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ORIGINAL ARTICLE

Genomic and proteomic analyses of the coral pathogen *Vibrio coralliilyticus* reveal a diverse virulence repertoire

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Vibrio coralliilyticus **has been implicated as an important pathogen of coral species worldwide. In this study, the nearly complete genome of *Vibrio coralliilyticus* strain P1 (LMG23696) was sequenced and proteases implicated in virulence of the strain were specifically investigated. The genome sequence of P1 (5 513 256 bp in size) consisted of 5222 coding sequences and 58 RNA genes (53 tRNAs and at least 5 rRNAs). Seventeen metalloprotease and effector (*vgrG, hlyA* and *hcp*) genes were identified in the genome and expressed proteases were also detected in the secretome of P1. As the VcpA zinc-metalloprotease has been considered an important virulence factor of *V. coralliilyticus,* a *vcpA* deletion mutant was constructed to evaluate the effect of this gene in animal pathogenesis. Both wild-type and mutant (*ΔvcpA*) strains exhibited similar virulence characteristics that resulted in high mortality in *Artemia* and *Drosophila* pathogenicity bioassays and strong photosystem II inactivation of the coral dinoflagellate endosymbiont (*Symbiodinium*). In contrast, the *ΔvcpA* mutant demonstrated higher hemolytic activity and secreted 18 proteins not secreted by the wild type. These proteins included four types of metalloproteases, a chitinase, a hemolysin-related protein RbmC, the Hcp protein and 12 hypothetical proteins. Overall, the results of this study indicate that *V. coralliilyticus* strain P1 has a diverse virulence repertoire that possibly enables this bacterium to be an efficient animal pathogen.**

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Introduction

Coral reefs are hotspots of productivity and biodiversity and therefore represent one of the most important marine biomes (Knowlton and Jackson, 2008; Kiessling *et al.*, 2010). Reefs also sustain significant fisheries worldwide, providing an important economic and food resource for local communities (Hughes *et al.*, 2003). The health of coral reefs are in decline, with recent assessments estimating that ~one-third of global reefs have been effectively destroyed or are under imminent threat of collapse (Wilkinson, 2008). The combined effects of global changes (for example, increase in sea water temperature) and local anthropogenic stressors (for example, water pollution, overfishing) have been implicated as contributing factors to the dramatic decline of coral reefs in recent decades (Rohwer and Youle, 2010). Coral diseases have also been implicated in the loss of reef habitat, especially in the Caribbean where diseases have fundamentally altered reef ecosystems (Sutherland *et al.*, 2004). Bacterial pathogens have been identified as the causative microbial agent of some coral diseases (Bourne *et al.*, 2009). For example, Rosenberg and colleagues demonstrated that the temperature-dependent necrosis of *Pocillopora damicornis* coral tissue and its zooxanthellae was caused by the bacterium *Vibrio coralliilyticus* (Ben-Haim *et al.*, 2003a). Infection occurred predominantly at high seawater temperatures (27–29 °C) and a single putative metalloprotease was identified as the key

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toxin in this disease process (Ben-Haim *et al.*, 2003b). At lower temperatures, this bacterium did not produce several putative toxins, most notably, a zinc-metalloprotease (Ben-Haim *et al.*, 2003b).

Investigations of white syndrome coral disease outbreaks in the Indo-Pacific between 2003 and 2006 isolated a number *V. coralliilyticus* strains from different coral species (*Pachyseris speciosa*, *Montipora aequituberculata* and *Acropora cytherea*) and identified them as the potential causative agents (Sussman *et al.*, 2008). The extracellular products derived from the *V. coralliilyticus* strain P1 (LMG23696) isolated from diseased *Montipora aequituberculata* colonies in Nelly Bay (Great Barrier Reef), caused photoinactivation of the *Symbiodiunium* (zooxanthellae) endosymbiont (Sussman *et al.*, 2008). Analysis of the extracellular products from strain P1 revealed a predominant zinc-metalloprotease that was found to be a homolog of the *Vibrio cholerae* hemagglutinin-protease (Sussman *et al.*, 2008). When purified from the other extracellular products, this zinc-metalloprotease was demonstrated to cause coral tissue damage within 18 h at 27 °C (Sussman *et al.*, 2009). Metalloproteases have several catalytic activities related to virulence but little is known about the diversity of these potential virulence factors in coral pathogenic bacteria. In the bivalve pathogen *V. splendidus*, the deletion of the Vsm metalloprotease resulted in the expression of other metalloproteases that were found to compensate for some of the functions fulfilled by the *vsm* gene product (Binesse *et al.*, 2008). In the fish pathogen *V. anguillarum*, mutants lacking a functional EmpA metalloprotease expressed two different proteases that were not detected in the wild-type strain suggesting that these proteases might compensate for the loss of EmpA (Milton *et al.*, 1992; Varina *et al.*, 2008). Similarly, a metalloprotease mutant of *Vibrio vulnificus* was found to express a serine protease in response that demonstrated comparable levels of proteolytic activity to the wild-type strain (Wang *et al.*, 2008).

Recent work has shown that vibrios, including *V. coralliilyticus*, are ubiquitous in coral mucus (Chimetto *et al.*, 2008; Alves *et al.,* 2010) and often dominate the microbial community of diseased corals (Bourne and Munn, 2005; Luna *et al.*, 2007). A similar pattern is observed at the whole reef scale as vibrios have been demonstrated to have much higher loads on unhealthy vs healthy coral reefs (Dinsdale *et al.*, 2008). Vega Thurber *et al.* (2008) have demonstrated that even when vibrios are at low-abundance on stressed corals, they were found to significantly alter the coral microbiome metabolism. Such studies suggest that a better understanding of the pathogenic potential of vibrios isolated from corals is required. In the present study, we sequenced the whole genome of the *V. coralliilyticus* strain P1 (LMG23696) with a specific focus on the diversity of metalloproteases in the genome, which can act as key virulence factors in the infection of corals. The expression of the collection of proteases from strain P1 was analyzed by protein and enzyme characterization assays. A mutant P1 strain (Δ*vcpA*) was generated to test if the zinc-metalloprotease VcpA is an essential virulence factor for *V. coralliilyticus* pathogenesis. The pathogenic potential of both the wild-type and mutant strains was evaluated in three model bioassays (*Artemia*, *Drosophila* and *Symbiodinium*) to test whether a single zinc metalloprotease (VcpA) is essential for the disease process. The comparative analyses of both mutant and wild-type strains enabled us to improve our current understanding of the role of VcpA in *V. coralliilyticus* animal pathogenesis.

Materials and methods

Bacterial strains, plasmids and media

The bacterial strains and plasmids used in this study are described in Supplementary Table S1. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium at 37 °C. *V. coralliilyticus* strains were grown at 28 °C in LB medium (Difco, Sparks, NV, USA), tryptone soy broth (Difco) or marine broth (Difco) supplemented with 0.5 M NaCl for the mutagenesis assays. Spectinomycin (100 mg l^{-1}), thymidine (0.3 mM) and diaminopimelic acid (DAP) (0.3 mM) were added as supplements when necessary. Induction of *ccdB* expression under the control of the PBAD promoter was achieved by the addition of 0.2% L-arabinose to the growth medium, and conversely, this activity was repressed by the addition of 1% D-glucose.

