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Two replication regions in the pJM1 virulence plasmid of the marine pathogen *Vibrio anguillarum*

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Abstract

Vibrio anguillarum is a fish pathogen that causes vibriosis, a serious hemorrhagic septicemia, in wild and cultured fish. Many serotype O1 strains of this bacterium harbor the 65kb plasmid pJM1 carrying the majority of genes encoding the siderophore anguibactin iron transport system that is one of the most important virulence factors of this bacterium. We previously identified a replication region of the pJM1 plasmid named *ori1*. In this work we determined that *ori1* can replicate in *E. coli* and that the chromosome-encoded proteins DnaB, DnaC and DnaG are essential for its replication whereas PolII, IHF and DnaA are not required. The copy number of the pJM1 plasmid is 1–2, albeit cloned smaller fragments of the *ori1* region replicate with higher copy numbers in *V. anguillarum* while in *E. coli* we did not observe an obvious difference of the copy numbers of these constructs which were all high. Furthermore, we were able to delete the *ori1* region from the pJM1 plasmid and identified a second replication region in pJM1 that we named *ori2*. This second replication region is located on ORF25 that is within the *trans*-acting factor (TAFr) region, and showed that it can only replicate in *V. anguillarum*.

Keywords

Vibrio anguillarum; Iron transport; Replication origin; *trans*-acting factor

1. Introduction

Vibrio anguillarum, commonly found in the aquatic environment, is the cause of vibriosis, a serious hemorrhagic septicemia of both wild and cultured fish (Actis et al., 2011; Naka and

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Crosa, 2011b). 23 serotypes have been reported in *V. anguillarum*, and serotypes O1 and O2 are the major causative agents of this disease (Sorensen and Larsen, 1986; Toranzo and Barja, 1990; Larsen et al., 1994). Many of the O1 serotype strains carry the 65kb plasmid pJM1, harboring the majority of genes involved in the siderophore anguibactin transport system, the most important virulence factor of this bacterium (Crosa, 1980; Crosa and Walsh, 2002; Di Lorenzo et al., 2003; Actis et al., 2011). In the pJM1 plasmid, there are several characteristic regions surrounded by transposon or insertion sequences (Fig. 1), and we proposed that pJM1 was acquired by *V. anguillarum* during evolution to gain the ability to compete for iron in environmental and/or host conditions where the amount of free iron is extremely limited (Tolmasky and Crosa, 1995; Di Lorenzo et al., 2003; Naka et al., 2008). The pJM1 iron transport operon (ITBO) carries the genes encoding enzymes for the biosynthesis of anguibactin (*angR* and *angT*) and transport (*fatABCD*), and also encodes antisense RNAs that influence the expression of the ITBO (Actis et al., 1985, 1988; Salinas et al., 1989; Farrell et al., 1990; Salinas et al., 1993; Tolmasky et al., 1993; Waldbeser et al., 1993; Actis et al., 1995; Salinas and Crosa, 1995; Waldbeser et al., 1995; Chen and Crosa, 1996; Chen et al., 1996; Wertheimer et al., 1999; Lopez et al., 2007; Stork et al., 2007; Naka et al., 2010) (Fig. 1). There is another region encoding the *trans*-acting factor (TAF) that is subdivided in two subregions TAFr and TAFb. TAFr is involved in the activation of the transcription of the ITBO operon via mechanisms that are still unknown, whereas TAFb carries the anguibactin biosynthesis genes, *angBCDE* (Tolmasky et al., 1988; Welch et al., 2000; Di Lorenzo et al., 2003) (Fig. 1). We identified a replication origin, named *ori1* between open reading frames ORF49 and 50 (Chen, 1995; Di Lorenzo et al., 2003) (Fig. 1), and in this study, we discuss results of an in depth analysis of this replication origin. Furthermore, we identified that homologues of replication related genes potentially encoding, DNA helicase, ParA and ParB. The latter two are located within TAFr and thus far from *ori1* (Di Lorenzo et al., 2003) (Fig. 1). In this work we also demonstrate that within TAFr there is a second origin of replication, *ori2*, that is able to replicate independently of *ori1* in *V. anguillarum* and that is inactive in *E. coli*.

2. Materials and Methods

2.1. Strains, plasmids and growth conditions

Strains, plasmids and primers used in this study are listed in Tables 1 and 2. *Vibrio anguillarum* strains were grown at 25°C in trypticase soy broth supplemented with 1% NaCl (TSBS) or on TSBS with 1.5% agar (TSAS). In some experiments, *V. anguillarum* was incubated in CM9 minimal medium (Naka and Crosa, 2011a). *E. coli* strains were grown at 37 °C in LB broth or M9 minimal medium (Sambrook and Russell, 2001), or on LB agar. When needed, antibiotics were added at the following concentrations, for *E. coli*, ampicillin (Amp) 100µg/ml, chloramphenicol (Cm) 30µg/ml and kanamycin (Km) 50µg/ml; for *V. anguillarum*, Cm 10µg/ml, Km 50µg/ml and rifampicin (Rif) 100µg/ml.

