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Characterization of the Secretomes of Two Vibrios Pathogenic to Mollusks

Stéphanie Madec, Vianney Pichereau, Annick Jacq, Mathieu Paillard, Claire Boisset, Fabienne Guérard, Christine Paillard, Jean-Louis Nicolas

1 Laboratoire Universitaire de Biodiversité et Ecologie Microbienne (EA3882), SFR48 ScinBio, Université de Bretagne Occidentale (UBO), UEB, ESIAB, Technopôle Brest Iroise, 29280, Plouzané, France, 2 Laboratoire des Sciences de l’Environnement Marin, UMR 6539 UBO/CNRS/IRD/Ifremer, Université de Bretagne Occidentale (UBO), Institut Universitaire Européen de la Mer, Technopôle Brest Iroise, 29280, Plouzané, France, 3 Institut de Génétique et de Microbiologie, UMR8621, CNRS-Université Paris-Sud, 91405, Orsay, France, 4 Centre de Recherche sur les macromolécules végétales, CERMAV-CNRS, BP53, 38041 Grenoble, France

Abstract

Vibrio tapetis causes the brown ring disease in the Japanese clam Ruditapes philippinarum while Vibrio aestuarianus is associated with massive oyster mortalities. As extracellular proteins are often associated with the virulence of pathogenic bacteria, we undertook a proteomic approach to characterize the secretomes of both vibrios. The extracellular proteins (ECPs) of both species were fractionated by SEC-FPLC and in vitro assays were performed to measure the effects of each fraction on hemocyte cellular parameters (phagocytosis and adhesion). Fractions showing a significant effect were subjected to SDS-PAGE, and proteins were identified by nano LC-MS/MS. 45 proteins were identified for V. aestuarianus and 87 for V. tapetis. Most of them belonged to outer membrane or were periplasmic, including porins or adhesins that were already described as virulence factors in other bacterial species. Others were transporter components, flagella proteins, or proteins of unknown function (14 and 15 respectively). Interestingly, for V. aestuarianus, we noted the secretion of 3 extracellular enzymes including the Vam metalloprotease and two other enzymes (one putative lipase and one protease). For V. tapetis, we identified five extracellular enzymes, i.e. two different endochitinases, one protease, one lipase and an adhesin. A comparison of both secretomes also showed that only the putative extracellular lipase was common to both secretomes, underscoring the difference in pathogenicity mechanisms between these two species. Overall, these results characterize for the first time the secretomes of these two marine pathogenic vibrios and constitute a useful working basis to further analyze the contribution of specific proteins in the virulence mechanisms of these species.

Introduction

Vibrios have frequently been associated with bivalve mortalities, essentially at the larval stage but also in adults [1–4]. Since 1987, several mortality events have been reported in clams (Ruditapes philippinarum) in different sites of the French coastline. Before death, clams go back to the sediment surface and display a brown deposit on the inner surface of the valves, between the pallial line and the edge of the shell [5]. This disease, named the Brown Ring Disease (BRD), was also described in Spain and Portugal, and affects both reared and wild clams. Bacteriological studies led to the identification of a new bacterial species, Vibrio tapetis, capable of reproducing the BRD in healthy animals [6].

In France, shellfish production is a well-established industry mainly relying on the commercial farming of the Pacific oyster (Crassostrea gigas). Annual mass summer mortalities of C. gigas have been reported since 1980 on the French coast. Several studies have demonstrated that these mortality outbreaks resulted from complex interactions between the physiological and/or genetic status of the oysters, environmental factors, and one or more infectious agents, among which the herpes virus OsHV1 [7], and Vibrio sp. [8]. Analyses of both moribund and healthy oyster hemolymph revealed that Vibrio aestuarianus was the most frequently disease-associated species [2] until 2008. Since then, a more virulent pathogenic herpes virus OsHV1, genotype microvar, emerged, reducing the occurrence of V. aestuarianus while V. splendidus strains are still frequently isolated [9].

The observed variable virulence of the isolates could be linked to the varying toxicity of the bacterial extracellular products (ECPs), allowing bacteria to escape the host immune defenses. In a previous study, the ECPs of the pathogenic strain V. aestuarianus 01/32 were shown to cause lethality in C. gigas, as well as morphological changes and immunosuppression in oyster hemocytes [10]. Further biochemical and genetic approaches evidenced the major role of the Vam extracellular metalloprotease in the
toxicity of *V. aestuarianus* ECPs and in the impairment of oyster hemocyte functions [11].

As for *V. tapetis*, the causative agent of BRD in adult clams, the pathogenicity process is not well established yet. As in the case of *V. aestuarianus*, *V. tapetis* isolates display variable levels of virulence [12]. This vibrio is known to decrease both hemocytes viability and phagocytic activities in *R. philippinarum* [13]. *In vitro* experiments showed adhesion capability of *V. tapetis* to the clam hemocytes and mantle cells [14], and its cytotoxic effects after phagocytosis resulted in cell rounding with loss of filipods [12].

It is recognized that the success of each step of the bacterial virulence process depends on the orchestrated activity of several specialized bacterial factors. In vibrios, such virulence factors have been more identified in human pathogens such as *V. cholerae*, *V. parahaemolyticus* and *V. vulnificus* [15–17] but also in *V. anguillarum*, *V. harveyi* and other fish, crustacean and mollusk pathogens [18]. Currently, the only virulence factor characterized in *V. aestuarianus* is the secreted zinc metalloprotease, Vam, a member of the thermolysin family [11]. No similar virulence factor has been described to date in *V. tapetis*, but a metalloprotease (Vsm), a homolog of Vam, was also identified as a major determinant of the toxicity of *V. splendidus* ECPs [19]. All this reinforced our objective to search for other secreted proteins potentially involved in the virulence of these two marine vibrios. So far only two vibrio secretomes have been described [20,21] and the importance of the extracellular compartment on host pathogen interaction led us to analyze more precisely the proteins of this compartment in both vibrios.

### Materials and Methods

1. **Bacterial strains, growth and culture conditions**

   *V. aestuarianus* 07/115 was isolated from the hemolymph of an oyster collected at Aber Benoît (Brittany, France) in September 2007. It was identified by the sequencing of the 16S rRNA and *gyrB* genes and was found to be highly virulent when injected in adult oysters [Jean-Louis Nicolas, unpublished results]. The *V. tapetis* CECT4600 strain was isolated in Aber Benoit (France) in Landeda (France) in 1995 from BRD diseased Manila clam (*Ruditapes philippinarum*) [22]. These strains were respectively grown in Difco marine broth 2216 (BD, Franklin Lakes, USA) and Zobell broth (HiMedia Laboratories, Mumbai, India), or on Difco marine agar and Zobell agar at 18°C.

