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Construction of a *Vibrio splendidus* Vsm metalloprotease mutant using a novel counter-selectable suicide vector

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Abstract:

splendidus is a dominant culturable *Vibrio* in seawater, but strains related to this group are also associated with mortalities in a variety of marine animals. The determinants encoding the pathogenic properties of these strains are still poorly known, however, the recent sequencing of the genome of *V. splendidus* LGP32, an oyster pathogen, offers the opportunity to decipher the basis of the virulence properties by the disruption of candidate genes. We have developed a novel suicide vector based on the *pir*-dependant R6K replicative origin, which can be transferred by RP4 based conjugation to, potentially, any *Vibrio* strain, and which also carries the plasmid F toxin *ccdB* gene under the control of the P_{BAD} promoter. We demonstrate that this genetic system allows the efficient counter-selection of integrated plasmids in presence of arabinose, both in *V. splendidus* and *V. cholerae*, and as such permits the efficient marker-less allelic replacement in these species. We used this technique to construct several mutants of *V. splendidus* LGP32, including a derivative deleted of a secreted metalloprotease gene, *vsm*. We show that this gene is essential for the LGP32 extracellular products toxicity when injected in oysters, but is not necessary for the virulence of bacteria in the model of oyster infection after bacterial injection.

1. Introduction

Vibrio splendidus is a dominant culturable *Vibrio* in coastal marine sediments, seawater and bivalves, namely oysters (23). The organism has long been considered as an environmental organism without any pathogenic significance. However, over the last years, different strains phenotypically related to this species have been associated with mortalities mainly in molluscs, shrimps, gorgonians and fishs (for review see 35). Compared to human pathogen species, little is known concerning *Vibrio* pathogenesis in marine animals and, despite descriptions of invasivity and extracellular products toxicity, no data are available for a *V. splendidus* related group (26, 37, 48, 56).

Among the different types of enzymatic activities that have been shown to play a role in the virulence of a variety of pathogenic bacteria, extracellular proteases are commonly involved, as for example in *V. cholerae* (7)*, V. vulnificus* (33) and *V. anguillarum* (42) even if a direct role of these proteases in the virulence has not been demonstrated. For example, it has been shown that the *V. cholerae* metalloprotease cleavage activity is essential for the activation of the A subunit of the cholera enterotoxin (12) as well as to degrade intestinal mucin and facilitate the action of cholera toxin (7). In the case of *V. vulnificus* infection, a metalloprotease has been shown to cause a hemorrhagic reaction by degrading type IV collagen in basement membranes (44). Finally, the *empA*-encoded metalloprotease of *V. anguillarum* has been involved in the invasive mechanism of this fish pathogen (49).

We have recently completed the sequencing of the genome of the *V. splendidus* strain LGP32, in order to gain access to its full gene repertoire (Mazel and coll in prep). The strain that we have chosen is an oyster (*Crassostrea gigas*) pathogen (23, 24). We have identified a gene, *vsm,* potentially encoding a Zinc-containing metalloprotease, which could play a role in pathogenesis (45). Interestingly, we found that the *vsm* predicted product shares 95% identity with the *V. anguillarum vam* gene, which has been involved in the virulence properties of this fish pathogen (42).

Gene knock-out is often essential for the formal demonstration of the predicted, or supposed, role of a gene candidate. However, such a strategy is still limited to the species in which the already available genetic tools can be used. Limitations can occur at several levels from the DNA delivery inside the cells, to the allelic exchange efficiency. DNA transformation, be it natural or artificial, is either inoperative or inefficient in numerous species. In *Vibrio splendidus*, plasmid transfer attempts using electroporation were ineffective (unpublished results) preventing the use of Wanner red-swap recombination strategy (14) to perform allelic exchange.

In many cases, exogenous DNA delivery can be achieved by using conjugation with broad host range plasmids and several systems have been described based upon the IncPα plasmid RP4 (RK2) transfer functions (see for example 54). The subsequent step of allelic replacement or integration of the incoming DNA is in most cases achieved through the use of a non-replicative DNA molecule. The most popular system for Gram-negative species is the one using conditionally replicative R6K plasmid derivatives, such as pGP704 (41). R6K replication is dependent on the binding of the *pir*-encoded Π protein and trans-complementation of a *pir*-dependent plasmid derivative by Π proteins expressed from another replicon can be performed (32). From this seminal observation, several plasmids carrying the R6Kγ origin of replication that can only be replicated in strains expressing *pir* have been constructed. When these plasmids also carry a RP4 transfer origin, they can be transferred to various bacterial cells through the broad-host range conjugation system of RP4. Since these plasmids behave as suicide vectors in *pir* $^-$ recipients, they have been successfully used to create mutants through gene disruption by insertion (41) or transposon mutagenesis (29). A wide range of Gram negative bacteria can be engineered with such tools and most of the proteobacteria can be used as recipients for conjugation (see for example 14 and references therein). Several counter-selectable markers have been described (reviewed in 48) and some of them have been successfully used in R6K-*oriT*$_{RP4}$ derivatives for the positive selection of replaced alleles (19, 51, 55).

One of these method consist of using a wild-type *rpsL* gene in a streptomycin-resistant *rpsL* mutant background (the streptomycin-sensitive wild-type is trans-dominant) (16). However, this strategy requires that cognate *rpsL* genes must be cloned and Sm R mutant strains be used.

Due to its general efficiency in Gram-negative bacteria and to the simplicity of the counter-selection protocol, the *Bacillus subtilis* levansucrase gene *sacB* has gained considerable notariety since 1985, when it was first introduced (25), and is now certainly the most commonly used of the different counter-selectable marker. *sacB* based suicide vectors, such as pCVD442 (19), have been successfully used for allelic replacement in *V. cholerae* (see for example 18). However, the use of *sacB* for allelic replacement

in many *Vibrio* species or in other marine bacterial species, even though it has been occasionally used with success, is seriously impeded by the obligatory absence of NaCl in the counter-selection medium (6). This is the case for example of *V. splendidus,* which cannot grow in the absence of NaCl in the medium.
In order to overcome this limitation we have developed a novel suicide vector, based on the pSW family (17) which can be mobilized by the RP4 transfer machinery, and which carries the *ccdB* gene of *E. coli* F plasmid under the control of the arabinose P_{BAD} promoter (52) as a counter-selection marker. We have demonstrated that this system allowed positive selection for the loss of vector sequences after homologous recombination in *V. splendidus* and *V. cholerae*, with a high efficiency. This strategy has been used to construct a *V. splendidus* strain deleted of the *vsm* gene in order to establish the contribution of this metalloprotease in the virulence properties of this strain in oyster infection.