Vibrio coralliilyticus *P1 genome sequencing and analysis*

The genome was sequenced by the Roche-454 pyrosequencing method according to Margulies *et al.* (2005) on the Genome Sequencer FLX system (Roche 454, Indianapolis, IN, USA). The high quality DNA extraction was performed as Pitcher *et al.*, 1989. The genome contigs and scaffolds were assembled with the GS *De Novo* Assembler, using the default setting (http://454.com/products-solutions/analysis-tools/gs-de-novo-assembler.asp) and resulted in ~12.3× coverage. An automated annotation was then performed using RAST (Rapid Annotation using Subsystem Technology) (Aziz *et al.*, 2008). The Blast atlas plots were constructed as described previously (Pedersen *et al.*, 2000; Hallin *et al.*, 2008). For building the Atlas, the genomes were automatically annotated and compared against the *V. cholerae* strain N16961 reference genome. The BLAST matrix perl script performed an all-against-all BLAST comparison of genomes from multiple organisms. For every combination, a protein BLAST was carried to identify all homologous proteins. For our purposes, we used the '50-50 rule', which requires the following

criteria: (1) at least 50% of the query protein must overlap in the alignment, and (2) at least 50% of the residues within the alignment must be identical. The genome sequence is available in Genbank (This Whole Genome Shotgun project is deposited at DDBJ/EMBL/GenBank under the accession AEQS00000000. The version described in this paper is the first version, AEQS01000000) and RAST.

Vector construction for the vcpA *deletion*

The *ΔvcpA* mutant was constructed by allelic exchange using the suicide vector pSW4426T described previously (Le Roux *et al.*, 2007). Alleles carrying an internal deletion were generated *in vitro* using a two-step PCR construction method (Binesse *et al.*, 2008). Using genomic DNA of the strain P1, two independent PCR amplifications of 500 bp regions encompassing *vcpA* (GeneBank accession number GQ452012) were performed using primer pairs: C1: 5′-gcccgaattcATGAAACAACGTCAAATG CTTTGGC-3′ and C2: 5′-CAAAACCTTTACGTACAT CCCAACGCAACAAAAAAGTCTACCATGTAAACG-3′; or C3: 5′-CGTTTACATGGTAGACTTTTTTGTTGCG TTGGGATGTACGTAAAGGTTTTG-3′ and C4: 5′-gcc cgaattcTTAGTCTAATCTTAGTGTCACGC-3′ (Lower nucleotides are EcoRI enzyme sites). After gel purification, 100 ng of the two PCR products were mixed and a final PCR amplification was carried out using the external primer pairs C1 and C4. The PCR product was referred to as the *ΔvcpA* allele and was *Eco*RI digested and ligated in an *Eco*RI linearized pSW4426T plasmid. The construct named pSWΔvcpAT was transferred to a chemiocompetent *E. coli* p3813 strain, and subsequently to *E. coli* β3914 donor cells. The conjugation experiments with *V. coralliilyticus* strain P1 were performed according to the filter-mating procedure using a donor/recipient ratio of 1/10 as previously described (Le Roux *et al.*, 2007). Selection against the *ΔdapA* donor *E. coli* β3914 was achieved by plating onto LB NaCl medium devoid of diaminopimelate (DAP), and supplemented with 1% glucose and 100 mg l^{-1} spectinomycin. Antibiotic-resistant colonies were grown in LB NaCl medium and spread on plates containing 0.2% arabinose. Mutants were screened by PCR amplification using the primer pair C1 and C4, amplifying a 1.8 kb- and a 1 kb bp-fragment in the wild-type and *ΔvcpA* strain, respectively. Sequencing through the *vcpA* gene locus confirmed its deletion in the *ΔvcpA* strain.

Pathogenicity tests

Two animal model assays (*Artemia* and *Drosophila*) and one whole cell assay consisting of the coral *Symbiodinium* endosymbiont (zooxanthellae) were used to test the pathogenicity of the wild-type and mutant *V. coralliilyticus* strains. *Drosophila* assays were performed as described by Alves *et al.* (2010). In brief, 10 wild-type Canton-S adult flies were placed in vials containing a cotton plug saturated with tryptone soy broth either alone or inoculated with 10^8 colony-forming units per ml of *V. coralliilyticus* strain P1 wild-type or mutant cells (Blow *et al.*, 2005). Each treatment in the *Drosophila* experiment was performed with five replicates at 24 °C. The *Artemia* bioassay was carried as described previously (Austin *et al.*, 2005). Briefly, 30 *Artemia* nauplii were placed in a sterile vial with *Vibrio coralliilyticus* wild-type or mutant (10^8 colony-forming units per ml) cells added in 20 ml sterile seawater. Each treatment of the *Artemia* experiment was performed in triplicate. Before use, *Artemia* cysts were pre-treated with sodium hypochlorite (2%) followed by several washes using sterile seawater. Cyst hatching was performed by incubation in sterile seawater with filtered aeration and light at 24 °C. *Drosophila* and *Artemia* survival were determined daily and the *Drosophila* and *Artemia* experiments were repeated three times.

Symbiodinium sp. type C1 was isolated from *Acropora tenuis* (Nelly Bay, Magnetic Island, Great Barrier Reef) tissue (15–20 cm in diameter) by air brushing and sequential washes (5 min at 1600 *g*) in 0.2 μm filtered seawater. Clean symbiont preparations were inoculated into sterile IMK (Wako Chemicals USA, Richmond, VA, USA) with antibiotics (penicillin, neomicin, streptomicin, nystatin, final (100 μg ml^{-1}) each, amphotericin final (2.5 μg ml^{-1}); plus GeO2 final (50 μM)) (Ishikura *et al.*, 2004). Cultures were maintained at 28 °C and 35 μmol quanta m^{-2} s^{-1}, under a 12:12 h light:-dark photoperiod. Cultures were genotyped by SSCP of the ITS1 region (van Oppen *et al.*, 2001). Growth was monitored periodically by hemocytometry to estimate log phase time. Before the protease challenge assay, cell density was recorded and adjusted to 1×10^6 cells per ml^{-1} in sterile filtered seawater. Symbionts were pipetted into 24-well plates and dark adapted for 20 min before the pulse-amplitude-modulated fluorescence readings.

Vibrio coralliilyticus strain P1 was grown in Luria-Bertani broth with 2% NaCl at 28 °C and 200 r.p.m. for 18 h. Absorbance (600 nm) of culture dilutions were measured in sterile microtitre 96-well plates using a Wallac Victor 2 1420 multi label counter spectrophotometer (Perkin Elmer, Waltham, MA, USA) and normalized to 1×10^9 cells per ml. Supernatants were obtained by centrifugation ($4750 \times g$, 10 min, 4 °C) and serial filtration through 0.45 mm and 0.22 μm filters (Millipore, Billerica, MA, USA) and proteolytic activity of the supernatants was measured by azocasein assay (Denkin and Nelson, 1999). Supernatants (1 ml) from the wild-type and mutant (three replicates per treatment) were added to *Symbiodinium* cultures in the wells and mixed by gentle pipetting. Control treatments comprised of sterile LB broth with 2% NaCl. Samples were maintained in the dark at 28 °C for the duration of the experiment. PS II

dark-adapted quantum yields (Fv/Fm) were measured by exposing the plates in a Maxi imaging-pulse amplitude-modulation (iPAM) fluorometer (Walz, Effeltrich, Germany) to a saturation light pulse.

