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A Metalloprotease Secreted by the Type II Secretion System Links *Vibrio cholerae* with Collagen

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***Vibrio cholerae* is autochthonous to various aquatic niches and is the etiological agent of the life-threatening diarrheal disease cholera. The persistence of *V. cholerae* in natural habitats is a crucial factor in the epidemiology of cholera. In contrast to the well-studied *V. cholerae*-chitin connection, scarce information is available about the factors employed by the bacteria for the interaction with collagens. Collagens might serve as biologically relevant substrates, because they are the most abundant protein constituents of metazoan tissues and *V. cholerae* has been identified in association with invertebrate and vertebrate marine animals, as well as in a benthic zone of the ocean where organic matter, including collagens, accumulates. Here, we describe the characterization of the *V. cholerae* putative collagenase, VchC, encoded by open reading frame VC1650 and belonging to the subfamily M9A peptidases. Our studies demonstrate that VchC is an extracellular collagenase degrading native type I collagen of fish and mammalian origin. Alteration of the predicted catalytic residues coordinating zinc ions completely abolished the protein enzymatic activity but did not affect the translocation of the protease by the type II secretion pathway into the extracellular milieu. We also show that the protease undergoes a maturation process with the aid of a secreted factor(s). Finally, we propose that *V. cholerae* is a collagenovorous bacterium, as it is able to utilize collagen as a sole nutrient source. This study initiates new lines of investigations aiming to uncover the structural and functional components of the *V. cholerae* collagen utilization program.**

Members of the genus *Vibrio* are important inhabitants of the marine coastal waters, estuaries, and ocean sediments, with some pathogenic species also causing wound infections, primary septicemia, gastroenteritis, and diarrhea in humans (1, 2). Among the more than 200 serogroups of *V. cholerae*, only O1 and O139 are recognized as agents of cholera, a potentially life-threatening diarrheal disease. Cholera remains a significant public health problem by affecting millions of people annually, predominantly in developing countries that have limited clean water supplies and poor sanitation (3). Infection of the human host occurs upon ingestion of water or food contaminated with the bacterium. Following colonization of the small intestine, *V. cholerae* utilizes a multicomponent secretory pathway, the type II secretion system (T2SS), to release cholera toxin. Cholera toxin induces acute diarrhea in people infected with cholera, enabling the bacteria to escape from the host into the aquatic reservoir (4). During interepidemic periods, *V. cholerae* persists freely or in association with various aquatic organisms, including copepods, insect egg masses, shellfish, and vertebrate fish (5–7). Extracellular proteins, including those secreted by the T2SS, have been implicated in facilitating the fitness of *V. cholerae* in both the human host and aquatic niches (4, 6, 8). The array of extracellular proteins, which rely on the T2SS to cross the outer membrane, is remarkably diverse. The T2S-dependent secretome is comprised of proteins involved in the utilization of chitin (different chitinases and chitin binding protein GbpA) as well as lipase, sialidase, cytolysin VCC, the biofilm-associated protein RbmA, Tarp, putative proteins VCA0738 and VC2298, serine proteases (VesA, VesB, and VesC), and metalloproteases (HapA, LapA, and LapX) (4).

Chitin and collagens are important sources of nutrients and energy in the marine environment, and their degradation by various microorganisms contributes to global carbon and nitrogen cycling (8–12). In *V. cholerae*, the chitin utilization program is a well-characterized, hierarchical process that involves bacterial chemotaxis toward the substrate, binding of the bacteria to abiotic or biotic chitinous surfaces mediated by several proteins, including GbpA, and finally the degradation of chitin, which is accomplished by extracellular chitinases ChiA-1 and ChiA-2, periplasmic hydrolases specific for chitin oligosaccharides as well as cytoplasmic enzymes (8, 13–15). The association of *V. cholerae* with chitinous zooplankton is one of the recognized environmental factors that contribute to the bacterial population dynamics and cholera epidemics and pandemics worldwide (8, 16). In contrast to the large body of studies examining the *V. cholerae*-chitin connection, scarce information is available about the factors employed by the bacteria for the interaction with collagens. Collagens might serve as biologically relevant substrates, because they are predominant protein constituents of metazoan tissues, including marine animals, and *V. cholerae* has been identified as resident in bivalves, fish skin, gills, muscle, and digestive tract, as well as in the benthic zone of the ocean, where organic matter containing col-

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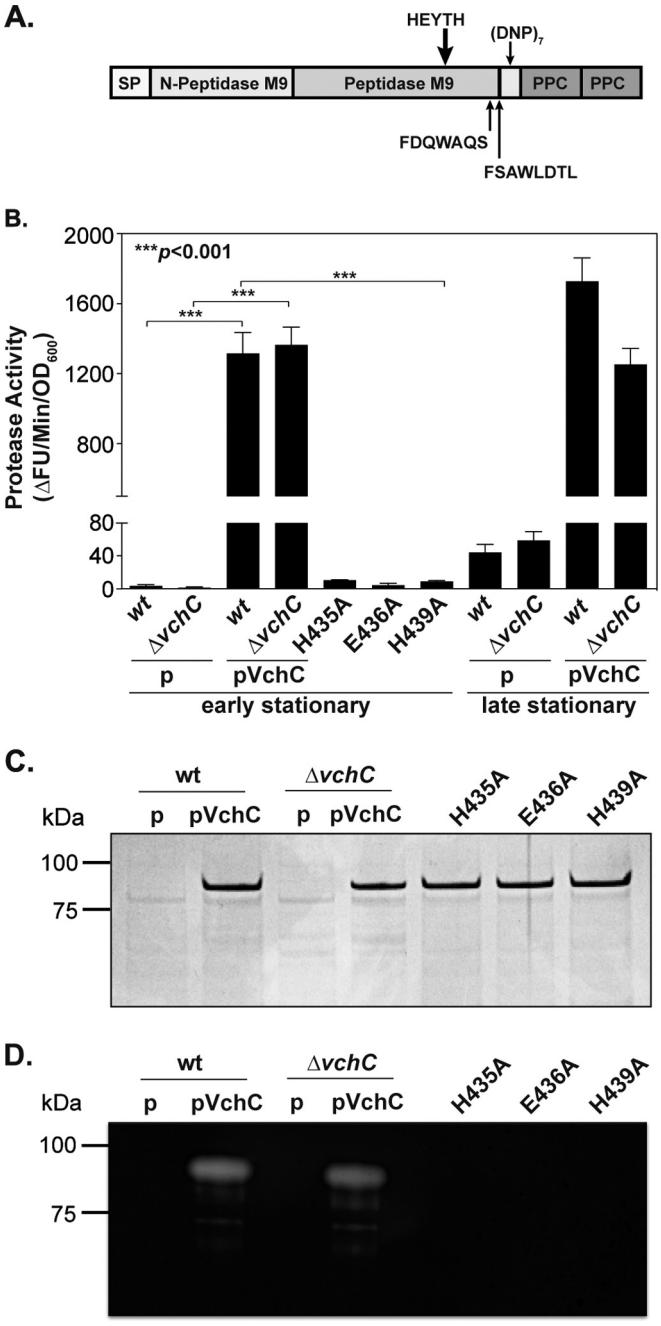

FIG 1 VchC is a secreted protein with conserved residues characteristic for the *Vibrio* clan II metalloproteases, which are critical for enzymatic activity but not for protease secretion. (A) Architecture of VchC from *Vibrio cholerae* N16961. The following individual domain and motif labels are used: SP, signal peptide; N-Peptidase M9, peptidase M9 with HEYTH motif and two potential collagen-binding motifs, FDQWAQS and FSAWLDTL; DNP, the variable-number sequence repeats of aspartic acid, asparagine, and proline; PPC, prepeptidase C-terminal domain. (B to D) The strains of *V. cholerae* N16961 wild type with pMMB67-EH (wt p), wild type with plasmid-borne VchC (wt pVchC), isogenic Δ*vchC* mutant with pMMB67-EH (Δ*vchC* p), Δ*vchC* mutant harboring pMMB67-EH-VchC (Δ*vchC* pVchC), and wild-type ectopically expressing mutated variants of VchC, namely, H435A, E436A, and H439A, were cultured at 37°C in LB medium supplemented with 100 μM IPTG to the early or late stationary phase of growth (as indicated). The supernatants were harvested and subjected to the following examinations. (B) The collagenolytic/gelatinolytic activity was measured using a fluorogenic substrate, DQ gelatin. The detected protease activity is expressed as a change in fluorescence (ΔFU) over time (Min) and normalized by optical density (OD_{600}) of corresponding bacterial cultures. All experiments were performed on at least three separate occasions in technical triplicates, and the means with corresponding standard errors of the means (SEM) are presented. (C) The protein profiles in harvested supernatants (as indicated above the gel) were assessed by 10% SDS-PAGE and staining with colloidal Coomassie. (D) Enzymatic activity of VchC was examined by in-gel zymography. Supernatants were diluted 100-fold, and samples normalized by the density of the culture (at the OD_{600}) were separated in a 10% Tris-glycine gel containing 0.1% gelatin as a substrate. A representative gel stained with colloidal Coomassie solution is shown. The migration of molecular mass markers is indicated.