2. **Preparation of extracellular products (ECPs) and fractionation by Size Exclusion Chromatography (SEC-FPLC) mode**

   ECPs were produced by the cellophane overlay method as previously described [10]. Total ECPs of *V. aestuarianus* and *V. tapetis* culture supernatants were filtered through 0.22 μm filter membranes and concentrated on an Amicon Ultra-4 membrane with a 10,000 molecular weight cut-off (MWCO) (Millipore, Billerica, MA, USA). The total protein content was quantified using a DC protein assay (Bio-Rad, Hercules, CA, USA) with 96-well micro-plates (Nunc) in a micro-plate reader (Bio-Tek Synergy HT) and the KC4 v3 software comparing the results with a calibration curve using standard proteins (Bovine Serum Albumin) provided with the DC protein assay kit. Then, ECPs were separated on an AKTA FPLC system (GE Healthcare, Piscataway, NJ, USA) directed by the Unicorn 5.1 software. Aliquots containing 1.4 mg of total proteins dissolved in mobile phase (isocratic elution mode in PBS: 10 mM Phosphate Buffer pH 7.4, 137 mM NaCl, and 2.7 mM KCl) and filtered on a 0.22 μm membrane was injected onto a Superdex S200 HR/10 30 gel filtration column from GE Healthcare (fractionation range of the column: 10–600 kDa) at a flow rate of 1 mL/min. Absorbance was monitored at 280 nm and 1 mL fractions were collected. The protein concentration of each fraction was determined and protease activity was assayed using azocasein as previously described [10]. Fractionated ECPs were conserved at −80°C until *in vitro* assays.

3. **In vitro assays : hemocyte cellular parameters**

   The effects of the obtained fractions were measured on oyster or clam hemocytes to assess the action of the ECPs on hemocyte adherence and phagocytosis capacities. Fractions showing inhibitory or stimulatory effects were compared to the negative control (FSSW: Filtered Sterile Sea Water). For both tests, ECPs of each bacterial species were tested at 32 μg.mL⁻¹ of proteins, following previously described procedures [10]. Briefly, for phagocytosis tests, a sub-sample (150 μl) of each hemolymph pool was distributed into a 5 ml polystyrene tube (Falcon, B-D Biosciences, San Jose, CA, USA), then underwent a two fold dilution with FSSW and was maintained on ice. Each sub-sample was subsequently combined with 30 μl of a fluorescent bead (2.00 μm in diameter, Fluoresbrite calibration grade, Polysciences, USA) working suspension (2% of the commercial suspension in FSSW, final concentration 1 x 10⁷ beads.mL⁻¹), and incubated at 18°C for 120 min. The cells were then analyzed on a flow cytometer (FACSCalibur, BD San Diego, USA). The results were expressed as the percentage of hemocytes containing three beads or more [10]. To estimate hemocyte adhesive capacity, the sub-samples (100 μl) of each hemolymph pool were distributed into 24-well microplates maintained on ice, as already described by Choquet et al. [12]. 100 μl of FSSW or ECPs was added in triplicate to each sub-sample. After three hours of incubation at 18°C, the cells were fixed by addition of 200 μl of a 6% formalin solution in FSSW. The supernatants were then transferred to cytometry tubes. The hemocyte number present in each supernatant was determined by flow cytometry. The results are expressed as average of non-adherent cells per ml i.e. an increase of the value compared to that of the negative control shows a cytotoxic effect of the tested ECPs.

4. **Proteins electrophoresis (SDS-PAGE)**

   The fractions showing a significant effect in *in vitro* on hemocyte phagocytosis or adherence were concentrated with Corning Spin-X UF Concentrators (Corning, Lowell, MA, USA) with a 10 kDa MWCO and applied on a Criterion precast acrylamide gradient gel 8–16% in Tris-HCl (Biorad, Hercules, CA, USA). After staining by Coomassie blue (Biosafe Coomassie, Biorad), the gel bands were cut out manually and conserved at −20°C before trypsin digestion.

5. **Protein identification**

   5.1. **In-gel digestions and peptides recovery.**

      Excised gel plugs were washed 3 times with water, 100 mM ammonium bicarbonate and 100% acetonitrile successively. Cysteins were reduced by a treatment with a 65 mM DTT solution for 15 minutes at 37°C followed by alkylation with 135 mM iodoacetamide at room temperature in the dark. Gel plugs were washed again with 100 mM ammonium bicarbonate/acetonitrile (1:1), 100% acetonitrile, 100 mM ammonium bicarbonate and 100% acetonitrile successively before being dried. Gel pieces were then re-swollen in a solution of trypsin (12.5 ng/μL in 50 mM ammonium bicarbonate; Promega), and digestion was performed overnight at 37°C. The resulting peptides were then extracted.
from the gel by sequential incubation in the following solutions: acetonitrile (ACN)/H2O/trifluoroacetic acid (TFA), 70:30:0.1 (v/v/v), 100% ACN and ACN/H2O/TFA, 70:30:0.1 (v/v/v), and extracts were eventually concentrated by evaporation to a final volume of 30 μL.

5.2. Mass spectrometry (MS) analysis. Peptide mixtures were separated on a nano-HPLC system (Ultimate 3000, Dionex, Jouy-en-Josas, France), with an injection volume of 22 μL. First, they were concentrated into a reversed-phase C18-PepMap trapping column (5 μm, 300 Å/300 μm i.d. x 5 mm, Dionex), and then eluted with a 75-min gradient of ACN (from 2 to 90%) in aqueous 0.05% formic acid, at a flow rate of 250 nL/min. The nano-LC apparatus was coupled on-line with an Esquire HCT Ultra PMT Discovery mass spectrometer (Bruker Daltonik, GmbH, Bremen, Germany), equipped with a nanoflow ESI source and an ion trap analyser (ITMS). The mass spectrometer was operated in the positive ionization mode. The EsquireControl software (Bruker Daltonik, GmbH) automatically alternated MS and CID MS-MS acquisitions with the following criteria: up to seven ions per MS scan with an intensity threshold of 30,000 and a dynamic exclusion of 15 sec.

