Abundance of Environmental *Vibrio* Species in Marine Environment and Characterization of Cryptic Plasmids

Ji-Dong Gu\(^1,2\)\(^*\), Ruifu Zhang \(^1\)

\(^1\)Laboratory of Environmental Microbiology and Toxicology, Department of Ecology & Biodiversity, The University of Hong Kong, Kadoorie Biological Sciences Building, Pokfulam Road, Hong Kong SAR, P.R. China;

\(^2\)The Swire Institute of Marine Science, The University of Hong Kong, Shek O, Cape d’Aguilar, Hong Kong SAR, P.R. China

*corresponding author (e-mail: jdgu@hkucc.hku.hk)

**Abstract**

Water samples were collected from several previously determined sampling sites at Mai Po Nature Reserve (22°29'N to 22°31'N and 113°59'E to 114°03'E) and Cape d’Aguilar Marine Reserve (22°12'N and 114°15'E) of Hong Kong. Fifty environmental isolates of *Vibrio* species were isolated from water samples of Mai Po Nature Reserve and the Cape d’Aguilar Marine Reserve in Hong Kong and they were screened for the presence of plasmids. Mai Po is a wastewater-impacted area while the Cape d’Aguilar Marine Reserve is a pristine marine water. Plasmids were found in *Vibrio* isolates from both sites at similar frequencies and each site had distinctive plasmid profiles. These plasmid-bearing *Vibrio* isolates were identified to be different species of the *Vibrio* species by both biochemical test and subsequent full-length 16S rRNA sequences. Antibiotic resistance test showed that all these plasmid-bearing *Vibrio* isolates showed multiple resistance to the 21 antibiotics tested. In addition, selective isolates also showed tolerance to 10 \(\mu\)M Hg\(^{2+}\) in culture medium and these isolates generally harbored large plasmid(s) (>30 kb). Among the plasmids detected, a novel cryptic plasmid, pVC, from an environmental isolate of *Vibrio cholerae* MP-1 from Mai Po Nature Reserve in Hong Kong has been isolated. The complete nucleotide sequence analysis (3,806 bp) revealed three major putative open reading frames (ORFs). Our results showed that high frequency of plasmid in *Vibrio* species of both polluted and pristine environments may be ecologically important to the survival of these bacteria in the environment. At the same time, public awareness of the associated problem with these bacteria should also be acknowledged. The specific functioning of the cryptic plasmids remains the focus of our current investigations.

**Key Words**: *Vibrio* species, pollution, antibiotics resistance, plasmids

**Introduction**

Mai Po Nature Reserve, the largest mangrove ecosystem in Hong Kong, is located at the northwestern New Territories of Hong Kong Special Administrative Region. It consists of extensive inter-tidal mudflats, dwarf mangroves, *gei wai* (traditional shrimp pond), reedbeds and fishponds, the Nature Reserve plays a very important role in supporting a wide range of wildlife including migratory birds, e.g., the endangered Black-faced Spoonbills (Tsim and Lock, 2002). This protected area is an important refueling station for winter birds on their way from Arctic and Northern China to Australia.

Mai Po Nature Reserve is threatened by increasing pollutions largely due to the recently economic development in the adjacent Shenzhen Special Economic Zone of the People’s Republic of China. Large quantities of domestic and industrial wastewaters have been discharged to the inter-tidal area without proper treatment. Water quality monitoring carried out by Hong Kong SAR Government indicated that
Mai Po Nature Reserve is one of the most polluted water bodies in Hong Kong (Lee, 1999). The status of pollution has not been improved in the last several years (Laboratory of Environmental Toxicology, 2003; 2004). In contrast, Cape d’Aguilar Marine Reserve of Hong Kong is also a protected area situated in Cape d’Aguilar Peninsula, southeast tip of Hong Kong, and the environment is pristine nature and clean (Morton and Harper, 1995). Though research has been conducted on the basic biology of flora and fauna in the area, investigation on microorganisms especially opportunistic water-borne pathogens has not been conducted before.