lagens accumulates (7, 17–20). Collagens are made up of helically coiled polypeptide fibrils, and due to their rigid structure, only a limited number of proteases have the ability to decompose these proteins (21). According to discrimination in the active sites, collagen-hydrolyzing enzymes can be classified as metalloproteases (M9 and M10 families), cysteine proteases (C1 family), and serine proteases (S1 and S8 families). Microbial proteases that belong to the MEROPS peptidase family M9 are comprised of predicted zinc-dependent metalloproteases with presumed collagenolytic activity (22). Based on differences in the deduced amino acid sequences and catalytic function, this family is further divided into M9A and M9B subfamilies containing proteases from *Vibrio* and *Clostridium*, respectively (22, 23). The clostridial collagenases have been investigated in far more detail than those produced by *Vibrio* species. The holotype enzymes in the M9B subfamily include class I and class II collagenases within M09.002 and M09.003 types, respectively, which were classified based on their relative activities toward natural and synthetic substrates, stability, as well as secondary and tertiary structures (23). The peptidase M9A subfamily contains enzymes clustered within two MEROPS groups, M09.001 and M09.004, with bacterial collagenase V from *V. alginolyticus* (X62635) and VMC peptidase from *V. mimicus* (AF004832) as holotype enzymes. The recent analysis of the sequences and domain architectures demonstrated that only these two classes of *Vibrio* metalloproteases should be considered collagenases. Overall, the *Vibrio* collagenases are multidomain proteins containing a signal peptide, a peptidase M9N domain (activator domain), a peptidase M9 domain, a polycystic kidney disease-like domain, and/or a bacterial prepeptidase C-terminal domain(s) (Fig. 1A) (23).

In this article, we describe, for the first time, the characterization of the *V. cholerae* putative collagenase encoded by open reading frame (ORF) VC1650 and belonging to the subfamily M9A peptidases. The molecular, biochemical, and functional approaches demonstrated that VC1650 encodes an extracellular T2SS-dependent collagenase-degrading native type I collagen extracted from fish skin and secreted from human fibroblasts. Moreover, alteration of the predicted catalytic residues coordinating zinc ions completely abolished the protein enzymatic activity but did not affect the translocation of the protease across the outer membrane into the extracellular milieu. We also have shown that the protease undergoes a maturation process that involves removal of the bacterial prepeptidase C-terminal domains with the aid of a secreted factor(s). Finally, we propose that *V. cholerae* is a collagenovorous bacterium, as it is able to survive in liquid media containing fish collagen as a carbon and nitrogen source. This study initiates new lines of investigations aiming to uncover the structural and functional components of the *V. cholerae* collagen utilization program.

(This study was presented in part as a poster at the 114th General Meeting of the American Society for Microbiology, Boston, MA, 17 to 20 May 2014 [24].)

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table S1 in the supplemental material. Bacteria were cultured at 37°C or 25°C in Luria-Bertani (LB) broth and M9 salts containing either Casamino Acids and 0.4% glucose or 0.4% fish collagen as indicated. The media were purchased from Difco. Antibiotics (Teknova, Amresco) were added to solid and liquid media at the following concentrations: carbenicillin, 50 μg/ml for plasmid expression; chloramphenicol, 30 μg/ml for plasmid expression in *Escherichia coli* and 4 μg/ml for chromosomal expression in *V. cholerae*; kanamycin, 50 μg/ml; streptomycin, 100 μg/ml; and polymyxin B sulfate, 100 U/ml.

Genetic manipulations. The *V. cholerae* O1 biovar El Tor N16961 genome sequence (NC_002505) was used as a template to design oligonucleotides. All oligonucleotide primers designed and used in this study are listed in Table S2 in the supplemental material. Chromosomal DNA isolated from strain N16961 (Promega Wizard genomic DNA purification kit) or purified plasmid DNA (GenCatch plasmid DNA mini-prep kit; Epoch Life Science), as indicated in the text, was utilized as the template in PCRs. PCRs were performed using Q5 high-fidelity DNA polymerase (New England BioLabs [NEB]) and primers synthesized by Integrated DNA Technologies. Restriction enzymes as well as T4 DNA ligase were purchased from New England BioLabs. Obtained constructs were sequenced at the Center for Genome Research and Biocomputing at Oregon State University. All plasmids were introduced into different strains of *V. cholerae* N16961, as defined in the text, via triparental mating using *E. coli* SY327λpir as the donor and *E. coli* MM294 as the plasmid-mobilizing helper strain (25). After conjugation, bacteria were plated onto a selective medium for *Vibrio* spp., thiosulfate-citrate-bile salts-sucrose (TCBS) agar (26), supplemented with appropriate antibiotics.

To place the native *vchC* gene under the control of the P_{TAC} promoter carried on the low-copy vector pMMB67-EH, a fragment of N16961 chromosomal DNA containing the VC1650 gene with its native *rbs* and an N-terminal signal peptide was amplified with oligonucleotides VchCF and VchCR. The PCR product was subcloned into the pCR-Script-Amp cloning vector (Stratagene) to generate pCR-Script-VchC. Subsequently, the EcoRI-PstI fragment containing *vchC* was cloned into a likewise-digested pMMB67-EH to yield pVchC.

Similarly, the recombinant version of VchC with a C-terminal 6× His tag, VchC-His, was engineered in a PCR using primers rVchCF and rVchCR. A sequence encoding the His tag was included within the primer rVchCR. The resulting PCR product was digested with EcoRI-XbaI and cloned into pMMB67-EH to generate a plasmid designated pVchC-His.

The Δ*vchC* knockout strain was constructed as follows. First, the cassette encoding chloramphenicol resistance (Cm^r) was amplified with primers CmF and CmR using plasmid pKD3 as the template. The PCR product was digested with NcoI and ligated into the NcoI site within the *vchC* carried on pCR-Script-VchC to generate pCR-Script-Δ*vchC*. The 3.5-kb fragment containing the insertionally inactivated *vchC* was then cut out from pCR-Script-Δ*vchC* using SalI-SacI and cloned into a suicide vector, pCVD442, digested with the same restriction enzymes to yield pCVDΔ*vchC*. pCVDΔ*vchC* was introduced into the N16961 strain of *V. cholerae* via triparental conjugation, the transconjugants were isolated on TCBS agar with chloramphenicol, and then they were counterselected as described previously (25). Bacterial colonies that were sensitive to carbenicillin and resistant to chloramphenicol were selected for PCR analysis and DNA sequencing to confirm the presence of the Cm^r cassette within the VC1650 gene on the *V. cholerae* chromosome.

Site-directed mutagenesis of the predicted catalytic triad of VchC. Mutagenesis of the predicted VchC catalytic residues H435, E436, and H439 into alanine was performed as described in the instructions provided by the manufacturer using the QuikChange site-directed mutagenesis kit (Agilent Technologies), appropriate primers listed in Table S2 (in the supplemental material), and pCRScript-VchC as the template. The presence of the desired mutations was verified by DNA sequencing. Subsequently, the individual mutated variants of VchC were cloned into pMMB67-EH digested with EcoRI-PstI to generate plasmid derivatives pVchC-H435A, pVchC-E436A, and pVchC-H439A and introduced into wild-type (wt) *V. cholerae* by triparental mating as explained above.

Purification of the C-terminally 6× His-tagged VchC. The overnight culture of *V. cholerae* N16961 harboring pVchC-His was diluted 1:100 into 1 liter of LB media supplemented with carbenicillin, and overproduction of VchC-His was induced with 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were grown with aeration at 37°C for 4 h until they reached the early stationary phase of growth (OD_{600} of about 4). The culture supernatant was separated from the cells by centrifugation. Subsequently, the supernatant was passaged through 0.22-μm filters (VWR), and the proteins were precipitated from solution with ammonium sulfate at 40% saturation. Following 1 h of incubation at 4°C, the sample was subjected to centrifugation at 10,000 × *g* for 40 min at 4°C. The pellet was solubilized in buffer A (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM imidazole), and the soluble fraction was subjected to dialysis against buffer A overnight at 4°C. Subsequently, the sample was centrifuged and the supernatant was applied to a 10-ml purification column (Thermo Scientific) containing nickel-nitrilotriacetic acid (Ni-NTA) resin equilibrated in buffer A. After washing with a solution containing 20 mM Tris-HCl, pH 8.0, 300 mM NaCl, and 10 mM imidazole, the protein was eluted with a buffer comprised of 20 mM Tris-HCl, pH 8.0, 500 mM NaCl, and 250 mM imidazole. The purified recombinant VchC-His was dialyzed against 20 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, and 10% glycerol and concentrated by ultrafiltration using a Microsep Advance centrifugal 5-ml tube with a molecular mass cutoff of 3 kDa (Pall Life Sciences). The concentration of a purified protein was determined using Bio-Rad assay (DC protein assay) according to the manufacturer's recommendations.

Preparation of human and fish collagen. Human collagen, naturally secreted from human neonatal fibroblast cells (VitroCol; Advanced BioMatrix), comprised of type I collagen (97%) and type III collagen (3%) solubilized in 0.1 N HCl, was subjected to dialysis against 20 mM Tris-HCl, pH 8.0, and 1 mM $CaCl_2$ using Amicon Ultra 0.5-ml filters. The sample then was diluted 2-fold in the same dialysis buffer and utilized in substrate specificity studies following the determination of protein concentration.