2. MaterialS and methods

2.1. Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in Tables 1 and 2. *V. splendidus* strain LGP32 was isolated from the hemolymph of oysters suffering from outbreaks of summer mortalities, and demonstrated to be pathogenic for oysters and clams (23). *V. cholerae* and *E. coli* strains were grown in Luria-Bertani (LB) or, in case of Π3813, Mueller-Hinton (MH) broth, at 37°C. *V. splendidus* strains were grown in LB-NaCl 0.5M, marine broth (MB) or marine agar (MA) at 20°C. All media were from Difco. Antibiotics were used at the following concentrations : ampicillin (Ap), 100 μg/ml ; chloramphenicol (Cm), 12,5 μg/ml ; erythromycin (Erm), 200 μg/ml ; kanamycin (Km), 25 μg/ml ; nalidixic acid (Nal), 30 μg/ml ; spectinomycin (Sp), 50 μg/ml ; tetracycline (Tc), 15 μg/ml. Thymidine (dT) and diaminopimelate (DAP) were supplemented when necessary to a final concentration of 0.3mM. Induction of *ccdB* expression under the control of P_{BAD} promoter was achieved by the addition of 0,2% L-arabinose to the growth media and repressed by 1% D-glucose.

2.2. Polymerase chain reaction (PCR)

PCR performed for plasmid assembly were done in 50 μl volumes using the Pfu DNA polymerase (Promega) following the manufacturer's instructions. Other PCR reactions were performed in 50 μl volumes using the Bioline Taq polymerase according to the manufacturer's instructions. Primers are listed in supplementary table 1 (online supplementary material). Conditions for amplification were as follows: 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, (Tm-10°C) for 30 s, and 72°C for 60 s per kb.

2.3. Construction of CcdB resistant *E. coli* strains

As mutation gyrA462 has been shown to prevent toxic interaction of the gyrase with CcdB (13), we chose to use such a genetic background to prevent selection of *ccdB* inactivating mutation. We previously developed a set of strains carrying *pir* alleles in different genetic contexts (17). One of these carry a copy-up allele (*pir116*) inserted in the thymidilate synthase gene (*thyA*) of *E. coli.*
The ΔthyA ::(erm-pir116) *E. coli* Π3813 strain was constructed by allelic replacement of the chromosomal *thyA* allele of B462 with the *ΔthyA* ::(*erm-pir116*), as described previously (17). Such thymidine auxotroph strain is CcdB resistant and permits the R6K vector replication.
The chromosomal *gyrA462* allele was moved from S17-1 to ΔdapA ::(erm-pir) *E. coli* strain β2163 by P1 transduction and tet^R selection of the cotransduced *zei298::Tn10* to construct strain β3914. To easily discriminate the transductant carrying the *gyrA462* allele from those still carrying *gyrA* we used a Nal^R/ Nal^S screening. Indeed, Nal resistance is linked to mutations in *gyrA,* but *gyrA462* does not confer this phenotype. Then, we first selected a spontaneous Nal^R derivative of β2163 and use it as recipient for the *gyrA462-zei298::Tn10* cotransduction. After transduction, the colonies which have acquired the *gyrA462* allele together with *zei298::Tn10* were expected to be Nal^S. In agreement with this, Tc^R Nal^S colonies were found to be insensitive to the CcdB toxicity. One clone was further used and named β3914.

2.4. Plasmids construction

2.4.1. pSU18T-P_{BAD}gfp

A 270 bp fragment harboring the RP4 origin of transfer (oriT$_{RP4}$) was amplified from pSW23T by PCR using primers OriT-Xba and OriT-Pst. After *XbaI-PstI* digestion, the generated fragment was cloned in the pSU18, a p15A derivative compatible with ColE1 and R6K derivatives, to lead to pSU18T. A 1978 bp fragment harboring the *araC* gene, the P_{BAD} promoter and the *gfp* gene was amplified from the P_{BAD}*gfp* plasmid by using primers P_{BAD}GFP-Kpn and P_{BAD}GFP-Xba. After *KpnI-XbaI* digestion, the generated fragment was cloned in pSU18T. GFP expression was confirmed by epifluorescence microscopy.

2.4.2. Suicide vectors

A 2645 bp fragment harboring a transcriptional terminator, the *aadA7* gene, the *araC* gene, the P_{BAD} promoter and *ccdB* gene was PCR amplified from GG784 DNA (52) as template using primers ccdB1 and ccdB2. After *XbaI-SacI* digestion, this fragment was ligated to the suicide vectors pSW23T and pSW29T, digested with the same restriction enzymes, leading to pSW4426T and pSW4427T, respectively.
Several *V. splendidus* genes, *luxU, luxM,* IS*Visp1 orfB, vsm and gyrA* as well as the *V. cholerae* superintegron integrase gene *intIA* were PCR amplified from genomic DNA using respectively primers luxU-1 + luxU-2, luxM-1 + luxM-2, ISvisp1-1 + ISvisp1-2, vsm-1+ vsm-2, gyrA-1 + gyrA-2 and intIA1 + intIA2. Amplicons were digested by *EcoR1* and cloned in *EcoR1* site of pUC18 (Pharmacia). The corresponding alleles carrying an internal deletion were constructed by inverse PCR using primers luxU-3 + luxU-4 (*ΔluxU* allele), luxM-3 + luxM-4 (*ΔluxM* allele), ISvisp1-3 + ISvisp1-4 (IS*visp1 ΔorfB* allele), vsm-3+ vsm-4 (*Δvsm* allele), gyrA-3 + gyrA-4 (*ΔgyrA* allele) and intIA3 + intIA4 (*ΔintIA* allele), *XhoI* digestion and self-ligation. The different alleles were then recovered after *EcoRI* digestion and gel extraction, and introduced by ligation in pSW4426T and/or pSW4427T previously linearized with *EcoRI*.

2.4.3. Vector construction for ectopic complementation

Complementation experiments were performed by introducing *vsm* under P_{BAD} promoter control, inside the non-essential gene coding for ISVisp1 transposase present in the chromosome, using the same strategy describe above. An inverse PCR was performed with primers GFP3 and GFP4, and pSU18T-P_{BAD}*gfp* as template. The resulting amplicon was digested by *EcoR1* and self-ligated, leading to pSU18T-P_{BAD}*gfp*2. The *vsm* gene was PCR amplified from *V. splendidus* genomic DNA, with primers vsm-1 and vsm-7, digested by *EcoR1-Xba1* and cloned in pSU18T-P_{BAD}*gfp*2 deleted of the *gfp* gene after *EcoR1-Xba1* digestion. This led to pSU18T-P_{BAD}*vsm.*
In order to do the ectopic complementation from the chromosome, the *araC*-P_{BAD}*vsm* amplicon was amplified from pSU18T-P_{BAD}*vsm* using primers araC-S-Xho + vsm-7, *XhoI* digested and cloned in the *XhoI* site of IS*Visp1 ΔorfB* allele carried by pSWδ6720T. This led to pSWδ6720T-*vsm.*

2.5. Conjugations

Overnight cultures of donor and recipient were diluted at 1:100 in culture media without antibiotic and grown at 30°C up an OD_{600nm} of 0.3. The different conjugations experiments were done by the filter mating procedure as described previously (5) with a donor/recipient ratio of 1/10. Conjugations were performed overnight on filter incubated on ML+DAP plates (+NaCl in case of *V. splendidus*), at 30°C. Counter-selection of *ΔdapA* donor was done by plating on a medium devoid of diaminopimelic acid (DAP), supplemented with 1% glucose and either chloramphenicol or kanamycin. First recombination frequency was calculated as the number of transconjugants from the total number of recipient. Antibiotic resistant colonies were isolated, grown in ML (+NaCl in case of *V. splendidus*) up to late logarithmic phase and spread on plate containing 0,2% arabinose. Mutants were screened by PCR using primers 5+6 flanking the different genes targeted.