Vibrio species are the natural inhabitants of aquatic environments. They live either freely in brackish water or in association with plankton (Islam et al., 1996). Consumption of insufficiently cooked seafood, accidental drinking of Vibrio-contaminated water or wound exposure to Vibrio-containing water is the most common means responsible for Vibrio-infections (Farmer and Hickman-Brenner, 1992). The co-dwelling of Vibrio species with marine animals may lead to possible transmission to humans. Vibrio species have also been listed as one of the leading bacterial pathogens in avian diseases. Vibrio mestchnikovii was isolated and identified from diseased domestic ducks and geese in Germany (Hinz et al., 1999). The enteritis in avian species like canaries and finches was caused by Vibrio parahaemolyticus (Lowrie and Borneman, 1999).

Comparative genomics study has demonstrated that environmental isolates of Vibrio species lack toxin-encoding genes in their bacterial chromosomes (Dziejman et al., 2002). However, due to the high similarity of genetic makeup of the environmental isolates and their pathogenic counterparts, the former may take up genes responsible for pathogenesis through acquiring plasmid, bacteriophage and integron (Chiang and Mekalanos, 1999). As a result, environmental isolates of Vibrio species may act as a reservoir for the potential spreading of virulence genes in the natural environment.

Bacterial resistance to antibiotics has become an emerging medical issue threatening the public health because of the widely availability of antibiotics and, sometimes, misuse without proper prescription (Davis and Amabile-Cuevas, 2003). More and more pathogenic bacteria have shown to resistant to one or a suite of antibiotics (Levy, 2001). In some cases, the virulent or pathogenic factors are carried by the same vectors responsible for antibiotic resistance. The ability of plasmid to be transferred from one bacterium to another horizontally, even occasionally between phylogenetically distant bacteria, has contributed greatly to the wide dissemination of antibiotic resistance genes in the environment (Dale and Park, 2004). Under the selection pressure from different antibiotics in the environment, exposed bacterial population may resist to a wide spectrum of antibiotics through multiple gene transfer and exchange process (Baquero and Blazquez, 1997). The antibiotic resistance profile displayed in pathogenic Vibrio species has been documented (Waldor et al., 1996; Hochhut et al., 2001; Beaber et al., 2002).

Heavy metals such as mercury are commonly found in areas with industrial activity, the occurrence of metal resistant bacteria in coastal marine sediments and waters has often been used as an indicator for pollution (Aviles et al., 1993, DeVincente et al., 1990). Correlation between environmental stress, e.g., pollution, bacteria resistance and increased plasmid incidence in marine bacterial populations has been observed (Burton et al., 1982, Baya et al., 1986, Hada and Sizemore, 1981, Glassman and

In this study, the plasmid distribution, antibiotic and mercury resistance were investigated using plasmid-bearing environmental Vibrio species isolated from two environments of Hong Kong.

Methods and Materials

Water sampling
Water samples were collected from several previously determined sampling sites at Mai Po Nature Reserve (22°29′N to 22°31′N and 113°59′E to 114°03′E) and Cape d’Aguilar Marine Reserve (22°12′N and 114°15′E) of Hong Kong. Relevant information can also be found elsewhere (Wang and Gu, 2005; Wang et al., 2004). Surface water was taken in 1 L plastic bottles when the tidal level was at approximately 1.5 m at Mai Po and Cape d’Aguilar. All the samples were transferred back to the laboratory immediately after sampling for processing. The samples used in this investigation were collected in May 2005.