The extraction of type I collagen from fish skin was carried out using a modified method described previously (27). A rainbow trout purchased from a local grocery store was thoroughly rinsed with double-distilled water (ddH_2O) and skinned. All subsequent steps were performed at 4°C. The skin was deproteinized by incubation in 0.1 N NaOH at a sample/alkali solution ratio of 1:10 (wt/vol) for 24 h and replaced with fresh sodium hydroxide solution every 6 h. The sample then was washed with ice-cold water until the mixture reached neutral pH. To remove fatty acids, the skin was incubated with stirring in 10% butyl alcohol at a sample/alcohol solution ratio of 1:10 (wt/vol) for 24 h. The solution was exchanged with fresh butyl alcohol every 6 h. The fish skin was washed again with ice-cold water and soaked for 24 h in 0.5 M acetic acid at a solid/solvent ratio of 1:15. The sample was centrifuged at 8,000 × *g* for 30 min. The supernatant was stored at 4°C, while the precipitate was subjected to reextraction for 16 h with gentle stirring in 0.5 M acetic acid at a sample/solvent solution ratio of 1:30 (wt/vol). To obtain the soluble collagen fraction, the suspension subsequently was centrifuged at 8,000 × *g* for 30 min. Both fractions were combined and precipitated with 0.05 M Tris-HCl, pH 7.5, and 2.6 M NaCl. The precipitate was centrifuged at 8,000 × *g* for 35 min and resuspended in 10 volumes of 0.5 M acetic acid. The solution was dialyzed against 15 volumes of 0.1 M acetic acid in a dialysis membrane with a molecular mass cutoff of 10 kDa (Thermo Scientific) for 24 h with a change of buffer every 4 to 8 h. The sample was further dialyzed against 15 volumes of ddH_2O with frequent changes of water until a neu-

tral pH was obtained (7 days). The purity of extracted collagen was examined by SDS-PAGE and colloidal Coomassie staining.

Enzymatic assays with a fluorescein-conjugated gelatin. Proteolytic activity of VchC was assessed using the recombinant VchC-His and in culture supernatants isolated from *V. cholerae*. To obtain the supernatants, the overnight cultures of *V. cholerae* N16961 carrying either empty vector pMMB67-EH (p), pVchC, pVchC-H435A, pVchC-E436A, or pVchC-H439A were diluted 1:100 into fresh LB supplemented with 100 μM IPTG. The samples were collected at different stages of bacterial growth as indicated. The supernatants were separated from bacterial cells by centrifugation as described previously (25). The enzymatic activity of VchC against a fluorescein-conjugated gelatin, DQ gelatin from pig skin (EnzCheck; Molecular Probes), was measured in culture supernatants and using purified VchC-His (100 fmol) by following the instructions provided by the manufacturer. The assays were conducted in 50 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, pH 7.6, and 25 μg/ml DQ gelatin for 10 min at 37°C using excitation and emission wavelengths of 485 ± 10 and 530 ± 15 nm, respectively. The detected change in fluorescence was normalized by either the optical density of the cultures (OD_{600}) or VchC concentration (28). The effect of different inhibitors, including 1,10-phenanothroline (5 mM), EDTA (5 mM), EGTA (5 mM), leupeptin (1 mM), phenylmethylsulfonyl fluoride (PMSF; 5 mM), *N*-methylmaleimide (5 mM), and benzamidine (5 mM), on VchC proteolytic activity also was studied. In these experiments, the purified protease and culture supernatants isolated from *V. cholerae* overexpressing native VchC, as specified, were treated with individual chemicals for 1 h at 37°C, and the ability of the enzyme to cleave DQ gelatin was examined. Experiments were conducted in technical duplicates and on at least three separate occasions. Means and standard errors of the means (SEM) are presented.

Subfractionation procedures. Overnight cultures of wt *V. cholerae* N16961 pVchC-His and the NΔ*eps* isogenic T2S mutant harboring pVchC-His were back diluted into fresh LB supplemented with 50 μM IPTG and grown with aeration for 4 h at 37°C. To imitate the cell envelope stress associated with the lack of a functional T2S system, the wild-type bacteria were treated with increasing concentrations (0, 50, 100, and 200 U/ml) of a membrane-perturbing agent, polymyxin B sulfate. The supernatants were separated from cells by centrifugation, and the periplasmic fractions were extracted by incubating *V. cholerae* with 2,000 U/ml of polymyxin B sulfate (25).

Substrate specificity assays. The ability of purified VchC-His to degrade a specific collagenase substrate, *N*-(3-(2-furyl)acryloyl)-Leu-Gly-Pro-Ala (FALGPA; Bachem AG), was performed according to the method described by Van Wart et al. (29). The negative and positive controls for the assays included modified trypsin (NEB) and collagenase D from *Clostridium histolyticum* (Roche), respectively. The reactions were initiated by the addition of the purified VchC-His, trypsin, or collagenase D (at 60 μg/ml) to samples containing 2.0 mM FALGPA in 50 mM Tricine, 400 mM NaCl, 10 mM $CaCl_2$, pH 7.5. The reaction mixtures were incubated at 37°C for 5 min, followed by monitoring the decrease in absorbance of the substrate at 345 nm for 1 h every 5 min at 30°C using the Synergy HT multimode microplate reader (BioTek). The amount of degraded FALGPA was calculated based on the calibration curve obtained by measurement of the absorbance of the reference samples (various concentrations of FALGPA in the reaction buffer). Experiments were performed in biological triplicates, and the means with corresponding SEM are reported.

In addition, type I collagen from fish skin and secreted from human neonatal fibroblast cells (prepared as described above), as well as fibronectin, lactoferrin, immunoglobulin A (IgA), porcine stomach mucin, and bovine albumin serum (BSA; Sigma-Aldrich), were examined as the candidate substrate proteins of VchC. In these assays, purified VchC (2 pmol) was incubated for 1 h at 37°C with individual protein substrates (2 μg; fibronectin at 0.2 μg) in a buffer containing 20 mM Tris-HCl, pH 8.0, and 1 mM $CaCl_2$. To demonstrate the specificity of VchC to collagen, titration assays were conducted by treating 5 μg of fish collagen with increasing amounts of the protease (0, 4, 10, 20, 40, 80, and 100 fmol). The VchC activity against fish collagen in the presence of 5 mM benzamidine, 5 mM 1,10-phenantroline, or the vehicle (DMSO) was examined by preincubating the protease (100 fmol) in the presence of a tested compound for 1 h at 37°C. Subsequently, 5 μg of fish collagen was added; the reaction mixtures were incubated for 1 h at 37°C and processed as described below. SDS-PAGE and colloidal Coomassie staining were utilized to evaluate the cleavage of all tested substrates. Experiments were performed in at least three independent trials, and representative results are shown.

Maturation of VchC. The late-stationary-phase cultures of wt *V. cholerae* bearing either p, pVchC, or pVchC-His were back diluted into fresh LB media supplemented with 100 μM IPTG and carbenicillin and then cultured with shaking for 16 h at 37°C. The samples were withdrawn at 4, 5, 6, 8, 10, and 16 h from back dilution. Supernatants were separated from bacterial cells by centrifugation and subjected to SDS-PAGE, followed by either staining with colloidal Coomassie or immunoblot analyses with monoclonal anti-His antisera as described below. SDS-PAGE gel images were scanned using a ChemiDoc system (Bio-Rad) and subjected to densitometric analysis using Image Lab 5.0 software (Bio-Rad). To quantify the intensity of the individual protein bands, a volume tool (rectangle), local background subtraction, and a linear regression method were used. The sum of adjusted volumes corresponding to 90-kDa and 66-kDa forms of VchC in each lane was set as 100%. The intensity of each band was calculated as a percentage of the total intensity for both bands at a given time point. The data represent the means with associated SEM obtained from three independent experiments.

To examine whether the processing of VchC depends on another extracellular factor(s), the purified protease (2 pmol) was incubated for 1 h at 37°C in the presence of supernatants isolated from *V. cholerae* N16961 wt p, NΔ*eps* p, Δ*vchC* p, and Δ*vchC* pVchC strains cultured to early stationary phase in LB at 37°C. The samples subsequently were resolved by SDS-PAGE, and the proteins were stained with Coomassie brilliant blue G-250. Controls were comprised of corresponding supernatants and LB without the addition of the protease and were subjected to the same treatment as the experimental samples. These experiments were performed in biological triplicates, and a representative SDS-PAGE gel is shown.

SDS-PAGE, immunoblotting, and zymography. Samples containing LDS loading buffer (Invitrogen) supplemented with dithiothreitol (50 mM) were separated by 10% SDS-PAGE and stained with colloidal Coomassie (Coomassie brilliant blue G-250). Periplasmic fractions and supernatants were matched by equivalent OD_{600} values. In immunoblot analysis, the proteins were transferred to nitrocellulose membrane (Pall Life Sciences) using Turbo blot (Bio-Rad). The membranes were blocked in 5% milk in phosphate-buffered saline (PBS; pH 7.0; Li-Cor) supplemented with 0.1% Tween 20 and probed with monoclonal anti-His antisera (1:1,000 dilution; Thermo Scientific), followed by incubation with goat anti-mouse-horseradish peroxidase (HRP) conjugate (1:1,000 dilution; Thermo Scientific). Immunoblots were developed using Clarity Western ECL substrate (Bio-Rad) on a Chemi-Doc MP system (Bio-Rad).