2.6. Immunoblot

Cells (2 ml of overnight culture) were centrifuged for 5 min at 5000 rpm, resuspended in 50 µl of bacterial protein extraction reagent (B-PER, Pierce) supplemented with protease inhibitor 1X (Complete, Roche),

vortexed vigorously for 1 min, and centrifuged for 5 min at 13000 rpm. As measured by the Bradford assay, equal amounts of protein were loaded and separated on a 10% polyacrylamide-sodium dodecyl sulfate (SDS) gel and transferred to a nitrocellulose membrane (Biorad) by electroblotting. The membrane was incubated with the primary antibody (rabbit polyclonal anti-GFP, Sigma) then with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Amersham). The proteins were visualized by means of the enhanced chemiluminescence kit (Amersham) as instructed by the supplier.

2.7. Preparation of V. splendidus extracellular products

Bacterial extracellular products (ECPs) were produced by the cellophane overlay method as described by Liu *et al* (36). Tubes containing 5 ml MB were inoculated with one bacterial colony of LGP32 wild type (WT) or mutant and incubated at 20°C for 24h. A volume of 500 µl of this culture was transferred onto a sterile cellophane film placed on the surface of each MA plate. After incubation at 20°C for 24h the cellophane overlay was transferred to an empty Petri dish. Cells were washed off the cellophane film using 2 ml of PBS and removed by centrifugation at 13000 rpm at 20°C for 30min. The supernatant containing the ECPs was sterilized by filtration (0,22 µm) and stored at –80°C until use. The protein concentration of the ECPs was measured by the method of Bradford (8) with bovine serum albumin (Sigma) as the standard and normalized.

2.8. Detection of protease activity

Protease activity was determined using azocasein (Sigma) as substrate. Briefly, crude ECPs (250 µl) were added to 250 µl of azocasein (5 $mg.ml^{-1}$ in 50 mM Tris–HCl buffer pH 8.0) and to 245 µl of distilled water. The mixture was incubated at 20°C for 10 min. The undigested substrate was precipitated by adding 500 µl of 10% trichloroacetic acid to the reaction mixture, followed by centrifugation at 13000 rpm and 4°C for 5 min. The supernatant (500 µl) was neutralized by the addition of an equal volume of 1 N NaOH. After mixing, the absorbance at 440 nm was measured for triplicate samples.
In addition, protease activity of separated proteins in an SDS-polyacrylamide gel was detected by copolymerizing 0,2% gelatin in the polyacrylamide matrix (30). After electrophoresis, the gel was soaked in 2,5% Triton X-100 for 2 h at room temperature, incubated overnight at 37°C in 50 mM Tris–HCl pH 7,5, 200 mM NaCl, 5 mM CaCl2, and then fixed and stained with 0,4% Coomassie brillant blue in 30% methanol-10% acetic acid and then destained in 30% methanol-10% acetic acid.

2.9. Virulence studies using oysters

Bacteria were grown under constant agitation at 20°C for 36 h in MB, harvested and resuspended in sterile seawater (121°C for 15 min) at an OD_{600} of 1. This OD corresponded to a bacterial concentration ranging from 10^9 to 2.10^9 CFU ml^{-1} as determined by conventional dilution plating on marine agar (data not shown). Oysters were intramuscularly injected with bacterial strains or ECPs (5 µg/gr oysters) as described previously (23, 34). After injection, the oysters were transferred to aquaria (15 to 20 oysters per aquarium of 2.5 l) containing aerated 5 µm-filtered seawater at 20°C, kept under static conditions and fed daily with a mixture of planktonic algae (*Isochrisis galbana* and *Chaetoceros calcitrans).* Each bacterial treatment was performed in duplicate and mortality was recorded daily.

2.10. Nucleotide sequence accession number

The nucleotide sequence of *luxU, luxM, vsm, gyrA,* IS*Visp1* genes and pSW4426T, pSW4427T cloning vectors have been deposited in the GenBank database under the accession number DQ987705, DQ987706, DQ987707, DQ987708, DQ987704, DQ995482 and DQ995483.

3. Results

3.1. Construction of a plasmid vector allowing controlled expression in V. splendidus

We constructed a p15A derivative plasmid, pSU18T-P_{BAD}*gfp,* carrying the green fluorescent protein gene under the control of the positively regulated arabinose-inducible P_{BAD} promoter and an origin of transfer from RP4. Conjugative transfer of this plasmid from *E. coli* to *V. splendidus* was observed at a frequency of 10^{-3} transconjugant per recipients. Western blot analysis using commercial polyclonal antibodies led us to detect GFP protein with a molecular mass of about 30 kDa from extracts of LGP32 transconjuguants when arabinose was added to the culture media while no signal was obtained when transconjuguants were grown in the presence of glucose or in case of LGP32 WT extracts (Fig.1). Thus the P_{BAD} promoter appears tighly and properly regulated in LGP32 by the *araC* gene product, which activates its transcription in response its natural inducer arabinose.

3.2. Development of a two step allelic replacement method using CcdB as a positive selection marker

CcdB is a very strong gyrase inhibitor, originally discovered in the post-seggregational killing operon carried on plasmid F, ccdBA (2). The *ccdB* gene has been employed as a potent counterselective marker in a number of commonly used applications (see for example (3, 4, 52).

Starting from pSW23T (Cm^R) and pSW29T (Km^R), both carrying an $oriV_{R6K\gamma}$ and an $oriT_{RP4}$, we constructed derivatives pSW4426T and pSW4427T, carrying the *ccdB* gene under the control of P_{BAD}-*araC.* In order to avoid the natural selection of inactive *ccdB* mutants due to leaking of the P_{BAD}, we established CcdB resistant *E. coli* [*pir*+] strains, Π3813 and β3914, by substitution of the *gyrA* gene with the *gyrA462* allele, which is insensitive to CcdB poisonning (2).

From pSW4426T we constructed three suicide vector derivatives bearing *luxU, luxM* and *gyrA* deleted alleles in order to create LGP32 mutants with mutation at these loci by allelic exchange. These Cm^R plasmids were designed to allow selection of strains in which the plasmid had been integrated into the target gene by a first homologous recombination (under *ccdB* repression) and to allow positive selection of a second recombination event resulting in suicide vector loss (*ccdB* expression) (Fig 2).

The conjugation+integration frequency of these plasmids in LGP32 genome was found to be 10^{-8} to 10^{-7} recombinant per recipients, depending on the size of the region flanking the deletion in the fragment (Table 3). Several Cm^R colonies were reisolated, grown overnight and 10^7 CFU were spread on plate containing 0,2% arabinose. One hundred colonies were obtained and found to be Cm^S. A screening for the mutants among the surviving colonies was performed by PCR using primers flanking the target gene in the genome. 40-50% mutants were obtained in the case of the *ΔluxU* and *ΔluxM* alleles replacement, whereas only wild type genotypes were detected in the case of the essential *gyrA* gene.