Environmental quality
Water quality at Mai Po was analyzed and it was 5.12 for dissolved oxygen (mg L⁻¹), 7.23 for pH, 65.3 for turbidity (NTU), 153.3 for suspended solid (mg L⁻¹), 3.90 for BOD₅ (mg L⁻¹), 8.66 for ammoniacal N (mg L⁻¹), 0.43 for nitrate-N (mg L⁻¹), 9.08 for total Kjeldahl N (mg L⁻¹), 0.71 for total phosphorus (mg L⁻¹) and 14.12 for chlorophyll a (g L⁻¹) (Laboratory of Environmental Toxicology, 2003; 2004; 2005). At Cape d’Aguilar, water quality at surface layer and bottom layer were 7.02 and 5.48 for dissolved oxygen (mg L⁻¹), 8.00 and 7.78 for pH, 1.06 and 2.28 for turbidity (NTU), 3.81 and 7.12 for suspended solid (mg L⁻¹), 0.91 and 1.04 for BOD₅ (mg L⁻¹), 0.095 and 0.093 for ammoniacal N (mg L⁻¹), 0.048 and 0.053 for nitrate-N (mg L⁻¹), 4.251 and 3.898 for total Kjeldahl N (mg L⁻¹), 0.780 and 0.757 for total phosphorus (mg L⁻¹) and 3.032 and 1.596 for chlorophyll a (g L⁻¹), respectively.

Selective isolation and purification of Vibrio species
Water samples (0.1ml) were directly spread on the Thiosulphate Citrate Bile Salts Sucrose (TCBS) agar plates (Difco Lab, Detroit, Michigan) and incubated at 30°C for about 12 hrs. Distinctive yellow colonies on agar plates were picked and streaked on new TCBS agar plates several times to purify bacterial isolates till pure cultures were obtained. Fifty isolates were randomly selected from each site for screening the presence of plasmids.

Extraction of plasmid
Small-scale extraction of plasmid DNA was carried out using alkaline lysis as described by Sambrook et al. (1989) and confirmed with QIAprep spin miniprep kit (Qiagen Inc., California). The plasmid DNAs were loaded onto 0.7% horizontal agarose gels. Gels were run at 5 V per cm, stained in ethidium bromide, destained in water and photographed on an UV transilluminator.

Identification of the plasmid-bearing Vibrio species
Biochemical identification was carried out using API 20NE System (Bio Merieux, France) according to the manufacture’s instructions for preliminary identification.
Total genomic DNAs of these plasmid-bearing strains were extracted using DNaseasy tissue kit (Qiagen Inc., California) as recommended by the manufacturer. 16S rRNA gene was amplified with the universal primers pA(5' - AGAGTTTGATCCTGGCTCAG-3'; E. coli bases 8 to 27') and reverse primer PC5B(5'-TACCTTGTTACGACTT-3'; E. coli bases 1507 to 1492) (Wilson et al., 1990). Amplification reaction mixtures contained 5 µl deoxynucleoside triphosphate mixture (20 mM), 5µl 10× Taq DNA polymerase buffer, 6 µl MgCl₂ (25 mM), 2 µl of each primer (25 pmol µl⁻¹), 3 µl of DNA template (20 ng µl⁻¹), 0.5 µl Taq polymerase (5U µl⁻¹) in a final reaction volume of 50 µl. PCR was conducted with a PTC-200 Peltier Thermal Cycler (MJ Research Inc., Massachusetts, 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 5min 72°C extension step after cycling was complete.

The amplified products were purified and the 1.5 kb fragments were ligated into the pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) by using T4 DNA ligase and incubated at 16°C overnight. Recombinant plasmids were transformed into competent E. coli JM109 cells, and positive colonies were identified by blue-white color selection on agar plates after incubated at 37°C for 12 hours.

The inserted fragments were sequenced with an ABI Prism model 377XL DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, California), initially by using pGEM-T vector specific primers, then the internal primers. Sequence was determined at Takara Biotechnology (Dalian) Co. Ltd (Dalian, China).

Sequences were initially analyzed by using BLAST (National Center for Biotechnology Information) to determine the closest available database sequences. Alignment of their 16S rRNA gene sequences and their closest sequences was accomplished by using Clustal X (Thompson et al., 1997). Neighbor-joining distance matrix analysis of sequences was performed by using the DNADIST and NEIGHBOR programs of the PHYLIP package (version 3.6; distributed by J. Felsenstein, University of Washington, Seattle). Phylogenetic tree was constructed by the neighbor-joining method using random sequence input orders and optimized by global rearrangement of branches. Bootstrap analysis was performed with 100 re-sampled data sets.