2.2. Determination of copy numbers of pJM1 and the *ori1*-containing derivatives

The pJM1 plasmid was digested with *Sau3AI*, *EcoRI*, *XhoI* or *BamHI*. The obtained fragments containing *ori1* were cloned into the appropriate restriction enzyme sites located at both ends of the 3kb Km resistance fragments. The plasmid copy number was determined by the method described by De Graaff et al. (1976). Briefly, 3–5 ml of exponential phase cultures of *V. anguillarum* H775-3 and *E. coli* strains harboring plasmids were labeled with [³H] thymine. Cell lysates were obtained by the modified method of Young and Sinsheimer (1967) by replacing sodium lauryl sulfate with 1.2% Sarkosyl. After adding cesium chloride and ethidium bromide in the cleared lysate, the refractive index was adjusted with the TES buffer to 1.3955. The samples were centrifuged in a Beckman type 50 rotor for longer than 40 hours, and the gradients were collected on Whatman filters. The DNA was precipitated

by trichloroacetic acid, and the radioactivity was measured in a liquid scintillation counter. Copy numbers were calculated by the following equation: copy number = [cpm of plasmid peak/cpm of chromosome peak] × [MWc (molecular weight of chromosome)/MWp (molecular weight of plasmid)].

2.3. Determination of essentiality of host factors for the *ori1* replication

To test whether any proteins synthesized from plasmid and/or chromosome were involved in the replication of Rep181, which was assessed by culturing *E. coli* HB101 harboring Rep181 with or without adding Cm, a protein synthesis inhibitor. pBR322 was used as a control since this plasmid can replicate exponentially in the presence of Cm (Clewell, 1972). *E. coli* HB101 carrying Rep181 or pBR322 were incubated overnight in M9 minimal medium with appropriate antibiotics. The overnight culture was diluted (50 times) into fresh M9 minimal medium, separated into two portions, and to one of the portions 170 µg/ml Cm were added. Samples were harvested at different time points, and plasmid DNA was extracted. Extracted DNA was linearized by digesting with *Pst*I and examined using a 0.8% agarose gel. The intensity of DNA bands on the gel was compared, and the data was normalized to the cell density (OD₆₀₀).

E. coli C2110 that is a DNA polymerase I (PolI) deficient strain was used to transform Rep181 to check the requirement of PolI for *ori1*. To test whether *ori1* require DnaA and integration host factor (IHF), Rep181 was transformed into the following strains: EH3896 (*dnaA*⁺) and EH3894 (*dnaA*[−]) for DnaA, and N99 (IHF⁺) and IHF mutants such as HN545 (*hip157*) and HN678 (*himA*) for IHF, and analyzed the existence of colonies after overnight incubation at 37°C. The necessity of DnaB, DnaC and DnaG for *ori1* replication was determined using the temperature sensitive *E. coli* strain mutants E107 (*dnaB* ts), PC1 (*dnaC* ts) and PC3 (*dnaG* ts), respectively. Rep181 was transformed into these strains, and incubated at 25°C. Obtained colonies were cultured into M9 minimal medium at 25°C until exponential phase and separated into two portions, one portion was incubated at 25°C and the other portion was incubated at 37°C. Plasmids were extracted from cells harvested at different time points and their banding profile and concentration were determined.

2.4. Construction of *V. anguillarum* 775(pJM1-Km)

A Km resistance gene was amplified by PCR with primers, Km-*Sma*I F and Km-*Sma*I R using pKD4 as a template, and cloned into pBluescript generating pBlue-Km-*Sma*I. The DNA fragment containing *angA* was PCR amplified by using primers, *angAp* Km *Spe*I and *angAp* Km *Sac*I, cloned into pCR-bluntII-TOPO (Invitrogen, Carlsbad, CA), and then subcloned into pBluescript. The plasmid was digested with *Stu*I, and the Km cassette (*Sma*I sites in both sides) from pBlue-Km-*Sma*I was ligated in the *Stu*I site. *angA*::Km thus obtained was moved into the *Spe*I and *Sac*I site of the pDM4 plasmid generating pDM4-*angA*::Km. This plasmid was conjugated into *V. anguillarum* 775(pJM1), and first recombinants were selected by Cm and Km resistance, and grew on TSAS supplemented with 15% sucrose and Km to obtain second recombinants which were confirmed by Cm sensitivity, pJM1 plasmid digestion pattern and PCR.

2.5. Construction of a deletion mutant of *ori1* in pJM1

Δ*ori1* has been constructed by the allelic exchange method. DNA fragments that locate upstream and downstream of *ori1* were amplified by PCR using primer sets, *ori1*-mut-up*Sac*I and *ori1*-mut-up*EcoRV*, and *ori1*-mut-dn*EcoRV* and *ori1*-mut-dn*Xho*I, respectively. The amplified fragments were separately cloned into pCR-BluntII-TOPO (Invitrogen), and subsequently cloned together in pBluescript. The inserted DNA digested with *Sac*I and *Xho*I was purified, and ligated into pDM4 generating pDM4-Δ*ori1*. This plasmid was conjugated into *V. anguillarum* by the method described before, and first recombinants were selected by

antibiotics resistance to 100 µg/ml Rif and 10 µg/ml Cm. Second recombinants were obtained as described before.

