Construction of a *Vibrio splendidus* Mutant Lacking the Metalloprotease Gene *vsm* by Use of a Novel Counterselectable Suicide Vector††

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*Vibrio splendidus* is a dominant culturable *Vibrio* in seawater, and strains related to this species are also associated with mortality in a variety of marine animals. The determinants encoding the pathogenic properties of these strains are still poorly understood; however, the recent sequencing of the genome of *V. splendidus* LGP32, an oyster pathogen, provides an opportunity to decipher the basis of the virulence properties by disruption of candidate genes. We developed a novel suicide vector based on the *pir*-dependent R6K replicative origin, which potentially can be transferred by RP4-based conjugation to any *Vibrio* strain and which also carries the plasmid F toxin *cdB* gene under control of the *P*~Pl~ promoter. We demonstrated that this genetic system allows efficient counterselection of integrated plasmids in the presence of abrinose in both *V. splendidus* and *Vibrio cholerae* and thus permits efficient markerless allelic replacement in these species. We used this technique to construct several mutants of *V. splendidus* LGP32, including a derivative with a secreted metalloprotease gene, *vsm*, deleted. We found that this gene is essential for LGP32 extracellular product toxicity when the extracellular products are injected into oysters but is not necessary for virulence of bacteria in the oyster infection model when bacteria are injected.

*Vibrio splendidus* is a dominant culturable *Vibrio* in coastal marine sediments, seawater, and bivalves, including oysters (23). This organism has long been considered to be an environmental organism without any pathogenic significance. However, over the last few years, different strains phenotypically related to this species have been associated with mortality mainly in mollusks, shrimps, gorgonians, and fish (for a review, see reference 35). Compared to human pathogen species, little is known about *Vibrio* pathogenesis in marine animals, and despite descriptions of invasiveness and extracellular product (ECP) toxicity, no data are available for a group related to *V. splendidus* (26, 37, 48, 56).

The different types of enzymatic activities that have been shown to play a role in the virulence of a variety of pathogenic bacteria include extracellular proteases; for example, such proteases have been described for *Vibrio cholerae* (7), *Vibrio vulnificus* (33), and *Vibrio anguillarum* (42), although a direct role of these proteases in virulence has not been demonstrated. For example, it has been shown that the *V. cholerae* metalloprotease cleavage activity is essential for activating the A subunit of the cholera enterotoxin (12), as well as for degrading intestinal mucus and facilitating 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 shown to be involved in the invasive mechanism of this fish pathogen (49).

We recently completed sequencing of the genome of *V. splendidus* strain LGP32 in order to obtain access to its full gene repertoire (F. Le Roux, M. Zouine, N. Chakroun, J. Binesse, D. Saulnier, L. Ma, C. Rusniok, C. Buchriser, and D. Mazel, unpublished data). The strain that we used is an oyster (*Crassostrea gigas*) pathogen (23, 24). We 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 exhibits 95% identity with the product of the *V. anguillarum* *vam* gene, which has been shown to be involved in the virulence properties of this fish pathogen (42).

Gene knockout is often essential for formal demonstration of the predicted or supposed role of a gene candidate. However, this strategy is limited to species in which the available genetic tools can be used. There can be limitations at several levels, from DNA delivery inside the cells to the allelic exchange efficiency. DNA transformation, whether it is natural or artificial, is either inoperative or inefficient in numerous species. In *V. splendidus*, attempts to transfer plasmids using electroporation were ineffective (unpublished results), which prevented use of the 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 based on the IncPα plasmid RP4 (RK2) transfer functions have been described (54). In most cases the subsequent step, allelic replacement or integration of the incoming DNA, is achieved through use of a nonreplicative DNA molecule. The most popular system for gram-negative species is the system using conditionally replicative R6K plasmid derivatives, such as pGP704 (41). R6K replication is dependent on binding of the *pir*-encoded II protein, and transcomplementation of a *pir*-dependent plasmid derivative by II proteins expressed from another replicon can be performed (32). Based on this seminal observation, several plasmids carrying the R6K* origin of rep-
lication that can be replicated only in strains expressing pir have been constructed. When these plasmids also carry an 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 probeorganisms can be used as recipients for conjugation (reference 14 and references therein). Several counterselectable markers have been described (for a review, see reference 48), and some of them have been successfully used in R6K-oriT derivatives for positive selection of replaced alleles (19, 51, 55).

