Virulence plasmid pJM1 prevents the conjugal entry of plasmid DNA into the marine fish pathogen *Vibrio anguillarum* 775

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Studies involving the introduction of cloned homologous genes into *Vibrio anguillarum* revealed that several plasmids could not be conjugally introduced into *V. anguillarum* 775(pJM1), but were transmissible to the pJM1-cured derivative H775-3. Recombinant pBR322 plasmids containing *V. anguillarum* genomic DNA inserts were mobilized from *Escherichia coli* donors, using pRK2013, into *V. anguillarum* H775-3 recipients at frequencies of 10^{-6} to 10^{-5} per recipient. When identical matings were performed with *V. anguillarum* 775(pJM1) recipients, the infrequent exconjugants recovered carried the pBR322-based plasmid but had lost the large virulence plasmid pJM1. Similar studies were carried out with plasmid RP4 and with recombinant derivatives of the closely related broad-host-range plasmid pRK290. While RP4 was transmissible from *E. coli* to *V. anguillarum* H775-3 at frequencies of 6.7×10^{-2} per recipient, transmission to *V. anguillarum* 775(pJM1) recipients occurred at frequencies of only 2.5×10^{-7} . When pRK290 contained *V. anguillarum* DNA inserts, the only exconjugants recovered had lost pJM1, or contained pJM1 and a deletion derivative of the recombinant pRK290 plasmid where all of the DNA insert had been deleted. The use of Dam⁻, Dcm⁻, or EcoK⁻ methylation-deficient *E. coli* donor strains failed to result in appreciable numbers of *V. anguillarum* 775(pJM1) exconjugants that contained the desired transferred plasmids. Following UV mutagenesis, a derivative of *V. anguillarum* 775(pJM1) was isolated that would accept conjugally transferred plasmid DNAs at frequencies similar to those observed when using *V. anguillarum* H775-3 recipients. These data suggest that virulence plasmid pJM1 mediates a restriction system that prevents conjugal transmission of plasmid DNA from *E. coli* donors into *V. anguillarum* 775(pJM1). This putative restriction system appears not to be directed towards Dam⁻, Dcm⁻, or EcoK-methylated DNA, and appears not to involve a Type II restriction endonuclease.

Introduction

Vibriosis, caused by the ubiquitous marine bacterium *Vibrio anguillarum*, results in a rapidly progressive haemorrhagic septicaemia that can account for high mortalities among marine animals, and especially among salmonids under conditions of high-density aquaculture. In Japan, where accurate data are available, losses of farmed finfish due to vibriosis alone exceed 20 million US dollars annually (Austin & Austin, 1987).

V. anguillarum serotype O1 is the serotype that predominates among cultured diseased fish, and especially among salmon and trout (Bolinches *et al.*, 1990; Larsen & Olsen, 1991; Larsen *et al.*, 1988; Myhr *et al.*,

1991; Toranzo & Barja, 1990; Toranzo *et al.*, 1987). Virulence of this serotype of *V. anguillarum* is nearly always due to the presence of 65–70 kb pJM1-like virulence plasmids that encode a high-affinity iron-uptake system (Crosa, 1989; Crosa *et al.*, 1977, 1985; Olsen & Larsen, 1990; Tolmasky *et al.*, 1988; Wiik *et al.*, 1989). Over the past seven years, 267 serotype O1 isolates from diseased fish around the world have been described; 260 carried a virulence plasmid that was similar or identical to pJM1, and only nine of these isolates carried additional plasmids (Bolinches *et al.*, 1990; Conchas *et al.*, 1991; Larsen & Olsen, 1991; Lemos *et al.*, 1988; Myhr *et al.*, 1991; Tolmasky *et al.*, 1988; Wiik *et al.*, 1989). Thus, it appears that in the case of fish-pathogenic serotype O1 strains of *V. anguillarum*, pJM1 may be a serospecific plasmid analogous to the 36 MDa plasmid associated with virulent *Salmonella enteritidis* isolates (Threlfall *et al.*, 1989).