3. Results

3.1. Determination of copy numbers of the pJM1 plasmid and its derivatives

The replication origin of the pJM1 plasmid of *V. anguillarum* 775 was first identified by our group within a stretch of 1.8 kbs (Chen, 1995). Wu et al. (2004) later, isolated the replication origin from pEIB1 that is highly related the pJM1 plasmid. The nucleotide sequence of the replication region is identical. We designated the replication origin as *ori1* to avoid confusion with another replication origin mentioned later. We have made a series of constructs, Rep181, Rep182 and Rep183, encompassing the *ori1* region (Fig. 1) that we cloned to the kanamycin (Km) resistance gene, and determined their copy number in *V. anguillarum* H775-3, a pJM1 cured strain, and *E. coli* HB101. Our results (Table 3) indicated that the copy number of the pJM1 plasmid in *V. anguillarum* is 1 to 2. Except for the shortest construct containing a 1.8 kb fragment encompassing *ori1* that showed a high copy number, 65, the rest of the derivatives showed medium copy numbers, 7 to 16 indicating that some factors located outside of the 1.8kb fragment control the copy number of the pJM1 plasmid. However, we did not observe this effect when we determined the copy number of the pJM1 derivatives in *E. coli* (all constructs showed similar copy number: 50–75) indicating that this copy number control specifically occurs in *V. anguillarum*.

3.2. Assessment of the essentiality of the chromosome encoded proteins for the replication of *ori1*

Two types of mechanisms for the initiation of plasmid replication have been reported. One mechanism found in ColE1 related plasmids, involves opening of the helix through an RNA transcript. The other mechanism found in other plasmids is that a plasmid-encoded Rep protein and/or chromosome-encoded factors bind to a specific region in an *ori*, and facilitate opening of the DNA helix (Kues and Stahl, 1989).

To test whether *ori1* requires protein synthesis from chromosome or the plasmid for its replication, we tested the replication of Rep181 in the presence or absence of chloramphenicol (Cm) that is an inhibitor of protein synthesis. As shown in Fig. 2 in the absence of Cm the copy number of Rep181 increases along with the incubation time while it remains low even after 16 hours incubation (panel A and C). The pBR322 plasmid in which replication can be processed without proteins was used as a control, and as we expected, the copy number of this plasmid increased in the presence of Cm (panel B and C). From these results, we can conclude that proteins from the plasmid or the chromosome are essential for the replication of the pJM1 replication origin *ori1*.

It has been reported that many plasmids require proteins originated from the chromosome-encoded genes (Scott, 1984; Tolmasky et al., 2010). The DnaA protein plays a key role on the initiation of replication of the bacterial chromosome as well as several plasmids in combination with plasmid-encoded Rep proteins by unwinding of the DNA in the *ori* region and helicase loading (Bramhill and Kornberg, 1988; Kaguni, 1997; Messer et al., 2001). The integration host factor (IHF) is a DNA-binding protein that enhances the bending of DNA in the *ori* region through its interaction with DNA to promote initiation of replication (Friedman, 1988; Rice, 1997), and IHF requires for the replication of plasmids such as pSC101 and R6K (Biek and Cohen, 1989; Dellis and Filutowicz, 1991). DnaB (helicase), DnaC (helicase accessory protein) and DnaG (primase) also involved in the replication of some plasmids such as RK2 and ColE1 plasmids (Pinkney et al., 1988; Konieczny and Helinski, 1997; Wang et al., 2004). In this study, we determined which chromosome-

encoded factors are essential for the replication of *ori1* in *E. coli*. Our results shown in Table 4 indicate that *ori1* replication requires DnaB, DnaC and DnaG while PolII, IHF and DnaA were not indispensable

3.3. The pJM1 plasmid can still replicate without *ori1*

Some plasmids carry several replication origins thus we determined whether *ori1* is essential for the replication of the pJM1 plasmid. First, we constructed pJM1-Km in which a Km resistance gene from pKD4 inserted into the *angA* gene that has been shown dysfunctional due to the lack of the N-terminal region, and then deleted the *ori1* replication region by the allelic exchange method. This experiment generated a pJM1 derivative in which the *ori1* region from the pJM1-Km plasmid was deleted demonstrating that there must be another replication origin in the pJM1 plasmid.

3.4. Identification and characterization of the second replication origin, *ori2* in pJM1

In the pJM1 plasmid there is a region that carries genes for replication and partition related proteins such as *parA*, *parB* and the DNA helicase gene homologues (Di Lorenzo et al., 2003), thus we suspected that the second replication origin could be located around this region. We PCR amplified several fragments around this region, and cloned into the suicide vector pDM4 that carries the R6K origin (Milton et al., 1996). The pDM4 derivatives thus obtained were conjugated into *V. anguillarum* H775-3, the pJM1-cured strain. Table 5 shows the constructs that were successfully conjugated and that replicated in this strain indicating that the second replication origin is indeed located within these regions. The common overlapping region in all of the constructs corresponds approximately to ORF25. We then cloned the ORF25 gene in pDM4 generating pDM4-ORF25. When pDM4-ORF25 was transformed into *E. coli* with or without λ pir that provides the π protein that R6K origin requires for the replication. Transformants were only obtained when *E. coli* carries λ pir demonstrating that ORF25 does not replicate in *E. coli* (Table 5). Furthermore, we were only able to transform pJM1-Km but not pJM1-Km Δ *ori1* into *E. coli* (Table 5). Next, we conjugated pDM4-ORF25 into *V. anguillarum* H775-3, and successfully obtained colonies while no colonies were obtained when empty pDM4 was attempted to be conjugated into H775-3 (Table 5). These results indicate that the second replication origin of the pJM1 plasmid is included within ORF25, and it replicates only in *V. anguillarum*. We designated this replication origin as *ori2* (Fig. 1). Furthermore, the stability of pDM4-ORF25 was tested, and the results indicated that the pDM4-ORF25 was very stable in *V. anguillarum* H775-3 as compared with pDM4-*ori1* that has been shown unstable in *V. anguillarum* (data not shown).