One of the methods consists of using a wild-type (WT) 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 be cloned and Sm' mutant strains be used.

Due to its general efficiency in gram-negative bacteria and to the simplicity of the counterselection protocol, the Bacillus subtilis levan sucrase gene sacB has gained considerable notoriety since 1985, when it was first introduced (25), and it is now certainly the most commonly used of the different counterselectable markers. sacB-based suicide vectors, such as pCVD442 (19), have been successfully used for allelic replacement in V. cholerae (18). However, even though sacB has occasionally been used successfully, the use of this gene for allelic replacement in many Vibrio species or in other marine bacterial species is seriously impeded by the necessary absence of NaCl in the counterselection medium (6). This is the case, for example, with V. splendidus, which cannot grow when NaCl is not present in the medium.

In order to overcome this limitation, we 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 the Escherichia coli F plasmid under control of the arabinose P_{BAD} promoter (52) as a counterselection marker. We demonstrated that this system allowed positive selection for the loss of vector sequences after homologous recombination in V. splendidus and V. cholerae, with high efficiency. This strategy was used to construct a V. splendidus strain with the vsm gene deleted in order to establish the contribution of the Vsm metalloprotease to the virulence properties of this strain during infection of oysters.

**MATERIALS AND METHODS**

**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 mortality and was demonstrated to be pathogenic for oysters and clams (23). V. cholerae and E. coli strains were grown in Luria-Bertani (ML) broth or, in case of I3813, Mueller-Hinton broth at 37°C. V. splendidus strains were grown in Luria-Bertani broth containing 0.5 M NaCl in marine broth (MB), or on marine agar (MA) at 20°C. All media were obtained from Difco. Antibiotics were used at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 12.5 μg/ml; erythromycin, 200 μg/ml; kanamycin, 25 μg/ml; nalidixic acid, 30 μg/ml; spectinomycin, 50 μg/ml; and tetracycline, 15 μg/ml. Thymidine and diaminopimelate were added when necessary at a final concentration of 0.3 mM. Induction of ccdB expression under control of the P_{BAD} promoter was achieved by addition of 0.2% L-arabinose to the growth media, and the expression was repressed by addition of 1% glucose.

**PCRs.** PCRs performed for plasmid assembly were done in 50-μl mixtures by using the Pfu DNA polymerase (Promega) and following the manufacturer's instructions. Other PCRs were performed in 50-μl mixtures using the Bioline Taq polymerase according to the manufacturer's instructions. The primers used are listed in Table S1 in the supplemental material. The conditions used for amplification were as follows: 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 10°C less than the melting temperature for 30 s, and 72°C for 60 s per kb.

**Construction of CcdB-resistant E. coli strains.** As the gyrA462 mutation has been shown to prevent toxic interaction of the gyrase with CcdB (13), we used this genetic background to prevent selection of a ccdB-inactivating mutation. We previously developed a set of strains carrying pir alleles in different genetic contexts (17). One of these strains carries a pir-116 allele inserted into the thymidylate synthase gene (thyA) of E. coli.

The gyrA462 allele was moved from S17-1 to ΔsacB::(erm-pir-116) E. coli strain I3813 strain was constructed by allelic replacement of the chromosomal thyA allele of B462 with ΔsacB::(erm-pir-116), as described previously (17). This thymidine auxotroph strain is CcdB resistant and peripbs R6K vector replication.