V. coralliilyticus *strain P1 protease activity*

V. coralliilyticus P1 wild-type and mutant strains were grown in tryptone soy broth NaCl at 28 °C for 18 h. Extracellular protease activity was evaluated via 10% SDS-polyacrylamide gel electrophoresis containing 0.1% gelatin incorporated as substrate (Heussen and Dowdle, 1980). Fifty ml of crude extracellular products were harvested from the *V. coralliilyticus* cultures and residual cells were removed by filtration (0.2 μm). The extracellular product proteins were concentrated 50-fold by dialysis (cut off 9 kDa) against polyethylene glycol 6000 for 24 h at 4 °C. Aliquots of 10–20 μl of the concentrated extracellular product were applied on a gelatin SDS-polyacrylamide gel electrophoresis gel. Inhibition tests were performed by adding protease inhibitors (EDTA at 20 mM; phenantrolin at 10 mM; phenylmethylsulfonyl fluoride at 1 mM; E-64 at 10 μM and pepstatin at 10 μM) to the samples before electrophoresis. After electrophoresis, the gel was soaked in 2.5% Triton X-100 for 2 h at room temperature, incubated overnight at 37 °C in 50 mM Tris-HCl (pH 7.5), fixed, stained with 0.4% Coomassie brilliant blue R-250 in methanol–acetic acid (30:10) and destained in the same solvent. The gels were dried, scanned and digitally processed.

Enzymatic properties of V. coralliilyticus *P1*

Bacterial cultures were tested for caseinase, phospholipase and hemolytic enzyme activity as described previously by Austin *et al*. (2005). Caseinase activity was observed on skimmed milk agar plates, which were prepared with tryptone soya agar mixed with an equal volume of 4% sterile skimmed milk (Oxoid, Cambridge, UK). A positive response was recorded as the presence of clear zones around the bacterial colonies after incubation at 24 °C for up to 5 days. Gelatinase activity was tested as previously described by Loghothetis and Austin (1996). Overnight cultures were spotted onto tryptone soya agar supplemented with 0.5% gelatin (Oxoid). After incubation at 24 °C for up to 7 days, the presence of zones of clearing around the colonies were recorded as a positive phenotype. Phospholipase activity was examined as previously described (Liu *et al.*, 1996). Overnight cultures were inoculated on tryptone soya agar supplemented with 1% egg yolk emulsion (Oxoid) with incubation at room temperature for up to 7 days. The development of opalescence around the bacterial colonies was recorded. Hemolysis was recorded using sheep blood agar (Probac, Sao Paulo, Brazil) and incubation at 24 °C for up to 4 days. Enzymatic activities were tested three times.

Secretome analyses

Secreted proteins were extracted from P1 cultures grown on tryptone soy broth and marine broth. Three replicate P1 cultures (500 ml, $OD_{550\,nm}$ 1.1) were used for protein extraction. Secreted proteins were precipitated using ammonium sulfate at a final concentration of 70% on ice. Precipitated proteins were dialyzed on dialysis tubing cellulose (33 × 21 mm; Sigma-Aldrich, St Louis, MO, USA) in 2 mM Tris-HCl solution, pH 8.0, for 16 h. Protein purification was carried out by using trichloroacetic acid at 20% followed by acetone washes. Protein quantification was performed using the Bradford methodology (Invitrogen, Carlsbad, CA, USA). To establish a 2D proteomic map of the *V. coralliilyticus* wild-type and mutant secretomes, isoelectric focusing was performed in an IPGphor III system (GE Healthcare Biosciences AB, Uppsala, Sweden). To determine the reproducibility of the 2D proteomic maps, three replicate analyses were performed beginning with the extraction from new cultures of both the wild-type and mutant. A total of 200 μg of protein and 125 μl rehydration solution (8 M urea, 2% CHAPS, 0.5% pharmalyte, 18 mM dithiothreitol, 0.002% bromo phenol blue) were added to a strip holder containing immobilized pH gradient strips with 7 cm and a pI range between 4 and 7. Isoelectric focusing was performed at 60 V for 12 h using an IPGPhor system (GE Healthcare Biosciences AB). Subsequently, proteins were treated with dithiothreitol (64 mM) and IAA (135 mM) for reduction and alkylation.

One-dimensional electrophoresis was performed to separate and identify the main secreted proteins of the *V. coralliilyticus* P1 wild-type and mutant strains. A total of 50 μg of dialyzed proteins were used in the One-dimensional electrophoresis gels. SDS-polyacrylamide gel electrophoresis was performed on a 12% polyacrylamide gel in a Ruby SE600 electrophoresis system (GE Healthcare Biosciences AB). All the bands were excised from three replicate gels as described previously (Hellman *et al.*, 1995; Coelho *et al.*, 2004). Sequencing grade porcine trypsin (Promega, Madison, WI, USA) was used for protein digestion. The reduction and alkylation were done just before digestion by incubation in 50 mM Tris solution (pH 7.4) plus dithiothreitol (64 mM) and then Tris plus IAA (135 mM) for 30 min each.

Protein identification was performed by liquid chromatography tandem mass spectrometry (LC-MS/MS) using a Q-TOF Ultima API mass spectrometer (Micromass, Manchester, UK) to analyze tryptic peptides according to Lery *et al*. (2008). A nanoflow ESI source was used with a lock spray source for lock mass measurements during all the chromatographic runs. Samples of ~0.5 mg of digested proteins were desalted using a Waters Opti-Pak C18 trap column (Waters, Milford, MA, USA). The mixture of trapped peptides was then separated by elution with a water/ACN 0.1% formic

acid gradient through a Nanoease C18 (75-mm id) capillary column. Data were acquired in data-dependent mode, and multiple charged peptides ions (+2 and +3) were automatically mass selected and dissociated in MS/MS experiments. Typical LC and ESI conditions were a 200 nl per min flow, a 3 kV nanoflow capillary voltage and a 100 V cone voltage. The MS/MS spectra were processed using the ProteinLynx 2.0 software (Waters) and the process (.pkl) file generated was submitted for identification against the NCBI database using MASCOT software with a mass accuracy better than 0.1 Da (Matrix Science Home, London, UK). Three independent gels were constructed. Identified proteins had a Mowse Score higher than 53 and identifications were confirmed at least three times.

Results

The Vibrio coralliilyticus *strain P1 genome*

Pyrosequencing and assembly of the P1 strain resulted in the generation of a draft genome sequence that contains a total of 229 contigs (5 513 256 bp in size and 46% GC). The genome was annotated using the RAST annotation system and the quality check using the RAST service was successfully completed. There were 5222 coding DNA sequences and 58 RNA genes (53 tRNAs and at least 5 rRNAs), representing one of the largest vibrio genomes sequenced to date. Because the genome is not closed, it is possible that some rRNA operons have been missed in the assembly. Around 40% of the coding DNA sequences (2082 open reading frames) were distributed in 340 different subsystems according to RAST. The remaining 3140 open reading frames (1629 of which were hypothetical open reading frames) did not belong to any of the known RAST subsystems.