4. Discussion

Many *V. anguillarum* O1 serotype strains carry the 65 kb plasmid pJM1 harboring genes encoding the siderophore anguibactin iron uptake system that is the most important virulence factor for this bacterium (Crosa, 1980; Crosa and Walsh, 2002; Di Lorenzo et al., 2003; Actis et al., 2011). We have identified two replication regions in the pJM1 plasmid. One is located between ORFs 49 and 50 (Chen, 1995), in this study we identified a second replication region on ORF25 that is located within the TAFr region involved in up-regulation of the iron transport and biosynthesis operon (ITBO) (Tolmasey et al., 1988; Welch et al., 2000; Di Lorenzo et al., 2003). ORF25 is located close to genes potentially encoding replication and partition related proteins such as DNA helicase (ORF19), ParB (ORF27) and ParA (ORF28). We previously reported that the region including ORF19-ORF45 in the pJM1 plasmid is flanked by two identical ISVme-like sequences (Di Lorenzo et al., 2003) (Fig. 1). This region includes *ori2* and replication related proteins, and many unknown or hypothetical proteins indicating that the *ori2* region might be acquired by a

pJM1 ancestor plasmid carrying *oriI* through transposition events and horizontal transfer. The Rep181 plasmid including the *oriI* region was not stable in *V. anguillarum* while the *ori2* plasmid, pDM4-ORF25 was stable in this bacterium suggesting that these replication and partition related proteins might be important for the stability of the *oriI* region in *V. anguillarum*.

The *oriI* region of the pJM1 plasmid can replicate in both *V. anguillarum* and *E. coli* and is similar to the replication origin found in pEBI1 (Wu et al., 2004), while the *ori2* region can only replicate in *V. anguillarum* indicating that some *V. anguillarum* chromosome-encoded proteins are required for the *ori2* replication in *E. coli*. We showed that the *oriI* replication origin of the pJM1 plasmid requires DnaB, DnaC and DnaG but not PolI, IHF and DnaA. Since *ori2* cannot replicate in *E. coli*, we were not able to test its requirement of chromosome-encoded proteins.

The copy number of the pJM1 plasmid was determined as 1–2 while shorter fragments carrying the *oriI* region exhibited higher copy numbers as compared to the whole pJM1 plasmid. The pJHC9-8 plasmid carrying *oriI*, *ori2* and possible replication proteins showed slightly higher copy number than the whole pJM1 plasmid and lower copy number than shorter constructs Rep181-184. Furthermore, the shortest constructs Rep181 showed much higher copy number as compared with rest of constructs. From these results we could speculate that some factors that negatively control copy number might exist in the region in Rep182 and Rep183 but absent from Rep181 increasing the copy number of Rep181. Also, *ori2* and/or possible replication proteins found in the pJM1 plasmid could affect the copy number because Rep181-4 in which *ori2* and possible replication proteins are missing showed higher copy number than pJHC9-8 that carries all *oriI*, *ori2* and possible replication protein genes. These copy number controls occurred only in *V. anguillarum* because all the constructs show similar copy number in *E. coli*. Experiments are being carried out to assess the contribution of these two origins and/or the putative partition and replication proteins to pJM1 replication and maintenance.

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References

- Actis LA, Potter SA, Crosa JH. Iron-regulated outer membrane protein OM2 of *Vibrio anguillarum* is encoded by virulence plasmid pJM1. J Bacteriol. 1985; 161:736–742. [PubMed: 3968037]
- Actis, LA.; Tolmasky, ME.; Crosa, JH. Vibriosis. In: Woo, PTK.; Bruno, DW., editors. Fish Diseases and Disorders, vol. 3: Viral, Bacterial, and Fungal Infections. 2. CABI International; Oxfordshire, UK: 2011. p. 570-605.
- Actis LA, Tolmasky ME, Crosa LM, Crosa JH. Characterization and regulation of the expression of FatB, an iron transport protein encoded by the pJM1 virulence plasmid. Mol Microbiol. 1995; 17:197–204. [PubMed: 7476205]
- Actis LA, Tolmasky ME, Farrell DH, Crosa JH. Genetic and molecular characterization of essential components of the *Vibrio anguillarum* plasmid-mediated iron-transport system. J Biol Chem. 1988; 263:2853–2860. [PubMed: 2830268]
- Biek DP, Cohen SN. Involvement of integration host factor (IHF) in maintenance of plasmid pSC101 in *Escherichia coli*: characterization of pSC101 mutants that replicate in the absence of IHF. J Bacteriol. 1989; 171:2056–2065. [PubMed: 2539358]
- Boyer HW, Roulland-Dussoix D. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J Mol Biol. 1969; 41:459–472. [PubMed: 4896022]
- Bramhill D, Kornberg A. A model for initiation at origins of DNA replication. Cell. 1988; 54:915–918. [PubMed: 2843291]

