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Metalloprotease Vsm Is the Major Determinant of Toxicity for Extracellular Products of *Vibrio splendidus*

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Abstract:

Genomic data combined with reverse genetic approaches have contributed to the characterization of major virulence factors of *Vibrio* species; however, these studies have targeted primarily human pathogens. Here, we investigate virulence factors in the oyster pathogen *Vibrio splendidus* LGP32 and show that toxicity is correlated to the presence of a metalloprotease and its corresponding *vsm* gene. Comparative genomics showed that an avirulent strain closely related to LGP32 lacked the metalloprotease. The toxicity of LGP32 metalloprotease was confirmed by exposing mollusk and mouse fibroblastic cell lines to extracellular products (ECPs) of the wild type (wt) and a *vsm* deletion mutant (Δ*vsm* mutant). The ECPs of the wt induced a strong cytopathic effect whose severity was cell type dependent, while those of the Δ*vsm* mutant were much less toxic, and exposure to purified protein demonstrated the direct toxicity of the Vsm metalloprotease. Finally, to investigate Vsm molecular targets, a proteomic analysis of the ECPs of both LGP32 and the Δ*vsm* mutant was performed, revealing a number of differentially expressed and/or processed proteins. One of these, the VSA1062 metalloprotease, was found to have significant identity to the immune inhibitor A precursor, a virulence factor of *Bacillus thuringiensis*. Deletion mutants corresponding to several of the major proteins were constructed by allelic exchange, and the ECPs of these mutants proved to be toxic to both cell cultures and animals. Taken together, these data demonstrate that Vsm is the major toxicity factor in the ECPs of *V. splendidus*.

1. Introduction

Vibrionaceae is a predominant family of Gram-negative bacteria found in aquatic environments (30). Bacteria within this family demonstrate a high genetic diversity and are able to colonize very different types of niches. They live freely as planktonic forms in the water column, or are associated in biofilms or with host organisms as pathogenic, commensal or mutualistic bacteria. To date, eight genome sequences are available from *Vibrionaceae*: *Vibrio cholerae* strains N16961 and 0395, *V. parahaemolyticus* RIMD2210633, *V. vulnificus* strains YJ016 and CMCP6, *V. fischeri* ES114, *V. harveyi* ATCC BAA-1116 and *Photobacterium profundum* SS9 (3, 10, 19, 24, 32). More recently, we have completed the sequencing of the genome of *V. splendidus* strain LGP32, an oyster (*Crassostrea gigas*) pathogen (Le Roux *et al.,* presented at the second conference on the biology of vibrios, Paris, 28 november to 1 december 2007).

Genomics data combined with reverse genetics have largely contributed to the characterization of main virulence factors in human pathogens: e.g., cholera toxin (CT) and the toxin coregulated pilus (TCP) in *V. cholerae;* the thermostable direct thermolysin (TDH) and the type III secretion system (TTSS) in *V. parahaemolyticus*; the capsular polysaccharide (CPS) in *V.vulnificus* (for a comprehensive review, see "The biology of Vibrios", 29). However, epidemiological studies have clearly established that virulence is most often multifactorial. For instance, although CT is the most important factor in the enteric disease cholera, CT-deficient strains of *V. cholerae* still elicit mild to severe diarrhea in human, indicating that other factors are likely to contribute to the pathogenesis of the disease (5, 11, 27, 28). Furthermore, the relative contribution of a given gene product may vary with strain, host and type of infection. In such a scheme, accessory virulence factors such as haemolysins, lipases and proteases may facilitate virulence.

Extracellular metalloproteases have a number of properties that suggest their involvement in pathogenesis including their cytotoxicity, tissue destructive activities and inhibitory effect on phagocytosis (9). Proteases may similarly interact with other virulence factors to potentiate their expression and/or effects on the host. For instance the hemaglutinin/protease of *V. cholerae* cleaves and activates the cholera enterotoxin (2), and it has been shown that the enterotoxic hemolysin was extracellularly processed into mature hemolysin after cleavage by various proteolytic enzymes including a metalloprotease (22). Recently, we have shown that Vsm, a metalloprotease of *V. splendidus* strain LGP32, is an essential component of lethality when extracellular products (ECP) are injected into oysters (15).

As with several other *Vibrio* species, *V. splendidus* has been associated with mortalities of marine invertebrates, causing major economic losses in aquaculture (for a review see 14). Compared to human pathogens the mechanisms involved in such invertebrate vibriosis are poorly understood. Such a lack of knowledge is in part a consequence of the absence of a standardised model for *in vivo* studies. Indeed, with no inbred mollusk lines, the genetic background of the experimental animals is heterogeneous. Because of these difficulties, *ex vivo* studies have become necessary to better characterize the several bacterial activities that appear to be involved in *V. splendidus* virulence. Virulence caused by factors such as the extracellular enzymes and stress proteins can be recognized by the loss of cell adherence, induction of cell lysis, or apoptosis. A clam primary cell culture, despite consisting of a heterogeneous cell population, has been used to study the interaction between cells and a pathogenic *Vibrio* species (4, 13). Indeed, Labreuche (12) showed that the level of lethality in animals treated with the ECP of another *Vibrio* species, *V. aestuarianus*, was correlated *ex vivo* to a significant inhibition of the phagocytic capacity of hemocytes as well as their ability to adhere to the substrate.

In the present study, the ECP of LGP32, a virulent strain of *V. splendidus*, was investigated to identify the products responsible for its toxicity. We first showed that the absence of toxicity toward oysters of LMG20012^T, a non-virulent strain closely related to LGP32, correlates with the absence of both a metalloprotease activity (designated Vsm) and its encoding gene. Toxicity of the ECP of LGP32, of Δ*vsm*, a metalloprotease deletion mutant of LGP32, and of the Vsm purified protein was tested on whole oysters, on Bge cells (a mollusk cell line) and on NIH 3T3 (a mouse fibroblastic cell line). Our data show that the ECPs of LGP32 induce a strong cytopathic effect whose severity is cell type dependent. Indeed, the purified Vsm protein is sufficient to induce *in vivo* and *ex vivo* toxicity while in contrast, the ECPs of Δ*vsm* strain is much less toxic. In order to identify Vsm target(s) in the ECPs, proteomic analysis was performed on the ECPs of both LGP32 and Δ*vsm*. Interestingly, these studies revealed a number of differentially expressed and/or processed proteins, of which VSA1062 was found to encode a metalloprotease sharing a significant identity with the immune-inhibitor A precursor, a virulence factor of *Bacillus thuringiensis*. New deletion mutants were constructed by allelic exchange

for three differentially expressed and/or processed extracellular proteins: VSA576, VS2864 and VSA1062. The ECPs of these mutants proved to be toxic both in cell culture and in intact animals, showing that none of those genes is essential for the LGP32 extracellular products toxicity when injected in oysters Altogether, our data demonstrate that Vsm is the major factor of toxicity in the ECP of *V. splendidus*.