**In vitro susceptibility of plasmid-bearing vibrio spp. bacteria to antibiotics**

Disc diffusion susceptibility testing method was used in this experiment. The antibiotic discs were purchased from MAST DIAGNOSTIC company (France). The culture medium used in this experiment was Tryptic Soy Broth (TSB) (Difco Lab., Detroit, Michigan), Tryptic Soy Agar plates for standard disc diffusion test were prepared by adding 15 g agar to 1 L TSB. The test bacterial strains were previously spread on the plates, and the discs impregnated with various antibiotics with known dosage will diffuse into the agar medium in a circle surrounding. All agar plates were incubated at 30°C for 14-16 hours. The diameter of inhibition zone was then measured and interpreted against the recommendations proposed by Kirby and Bauer (Bauer et al., 1966; Benson, 2002). Agar plates inoculated only with bacterial test isolates without introduction of antibiotic disks served as controls.
Effects of mercury ion on the growth of plasmid-bearing vibrio spp. bacteria

TSB was supplemented with appropriate amount of HgCl$_2$ stock solution (50 mM) to achieve 0, 10 and 50 µM Hg$^{2+}$, over-night bacteria culture (adjusted to OD$_{600}$ 0.8) of the test bacterial strains were inoculated by one percent into these different treatments, non-inoculated blanks were also included for the investigation. All plate wells were incubated at 30°C. After 24 hrs, OD$_{600}$ values were recorded. All treatments replicate 3 times.

Nucleotide sequences accession numbers

The 16S rRNA gene sequences determined in this study of these plasmid-bearing Vibrio spp. bacteria have been deposited in the NCBI database under Accession No. AY911390 to AY911397.

Results and Discussion

Isolation of plasmid-bearing Vibrio species and their native plasmid profiles

Fifty isolates were randomly selected from each site of Mai Po Nature Reserve and Cape d’Aguilar Marine Reserve using TCBS selective agar plates, and they were preliminary identified as members of Vibrio genus by the results of API 20NE biochemical identification (data not shown). These isolates were used to screen for the presence of plasmids. The plasmid-bearing Vibrio strains isolated from Mai Po Nature Reserve were designated MP-1, MP-2, MP-3 and MP-4, and plasmid-bearing strains from Cape d’Aguilar Marine Reserve were designated SW-1, SW-2, SW-3 and SW-4, respectively. Their plasmid distribution patterns are shown in Table 1, and their plasmid profiles are illustrated in Fig.1.

Because Vibrio species are potential pathogens of humans, indigenous animals and migratory birds, the occurrence of Vibrio species at both sites suggested the potential threat from the presence of these bacteria in the coastal environment. It is well known that plasmid is one of the most important mediators facilitating the fast spreading of virulence and antibiotic resistance among bacteria (Dale and Park, 2004). Numerous studies have revealed a correlation between environmental stress, e.g. pollution, bacteria resistance and increased plasmid incidence in culturable marine bacterial populations (Burton et al., 1982, Baya et al., 1986, Hada and Sizemore, 1981, Glassman and McNicol, 1981). Both sampling sites are located at different parts of Hong Kong coastal marine environments, the occurrence rates of plasmid-bearing Vibrio isolates of the two sites were very similar, but their plasmid profiles were distinctively different from each other, suggesting that intrinsic biological properties may be associated with the Vibrio species isolated from different sites. Because of the unavailability of larger supercoiled DNA markers, the exact sizes of these plasmids could not be determined at this time, their sizes were approximately between several kb to more than 30 kb.
Identification of the plasmid-bearing *Vibrio* species isolates

From the results of API 20NE System biochemical identification (data not shown) and phylogenetic analysis (*Fig 2*), their taxonomies were confirmed. MP-1, MP-2, MP-3 and MP-4 were *Vibrio cholerae*, *Vibrio parahaemolyticus*, *Vibrio shilonii*, and *Vibrio vulnificus*, respectively, while SW-1, SW-2, SW-3 and SW-4 were *Vibrio parahaemolyticus*, *Vibrio shilonii*, *Vibrio harveyi*, and *Vibrio harveyi*, respectively. All these eight plasmid-bearing *Vibrio* isolates were members of potential human or marine animal pathogens. *V. harveyi* is responsible for coagglutination in prawn plasma which leads to unrecoverable hemolymph (Lee et al., 1999). *V. shilonii* is believed to be the causative agent of coral bleaching in the Mediterranean Sea area (Banin et al., 2000). Strains of *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* are the most life-threatening human public health threats (Tison, 1999) and were also reported from another investigation (Wang et al., 2004; Wang and Gu, 2005).