Zymography. The proteolytic activity against 0.1% gelatin in a 10% Tris-glycine gel (Novex; Invitrogen) was examined in supernatants isolated from different strains of *V. cholerae* at distinct time points of bacterial growth from the time of back dilution, as specified in the text. Samples matched by equivalent OD_{600} values were separated on zymogram gels, and protein renaturation was performed according to the instructions provided by the manufacturer. The activity of VchC was observed as a clear band, where gelatin was digested by the protease, against a dark background stained with colloidal Coomassie.

Bioinformatics. Amino acid sequence of VchC from *V. cholerae* N16961 was used to perform a BLAST similarity search using the UniProt database (http://www.uniprot.org/blast/). Homologous proteins from the genus *Vibrio* were downloaded as FASTA files and aligned using Clustal Omega (www.ebi.ac.uk/Tools/msa/clustalo/). All searches and alignments were performed using default settings.

Statistical analyses. The statistical analyses were performed using GraphPad Prism software (version 6.0a). An unpaired Student's *t* test or

Mann-Whitney test, as indicated, was used to analyze the data and the statistical significance ($P < 0.05$).

RESULTS

VC1650 is annotated as a putative collagenase. We are interested in the discovery and characterization of the complete array of the T2S-dependent exoproteins to gain better insights into the contribution of this secretion pathway to the fitness of *V. cholerae* in the human host and in the aquatic environment. While inspecting the chromosomal location of the gene encoding one of the recently identified T2S-dependent serine proteases, VesC (30), we found that the neighboring gene located in an opposite orientation, VC1650, encodes a putative metalloprotease, collagenase. This putative protein consists of 818 amino acids and has a calculated molecular mass of 92.997 kDa. According to the MEROPS protease database classification (http://merops.sanger.ac.uk/cgi-bin/famsum?family=m9), VC1650 belongs to the M9 family within the M9A clan of metalloproteases. Analysis of the deduced amino acid sequence of VC1650 revealed the presence of an N-terminal signal peptide (residues 1 to 29), an N-terminal peptidase M9 domain (activator domain; amino acids 31 to 218), and a peptidase M9 domain (residues 268 to 558) with three residues that are characteristic for the catalytic site present in class II *Vibrio* metalloproteases (underlined), HEYTH, located at amino acids 435, 436, and 439, respectively (Fig. 1A) (31). In addition, two FAXWXXT motifs, **F**DQ**W**AQS and **F**SA**W**LD**T** (conserved residues are in boldface), which are implicated in collagen binding (32), and two bacterial prepeptidase C-terminal (PPC) domains also were identified at amino acids 537 to 543, 555 to 561, 612 to 686, and 731 to 804, respectively. A comparison of the amino acid sequences of class II metalloproteases in different *Vibrio* species showed that VC1650 displays the highest similarity (over 70%) to the 61-kDa collagenase VMC from *V. mimicus* and 63-kDa PrtVp protease (VPPRT) from *V. parahaemolyticus* 93 (31–34).

We compared the predicted amino acid sequences of VC1650 orthologs among 380 proteomes of *Vibrio* isolates deposited in the UniProt database using Clustal Omega. These studies revealed that only the *V. cholerae* putative collagenase contains tandem repeats of triplet amino acids: aspartic acid, asparagine, and proline (a DNP motif) (Fig. 1A). This motif is located three residues downstream from the second putative collagen-binding site (Fig. 1A). Interestingly, the amount of DNP triplets varies from 2 to 9 in different *V. cholerae* strains. These simple sequence repeats, also designated VNTRs (for variable number of tandem repeats), were among a group of different VNTRs identified in a few additional loci of *V. cholerae* that recently have been utilized for strain typing and phylogeny studies of environmental and clinical isolates (35–37).

In the reports describing the characterization of collagenases from *V. parahaemolyticus* and *V. mimicus* by Kim et al. (31) and Lee et al. (32), respectively, the authors referred to VC1650 as VCC; however, this name has been used for the *V. cholerae* cytolysin/hemolysin (38, 39). Thus, we designated VC1650 *Vibrio cholerae* collagenase, or VchC.

VchC is a secreted protein that displays proteolytic activity. To initiate the functional characterization of VchC, we wanted to establish whether the ORF VC1650 encodes a secreted protease with collagenolytic activity. The inactivation of *vchC* was accomplished in *V. cholerae* N16961 using homologous recombination as described in Materials and Methods. Subsequently, the wild-type (wt) and isogenic Δ*vchC* mutant bacteria, both bearing the empty vector pMMB67-EH (p), were cultured in LB medium at 37°C and harvested at 4 h and 16 h from back dilution, which correspond to the early and late stationary phases of growth, respectively. Subsequently, the enzymatic activity of VchC was tested using as a substrate a highly quenched, fluorescein-labeled gelatin from pig skin, DQ gelatin. The proteolytic activity was measured as the change in fluorescence (ΔFU) over time and normalized by the density of the culture. At the early stationary phase, the proteolytic activities in the wt p and Δ*vchC* p supernatants were close to the background level, whereas at the late stationary stage the detected activities did not differ significantly between both strains and were 44 ± 9.8 ΔFU and 59 ± 10 ΔFU (means ± SEM), respectively (Fig. 1B). These experiments indicated that the lack of VchC did not affect the measured extracellular protease activity against DQ gelatin and suggested that VchC is not produced under standard laboratory growth conditions.

To further examine whether VC1650 encodes an active protease, the gene was cloned under the control of an IPTG-inducible promoter located on pMMB67-EH. The resulting plasmid, pVchC, was introduced via conjugation into the wt and isogenic Δ*vchC* strains of *V. cholerae* N16961. The production of VchC was induced with 100 μM IPTG, and the supernatants were separated from *V. cholerae* cells at the early and late stationary phases of growth. The overexpression of *vchC* resulted in a dramatic increase in the proteolytic activity, reaching 1,317 ± 118 and 1,365 ± 101.9 (ΔFU; means ± SEM) in the wt and Δ*vchC* mutant supernatants, respectively (Fig. 1B). Similar levels of VchC activity, 1,727 ± 134.2 and 1,254 ± 92.17 (ΔFU; means ± SEM), were detected in the wt and Δ*vchC* strains, respectively, when the supernatants were harvested at the late stationary phase of growth (Fig. 1B). Throughout our subsequent studies, unless specified otherwise, we used supernatants isolated from *V. cholerae* at the early stationary phase of growth.

To assess the presence of VchC in the extracellular milieu, supernatants of wt p and Δ*vchC* p strains, as well as both strains carrying pVchC, were resolved by SDS-PAGE, and the protein profiles were visualized by staining with colloidal Coomassie (Fig. 1C). As expected based on the enzymatic studies, VchC was not detected in the supernatants isolated from wt p and Δ*vchC* p strains, whereas an apparent band of approximately 90 kDa, which migrated according to the molecular weight of VchC lacking the predicted signal peptide, was present in the samples containing supernatants derived from both strains of *V. cholerae* overproducing VchC (Fig. 1C).

The proteolytic activity of VchC also was assessed by in-gel zymography, because this technique (in contrast to the enzymatic assay with DQ gelatin) enables examination of molecular weight and the visualization of variant forms of the hydrolases present in a sample (40). The supernatants isolated from wt p, Δ*vchC* p, wt pVchC, and Δ*vchC* pVchC strains were separated in 10% Tris-glycine gels copolymerized with 0.1% gelatin as a protease substrate. After the refolding of proteins, the zones of gelatinolytic activity were revealed by staining the gel with Coomassie brilliant blue G-250. A prominent clearing zone of 90 kDa and a barely visible band of about 66 kDa were observed solely in the extracellular fractions harvested from wt and Δ*vchC* strains ectopically overexpressing *vchC* (Fig. 1D). Together, these results demonstrated that VchC is an active, extracellular protease capable of

cleaving gelatin and is not produced by *V. cholerae* under standard laboratory growth conditions.

The consensus zincin motif is crucial for VchC activity but not for protein secretion. The conserved HEXXH motif, which is characteristic for the catalytic center of metalloproteases belonging to the zincin superfamily (41), lies within the peptidase M9 domain of VchC and contains the HEYTH sequence (Fig. 1 A). In other studied zincins, the two histidine residues coordinate a zinc ion while the glutamate residue acts as a catalytic base (1, 21, 41). To evaluate the importance of these residues in VchC, a site-directed mutagenesis approach was utilized and individual amino acids were replaced with alanine. Subsequently, the respective mutated forms of VchC (H435A, E436A, and H439A) were cloned into pMMB67-EH, introduced into wt *V. cholerae*, and overexpressed by the addition of 100 μM IPTG, and the effect of these point mutations on VchC enzymatic activity was examined in the harvested culture supernatants (Fig. 1B). Compared to wild-type VchC, the mutant enzymes VchC H435A, VchC E436A, and VchC H439A displayed protease activity reduced to 0.76, 0.32, and 0.68%, respectively.

To verify whether the alteration of the HEYTH motif affects secretion of VchC, the supernatants were resolved by 10% SDS-PAGE and the protein profiles were revealed by staining with colloidal Coomassie (Fig. 1C). A 90-kDa VchC band was present in all samples, indicating that while the two histidine and glutamate residues are crucial for VchC enzymatic function, they do not affect protease secretion. In addition, in-gel zymography confirmed the lack of a detectable gelatinolytic activity of mutated versions of VchC (Fig. 1D).