2. Materials and methods

2.1. Bacterial cultures

The bacterial strains used in this study and their relevant properties are described in Table 1. *Escherichia coli* strains were grown in Luria-Bertani (LB) or, for strain Π3813, Mueller-Hinton (MH) broth, at 37°C. *Vibrio* strains were grown in LB + NaCl 0.5M, marine broth (MB) or marine agar (MA) at 20°C. All media were from Difco. Chloramphenicol (12.5 $mg.l^{-1}$), Thymidine (0.3 mM) and diaminopimelate (0.3 mM) were added as supplements when necessary. 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 conversely, this activity was repressed by the addition of 1% D-glucose.

2.2. PCR amplification

PCR amplifications for plasmid construction were performed in 50 µl reactions containing *Pfu* DNA polymerase (Promega). For long-range PCR, we used the Herculase II fusion enzyme (Stratagene). Routine PCRs were performed in 50 µl reactions using the Bioline *Taq* polymerase according to the manufacturer's instructions. Conditions for amplification were as follows: 94°C for 3 min, 30 cycles at 94°C for 30 s, (Tm-10°C) for 30 s, and 72°C for 60 s per kb.

2.3. Gene annotation and comparative genomic

The whole genome sequence of *V. splendidus* LGP32, as the genome of *V. cholerae*, *V. parahaemolyticus*, *V. vulnificus* and *V. fischeri,* has been transferred to the annotation database MaGe (Magnifying Genome), which allows dynamic and permanent update of genome annotation (http://www.genoscope.cns.fr/agc/mage). The MaGe system offers a set of graphical interfaces that allow relevant expert annotation of microbial genomes to be performed. One of the easily accessed features of this system is the ability to explore gene context by searching for conserved synteny. Putative orthologous relationships between two genomes are defined as gene couples satisfying the BBH criterion or an alignment threshold (a minimum of 30% sequence identity on 80% of the length of the smallest protein). These relations are subsequently used to search for conserved gene clusters, e.g., synteny groups among several bacterial genomes. From these comparisons, we defined a species-specific gene as a gene having no ortholog in the compared species (significant similarities were not detected).

2.4. Suicide vector construction

Alleles carrying an internal deletion were generated *in vitro* using a two-step PCR construction method (20). Using genomic DNA of the strain LGP32, independent PCR amplifications of the regions encompassing ORF VSA1062, VS2864 and VSA576 were performed using two primer pairs: (1062-1, -2) (457 bp) and (1062-3, -4) (447 bp); (2864-1, -2) (341 bp) and (2864-3, -4) (341 bp); (576-1, -2) (500 bp) and (576-3, -4) (465 bp). Primer sequences are listed on supplementary Table 1 (online supplementary material). After gel purification, 100 ng of the two PCR products were mixed for each ORF and a final PCR amplification was carried out using the most external primer pairs: (1062-1, -4) (2864-1, -4) and (576-1, -4). After gel purification, the PCR products, referred to as the *Δ1062, Δ2864* and *Δ576* alleles, were *Eco*RI digested and ligated in a *Eco*RI linearized pSW4426T plasmid described previously (15).

2.5. Conjugation

We have previously developed a suicide vector based on the *pir*-dependent R6K replicative origin, which can be transferred by RP4-based conjugation to *V. splendidus,* and which also carries the plasmid F toxin gene (*ccdB*) gene under the control of the P_{BAD} promoter (15). This genetic system allows the efficient counter-selection of integrated plasmids in the presence of arabinose in *V. splendidus.* As such this approach permits efficient marker-less allelic replacement in this species. The different conjugation experiments were performed according to the filter-mating procedure using a donor/recipient ratio of 1/10 as previously described (1). Selection against the *⊿dapA* donor was achieved by plating on a medium that was devoid of diaminopimelic acid (DAP), and supplemented with 1% glucose and chloramphenicol. Antibiotic-resistant colonies were grown in LB + NaCl and spread on plates containing 0.2% arabinose. Mutants were screened by PCR amplification as previously described (15) using the primer pairs: (1062-5, -6), (2864-5, -6) and (576-5, -6).

2.6. Preparation of the extracellular products

Bacterial extracellular products (ECPs) were obtained following the cellophane overlay method as described by Liu *et al* (16). Briefly, 500 µl of an overnight culture of bacteria were spread on a marine agar plate covered by a cellophane film. After 24h incubation at 20°C, bacteria were collected using 1 ml of cold PBS 1X. After a centrifugation of 10 min at 4000g, the supernatant (ECP) was filtrated on 0,22 microm pore-sized filter. Protein concentration of ECP (ranging from 100 to 500 $mg.l^{-1}$) was estimated using the Bradford method according to the manufacturer's instructions (Coomassie Protein Assay Reagent, Pierce Biotechnology, Rockford, IL). Proteins were separated on a 10% SDS PAGE, and were stained using Coomassie blue (Biorad).

2.7. Protease activity measurement

Protease activity was determined using azocasein (Sigma) as a 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); The mixture was incubated at 20°C for 1 hour. The undigested substrate was precipitated by adding 500 µl of 10% trichloroacetic acid to the reaction mixture, followed by centrifugation at 12000 g and 4°C for 5 min. Supernatant (500 µl) was neutralized by addition of an equal volume of 1 N NaOH. After mixing, the absorbance at 440 nm was measured on triplicate samples.

2.8. Purification of the metalloprotease

ECPs of LGP32 were concentrated 5 times at 4°C by ultrafiltration (stirred cell system with a 10 kDa molecular weight cutoff, Pall). 15 ml of concentrated ECPs were applied onto a Hiload 26/60 Superdex 75 pg column (GE Healthcare) equilibrated in Tris-HCl 50 mM pH 7,5. Fractions of 3,5 ml were collected at flow rate of 0,5 ml/min. Fractions containing the Vsm were checked on a 10% SDS PAGE, quantified by the Bradford method and tested for their protease activity as described previously.

2.9. 2D electrophoresis and peptide mass fingerprinting protein identification

Separation of extracellular proteins was performed as previously described (26) ; briefly, 350 µg of proteins were loaded on each strip. Isoelectric focusing was carried out using immobilised pH gradients (pH 4-7, Bio-Rad Laboratories, Hercules, California) in a Protean IsoElectric Focusing Cell II (Bio-Rad). Strips were subsequently loaded onto 10% SDS-PAGE gels, and electrophoresis was performed in a Protean II xi cell (Bio-Rad). Experiments were conducted in triplicate. Proteins were visualized by staining with Bio-Safe Coomassie (Bio-Rad), and identified by Peptide Mass Fingerprinting after trypsinolysis using Matrix-Assisted Laser Desorption Ionization – Time of Flight / Mass Spectrometry (MALDI-TOF/MS) at the *Plateau d'Analyses Protéomiques par Séquençage et Spectrométrie de Masse* (INRA, Jouy-en-Josas, France). Protein identification was achieved using the MS-Fit software (UCSF Mass Spectrometry Facility, USA ; http://prospector.ucsf.edu). All searches were performed against the database of *V. splendidus* LGP32 genome sequence (Le Roux *et al.,* presented at the second conference on the biology of Vibrios, Paris, 28 november to 1 december 2007).