The chromosomal gyrA462 allele was moved from S17-1 to ΔsacB::(erm-pir) E. coli strain B2163 by P1 transduction and Tet’ selection of cotransduced ze298::Tn10 to construct strain B3914. To easily discriminate the transductant carrying the gyrA462 allele from transductants still carrying gyrA, we used NaI/NaI screening. Indeed, NaI resistance is linked to mutations in gyrA, but gyrA462 does not confer this phenotype. Then we selected a spontaneous NaI derivative of B2163 and used it as the recipient for gyrA462-ze298::Tn10 cotransduction. After transduction, the colonies which had acquired the gyrA462 allele together with ze298::Tn10 were expected to be NaI+. In agreement with this, Ze NaI colonies were found to be sensitive to CcdB toxicity. One clone was designated B3914 and used in this study.

### Table 1. Bacterial strains used and constructed in this study

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>Description</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>LGP32</td>
<td>Vibrio splendidus</td>
<td>24</td>
</tr>
<tr>
<td>N16961</td>
<td>Vibrio cholerae</td>
<td>28</td>
</tr>
<tr>
<td>DHS5</td>
<td>F' supE44 ΔlucU169 (680arZAM15) ΔargF</td>
<td>Laboratory</td>
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<tr>
<td>B462</td>
<td>lacI thi-1 supE44 endA1 recA1 lasR17</td>
<td>L. Van Melderen</td>
</tr>
<tr>
<td>I3813</td>
<td>B462 ΔsacB::(erm-pir-116) (Erm’)</td>
<td>This study</td>
</tr>
<tr>
<td>S17-1</td>
<td>F’ RP4-2-Tc::Mu aph::Tn7 recA (Sm’)</td>
<td>54</td>
</tr>
<tr>
<td>p2163</td>
<td>F’ RP4-2-Tc::Mu Aph::Tn7 (erm-pir)</td>
<td>17</td>
</tr>
<tr>
<td>E3914</td>
<td>B2163 gyrA462 zei-298::Tn10 (Km’ Em’ Tc’)</td>
<td>This study</td>
</tr>
<tr>
<td>GG784</td>
<td>Tgl F’ ΔaccA::speC recBAD ccdB (Sp’R)</td>
<td>52</td>
</tr>
<tr>
<td>82989</td>
<td>LGP32 Δvsm</td>
<td>This study</td>
</tr>
<tr>
<td>64751</td>
<td>LGP32 ΔaurU</td>
<td>This study</td>
</tr>
<tr>
<td>63343</td>
<td>LGP32 ΔaurM</td>
<td>This study</td>
</tr>
<tr>
<td>66720-vsm</td>
<td>82998 ΔIS51 orfB::araC-P_{BAD}vsm</td>
<td>This study</td>
</tr>
<tr>
<td>šintIA</td>
<td>N16961 ΔintIA</td>
<td>This study</td>
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### Table 2. Plasmids used and constructed in this study