To validate the use of our strategy in another *Vibrio* species, *V. cholerae,* we constructed an integron integrase allele *ΔintIA* and cloned it pSW4427T, a suicide vector containing Km^R gene instead of Cm^R. In *V. cholerae* N16961, the frequency of conjugation + integration was found to be 10^{-6} recombinant/recipient. Several isolates were grown without selection and colonies were selected on arabinose-supplemented medium. Thirty Km^S colonies were then tested by PCR and nine isolates were found to carry the deleted allele while the rest carried WT *intIA* gene (data not shown).

3.3. Construction and characterization of Δvsm V. splendidus mutant

We constructed pSWδ2989T, a suicide vector bearing an internal deletion within *vsm* gene to create a LGP32 mutant with a mutation at this locus by allelic exchange. After conjugative transfer and selection on arabinose, a screening for the mutants among the surviving colonies was performed by PCR using primers flanking the *vsm* gene in the genome and amplifying 1800 bp from wild type and 1300 bp from mutant (Table 3). Surprisingly all tested colonies (18/18) were found to carry the mutated allele instead of the 50% theoretically expected, when using mutant constructions having 5' and 3' flanking regions of similar size as recombination targets. One of these isolate was further used and named δ2989.

The mutant δ2989 was tested for the loss of metalloprotease activity. The proteolytic activity of δ2989 ECP (Fig. 3A, lanes 3 and 4) was determined by azocasein assay and shown to be 8 times lower than the wild type (Fig.3A lanes 1 and2). In addition a gelatin-SDS-polyacrylamide gel was used to detect extracellular

proteins with protease activity. In the case of LGP32 ECP, two predominants protease bands (estimated at 30-40 KDa) and one minor band (estimated at 70 KDa) were seen as strong zones of clearing (Fig.3B, lanes 1 and 2). These bands were missing from δ2989 (Fig.3B, lane 3 and 4).

3.4. Ectopic complementation of the *vsm* mutation

IS*Visp1* is a insertion sequence specific of *V. splendidus* (deposited at the IS database (http://www-is.biotoul.fr/is.html) and found at one copy in the LGP32 genome (Mazel and coll. in prep). As this locus is likely unessential, we chose to use it as a platform for ectopic expression in trans-complementation experiment of the *Δvsm* mutant. The *vsm* gene under the control of *araC*-P_{BAD}, was cloned inside the IS*Visp1 ΔorfB* allele carried by the pSWδ6720T ($Cm^{R)}$). The resulting plasmid was transferred by conjugation to the strain δ2989, in which it could integrate the genome through homologous recombination with the IS*Visp1 orfB* gene. Such Cm^R clones were obtained, among which, four were grown without selection and then submitted to selection on arabinose-supplemented medium. Ten colonies showing the Cm^S phenotype expected in case of a second recombination event, were tested by PCR, half were found to carry the *ΔorfB::araC*-P_{BAD}-*vsm* allele. One of these isolates was further used and named δ6720-*vsm*
As shown in Figure 3A, the metalloprotease activity was restored by this ectopic complementation as the activity detected in the δ6720-*vsm* ECP, when grown in presence of arabinose was similar to that from LGP32 WT, and clearly higher than in the δ2989 ECP. Furthermore, gelatin-SDS-polyacrylamide gel analysis of δ6720-*vsm* ECPs showed the same two predominant protease bands than those observed in LGP32 WT, but only when arabinose was added in the media (Fig.3B, lane 6).

3.5. Oyster experiments

In order to evaluate their toxicity, the ECPs (5 μg/gr oysters) were injected into oyster adductor muscle (Fig.4A). Two days post injection, 68% mortalities were obtained in case of LGP32 and 15% in case of δ2989. The ectopic expression of *vsm* driven by arabinose was found to restore the toxicity of ECP as mortality rates similar to those from LGP32 WT were obtained from δ6720-*vsm*. These data demonstrate the role of the Vsm metalloprotease in the ECP toxicity. However when virulence of LGP32 and δ2989 strains were compared after injection of living bacteria to oyster, similar mortality rates were obtained for both strains (Fig.4B). These results suggest that *vsm* expression is not essential for full bacterial virulence.

4. DISCUSSION

Since the first report on the complete genome sequence of *Haemophilus* influenzae in 1995 more than 300 other prokaryotic genome sequences have been completed and another 750 projects are underway (22). Genomics-based approaches have significantly advanced our understanding of the physiology and pathogenicity of many microbes and provided insights into the mechanisms and history of genome evolution. Paradoxically, only a limited number of bacterial species are amenable to genetic manipulation, which is often essential for the demonstration of the proposed or suspected function of a gene candidate.
In parallel to the complete sequencing of the *V. splendidus* genome, we have developed a gene knock-out strategy which has been used here to investigate the role of a secreted metalloprotease in the toxicity of extra-cellular products and in the virulence *in vivo.*
We have, as first step, established the conditions to transfer a mobilizable plasmid in *V. splendidus*, through the RP4 conjugative machinery, and the functionality of the P_{BAD} promoter in this strain using a replicative vector expressing the GFP.
The second step was to establish a genetic system for allelic replacement in *V. splendidus.* In 1988, Miller and Mekalanos set up a system allowing single gene disruption through a homologous recombination event between a targeted gene and a trucated version of it carried by a suicide vector introduced in the strain by conjugation (41). This strategy has since had considerable success. However, it has several important limitations, due first to the fact that mutants constructed by this technique carry in their chromosome a partial duplication of the targeted gene, which can be the source of reversion events at high frequency. Another limitation is due to the presence of a copy of the vector backbone, which prevent

further mutant construction by the same method in these strains, as recombination between the incoming vector and the chromosomal copy cannot be counterselected. Finally, such mutations through vector integration can also have polar or other uncontrolled effects on the genes located in the neighborhood of the targeted gene.

To overcome these limitations Donnenberg and Kaper developed a two steps strategy to allow the positive selection of clones in which a second recombination event leading to the loss of the vector and either the mutated or the WT allele, had occurred, after the first integration event (19). The positive selection was linked to the presence in the vector backbone of a copy of the *B. subtilis sacB* gene. *sacB* encodes an enzyme, the levan-sucrase, and its activity had been shown to be toxic for gram negative organisms when grown in presence of sucrose (25). When a mutant which contains an integrated copy of a suicide vector that also carries *sacB* through recombination into a target locus, upon exposure to sucrose, the daughter cells that had undergone a second recombination event resulting in the loss of the suicide vector are the only ones to thrive. If the targeted gene is not vital, the second recombination is expected to lead to either restoration of the WT allele or to allelic substitution in a 1:1 ratio. If the gene is essential, the sucrose-surviving isolates will carry the WT allele. However, it is known that the SacB toxicity is susceptible to the presence of sodium chloride in the selective medium (6). As the addition of sodium chloride in media is absolutely necessary for many *Vibrio* species growth, such as *V. splendidus*, we had to use and develop a different counter-selective marker to be able to use a similar strategy for mutant construction in such species.