2.10. Virulence assays

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, which 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 as described previously (6, 12). 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 treatment was performed in duplicate and mortality was recorded daily.

2.11. Cell culture and microscopy

Bge cells from the tropical snail *Biomphalaria glabrata* (ATCC n° CRL-1494) were grown at 26°C on a 22% Schneider medium complemented with lactalbumine hydrolysate (4.5 $g.l^{-1}$), D galactose (1.3 $g.l^{-1}$) and 10% heat-inactivated fetal calf serum. NIH 3T3 cells were maintained at 37°C in a CO_2 atmosphere, on DMEM with 10% heat-inactivated fetal calf serum. All cell culture reagents were from Invitrogen.
For microscopy, cells were seeded on coverslips. Fixation was carried out with 3.7% Paraformaldehyde in PHEM buffer (for 1 l add the following: PIPES 18.14 g; HEPES 6.5 g; EGTA 3.8g; $MgSO_4$ 0.99 g and pH to 7.0 with 10M KOH). Observation of the formazan needle-like crystals present at the cell surface after MTT treatment was performed under Diffraction Interference Contrast (DIC) microscopy. Immunostaining of the microtubule cytoskeleton was performed using a primary rabbit polyclonal anti- ß tubulin antibody and a Texas Red conjugated secondary antibody (Molecular Probes). Actin staining was carried out using Texas Red conjugated phalloidin. Cells were then mounted in Moviol mounting media before analysis.

2.12. Cytotoxicity assay

Non-confluent cell cultures were obtained by seeding 65 000 (Bge) or 40 000 (NIH) cells, respectively, with 300 µl of medium in each well of a 24-well plate, followed by an overnight incubation of the cells in their respective culture conditions. A final protein concentration of 10 $µg.ml^{-1}$ of the ECPs (normalized for their content in proteins), were added to the cells and left in contact from 3 h to 90 h before adding 33 µl of a MTT solution (5 $mg.ml^{-1}$) using the Vybrant MTT Cell Proliferation Assay (Molecular Probes, NL). For quantitative assays, cells were further incubated for 4 h, and were then lysed with a 0.5% SDS solution and incubated at 37°C for 16 h according to manufacturers recommendations. Optical density was determined at 570 nm on triplicate samples.

2.13. Nucleotide sequence accession number

The nucleotide sequence of *VSA1062, VSA576 and VS2864* genes have been deposited in the GenBank database under the accession number EU349011, EU349012 and EU349013.

3. RESULTS

3.1. The non-virulent LMG20012^T vibrio is devoid of Vsm metalloprotease

A preliminary screening to test for the virulence of live bacteria belonging to several strains closed to *V. splendidus* indicated that the LMG20012^T strain carried essentially no virulence (i.e. bacterial injection gave results similar to the seawater treatment (data not shown). Similarly, the injection of LGP32 and LMG20012^T ECPs, instead of bacteria, in oysters (5 mg.gr^{-1} of flesh) revealed a 93,3% mortality for LGP32 three days post-injection, and 3,3% mortality for LMG20012^T (Table 2). In addition, no protease activity could be detected using the azocasein assay in the LMG20012^T ECP (Table 2) thus further suggesting a role for the Vsm metalloprotease in *V. splendidus* virulence (15). Indeed, PCR amplification on LMG20012^T genomic DNA using primers framing the *vsm* gene-containing region (11,4 kbp) produced a DNA fragment lacking a 6.1 kbp region that most notably carries *vsm* in *V. splendidus* LGP32, and 4 other ORFs encoding conserved proteins of unknown function (Fig. 1). Altogether, these results suggest that the absence of *vsm* gene in strain LMG20012^T and thus of its metalloprotease activity, might explain part of its non virulence.

3.2. Vsm is lethal for oyster

To purify the Vsm metalloprotease, the ECPs from LGP32 were subjected to Gel filtration chromatography. The collected fractions 39-41, contained a single band as shown on SDS PAGE while displaying the highest protease activity (data not shown). As shown on Table 2, injection of 10 μg per oyster of this purified Vsm protein induced a 56% mortality within 24h post-challenge while the injection of LGP32 and Δ*vsm* ECPs induced 93% or 16% mortalities, respectively (Table 2). The fact that purified Vsm appear slightly less toxic than crude LGP32 ECP is correlated with a decrease of the proteolytic activity between LGP32 ECPs (50%) and the purified Vsm (25%) (Table 2) suggesting that purification step altered the protease activity or that a cofactor, present in ECP, is require for a full Vsm activity. Taking together, these results suggest that the metalloprotease Vsm is necessary and sufficient to induce oysters mortalities.

3.3. Distinct proteins in the ECP of Δ*vsm* and LGP32

Because Vsm metalloprotease might act indirectly by cleaving other virulence factor (s) in the LGP32 ECP, SDS-PAGE electrophoresis was carried out to compare the protein profiles of ECPs of LGP32 and its previously described derivative Δ*vsm* (15). SDS PAGE analysis revealed a predominant band (approx. 35-37 kDa) in LGP32 ECP that was missing in the ECP of Δ*vsm* (Fig. 2A, lane 2). In addition, Δ*vsm* ECP displayed two bands of weak intensity migrating at 100 kDa and one band at 55 kDa (Fig. 2A, lane 3). 2D gel analysis in a 4 to 7 pI range, performed on ECPs of LGP32 and Δ*vsm* revealed six major differential spots referred to as spot 1-6 on Figure 2 B. MALDI-TOF MS analysis and peptide mass fingerprinting using the LGP32 database (Le Roux *et al.,* presented at the second conference on the biology of vibrios, Paris, 28 november to 1 december 2007) allowed the identification of 56/126 spots with high matching scores (Table 2, online supplementary data). Only six spots were found to be constantly different between both ECPs, and they corresponded to the Vsm metalloprotease (Fig. 2B, spots 1- 3), VSA576 (Fig. 2B, spot 4), VSA1062 (Fig. 2B, spot 5) and VS2864 (Fig. 2B, spot 6). VSA576 and VS2864 ORFs encode two predicted proteins of unknown function, which are proteins of 73.7 and 94.6 kDa with isoelectric points of 5.31 and 4.64, respectively. VSA1062 gene encodes a 101 kDa metalloprotease that belongs to the M6 peptidase family, according to the MEROPS classification (http://merops.sanger.ac.uk). Interestingly, this protein shares a significant identity (41%) with the immune-inhibitor A precursor, a virulence factor encoded by the *ina* gene of *Bacillus thuringiensis* (18). Thus, one of the proteins expressed in Δ*vsm* is a metalloprotease that might compensate for the missing Vsm.

