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 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repertoire (F. Le Roux, M. Zouine, N. Chakroun, J. Binesse, D. Saulnier, L. Ma, C. Rusniok, C. Buchriser, and D. Mazel, unpublished data).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* vsm 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 R6Kc origin of rep-

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llication that can be replicated only in strains expressing pisr 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 pisr 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 proteobacteria 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 transdominant) (16). However, this strategy requires that cognate rpsL genes be cloned and Smr mutant strains be used.

Due to its general efficiency in gram-negative bacteria and to the simplicity of the counterselection protocol, the Bacillus subtilis levensuсrase gene sacB has gained considerable notoriety since 1985, when it was first introduced (25), and it is now the simplest of the counterselection protocols, the source of the counterselection medium, the source of the counterselection medium, the source of the counterselection medium, the source of the counterselection medium, and the expression was repressed by addition of 1%D-glucose. The chromosomal pisr allele was moved from S17-1 to R6K-oriT derivatives for positive selection of replaced alleles (19, 51, 55).

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 3813, 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 medium, and the expression was repressed by addition of 1% D-glucose.

**PCR.** 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.** 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 pisr alleles in different genetic contexts (17). One of these strains carries a pisr allele inserted into the thyminidylate synthase gene (thyA) of E. coli.

The CcdB-pol gene of E. coli was cloned into pUC18 and used as a source for pisr alleles in different genetic contexts. The pH28-1-166 allele of B462 with armsA-P_{BAD} expression was used for allelic replacement in V. cholerae and V. splendidus. The pH28-1-166 allele was constructed by allelic replacement of the chromosomal thyA allele of B462 with armsA-P_{BAD} expression, as described previously (17). This thyminidylate synthase strain is CcdB resistant and permits R6K vector replication.

The chromosomal gyrA462 allele was moved from S17-1 to R6K-oriT derivatives for positive selection of replaced alleles in different genetic contexts. One of these strains carries a pisr allele inserted into the thyminidylate synthase gene (thyA) of E. coli. The DthymiD synthase is encoded by the chromosomal thyA allele of B462 with armsA-P_{BAD} expression, as described previously (17). This thyminidylate synthase strain is CcdB resistant and permits R6K vector replication.

**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>B462</td>
<td>lacI:H9241 endA1 araA96 thi-1 relA1 collection</td>
<td>L. Van Melderen</td>
</tr>
<tr>
<td>I18313</td>
<td>B462::SacB:erm-pir-116 (Ermr)</td>
<td>This study</td>
</tr>
<tr>
<td>S17-1</td>
<td>F- RP4-2-Tc::Mu aph::Tn7 recA3 (Smr)</td>
<td>54</td>
</tr>
<tr>
<td>j2163</td>
<td>F- RP4-2-Tc::Mu leu2::pisr (erm-pir)</td>
<td>17</td>
</tr>
<tr>
<td>B3914</td>
<td>B2163 gyrA462 zei-298::Tn10 (Km' Em' Tc)</td>
<td>This study</td>
</tr>
<tr>
<td>G7784</td>
<td>TGI F- ( đặcA::speC rexA::BAD ccdB) (Sp')</td>
<td>52</td>
</tr>
<tr>
<td>62899</td>
<td>LGP32 Δvsm</td>
<td>This study</td>
</tr>
<tr>
<td>63453</td>
<td>LGP32 ΔluxM</td>
<td>This study</td>
</tr>
<tr>
<td>66720::vsm</td>
<td>62899 ΔIS151 orf2::araC-P_{BAD}vsm</td>
<td>This study</td>
</tr>
<tr>
<td>8119A</td>
<td>N16961 ΔluxA</td>
<td>This study</td>
</tr>
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</table>