5.3. Protein identification. The DataAnalysis 3.4 software (Bruker Daltonik, GmbH) was used to create the peak lists from raw data. For each acquisition, a maximum of 2,000 MS/MS spectra were detected with an intensity threshold of 30,000 and the charge state of precursor ions was automatically determined by resolved-isotope deconvolution. The proteinScape 2.0 software (Bruker Daltonik, GmbH) was used to submit the MS/MS data to the genomic V. aestuarianus 02/041 database (3693 CDS sequences; 1125373 residues, unpublished results), the only V. aestuarianus sequences available at that time. Peptide sequences were found to be 100% identical to the identified proteins in the database. Similarly, the MS/MS data for V. tapetis were submitted to the V. tapetis CECT4600 database (5498 sequences; 1633991 residues, unpublished). Submission to randomized versions of these databases (decoy) was used to determine the false positive rate (FPR), defined as the number of validated decoy hits/(number of validated target hits + number of decoy hits)*100, using the Mascot algorithm (Mascot server v2.2.07; http://www.matrixscience.com). Trypsin was selected as the cleaving enzyme with one allowed missed cleavage. In addition, carbamidomethylation of cysteins was set as fixed modifications and methionine oxidation were considered as variable modifications. The mass tolerance for parent and fragment ions was set to 0.6 and 0.5 Da, respectively. Peptide identifications were accepted if the individual ion Mascot scores were above 25 or the identity threshold (the ion score is $2^{10\log(P)}$, where P is the probability that the observed match is a random event, p-value<0.05). In case of ambiguous assignments (one compound fitting more than one peptide), the peptide sequence with the highest score was retained. The compilation of peptides identified to proteins was performed with the ProteinExtractor algorithm [23], so that every protein reported was identified by at least one peptide with a significant ion Mascot score (above the identity threshold) that could not be mapped to a higher-ranking protein already in the result list. This means that the final protein lists contain only those proteins and protein

Figure 1. UV spectrum of total ECPs of V. aestuarianus and V. tapetis on a Superdex S200 10/30 column. Eluted fractions were collected with a flow of 1 mL/min. Fractions are numbered according to their elution time (top and bottom X-axes, respectively). Gel filtration profile was expressed in milliabsorbance units (mAU).

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variants that could be distinguished directly by MS/MS. For every protein reported in the identification lists, a combined protein score (metascore) was calculated from the peptide scores with the ProteinExtractor algorithm. Finally, protein identifications were accepted if the False Positive Rate of the search was lower than 1%.

5.4. Bioinformatics. For each result of proteomic identification, we used various softwares and algorithms to determine i/a score of identification; this score was given by the MASCOT software, ii/the presence or not of a signal peptide and the predicted position of the cleavage site; the algorithm SignalP 3.0 (probability>0.95) was used except in the case of TolC for which SignalP 4.01 was used instead [http://www.cbs.dtu.dk/services/SignalP/] and iii/the subcellular localization using PsortB and Psort Gram negative bacteria [http://psort.hgc.jp/form.html]; in case of ambiguity (score above threshold for two locations), the highest score was chosen. Lipoproteins and their localisation (outer membrane associated versus inner membrane associated) were predicted using LipoP1.0 [http://www.cbs.dtu.dk/services/LipoP/]. In general, lipoproteins are periplasmic but anchored to one or the other membrane by their acyl moiety (indicated by P/OM for instance). In most cases, they were associated with the OM. In some cases, they could be associated with the OM and facing outward.

Figure 2. Effect of *V. aestuarianus* 07/115 (right bars) and *V. tapetis* CECT 4600 (left bars) total ECPs on oyster and clam, respectively, hemocyte phagocytosis capability (top panel) and hemocyte adhesion (bottom panel). Tests were carried out in triplicates as described in M&M and the error bars correspond to SD. Incubation of hemocytes with sterile sea water (SSW) was used as a negative control.

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Results and Discussion

1. Preparation and fractionation of *V. aestuarianus* 07/115 and *V. tapetis* CECT4600 ECPs

The proteins from the extracellular compartment are of particular interest for functional investigation of bacterial pathogen virulence, because they come into direct contact with host tissues and are often effectors of pathogenicity. Several lines of evidence highlight an important role of ECPs in the virulence of pathogenic vibrios. For example, a previous study on *V. aestuarianus* 01/032 showed that its ECPs displayed immuno-suppressive activities on oyster hemocyte functions [10]. Similar effects were described in *V. tapetis*, in which ECPs were shown to significantly decrease adhesive- [12] and phagocytic- [13] activities of clam hemocytes. However, although the biological activity of *V. aestuarianus* ECPs has been associated with the secretion of the zinc metalloprotease, Vam [11], few studies have been carried out to date in *V. tapetis* and nothing is known about the molecular components responsible for the biological activity of the *V. tapetis* ECPs.

The extraction of secreted proteins was performed under conditions known to induce virulence [11,12]. ECPs were fractionated, their biological activity against hemocytes was assayed, and their protein contents were analyzed, as described in Materials and Methods.

In the case of *V. aestuarianus*, fractionation of total ECPs gave four major peaks (Fig. 1). A first symmetrical peak eluted in the void volume of the column, suggesting that it was composed of a mixture of protein aggregates or complexes larger than 600 kDa. Three poorly resolved additional peaks eluted at 16, 18 and 22 minutes, respectively. The elution diagram obtained with *V. tapetis* ECPs comprised a first peak also eluting in the void volume, and a second broad peak, lower in absorbance than the three peaks of *V. aestuarianus*, but exactly superimposed. The fractions were recovered every minute and numbered according to the elution time. Determination of fraction protein contents allowed us to select a set of fractions (8, 9, 16 to 23 for *V. aestuarianus* ECPs and 8, 9, 14 to 21 for *V. tapetis* ECPs) showing a minimal concentration of 0.3 mg/ml of protein, to carry out further analyses.

2. Effects of *V. aestuarianus* and *V. tapetis* ECPs on phagocytosis and adherence activities of oyster and clam hemocytes respectively

We first assayed the activities of unfractionated ECPs. In both cases, the biological parameters assayed were hemocyte adhesion and phagocytosis. We found that *V. aestuarianus* ECPs induced a decrease of phagocytosis and adherence properties of oyster hemocytes as shown in Fig. 2. This result is in keeping with previous results obtained by Labreuche et al. [10]. Similarly, *V. tapetis* ECPs triggered a decrease of hemocytes adherence as previously described [12]. However, *V. aestuarianus* total ECPs did not impact the phagocytic ability of clam hemocytes, contrary to what was found by Allam and Ford [13] who previously described a decrease in phagocytosis after treatment by bacterial supernatants obtained from liquid cultures. This discrepancy may be due to the different conditions used to prepare the ECPs (liquid culture versus cellophane overlay on plate).
The different FPLC fractions of ECPs previously obtained were then similarly tested for biological activity towards oysters (V. aestuarianus) and clam (V. tapetis) hemocytes. The results presented in Fig. 3 showed that all the assayed fractions obtained from V. aestuarianus decreased the adhesive capacities of oyster hemocytes, with an increase of non-adherent hemocytes ranging from a factor 1.8 (fraction 16) to 2.5 (fraction 20). The only extracellular virulence factor described to date for V. aestuarianus is the Vam metalloprotease, which causes aggregation and the loss of pseudopods of oyster hemocytes [11]. Only the fractions 16 and 17 contained an azocaseinase activity (data not shown), suggesting that Vam is not responsible for this loss of adhesion and that V. aestuarianus 07/115 extracellular products, in particular in fraction 20, contain additional factors playing a role in adherence decreasing.