3.4. The differentially expressed proteins are not involved in virulence

Deletion mutants were obtained for the differentially expressed and/or processed proteins, VSA1062, VSA576, and VS2864, using method we previously described (15). The metalloprotease activity of the mutant ECPs, determined using the azocasein assay, was found to be in the range of the wild-type strain for the Δ1062, Δ2864 and Δ576 mutants (Table 2). In contrast, the ECP of the Δ*vsm* mutant displayed a dramatically reduced protease activity (20% of the LGP32 protease activity), while the

double mutant Δ*vsm*-1062 had essentially no activity. Twenty-four hours post-injection into the oyster adductor muscle (5 μg.g^{-1} of flesh), the observed mortality was in the same range for the wild type as for the Δ1062 mutant ECPs (93%). It was slightly decrease for the Δ2864 and Δ576 mutant ECPs (86,7 and 80%, respectively) while it was dramatically reduced for the Δ*vsm* mutant ECP (16,7%) and for the double mutant Δ*vsm*-1062 (3,3%) (Table 2). In live oysters experimental variation is high, therefore we consider that a difference of 10% in mortality rate is a minor or non significant effect. In addition, and in agreement with our previously report (15), the same mortality rate was observed for the different mutants as for LGP32 when live bacteria were injected into oysters instead of ECPs (data not shown). These data show that these factors, that are differentially expressed and/or processed between LGP32 and Δ*vsm*, are not, or only marginally, involved in virulence for the oyster. Moreover, these results suggest that Vsm toxicity is not mediated through these factors.

3.5. Virulent ECPs alter cell morphology

Non-confluent Bge cells, a mollusk cell line, were exposed to ECPs at a final protein concentration of 10 μg.ml^{-1}. Observation in Diffraction Interference Contrast (DIC) microscopy revealed the retraction of cytoplasmic extensions and cell rounding within 3 hours after ECP treatment, suggesting a cytopathic effect (CPE) for LGP32 (Fig. 3, panels A and B). Interestingly, this CPE was reproduced by using 10 μg.ml^{-1} of purified Vsm. In contrast, the ECPs of either LMG20012^{T} or Δ*vsm* had no apparent effect even after 24 or 48 h of exposure (data not shown). After 8 h, cells treated with LGP32 ECPs were floating mostly as groups of 15 to 20 closely associated rounded cells (Fig. 3, panel B). Immunostaining of microtubules revealed a strong alteration of the microtubule cytoskeleton (Fig. 3, panels C and D), thus corroborating the LGP32 ECP-treated cell morphology. In contrast, cells incubated with LMG20012^{T} or Δ*vsm* ECPs, did not display any alteration of their microtubule cytoskeleton (data not shown).

The experiment was then repeated on non-confluent NIH 3T3, a mouse fibroblast cell line. Cells presented a round morphology as early as 1 h after treatment with the LGP32 ECPs or purified Vsm, while 2 h later they were already floating in the culture medium (data not shown). Labelled phalloidin revealed intensely stained stress fibers in untreated NIH cells (Fig. 3, panel E) while LGP32 ECPs-treated cells displayed large speckles of aggregated actin in the retracted cytoplasm (Fig. 3, panel F). Similar observations were made when using the Δ1062, Δ576 and Δ2864 mutants, while Δ*vsm* and Δ*vsm*-1062 did not induce any apparent modification of the cell morphology or actin cytoskeleton (data not shown). The ECP of the virulent LGP32 *Vibrio*, in contrast to those from Δ*vsm*, induces cells to change of morphology and then to detach of the extracellular matrix and these alterations are much stronger and faster on mammalian NIH cells than on mollusk Bge cells. This activity has to be attributed to Vsm, as the purified protein is sufficient to induce cell rounding.

3.6. ECP alteration of viability is cell dependent

To determine whether the ECP treatment altered Bge physiology, cell viability was estimated using the MTT assay that requires MTT endocytosis, reduction and polymerization, and exocytosis as needle-like crystals (17). Indeed, this assay revealed a 27% decrease of the MTT reduction as early as 3 h after treatment with LGP32 ECP (Fig. 5, panel A). Interestingly, kinetics showed no further alteration of the rate of MTT reduction when the LGP32 ECP treatment duration was increased to 48 h (Fig. 5, panel A). DIC microscopy on ECP-treated cells revealed long and abundant needle-like crystals at the periphery of Bge cells (Fig. 4), even when MTT incubation was performed as late as 90 h post-ECP treatment (data not shown) a time when most cells were associated in groups and in suspension. When performed on NIH 3T3, the MTT assay revealed a significant alteration of cell metabolism, with a 45%, 75% and 85% decrease after exposure to LGP32 ECP for 3 h, 24 h and 48 h, respectively (Fig. 5, panel A). In addition, the ECPs of Δ576 and Δ2864 (data not shown) and Δ1062 (Fig. 5 panel B) were as cytotoxic for NIH cells as LGP32 ECP while the ECP of Δ*vsm*, although devoid of Vsm protease, still induced a moderate degree of cytotoxicity (Fig. 5, panel B). These data indicate that additional cytotoxic factor(s) are present in Δ*vsm* and, thus, are likely to be present in LGP32 ECPs. To confirm this hypothesis, the double-deletion mutant Δ*vsm*-1062 was constructed as above, and its ECP was shown to be devoid of metalloprotease activity by the azocasein assay (Table 2). Nevertheless, when assayed on NIH cells, the ECP of the double mutant Δ*vsm*-1062 was shown to be as cytotoxic as Δ*vsm* (Fig. 5, panel B), thus confirming that the putative VSA1062 metalloprotease carries little or no cytotoxicity. Again, similar results were obtained using purified Vsm instead of LGP32 ECPs (data not shown). Untreated NIH cells were then either seeded in 24 well-plates or left in suspension for extensive periods of time and viability was tested using the MTT assay at 3 h, 24 h and

48 h. Our data, expressed as a percentage of the attached cells activity, indicate that the ability of unattached NIH cells to metabolize MTT decreased at a rate similar to that of the LGP32 ECP treated NIH cells (Fig. 5, panel B). It thus suggests that the difference in the rate of MTT reduction between ECP treated Bge and NIH cells, reflects the ability of Bge, but not NIH cells, to survive in suspension for extensive periods of time (our unpublished observation).