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The observation that similar or identical virulence plasmids exist in biochemically, geographically and genetically distinct backgrounds of *V. anguillarum* throughout the world raises intriguing questions concerning the dissemination of virulence determinants among bacteria in the marine environment, and between *V. anguillarum* strains in particular. While transposition has been suggested as a possible explanation for the dissemination of the 25 kb iron-uptake virulence region of pJM1 DNA (Crosa *et al.*, 1985), this mechanism cannot account for the high degrees of similarity observed among pJM1-like plasmids, often encompassing 60–65 kb of DNA. Thus far, conjugal transmission of pJM1 has not been reported, nor has it been possible to transduce pJM1 with vibriophage ϕ As3, or to demonstrate transposition or transformation in the laboratory (J. T. Singer, unpublished observations).

To examine more thoroughly the transmissibility of pJM1 virulence determinants, we attempted first to tag virulence DNA regions with an easily selectable 1.2 kb kanamycin-resistance determinant via marker exchange, mediated by homologous recombination (Singer *et al.*, 1991a, b). However, all initial attempts to introduce plasmid DNA into *V. anguillarum* 775(pJM1) by conjugation failed. We present evidence here for the presence of a pJM1-mediated restriction system as an explanation for the initial difficulties in introducing plasmid DNA into *V. anguillarum* 775(pJM1). This explanation might also serve to reconcile the observation that pJM1-like plasmids rarely occur in nature in the presence of other plasmids.

Methods

Bacterial strains. Rifampin-resistant *V. anguillarum* 775R1(pJM1) and the pJM1-cured strain *V. anguillarum* H775-3 were described previously (Singer *et al.*, 1991a, b). *Escherichia coli* strains used in this study and their relevant characteristics included HB101 [r_{f} , m_{g} , Mar^- , McrB^- , EcoK^-] (Raleigh & Wilson, 1986), HB101(pRK2013) and HB101(pRK290) (Ditta *et al.*, 1980), J53(RP4) (Thomas, 1981), GM2929 [$\text{dam-13}::\text{Tn9 dcm-6 hsdR2 mcrA mcrB1}$] (from Michael G. Marinus, Department of Pharmacology, University of Massachusetts Medical School, Worcester, Massachusetts 01655, USA), and ER1647 [$\text{mcrA1272}::\text{Tn10}$, $\Delta(\text{mcrCB-hsdSMR-mrr})2\text{-Tn10}$] (from Elisabeth A. Raleigh, New England Biolabs, Beverly, Massachusetts 01915, USA).

Plasmid-encoded phenotypes and incompatibility groups. Plasmid pRK2013 (Inc Ia) encodes kanamycin resistance and is a restricted-host-range, PolA-dependent helper plasmid that carries plasmid RK2 transfer genes cloned into plasmid ColE1 (Figurski & Helinski, 1979). Plasmid pRK290 (Inc P) encodes tetracycline resistance and is a 20 kb mobilizable RK2-based broad-host-range cloning vector for use in Gram-negative organisms (Ditta *et al.*, 1980). RP4 (Inc P) is a broad-host-range conjugative plasmid highly similar to RK2 that encodes resistance to ampicillin, tetracycline and kanamycin (Thomas, 1981). Plasmid pBR322 (Inc Ia) is a restricted-host-range pMB1-based vector encoding ampicillin and tetracycline resistance (Balbas *et al.*, 1988). Virulence plasmid pJM1 carries no known antibiotic resistance

determinants, but codes for the production of the siderophore anguibactin and its cognate ferric-anguibactin transport components (Crosa, 1989). pJM1 has been described only from *V. anguillarum* isolates and has not been assigned an incompatibility group. However, Crosa *et al.* (1977) demonstrated that pJM1 shares little or no ($\leq 1\%$) sequence homology with plasmids from incompatibility groups FI, FII, P, X, N, W, I and O. When transformed into *E. coli* hosts, selectable pJM1-*kan* plasmids (Singer *et al.*, 1991b) are compatible with plasmids pBR322, RP4, and with their derivatives (J. T. Singer, unpublished observations).