**Table 2. Plasmids used and constructed in this study**

<table>
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<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source or reference</th>
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<tr>
<td>pUC18</td>
<td>ori ColE1 (Ap')</td>
<td>Pharmacia</td>
</tr>
<tr>
<td>pKOBEGA</td>
<td>psC101::pBAD-gfp-89a (Ap')</td>
<td>10</td>
</tr>
<tr>
<td>pSU18</td>
<td>ori p15A (Cm')</td>
<td>1</td>
</tr>
<tr>
<td>PBA640</td>
<td>gfp cassette (S6St, F64L) (Ap')</td>
<td>J. M. Ghigo</td>
</tr>
<tr>
<td>pSU18T-P_{BAD}dft</td>
<td>pSU18T::oriT::araC-P_{BAD}gfp (Cmr)</td>
<td>This study</td>
</tr>
<tr>
<td>pSU18T-P_{BAD}f2</td>
<td>pSU18T::oriT::araC-P_{BAD}gfp (Cmr)</td>
<td>This study</td>
</tr>
<tr>
<td>pSU18T-P_{BAD}luxM</td>
<td>pSU18T::oriT::araC-P_{BAD}luxM (Cmr')</td>
<td>This study</td>
</tr>
<tr>
<td>pSW23T</td>
<td>oriV_{SacB}::P_{BAD} (Smr)</td>
<td>17</td>
</tr>
<tr>
<td>pSW29T</td>
<td>oriV_{SacB}::P_{BAD} (Km')</td>
<td>This study</td>
</tr>
<tr>
<td>pSW4426T</td>
<td>pSW23T::aadA7-araC-P_{BAD}cCdB (Sp' Cm')</td>
<td>This study</td>
</tr>
<tr>
<td>pSW5407T</td>
<td>pSW23T::aadA7-araC-P_{BAD}cCdB (Sp' Kmr')</td>
<td>This study</td>
</tr>
<tr>
<td>pSW62899T</td>
<td>pSW4426T, ΔluxM (Sp' Cm')</td>
<td>This study</td>
</tr>
<tr>
<td>pSW6345T</td>
<td>pSW4426T, ΔluxM (Sp' Cm')</td>
<td>This study</td>
</tr>
<tr>
<td>pSW6597T</td>
<td>pSW4426T, ΔluxA (Sp' Cm')</td>
<td>This study</td>
</tr>
<tr>
<td>pSW69097T</td>
<td>pSW4426T, ΔluxA (Smr' Cm')</td>
<td>This study</td>
</tr>
<tr>
<td>pSW66720::vsm</td>
<td>pSW4426T, ΔIS151 orf2::araC-P_{BAD}vsm</td>
<td>(Sm' Cm')</td>
</tr>
</tbody>
</table>

**Acknowledgments:** This work was supported by grants from the National Institutes of Health (AI35247) and the National Science Foundation (MCB-9531643). We thank D. Skelly, R. L. Schmid, and K. T. Doi for critical reading of the manuscript.
Plasmid construction. (i) pSU1ST-PBADgfp. A 270-bp fragment harboring the RP4 origin of transfer (oriT) was amplified from pSW23T by PCR using primers OriT-Xba and OriT-Pst. After XbaI-PstI digestion, the fragment was cloned in pSU18, a p15A derivative compatible with ColE1 and R6K derivatives, yielding pSU18GFP. A 1,978-bp fragment harboring the acc gene, the P_{AaPS} promoter, and the gfp gene was amplified from the pSU18GFP plasmid by using primers P{BAD}GFP-Kpn and P_{BAD}GFP-Xba. After KpnI-XbaI digestion, the fragment was generated in cloned pSU18. Green fluorescent protein (GFP) expression was confirmed by epifluorescence microscopy.

(ii) Suicide vectors. A 2,645-bp fragment harboring a transcriptional terminator, the adaA7 gene, the acc gene, the P_{AaPS} promoter, and the ccdB gene was PCR amplified using GGT784 DNA (52) as the template and primers ccdB1 and ccdB2. After XbaI-Sacl 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, ISvSp1 orfB, visp1, and gyrA, as well as the V. cholerae superintegrase integrase gene intI1, were PCR amplified from genomic DNA using primers luxU-1 and luxU-2, primers luxM-1 and luxM-2, primers Visp1-1 and Visp1-2, primers visp1-1 and visp1-2, primers gyrA-1 and gyrA-2, and primers intI1-1 and intI1-2, 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 Visp1-3 and Visp1-4 (Δvisp1 allele), primers visp1-3 and visp1-4 (Δvisp1 allele), primers gyrA-3 and gyrA-4 (ΔgyrA allele), and primers intI1-3 and intI1-4 (ΔintI1 allele). XhoI digestion, and self-ligation. The different alleles were then recovered after EcoRI digestion and gel purification and ligated into pSU4426T and/or pSU4427T previously linearized with EcoRI.