4. Discussion

In this report, we investigated the virulence of *V. splendidus* LGP32, a strain that was isolated in 2004 from the hemolymph of oysters suffering from outbreaks of summer mortalities, and which later proved to be pathogenic for oysters and clams (6). Taxonomic analyses failed to identify markers associated with *V. splendidus* pathogenicity (6). Therefore, virulence of the isolated strains had to be assessed by experimental challenge. Analysis of the data provided by this approach is hampered by the variability of the physiological state of the animals, which depended upon a continuously changing environment (food, temperature, salinity, pollutants) and to a heterogeneous genetic back-ground (25). Hence there is a need for the development of cellular and/or molecular tests to evaluate the virulence of a strain. Indeed, standardized *in vitro* assays are necessary to screen for distinct activities covered by the generic term virulence, such as adherence or cytolytic effects. However, marine invertebrate cell lines do not exist and so far the experiments to apprehend interactions between cells and pathogenic vibrios rely on primary lines of haemocytes maintained in survival conditions (4, 8, 23). Nevertheless, the use of such a cell population has drawbacks. The haemocyte population is made of distinct cell types that remain to be fully characterized. Moreover, each animal provides a limited number of cells (about 10^6), and this procedure requires pooling haemocytes from different individuals with heterogeneous genetic backgrounds. Even more disturbing is the fact that the oyster hemolymph is naturally contaminated with bacteria that can confound the conclusions, especially if the experiment lasts long enough to allow bacteria proliferation. Interestingly, a recent report describes the use of a flounder gill cell line as a test for the cytotoxicity of the recombinant metalloprotease of *V. anguillarum*, a fish vibrio (33).

To investigate the role of the secreted metalloprotease of *V. splendidus* as well as those of other proteins present in the ECP, new genetic tools were developed to establish knockout mutants in this species (15). ECPs of these mutants were then tested through animal challenges performed in parallel with cytotoxicity assays on cell lines.

We first tested the ECPs on Bge cells, the unique mollusc cell line available to date. DIC microscopy revealed that the ECP produced by the LGP32 virulent strain alters Bge cell morphology as soon as 3 h post treatment and then later on, it induces cells to detach from the extracellular matrix. This cytotoxic effect was not observed with the ECPs of LMG20012^T, a non-virulent vibrio, or the ECPs of Δ*vsm*, a *vsm* deleted mutant whereas the purified Vsm induced the detachment. These data demonstrated a main role for the Vsm metalloprotease in the rounded cell morphology. However, when using the MTT reduction assay, only a moderate alteration of Bge cell metabolism was observed even when the assay was performed as late as 90 h of incubation with LGP32 ECPs or purified Vsm. These results indicated that despite an inhibition of cell anchorage, Vsm only slightly alters Bge cell viability and metabolic functions. It is thus apparent that cytoplasmic retraction and cell rounding as observed by DIC microscopy might not *per se* be sufficient to determine the virulence of an ECP or of a protein.

Because this observation might be cell-type dependent, the LGP32 ECP cytotoxicity and purified Vsm were also assessed on NIH3T3 cells, a fibroblastic mouse cell line sensitive to extracellular matrix attachment. Our data show a strong correlation between the alteration of the NIH cell morphology accompanied by a quick detachment of the plate floor and the alteration of cell metabolism (see Fig. 5). These dramatic changes were accompanied by a total de-structuralization of the actin cytoskeleton, suggesting the non-viability of most of the cells. Indeed, the MTT assay revealed a strong alteration of NIH metabolism resulting in a 75% inhibition 24 h after ECP treatment (Fig.5B). This clear discrepancy between the responses of the two cell types suggests that the Vsm activity promotes the NIH cell death by inducing cell detachment. Indeed, we have shown that when left in suspension for extended periods of time, the MTT metabolism of NIH cells decreased as if they were ECP treated (Fig. 5B). On the other hand, Bge cells are thought to be of hematopoietic origin and haemocytes are circulating cells involved in the invertebrate immune defense and their survival is not attachment dependent. Indeed, Bge cells maintained for 96 h in suspension were still metabolically active and capable of rapidly forming a monolayer (our unpublished observation). The striking rate of

oyster mortality induced by LGP32 ECP indicates that the Vsm metalloprotease is not likely to act on haemocytes but instead that it actually targets a most sensitive cell type(s), which alteration quickly leads to the death of the organism.
The second part of this study was aimed at identifying additional cytotoxic proteins by studying some of the ECP proteins that are differentially expressed between LGP32 and Δ*vsm*, the mutant lacking Vsm. 2D gel analysis of LGP32 and Δ*vsm* ECPs revealed six differentially produced spots that were identified using MALDI-TOF MS analysis and peptide mass fingerprinting. The Δ*vsm* ECP was found to contain a predicted protein of unknown function VS2864 and, more interestingly, VSA1062, an additional metalloprotease of the M6 family, while LGP32 contained an unknown protein, VSA576, in addition to three spots corresponding to Vsm. Results of the azocasein assay showed that while the deletion mutants Δ576, Δ2864 and Δ1062 express a level of metalloprotease activity similar to LGP32, the level of Δ*vsm* is down to 20% while the double mutant Δ*vsm*-1062 has no detectable activity (see Table 2). Moreover, the Δ576, Δ2864 and Δ1062 deletion mutants proved to be as cytotoxic as LGP32 on NIH cells as shown by DIC microscopy and MTT assay. It was tempting to propose that *V. splendidus* metalloprotease VSA1062, which is detected in Δ*vsm* ECP but not in LGP32, could compensate for some of the functions of the deleted *vsm* gene. Indeed, Milton et al., (21) showed that *V. anguillarum* mutants lacking a functional metalloprotease, carried two different proteases which are not detected in the wild type strain suggesting that these proteases might compensate for the loss of the *empA* protease. Strinkingly, while the double mutant Δ*vsm*-1062 displays no detectable metalloprotease activity, its ECP cytotoxicity is similar to that of Δ*vsm* (Fig.5B). These data suggest that although VSA1062 metalloprotease shares a significant identity (41%) with the immune-inhibitor A precursor (InHA), a virulence factor encoded by the *ina* gene of *Bacillus thuringiensis* (18), it is not, or only marginally, involved in the ECP toxicity. Instead, like InHA which is involved in antibacterial peptide resistance, VSA1062 might similarly play a role in bacterial resistance against the oyster immune response, a possibility that will be the focus of future investigations. Altogether, genome analysis indicating no additional metalloprotease gene in *V. splendidus* (Le Roux *et al.,* presented at the second conference on the biology of Vibrios, Paris, 28 november to 1 december 2007) and the data presented in this report show that although Vsm is the main factor of toxicity in the ECP, additional factors(s) may have a significant contribution to the degree of virulence of this pathogen.