Media and growth conditions. *V. anguillarum* strains were grown in L broth containing 2% (w/v) NaCl at 26 °C, or on L agar-2% NaCl plates containing 1.6% (w/v) agar. *E. coli* strains were grown in L broth or on L agar plates at 37 °C. Where required, antibiotics were used at the following final concentrations: ampicillin, 100 or 400 $\mu\text{g ml}^{-1}$; kanamycin, 50 $\mu\text{g ml}^{-1}$; tetracycline, 5 $\mu\text{g ml}^{-1}$; and rifampin, 100 $\mu\text{g ml}^{-1}$.

Molecular biological techniques. Plasmid DNA isolations, purification, digestions, ligations, transformation, and conditions for agarose gel electrophoresis were as previously described (Singer & Earley, 1989).

Construction of pCW1 and pCW2. Plasmid pSE6 (Singer & Earley, 1989) contains a 13.6 kb *XhoI* fragment of pJM1 iron-uptake DNA inserted into the compatible *SaII* site of pBR322, thereby destroying both *XhoI* and *SaII* recognition sequences at the junctions of insert and vector DNAs. A 1.2 kb *SaII* DNA fragment encoding kanamycin resistance was purified (Lizardi *et al.*, 1984) from plasmid pUC4-K (Vieira & Messing, 1982) and was inserted into the unique *SaII* site of cloned pJM1 DNA, resulting in plasmid pSE6-*kan1* (Singer *et al.*, 1991b). A 10.8 kb *EcoRI* DNA fragment containing the central portion of cloned pJM1 iron-uptake DNA along with the *kan* insert was purified from pSE6-*kan1* (Singer *et al.*, 1991b) and was ligated into the unique *EcoRI* site within plasmids pBR322 and pRK290, resulting in plasmids pCW2 (15.2 kb) and pCW1 (30.8 kb), respectively.

Conjugal matings. Triparental filter matings using the helper plasmid pRK2013 were used to mobilize pBR322 and pRK290 derivatives from *E. coli* donors into *V. anguillarum* recipients. Overnight 5 ml cultures of donor and recipient cells, grown under selection, were harvested by centrifugation and suspended in 5 ml of L broth or L broth-2% NaCl to remove traces of antibiotic. Equal volumes (1–1.5 ml) of donor and recipient cultures were mixed, and filtered through 47 mm 0.45 μm membrane filters; the filters were placed on the surface of L agar plates containing 1.0% (w/v) NaCl and were incubated at 26 °C for 18 h. Cells were then resuspended and diluted in L broth-2% NaCl, and dilutions were plated onto L agar-2% NaCl plates supplemented with rifampin and additional appropriate antibiotics to select exconjugants of *V. anguillarum*. Following a 48 h incubation at 26 °C, exconjugants were quantified and transfer frequencies were calculated per recipient plated.

Restriction endonuclease purification. Putative restriction endonuclease activity was purified from *V. anguillarum* 775R1(pJM1) and 775R1Rsut(pJM1) according to Makula & Meagher (1980). Enzyme activity was assayed according to Makula & Meagher (1980) or by the method of Schleif (1980).

Results and Discussion

pJM1-mediated restriction

Results presented in Table 1 show that plasmid pBR322, and pBR322-based recombinant plasmids such as

Table 1. *Conjugal transfer of restricted-host-range and broad-host-range plasmids to V. anguillarum*

Filter matings were performed as described in the text. Plasmid transfer frequencies are the mean $\pm$ SD for triplicate determinations. ND, None detected. Normal limit of detection was approximately 10^{-9} exconjugants per recipient plated.