(iii) Vector construction for ectopic complementation. Complementation experiments were performed by introducing visp1 under P_{AaPS} promoter control into the nonessential gene coding for ISvSp1 transposase present in the chromosome, using the strategy described above. An inverse PCR was performed with primers GFP3 and GFP4 using pSU1ST-PBADgfp as the template. The resulting amplon was used as template for EcoRI and self-ligation into pSU18T (Pharmacia). The resulting amplified was cloned into pSU18, a p15A derivative compatible with ColE1 and R6K derivatives. The vsm gene was PCR amplified from V. splendidus genomic DNA with primers vsm-1 and vsm-2, digested by EcoRI/XhoI, and cloned in pSU1ST-PBADgfp with the gfp gene deleted after EcoRI-XbaI digestion. This yielded pSU1ST-PBADvisp1 vsm. In order to perform ectopic complementation from the chromosome, the acc-P_{AaPS} amplicon was amplified from pSU1ST-PBADgfp vsm using primers araC-S-XhoI and vsm-1 and vsm-2 digested and cloned in XhoI in the site of the ISvSp1 ΔgyrA allele carried by pSW66720T. This yielded pSU1ST-PBADvisp1 araC vsm.

Conjugation. Overnight cultures of a donor and a recipient were diluted 1:100 in culture media without antibiotic and grown at 30°C to an optical density at 600 nm (OD_{600}) of 0.3. The different conjugation experiments were performed by a filter mating procedure as described previously (5) with a donor/recipient ratio of 1:10. Conjugation was performed overnight on plates incubated on plates containing LB supplemented with diaminopimelic acid (and containing NaCl in the case of V. splendidus) at 30°C. Selection of a ΔgyrA donor was performed by plating on a medium lacking diaminopimelic acid but supplemented with 1% glucose and either chloramphenicol or kanamycin. The first recombination frequency was calculated by using the number of transconjugants and the total number of recipients. Antibiotic-resistant colonies were isolated, grown in ML (containing NaCl in the case of V. splendidus) to the late logarithmic phase, and spread on plates containing 0.2% arabinose. Mutants were screened by PCR using primers 5 and 6 and flanking the different genes targeted.

Immunoblotting. Cells (2 ml of an overnight culture) were centrifuged for 5 min at 5,000 rpm, resuspended in 50 ml of bacterial protein extraction reagent (Pierce) supplemented with 1× protease inhibitor (Complete; Roche), vortexed vigorously for 1 min, and centrifuged for 5 min at 13,000 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 (Bio-Rad) by electroblotting. The membrane was incubated with the primary antibody (rabbit polyclonal anti-GFP [Sigma]) and then with horseradish peroxidase-conjugated goat anti-rabbit immunoglobulins (Amer sham). The proteins were visualized with an enhanced chemiluminescence kit (Amersham) as instructed by the supplier.

Preparation of V. splendidus extracellular products. Bacterial ECPs were produced using the cellophane overlay method described by Liu (36). Tubes containing 5 ml MB were inoculated with one colony of the LGBP32 wild type or mutant and incubated at 20°C for 24 h. After 500 μl of each culture was transferred onto a sterile cellophane film placed on the surface of an MA plate. After incubation at 20°C for 24 h, the cellophane overlay was transferred to an empty petri dish. Cells were washed off the cellophane film using 2 ml of phosphate-buffered saline and were removed by centrifugation at 13,000 rpm at 20°C for 30 min. The supernatant containing the ECPs was sterilized by filtration (0.22 μm) and stored at −80°C until it was used. The protein concentration of the ECPs was determined by the method of Bradford (8) with bovine serum albumin (Sigma) as the standard and normalized.

Detection of protease activity. Protease activity was determined using azocasein (Sigma) as the substrate. Briefly, crude ECPs (250 μl) were added to 250 μl of azocasein (5 mg/ml in 50 mM Tris-HCl buffer, pH 8.0) and 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 13,000 rpm and 4°C for 5 min. The supernatant (500 μl) was neutralized by addition of an equal volume of 1 N NaOH. After mixing, the absorbance at 400 nm was determined for triplicate samples.

In addition, the 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 brilliant blue in 30% methanol-10% acetic acid and destained in 30% methanol-10% acetic acid.