5. Conclusion

In this report we show that Vsm, a metalloprotease, is the main factor of toxicity in the ECP of a virulent strain of *V. splendidus*. Our data, obtained on two distinct cell lines and through animal challenge, illustrate the multi-criteria definition of virulence. The term cytotoxicity is used when referring to the alteration of cell morphology or of cell viability. Cell morphology is certainly a quick and valid test for cytotoxicity since it was corroborated by animal challenges. Nevertheless, viability assays have shown for Bge cells that morphology can be durably altered without implying cell death. The mechanism of cytotoxicity of the metalloprotease Vsm remains to be explored and the observed difference regarding mollusk and mammalian cells are of particular interest.

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TABLES

Table 1: Bacterial strains and plasmids used in this study

Bacterial strains	Description	Reference
π3813	*lacIQ, thi1, supE44, endA1, recA1, hsdR17, gyrA462, zei298::Tn10 ΔthyA::(erm-pir116)* [Tc^R Erm^R]	(15)
β3914	(F ⁻) RP4-2-Tc::Mu dapA ::(erm-pir) *gyrA462, zei298::Tn10* [Km^R Em^R Tc^R]	(15)
LGP32	*Vibrio splendidus*	(6)
LMG20012[T]	*Vibrio tasmaniensis*	(31)
Δ*vsm*	LGP32 *Δvsm* (M4 metalloprotease)	(15)
Δ1062	LGP32 *Δ1062* (M6 metalloprotease)	This study
Δ2864	LGP32 *Δ2864*	This study
Δ576	LGP32 *Δ576* (unknown function)	This study
Δ*vsm*-1062	LGP32 *Δvsm Δ1062*	This study
Plasmids	Description	Reference
pSW4426T	$oriV_{R6K\gamma}$; $oriT_{RP4}$; *araC*-P_{BAD}*ccdB;* [Sp^R, Cm^R]	(15)
pSWΔ1062T	pSW4426T*;Δ1062;* [Sp^R, Cm^R]	This study
pSWΔ2864T	pSW4426T*;Δ2864;* [Sp^R, Cm^R]	This study
pSWΔ576T	pSW4426T*;Δ576;* [Sp^R, Cm^R]	This study

Table 2: Oyster mortality 24h post ECPs or purified Vsm protein (underline) or sterile sea water (SSW) injection and proteolytic activity (protease unit/mg protein)

	LGP32	LMG20012T	Vsm	Δ*vsm*	Δ1062	Δ2864	Δ576	Δ*vsm*-1062	SSW
Oyster mortality	93.3 ± 9.4%	3.3 ± 4.7%	56,1 ± 13,3%	16.7 ± 4.7%	93.3 ± 9.4%	86,7	80	3.3 ± 4.7%	0
Proteolytic activity	50	0	25,6	11	46	52	46	0	0

FIGURES

Figure 1

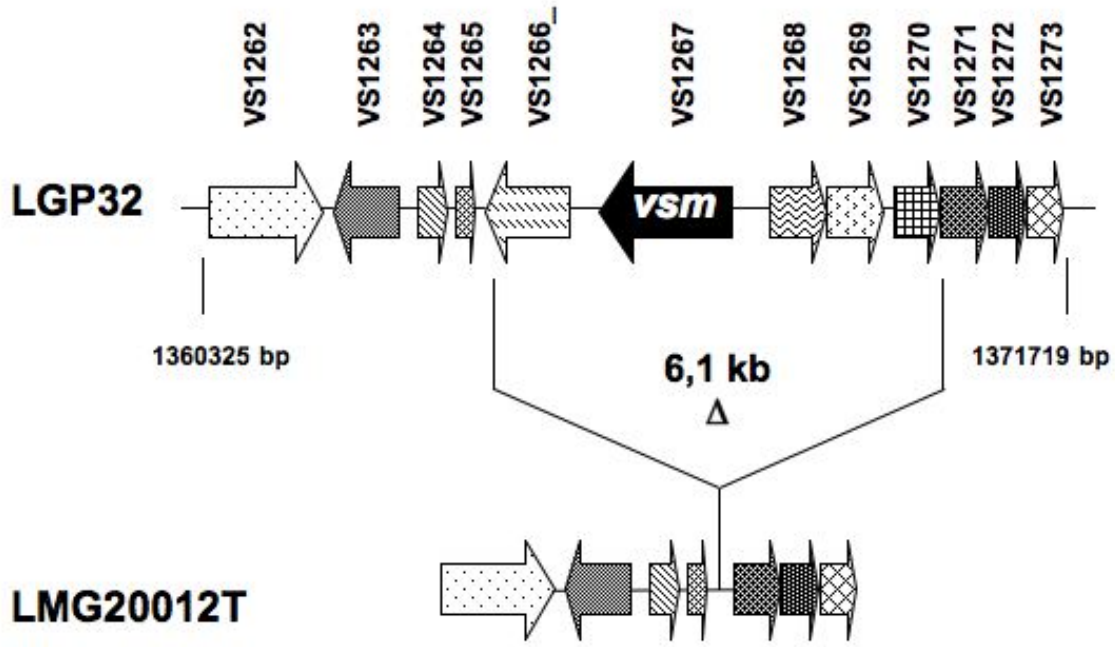

Fig. 1. Genetic organization of the vsm gene region. The region flanking vsm, localized in the chromosome 1 of V. splendidus LGP32, was PCR amplified in LMG20012T and sequenced. Genes found in LMG20012T and LGP32 are represented by arrows with the same texture. The absence in LMG20012T of a genomic region of 6.1 kb containing the vsm gene is indicated ( ). ORFs were annotated as: VS1267, Vsm extracellular zinc metalloprotease; VS1262, tryptophanase; VS1263, putative HTH-type transcriptional activator; VS1264, VS1265, VS1266, VS1269, VS1271, VS1272 and VS1273, conserved hypothetical proteins; VS1268 and VS1269 hypothetical proteins.