**In vitro susceptibility of plasmid-bearing Vibrio spp. bacteria to antibiotics**

Inhibition zone diameters were compared against the standard in Kirby-Bauer method in antibiotic resistance profile test using disc diffusion susceptibility method (Bauer et al., 1966; Benson, 2002). The final interpretation of measurements, grouped into three categories, namely sensitive, intermediate and resistance.

All these plasmid-bearing *Vibrio* isolates showed multiple-resistance to the 21 antibiotics tested in this study. Although they belong to different species of the *Vibrio* genera, they displayed some common antibiotic resistance profile, all isolates were resistant to ampicillin, carbencillin, cephalothin, clindamycin, colistin sulphate, erythromycin, fusidic acid, methicillin, nitrofurantoin and penicillin G. The presence of plasmid(s) in *V. cholerae*, mainly in pathogenic strains during the cholera outbreaks is not rare. *V. cholerae* O1 El Tor from Bangladesh was found to carry multiple antibiotic resistance genes (Threlfall et al., 1980). Petroni et al. (2002) reported an antibiotic resistant plasmid in *V. cholerae* O1 El Tor was transferable to *E. coli*, suggesting the potential wide spread of resistance genes among environmental bacteria. The reported transferable R plasmids were usually as big as 30 kb, because those indispensable components of a conjugative plasmid make it rather big in size.
The presence of both resistance of antibiotics and the large plasmid in *Vibrio* isolates may have significant ecological and public health implications.

**Fig. 2.** The unroot phylogenetic tree of these plasmid-containing *Vibrio* species strains and their relatives. The phylogenetic trees were constructed by using neighbor-joining method using random sequence input orders and optimized by global rearrangement of branches with PHYLIP package. Boot-strap analysis was performed with 100 re-sampled data sets.

**Effects of mercury ion on the growth of plasmid-bearing vibrio spp. bacteria**

In this study, maximum biomass (A) achieved in terms of the OD\textsubscript{600} values of these plasmid-bearing *Vibrio* isolates were significantly different for the Hg\textsuperscript{2+} concentrations tested (\(p<0.01\)). When the exogenous Hg\textsuperscript{2+} concentration was 50 µM in the culture medium, none of the isolates tested showed any detectable growth, but strain MP-2, SW-1, SW-2 and SW-4 showed tolerance to 10 µM Hg\textsuperscript{2+}. All mercury resistant isolates in this study had large plasmid(s) (Fig 1). While abiotic reduction of Hg\textsuperscript{2+} may occur, biotic reduction of Hg\textsuperscript{2+} is the predominant mechanism of Hg\textsuperscript{0} formation in seawater and freshwater environments (Mason et al., 1995). To date, the majority of bacteria adapted to survive and proliferate in the presence of mercury have been shown to reduce Hg\textsuperscript{2+} to its volatile form Hg\textsuperscript{0} via the well-characterized mer operons (Barkay, 1992, Jeffery et al., 1996, Liebert et al., 1997, Rochelle et al., 1991, Silver and Walderhaug, 1992). Mercury resistant genes were reported to be located on large plasmids (Rani and Mahadevan, 1994, Summers and Silver, 1992, Silver, 1996). In addition, other mercury resistance mechanism such as efflux systems in marine bacteria was reported plasmid-encoded (Reyes et al., 1999). In our study, the location of the mercury resistant genes needs to be identified in the future research.

In conclusion, *Vibrio* species are widely occurring in the environments and a significant portion of the bacterial isolates has plasmid. Some of them may have multiplasmids. These plasmids are responsible for their survival through resistant to
high concentrations of antibiotics or pollutants. The ecological function and evolutionary role of plasmids remain to be elucidated.

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References


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