Purification of a secreted, chimeric VchC from *V. cholerae* culture supernatants. Several different attempts have been made to express and purify a recombinant version of VchC containing a C-terminal 6× His tag using *E. coli* BL21(DE3) as a heterologous host. However, the protein was not produced in sufficient quantities and localized mainly to the insoluble fraction (data not shown). Therefore, as an alternative approach, we engineered by PCR and cloned into a broad-host-range vector (pMMB67-EH) a version of VchC containing the native signal peptide and ribosome binding site as well as the C-terminal 6× His epitope. The resulting plasmid, pVchC-His, subsequently was introduced via triparental mating into wt *V. cholerae* N16961, and the overproduction of VchC-His was conducted at 37°C in LB supplemented with 100 μM IPTG. Supernatant containing the secreted, chimeric VchC-His was harvested when the bacterial culture reached an early stationary stage of growth (OD_{600} of about 4), and the proteins were precipitated by 40% saturation with ammonium sulfate. After dialysis, VchC-His was purified to homogeneity using Ni^{2+}-NTA chromatography. The protease migrated in SDS-PAGE as an approximately 90-kDa protein according to its deduced amino acid sequence, which lacked signal peptide and contained the histidine epitope (Fig. 2A) and was capable of digesting DQ gelatin in a dose-dependent manner (Fig. 2B). The zymography analysis enabled detection of a faint gelatinolytic band with a molecular mass of about 66 kDa (Fig. 2C), in addition to a dominant clearing zone corresponding to the 90 kDa form of VchC. This clearing area likely was derived from the mature form of VchC, which lacks the PPC domains (see Fig. 5).

The activity of VchC is inhibited in the presence of compounds chelating divalent metal ions. To further characterize the molecular properties of VchC, metal ion chelators as well as protease inhibitors of different specificities were utilized. In these studies, the purified VchC-His (100 fmol) and culture supernatants collected from *V. cholerae* harboring pVchC were incubated with individual compounds for 1 h at 37°C, followed by the assessment of the protease activity toward DQ gelatin (Fig. 3A and B, respectively). The proteolytic activity of VchC was not significantly affected in the presence of serine (PMSF, benzamidine, and leupeptin) and cysteine protease inhibitors (leupeptin and *N*-ethylmaleimide). In contrast, the ability to digest DQ gelatin was markedly diminished by chelators of divalent metal ions (EDTA), an agent more selective toward Ca^{2+} (EGTA), and completely inhibited by the Zn^{2+} chelator (1,10-phenanthroline). Collectively, these experiments confirmed that VchC is a metalloprotease that requires divalent cations, including zinc, for its activity.

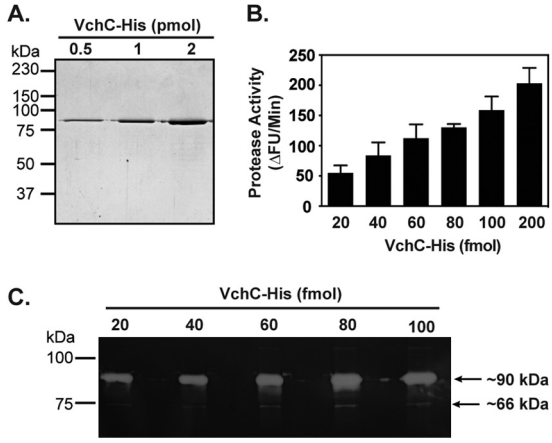

FIG 2 Purification of recombinant VchC-His secreted by *V. cholerae*. The engineered recombinant version of VchC containing native ribosome binding site and signal peptide, as well as an engineered C-terminal 6× His epitope, was cloned under the control of the P_{TAC} promoter carried on pMMB67-EH to create pVchC-His and introduced into wt *V. cholerae* N16961. The bacteria were grown at 37°C until the early stationary phase of growth in LB media supplemented with 100 μM IPTG. The supernatant was separated from bacterial cells by centrifugation, and the proteins were precipitated using ammonium sulfate. The precipitate was solubilized and dialyzed in 20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM imidazole, and subsequently VchC-His was purified using Ni^{2+}-NTA affinity chromatography. (A) The purity of eluted and concentrated samples containing 0.5, 1, and 2 pmol VchC-His was assessed by 10% SDS-PAGE and colloidal Coomassie staining. (B) The proteolytic activity of VchC-His against DQ gelatin was examined using increased amounts of the enzyme (as indicated). The means ± SEM are presented (n = 3). (C) The purified VchC-His was examined by in-gel zymography using 10% Tris-glycine gel copolymerized with 0.1% gelatin. Arrows indicate two detectable forms of VchC. The migration of molecular mass markers is presented on the left of each panel.

Secretion of VchC is affected in *V. cholerae* lacking a functional T2S pathway. We hypothesized that VchC is a T2S-dependent exoprotein. Preliminary studies using gelatin zymography as well as DQ gelatin hydrolysis established that there were dramatic differences in the extracellular protease activity detected in the supernatants isolated from the late-stationary-phase cultures of wt *V. cholerae* and isogenic T2SS mutants (see Fig. S1 in the supplemental material). We concluded that the proteolytic activity observed in wt *V. cholerae* was not associated with VchC based on findings that the inactivation of *vchC* had no effect on the degradation of the gelatin (Fig. 1B; also see Fig. S1A in the supplemental material). The observed collagenolytic/gelatinolytic activity likely

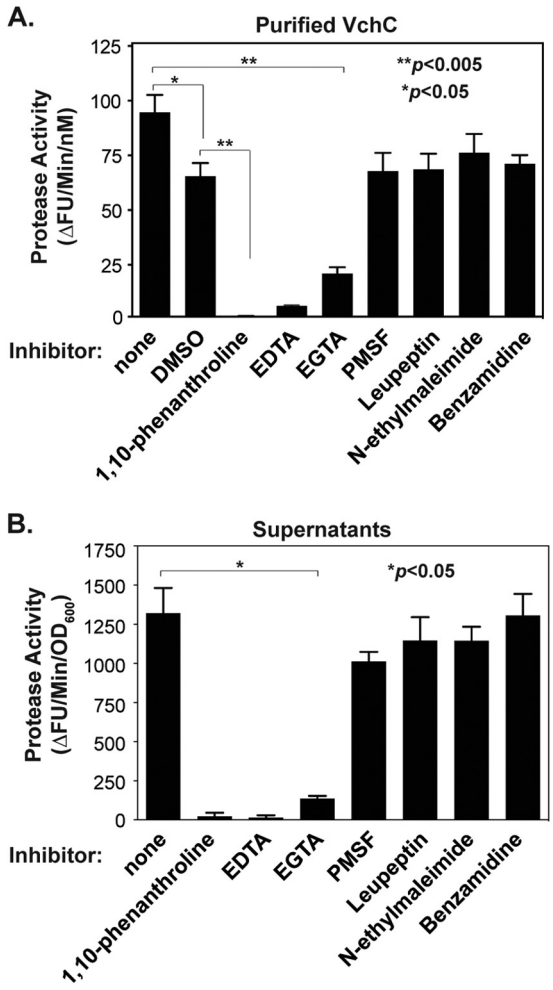

FIG 3 Presence of divalent metal chelators is detrimental to VchC activity. The effect of various inhibitors, including 1,10 phenanothroline (5 mM), EDTA (5 mM), EGTA (5 mM), leupeptin (1 mM), phenylmethylsulfonyl fluoride (5 mM), *N*-methylmaleimide (5 mM), and benzamidine (5 mM) on VchC proteolytic activity was studied using a purified protein (A) and in culture supernatants (B). (A) The purified, recombinant VchC-His (100 fmol) was incubated for 1 h at 37°C in the presence and absence of protease inhibitors (as indicated), followed by the assessment of enzymatic activity toward DQ gelatin. (B) Supernatant isolated at the early stationary phase of growth from *V. cholerae* N16961 wt pVchC was incubated for 1 h at 37°C alone and with either metallo-, serine-, or cysteine protease inhibitors. Subsequently, VchC activity was examined using DQ gelatin. Experiments were performed in technical triplicates on at least three separate occasions, and the bar graphs with error bars represent the means ± SEMs.