However, although total V. aestuarianus ECPs decreased the phagocytic activity of hemocytes (Fig 2), none of the V. aestuarianus ECP fractions affected the oyster hemocyte phagocytic activity (Fig 3A). This result suggests that phagocytosis inhibition by ECPs may involve the joined activity of several factors that have been eluted in separate fractions.

In the case of V. tapetis ECPs (Fig 3B), a reduction in clam hemocyte phagocytosis capacity was recovered in fractions 19–20–21, in accordance with previously published results [13]. This suggests that an inhibitor of this activity is present in the total V. tapetis ECPs, which was separated during fractionation. As in the case of V. aestuarianus, all the recovered fractions displayed an effect on hemocyte adhesion, but with more variations amongst fractions. For example, fraction 14 triggered in excess of a 4-fold increase in non-adherent hemocytes whereas fraction 8 had only a 2-fold effect (Fig. 3D).

In summary, our results indicate that both V. tapetis and V. aestuarianus ECPs have major effects on hemocyte properties including loss of adherence and inhibition of phagocytosis, especially in the case of V. tapetis. In the case of V. aestuarianus, inhibition of adhesion is independent of Vam, and is maximal in fraction 20. In the case of V. tapetis, we could partly separate adhesion inhibition activity (maximal in fraction 14) and phagocytosis inhibition activity (maximal in fractions 19–21). In contrast, the observed phagocytosis inhibition in V. aestuarianus was lost upon fractionation, suggesting that it requires several factors acting in a complementary way while with V. tapetis, it was detected only after fractionation, suggesting the presence of an inhibitor in total ECPs.

3. Proteomic analysis of the two secretomes

Fractions combining both a significant effect in vitro on hemocytes, and sufficient protein amounts were further characterized by proteomic analysis. Accordingly, fractions 8 and 16 to 23 for V. aestuarianus and 9, 14 to 17 and 19 to 21 for V. tapetis were subjected to SDS-PAGE for further protein identification by nano LC-MS/MS. Several fractions (18 to 22 for V. aestuarianus and 19, 20, 21 for V. tapetis) did not show any band after Coomassie Blue staining, but were directly trypsinolyzed and analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) starting from a total protein content of 5 μg. As shown in Fig. 4, active fractions from both bacteria contained multiple proteins. In order to identify them, 16 and 43 distinct bands were excised for V. aestuarianus and V. tapetis, respectively, and analyzed by mass spectrometry, as described in Materials and Methods. Using the known proteome from both species, our proteomic analysis of V. aestuarianus and V. tapetis secretomes led to the unambiguous identification of 45 and 87 proteins, respectively (Tables 1 and 2). Only five proteins in the ECPs of V. tapetis and none in the V. aestuarianus secretome were predicted to be cytoplasmic, emphasizing the quality of our protocol and the absence of cell lysis.

98% and 70% of the proteins in the V. aestuarianus and V. tapetis secretomes, respectively, were predicted to have a signal peptide (see Materials and Methods for the algorithms used), indicating that they are periplasmic or outer membrane components (see Tables 1 & 2). Most of the proteins appeared to be normal components of the outer membrane and the periplasmic space, suggesting that they were released in the medium most probably as membrane vesicles, as was previously described for other bacteria [24]. In accordance with this hypothesis, 98% of the...
**Table 1. Proteins identified in the *V. aestuarianus* secretome.**