Of the subsystem partitioned genes, most belonged to basic metabolic processes such as the metabolism of carbohydrates, amino acids and derivatives, nucleosides and nucleotides, proteins, vitamins and cofactors. *V. coralliilyticus* has putative genes for complete glycolysis and Entner–Doudoroff pathways, complete tricarboxylic acid and reductive pentose phosphate cycles and butanoate, propanoate and folate metabolism. Genes-encoding enzymes associated with nitrogen metabolism (for example, ammonia assimilation (*glnA*) and denitrification) were also identified. There were predicted genes for the synthesis of most essential amino acids and for the utilization of chitobiose, *N*-acetylglucosamine, maltose, cellobiose, galactose, fructose and mannose. Complete sec pathways and large numbers of ATP-binding cassette transporters were also found.

The RAST annotation of *V. coralliilyticus* strain P1 also identified a variety of genes belonging to the virulence subsystem. The genome harbors several quorum sensing and biofilm production-related genes, including the *hapR* and *luxT* regulators, the *lux* and *las* genes for autoinducer-2 (AI-2), the polysaccharide biosynthesis set (*vsp, esp, rbm* genes and others) and MSHA type IV pilus biogenesis genes. The *syp* genes that are associated with symbiotic colonization were also found. Genes belonging to the type IV secretion system (the *flp* pilin and *tad* genes) and the type VI secretion system (virulence-associated secretion (VAS)) were detected, as well as the *tfoX* gene and the *hcp* and *vgrG* genes encoding the effector hemolysin-corregulated proteins. Several auxiliary colonization factors (*acfABCD*) and *tagE* were also found in the genome. Genes-encoding proteins involved in iron uptake (transport systems Ton and Tol, Fur repressor gene) and in particular heme receptors and transporters were found. In addition, several stress response factors related to the oxidative stress response, such as glutaredoxins, superoxide dismutases, catalase, peroxidase, stress response systems via glutathione biosynthesis and the central regulators involved in the stress response were also detected in the genome. Flagellar components (*fla* and *flg*) and methyl-accepting chemotaxis proteins I-IV and other chemotaxis systems (*che* and *fli* genes and *cheY* regulator) were also present.

Importantly, several virulence genes were not allocated to any known subsystem according to RAST. This is the case for some hemolysins (the pore forming toxin *hlyA*), proteins of the hemolysin family (the hemolysin-related protein RbmC), a putative *rtx* toxin and Rtx transporters, the toxin co-regulated pilus (*tcp*) biogenesis genes, the *toxT* gene, three copies of *toxR* regulators, the type III secretion system, zona occludens toxin (*zot*), accessory cholera enterotoxin (*ace*), attachment to host cells and virulence (*attT* protein), cytolysin precursor, and the cytolysin secretion protein.

The Blast atlas comparative genomic analysis illustrated the relative similarity in gene content among *V. coralliilyticus* strain P1 and other vibrios (Figure 1). There was a clear conservation of gene content between P1, the type strain of *V. coralliilyticus* (ATCC BAA-450 = LMG20984T), *V. splendidus* LGP32 and *V. vulnificus* CMCP6, *V. parahaemolyticus* RIMD, *V. campbellii* BAA-1116 and the reference strain *V. cholerae* N16961. There were many areas corresponding to conserved genes in the genomes (denoted by strong colors). Four zones (R1-R4), which denoted highly conserved regions, were observed on the Blast Atlas, including genes of the replication, transcription and transduction processes and some flagellar components and transporters (Supplementary Table S2). Some regions (light colored gaps) indicated low conservation in gene content between the reference strain N16961 and all other vibrio strains. There were several differences particularly in chromosome II, including the hypervariable superintegron region (midpoint 375 Kb) that had high values of global direct repeats and percentage AT content (Figure 1b). A BLASTP

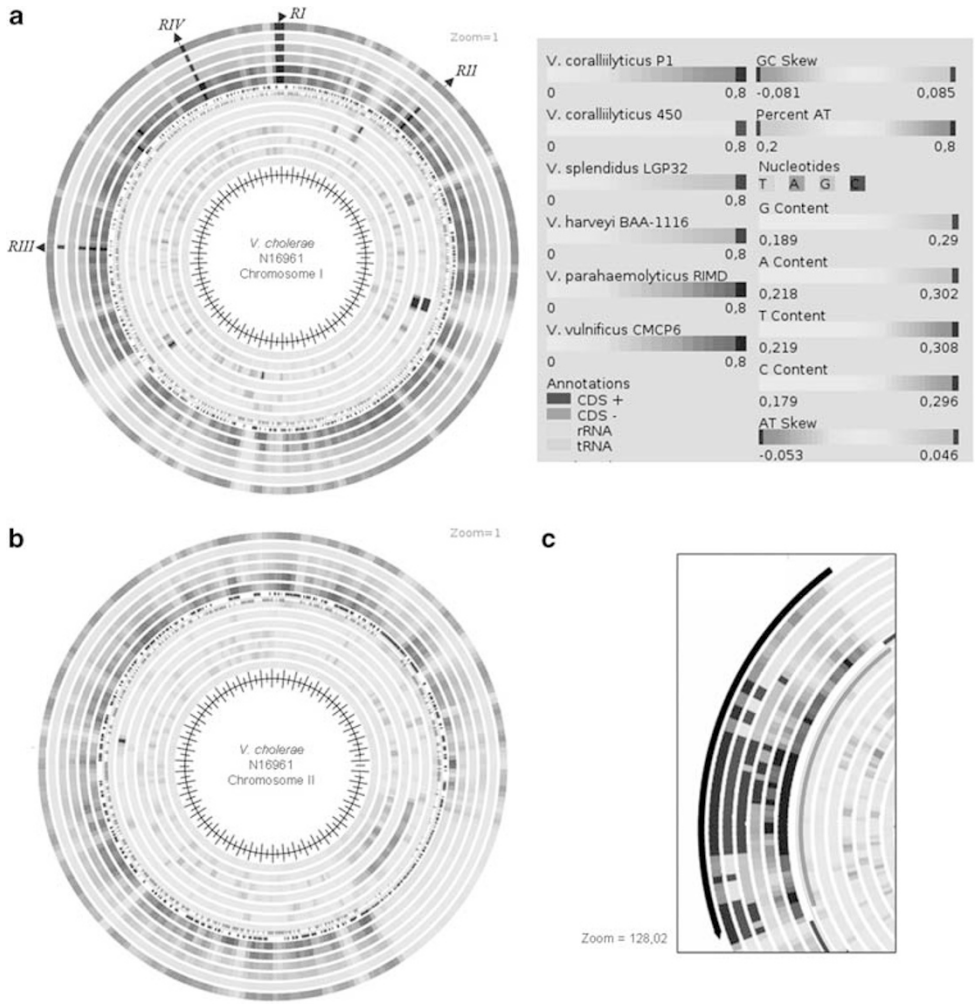

Figure 1 Blast atlas of the *V. coralliilyticus* P1 genome. General visualization of differences in gene content in chromosome I (**a**) and chromosome II (**b**) using *V. cholerae* N16961 as the reference genome. The enlarged region (**c**) depicts the *vcpA* gene locus (inner red circle) and its putative homologs. Light colored regions indicate low conservation in gene content, whereas dark colored regions indicate high conservation.

analysis between the *Vibrio coralliilyticus* LMG20984T type strain (5344 Mb, 5,222 CDS and 122 RNAs) and *V. coralliilyticus* strain P1 revealed 4,509 shared predicted coding genes (>70% AA identity) and 462 strain-specific genes on the P1 genome (114 functional proteins and 348 hypothetical proteins). Among the strain-specific genes of strain P1 were several phage genes, membrane proteins (including transporters), regulators, a type IV restriction endonuclease Mrr and putative effectors, the accessory colonization factor AcfD precursor, the internalin-like protein, lyzozymes and toxin-antitoxin system proteins Doc-Phd (Supplementary Table S3).

