A New ColE1-like Plasmid Group Revealed by Comparative Analysis of the Replication Proficient Fragments of *Vibrionaceae* Plasmids

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Plasmids play important roles in horizontal gene transfer among *Vibrionaceae*, but surprisingly little is known about their replication and incompatibility systems. In this study, we successfully developed a bioinformatics-assisted strategy of experimental identification of seven *Vibrio* plasmid replicons. Comparative sequences analysis of the seven *Vibrio* plasmid replicons obtained in this study together with eight published *Vibrionaceae* plasmid sequences revealed replication-participating elements involved in the ColE1 mode of replication initiation and regulation. Like plasmid ColE1, these *Vibrionaceae* plasmids encode two RNA species (the primer RNA and the antisense RNA) for replication initiation and regulation, and as a result, the 15 *Vibrionaceae* plasmids were designated as ColE1-like *Vibrionaceae* (**CLV**) plasmids. Two subgroups were obtained for the 15 CLV plasmids, based on comparison of replicon organization and phylogenetic analysis of replication regions. Coexistence of CLV plasmids were demonstrated by direct sequencing analysis and Southern hybridization, strongly suggesting that the incompatibility of CLV plasmids is determined mainly by the RNA I species like the ColE1-like plasmids. Sequences resembling the conserved Xer recombination sites were also identified on the CLV plasmids, indicating that the CLV plasmids probably use the host site-specific recombination system for multimer resolution like that used by ColE1-like plasmids. All the results indicated that the 15 plasmids form a new ColE1-like group, providing a basis for the rapid characterization and classification of *Vibrionaceae* plasmids.

**Keywords:** *Vibrio*, plasmids, replication, RNA, compatibility, replication initiation

Members of *Vibrionaceae*, belonging to the Gram-negative Gammaproteobacteria, are highly abundant in aquatic environments [1, 21, 32, 50, 73]. The significant genome plasticity enables vibrios to possess greater flexibility to adapt to variable ecological niches [73]. Examination of the complete genomes of prokaryotes revealed that horizontal gene transfer appears more frequently in *Vibrionaceae* than in the other prokaryotic species [28, 48]. Plasmids, one type of mobile genetic elements, have proven to play an important role in genetic exchange among diverse vibrios [31]. In addition, *Vibrionaceae* plasmids specify some ecologically and medically important traits, such as antibiotic resistance, virulence, and toxin, which greatly facilitate environmental adaptation of *Vibrionaceae* [2, 12, 23, 24, 38, 40, 51, 54, 65, 74, 78]. In contrast to the demonstrated contributions of *Vibrionaceae* plasmids to horizontal gene transfer, much less is known about their replication and incompatibility systems.

A DNA hybridization-based study has demonstrated that *Vibrionaceae* plasmids and even marine plasmids contained replicons unrelated to the well-characterized bacterial plasmids [73]. This is consistent with the results from comparative analysis of the increasing plasmid sequences reported for *Vibrionaceae* [9, 16, 23, 24, 26, 31, 33, 40, 42, 53, 55, 79, 81, 84, 85]. Low or even no sequence similarity with characterized plasmid replication systems was observed for many *Vibrionaceae* plasmids [9, 16, 23, 24, 26, 31, 33, 40, 42, 53, 55, 79, 81, 84, 85]. So far, only a few studies have reported the identification and characterization of *Vibrio* plasmid replication and incompatibility loci [64, 84, 85]. The replication regions experimentally isolated from *Vibrionaceae* plasmids include pMP1, pVC, pTM121, pTM41, and pTM172, all of which have been predicted to replicate requiring a plasmid-encoded replication protein [64, 84, 85]. The putative Rep proteins encoded by pTM121, pTM41, and pTM172, all of which have been predicted to replicate requiring a plasmid-encoded replication protein [64, 84, 85]. The putative Rep proteins encoded by pTM121, pTM41, and pTM172 [64] showed only low similarity with those encoded by plasmids R6k [66], pCFC1 [15], and pYV [35], respectively, whereas the putative Rep proteins of pMP1 and pVC showed no homologs in the public databases [84, 85]. It is therefore necessary to isolate more
replication loci of *Vibrionaceae* plasmids to better understand their unique replication systems.

ColE1 is a small *Escherichia coli* plasmid, and its replication and stability systems have been extensively studied [3, 13, 14, 19, 20, 36, 41, 44, 45, 61, 68, 72, 75–77]. ColE1-like plasmids specify two RNA species instead of a plasmid-encoded replication initiation protein for replication and regulation [36]. The plasmid-specific primer RNA (RNA II) and antisense RNA (RNA I) are transcribed from two opposite arranged conserved promoters on all the ColE1-like plasmids [13, 14, 20, 61]. RNA II is the only plasmid-specified element essential for replication initiation in the ColE1-like plasmids, which form a persistent hybrid with the template DNA at the replication origin [20, 36, 45]. The approximately 108 nt-long RNA I is the primary determinant of incompatibility and copy number of ColE1-like plasmids [13, 20, 44, 72, 75, 76, 77]. ColE1 contains a 250 bp cer site for the Xer system, which is the site-specific recombination system catalyzing conversion of plasmid or chromosome dimers into monomers [3, 19, 41, 68]. Four chromosome-encoded proteins, namely ArgR [68, 69], PepA [67], XerC [19], and XerD [4], are involved in the recombination at the ColE1 cer site.