Virulence studies using oysters. Bacteria were grown with 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_{600} corresponded to a bacterial concentration of 10^8 to 2 × 10^9 CFU ml^-1 as determined by conventional dilution plating on marine agar (data not shown). Oysters were inoculated intramuscularly with bacterial strains or ECPs (5 μg/g of oyster) as described previously (23, 34). After injection, the oysters were transferred to aquaria (15 to 20 oysters per 2.5-liter aquarium) containing aerated 5-multertain marine seawater at 20°C, kept under static conditions, and fed daily with a mixture of planktonic algae (Isochris is galbana and Chaetoceros calcitrans). Each bacterial treatment was performed in duplicate, and mortality was recorded daily.

Nucleotide sequence accession numbers. The nucleotide sequences of the luxU, luxM, vsm, visp1, and visp2 genes and the pSU4426T and pSU4427T cloning vectors 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. Conjugative 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 LGBP32 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 LGBP32, WT extracts (Fig. 1). Thus, the P_{BAD} promoter appeared to be tightly and properly regulated in LGBP32 by the araC gene product, which activated transcription in response to its natural inducer, arabinose.

Development of a two-step allelic replacement method using CdcB as a positive selection marker. CdcB is a very strong gyrase inhibitor and was originally discovered in the postregional killing operon carried on plasmid F, ccdB (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 orf{BAD} or orf{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-
null
for ectopic expression in a transcomplementation experiment with the visp1 conjugation to strain gene. Cmr clones were obtained, and four of these clones orfB Cms phenotype expected after a second recombination event on arabinose-supplemented medium. Ten colonies having the were grown without selection and then subjected to selection with the for ectopic expression in a transcomplementation experiment as described in Materials and Methods. Bacterial ECPs were produced by the cel-ephane overlay method as described by Liu (36). (A) The error bars indicate standard deviations. Data from each experiment were statis-
tically analyzed using the chi-square test and StatView software. The differences were significant only for comparisons of ECPs of LGP32 and the 52989 mutants (χ² = 27.62, P < 0.0001) and for comparisons of ECPs of the 56720-vsm and 82989 mutants (χ² = 23.69, P < 0.0001). Sterile seawater was injected as a negative control, and no mortality was observed (data not shown).

**FIG. 4.** Comparison of levels of oyster mortality after intramuscu-
lar injection of ECPs (5 µg/g of oysters) from wild-type strain LGP32 and the 52989 and 56720-vsm mutants (A) or of living organisms (10⁹ to 2 × 10⁸ CFU ml⁻¹). Experiments were performed in triplicate (15 to 20 oysters per aquarium). The mean mortality rates were deter-
mimed 2 days after injection of ECPs (A) or strains (B). The error bars indicate standard deviations. Data from each experiment were statis-
tically analyzed using the chi-square test and StatView software. The differences were significant only for comparisons of ECPs of LGP32 and the 52989 mutants (χ² = 27.62, P < 0.0001) and for comparisons of ECPs of the 56720-vsm and 82989 mutants (χ² = 23.69, P < 0.0001). Sterile seawater was injected as a negative control, and no mortality was observed (data not shown).

**DISCUSSION**

Since the first report on the complete genome sequence of Haemophilus influenzae in 1995, more than 300 other prokary-
otic genome sequences have been completed and another 750 projects are under way (22). Genomics-based approaches have signifi-
cantly increased our understanding of the physiology and pathogenicity of many microbes and have provided insights into the mechanisms and history of genome evolution. Para-
doxically, only a limited number of bacterial species are ame-
nable 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 ECP toxicity. However, when the levels of virulence of the LGP32 and 82989 strains were compared after injection of living bacteria into oysters, similar mortality rates were obtained for the two strains (Fig. 4B). These results suggest that vsm expression is not essential for full bacterial virulence.
truncated version of it carried by a suicide vector introduced into a strain by conjugation. 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 second 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. When a mutant which carries 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 survive. If the targeted gene is not vital, the second recombination is expected to lead either to 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. 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, 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<sub>BAD</sub> promoter. 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. We constructed two vectors, pSW4426T, which carries a Km<sup>R</sup> marker and the araC-P<sub>BAD</sub>-ccdB cassette, and pSW4427T, which is identical to pSW4426T except that it carries a Km<sup>R</sup> 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 intII [40]) as the target. The ΔintI4 allele was cloned into the Kmr<sup>R</sup> 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 vam gene, which has been associated with virulence properties of this fish pathogen.

**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, GXXNXXSD, 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 autoproteolytic cleavage has been described in other Vibrio species. 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.

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\(^+\) 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 Δvsm 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\(visp\)\(_1\) 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 V\(ibrio\), 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 V\(ibrio\) 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 cytotoxin/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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