| Protein function                                                                 | Genbank accession Nr | MW (kDa) | Score* | Nr of peptides | coverage | FPLC fraction | Signal peptide cleavage site ** | Predicted localization* *** |
|---------------------------------------------------------------------------------|----------------------|----------|--------|----------------|----------|---------------|---------------------------------|-----------------------------|
| Structural component, envelope biogenesis and quality control, stress response  |                      |          |        |                |          |               |                                 |                             |
| OmpU: Outer membrane protein U                                                   | KM588597             | 37       | 1682   | 20             | 68       | 8             | 21–22 OM                        |                             |
| Lpp: Major outer membrane lipoprotein                                            | KM588630             | 9        | 335    | 4              | 62       | 8             | 22–23 P/OM                      |                             |
| OmpA: Outer membrane protein A                                                   | KM588598             | 35       | 327    | 5              | 22       | 8             | 21–22 OM                        |                             |
| BamA/YaeT: Outer membrane protein assembly factor                                | KM588599             | 91       | 897    | 17             | 23       | 8             | 20–21 OM                        |                             |
| Tsp: carboxy-terminal protease for penicillin-binding protein 3                  | KM588600             | 75       | 198    | 5              | 8        | 8             | 23–24 P/OM                      |                             |
| Peptidoglycan-associated outer membrane lipoprotein Pal                          | KM588601             | 19       | 100    | 2              | 15       | 8             | 25–26 P/OM                      |                             |
| Periplasmic component of the Tol-Pal system, YbgF-like                           | KM588602             | 29       | 453    | 6              | 25       | 8             | 23–24 P                         |                             |
| Thiol-disulfide isomerase DsbA                                                    | KM588603             | 22       | 146    | 4              | 20       | 8             | 19–20 P                         |                             |
| LptD: LPS-assembly protein                                                       | KM588604             | 52       | 52     | 2              | 3        | 8             | 24–25 OM                        |                             |
| Putative lipoprotein LpoA, activator of penicillin binding protein 1A            | KM588605             | 67       | 226    | 5              | 9        | 8             | 26–27 P/OM                      |                             |
| Putative penicillin-binding protein activator LpoB-like, outer-membrane lipoprotein | KM588606             | 22       | 894    | 12             | 71       | 8             | 25–26 P/OM                      |                             |
| NlpI lipoprotein                                                                | KM588607             | 37       | 334    | 7              | 19       | 8             | 37–38 P/OM                      |                             |
| Energetic metabolism                                                            |                      |          |        |                |          |               |                                 |                             |
| TorA: trimethylamine-N-oxide reductase (TMAO reductase)                          | KM588608             | 92       | 119    | 3              | 4        | 8             | 33–34 P                         |                             |
| NapA: nitrate reductase, periplasmic, large subunit                              | KM588631             | 93       | 1012   | 19             | 24       | 8             | 29–30 P                         |                             |
| Transporter components                                                           |                      |          |        |                |          |               |                                 |                             |
| LamB: Maltoporin                                                                | KM588632             | 43       | 800    | 12             | 43       | 8             | 22–23 OM                        |                             |
| Putative outer membrane porin; locus of qsr prophase                             | KM588610             | 35       | 117    | 3              | 12       | 8             | 19–20 OM                        |                             |
| HisU: ABC type histidine transporter periplasmic histidine binding protein       | KM588611             | 28       | 1169   | 19             | 72       | 8             | 21–22 P                         |                             |
| AapJ: ABC type L Aminoacid transporter subunit; periplasmic-binding              | KM588611             | 37       | 720    | 13             | 49       | 8             | 25–26 P                         |                             |
| PtsS1: ABC type phosphate transporter, periplasmic phosphate binding protein     | KM588633             | 29       | 613    | 11             | 42       | 8             | 22–23 P                         |                             |
| TRAP-type uncharacterized transport system, periplasmic component                | KM588612             | 34       | 524    | 12             | 32       | 8             | 24–25 P                         |                             |
| MglB: methylgalactoside ABC transporter periplasmic-binding protein               | KM588613             | 35       | 460    | 7              | 21       | 8             | 22–23 P                         |                             |
| Protein function | Genbank accession Nr | MW (kDa) | Score* | Nr of peptides | coverage | FPLC fraction | Signal peptide cleavage site ** | Predicted localization*** |
|------------------|----------------------|----------|--------|----------------|----------|---------------|---------------------------------|--------------------------|
| Putative ABC type dipeptide transporter, periplasmic peptide-binding protein | KMS88637 | 57 | 266 | 6 | 11 | 8 | 23–24 | P |
| Putative ABC type tungstate transport system, substrate binding protein | KMS88614 | 30 | 78 | 2 | 8 | 8 | 23–24 | P |
| Pts2: ABC type phosphate transport system, periplasmic component | KMS88635 | 30 | 68 | 2 | 7 | 8 | 21–22 | P |
| TolC: outer membrane efflux channel of type I secretion system | KMS88615 | 48 | 64 | 2 | 5 | 8 | 22–23 | OM |
| FadL2: Long-chain fatty acid outer membrane porin; bacteriophage T2 receptor | KMS88616 | 46 | 225 | 4 | 15 | 8 | 25–26 | OM |
| Putative YceI-like protein: lipid/polyisoprenoid-binding protein | KMS88636 | 20 | 89 | 2 | 8 | 8 | 22–23 | P |
| Motility/Flagella | | | | | | | | |
| FliD: Flagellar hook-associated protein 2 | KMS88617 | 71 | 123 | 3 | 6 | 8 | - | E |
| Extracellular proteins | | | | | | | | |
| Vam: secreted Zinc Metalloprotease Vam | KMS88637 | 66 | 1011 | 14 | 27 | 16, 17 | 25–26 | E |
| Putative extracellular triacylglycerol lipase | KMS88638 | 83 | 631 | 12 | 16 | 8 | 20–21 | E |
| Vpp: Vam protease processing enzyme | KMS88639 | 101 | 422 | 6 | 8 | 8 | 23–24 | E |
| Unknown function | | | | | | | | |
| Lipoproteins | | | | | | | | |
| Putative outer membrane lipoprotein | KMS88618 | 21 | 469 | 6 | 39 | 8 | 17–18 | P/OM |
| Maltese operon periplasmic protein (MalM), outer membrane associated lipoprotein | KMS88640 | 71 | 139 | 4 | 12 | 8 | 24–25 | P/OM |
| Conserved lipoprotein of unknown function | KMS88619 | 14 | 193 | 4 | 33 | 8 | 21–22 | P/OM |
| Putative outer membrane lipoprotein | KMS88620 | 20 | 51 | 1 | 7 | 8 | 27–28 | P/OM |
| Conserved lipoprotein of unknown function | KMS88621 | 12 | 155 | 3 | 27 | 8 | 22–23 | P/OM |
| Conserved lipoprotein of unknown function | KMS88622 | 14 | 78 | 1 | 15 | 8 | 21–22 | P/OM |
| Putative outer membrane lipoprotein | KMS88623 | 28 | 190 | 3 | 18 | 8 | 20–21 | P/OM |
| Conserved outer membrane lipoprotein of unknown function | KMS88641 | 21 | 362 | 9 | 62 | 8 | 17–18 | P/OM |
| Others | | | | | | | | |
| Putative outer membrane protein | KMS88624 | 17 | 110 | 2 | 13 | 8 | 19–20 | OM |
| Outer membrane proteins | KMS88625 | 84 | 110 | 4 | 5 | 8 | 26–27 | OM |
| Conserved outer membrane of unknown function | KMS88626 | 25 | 83 | 1 | 6 | 8 | 23–24 | OM |
proteins in the case of V. aestuarianus and 78% in this of V. tapetis came from the FPLC fractions eluted in the column void volume, corresponding to materials larger than 600 kDa. Proteomic composition of these fractions appears to reflect mostly the native composition of the bacterial envelope, with no obvious specific enrichment. However, it is also possible that some of this material correspond to aggregates rather than vesicles.

The identified proteins were classified according to their biological functions (Tables 1 and 2) (1) Structural components, envelope biosynthesis and quality control, stress response, (2) energetic metabolism, (3) transporter components, (4) iron acquisition (except in V. aestuarianus), (5) catabolism, including chitin utilization, (6) motility, flagellar genes, (7) extracellular proteins, (8) unknown function.

4. Identification of known and potential virulence factors in the vibrio secretomes

The only extracellular virulence factor characterized to date in V. aestuarianus is the secreted zinc metalloprotease, Vam, which was shown to cause lethality of C. gigas oysters [11]. This protein was clearly identified and found to be quantitatively dominant in two active soluble fractions (16 and 17) in our study. More interestingly, we also identified a second extracellular protease in the V. aestuarianus secretome which we named Vpp (for Vam processing protease). Vpp is a homologue of Epp, a secreted protease which processes the secreted metalloprotease EmpA in Vibrio anguillarum [25], EmpA being a homologue of Vam. Hence, Vpp might be the Vam processing enzyme. Vpp is also a homologue of PrtV of V. cholerae. In V. cholerae, PrtV was found to play a role in resistance to grazing by natural predator, outside the host, rather than in pathogenicity to humans [26]. Further studies should clarify the role of Vpp in V. aestuarianus, especially as fraction 8 that contains Vpp was found to decrease oysters hemocyte adherence.

Up to now, no secreted virulence factors have been described in V. tapetis. The only virulence factor characterized to date is the inner membrane protein DjlA, which was shown to be required for cytotoxicity towards clam hemocytes [27]. In contrast to V. aestuarianus, no metalloprotease was found in the V. tapetis secretome, suggesting different virulence mechanisms between the two species. However, two serine proteases (i.e. KM596588 and KM596661) have been identified in two different fractions. As secreted serine protease was already shown to be involved in the virulence of several pathogenic bacteria [28], these two proteins could also play a role in the pathogenesis of V. tapetis.