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td>pUC18</td>
<td>ori ColE1 (Ap’)</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pKOBEGA</td>
<td>pSC101::pCEd2-amb3 (Ap’)</td>
<td>10</td>
</tr>
<tr>
<td>pSU17</td>
<td>ori p15A (Cm’)</td>
<td>J. M. Ghigo</td>
</tr>
<tr>
<td>pBADgfp</td>
<td>gfp cassette (S6ST, F64L) (Ap’)</td>
<td>This study</td>
</tr>
<tr>
<td>pSU18T-5PAM6Rf2</td>
<td>pSU18T-oriRNAa::araC-P_{BAD}gfp (Cm’)</td>
<td>This study</td>
</tr>
<tr>
<td>pSU18T-5PAM6Rf3</td>
<td>pSU18T-oriRNAa::araC-P_{BAD}gfp (Cm’)</td>
<td>This study</td>
</tr>
<tr>
<td>pSW23T</td>
<td>oriV_{BAD}, oriP15A (Cm’)</td>
<td>17</td>
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<tr>
<td>pSW29T</td>
<td>oriV_{BAD}, oriP15A (Km’)</td>
<td>17</td>
</tr>
<tr>
<td>pSW4426T</td>
<td>pSW23T::Δafl-aacC-P_{BAD}cddB (Sp’ Cm’)</td>
<td>This study</td>
</tr>
<tr>
<td>pSW4427T</td>
<td>pSW23T::Δafl-aacC-P_{BAD}cddB (Sp’ Kmr)</td>
<td>This study</td>
</tr>
<tr>
<td>pSW62989T</td>
<td>pSW4426T, ΔaflU (Sp’ Cm’)</td>
<td>This study</td>
</tr>
<tr>
<td>pSW64175T</td>
<td>pSW4426T, ΔaflU (Sp’ Kmr)</td>
<td>This study</td>
</tr>
<tr>
<td>pSW63453T</td>
<td>pSW4426T, ΔaflM (Sp’ Cm’)</td>
<td>This study</td>
</tr>
<tr>
<td>pSW6579T</td>
<td>pSW4426T, ΔaflA (Sp’ Cm’)</td>
<td>This study</td>
</tr>
<tr>
<td>pSW69910T</td>
<td>pSW4426T, ΔaflA (Sp’ Kmr)</td>
<td>This study</td>
</tr>
<tr>
<td>pSW66720-vsm</td>
<td>pSW4426T, ΔIS51 orfB::araC-P_{BAD}vsm</td>
<td>(Sp’ Cm’)</td>
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</table>
Plasmid construction. (i) pSU1ST-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 fragment was cloned in pSU18, a pUC derivative compatible with CoE1 and RskK derivatives, yielding pSU1ST. A 1,978-bp fragment harboring the **arcC** gene, the **P_{BAD}** promoter, and the **gfp** gene was amplified from the pSU18 gfp plasmid by using primers P_{BAD}GFP-Kpn and P_{BAD}GFP-Xba. After KpnI-XbaI digestion, the fragment was generated in pSU18T. Green fluorescent protein (GFP) expression was confirmed by epifluorescence microscopy. (ii) Suicide vectors. A 6,265-bp fragment harboring a transcriptional terminator, the **aada7** gene, the **arcC** gene, the **P_{BAD}** promoter, and the **ccdB** gene was PCR amplified using GG784 DNA (32) as the template and primers ccdB1 and ccdB2. After XbaI-SacI digestion, this fragment was ligated to the suicide vectors pSW23T and pSW29T and digested with the same restriction enzymes, yielding pSW4426T and pSW4427T, respectively.

Several *V. splendidus* genes, including luxU, luxM, ISVisp1 orfB, vsm, and gycA, as well as the *V. cholerae* superintegron integrase gene intIA, were PCR amplified from genomic DNA using primers luxU-1 and luxU-2, primers luxM-1 and luxM-2, primers ISvisp1-1 and ISvisp1-2, primers vsm-1 and vsm-2, primers gycA-1 and gycA-2, and primers intIA1 and intIA2, respectively. Amplicons were digested with EcoRI and cloned in the EcoRI site of pUC18 (Pharmacia). The corresponding alleles carrying an internal deletion were constructed by inverse PCR using primers luxU-3 and luxU-4 (ΔluxU allele), primers luxM-3 and luxM-4 (ΔluxM allele), primers ISvisp1-3 and ISvisp1-4 (ΔISvisp1::ΔorfB allele), primers vsm-3 and vsm-4 (Δvsm allele), primers gycA-3 and gycA-4 (ΔgycA allele), and primers intIA3 and intIA4 (ΔintIA allele), XhoI digestion, and self-ligation. The different alleles were then recovered after EcoRI digestion and gel purification. In vitro PCR amplification by PCR ligation into pSW4426T and/or pSW4427T previously linearized with EcoRI. (iii) Vector construction for ectopic complementation. Complementation experiments were performed by introducing vsm under **P_{BAD}** promoter control into the nonessential gene coding for ISVisp1 transposase present in the chromosome, using the strategy described above. An inverse PCR was performed with primers GFP3 and GFP4 using pSU18T-P_{BAD}gfp as the template. The resulting ampiclon was sequenced using EcoRI and self-ligation. The primers have been deposited in the GenBank database under accession numbers DQ987705, DQ987706, DQ987707, DQ987708, DQ987704, DQ995482, and DQ995483.