- Carl PL. *Escherichia coli* mutants with temperature-sensitive synthesis of DNA. *Mol Gen Genet*. 1970; 109:107–122. [PubMed: 4925091]
- Chen, Q. PhD thesis. Oregon Health and Science University; Portland, Oregon: 1995.
- Chen Q, Crosa JH. Antisense RNA, *fur*, iron, and the regulation of iron transport genes in *Vibrio anguillarum*. *J Biol Chem*. 1996; 271:18885–18891. [PubMed: 8702549]
- Chen Q, Wertheimer AM, Tolmasky ME, Crosa JH. The AngR protein and the siderophore anguibactin positively regulate the expression of iron-transport genes in *Vibrio anguillarum*. *Mol Microbiol*. 1996; 22:127–134. [PubMed: 8899715]
- Clewell DB. Nature of Col E 1 plasmid replication in *Escherichia coli* in the presence of the chloramphenicol. *J Bacteriol*. 1972; 110:667–676. [PubMed: 4336693]
- Crosa JH. A plasmid associated with virulence in the marine fish pathogen *Vibrio anguillarum* specifies an iron-sequestering system. *Nature*. 1980; 284:566–568. [PubMed: 7366725]
- Crosa JH, Walsh CT. Genetics and assembly line enzymology of siderophore biosynthesis in bacteria. *Microbiol Mol Biol Rev*. 2002; 66:223–249. [PubMed: 12040125]
- De Graaff J, Elwell LP, Falkow S. Molecular nature of two beta-lactamase-specifying plasmids isolated from *Haemophilus influenzae* type b. *J Bacteriol*. 1976; 126:439–446. [PubMed: 770429]
- Dellis S, Filutowicz M. Integration host factor of *Escherichia coli* reverses the inhibition of R6K plasmid replication by pi initiator protein. *J Bacteriol*. 1991; 173:1279–1286. [PubMed: 1991721]
- Di Lorenzo M, Stork M, Tolmasky ME, Actis LA, Farrell D, Welch TJ, Crosa LM, Wertheimer AM, Chen Q, Salinas P, Waldbeser L, Crosa JH. Complete sequence of virulence plasmid pJM1 from the marine fish pathogen *Vibrio anguillarum* strain 775. *J Bacteriol*. 2003; 185:5822–5830. [PubMed: 13129954]
- Farrell DH, Mikesell P, Actis LA, Crosa JH. A regulatory gene, *angR*, of the iron uptake system of *Vibrio anguillarum*: similarity with phage P22 *cro* and regulation by iron. *Gene*. 1990; 86:45–51. [PubMed: 2311935]
- Friedman DI. Integration host factor: a protein for all reasons. *Cell*. 1988; 55:545–554. [PubMed: 2972385]
- Hansen EB, Yarmolinsky MB. Host participation in plasmid maintenance: dependence upon *dnaA* of replicons derived from P1 and F. *Proc. Natl Acad Sci U S A*. 1986; 83:4423–4427.
- Kaguni JM. *Escherichia coli* DnaA protein: the replication initiator. *Mol Cells*. 1997; 7:145–157. [PubMed: 9163725]
- Konieczny I, Helinski DR. Helicase delivery and activation by DnaA and TrfA proteins during the initiation of replication of the broad host range plasmid RK2. *J Biol Chem*. 1997; 272:33312–33318. [PubMed: 9407123]
- Kues U, Stahl U. Replication of plasmids in gram-negative bacteria. *Microbiol Rev*. 1989; 53:491–516. [PubMed: 2687680]
- Larsen JL, Pedersen K, Dalgaard I. *Vibrio anguillarum* serovars associated with vibriosis in fish. *J Fish Dis*. 1994; 17:259–267.
- Lopez CS, Alice AF, Chakraborty R, Crosa JH. Identification of amino acid residues required for ferric-anguibactin transport in the outer-membrane receptor FatA of *Vibrio anguillarum*. *Microbiology*. 2007; 153:570–584. [PubMed: 17259629]
- Messer W, Blaesing F, Jakimowicz D, Krause M, Majka J, Nardmann J, Schaper S, Seitz H, Speck C, Weigel C, Wegrzyn G, Welzeck M, Zakrzewska-Czerwinska J. Bacterial replication initiator DnaA. Rules for DnaA binding and roles of DnaA in origin unwinding and helicase loading. *Biochimie*. 2001; 83:5–12. [PubMed: 11254968]
- Milton DL, O'Toole R, Horstedt P, Wolf-Watz H. Flagellin A is essential for the virulence of *Vibrio anguillarum*. *J Bacteriol*. 1996; 178:1310–1319. [PubMed: 8631707]
- Naka H, Crosa JH. Identification and characterization of a novel outer membrane protein receptor FetA for ferric enterobactin transport in *Vibrio anguillarum* 775 (pJM1). *Biometals*. 2011a; 10.1007/s10534-011-9488-4
- Naka H, Crosa JH. Genetic determinants of virulence in the marine fish pathogen *Vibrio anguillarum*. *Fish Pathol*. 2011b; 46:1–10. [PubMed: 21625345]