The present study was undertaken to characterize the replication loci of seven small *Vibrionaceae* plasmids. A PCR-based replicon rescuing method was developed to enable the rapid isolation of minimal replication proficient fragments. Comparative sequence analysis of the seven replication proficient fragments identified in this study with eight published *Vibrionaceae* plasmid sequences revealed a group of *Vibrionaceae* plasmids specifying replication participating elements for the ColE1 mode of replication initiation and regulation. Thus, the 15 *Vibrionaceae* plasmids were designated CLV (ColE1-like *Vibrionaceae*) plasmids. The CLV plasmids can encode an ~70 nt RNA I specifying two stem-loop structures instead of three for the ~108 nt RNA I molecule encoded by ColE1-like plasmids. The coexistence of CLV plasmids indicated that their incompatibility mechanism is specified mainly by the RNA I species. Sequences resembling the conserved Xer recombination sites were identified on the CLV plasmids. Phylogenetic analysis of the CLV plasmid replicons revealed their evolutionary relatedness with the ColE1-like plasmid replicons. To our knowledge, this is the first report of the ColE1 mode of replication adopted by *Vibrionaceae* plasmids.

**Materials and Methods**

**Bacterial Strains, Culture Media, and DNA Isolation**

The bacterial strains and the plasmids used in this study are listed in Table 1. One g of sediment sample collected from the Mai Po Nature Reserve, Hong Kong in August 2006 was serially diluted in artificial seawater (37 g of sea salts per liter of distilled water; Sigma Chemicals Co., St. Louis, MO, U.S.A.) and the dilutions were spread onto TCBS agar plates and incubated overnight at 30°C. Yellow or green colonies were picked from the plates and restreaked at least three times on the same medium for purification. The purified TCBS isolates were routinely grown at 30°C in Luria–Bertani broth amended with NaCl to 10% of salinity. *Escherichia coli* strains were grown at 37°C in Luria–Bertani broth supplemented with 100 µg/ml of ampicillin.

One hundred purified TCBS isolates were screened for the presence of plasmids by the standard techniques [60]. Fifty isolates carrying plasmids were used in this study. Plasmid DNA from strains VP1 to VP11 (Table 1) was extracted by using the Qiagen Plasmid Miniprep Kit (Qiagen) according to the manufacturer’s instruction. Plasmid DNA was purified from agarose gel with the Viogene Gel-M Gel Extraction System (Viogene) according to the manufacturer’s instruction. Genomic DNA of purified Vibrio isolates was extracted by using the Qiagen DNeasy Tissue Kit (Qiagen) according to the manufacturer’s instruction.

**PCR Amplification**

DNA fragments of 16S rRNA genes were amplified from genomic DNA by using the primer pair 27f/1492r as previously described [30]. The PCR conditions were 10 min for activating the polymerase at 95°C and then 35 cycles consisting of 1 min at 95°C, 1 min at 54°C, and 1 min at 72°C, and finally 7 min of extension at 72°C. The PCR condition for primer sets VCf/r was as follows: step 1, 5 min at 95°C; step 2, 25 cycles consisting of 0.5 min at 95°C, 1 min at 47°C, and 1 min at 72°C; step 3, 10 min of extension at 72°C. For primer sets ApR (F/R), ApP (F/R), and Pro (F/R), amplification was conducted for 35 cycles under the following conditions: 0.5 min at 95°C, 1 min at 55°C, and 2 min at 72°C, with initial incubation at 95°C for 5 min and 10 min of extension at 72°C. Each 25-µl PCR reaction mixture contained 100 ng of template DNA, 25 pmol of each primer, 5 mM each deoxynucleoside triphosphate (dNTP), and 2.5 U of Taq DNA polymerase (Promega) in the buffer provided by the manufacturer. Amplified products were electrophoresed in 1.0% agarose gels containing 0.5 mg/ml of ethidium bromide. PCR products were directly sequenced by using the corresponding PCR primers or cloned into the pMD18-T vector (TAKARA, Dalian, China) for sequencing by the M13 universal primer pair (Tech Dragon Limited, Hong Kong).

**Cloning and Sequencing the Replication Proficient Fragments**

The replicon rescuing method of Sobecky et al. [64] was used with further modification in this study. The ampicillin-resistant gene with its promoter sequence was amplified from plasmid vector pBR322 with primer pair ApR (F/R) and cloned into vector pMD18-T. As previously described [64], approximately 1 µg of plasmid DNA was partially digested with restriction endonuclease Sau3AI for 1, 10, and 30 min at 37°C, and the partially digested plasmid DNA was ligated into the Ampf gene isolated as a BamHI fragment from pMD18-T-Ap (Table 1) with T4 DNA ligase, as recommended by the manufacturer (Promega, Madison, WI, U.S.A.). The ligation mixture was transformed into *E. coli* DH5α, which was grown on Luria–Bertani agar (Gibco Scientific, Grand Island, NY, U.S.A.) supplemented with 100 µg/ml of ampicillin at 37°C for 12 to 16 h. Colonies PCR was performed with the primer pair ApR (F/R). Reverse PCR with primer pair ApP (F/R) was performed to amplify...