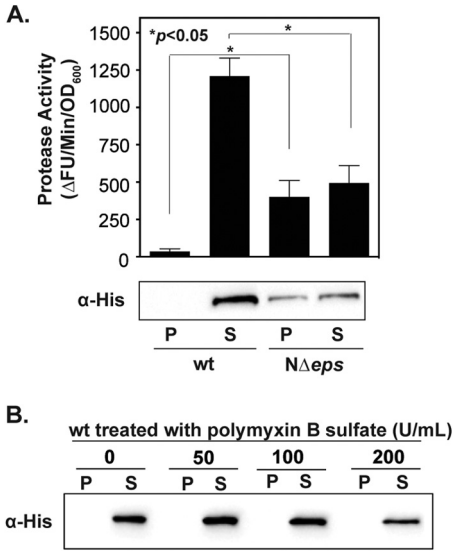

FIG 4 Extracellular localization of VchC is affected by the lack of a functional T2SS and not by cell envelope stress. (A) The activity and localization of VchC-His was examined in the periplasmic (P) and the supernatant (S) fractions isolated at the early stationary stage of growth from *V. cholerae* N16961 wt and the NΔ*eps* isogenic T2SS knockout strain, harboring pVchC-His. Protease activity assays using DQ gelatin as a substrate were performed in technical triplicates in four independent experiments, and means ± SEMs are presented. The samples containing periplasmic and supernatant fractions were normalized by the OD_{600} and resolved by 10% SDS-PAGE, and the presence of VchC-His was analyzed by Western blotting with monoclonal anti-His antibodies. (B) To induce cell envelope stress, wt *V. cholerae* N16961 carrying pVchC-His was treated with increasing concentrations of a membrane integrity-perturbing agent, polymyxin B sulfate (U/ml). Subsequently, supernatants (S) and periplasmic (P) fractions were harvested, matched by equivalent OD_{600} units, and resolved by 10% SDS-PAGE, followed by immunoblot analysis with anti-His antisera. Representative immunoblots are shown.

was associated with another protease(s) secreted in a T2S-dependent manner.

The genetic inactivation of the T2SS results in pleiotropic effects on *V. cholerae* physiology, including growth defects and pronounced alterations in the outer membrane, which leads to the leakage of 55 to 65% of the periplasmic constituent and various amounts of different T2S exoproteins to the culture media (25, 30, 42). Therefore, the evaluation of potential T2S substrates imposes an experimental challenge. To test further whether the T2S complex is required for the transport of VchC to the extracellular milieu, the wt and an isogenic mutant lacking the entire T2S apparatus, the NΔ*eps* strain, both bearing pVchC-His, were utilized. In the initial studies, as in our experiments with T2S-dependent serine proteases VesA, VesB, and VesC (30), the overproduction of VchC-His was induced with 100 μM IPTG, and the cultures were maintained until the late stationary phase of growth. Under these conditions, however, the NΔ*eps* strain harboring pVchC-His repeatedly failed to propagate, whereas the corresponding strain carrying the empty vector reached a cell density similar to that of the wt strain (data not shown). This observation suggested that the overexpression of *vchC* had deleterious effects on the viability of the T2S mutant, likely due to the accumulation of the protease in the periplasm. Finally, under optimized conditions, which included adjusting the initial density of the culture to an OD_{600} of ~0.4, inducing expression of *vchC* with 50 μM IPTG, and harvesting the bacteria at the early stationary phase of growth, the NΔ*eps* strain multiplied sufficiently and produced detectable amounts of VchC-His. At this point in the experiment, the supernatants and periplasmic fractions were extracted from both the wt and NΔ*eps* strains, and the samples were separated by SDS-PAGE followed by immunoblot analysis with monoclonal anti-His antisera (Fig. 4A). As expected, in wt *V. cholerae*, VchC-His localized entirely to the extracellular milieu. In contrast, in the NΔ*eps* strain, the protein was detected at similar levels in both the periplasmic and the supernatant fractions, suggesting that the transport of the protease across the outer membrane was affected. Supporting these findings, the activity of VchC-His against DQ

gelatin showed a 9.67-fold increase and 2.6-fold reduction, respectively, in the periplasm and the supernatant fractions isolated from the NΔ*eps* strain compared to the corresponding samples of wt *V. cholerae* (Fig. 4A).

We also considered that the observed accumulation of VchC in the periplasm of the NΔ*eps* strain might not be directly caused by the lack of T2SS but was related to the alterations of the outer membrane, which in turn could have a negative impact on the transport of the protease via a different secretion mechanism. To address this possibility, a chemical treatment of wt *V. cholerae* with polymyxin B sulfate was utilized. This antimicrobial peptide induces perturbations in cell envelope homeostasis that closely resemble the phenotypes observed in the *V. cholerae* T2S mutants, including stimulation of RpoE-driven cell envelope stress response, increased sensitivity to oxidants, and dysregulation of iron metabolism (43). We have established that although the El Tor *V. cholerae* N16961 is quite resistant to treatment with polymyxin B, the extracytoplasmic and oxidative stress responses already are triggered by 100 U/ml of the compound without affecting bacterial viability (25, 43). In particular, the concentrations of 100 and 200 U/ml of polymyxin B sulfate result in a more than 10-fold upregulation of RpoE (43). Accordingly, to stimulate the cell envelope stress, wt *V. cholerae* was cultured in the presence of an increasing concentration of the antimicrobial peptide and the localization of VchC-His was examined in the periplasmic and supernatant fractions by SDS-PAGE and immunoblotting (Fig. 4B). These studies demonstrated that VchC-His was present solely in the extracellular milieu, albeit at lower levels at the highest tested compound concentration. Taken together, these results support the hypothesis that T2SS is required to translocate VchC from the periplasmic space to the extracellular environment.

VchC undergoes a maturation process. Bacterial extracellular proteases often undergo multiple proteolytic cleavage steps that lead to the conversion of a pre-proenzyme (an inactive precursor) into a mature protein (44). In *V. cholerae*, both well-characterized metalloproteases, HapA and PrtV, are subjected to a maturation process that includes several N- and C-terminal modification steps during translocation across the cell envelope and prolonged incubation (45–47).

To assess the processing of VchC, cultures of wt *V. cholerae* carrying either pVchC-His or the empty vector pMMB67-EH (p), which served as a control for the experiment, were grown at the same time in LB medium supplemented with 100 μM IPTG, and the supernatants were isolated at 4, 5, 6, 8, 10, and 16 h from back dilution. Apparent changes in the abundance of 90-kDa and 66-kDa proteins were revealed by SDS-PAGE coupled with colloidal Coomassie staining and densitometry analysis in the extracellular fractions isolated from the wt overproducing VchC-His (Fig. 5A and B). At the early stationary phase of growth, as expected from our previous studies (Fig. 1B and 2A), the 90-kDa form of VchC constituted 94.55% ± 2.11% (means ± SEM) of secreted VchC (Fig. 5A and B). In addition, a very faint 66-kDa protein, which was absent from the control supernatants, also was detected. A progressive inversion of the distribution of these two proteins was observed in samples collected between 5 and 8 h of growth, and finally after 10 h the 66-kDa protein accounted for 91.23% ± 2.11% of VchC (Fig. 5A and B). In-gel zymography showed a clearing zone pattern that mirrored these observations (Fig. 5C), confirming that the 66-kDa protein corresponded to the processed form of VchC. The proteolytic activity toward DQ gelatin did not differ significantly throughout the time course of this experiment, suggesting that these two different forms of VchC displayed similar levels of protease activity (Fig. 5C).

Based on the analysis of the molecular weight of the mature protease, we hypothesized that the PPC domains were cleaved off from VchC to form the 66-kDa protein. To test this hypothesis, monoclonal anti-His antisera were utilized to detect the chimeric version of the protease in culture supernatants isolated from *V. cholerae* bearing pVchC-His (Fig. 5D). The antisera cross-reacted with the 90-kDa form of VchC-His in supernatants collected at 4 and 5 h of growth (Fig. 5D). However, in samples harvested just 1 h later, the full-length form of VchC-His began to vanish and became untraceable by 10 h, which also supports the densitometry analysis (Fig. 5B). Because the His epitope was engineered to the C terminus of the protein, we concluded that the PPC domains were cleaved from VchC.

Finally, to dissect whether the maturation of VchC is an autocatalytic process and/or depends on an additional extracellular factor(s), including exoproteins secreted by the T2SS, the purified 90-kDa VchC-His was added to the supernatants isolated from the early stationary cultures of wt, NΔ*eps*, Δ*vchC* p, and Δ*vchC* pVchC strains. After incubation for 1 h at 37°C, the samples were resolved by SDS-PAGE and the forms of VchC were detected by staining with colloidal Coomassie dye solution (Fig. 5E). The vast majority of the protease in these samples was in the 66-kDa form, whereas in the control sample containing the purified VchC-His incubated with LB media alone, the protease was mostly unprocessed (Fig. 5E).

Together, these studies suggest a scenario of VchC maturation that includes the removal of an N-terminal signal peptide during transport of the protease across the inner membrane and the cleavage of the PPC domains in the extracellular milieu, with the latter step being promoted by the presence of an additional factor(s) that does not depend on the T2S pathway (Fig. 5F).

Assessment of the substrate specificities reveals type I collagen as a potential biological target of VchC. To gain insights into the biological functions of VchC, we aimed to identify its potential target(s). First, we examined the ability of the protease to digest FALGPA, a synthetic peptide substrate that is specifically cleaved by bacterial collagenases (29). The activity of the purified VchC-His was monitored as a decrease in absorbance, which is associated with the cleavage of FALGPA, and the amount of processed substrate during 1 h of incubation was calculated (Fig. 6A). The VchC collagenolytic activity was evident compared to that of the positive control, represented by collagenase D from *C. histolyticum* and the negative control, trypsin. Both proteases, VchC and collagenase D, degraded 1.22 ± 0.014 and 1.22 ± 0.030 mM FALGPA (means ± SEM) within 30 and 45 min, respectively. As expected, no change in the FALGPA concentration was detected in samples containing trypsin.