Metalloprotease diversity

A search for metalloprotease genes within the derived genome data identified 17 metalloproteases, zinc proteases or putative proteases (Table 1). They were dispersed in different contigs and most were inserted in regions with low conservation among the compared vibrio genomes. With respect to putative localization, some of the proteases were predicted to be in the membrane, others in the cytoplasm and nine were secreted. Among these genes, there were three metalloprotease genes from the M4 peptidase family. This family comprises metalloproteases described as virulence factors, including the *V. cholerae* hemagglutinin/proteases and thermolysins, which were orthologous to the *vtpA, vtpB* and *vthA* genes from *V. tubiashii* (*V. coralliilyticus* nomenclature—*vcpA*, *vcpB* and *vchA*, respectively). Other proteases were found to belong to peptidase families such as the U32 collagenase group, the M6 metalloprotease, which is a homolog to the immune inhibitor A found in *V. cholerae*, the M50 membrane metalloproteases and the M48 Zn-dependent proteases with chaperone function (Table 1). This diverse metalloprotease set and the other virulence-related effectors found in the genome indicate a high diversity of pathogenic systems in

Table 1 Putative metalloprotease genes found in the genome of *V. coralliilyticus* strain P1

Gene description	*Peptidase/ domain family*[a]	*Protein characterization*[b]	*ID*[c]
Putative protease	U32	Collagenase	fig|6666666.585.peg.241
Vibriolysin, extracellular zinc protease (*vcp*A)[d]	M4	Hemagglutinin/protease	fig|6666666.585.peg.383
Vibriolysin, extracellular zinc protease (*vcpB*)	M4	Zinc metalloprotease, thermolysin	fig|6666666.585.peg.493
Bacterial leucyl aminopeptidase	M20	Aminopeptidase	fig|6666666.585.peg.494
Putative protease	U32	Collagenase	fig|6666666.585.peg.638
Putative protease	U32	Collagenase	fig|6666666.585.peg.639
Metalloprotease, putative zinc-binding domain	M6	Secreted metalloprotease	fig|6666666.585.peg.2348
Zinc protease	M16	Zinc protease	fig|6666666.585.peg.2982
Membrane-associated zinc metalloprotease	M50	Metalloprotease	fig|6666666.585.peg.3042
Bacterial leucyl aminopeptidase	M20	Aminopeptidase	fig|6666666.585.peg.3342
Zinc metalloprotease[e]	PCC domain[e]	Leucine aminopeptidase-related protein	fig|6666666.585.peg.3343
Vibriolysin, extracellular zinc protease (*vchA*)	M4	Hemagglutinin/protease	fig|6666666.585.peg.3360
Exported zinc metalloprotease YfgC precursor	M48	Zinc metalloprotease	fig|6666666.585.peg.3932
Zinc-dependent protease with chaperone function	M48	Zinc-dependent protease with chaperone function	fig|6666666.585.peg.4797
Zinc-dependent protease	M50	Membrane metalloprotease	fig|6666666.585.peg.4798
Inactive homolog of metal-dependent proteases, putative molecular chaperone	M22	Glycoprotease	fig|6666666.585.peg.4911
Zinc-dependent protease with chaperone function	M48	Zinc-dependent protease with chaperone function	fig|6666666.585.peg.4997

[a]Peptidase or domain families following BLAST/NCBI results.
[b]Protease characterization according to descriptions from orthologous proteins.
[c]Gene identification on *V. coralliilyticus* strain P1 genome according to RAST.
[d]The *vcpA* gene that was deleted to generate the Δ*vcpA* mutant.
[e]Indicates a protease not classified in a peptidase family with only the signal for secretion PCC domain.

V. coralliilyticus P1. The comparison between the *vcpA* of P1 and its orthologs in other vibrio genomes revealed some interesting features (Figure 1c; Supplementary Figure S1). The two strains of *V. coralliilyticus* (P1 = LMG23696 and ATCC BAA-450 = LMG20984T), *V. splendidus* LGP32 and *V. vulnificus* CMCP6 had a central region of high conservation. On the other hand, the orthologous genes from *V. parahaemolyticus* RIMD and *V. campbellii* BAA-1116 had a distinct central region. On alignment analysis, the *vcpA* sequence was identical to the *vtpA* metalloprotease, the major virulence factor of *V. tubiashii* pathogenesis.

V. coralliilyticus *P1* vcpA *zinc-metalloprotease mutant*

The *V. coralliilyticus* P1 strain possessed a hemagglutinin/protease gene, designated as *vcpA*, similar to a major virulence factor of several vibrios, including *V. tubiashii*, *V. splendidus* and *V. cholerae* (Supplementary Figures S1; Supplementary Online Material). A *vcpA* deletion mutant was constructed using the allelic exchange strategy (see Materials and methods). The PCR amplicon size of the *vcpA* gene was ~1800 bp in the restored wild type and 1000 bp in the mutants obtained by allelic exchange, indicating the internal deletion of the target gene. Approximately 50% of the colonies were positive for the allelic exchange. Sequencing of the mutants and wild-type *vcpA* genetic locus showed an internal deletion only in the mutant. The pathogenic potential, enzymatic activities and secretome of one selected mutant, named Δ*vcpA*, and the wild-type P1 strain was evaluated.

Assessment of strain pathogenicity

The pathogenic potential of the *V. coralliilyticus* P1 wild-type and Δ*vcpA* strains as tested in two animal assays (*Drosophila* and *Artemia)*, were indistinguishable as judged by the mortality of the animals (Figure 2). Both the wild-type and mutant strains killed ~60% of the *Drosophila* and 40% of the *Artemia* after 48 h (Figure 2). The *Drosophila* and *Artemia* mortality rates in the control treatments were ~10% after 48 h.

Pathogenicity assays performed on coral-derived *Symbiodinium* Clade C1 cultures also indicated that both the wild-type and the mutant strain of *V. coralliilyticus* P1 had the same pathogenic potential (Figure 3). Both strains caused a similar level of photoinactivation of Photosystem II (PS II) of the *Symbiodinium* cultures in the 8 h following inoculation. Total (100%) photoinactivation was measured after 4 days. In contrast, *Symbiodinium* cultures in the control treatments remained unaffected, showing only a 1.9% average decrease in PS II dark-adapted quantum yields after 4 days.