We developed a novel suicide vector, based on the pSW family (17), which can be mobilized by the RP4 transfer machinery, and which carry the *ccdB* gene of the *E. coli* F plasmid under the control of the arabinose P_{BAD} promoter (52) as a counter-selective marker. Indeed, CcdB encodes a very efficient gyrase inhibitor, which has been observed to work on a broad spectrum of bacteria, and has been used as counter-selective marker for the development of several cloning vector (see for example 3, 4). We constructed two vectors, pSW4426T which carry a CmR marker and the *araC-*P_{BAD}*-ccdB* cassette, and pSW4427T, which is identical to pSW4426T but carry a KmR marker. Using these plasmids, once they carries the mutated allele, the allelic replacement can be assayed in a two step procedure, first through the selection, via the plasmid resistance markers, of clones of the recipient strain that have integrated the suicide plasmid through homologous recombination with the WT allele, and second, transfer of the selected clones in a medium supplemented with arabinose to induce the expression of the lethal CcdB. As described above, if the targeted gene is not vital, the second recombination will lead to either the WT allele restoration or to the allelic substitution in identical proportion, 50%, while if the gene is essential, the surviving isolates will only carry the WT allele.

In order to validate our technique, *V. splendidus* Δ*luxU,* Δ*luxM* and Δ*gyrA* alleles were constructed and cloned in pSW4426T and allelic replacement was tested for each of them following the protocol described above. As expected, the Δ*luxU* and Δ*luxM* alleles were successfully substituted to the WT alleles at a frequency of about 50% in the arabinose surviving cells, while in the case of the essential *gyrA* gene, the arabinose surviving cells were only found to carry the WT allele. We also observed that the frequency of conjugation plus insertion correlated with the size of the DNA allowing the first recombination event.

We tested this two-step knock out strategy in another *Vibrio* species, *V. cholerae*, using to the superintegron integrase gene *intIA* (formerly called *intI4*, 40), as target. The Δ*intIA* allele was cloned into the KmR vector pSW4427T and allelic replacement was successfully assayed in strain N16961. As for the *luxU* and *M* gene replacement in *V. splendidus,* arabinose surviving clones were found to carry either the WT or the Δ*intIA* allele in nearly identical proportion, showing that IntIA is not essential in *V. cholerae* in laboratory culture conditions.

4.1. Construction and characterization of a *V. splendidus* metalloprotease mutant

As mentioned in the introduction, we have now completed the sequence of the *V. splendidus* LGP32 genome (Mazel et coll in prep). Among the genes which could play a role in the pathogenesis, we identified a Zinc-containing metalloprotease gene, *vsm*, the predicted product of which shares 95% identity with the *V. anguillarum vam* gene, which had been involved in the virulence properties of this fish pathogen (42).

Vibrio Zinc-containing metalloproteases are classified into 3 distinct categories according to their amino acid sequences (for review see 45). The class I *Vibrio* metalloproteases contain a large signal peptide region and a zinc-binding motif that includes an extra glutamic acid located 19 bases downstream from

the second histidine residue (HEXXH~19 aa~E), whereas metalloproteases belonging to classes II et III only have a HEXXH motif. The *V. splendidus vsm* gene product carried the characteristic class I metalloproteases zinc-binding motif. In addition to this HEXXH~19 aa~E motif, analysis of the primary structure of the predicted Vsm protein through sequence alignments predicted the existence of the second consensus sequence GXXNEXXSD, which when associated with the HEXXH motif, constitutes the features that define the thermolysin family.

The metalloproteases of the thermolysin family are synthesized as inactive precursors, which are further maturated by successive processing stages. According to literature, a N-terminal peptide is cleaved during its passage through the inner membrane in a signal peptide-dependant manner. In the periplasm the N-terminal propeptide is then cleaved by an autoproteolytic mechanism and the mature protein is generated. A second processing at the carboxy terminus by autocatalytic cleavage has been described in other *Vibrio* species (15, 27, 33, 42). In these species, the mature protease has been proposed to consist of 2 domains, an N-terminal domain mediating the proteolytic action and a C-terminal domain that may be implicated in attachment to protein substrates (46).

In order to characterize the role of the thermolysin related Vsm protease, we constructed a *Δvsm* mutant strain using the technique described above with plasmid pSWδ2989T. Cm^S arabinose-resistant isolates were obtained with frequency similar to those obtained with the *lux* alleles used in the validation process. However, PCR analysis and DNA sequencing revealed that surviving clones were *Δvsm* mutants in all cases, and that none of them corresponded to the WT *vsm* allele restoration. The probability of this happening by chance is extremely low (1 in 262,144). This could be an indication of the fact that *vsm* expression is somehow deleterious in laboratory culture conditions and that the deleted allele has a strong selective advantage in these conditions compared to the wild type allele. Interestingly, we did not observe any recombinational bias when we constructed the ectopically complemented *vsm* strain (see results). In this case, we reintroduced the *vsm* gene under the P_{BAD} promoter control, thus in the absence of arabinose, the ectopic *vsm* is silent, likely relieving any selective pressure against the recombinant**.**

The complete open reading frame of *vsm* encodes a 610 aa polypeptide, corresponding to a putative preproprotein with a theorical molecular mass of 67 kDa. The cleavage of the preproprotein at the N-terminal amino acid side should led to a mature protein of 412 aa long, with a calculated molecular mass of 41.3 kDa. A second processing at the carboxy terminus could led to a shorter protein of about 30 kDa

Analysis of proteins with protease activity in LGP32 ECP performed using gelatin-SDS-polyacrylamide gel, revealed three strong zones of clearing, two predominants bands corresponding to proteases of aproximatively 30-40 KDa and a minor band corresponding to a protease of about 70 KDa. Those bands could correspond to the different processing stages of Vsm, as they were not detected in the mutant strain δ2989. However the alternative hypothese that these bands could correspond to other proteases, whose processing is controlled by Vsm, cannnot be excluded.

In order to demonstrate that this phenotype was due to the *vsm* deletion, and not to an indirect effect of the deletion, we performed an ectopic complementation through the expression of the *vsm* gene under the control of p_{BAD} promoter from another locus of the *V. splendidus* genome, the single copy IS*Visp1*. We observed that when grown in presence of arabinose, this strain displayed an ECP protease activity identical the WT ECP, demontrating the direct relationship between *vsm* deletion and the ECP activity.

In the past, the pathogenesis of bacterial infections has been frequently associated with the production of extracellular proteases (38, 39, 57). The most generally-accepted belief is that these proteases facilitate the spread of the pathogen into the host by causing extensive tissue damage, and up-regulate bacterial growth by degrading numerous host proteins to provide readily available nutrients. Among the bacteria of the genus *Vibrio*, different proteases have already been characterized and reported to play important role in the pathogenicity of *V. cholerae* (20) *V. anguillarum* (49)*, V. vulnificus* (43) and *V. mimicus* (11) to name a few.

As previous work demonstrated that ECPs from *V. splendidus* were implicated in the virulence process (26), we hypothesized that Vsm might directly contribute to oyster toxicity, by analogy with extracellular proteases produced by other shellfish-pathogenic *Vibrio* (9, 50).

When injected to oysters, the LGP32 ECP displayed lethality suggesting that they contained one or more toxic factors, responsible either directly or indirectly for some of the pathological processes observed during infection. This lethal effect was dramatically reduced in the mutant δ2989, and restored by ectopic complementation, suggesting a role of the Vsm metalloprotease in the ECPs toxicity. However similar mortality rates were obtained when strain LGP32 or δ2989 were injected to oysters, showing that *vsm* expression is not necessary for bacterial virulence in this infection model.