Donor	Recipient	Selection	Exconjugants per recipient plated
HB101(pBR322) + HB101(pRK2013)	H775-3 775R1(pJM1)	Rif + Ap Rif + Ap	$2.2 \pm 0.5 \times 10^{-5}$ ND
HB101(pCW2) + HB101(pRK2013)	H775-3 775R1(pJM1)	Rif + Ap Rif + Ap	$1.6 \pm 0.7 \times 10^{-6}$ ND*
J53(RP4)	H775-3 775R1(pJM1)	Rif + Ap Rif + Ap	$6.7 \pm 2.7 \times 10^{-2}$ $2.5 \pm 0.6 \times 10^{-7}$
HB101(pRK290) + HB101(pRK2013)	H775-3 775R1(pJM1)	Rif + Tc Rif + Tc	$3.8 \pm 0.7 \times 10^{-6}$ ND
HB101(pRK290, pRK2013)	H775-3 775R1(pJM1)	Rif + Tc Rif + Tc	$3.3 \pm 0.7 \times 10^{-5}$ ND
HB101(pCW1, pRK2013)	H775-3 775R1(pJM1)	Rif + Tc Rif + Tc	$1.4 \pm 0.4 \times 10^{-5}$ ND†

* Two Rif^r Ap^r exconjugants were isolated; both contained pCW2 but were cured of pJM1.

† Four Rif^r Tc^r clones were isolated; one carried pCW1 but was cured of pJM1, and three carried pJM1 but contained deletion derivatives of pCW1 in which all of the cloned pJM1 DNA had been deleted.

Table 2. *Conjugal transfer of plasmids from methylase-deficient donors to V. anguillarum*

Filter matings were performed as described in the text. Plasmid transfer frequencies are the mean $\pm$ SD for triplicate determinations. ND, None detected. Normal limit of detection was approximately 10^{-9} exconjugants per recipient plated.

Donor	Recipient	Selection	Exconjugants per recipient plated
GM2929(pBR322) + HB101(pRK2013)	H775-3 775R1(pJM1)	Rif + Ap Rif + Ap	$1.2 \pm 0.4 \times 10^{-6}$ ND
ER1647(pBR322) + HB101(pRK2013)	H775-3 775R1(pJM1)	Rif + Ap Rif + Ap	$2.2 \pm 0.4 \times 10^{-5}$ $4.0 \pm 3.0 \times 10^{-9}$

pCW2, could be introduced into plasmid-free *V. anguillarum* H775-3, but not into *V. anguillarum* 775R1 carrying virulence plasmid pJM1. The presence of pJM1 also appeared to decrease conjugal transmission of broad-host-range plasmid RP4 by a factor of $>10^5$. Similar effects were also observed when the broad-host-range cloning vector pRK290 was mobilized into *V. anguillarum* 775R1(pJM1) (Table 1).

The results in Table 1 suggest that virulence plasmid pJM1 mediates a restriction or exclusion system that prevents the conjugal transmission of plasmid DNA into *V. anguillarum*. This system probably involves an endonuclease/exonuclease activity, rather than surface exclusion, based upon results obtained with crosses between *E. coli* HB101(pCW1, pRK2013) and *V. anguillarum* 775R1(pJM1) (Table 1). From several of these crosses, a total of four exconjugants were eventually isolated. One contained pCW1 but was cured of pJM1.

The remaining three exconjugants contained full-length pJM1 plasmids, as determined by restriction endonuclease analysis, but contained 17–19 kb deletion derivatives of pCW1 (30.8 kb) in which all of the cloned DNA had been deleted, and in which deletions extended into vector DNA sequences to varying degrees (not shown). These results support the existence of a pJM1-encoded or pJM1-controlled enzymic activity responsible for degradation of the incoming plasmid DNAs.

Use of methylase-deficient donor strains

Since methyl-specific restriction endonucleases might be active against DNAs that display specific patterns of DNA methylation (Raleigh *et al.*, 1991), *E. coli* donors that were methylation deficient were tested to determine whether plasmid transfer frequencies might be increased. The results in Table 2 show that GM2929 was

Table 3. *Conjugal transfer of plasmids to restriction-deficient V. anguillarum*

Filter matings were performed as described in the text. Plasmid transfer frequencies are the mean $\pm$ SD for triplicate determinations.