- Naka H, Lopez CS, Crosa JH. Reactivation of the vanchrobactin siderophore system of *Vibrio anguillarum* by removal of a chromosomal insertion sequence originated in plasmid pJM1 encoding the anguibactin siderophore system. *Environ Microbiol.* 2008; 10:265–277. [PubMed: 18005167]
- Naka H, Lopez CS, Crosa JH. Role of the pJM1 plasmid-encoded transport proteins FatB, C and D in ferric anguibactin uptake in the fish pathogen *Vibrio anguillarum*. *Environ Microbiol Rep.* 2010; 2:104–111. [PubMed: 21304833]
- Pinkney M, Diaz R, Lanka E, Thomas CM. Replication of mini RK2 plasmid in extracts of *Escherichia coli* requires plasmid-encoded protein TrfA and host-encoded proteins DnaA, B, G DNA gyrase and DNA polymerase III. *J Mol Biol.* 1988; 203:927–938. [PubMed: 2850370]
- Rice PA. Making DNA do a U-turn: IHF and related proteins. *Curr Opin Struct Biol.* 1997; 7:86–93. [PubMed: 9032059]
- Salinas PC, Crosa JH. Regulation of *angR*, a gene with regulatory and biosynthetic functions in the pJM1 plasmid-mediated iron uptake system of *Vibrio anguillarum*. *Gene.* 1995; 160:17–23. [PubMed: 7543065]
- Salinas PC, Tolmasky ME, Crosa JH. Regulation of the iron uptake system in *Vibrio anguillarum*: evidence for a cooperative effect between two transcriptional activators. *Proc Natl Acad Sci U S A.* 1989; 86:3529–3533. [PubMed: 2542936]
- Salinas PC, Waldbeser LS, Crosa JH. Regulation of the expression of bacterial iron transport genes: possible role of an antisense RNA as a repressor. *Gene.* 1993; 123:33–38. [PubMed: 7678569]
- Sambrook, J.; Russell, DW. *Molecular cloning: a laboratory manual.* 3. Cold Spring Harbor Laboratory Press; Cold Spring Harbor, NY, USA: 2001.
- Scott JR. Regulation of plasmid replication. *Microbiol Rev.* 1984; 48:1–23. [PubMed: 6201704]
- Simon R, Priefer U, Puhler A. A broad host range mobilization system *in vivo* genetic engineering transposon mutagenesis in gram negative bacteria. *Bio/Technology.* 1983; 1:787–796.
- Sorensen UB, Larsen JL. Serotyping of *Vibrio anguillarum*. *Appl Environ Microbiol.* 1986; 51:593–597. [PubMed: 3963811]
- Stork M, Di Lorenzo M, Welch TJ, Crosa JH. Transcription termination within the iron transport-biosynthesis operon of *Vibrio anguillarum* requires an antisense RNA. *J Bacteriol.* 2007; 189:3479–3488. [PubMed: 17337574]
- Tolmasky ME, Actis LA, Crosa JH. Genetic analysis of the iron uptake region of the *Vibrio anguillarum* plasmid pJM1: molecular cloning of genetic determinants encoding a novel trans activator of siderophore biosynthesis. *J Bacteriol.* 1988; 170:1913–1919. [PubMed: 2832388]
- Tolmasky ME, Actis LA, Crosa JH. A single amino acid change in AngR, a protein encoded by pJM1-like virulence plasmids, results in hyperproduction of anguibactin. *Infect Immun.* 1993; 61:3228–3233. [PubMed: 8335354]
- Tolmasky, ME.; Actis, LA.; Crosa, JH. Plasmid DNA replication. In: Flickinger, M., editor. *Encyclopedia of Bioprocess Technology.* Wiley Online Library; New York, NY: 2010. p. 3931-3953.
- Tolmasky ME, Crosa JH. Iron transport genes of the pJM1-mediated iron uptake system of *Vibrio anguillarum* are included in a transposonlike structure. *Plasmid.* 1995; 33:180–190. [PubMed: 7568465]
- Tolmasky ME, Salinas PC, Actis LA, Crosa JH. Increased production of the siderophore anguibactin mediated by pJM1-like plasmids in *Vibrio anguillarum*. *Infect Immun.* 1988; 56:1608–1614. [PubMed: 3372022]
- Toranzo AE, Barja JL. A review of the taxonomy and seroepizootiology of *Vibrio anguillarum*, with special reference to aquaculture in the northwest Spain. *Dis Aquat Org.* 1990; 9:73–82.
- Waldbeser LS, Chen Q, Crosa JH. Antisense RNA regulation of the fatB iron transport protein gene in *Vibrio anguillarum*. *Mol Microbiol.* 1995; 17:747–756. [PubMed: 8801428]
- Waldbeser LS, Tolmasky ME, Actis LA, Crosa JH. Mechanisms for negative regulation by iron of the *fata* outer membrane protein gene expression in *Vibrio anguillarum* 775. *J Biol Chem.* 1993; 268:10433–10439. [PubMed: 7683679]
- Walter MA, Potter SA, Crosa JH. Iron uptake system mediated by *Vibrio anguillarum* plasmid pJM1. *J Bacteriol.* 1983; 156:880–887. [PubMed: 6313622]

- Wang Z, Yuan Z, Hengge UR. Processing of plasmid DNA with ColE1-like replication origin. *Plasmid*. 2004; 51:149–161. [PubMed: 15109822]
- Wechsler JA, Gross JD. *Escherichia coli* mutants temperature-sensitive for DNA synthesis. *Mol Gen Genet*. 1971; 113:273–284. [PubMed: 4946856]
- Welch TJ, Chai S, Crosa JH. The overlapping *angB* and *angG* genes are encoded within the *trans*-acting factor region of the virulence plasmid in *Vibrio anguillarum*: essential role in siderophore biosynthesis. *J Bacteriol*. 2000; 182:6762–6773. [PubMed: 11073922]
- Wertheimer AM, Verweij W, Chen Q, Crosa LM, Nagasawa M, Tolmasky ME, Actis LA, Crosa JH. Characterization of the *angR* gene of *Vibrio anguillarum*: essential role in virulence. *Infect Immun*. 1999; 67:6496–6509. [PubMed: 10569768]
- Wu H, Ma Y, Zhang Y, Zhang H. Complete sequence of virulence plasmid pEIB1 from the marine fish pathogen *Vibrio anguillarum* strain MVM425 and location of its replication region. *J Appl Microbiol*. 2004; 97:1021–1028. [PubMed: 15479418]
- Young, ETn; Sinsheimer, RL. Vegetative bacteriophage lambda-DNA. II Physical characterization and replication. *J Mol Biol*. 1967; 30:165–200. [PubMed: 4865143]

Highlights

Two replication regions were identified in the *Vibrio anguillarum* pJM1 plasmid.