Previous studies have examined the contribution in the virulence of various *Vibrio* metalloproteases in animal experimental models by using mutants deleted at the protease gene (20, 31, 42, 53). No conclusive evidence about the role of the protease in virulence was found, since mutants deficient in protease showed comparable virulence levels than their parental strains. In *V. vulnificus,* Shao and Hor suggested that others factors may be overactive in the absence of the metalloprotease and proposed a possible multifactor interaction in bacterial virulence, involving the protease to an non defined extent. To date, it is not known whether the Vsm metalloprotease is preponderant or not during oyster infection or whether additional and/or coregulated virulence factors might be involved in the pathogenesis. There are only a few examples of toxins (such as diphtheria or tetanus) which act as single determinants to produce disease. Microbial pathogenesis is often multifactorial, and pathogens use several biochemical mechanisms operating in concert to produce infection and disease (21). For instance, the HA/P metalloprotease from *V. cholerae* was reported to activate proteolytically both the El Tor cytolysin/haemolysin (47) and the cholera toxin CT, an ADP-ribosylating enterotoxin inducing a highly secretory diarrhea (7). It could be thus hypothesized that Vsm metalloprotease may similarly interact with other virulence factors produced within *V. splendidus* ECPs to potentiate their expression and/or effects on the host. Research is now ongoing to identify the protein targets that are processed by Vsm in the ECP fraction.

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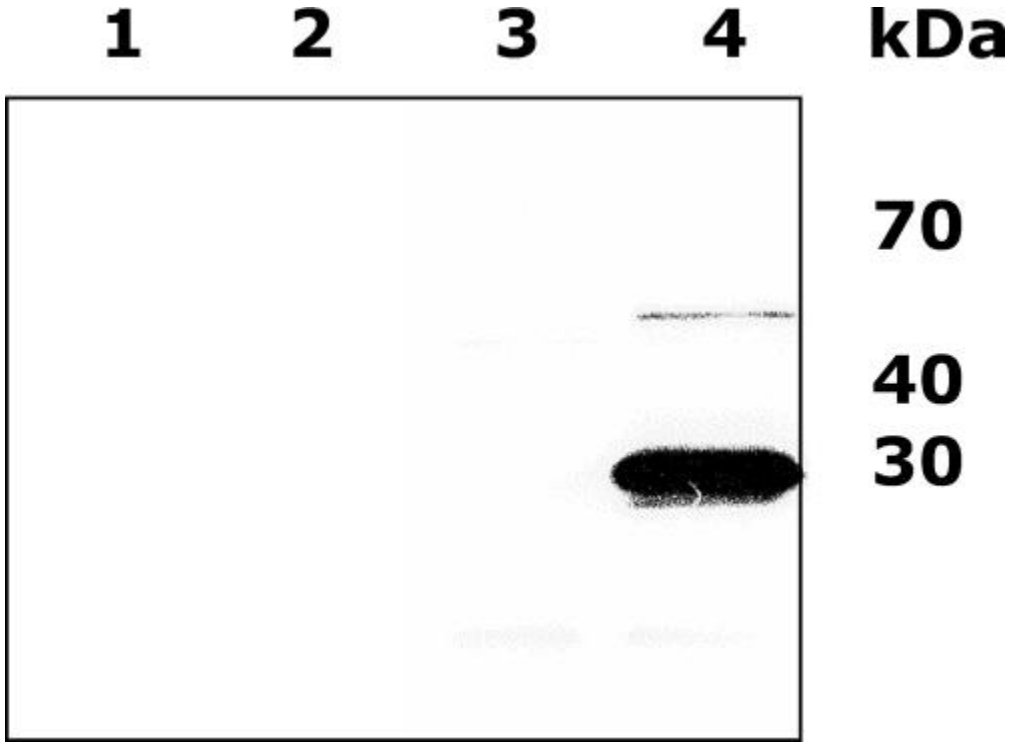

Fig.1: Detection of GFP by immunoblot from protein extract of LGP32 WT (1) or transconjugants containing the plasmid pSU18T-p_{BAD}*gfp* (2-4). GFP expression is repressed in absence (2) or presence of 1% glucose (3) and induced in presence of 0,2% arabinose (4). Cells were grown overnight before protein extraction and GFP detection. The expression of GFP in strains was confirmed in epifluorescence microscopy

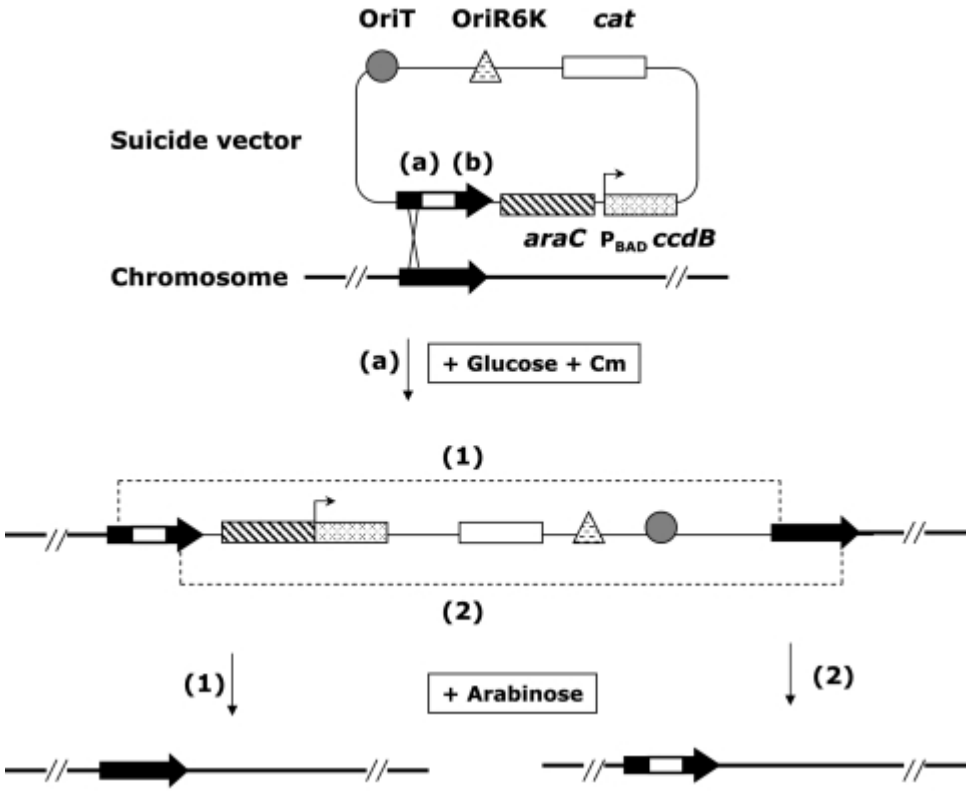

Fig.2: Schematic representation of the two-steps allelic exchange procedure. Integration can occur by recombination on either side of the plasmid allele of interest (a or b). For excision to lead to a successful allelic exchange recombination must occur in the second region of homology. Only strains in which the p_{BAD}ccdB containing plasmid has been excised can survive on arabinose containing medium. When the 5' and 3' regions, which flank the deletion in the chosen gene, are of similar size, selection for plasmid loss should result in a 50:50 ratio of wt:mutant when targeting non essential genes.