RESULTS

Construction of a plasmid vector allowing controlled expression in *V. splendidus*. We constructed a p15A derivative plasmid, pSU1ST-P_{BAD}gfp, carrying the green fluorescent protein gene under control of the positively regulated arabinose-inducible **P_{BAD}** promoter and an origin of transfer from RP4. Conjugal transfer of this plasmid from *E. coli* to *V. splendidus* was observed at a frequency of 10^{-3} transconjugant per recipient. Western blot analysis using commercial polyclonal antibodies resulted in detection of GFP with a molecular mass of about 30 kDa in extracts of LGP32 transconjugants when arabinose was added to the culture media, while no signal was obtained when transconjugants were grown in the presence of glucose or, in the case of LGP32, WT extracts (Fig. 1). Thus, the **P_{BAD}** promoter appeared to be tightly and properly regulated in LGP32 by the arac**C** gene product, which activated transcription in response to its natural inducer, arabinose.

Development of a two-step allelic replacement method using CdB as a positive selection marker. CdB is a very strong gyrase inhibitor and was originally discovered in the postregulative killing operon carried on plasmid F, *cdBA* (2). The **ccdB** gene has been employed as a potent counterselection marker in a number of commonly used applications (3, 4, 52).

Starting with pSW23T (Cm') and pSW29T (Km'), both carrying *oriT_{RKO}* and *oriT_{RP4}*, we constructed the derivatives pSW4426T and pSW4427T carrying the **ccdB** gene under control of **P_{BAD}**-araC. In order to avoid natural selection of inac...
The expression of GFP in strains was confirmed by epifluorescence microscopy.

tive ccdB mutants due to leaking of \( P_{BAD} \) we obtained CcdB-resistant \( E. coli \) (pir') strains \( \Pi3813 \) and \( \beta3914 \) by replacement of the \( \text{gyrA} \) gene with the \( \text{gyrA}462 \) allele, which is not sensitive to CcdB poisoning (2).

Using pSW4426T, we constructed three suicide vector derivatives with the luxU, luxM, and \( \text{gyrA} \) alleles deleted in order to create LGP32 mutants with mutations at these loci by allelic exchange. These \( \text{Cm}^r \) plasmids were designed to allow selection of strains in which the plasmid was integrated into the target gene by a first homologous recombination event (with ccdB repression) and to allow positive selection of a second recombination event resulting in suicide vector loss (ccdB expression) (Fig. 2).

The conjugation and integration frequencies of these plasmids in the LGP32 genome were found to be \( 10^{-8} \) to \( 10^{-7} \). Integration can occur by recombination on either side of the plasmid allele of interest (a or b). For excision to lead to successful allelic exchange, recombination must occur in the second region of homology. Only strains in which the \( P_{BAD}\text{ccdB} \)-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 similar sizes, selection for plasmid loss should result in a 50:50 ratio of wild type to mutant when nonessential genes are targeted.

Table 3 shows the insert size dependence for integration efficiency and proportion of mutants obtained using the two-step allelic replacement procedure.

<table>
<thead>
<tr>
<th>Open reading frame</th>
<th>Size (bp)</th>
<th>Integration efficiency</th>
<th>Proportion of mutants</th>
</tr>
</thead>
<tbody>
<tr>
<td>luxU</td>
<td>140</td>
<td>( 10^{-8} )</td>
<td>3/8</td>
</tr>
<tr>
<td>luxM</td>
<td>570</td>
<td>( 2 \times 10^{-7} )</td>
<td>4/8</td>
</tr>
<tr>
<td>vsm</td>
<td>600</td>
<td>( 5 \times 10^{-7} )</td>
<td>18/18</td>
</tr>
<tr>
<td>gyrA</td>
<td>600</td>
<td>( 10^{-8} )</td>
<td>0/8</td>
</tr>
</tbody>
</table>

recombinant per recipient, depending on the size of the region flanking the deletion in the fragment (Table 3). Several \( \text{Cm}^r \) colonies were reisolated and grown overnight, and \( 10^7 CFU \) was spread on a plate containing \( 0.2\% \) arabinose. One hundred colonies were obtained and found to be \( \text{Cm}^r \). Screening for mutants among the surviving colonies was performed by PCR using primers flanking the target gene in the genome; 40 to 50% of the colonies were mutants in the case of \( \Delta\text{luxU} \) and \( \Delta\text{luxM} \) allele replacement, whereas only wild-type genotypes were detected in the case of the essential \( \text{gyrA} \) gene.