The copy number of pJM1 is 1 to 2.

ori1 requires plasmid or host proteins for its replication.

The *ori1* region replicates in both *E. coli* and *V. anguillarum*.

The *ori2* region only replicates in *V. anguillarum*.

Fig. 1. Schematic map of the pJM1 plasmid

Predicted ORFs are shown as arrows. Genes and antisense RNAs (*rnaA* encoding RNA α , and *rnaB* encoding RNA β) are depicted as black-filled arrows and blocks, respectively. The grey-filled arrows represent insertion sequence or transposon related genes. *ori1* and *ori2* regions are surrounded by dotted lines. *trans*-acting factor (TAF) regions composed of TAFb and TAFr, are also shown by dotted lines. Lines inside the plasmid map denote the DNA fragments used to construct Rep181, Rep182, Rep183 and pJHC9-8.

Fig. 2. The effect of chloramphenicol on Rep181 replication in *V. anguillarum*
 The presence or absence of chloramphenicol (Cm) is indicated with Cm⁺ or Cm⁻, respectively. Panel A, detection of *Pst*I-linearized Rep181 plasmid DNA levels on 0.8% agarose gel. Panel B, detection of *Pst*I-linearized pBR322 plasmid DNA levels on 0.8% agarose gel. Panel C, comparison of DNA replication efficiency of plasmid Rep181 and pBR322 in the presence and absence of Cm. The amounts of linearized DNAs were normalized with the cell density at each time point. This experiment was carried out in triplicate and the standard deviations are shown in this panel.

Table 1

Strains and plasmids used in this study

Strains and plasmids	Characteristics	Reference or source
<i>V. anguillarum</i> strains		
775(pJM1)	Wild type, Washington	Laboratory stock
H775-3	Plasmidless derivative of 775(pJM1)	Laboratory stock
775(pJM1-Km)	The Km cassette in <i>angA</i>	This study
775(pJM1-Km Δ <i>oriI</i>)	<i>oriI</i> region deletion mutant of 775(pJM1-Km)	This study
<i>E. coli</i> strains		
HB101	<i>supE44 hsd20 (r^{-B}m^{-B}) recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1</i>	Boyer and Roulland-Dussoix (1969)
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 deoR</i> <i>recA1 ara</i> Δ 139 Δ (<i>ara-leu</i>) 7697 <i>galUgalK</i> rpsL (Str ^r) <i>endA1 nupG</i>	Invitrogen
S17-1 λ pir	<i>thi pro hsdR</i> hsdM ⁺ <i>recA</i> RP4-2-Tc::Mu-Km::Tn7 λ pir	Simon et al. (1983)
DH5 α	F ⁻ , ϕ 80 <i>lacZ</i> Δ M15, <i>endA1, recA1, hsdR17, (r_K⁻m_K⁺), supE44, thi-1, gyrA96, relA1, Δ(<i>lacZYA-argF</i>)U169, λ⁻</i>	Laboratory stock
JM109 λ pir	<i>recA1 endA1 gyrA96 thi hsdR17 supE4relA1</i> Δ (<i>lac-proAB</i> ⁺) (F' <i>traD36 proABlac</i> ⁺ <i>lacZ</i> Δ M15) λ pir	Laboratory stock
EH3896	CM1565 <i>zia</i> ::pKN500 <i>dnaA</i> ⁺ <i>mad-1 tnaA</i> ::Tn10	Hansen and Yarmolinsky (1986)
EH3894	CM1565 <i>zia</i> ::pKN500 Δ <i>dnaA</i> <i>mad-1 tnaA</i> ::Tn10	Hansen and Yarmolinsky (1986)
E107	F ⁻ , <i>thr-1, leuB6</i> (Am), <i>fluA21, lacY1, glnV44</i> (AS), λ ⁻ , <i>rfbC1, thyA6, rpsL67</i> (strR), <i>thi-1, dnaB107</i> (ts), <i>deoC1</i>	Wechsler and Gross (1971)
PC1	F ⁻ , <i>leuB6</i> (Am), λ ⁻ , <i>thyA47, rpsL153</i> (strR), <i>dnaC1</i> (ts), <i>deoC3</i>	Carl (1970); Wechsler and Gross (1971)
PC3	F ⁻ , <i>leuB6</i> (Am), λ ⁻ , <i>thyA47, dnaG3</i> (ts), <i>rpsL153</i> (strR), <i>deoC3</i>	Carl (1970); Wechsler and Gross (1971)
Plasmids		
Rep181	<i>Sau3A</i> I partially digested fragment of pJM1, containing <i>oriI</i> , combined with a 3kb Km resistance fragment	This study
Rep182	<i>EcoR</i> I digested fragment of pJM1, containing <i>oriI</i> , combined with a 3kb Km resistance fragment	This study
Rep183	<i>Xho</i> I digested fragment of pJM1, containing <i>oriI</i> , combined with a 3kb Km resistance fragment	This study
Rep184	<i>Bam</i> HI digested fragment of pJM1, containing <i>oriI</i> , combined with a 3kb Km resistance fragment	This study
pJHC9-8	pJM1 derivative lacking the anguibactin biosynthesis and transport region.	Walter et al. (1983); Tolmasky et al. (1988)
pDM4	Suicide plasmid <i>sacB</i> gene, R6K origin, Cm ^r	Milton et al. (1996)
pCR-blunt2-TOPO	T-vector, Km ^r Amp ^r	Invitrogen
pBluescript II	Cloning vector, Amp ^r	Stratagene
pDM4- Δ <i>oriI</i>	pDM4 harboring Δ <i>oriI</i>	This study
pDM4- <i>oriI</i>	pDM4 harboring the <i>oriI</i> region (53803-55425 [*]) of pJM1	This study
pDM4-ORF25	pDM4 harboring ORF25 (the <i>ori2</i> region) (32297-33526 [*]) of pJM1	This study
pDM4- <i>angA</i> ::Km	pDM4 harboring <i>angA</i> interrupted by the Km resistance cassette.	This study
pBlue-Km- <i>Sma</i> I	pBluescript II carrying the Km resistance cassette with <i>Sma</i> I recognition sites in both sides.	This study