Figure 2

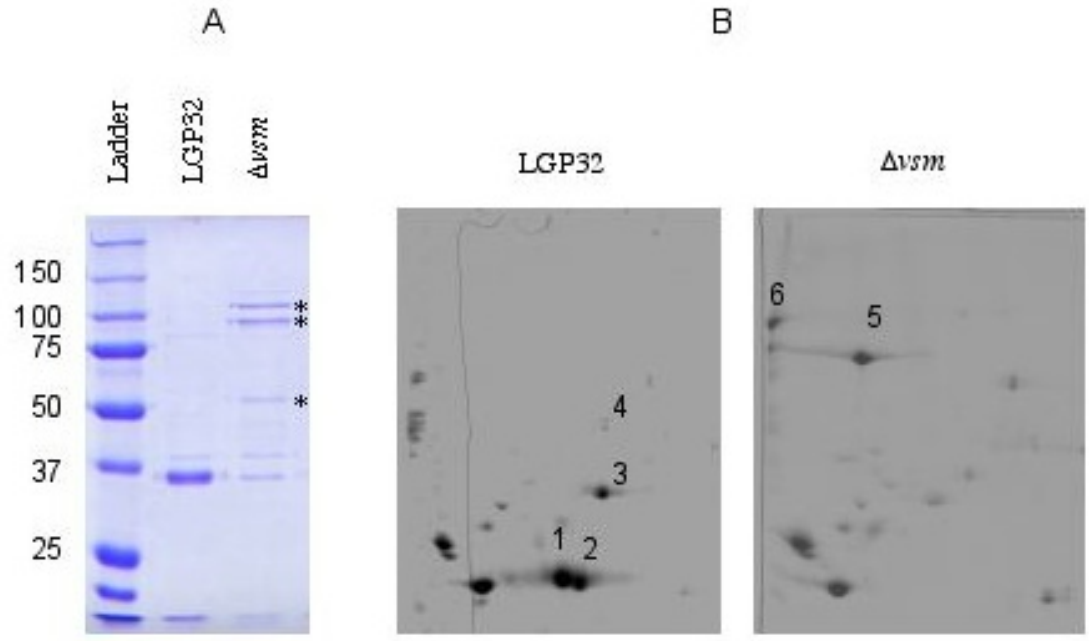

Fig. 2. ECP protein profiles for LGP32 and Δ*vsm*. (A) ECP analysis through a Coomassie blue stained 10% SDS PAGE. Bands corresponding to differentially expressed and/or processed proteins in Δ*vsm* ECP are indicated (*). (B) 2D analysis of the ECPs. The most significant differential spots are numbered (1-3) for Vsm, (4) for VSA576, (5) for VSA1062 and (6) for VS2864.

Figure 3

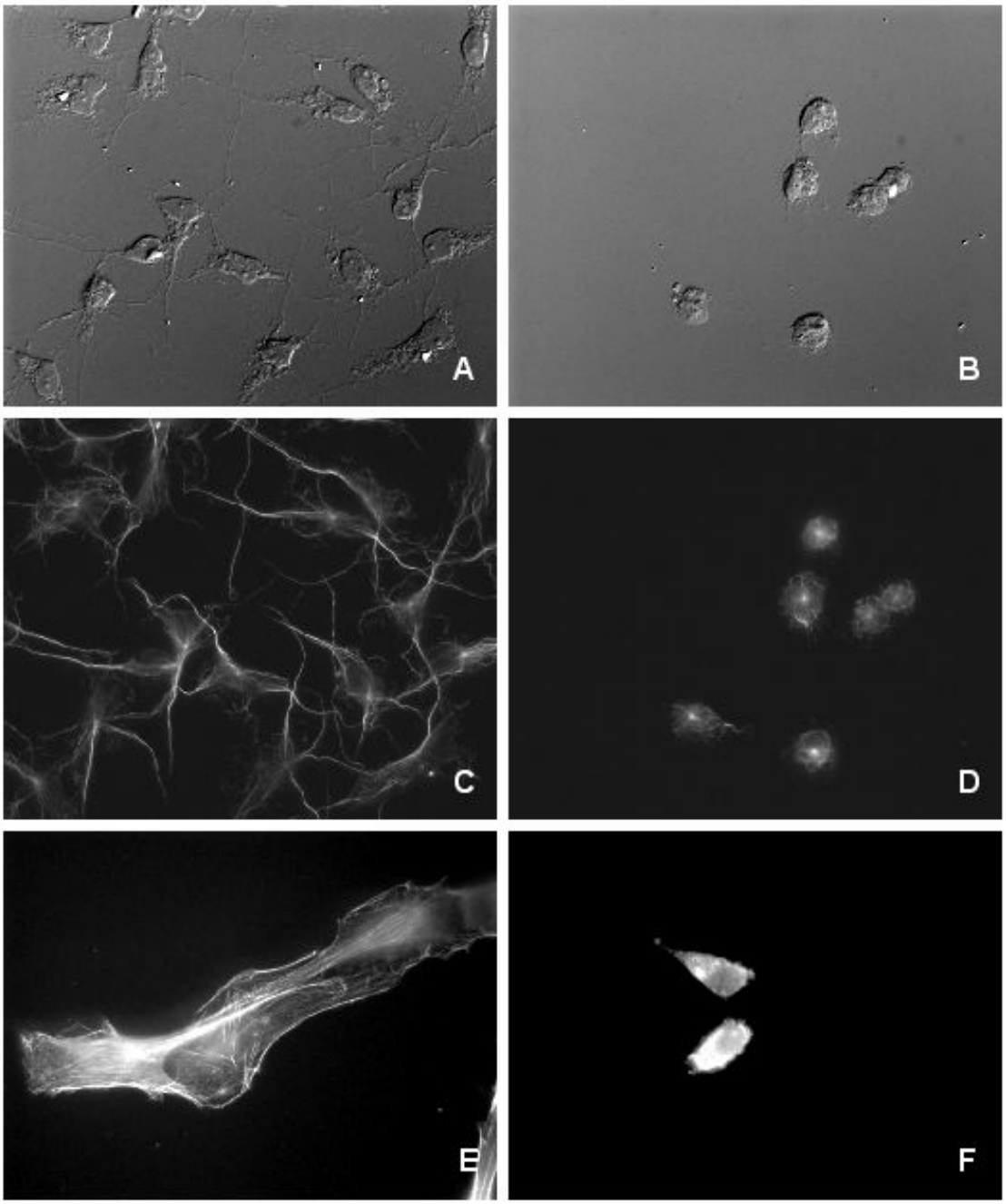

Fig. 3. ECP alteration of cell morphology. Non-confluent cell cultures were obtained by seeding coverslips with 65 000 Bge or 40 000 NIH cells in 300 µl of medium in 24-well plates and were grown overnight in their respective culture conditions. ECPs were added to the cells at a final protein concentration of 10 µg.ml^{-1} and cells were further incubated for 6 hours. (A-B) DIC microscopy. ECP treated Bge cells (B) showing cytoplasmic retraction and cell rounding when compared to untreated

Bge cells (A). (C-F) Fluorescent microscopy. Immunostaining of ß tubulin on Bge cells showing the retraction of microtubule cytoskeleton in ECP treated cells (D) when compared to untreated cells (C). (E-F) NIH cells. Actin labelling showing the disruption of the actin cytoskeleton in ECP treated cells (F). Untreated cells (E).