Enzymatic properties of V. coralliilyticus *P1 wild type and mutant*

The wild-type *V. coralliilyticus* P1 strain had higher caseinase, gelatinase and phospholipase activities,

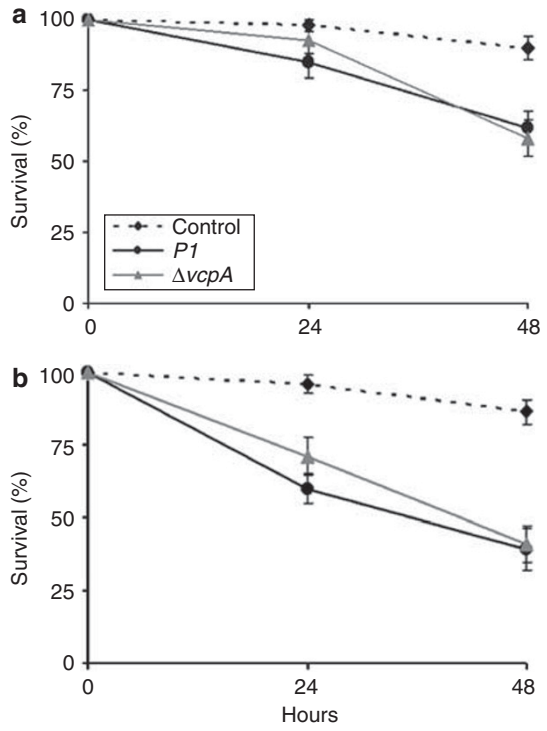

Figure 2 Pathogenic potential of the *V. coralliilyticus* wild-type and Δ*vcpA* strains. Pathogenicity tests using (**a**) *Drosophila* and (**b**) *Artemia nauplii*. The error bars indicate the mean ± s.d. The control treatments do not contain bacteria.

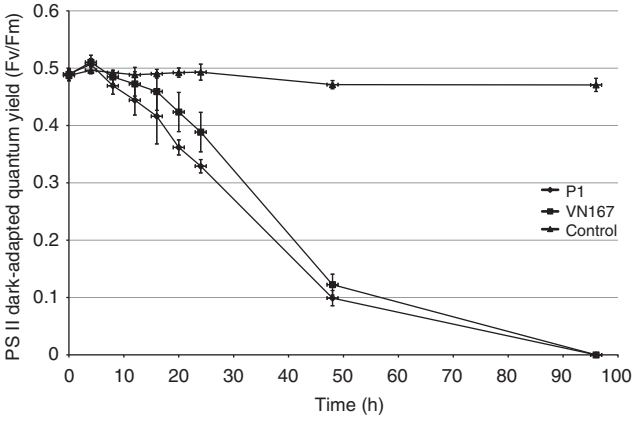

Figure 3 Photosystem II inactivation of *Symbiodinium* Clade C1 cultures by *V. coralliilyticus* wild-type and Δ*vcpA* strain supernatants. The error bars indicate the mean ± s.d.

whereas the Δ*vcpA* mutant had higher hemolysin activity (Table 2). The distinct phenotypes (decreased production of phospholipase and increased production of hemolysin) point to a change in the enzymatic expression in the mutant in response to the *vcpA* deletion.

The proteolytic activity of the secretome of the mutant and the wild type were analyzed by gelatin SDS-polyacrylamide gel electrophoresis (Supplementary Figure S2). The extracellular protease fingerprint of the P1 wild type revealed seven major proteases with a molecular mass range between 20 and 105 kDa. These bands were also present in the mutant, except for one strong zone of clearing (at estimated molecular mass of 66 kDa) (Supplementary Figure S3). This band corresponded to the VcpA zinc-metalloprotease as this protease was not present in the mutant. Specific enzyme inhibitors were used to characterize the different bands. EDTA treatment inhibited all the higher bands (115, 66, 51 and 45 kDa). Phenantroline, a metalloprotease inhibitor, inactivated the 20 kDa band and greatly reduced the activity of the VcpA zinc-metalloprotease, a large protease (66 kDa). The serine protease inhibitor PMSF inactivated an upper band (115 kDa) and one band of 51 kDa (Supplementary Figure S3). A preparative gel containing concentrated *V. coralliilyticus* P1 extracellular products showed high levels of serine protease activity. The E-64, pepstatin, cysteine and aspartic acid peptidase inhibitors did not inhibit any protease bands (data not shown).

Secretome of Vibrio coralliilyticus *wild-type and mutant strains*

The wild-type and Δ*vcpA* mutant strains clearly displayed different secretomes as assessed by 2-D electrophoreses (Supplementary Figure S3). At least 11 spots were more intense or only present in the mutant secretome, whereas eight spots were specific to the wild type. Further analysis of the secretomes through 1-D SDS-polyacrylamide gel electrophoresis analysis demonstrated fewer protein bands for the wild type when directly compared with the mutant strain (Supplementary Figure S4). Mass spectrometry of visible bands excised from the gels indicated that the wild type and the mutant secreted distinct groups of proteins (Table 3), possibly in response to the *vcpA* deletion. The analysis of the wild type and the mutant secretomes in two culture media (Marine and Tryptone Soy broths) confirmed the loss of the VcpA metalloprotease expression in the mutant but not in the wild type. In addition, the mutant secreted 18 exclusive proteins in tryptone soy broth, including four types of metalloproteases found previously on the genome (VchA and bacterial leucyl aminopeptidase proteins). The mutant also expressed a chitinase, a hemolysin-related protein RbmC, the Hcp protein and 12 hypothetical proteins (Table 3). In marine broth, the expression of the chitinase and the VthA protein was only seen in the mutant but the three aminopeptidases, the VcpB thermolysin and an alkaline serine protease were detected in both strains.

Discussion

Sequencing of the genome of the coral pathogen *V. coralliilyticus* strain P1 (LMG23696), originally isolated from diseased corals on the Great Barrier

Table 2 Proteolytic characteristics of the *V. coralliilyticus* wild type and Δ*vcpA* strains

Culture	*Caseinase*	*Gelatinase*	*Phospholipase against egg yolk*	*Haemolysin (Sheep)*
V. coralliilyticus P1	+++	++	+++	+ (β)
V. coralliilyticus Δ*vcpA*	++	+	+	+++ (β)

Abbreviations: β, β-haemolysis; +, zone of clearing/opalescence of 1–2 mm; ++, zone of clearing/opalescence of 4–6 mm; +++, zone of clearing/opalescence of ⩾7 mm.

Reef, was shown to possess a diverse repertoire of virulence factors. Most notably, 17 putative metalloproteases belonging to distinct peptidase families were identified and importantly some of these metalloproteases were also detected in the secretome of P1. Moreover, coding genes that potentially improve *V. coralliilyticus* fitness (such as stress response, biofilm formation, quorum sensing, flagellar proteins and chemotaxis systems) were identified in the genome. The presence of the *tfoX* regulator and type IV and VI secretion systems, and *vgrG* and *hcp* effectors illustrate the genomic versatility and additional factors that are likely important in *V. coralliilyticus* pathogenicity. In addition, several genes responsible for colonization were found in the P1 genome. The *syp* genes, a group of genes described in *V. fischeri* that promote symbiotic colonization and biofilm formation via Sigma54 regulation (Yip *et al.*, 2005), and the *flp* genes for biogenesis of type IV Flp pilus described in *Actinobacillus actinomycetemcomitans* (Boyd *et al.*, 2008), may represent important loci for the process of coral colonization. In addition, strain-specific genes found in the P1 genomes revealed the importance of phage genes driving intra-species diversification. The P1 genome had several phage genes that were not present in the LMG 20984T genome, indicating that phages may allow genome diversification within the species *V. coralliilyticus*.