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

Construction and characterization of \( V. splendidus \) \( \Delta\text{vsm} \) mutant. We constructed pSW62989T, a suicide vector having an internal deletion in the \( \text{vsm} \) gene, to create an LGP32 mutant with a mutation at this locus by allelic exchange. After conjugal transfer and selection with arabinose, screening for mutants among the surviving colonies was performed by PCR using primers flanking the \( \text{vsm} \) gene in the genome and amplifying 1,800 bp from the wild type and 1,300 bp from mutants (Table 3). Surprisingly, all 18 colonies tested were found to carry the mutated allele, instead of the 50% theoretically expected, when mutant constructs having 5' and 3' flanking regions that were similar sizes were used as recombination targets. One of these isolates was designated 82989 and used in this study.

The 82989 mutant was tested for the loss of metalloprotease activity. The proteolytic activity of 82989 ECPs (Fig. 3A, lanes 3 and 4) was determined by an azocasein assay and was shown to be eightfold lower than that of the wild type (Fig. 3A, lanes 1 and 2). In addition, a gelatin-SDS-polyacrylamide gel was used to detect extracellular proteins with protease activity. In the case of LGP32 ECPs, two predominant protease bands (at estimated molecular masses of 30 to 40 kDa) and one minor band (at an estimated molecular mass of 70 kDa) were observed as strong zones of clearing (Fig. 3B, lanes 1 and 2). These bands were missing from the 82989 gel (Fig. 3B, lanes 3 and 4).

Ectopic complementation of the \( \text{vsm} \) mutation. ISvisp1 is an insertion sequence specific for \( V. splendidus \) (deposited in the IS database at http://www-is.biotoul.fr/is.html), and one copy is present in the LGP32 genome (Mazel et al., unpublished data). As this locus is probably not essential, we used it as a platform...
for ectopic expression in a transcomplementation experiment with the Δvsm mutant. The vsm gene under control of araC-PRAD was cloned in the ISvisp1 ΔorfB allele carried by pSW66720T (Cm'). The resulting plasmid was transferred by conjugation to strain Δ2989, in which it could integrate into the genome through homologous recombination with the ISvisp1 orfB gene. Cm' clones were obtained, and four of these clones were grown without selection and then subjected to selection on arabinose-supplemented medium. Ten colonies having the Cm' phenotype expected after a second recombination event were tested by PCR, and one-half of them were found to carry β-galactosidase overlay method as described by Liu (36). (A) The error bars indicate standard deviations. Data from each experiment were statistically analyzed using the chi-square test and StatView software. The differences were significant only for comparisons of ECPs of LGP32 and the δ2989 and δ6720-vsm mutants (A) or of living organisms (10^7 to 2 x 10^7 CFU ml^-1). Experiments were performed in triplicate (15 to 20 oysters per aquarium). The mean mortality rates were determined 2 days after injection of ECPs (A) or strains (B). The error bars indicate standard deviations. (B) The molecular masses on the right indicate the positions of molecular mass markers migrating in the nondenaturing conditions used.

**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 under way (22). Genomics-based approaches have significantly increased our understanding of the physiology and pathogenicity of many microbes and have 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 demonstration of the proposed or suspected function of a gene candidate.

Parallel to the complete sequencing of the *V. splendidus* genome, we developed a gene knockout strategy which was used here to investigate the role of a secreted metalloprotease in the toxicity of extracellular products and in virulence in vivo.