The secretomes of both vibrio species contained an extracellular triacylglycerol lipase (Table 1). This protein belongs to the same family as the phospholipase Pla1, a secreted virulence factor of Aeromonas hydrophila [29] and Cef, a toxin with cell elongation activity produced by Vibrio hollisae, which causes diarrhea in humans [30]. Phospholipases can act as potent membrane destructors and can manipulate host signalling pathways [31].

Another protein of interest is KM596634, identified in fraction 16 of the V. tapetis secretome, which contains the signatures of RTX toxins and autotransporters. Autotransporters are bacterial virulence factors that contain an N-terminal extracellular (“passenger”) domain and a C-terminal β barrel (“β”) domain that anchors the protein to the outer membrane. Upon autocleavage, the passenger domain is secreted. RTX (Repeat in toxins) toxins are virulence factors containing glycine- and aspartate-rich repeats binding Ca(2+) ions [32]. Such proteins were shown as virulence factors in other vibrio species [33,34].

Table 1. Cont.

| Protein function | Genbank accession Nr | MW (kDa) | Nr of peptides | Score* | FPLC fraction | Signal peptide cleavage site** | Predicted localization*** |
|------------------|---------------------|--------|--------------|--------|---------------|-----------------------------|-------------------------|
| Conserved exported protein of unknown function | KM588627 | 28 | 356 | 7 | 41 | 18-19 | P |
| Conserved outer membrane protein of unknown function | KM588628 | 35 | 137 | 2 | 7 | 20-21 | OM |
| Conserved outer membrane protein of unknown function | KM588629 | 20 | 63 | 2 | 12 | 19-20 | OM |
| doi:10.1371/journal.pone.0113097.t001 | | | | | | | |
### Proteins identified in the *V. tapetis* secretome.