Subsequently, to examine the ability of VchC to digest native collagen, the type I collagen was extracted from fish skin and incubated with VchC-His for 1 h at 37°C. As shown in Fig. 6B, complete degradation of 2 μg of collagen was observed in the presence of 2 pmol VchC. Similar results were obtained when type I collagen secreted from human fibroblasts was incubated with the protease. The specificity of VchC toward collagen as a natural substrate was further assessed in the titration experiments (Fig. 6C). The degradation of 5 μg of collagen was initiated by 4 fmol VchC and completely digested with 100 fmol of the protease. Corroborating our earlier observations (Fig. 3), the collagenolytic ac-

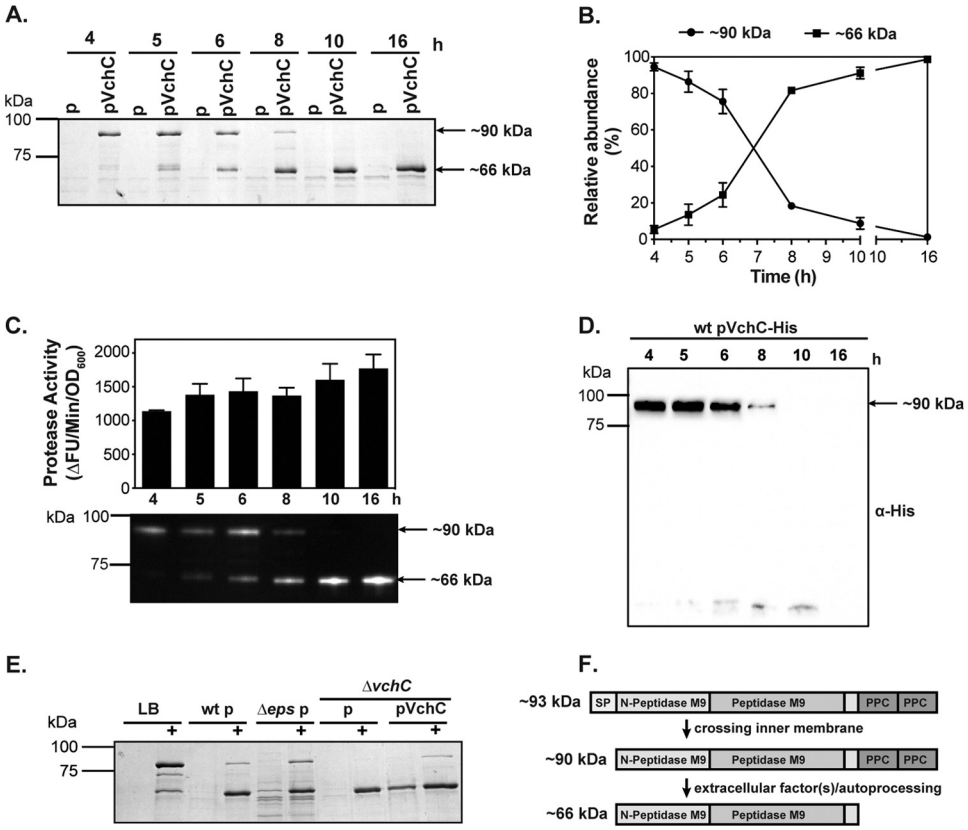

FIG 5 Elucidating maturation of VchC. (A to D) Time course analysis of VchC processing. The overnight cultures of *V. cholerae* N16961 carrying either p or pVchC-His were back diluted into fresh LB media supplemented with 100 μM IPTG and incubated with shaking at 37°C. At different time points from back dilution (4, 5, 6, 8, 10, and 16 h), samples were withdrawn and the supernatants were isolated and examined as follows. (A) Samples of harvested supernatants matched by equivalent OD_{600} units were separated using 10% SDS-PAGE, and the proteins were visualized with colloidal Coomassie staining. (B) The gels were scanned and the protein bands were subjected to densitometric analysis as described in Materials and Methods. The intensity of 90- and 66-kDa forms of VchC was calculated as a percentage of the total intensity for both protein bands at a given time point. The data represent the means with associated SEM obtained from three independent experiments. (C) The proteolytic activity against DQ gelatin (bar graph) or in-gel zymography using gelatin as a substrate was assessed in culture supernatants collected from wt pVch-His as described above. The differences in the measured protease activity between samples examined at the distinct times of the experiment were not statistically significant, as determined by Student's *t* test. (D) The supernatants obtained from wt pVch-His were resolved by 10% SDS-PAGE and transferred, and the presence of VchC-His was detected in immunoblot analysis using monoclonal anti-His antisera as primary antibodies. A representative immunoblot is shown. (E) To study the mode of maturation of VchC, the purified VchC (2 pmol) was incubated for 1 h at 37°C in the presence of either LB alone or supernatants isolated from early-stationary-phase cultures of wt p, Δ*eps* p, Δ*vchC* p, and Δ*vchC* pVchC strains. The reactions subsequently were separated by 10% SDS-PAGE, and the proteins were stained with colloidal Coomassie. A plus sign denotes a reaction mixture containing purified VchC-His. (F) A proposed scenario of VchC maturation. Signal peptide (SP) is cleaved upon translocation of the protein to the periplasm. Following the secretion of VchC into the extracellular milieu, the two prepeptidase C-terminal domains (PPC) are cleaved off from the protein with the aid of another secreted factor(s).

tivity of VchC against fish collagen was abolished in the presence of the zinc chelator, 1,10-phenanthroline, while the serine protease inhibitor (benzamidine) had no effect on protease activity. In contrast, the protease (2 pmol) failed to degrade other tested protein candidates, including mucin, fibronectin, lactoferrin, IgA, and BSA (Fig. 6D). Cumulatively, these studies demonstrated that VchC is a new zinc-dependent metalloprotease of *V. cholerae* with a specific activity toward collagen.

Assessment of VchC function in intestinal colonization and transmission. To examine the role of VchC in *V. cholerae* intestinal colonization in the mammalian and fish hosts, infant mouse and zebrafish models, respectively, have been utilized. In the biological replicate experiments, groups of six mice were separately inoculated with either the wt N16961 strain or the isogenic Δ*vchC* mutant. Following intestinal colonization, 8.78×10^6 and 6.10×10^6 CFU were recovered for the corresponding strains (see Fig. S2A in the supplemental material), demonstrating that the lack of VchC did not diminish the ability of *V. cholerae* to survive and colonize the gastrointestinal tract of the mice. Similarly, there was no statistically significant difference between colonization efficacies of wt and Δ*vchC* strains in the zebrafish model as well as in the transmission of individual strains between infected and naive fish (see Fig. S2B and C, respectively). Together, these studies suggested that under the tested conditions, VchC does not contribute to the *V. cholerae* N16961 colonization or transmission.

***V. cholerae* is collagenovorous.** Finally, we examined whether *V. cholerae* can utilize collagen as a sole carbon and nitrogen source. In these studies, the wt p, Δ*vchC* p, and Δ*vchC* pVchC *V. cholerae* N16961 strains were cultured in a solution of M9 minimal salts containing either 0.4% fish collagen or Casamino Acids and 0.4% glucose. Bacteria were maintained at the temperature encountered in the aquatic environment (25°C) and the human host (37°C), and their survival was assessed every 24 h by the determination of the number of CFU recovered on LB agar plates. Overall,