In the first step, we established conditions for transferring a mobilizable plasmid in *V. splendidus* through the RP4 conjugative machinery and the functionality of the P_RAD 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
truncated version of it carried by a suicide vector introduced into a strain by conjugation (41). Since then, workers have had considerable success with this strategy. However, it has several important limitations. First, the chromosomes of mutants constructed by this technique carry a partial duplication of the targeted gene, which can be the source of reversion events at a high frequency. Second, the presence of a copy of the vector backbone prevents further mutant construction by the same method in these strains, as recombination between the incoming vector and the chromosomal copy cannot be counterselected. Finally, the mutations obtained 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-step strategy that allowed 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 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, levansucrase, and its activity has been shown to be toxic for gram-negative organisms when they are grown in the presence of sucrose (25). When a mutant which contains an integrated copy of a suicide vector also carries sacB due to recombination into a target locus, upon exposure to sucrose, the daughter cells that underwent a second recombination event resulting in loss of the suicide vector are the only cells that 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 at a 1:1 ratio. If the gene is essential, the isolates that survive in the presence of sucrose carry the WT allele. However, it is known that SacB toxicity is susceptible to the presence of sodium chloride in the selective medium (6). As addition of sodium chloride to media is absolutely necessary for the growth of many Vibrio species, such as V. splendidus, we had to develop and use a different counterselective marker in order 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 carries the ccdB gene of the E. coli F plasmid under control of the arabinose P_{BAD} promoter (52) as a counterselective 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 a counterselective marker for the development of several cloning vectors (3, 4). We constructed two vectors, pSW4426T, which carries a Cm\(^{\prime}\) marker and the araC-P_{BAD}-ccdB cassette, and pSW4427T, which is identical to pSW4426T except that it carries a Km\(^{\prime}\) marker. When these plasmids carry the mutated allele, allelic replacement can be assayed in a two-step procedure, first through 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 through transfer of the selected clones in a medium supplemented with arabinose to induce the expression of the lethal CcdB protein. As described above, if the targeted gene is not vital, the second recombination leads to either WT allele restoration or to allelic substitution with the same proportion (50%); if the gene is essential, the surviving isolates carry only the WT allele.

In order to validate our technique, V. splendidus ΔluxU, ΔluxM, and ΔgyrA alleles were constructed and cloned in pSW4426T, and allelic replacement was tested for each of them using the protocol described above. As expected, the ΔluxU and ΔluxM alleles were successfully substituted for the WT alleles at a frequency of about 50% in the cells that survived in the presence of arabinose, while in the case of the essential gyrA gene, the cells that survived in the presence of arabinose were found to carry only 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 knockout strategy with another Vibrio species, V. cholerae, using to the superintegron integrase gene intI4 (formerly called intI4 [40]) as the target. The ΔintI4 allele was cloned into the Km\(^{\prime}\) vector pSW4427T, and allelic replacement was successfully assayed in strain N16961. As observed for luxU and luxM gene replacement in V. splendidus, clones that survived in the presence of arabinose were found to carry the WT and ΔintI4 alleles at nearly identical proportions, showing that IntI4 is not essential in V. cholerae in laboratory culture conditions.

**Construction and characterization of a V. splendidus metalloprotease mutant.** As mentioned above, we have completed sequencing of the V. splendidus LGP32 genome (Mazel et al., unpublished data). Among the genes which could play a role in pathogenesis, we identified a zinc-containing metalloprotease gene, vsm, the predicted product of which exhibits 95% identity with the product of the V. anguillarum vsm gene, which has been associated with the virulence properties of this fish pathogen (42).

**Vibrio** zinc-containing metalloproteases are classified into three distinct categories according to their amino acid sequences (for a review, see reference 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 amino acids-E), whereas metalloproteases belonging to classes II and III have only a HEXXH motif. The V. splendidus vsm gene product contains the characteristic class I metalloprotease zinc-binding motif. In addition to this HEXXH-19 amino acid-E motif, analysis of the primary structure of the predicted Vsm protein through sequence alignment predicted the existence of a second consensus sequence, GXXNEXXSD, which, when associated with the HEXXH motif, constitutes the features that define the thermolysin family.