The VcpA zinc-metalloprotease has been identified in previous studies as the key virulence factor likely important in the bacterial infection of corals and implicated in coral tissue necrosis (Ben-Haim *et al.*, 2003a, b; Sussman *et al.*, 2008). The *V. coralliilyticus vcpA* gene is orthologous to the *vtpA* gene from the oyster pathogen *Vibrio tubiashii* and similarly identified as central for strain virulence (Hasegawa *et al.*, 2008). A Δ*vcpA V. coralliilyticus* P1 mutant demonstrated the same pathogenic potential as the wild-type strain in two animal models and a whole cell *Symbiodiunium* bioassay evaluated in this study, highlighting that the pathogenicity of vibrios in marine animals is a complex interplay of multiple genetic factors and unlikely the result of one determinant. We suggest that coral infection by *V. coralliilyticus* depends on several toxins that act in concert to promote bacterial pathogenicity. The mutant has lower caseinase and phospholipase activities, but higher hemolysin activity. High levels of serine protease and metalloprotease activities were also observed in the wild type. The 66 kDa protein corresponds to the VcpA zinc-metalloprotease whose gene was deleted in the Δ*vcpA* mutant. Isoforms of the VcpA metalloprotease may also occur in the secretome although it is not clear if isoforms have some proteolytic activity (Miyoshi *et al.*, 1997; Park *et al.*, 2008).

The VcpA loss may activate an alternative mechanism for pathogenesis in Δ*vcpA* mutants. The activation of two putative duplicates of this metalloprotease may be one potential alternative mechanism (Figure 4). We suggest that *vcp*A, *vcp*B, and *vch*A are paralogous genes. VcpB and VchA (both from M4 family) may enable P1 to cause disease as this family is known to be thermolysins with high proteolytic activity in vibrios (Hasegawa *et al.*, 2008). In addition, the collagenase metallopeptidases (U32) degrade type I collagens and help in the invasion of the host. They are homologs of the *prtC* gene of *Bacteroides gingivalis* (Kato *et al.*, 1992). The M6 peptidase family comprises the immune inhibitor A from the insect pathogenic Gram-positive *Bacillus thuringiensis*. This protein cleaves host antibacterial proteins. Homologs of the immune inhibitor A, named PrtV, have been found in *V. cholerae* and *V. anguillarum* (Vaitkevicius *et al.*, 2006, 2008). PrtV is required for killing *C. elegans* and has a cytotoxic effect, leading to cell death. PrtV can degrade extracellular matrix components. A *Vibrio anguillarum* M3 mutant, defective for *prtV*, exhibited decreased growth in turbot intestinal mucus, reduced hemolytic activity on turbot erythrocytes, and increased LD50 in infection experiments (Mo *et al.*, 2010). Homologs of the immune inhibitor A have also been found in other genomes (*Aeromonas hydrophila* ATCC 7966 and *Bacillus thuringiensis* BMB171). The presence of a leucine (leucyl) aminopeptidase (LAP) and a (LAP)-related protein in the genome and secretome is an important finding. LAPs are often viewed as cell maintenance enzymes with critical roles in turnover of peptides (Rawlings *et al.*, 2004). Some LAPs may bind DNA enabling them to serve as transcriptional repressors that control pyrimidine, alginate and cholera toxin biosynthesis, as well as mediate site-specific recombination events in plasmids and phages (Matsui *et al.*, 2006). LAPs have attracted additional interest due to their applicability in industry as whole-cell biocatalysts for the selective hydrolysis and enzymatic resolution of DL-amino

Table 3 Secreted proteins identified from the *V. coralliilyticus* wild type and Δ*vcpA* strains

Protein band[a]	*ID*[b]	*Description*	*Protein mass*[c]	*P1*	*ΔvcpA*
1	fig|6666666.585.peg.2341	3-ketoacyl-CoA thiolase	41 708	X	
1	fig|6666666.585.peg.1263	ABC transporter, periplasmic spermidine putrescine-binding protein PotD	39 005	X	
1	fig|6666666.585.peg.33	Amino acid ABC transporter, periplasmic amino acid-binding portion	60 828	X	
1	fig|6666666.585.peg.4340	Aminoacyl-histidine dipeptidase (peptidase D)	53 915	X	
1	fig|6666666.585.peg.3512	Arginine deiminase	45 475	X	
1	fig|6666666.585.peg.1603	Aspartate-semialdehyde dehydrogenase	39 344	X	
1	fig|6666666.585.peg.3784	D-3-phosphoglycerate dehydrogenase	44 631	X	
1	fig|6666666.585.peg.3679	Dihydrolipoamide dehydrogenase of pyruvate dehydrogenase complex	51 256	X	
1	fig|6666666.585.peg.3268	Glutamine synthetase type I	51 623	X	
1	fig|6666666.585.peg.4475	Heat shock protein 60 family chaperone GroEL	57 590	X	
1	fig|6666666.585.peg.2448	Maltoporin	43 893	X	
1	fig|6666666.585.peg.383	Vibriolysin (neutral protease precursor) VcpA[d]	65 881	X	
1	fig|6666666.585.peg.3939	Outer membrane protein NlpB, lipoprotein component of the protein assembly complex	38 529	X	
1	fig|6666666.585.peg.5024	Oligopeptide ABC transporter, periplasmic oligopeptide-binding protein OppA	60 828	X	
1	fig|6666666.585.peg.3514	Ornithine carbamoyltransferase	38 107	X	
1	fig|6666666.585.peg.667	Outer membrane stress sensor protease DegQ (serine protease)	48 032	X	
1	fig|6666666.585.peg.158	Outer membrane vitamin B12 receptor BtuB	67 400	X	
1	fig|6666666.585.peg.3681	Pyruvate dehydrogenase E1 component	99 539	X	
1	fig|6666666.585.peg.3925	UDP-sugar hydrolase	61 372	X	
3	fig|6666666.585.peg.2503	Hemolysin-related protein RbmC	103 393		X
3	fig|6666666.585.peg.796	Hypothetical protein (VIC_004311)	35 219		X
3	fig|6666666.585.peg.3372	Hypothetical protein (putative hydroxymethyltransferase)	110 770		X
4	fig|6666666.585.peg.2748	Chitinase	91 709		X
4	fig|6666666.585.peg.3507	Extracellular deoxyribonuclease Xds	94 471		X
4	fig|6666666.585.peg.761	Flagellar hook-associated protein FliD	73 020		X
4	fig|6666666.585.peg.3361	Hypothetical protein (VIC_001630)	68 635		X
4	fig|6666666.585.peg.203	Hypothetical protein (VIC_002088)	84 685		X
4	fig|6666666.585.peg.526	Acyl-CoA dehydrogenase, short-chain specific (peptidase S8 and S53 subtilisin kexin sedolisin)	71 910		X
5	fig|6666666.585.peg.494	Bacterial leucyl aminopeptidase precursor (aminopeptidase)[e]	56 432		X
5	fig|6666666.585.peg.3342	Bacterial leucyl aminopeptidase precursor[e]	54 368		X
5	fig|6666666.585.peg.2006	Endonuclease I	59 494		X
5	fig|6666666.585.peg.1918	Hypothetical protein (VIC_003980)	56 620		X
5	fig|6666666.585.peg.3343	Zinc metalloprotease (leucine aminopeptidase-related protein)[e]	52 531		X
6	fig|6666666.585.peg.3360	Vibriolysin (hemolysin/cytolysin) VchA	53 487		X
6	fig|6666666.585.peg.4379	Peptidyl-prolyl cis-trans isomerase SurA	48 390		X
7	fig|6666666.585.peg.2756	Hypothetical protein (VIC_002899)	45 749		X
8	fig|6666666.585.peg.1029	Flagellar hook protein FlgE	46 945		X
8	fig|6666666.585.peg.832	TraF-related protein (hypothetical protein VIC_003016)	40 308		X
8	fig|6666666.585.peg.3345	Spindolin-related protein	43 472		X
9	fig|6666666.585.peg.55	Hypothetical protein (VIC_000374)	36 006		X
9	fig|6666666.585.peg.1802	Hypothetical protein (VIC_003092)	37 704		X
9	fig|6666666.585.peg.3320	Putative outer membrane protein (nonspecific porin)	37 176		X
1 and 9	fig|6666666.585.peg.5035	Alanine dehydrogenase	40 075	X	X
1 and 9	fig|6666666.585.peg.759	Flagellin protein FlaA (flagellin B)	39 710	X	X
1 and 9	fig|6666666.585.peg.3950	Flagellin protein FlaC	40 232	X	X
1 and 9	fig|6666666.585.peg.3951	Flagellin protein FlaD	39 782	X	X
1 and 9	fig|6666666.585.peg.2588	Hypothetical protein (VIC_003990)	41 373	X	X
1 and 9	fig|6666666.585.peg.1548	Hypothetical protein (VIC_004396)	38 409	X	X
1 and 9	fig|6666666.585.peg.621	Outer membrane protein OmpU	37 027	X	X
1 and 9	fig|6666666.585.peg.2406	Protease precursor (serine protease/subtilase peptidase)	62 976	X	X
1 and 9	fig|6666666.585.peg.659	Type I secretion TolC precursor	47 435	X	X
10	fig|6666666.585.peg.2563	Hypothetical protein (VIC_001560)	29 960		X
11	fig|6666666.585.peg.4653	Hcp	19 444		X
11	fig|6666666.585.peg.3359	Hypothetical protein (VIC_001628)	31 874		X
12	fig|6666666.585.peg.5132	Hypothetical protein (VIC_003927)	18 030		X