Donor	Recipient	Selection	Exconjugants per recipient plated
J53(RP4)	H775-3	Rif + Ap	$6.7 \pm 2.7 \times 10^{-2}$
	775R1Rsut(pJM1)	Rif + Ap	$1.1 \pm 0.3 \times 10^{-1}$
HB101(pBR322) +	H775-3	Rif + Ap	$2.1 \pm 0.4 \times 10^{-5}$
HB101(pRK2013)	775R1Rsut(pJM1)	Rif + Ap	$1.7 \pm 0.4 \times 10^{-5}$
ER1647(pBR322) +	H775-3	Rif + Ap	$2.3 \pm 0.5 \times 10^{-5}$
HB101(pRK2013)	775R1Rsut(pJM1)	Rif + Ap	$5.0 \pm 0.4 \times 10^{-5}$
HB101(pCW2) +	H775-3	Rif + Ap	$1.8 \pm 0.4 \times 10^{-6}$
HB101(pRK2013)	775R1Rsut(pJM1)	Rif + Ap	$1.7 \pm 0.4 \times 10^{-6}$

even a lower efficiency donor than HB101 when *V. anguillarum* H775-3 was the recipient; when *V. anguillarum* 775R1(pJM1) was the recipient, no exconjugants were detected. However, the use of ER1647 as a donor strain in matings resulted in exconjugants of both *V. anguillarum* H775-3 and 775R1(pJM1). While exconjugants of *V. anguillarum* 775R1(pJM1) were isolated only at very low frequency, ten exconjugants that were examined carried both full-length pJM1 and pBR322 plasmids.

These results indicate that the pJM1-mediated restriction system is not targeted towards Dam-, Dcm-, or *EcoK*-methylated DNA. In view of their similar genotypes with respect to methylation, it is difficult to explain the observation that ER1647 acts as a low-frequency donor while HB101 does not. It is possible that the escape from restriction observed with ER1647 donors was due to the deletion of additional sequences upstream or downstream from *hsdRMS* that may influence methylase activities in an unknown fashion.

Isolation of restriction-defective mutants of *V. anguillarum*

Since mating frequencies using ER1647 donors and *V. anguillarum* 775R1(pJM1) recipients were still too low to be generally useful in the laboratory, we attempted to isolate a restriction-defective mutant of *V. anguillarum* 775R1(pJM1). Cells were grown in M9 minimal medium, supplemented as previously described (Singer & Earley, 1989), to an OD₆₀₀ of 0.5 and were chilled on ice. A 5.0 ml culture sample was irradiated (3 J m^{-2}) at 254 nm in the bottom of a standard glass Petri dish, in the dark with gentle shaking (50 r.p.m.). Surviving cells were then allowed to grow overnight in the dark and cells in the culture were used as recipients in matings with HB101(pBR322) and HB101(pRK2013).

Rare *V. anguillarum* exconjugants were selected on medium containing rifampin and ampicillin (400 µg

ml⁻¹), and were then tested for the presence of pJM1 by screening on M9 minimal medium plates containing the iron chelator ethylenediamine di-(*o*-hydroxyphenylacetic acid) at a concentration of 10 µM (Singer & Early, 1989). Of the 17 Rif^r Ap^r exconjugants tested, only three still retained pJM1, as evidenced by growth in the presence of ethylenediamine di-(*o*-hydroxyphenylacetic acid). These strains were chosen for further study, and were grown in the absence of selection to permit segregation of unstable pBR322 plasmids. Ap^s clones were then re-tested as recipients in conjugal matings with HB101(pBR322) and HB101(pRK2013). One clone that appeared to act as an efficient recipient was designated *V. anguillarum* R1Rsut(pJM1) and was characterized further.