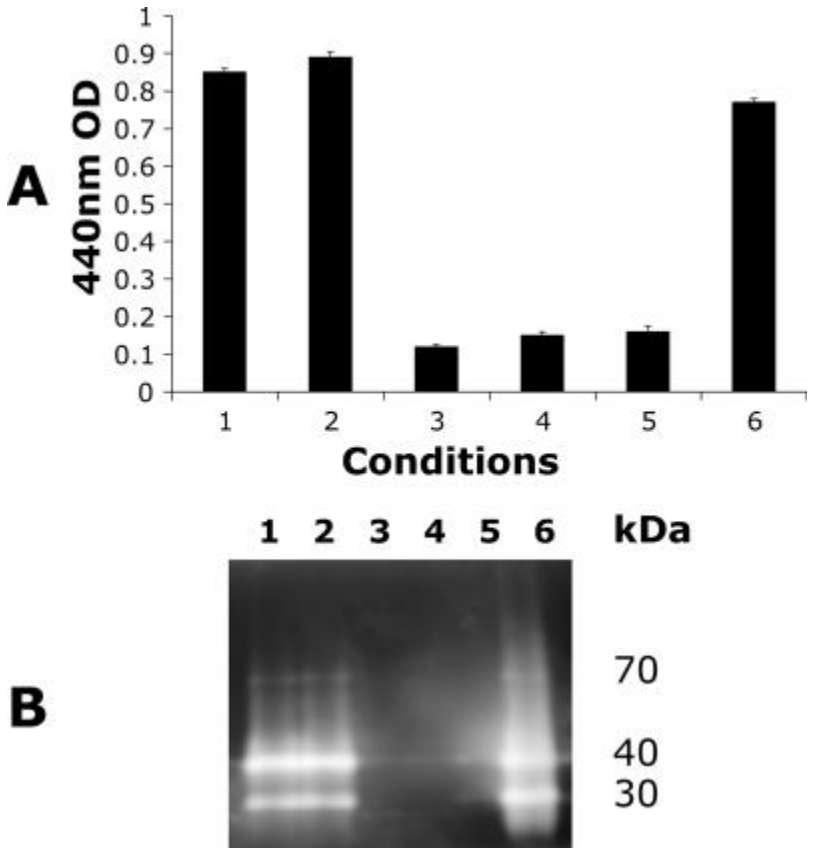

Fig.3: The proteolytic activity determination of ECP with azocasein assay (A: absorbance at 440 nm) and gelatin-SDS-polyacrylamide gel (B: protease which degraded the gelatin are detected by zones of clearing). ECP of LGP32 (lane 1 and 2), δ2989 (lane 3 and 4) and δ6720-*vsm* (lane 5 and 6) were prepared in MA (lane 1, 3, 5) or MA+ara (Lanes 2, 4, 6) according to the material and method section. Bacterial extracellular products (ECPs) were produced by the cellophane overlay method as described by Liu *et al* (36). Bars indicate standard deviations (A). The given molecular weights are only indicative as they correspond to molecular mass markers migration in these non-denaturing conditions (B).

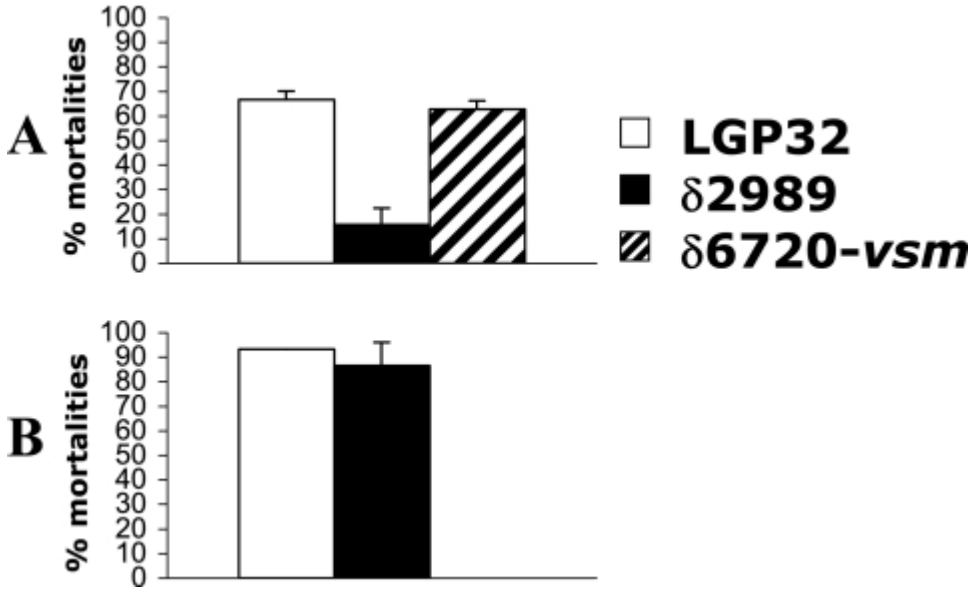

Fig.4: Comparison of oyster mortality after intramuscular injection of ECP (5 μg/gr oysters) from wild type LGP32, δ2989 and δ6720-*vsm* mutants (A) or of living strains (10^9 to 2.10^9 CFU ml^{-1}). Experiments were performed in triplicate (15 to 20 oysters per aquarium). Mean mortality rates (%) are given at two days post oysters injection of ECP (A) or strains (B). Bars indicate standard deviations. Data from each experiment were statistically analysed using Chi-square test and StatView® software. Significant results were obtained only after ECP injections between LGP32 and δ2989 mutants and between δ6720-vsm and δ2989 mutants (χ^2 = 27.62, p<0.0001 and χ^2 = 23.69, p<0.0001 respectively). Sterile seawater was injected as negative control and gave no mortality (data not shown).

Table 1 : Bacterial strains used and constructed in this study

Bacterial strains	**Description**	**Reference**
LGP32	5. Vibrio splendidus	(24)
N16961	*Vibrio cholerae*	(28)
DH5α	(F $^-$) *supE44 ΔlacU169 (Φ80lacZΔM15)ΔargF hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
B462	*lacIQ, thi1, supE44, endA1, recA1, hsdR17, gyrA462, zei298::Tn10* [Tc^R]	Provided by L. Van Melderen
Π3813	B462 *ΔthyA::(erm-pir116)* [Erm ^{R}]	This study
S17-1	(F $^-$) RP4-2-Tc ::Mu *aph* ::Tn7 *recA* [Sm^R]	(54)
β2163	(F $^-$) RP4-2-Tc::Mu ΔdapA ::(erm-pir) [Km ^{R} Em ^{R}]	(17)
β3914	β2163 *gyrA462, zei298::Tn10* [Km ^{R} Em ^{R} Tc^R]	This study
GG784	TG1 F' *(ΔccdA ::spec RexBADccdB*) [Sp^R]	(52)
δ2989	LGP32 *Δvsm*	This study
δ4175	LGP32 *ΔluxU*	This study
δ3453	LGP32 *ΔluxM*	This study
δ6720–vsm	δ2989 *ΔISVs1 orfB::araC-p$_{BAD}$vsm*	This study
δintIA	N16961 *ΔintIA*	This study