Abbreviation: ABC, ATP-binding cassette transporters. Reference numbers denoted in parentheses in protein descriptions refer to the location on the *Vibrio coralliilyticus* ATCC BAA-450 genome. X symbol denotes the identification of proteins in each secretome sample.
[a]Number of the gel bands (Supplementary Figure S4) whose proteins were identified. Proteins were identified from secretomes of the P1 and the DvcpA mutant grown on TSB.
[b]Gene identification on *V. coralliilyticus* P1 genome according to RAST.
[c]Theoretical protein mass (daltons) provided by MASCOT Search with the use of the Expasy Tool Compute software.
[d]Symbol points to the protein corresponding to the *vcpA* gene, which was deleted to generate the Δ*vcpA* mutant.
[e]Proteins identified in P1 and the mutant secretome when grown in marine broth.

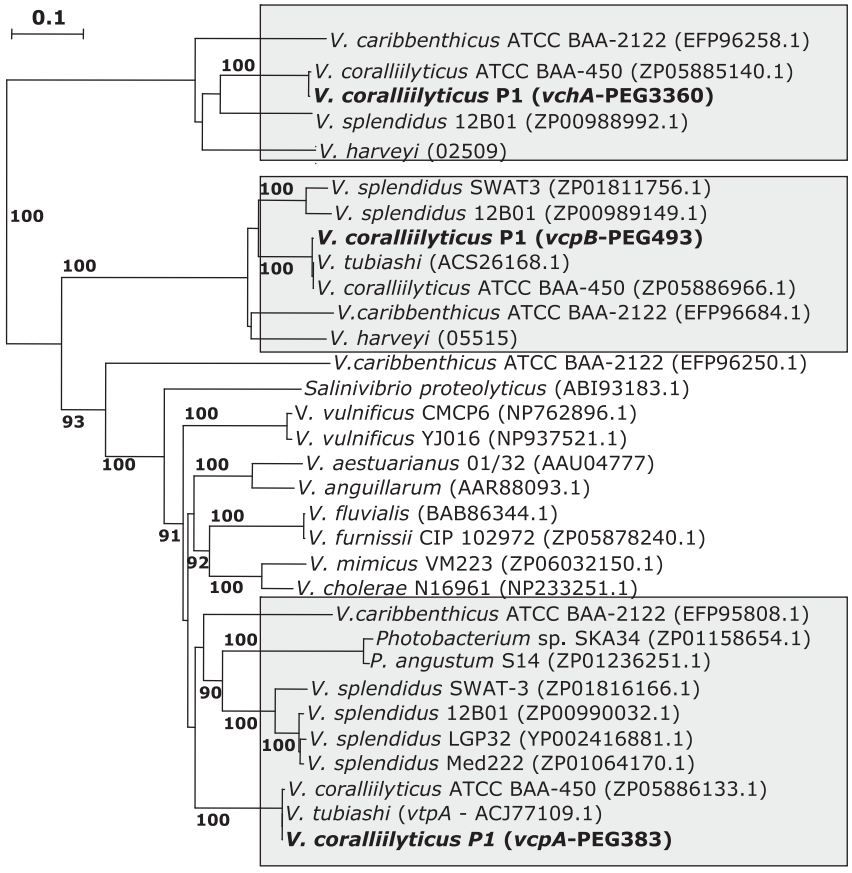

Figure 4 Phylogenetic tree based on the 600 AA sequence of VcpA and its homologues. The tree was constructed using the poison and neighbor joining methods. The scale bar indicates 10% sequence divergence. The bootstrap values of 1000 repetitions are indicated at the nodes.

acid amide racemates (Kamphuis *et al*., 1992; Hermes *et al*., 1993).

The comparative analysis of the secretome of wild-type and mutant *V. coralliilyticus* P1 strains identified distinct differences in protein secretion as a result of the deletion of the *vcpA* gene. This observation indicates that the VcpA zinc-metalloprotease might have a regulatory role on *V. coralliilyticus* P1 gene expression. The lack of *vcpA* expression in the mutant and the expression of several proteins (chitinase, hemolysin/cytolysin VthA, aminopeptidases (LAPs), leucine aminopeptidase-related protein, hemolysin-related protein RbmC, peptidase S8 and S53 subtilisin kexin sedolisin, Hcp protein) only by the mutant supports this conclusion. In addition, the presence of the hemolysin VchA in the mutant secretome, corroborated by its increased hemolytic activity in the enzymatic tests may suggest regulation of hemolysin production by the deleted zinc-metalloprotease. In *V. tubiashii*, the zinc-metalloprotease VtpA regulates VthA hemolysin expression by means of a posttranscriptional mechanism (Hasegawa and Häse, 2009). The VtpA mutant of *V. tubiashii* had decreased pathogenic potential in *C. gigas* larvae (Hasegawa *et al*., 2008). The hyper expression of VthA did not increase the pathogenic potential of *V. tubiashii* in *C. gigas*. Further work is required to determine the exact role of VcpA in gene expression regulation in *V. coralliilyticus* P1.

Coral disease is a complex multifactorial process involving environmental triggers and holobiont responses. The study highlights that bacterial infection of corals and the virulence (protease) factors involved are highly complex and that coral disease is likely the result of an interplay of multiple factors rather than one determinant. The high diversity and activity of proteases observed in *V. coralliilyticus* P1 linked with its rapid growth capacity under optimum environmental conditions indicates that it may have a central role in the homeostasis and health status of the reef systems.

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