| Protein function | Genbank accession Nr | MW (kDa) | Score* | Nr of peptides |_coverage | FPLC fraction | Signal peptide cleavage site** | Predicted localization*** |
|------------------|----------------------|----------|--------|----------------|----------|---------------|-------------------------------|--------------------------|
| **Structural components, envelope biogenesis and quality control, stress response** | | | | | | | | |
| OmpH: Outer membrane porin H | KM596581 | 35 | 2838 | 33 | 71 | 16 | 19–20 | OM |
| OmpU: Outer membrane protein U | KM596582 | 37 | 2318 | 24 | 77 | 16 | 21–22 | OM |
| OmpV: Outer membrane protein V | KM596645 | 28 | 303 | 6 | 29 | 16 | 23–24 | OM |
| BamA/YaeT: Outer membrane protein assembly factor | KM596583 | 90 | 204 | 3 | 4 | 9 | 20–21 | OM |
| **Outer membrane protein** | | | | | | | | |
| OmpA: Outer membrane protein A | KM596646 | 35 | 665 | 9 | 27 | 9 | 20–21 | OM |
| Lpp : major outer membrane lipoprotein | KM596647 | 10 | 54 | 1 | 12 | 9 | 22–23 | OM |
| MltC : membrane-bound lytic murein transglycosylase C | KM596584 | 27 | 93 | 2 | 7 | 9 | 18–19 | OM |
| **Periplasmatic component of the Tol-Pal system, YbgF-like** | | | | | | | | |
| ToIβ : periplasmatic component of the Tol-Pal system | KM596584 | 49 | 787 | 17 | 34 | 9 | - | P |
| **Energetic metabolism** | | | | | | | | |
| TorA: trimethylamine N-oxide (TMAO) reductase I catalytic subunit | KM596596 | 92 | 754 | 12 | 14 | 14 | 33–34 | P |
| **Transporter components** | | | | | | | | |
| Putative outer membrane efflux channel of type I secretion system | KM596598 | 48 | 136 | 3 | 8 | 9 | 22–23 | OM |
| Putative outer membrane efflux channel of type I secretion system | KM596599 | 51 | 55 | 2 | 4 | 9 | 22–23 | OM |
| Protein function                                                                 | Genbank accession Nr | MW (kDa) | Score* | Nr of peptides | coverage | FPLC fraction | Signal peptide cleavage site** | Predicted localization*** |
|---------------------------------------------------------------------------------|----------------------|----------|--------|----------------|----------|---------------|--------------------------------|--------------------------|
| TolC: outer membrane efflux channel of type I secretion system                  | KM596600            | 47       | 1434   | 21             | 54       | 9             | 22–23 OM                        |                          |
| LamB: maltoporin                                                                | KM596650            | 46       | 249    | 5              | 16       | 9             | 24–25 OM                        |                          |
| Outer membrane porin                                                            | KM596601            | 37       | 143    | 2              | 7        | 9             | 19–20 OM                        |                          |
| Putative outer membrane porin                                                   | KM596602            | 40       | 486    | 11             | 34       | 9             | 22–23 OM                        |                          |
| AapJ: ABC type L Aminoacid transporter subunit; periplasmic-binding             | KM596603            | 36       | 1004   | 17             | 47       | 9             | 25–26 P                         |                          |
| TRAP-type uncharacterized transport system, periplasmic component                | KM596604            | 35       | 996    | 17             | 44       | 9             | 24–25 U                         |                          |
| BtuB: Vitamin B12/cobalamin outer membrane transporter, TonB dependent          | KM596605            | 68       | 747    | 13             | 23       | 9             | 22–23 OM                        |                          |
| Putative chitoporin                                                             | KM596606            | 50       | 1046   | 14             | 39       | 9             | 23–24 OM                        |                          |
| Putative [GlcNAc]2 periplasmic substrate-binding protein of ABC transporter      | KM596607            | 63       | 667    | 13             | 23       | 9             | 27–28 P                         |                          |
| Putative [GlcNAc]2 ABC transporter, periplasmic substrate-binding protein of ABC transporter | KM596608          | 63       | 152    | 4              | 7        | 9             | 23–24 P                         |                          |
| Putative substrate binding protein component of oligopeptide/dipeptide ABC transporter | KM596609         | 66       | 643    | 14             | 27       | 9             | 20–21 P                         |                          |
| Putative ABC-type oligopeptide/dipeptide transport system, substrate-binding periplasmic component | KM596651          | 69       | 151    | 4              | 7        | 9             | 20–21 P                         |                          |
| Fad L1: Long-chain fatty acid outer membrane porin                              | KM596652            | 44       | 164    | 4              | 16       | 9             | 21–22 OM                        |                          |
| FadL2: Long-chain fatty acid outer membrane porin                               | KM596610            | 43       | 441    | 6              | 18       | 9             | 25–26 OM                        |                          |
| ABC-type sugar transport system, periplasmic component                           | KM596611            | 45       | 326    | 7              | 24       | 9             | 21–22 P                         |                          |
| Putative outer membrane protein of unknown function                             | KM596653            | 40       | 326    | 6              | 15       | 9             | 21–22 OM                        |                          |
| Putative ABC-type transport system, periplasmic substrate-binding component      | KM596612            | 33       | 270    | 5              | 25       | 9             | 25–26 P                         |                          |
| TRAP-type C4-dicarboxylate transport system, periplasmic component               | KM596613            | 37       | 228    | 4              | 18       | 9             | 22–23 P                         |                          |
| Putative TonB-dependent receptor protein                                         | KM596654            | 77       | 91     | 2              | 3        | 9             | 22–23 OM                        |                          |
| Spermidine/putrescine ABC transporter; substrate binding periplasmic protein PotD2 | KM596614          | 39       | 61     | 1              | 4        | 9             | 24–25 P                         |                          |
| Putative oligogalacturonate-specific porin                                      | KM596615            | 26       | 522    | 10             | 26       | 9             | 20–21 OM                        |                          |
| Putative Rhs family protein, may bind carbohydrates                            | KM596616            | 259      | 54     | 1              | 1        | 14            | -                                | IM                       |
| Iron acquisition                                                               | Putative outer membrane siderophore receptor | KM596655 | 65 | 65 | 2 | 3 | 9 | 25–26 OM |
| Protein function                                                                 | Genbank accession Nr | MW (kDa) | Score* | Nr of peptides | coverage | FPLC fraction | Signal peptide cleavage site** | Predicted localization*** |
|---------------------------------------------------------------------------------|----------------------|----------|--------|----------------|----------|---------------|--------------------------------|--------------------------|
| FbpA: Periplasmic ferric iron-binding protein of ABC transporter                | KM596617             | 37       | 302    | 6              | 18       | 9             | 21–22                          | P                        |
| Putative TonB-dependent vibriobactin receptor                                    | KM596656             | 71       | 75     | 2              | 4        | 9             | 29–30                          | OM                       |
| Putative iron-regulated protein with peptidase M75 domain                     | KM596618             | 46       | 235    | 4              | 9        | 9             | 24–25                          | OM                       |
| **Catabolism**                                                                  |                      |          |        |                |          |               |                                |                          |
| **Chitin utilization**                                                           |                      |          |        |                |          |               |                                |                          |
| GbpA: N-acetyl glucosamine (GlcNAc) binding protein                             | KM596619             | 54       | 358    | 7              | 14       | 17            | 24–25                          | E                        |
| Chitinase                                                                       | KM596657             | 53       | 472    | 8              | 20       | 15            | 19–20                          | U                        |
| ChiA: endochitinase A                                                            | KM596620             | 89       | 367    | 6              | 6        | 14            | 21–22                          | E                        |
| Endochitinase                                                                   | KM596621             | 88       | 935    | 11             | 12       | 16            | 22–23                          | E                        |
| Putative chitinase                                                              | KM596658             | 70       | 1876   | 26             | 35       | 16            | 28–29                          | OM                       |
| Putative chitinase                                                              | KM596659             | 48       | 192    | 4              | 9        | 9             | 20–21                          | P                        |
| **Others**                                                                       |                      |          |        |                |          |               |                                |                          |
| Aryl/Alkyl sulfatase                                                            | KM596660             | 73       | 251    | 6              | 12       | 9             | 19–20                          | P                        |
| Ggt: Gamma-glutamyltranspeptidase                                               | KM596622             | 63       | 212    | 6              | 11       | 16            | 24–25                          | P                        |
| PepD: aminocarboxyl-histidine dipeptidase (peptidase D)                         | KM596623             | 53       | 162    | 5              | 10       | 14            | -                              | C                        |
| CpdB: 2',3'-cyclic-nucleotide 2'-phosphodiesterase                             | KM596624             | 72       | 67     | 2              | 2        | 9             | 25–26                          | P                        |
| UshA: bifunctional UDP-sugar hydrolase and 5'-nucleotidase, outer membrane associated lipoprotein | KM596625             | 61       | 1949   | 32             | 50       | 9             | 25–26                          | P/OM                     |
| **Motility/Flagella**                                                           |                      |          |        |                |          |               |                                |                          |
| Putative flagellar hook-length control protein FliK                            | KM596626             | 71       | 1359   | 16             | 31       | 9             | -                              | IM                       |
| Flagellin                                                                       | KM596627             | 40       | 300    | 6              | 17       | 9             | -                              | E                        |
| FlgJ: peptidoglycan hydrolase                                                  | KM596628             | 34       | 650    | 13             | 44       | 9             | -                              | P                        |
| FlgF: Flagellar basal body rod protein                                         | KM596629             | 27       | 147    | 3              | 19       | 9             | -                              | P                        |
| FlgE: Flagellar hook protein                                                   | KM596630             | 47       | 1313   | 20             | 45       | 9             | -                              | OM                       |
| FlgD: Flagellar basal body rod modification protein                            | KM596631             | 25       | 781    | 10             | 55       | 9             | -                              | P                        |
| FlgC: Flagellar component of cell-proximal portion of basal-body rod           | KM596632             | 15       | 656    | 7              | 72       | 9             | -                              | P                        |
| FlgB: Flagellar basal body rod protein                                          | KM596633             | 14       | 500    | 7              | 63       | 9             | -                              | P                        |
| **Extracellular proteins**                                                      |                      |          |        |                |          |               |                                |                          |
| Putative extracellular protease                                                | KM596661             | 38       | 387    | 7              | 29       | 9             | 24–25                          | E                        |
| Putative extracellular lipase                                                   | KM596662             | 90       | 1006   | 16             | 23       | 9             | 22–23                          | E/OM                     |
| Putative autotransporter adhesin/RTX toxin                                      | KM596634             | 136      | 936    | 12             | 11       | 16            | 27–28                          | E/OM                     |
### Table 2. Cont.

