.J. Ruiz-Echevarria, M. Espinosa, and R. Diaz-Orejas. 1998. Replication and control of circular bacterial plasmids. *Microbiol. Mol. Biol. Rev.* 62, 434-464.

Farmer, J.J. III. 1992. The family *Vibrionaceae*, p. 2398-2952. *In* A. Balows, H.G. Trüper, M. Dworkin, and W. Harder (eds), The Prokaryotes: a Handbook on the Biology of Bacteria: Ecophysiology, Isolation, Identification, Applications. Springer-Verlag, New York, USA.

Faruque, S.M., M.J. Albert, and J.J. Mekalanos. 1998. Epidemiology, genetics, and ecology of toxigenic *Vibrio cholerae*. *Microbiol. Mol. Biol. Rev.* 62, 1301-1314.

Fuller, R.S., B.E. Funnell, and A. Kornberg. 1984. The dnaA protein complex with the *E. coli* chromosomal replication origin (*oriC*) and other DNA sites. *Cell* 38, 889-900.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95-98.

Heidelberg, J.F., J.A. Eisen, W.C. Nelson, R.A. Clayton, M.L. Gwinn, R.J. Dodson, D.H. Haft, E.K. Hickey, J.D. Peterson, L. Umayam, S.R. Gill, K.E. Nelson, T.D. Read, H. Tettelin, D. Richardson, M.D. Ermolaeva, J. Vamathevan, S. Bass, H.Y. Qin, I. Dragoi, *et al.* 2000. DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature (London)* 406, 477-483.

Helinski, D.R., A.E. Toukdarian, and R.P. Novick. 1996. Replication control and other stable maintenance mechanisms of plasmids, p. 217-261. *In* F.C. Neidhardt (ed.), *Escherichia coli* and *salmonella*: Cellular and Molecular Biology, vol. 1. American Society for Microbiology Press, Washington, D.C., USA.

Holtwick, R., A. von Wallbrunn, H. Keweloh, and F. Meinhardt. 2001. A novel rolling-circle-replicating plasmid from *Pseudomonas putida* P8: molecular characterization and use as vector. *Microbiol.* 147, 337-344.

Islam, M.S., B.S. Drasar, and R.B. Sack. 1996. Ecology of *Vibrio cholerae*: role of aquatic fauna and flora, p. 187-227. *In* B.S. Drasar and B.D. Forrest (eds), Cholera and the Ecology of *Vibrio choleae*. Chapman and Hall, London, UK.

Laboratory of Environmental Toxicology. 2004. Baseline Ecological Monitoring Program for Mai Po and Inner Deep Bay Ramsar Site 2002-2003 Final Report, p. 291. Department of Ecology & Biodiversity, The University of Hong Kong. Hong Kong.

Lawrence, J. and J. Roth. 1998. Roles of horizontal transfer in bacteria in bacterial evolution, p. 208-228. *In* M. Syvanen and C.I. Kado (eds), Horizontal Gene Transfer. Chapman and Hall, New York, USA.

Rost, B. and C. Sander. 1993. Prediction of protein structure at better than 70% accuracy. *J. Mol. Biol.* 232, 584-599.

Rubin, E.J., W. Lin, J.J. Mekalanos, and M.K. Waldor. 1998. Replication and integration of a *Vibrio cholerae* cryptic plasmid linked to the CTX prophage. *Mol. Microbiol.* 28, 1247-1254.

Said, B. and B. Drasar. 1996. *Vibrio cholerae*, p. 1-17. *In* B.S. Drasar and B.D. Forrest (eds), Cholera and the Ecology of *Vibrio choleae*. Chapman and Hall, London, UK.

Salyers, A.A. and D.D. Whitt. 2002. Bacterial Pathogenesis: A Molecular Approach, (2nd ed.). p. 363-380. American Society for Microbiology Press, Washington D.C., USA.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual, (2nd ed). p. 1.25-1.31. Cold Spring Harbor Laboratory Press, New York, USA.

Sinha, V.B. and B.S. Srivastava. 1978. Plasmid-induced loss of virulence in *Vibrio cholerae*. *Nature (London)*, 276, 708-709.

Sobecky, P.A. 1999. Plasmid ecology of marine sediment microbial communities. *Hydrobiologia* 401, 9-18.

Sobecky, P.A. 2002. Approaches to investigating the ecology of plasmids in marine bacterial communities. *Plasmid* 48, 213-221.

Thompson, J.D., T.J. Gibson, F. Plewniak, F. Jeanmougin, and D.G. Higgins. 1997. The CLUSTAL X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* 24, 4876-4882.

Wang, Y., P.C. Leung, P. Qian, and J.-D. Gu. 2004. Effects of UV, H_2O_2 and Fe^{3+} on the growth of four environmental isolates of *Aeromonas* and *Vibrio* species isolated from a mangrove environment. *Microb. Environ.* 19, 163-171.

Wang, Y., P.C. Leung, P. Qian, and J.-D. Gu. 2006. Antibiotic resistance and plasmid profile of environmental isolates of *Vibrio* species from Mai Po Nature Reserve, Hong Kong. *Ecotoxicol.* 15, 371-378.

Wang, Y.L. and J.-D. Gu. 2005. Influence of temperature, salinity and pH on the growth of environmental *Aeromonas* and *Vibrio* species isolated from Mai Po and the Inner Deep Bay Nature Reserve Ramsar Site of Hong Kong. *J. Basic Microbiol.* 45, 83-93.

Wilson, K.H., R.B. Blitchington, and R.C. Greene. 1990. Amplification of bacterial 16S ribosomal DNA with polymerase chain reaction. *J. Clin. Microbiol.* 28, 1942-1946.

Xu, X.R., H.B. Li, and J.-D. Gu. 2005. Degradation of *n*-butyl benzyl phthalate by *Pseudomonas fluorescens* B-1 isolated from mangrove sediment. *J. Microbiol. Biotechnol.* 15, 946-951.

Zhang, R., Y. Wang, and J.-D. Gu. 2006. Identification of environmental plasmid-bearing *Vibrio* species isolated from polluted and pristine marine reserves of Hong Kong and resistance to antibiotics and mercury. *Antoine van Leeuvenhoek* 89, 307-315.