Characterization of a restriction-defective mutant of *V. anguillarum*

The results presented in Table 3 show that *V. anguillarum* R1Rsut(pJM1) was able to act as a conjugal recipient in triparental matings as efficiently as the pJM1-cured strain *V. anguillarum* H775-3. In addition, plasmid transfer frequencies with *V. anguillarum* R1Rsut(pJM1) recipients were similar regardless of whether the donor was *E. coli* HB101 or ER1647. These results suggest that *V. anguillarum* R1Rsut(pJM1) is a restriction-defective mutant that arose as a result of UV mutagenesis. The putative mutagenic event did not result in the detectable deletion of pJM1 plasmid DNA, nor in its rearrangement, as plasmid fingerprints with *EcoRI*, *HindIII*, *XhoI* and *PstI* were still indistinguishable for pJM1 DNA purified from *V. anguillarum* R1Rsut(pJM1) and from wild-type *V. anguillarum* R1(pJM1).

If a mutation is responsible for the apparent restriction-defective phenotype of *V. anguillarum* R1Rsut(pJM1), then it should be possible to quantify differences in endonuclease activities between *V. anguillarum* R1Rsut(pJM1) and wild-type *V. anguillarum*

R1(pJM1). Each strain was tested for the presence of type II restriction endonucleases by the method of Schleif (1980), with uniformly negative results. This was unexpected, since some *V. anguillarum* strains were previously shown to produce *VanI*, an isoschizomer of *BglI* (Wood, 1988). Both strains did, however, produce unusually high levels of non-specific exonuclease activity that could easily have masked any specific type II activity. Because of this potential masking, putative restriction endonucleases were purified from 3-litre cultures of each strain and were fractionated as previously described using Phosphocellulose P11 (Whatman) and Hydroxylapatite C (Clarkson Chem. Co.) (Makula & Meagher, 1980). Partial purification and fractionation revealed no type II restriction endonuclease activities in purified fractions from either of the above two strains. There was, however, a pronounced difference in the levels of nonspecific nuclease activity produced by each strain, with *V. anguillarum* R1Rsut(pJM1) producing only about 30–35% of the nuclease activity exhibited in fractions from *V. anguillarum* R1(pJM1). *V. anguillarum* H775-3 produced levels of nuclease activity similar to those observed with *V. anguillarum* R1Rsut(pJM1) (Ronald A. Makula, personal communication).

Based on the above data, it is tempting to speculate that a UV-induced mutation has resulted in a decrease in a pJM1-mediated type I-like restriction activity in *V. anguillarum* R1Rsut(pJM1). However, we have no direct genetic evidence linking relief from restriction with a specific mutation, or with the apparent decrease in observed nuclease activity. In addition to the possibility that pJM1 encodes a restriction-modification-like system, it is also possible that pJM1 encodes a *trans*-acting factor that activates expression of a chromosomally-encoded restriction-modification system. However, we have been unable to demonstrate any restriction activity in *E. coli* transformants harbouring cloned pJM1 DNAs, or in *V. anguillarum* H775-3 exconjugants carrying cloned pJM1 DNA fragments.

Regardless of the restriction mechanism involved, other investigators have also been unsuccessful using *V. anguillarum* as a conjugal recipient in matings performed by a variety of techniques and with a variety of Gram-negative bacterial donors (Toranzo & Barja, 1985). In addition, pJM1-like plasmids have only rarely been observed in the presence of other plasmids in natural isolates of *V. anguillarum* (Conchas *et al.*, 1991; Crosa *et al.*, 1977; Larsen & Olsen, 1991; Myhr *et al.*, 1991; Olsen & Larsen, 1990; Tolmasky *et al.*, 1988; Wiik *et al.*, 1989), and previous to our earlier studies (Singer *et al.*, 1991a, b), only broad-host-range plasmids related to RP4 were transferred to *V. anguillarum* 775(pJM1). It is possible that a pJM1-mediated restriction system is responsible

for the above observations, and that such a system may limit the spread of plasmids to *V. anguillarum* under natural conditions. If this putative restriction system functions in the marine environment, it could be advantageous to aquaculturists in limiting the spread of R-factors among populations of *V. anguillarum* selected by antibiotic chemotherapy used to treat or prevent vibriosis.

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