Table 2 : Plasmid used and constructed in this study

Plasmid	**Description**	**Reference**
PUC 18	Ori ColE1, [Ap^R]	Pharmacia
pKOBEG A	pSC101ts::P_{bad}*red*$\gamma\delta\beta\alpha$; [Ap^R]	(10)
pSU18	Ori p15A [Cm^R]	(1)
pBAD*gfp*	*gfp* cassette (S65T, F64L) [Ap^R]	Provided by J. M. Ghigo
PSU18T-P_{BAD}*gfp*	pSU18::*oriT*$_{RP4}$-*araC*-P_{BAD}*gfp;* [Cm^R]	This study
PSU18T-P_{BAD}*gfp2*	pSU18::*oriT*$_{RP4}$-*araC*-P_{BAD}*gfp;* [Cm^R]	This study
PSU18T-P_{BAD}*vsm*	pSU18::*oriT*$_{RP4}$-*araC*-P_{BAD}*vsm;* [Cm^R]	This study
pSW23T	*oriV*$_{R6K\gamma}$; *oriT*$_{RP4}$; [Cm^R]	(17)
pSW29T	*oriV*$_{R6K\gamma}$; *oriT*$_{RP4}$; [Km^R]	(17)
pSW4426T	pSW23T::*aadA7-araC*-P_{BAD}*ccdB;* [Sp^R, Cm^R]	This study
pSW4427T	pSW29T::*aadA7-araC*-P_{BAD}*ccdB;* [Sp^R, Km^R]	This study
pSWδ2989T	pSW4426T*;Δvsm;* [Sp^R, Cm^R]	This study
pSWδ4175T	pSW4426T*;ΔluxU;* [Sp^R, Cm^R]	This study
pSWδ3453T	pSW4426T*;ΔluxM;* [Sp^R, Cm^R]	This study
pSWδ5679T	pSW4426T*;ΔgyrA;* [Sp^R, Cm^R]	This study
pSWδintIAT	pSW4427T*;ΔintIA;* [Sp^R, Km^R]	This study
pSWδ6720T-*vsm*	pSW4426T*;ΔISVs1 orfB::araC*-P_{BAD}*vsm;* [Sp^R, Cm^R]	This study

Supplementary Table 1 : Oligonucleotides used in this study.

Oligonucleotide	Sequence	Size	
		WT	Mutant
Thy1	TAAGAATTCCGTTTGGTCTTGGTGCCG	3012	
Thy2	CGGGAATTCACGTAAATAGAGCAAATAGTCC		
OriT-Xba1	CGGGTCTAGACAGCGCTTTTCCGCTGCATAAC	270	
OriT-Pst1	CGGGCTGCAGCCGGCCAGCCTCGCAGAGCA		
P_{BAD}GFP-Kpn1	GCCCGGTACCAATTATGACAACTTGACGGC	1978	
P_{BAD}GFP-Xba1	GCCCTCTAGACTATTTGTATAGTTCATCCATGCC		
GFP3	GCCCGAATTCATGAGTAAAGGAGAAGAACTTTTC	4437	
GFP4	GCCCGAATTCTTCCTCAATTGCAATCGCCATCGTTTC		
ccdB1	GCCCTCTAGAAAAAAAAGCCCGCTCATTAGGCGGGCTCGAATAAA TACCTCATTGGCTGGCACCAAGCAG	2645	
ccdB2	GCCCGAGCTCTTATATTCCCCAGAACATCAGG		
luxU-1	GCCCGAATTCTAGGGCAAGAGAATGTCCCAGTGT	277	
luxU -2	GCCCGAATTCTGCTGGTACTCTTGGTAGGTGGAT		
luxU-3	GCCCCTCGAGTTTGGTGCGGACTCTTTGTG	4692	
luxU-4	GCCCCTCGAGCACTACTCTTAAGTGCATGGC		
luxU-5	GTCTGTCGAAGAAGGCAAGG	927	918
luxU-6	GAATTGCGTGACTACTGACC		
luxM-1	GCCCGAATTCAGGTTCACTGTTGGCTAGCA	1132	
luxM-2	GCCCGAATTCGAGGCACCGTCTATAGTCTCGAAA		
luxM-3	GCCCCTCGAGAACACCCAACAGATGAAGCC	5507	
luxM-4	GCCCCTCGAGCATCTCATACCACTTCTCACC		
luxM-5	TGCATCCTGCTAACACAGCG	1874	1825
luxM-6	GGCATTACTGCTGATCCACG		
ISvisp1-1	GCCCGAATTCCTTGCTAAAAAAGTGGCAACG	695	
ISvisp1-2	GCCCGAATTCCGATTCATACTTGGGACAATTCC		
ISvisp1-3	GCCCCTCGAGGGCCAAGCTACATCGGTTC	5069	
ISvisp1-4	GCCCCTCGAGGATCCGAGCTACACGCTTGC		
ISvisp1-5	GTCTAGTCGGATTGCAGAGC	770	550
ISvisp1-6	TCAGTGCAATGACCTGGACG		
vsm-1	CGGGGAATTCATGAACCAACAACGTCAACTAAGCTGGAAAATAGCA GCTATAGCAGC	1824	
vsm-2	GCCCGAATTCTCAATCTAAACGTAACGTTAGGC		
vsm-3	GCCCCTCGAGGCGGTGTTTATAACCGCGCC	5487	
vsm-4	GCCCCTCGAGCGTTCTGGCATAGAGGATGC		
vsm-5	CTTCCTATAAAGCCGTTGGG	1902	1379
vsm-6	CTTGTTTGAGAGGCTCGCTCC		
vsm-7	CGGGTCTAGACTCGAGCTCCCGGGTCAATCTAAACGTAACGTTAG G	3105	
araCSxho	GCCCCTCGAGGATCTAATTATGACAACTTGACGGC		
gyrA-1	GCCCGAATTCATGTCTAACGAAATTACATATGATGG	2259	
gyrA-2	GCCCGAATTCCTAAGCGTCACTAGGTTCATC		
gyrA-3	GCCCCTCGAGGTGAGCCAATTACAGGCCGC	5508	
gyrA-4	GCCCCTCGAGCCTACCGCGATACCAGTAATACC		
gyrA-5	TAACGGTGATATGGCCGAGG	2349	1247
gyrA-6	AACCCAGCTCTTAAGCTGGG		
intIA1	GCCCGAATTCATGAAATCCCAGTTTTTGTTAAG	963	
intIA2	GCCCGAATTCCTAAAGACGGGATAATGGGC		
intIA3	GCCCCTCGAGGCAAAAGAACTCTATCCGCATC	5279	
intIA4	GCCCCTCGAGCCCCGAACCGTAGAGTAAC		
intIA5	CATGATTTCGCAAAGAACCCATG	1012	922
intIA6	CCAGTTAAATATTCAGTGAGAAC		