| Protein function                        | Genbank accession Nr  | MW (kDa) | Score* | Nr of peptides | Coverage | FPLC fraction | Signal peptide cleavage site** | Predicted localization*** |
|-----------------------------------------|-----------------------|----------|--------|----------------|----------|---------------|--------------------------------|--------------------------|
| **Unknown function**                    |                       |          |        |                |          |               |                                |                          |
| **Lipoproteins**                        |                       |          |        |                |          |               |                                |                          |
| Conserved outer membrane lipoprotein of unknown function | KMS96635             | 12       | 474    | 8              | 56       | 9             | 24–25                          | P/OM                     |
| Conserved outer membrane lipoprotein of unknown function | KMS96667             | 15       | 312    | 4              | 32       | 9             | 27–28                          | P/OM                     |
| Putative outer membrane associated lipoprotein | KMS96636             | 51       | 1626   | 29             | 58       | 15            | -                              | P/OM                     |
| Putative outer membrane associated lipoprotein | KMS96637             | 16       | 121    | 2              | 18       | 9             | 24–25                          | P/OM                     |
| Conserved outer membrane lipoprotein of unknown function | KMS96638             | 14       | 240    | 4              | 35       | 9             | 18–19                          | P/OM                     |
| Conserved outer membrane lipoprotein of unknown function | KMS96663             | 24       | 104    | 1              | 8        | 9             | 22–23                          | P/OM                     |
| Conserved outer membrane lipoprotein of unknown function | KMS96639             | 24       | 66     | 1              | 5        | 9             | 24–25                          | OM                       |
| **Others**                              |                       |          |        |                |          |               |                                |                          |
| Conserved outer membrane protein of unknown function | KMS96640             | 25       | 284    | 3              | 14       | 9             | 22–23                          | OM                       |
| Conserved outer membrane protein of unknown function | KMS96641             | 36       | 86     | 2              | 12       | 9             | 23–24                          | OM                       |
| Conserved outer membrane protein of unknown function | KMS96642             | 84       | 352    | 6              | 11       | 9             | 29–30                          | OM                       |
| Conserved outer membrane protein of unknown function | KMS96664             | 19       | 564    | 6              | 29       | 17            | 23–24                          | OM                       |
| Conserved putative inner membrane protein of unknown function | KMS96643             | 67       | 504    | 10             | 16       | 15            | 23–24                          | IM                       |
| Conserved outer membrane protein of unknown function | KMS96644             | 46       | 407    | 7              | 16       | 9             | 18–19                          | OM                       |
| Conserved protein of unknown function   | KMS96655             | 19       | 68     | 1              | 6        | 9             | -                              | C                        |
| Conserved protein of unknown function   | KMS96666             | 56       | 417    | 6              | 19       | 16            | 21–22                          | OM                       |

* Score given by the MASCOT software, ** as given by the algorithm SignalP 3.0, *** algorithm used for subcellular localization: PsortB or Psort Gram negative bacteria. Lipoproteins localisation were predicted using LipoP1.0 (see details in materials and methods, bioinformatics). (OM: outer membrane, IM: inner membrane, P: periplasmic, E: extracellular, C: cytosolic, U: unknown)

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Finally, it should be noted that, contrary to *V. aestuarianus*, the *V. tapetis* secretome contains one receptor (GbpA) and several chitinases, underscoring the role of chitin as a carbon source in the environment. Besides, chitinases have already been shown to be bacterial virulence factors, eg in *Listeria monocytogenes* [35], and *Legionella pneumophila* [36]. Chitin is also a component of the shell organic matrix, and *V. tapetis* as a pathogen forms biofilms on the inner surface of the shell [37]. Hence, chitin use may be especially relevant to *V. tapetis* pathogenicity.

5. Proteins common to the *V. aestuarianus* and *V. tapetis* secretomes

Finally, the sequence of each secretome protein of a given species was compared *in silico* (using blastP) to the full proteome of the other species, allowing us to identify 21 common proteins. The results are presented in Table 3. The only potential virulence factor is the putative extracellular lipase (Pla1) already mentioned above. The other proteins corresponded to normal components of the envelope in gamma proteobacteria, and/or in the *Vibrio* genus.

### Table 3. Proteins found in both the *V. aestuarianus* and *V. tapetis* secretomes (based on Blast of each secretome against the other).

| *V. tapetis* Genbank accession Nr | Protein name/function | *V. aestuarianus* Genbank accession Nr |
|-----------------------------------|-----------------------|----------------------------------------|
| KM596604                          | Putative TRAP-type transport system | KM588612 |
| KM596594                          | LptD                   | KM588604 |
| KM596600                          | TolC                   | KM588615 |
| KM596589                          | Putative Lipoprotein LpoA, activator of penicillin binding protein 1A | KM588605 |
| KM596596                          | TorA                   | KM588608 |
| KM596586                          | Tsp                    | KM588600 |
| KM596603                          | AapJ                   | KM588611 |
| KM596585                          | Periplasmic component of the Tol-Pal system, YbgF-like | KM588602 |
| KM596635                          | Conserved lipoprotein of unknown function | KM588621 |
| KM596592                          | Nlp                    | KM588607 |
| KM596582                          | OmpU                   | KM588597 |
| KM596638                          | Conserved lipoprotein of unknown function | KM588619 |
| KM596583                          | BamA (YaeT)            | KM588599 |
| KM596640                          | Conserved outer membrane protein of unknown function | KM588626 |
| KM596642                          | Putative outer membrane protein of unknown function | KM588625 |
| KM596650                          | LamB                   | KM588632 |
| KM596647                          | Lpp                    | KM588630 |
| KM596662                          | Putative extracellular lipase | KM588638 |
| KM596652                          | Long-chain fatty acid outer membrane porin FadL | KM588616 |
| KM596646                          | OmpA                   | KM588598 |
| KM596641                          | Conserved outer membrane protein of unknown function | KM588628 |

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### Conclusion

Extracellular products, especially secreted proteins, enter in direct contact with the host cells, and play a major role in the virulence of pathogenic bacteria. As a consequence, secretomic approaches are of particular relevance to identify the proteins involved in the infection process, and several studies have been carried out for different pathogens in recent years [38–41]. It should be noted that to date, only two secretomes of vibrios have been reported in the literature, i.e. those of *V. coralliilyticus* [20] and *V. cholerae* [42].

In this paper, we characterized the extracellular proteome of *V. aestuarianus* and *V. tapetis*, two vibrio species pathogenic to mollusks, as a first step towards the identification of new potential virulence factors. Although the extracellular products from both species were shown to be involved in bacterial virulence, only one extracellular virulence factor has been characterized to date, in the case of *V. aestuarianus*, the Vam zinc metalloprotease [11].

This protein appeared as a major component of the *V. aestuarianus* secretome. However, we showed that a metalloprotease-free fraction (fraction 8) also displayed biological activity to hemocytes, thus suggesting the occurrence of other potential virulence factors in this species.
As the V. tapetis secretome does not contain any metalloprotease, the virulence mechanisms in this species are necessarily different from those in V. aestuarianus. In addition, we showed here that several chromatographic fractions of ECPs displayed biological activity towards oyster- and clam-hemocytes, for V. aestuarianus and V. tapetis, respectively, indicating that other factors are also responsible for the biological effects on hemocytes. Overall, we could identify 44 and 87 different proteins in the active fractions of V. aestuarianus and V. tapetis secretomes. Our data constitute the first valuable resource to further investigate the virulence factors of these two marine pathogen vibrios. Future works will aim at assessing the actual role of specific secreted proteins in the virulence.

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Author Contributions

Conceived and designed the experiments: SM VP CB CP JLN. Performed the experiments: SM VP MP. Analyzed the data: SM VP AJ FG CP. Wrote the paper: SM VP CB CP JLN. Contributed reagents/materials/analysis tools: VP CB CP JLN. Contributed to the writing of the manuscript: SM VP AJ JLN.
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