* Nucleotide coordinates in pJM1 (accession number: NC_005250)

Table 2

Primers used in this study

Primer name	Nucleotide sequence (5'-3')
ORF25- <i>SpeI</i> -F	<u>ACTAGT</u> TTACTCGCTGAGTTGTAGCAACTG
ORF25- <i>SpeI</i> -R	<u>ACTAGT</u> ATGCAGAGAACTAGAATGCAAATAAA
<i>oriI</i> - <i>Sall</i> -F	GTCGACGCCAAGCTTCCAGTGTGATGG
<i>oriI</i> - <i>SacI</i> -R	<u>GAGCTC</u> AGCAAAAATTAGGGCGGATC
<i>oriI</i> -mut-up <i>SacI</i>	<u>GAGCTC</u> TGGAGAGTTGATCTTATTCTTCG
<i>oriI</i> -mut-up <i>EcoRV</i>	<u>GATATC</u> ATTTACCAAGTAGTTGCATTGCC
<i>oriI</i> -mut-dn <i>EcoRV</i>	<u>GATATC</u> ATAAACGGTCATACTGGACACTGC
<i>oriI</i> -mut-dn <i>XhoI</i>	<u>CTCGAG</u> GATTAAAGAGATTGCAGAGCAAG
<i>angAp</i> Km <i>SpeI</i>	<u>ACTAGT</u> TTAATAACTGAACAACCTTTGGGCTG
<i>angAp</i> Km <i>SacI</i>	<u>GAGCTC</u> ATAGCACAAAGCTCTACATAATGCAG
Km- <i>SmaI</i> F	<u>CCCGGG</u> GTGTAGGCTGGAGCTGCTTCGAAG
Km- <i>SmaI</i> R	<u>CCCGGG</u> CATATGAATATCCTCCTTAGTTCC

Underlines indicate restriction enzyme recognition sites

Table 3Copy number of plasmids derived from pJM1 in *V. anguillarum* H775-3 and *E. coli* HB101

Plasmid	Cloned fragment	Size of plasmids	Copy number	
			H775-3	HB101
Rep181	<i>Sau</i> 3AI fragment in the <i>oriI</i> region	4.4	65	60
Rep182	<i>Eco</i> RI fragment	13	13	50
Rep183	<i>Xho</i> I fragment	15.4	16	73
Rep184	<i>Bam</i> HI fragment	17	N.D.	75
pJHC9-8	A deletion derivative of pJM1	45	7	50
pJM1	Wild type	65	1–2	N.D.

The size of Rep181, Rep182, Rep183 and Rep184 include the 3kb Km resistance fragments while the size of pJHC9-8 includes transposon Tn3-HoHo1. The copy number is the number of copies of a plasmid per cell or chromosome equivalent. Plasmid copy number was estimated by centrifuging a [³H] thymine-labeled total-cell-lysate in an ethidium-cesium chloride gradient. The copy number of plasmids were calculated using the following formula: copy number = (cpm of plasmid peak/cpm of chromosome peak) x (molecular weight of chromosome/molecular weight of plasmid). N.D., Not determined.

Table 4
Requirement of *E. coli* chromosome proteins for the replication of the Rep181 plasmid

	PolI	IHF	DnaA	DnaB	DnaC	DnaG
Rep181 replication	-	-	-	+	+	+

+, requires; -, does not require

Table 5Replication of plasmid derivatives of pJM1 in *V. anguillarum* and *E. coli*

	<i>V. anguillarum</i> H775-3	<i>E. coli</i> DH5a	<i>E. coli</i> JM109 λ pir
pJM1-Km	+	+	N.D.
pJM1-Km $\Delta ori1$	+	–	N.D.
pDM4- <i>ori1</i>	+	+	+
pDM4-ORF25	+	–	+

Plasmids were conjugated into *V. anguillarum* H775-3 or transformed into *E. coli* with or without λ pir, and the presence of conjugants or transformants were considered that plasmids can replicate in those hosts. +, replicates; –, does not replicate; N.D., not determined.