Figure 4

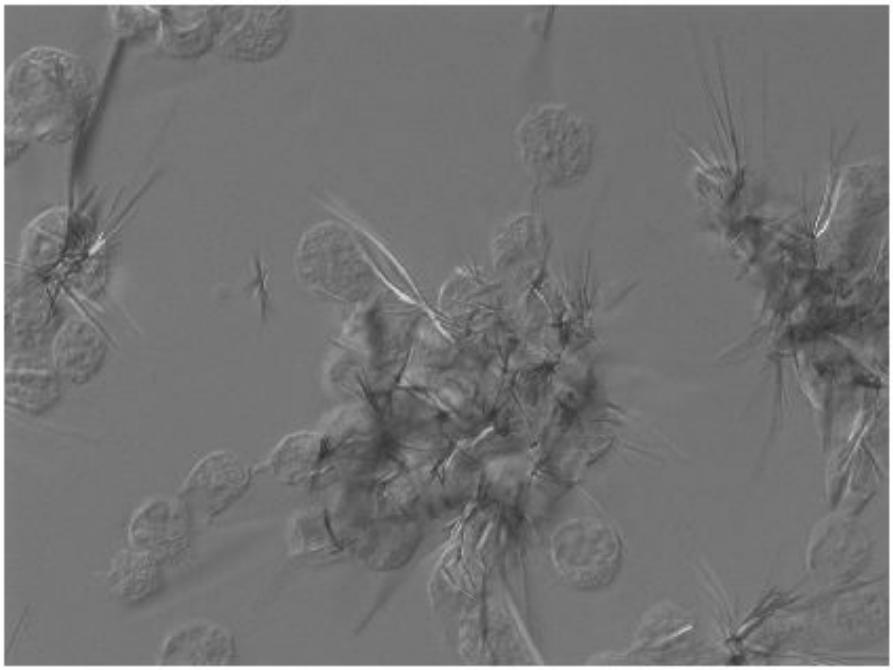

Fig. 4. Rounded ECP treated Bge cells with formazan crystals.
Bge cells were seeded on coverslips and treated with LGP32 ECP for 24 h, then incubated for 4 hr with MTT and fixed with PFA as mentioned above. DIC microscopy revealed groups of rounded and closely associated cells. These cells display long crystals of formazan indicating an active MTT metabolism despite the ECP treatment.

Figure 5

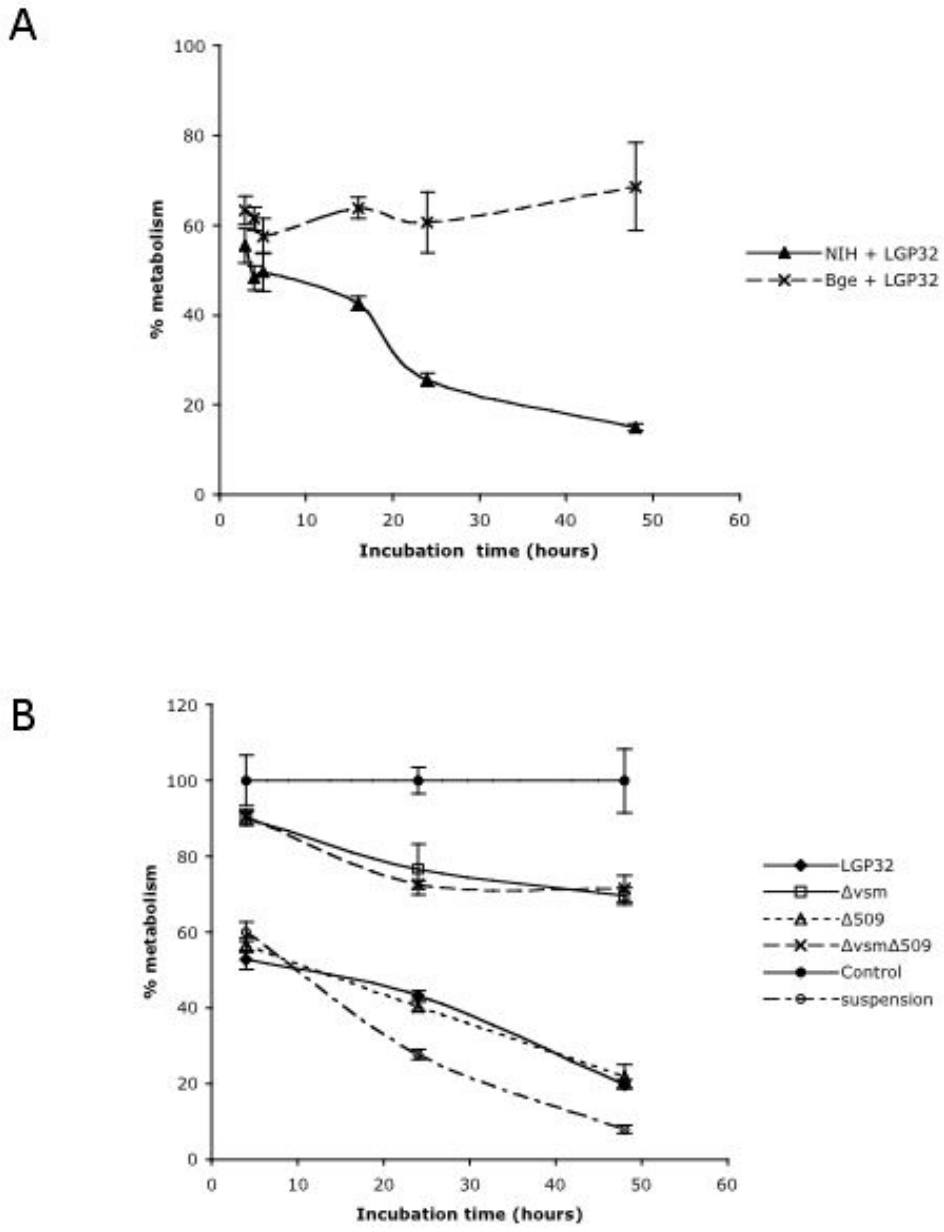

Fig. 5. Viability test on ECP treated cells.
Cells were treated as mentioned on Fig.3 and were then incubated for 3 h to 48 h before adding 33 µl of a MTT solution (5 $mg.ml^{-1}$) using the Vybrant MTT Cell Proliferation Assay (Molecular Probes, NL). Optical density was determined at 570 nm on triplicate samples. Cell viability is expressed as a mean

percentage of the MTT reduction as formazan, 100% being defined as the activity of untreated cells at each time point. (A) Kinetics of cytotoxicity of LGP32 ECP on Bge and NIH cells. The upper curve shows that despite being rounded after ECP treatment, Bge cell capacity to metabolise MTT was only slightly altered. Instead, the ECP induced a continuous decrease of MTT metabolism on NIH cells.
(B) Kinetics of cytotoxicity of mutant ECPs on NIH cells. Cells were incubated with the same amount of ECPs normalized for their content of total protein and treated with MTT as above. Δ*vsm* and the double mutant Δ*vsm*-1062 display a moderate cytotoxicity indicating that VSA1062 is not a factor of virulence in the ECP. Indeed, the ECPs of Δ1062 and LGP32is are equally cytotoxic. Interestingly, the MTT reduction assay provides essentially the same percentage of viability for NIH cells maintained in suspension as for cells treated with virulent ECPs.