The metalloproteases belonging to the thermolysin family are synthesized as inactive precursors, which mature through successive processing stages. According to previous reports, an N-terminal peptide is cleaved during passage through the inner membrane in a signal peptide-dependent 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 two 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 pSW62989T. Cm\(^r\) arabinose-resistant isolates were obtained at a frequency similar to the frequencies of the lux alleles used in the validation process. However, PCR analysis and DNA sequencing revealed that surviving clones were Δ\(vsm\) mutants in all cases and that none of them exhibited WT \(vsm\) allele restoration. The probability of this happening by chance is extremely low (1 in 262,144).

This could be an indication 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 P\(_{BAD}\) promoter control; thus, in the absence of arabinose, the ectopic \(vsm\) gene is silent, likely relieving any selective pressure against the recombinant.

The complete open reading frame of \(vsm\) encodes a 610-amino-acid polypeptide corresponding to a putative preproprotein with a theoretical molecular mass of 67 kDa. Cleavage of the preproprotein at the N-terminal amino acid side should lead to a mature protein that is 412 amino acids long and has a calculated molecular mass of 41.3 kDa. A second processing at the carboxy terminus could lead to a shorter, approximately 30-kDa protein.

Analysis of proteins with protease activity in LGP32 ECPs performed using a gelatin-SDS-polyacrylamide gel revealed three strong zones of clearing, two predominant bands corresponding to proteases having molecular masses of approximately 30 to 40 kDa and a minor band corresponding to a protease having a molecular mass of approximately 70 kDa. These bands could correspond to the different processing stages of Vsm, as they were not detected in mutant strain 62989. However, an alternative hypothesis, that these bands could correspond to other proteases whose processing is controlled by Vsm, cannot 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 ectopic complementation through expression of the \(vsm\) gene under control of the P\(_{BAD}\) promoter from another locus of the \(V. splendidus\) genome, the single-copy IS\(V_{esp}\)I gene. We observed that when grown in presence of arabinose, this strain displayed ECP protease activity identical to the WT ECP protease activity, demonstrating the direct relationship between \(vsm\) deletion and the ECP activity.

In the past, the pathogenesis of bacterial infections has frequently been 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 in the genus \(Vibrio\), different proteases have been characterized and reported to play important roles 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 vibrios (9,50).

When injected into oysters, the LGP32 ECPs exhibited lethality, suggesting that they contained one or more toxic factors responsible either directly or indirectly for some of the pathological processes observed during infection. The lethal effect was dramatically reduced in mutant 62989 and was restored by ectopic complementation, suggesting that the Vsm metalloprotease has a role in ECP toxicity. However, similar mortality rates were obtained when strain LGP32 or strain 62989 was injected into oysters, showing that \(vsm\) expression is not necessary for bacterial virulence in this infection model.

In previous studies workers have examined the contribution to virulence of various \(Vibrio\) metalloproteases in animal experimental models by using mutants with deletions at the protease gene (20,31,42,53). No conclusive evidence concerning the role of the protease in virulence was found, since mutants deficient in protease exhibited levels of virulence comparable to those of their parental strains. Shao and Hor suggested that in \(V. vulnificus\) other factors may be overactive in the absence of the metalloprotease and proposed a possible multifactor interaction in bacterial virulence, involving the protease to an undefined extent. So far, it is not known whether the Vsm metalloprotease is predominant during oyster infection or whether additional and/or coregulated virulence factors are involved in the pathogenesis. There are only a few examples of toxins (such as diphtheria or tetanus toxin) 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 cytolsin/hemolysin (47) and cholera toxin, an ADP-ribosylating enterotoxin inducing a highly secretory diarrhea (7). Thus, it could be hypothesized that Vsm metalloprotease may similarly interact with other virulence factors in \(V. splendidus\) ECPs to potentiate their expression and/or effects on the host. Research is now under way to identify the protein targets that are processed by Vsm in the ECP fraction.

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