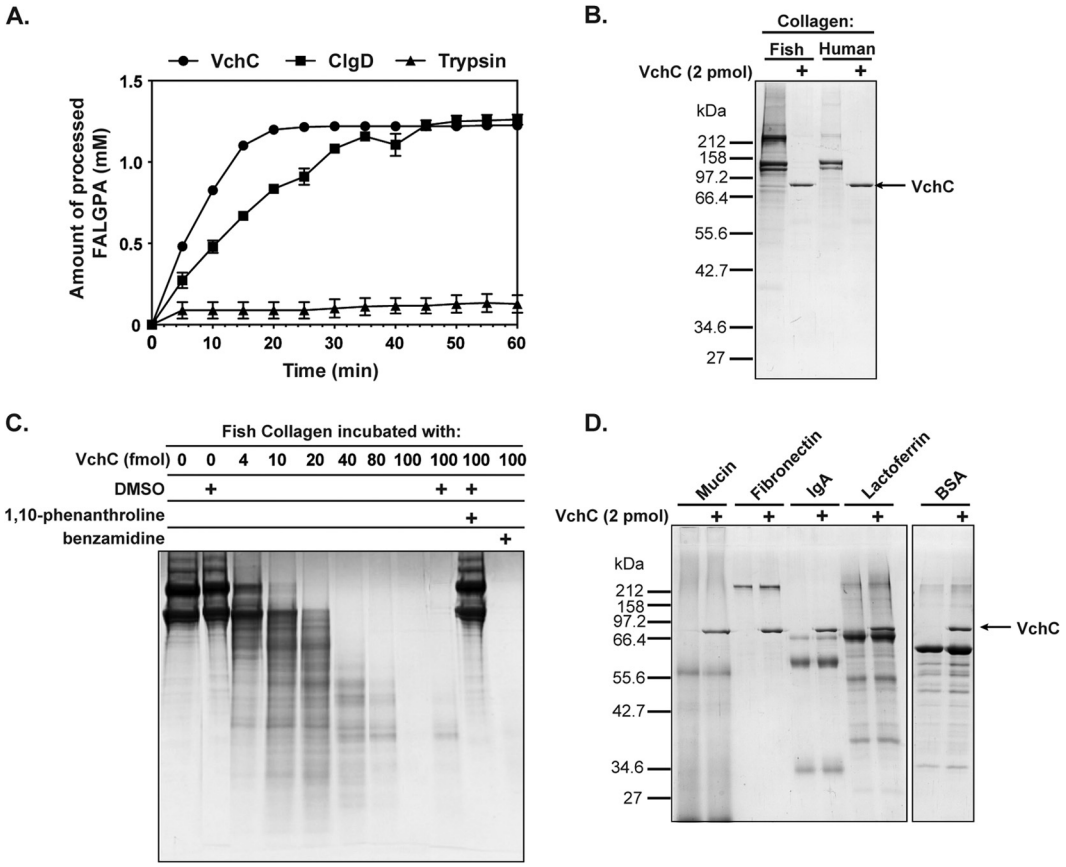

FIG 6 Analysis of the substrate specificity of VchC. (A) The ability of VchC to cleave a collagenase-specific substrate, synthetic peptide N-(3-(2-furyl)acryloyl)-Leu-Gly-Pro-Ala (FALGPA), was tested by adding the purified VchC-His at 60 μg/ml to 2.0 mM FALGPA in 50 mM Tricine, 400 mM NaCl, 10 mM $CaCl_2$, pH 7.5. The reaction mixtures were incubated at 37°C for 5 min, followed by monitoring the change in absorbance at 345 nm for 1 h every 5 min at 30°C. The amount of degraded FALGPA was calculated based on the calibration curve. Purified trypsin and collagenase D from *C. histolyticum* (ClgD) served as a negative and a positive control for the assay, respectively. All experiments were performed on three separate occasions, and means ± SEMs are presented. (B) Purified type I collagens from fish skin and commercially available, naturally secreted collegens from human fibroblasts, as indicated, were incubated with (+) and without purified VchC (2 pmol) for 1 h at 37°C. The reactions subsequently were examined by 10% SDS-PAGE coupled with colloidal Coomassie staining. (C) To demonstrate the specificity of VchC toward collagen, various amounts of the purified protease (as indicated above the gel) were added to the type I collagen extracted from fish skin. In addition, the ability of VchC to proteolytically cleave collagen was tested in the presence of a metalloprotease inhibitor (1,10-phenanthroline) and serine protease inhibitor (benzamidine). DMSO was added to the reaction mixture as a vehicle control for 1,10-phenanthroline. (D) Examining the ability of VchC to degrade mucin, fibronectin, immunoglobulin A (IgA), lactoferrin, and bovine serum albumin (BSA). Candidate substrate proteins (2 μg) were incubated with (+) and without the addition of purified VchC (2 pmol) for 1 h at 37°C. Subsequently, all reactions were separated by 10% SDS-PAGE and the proteins were stained with colloidal Coomassie. The migration of molecular mass markers is indicated. Arrows denote the visible, approximately ~90-kDa form of VchC.

the proliferation of *V. cholerae* in the presence of fish collagen was not as robust as that in the complete M9 medium (see Fig. S3A in the supplemental material). However, after 24 h there was a substantial (100-fold) increase in the number of recovered CFU of each of the bacterial strains grown at 25°C compared to the starting inoculum of 1,000 CFU/ml. Interestingly, in the corresponding cultures maintained at 37°C, the only significant increase of the CFU was observed in the Δ*vchC* pVchC strain at 72 h of growth. In addition, under all tested growth conditions, there was no statistically significant difference in the survival between wt and Δ*vchC* strains. The low detection limit of 7 ng/ml (2×10^{-3} U/ml) precluded the identification of collagenolytic/gelatinolytic activity against DQ gelatin in the supernatants isolated from all cultures at 48 h (data not shown). Subsequent in-gel zymography with gelatin as a substrate revealed the presence of several hydrolases in the supernatants of wt and Δ*vchC* strains grown in complete M9 medium, but none of them corresponded to the molecular weight of VchC (see Fig. S3B). Despite the higher sensitivity (5×10^{-6} U/ml) than that in the DQ gelatin assay, this method also failed to detect any proteolytic activity against gelatin in the cultures grown in the presence of fish collagen. This likely was due to notably lower cell densities and, as a result, minute amounts of secreted proteins.

These experiments demonstrated that *V. cholerae* is collagenovorous, as it is able to use collagen as a sole source of nutrients, and suggest the exciting possibility that in addition to VchC, another temperature-regulated factor(s) contributes to the degradation of fish collagen.

DISCUSSION

In aquatic ecosystems, the degradation of insoluble polymers, such as chitin and collagens, are critical for global carbon and nitrogen cycling (8). The members of the genus *Vibrio*, including

V. cholerae, are well-recognized participants in the chitin utilization program (8, 9, 13, 15, 17, 48–50). However, the mechanisms underlying the presumed collagenolytic cascade that would involve sensing, attachment, and degradation of collagens by *V. cholerae* and other marine microbes remain largely unknown. Collagens are the primary components of the extracellular matrices and the most abundant proteins in skin, cartilage, blood vessels, bone, dentin, and other organs in animals (23, 51–53). The degradation of collagens occurs during many physiological and pathological conditions. However, due to the complicated, hierarchical structure and insolubility in water, only a few proteases with unique features possess the ability to degrade collagens.

The true collagenases are enzymes that directly hydrolyze native collagen molecules with unique specificity and should be differentiated from other collagen-degrading proteases and from gelatinases, which decompose gelatin, a denatured form of collagen (21, 23, 54). The diversity of the structure of collagens makes it difficult to discriminate between these three types of enzymes. In addition, many bacterial collagenases are capable of digesting both collagen and gelatin and also are less specific than those from animal origin (21, 23, 55). Duarte et al. recently proposed a classification scheme to distinguish microbial collagenases by establishing a parallelism with vertebrate matrixins from the family M10 metalloproteases (23). Following this classification, the *V. cholerae* collagenase VchC, the subject of studies in this report, belongs to the zincin family of metallopeptidases, MEROPS MA(E), containing the conserved HEXXH signature (Fig. 1A). Based on the predicted amino acid sequence around the zinc binding residues, VchC falls into the gluzincin subclan of peptidases, as it contains a third zinc ligand, E465. Further, the multidomain architecture of VchC strongly suggests that the protease belongs to the M9A peptidase family (Fig. 1A).

The insights into mechanisms of collagen degradation by bacterial collagenases originate predominantly from studies on terrestrial collagenases from *C. histolyticum* (M9B family), whereas only isolated information is available regarding the structure-function relationship and the specificity of the *Vibrio* collagenases in the subfamily M9A. Accordingly, by applying a site-directed mutagenesis approach, we have demonstrated that, indeed, the residues H435, E436, and H439 within a predicted zinc binding motif, HEYTH in VchC, are critical for the protease activity but not for protein recognition and secretion by the T2SS (Fig. 1). Subsequent studies with different metal chelators and protease inhibitors confirmed that the collagenolytic activity of VchC depends on the presence of zinc and likely also calcium ions (Fig. 3 and 6). To the best of our knowledge, the role of the predicted catalytic residues as well as the route of the *Vibrio* collagenase secretion were experimentally verified for the first time. Further, several lines of evidence presented here suggested that in addition to VchC, the T2SS in *V. cholerae* secretes another collagenolytic/gelatinolytic protease(s) (Fig. 4; also see Fig. S1 in the supplemental material). This perhaps is expected, considering that the T2SS in many bacteria translocates to the extracellular milieu hydrolytic enzymes, such as cellulases, xylanases, and chitinases, which are responsible for digestion of insoluble polymers (4, 56–59).

Our studies suggest that expression of VchC is tightly regulated. Unless ectopically overexpressed, the activity of the protease could not be detected under any of the tested growth conditions (Fig. 1; also see Fig. S1 and S3 in the supplemental material). Based on sequence analysis of the ORF VC1650, the translation of VchC starts from the GUG. Among the total of 4,024 genes, only 161 possess this translation initiation codon, which implies its sparse usage by *V. cholerae* (60). One of the intriguing features of VchC compared to homologous proteins in *Vibrio* is the presence of different numbers of VNTRs encoding the DNP motif (Fig. 1A), which are distributed solely in various *V. cholerae* isolates. The VNTRs within coding regions, such as those observed in VchC, might serve beneficial functions in many aspects of adaptive bacterial behavior in response to changing environmental conditions. In addition, VNTRs are enriched in genes encoding cell surface proteins and function to modulate cell-cell adhesion, surface exposition of active protein domains, and antigenic variation to enable an evasion of the host immune system (61, 62). The function of VNTRs within VchC remains to be elucidated, but their presence solely in *V. cholerae* suggests a role(s) in the interaction between the bacterium and its host(s). In general, extracellular proteases produced by human-pathogenic *Vibrio* are considered virulence factors (1). Although the pathological functions were not studied in detail, it has been proposed that collagenases of two species causing wound infections, *V. parahaemolyticus* and *V. alginolyticus*, contribute to the spread of the bacteria by digestion of the components of the extracellular matrix (1). VchC did not contribute to the ability of *V. cholerae* N16961 to colonize the mouse and zebrafish intestines or to the transmission of the bacteria from infected to naive fish (see Fig. S2). This could be due to functional redundancy, as *V. cholerae* produces an additional collagenolytic/gelatinolytic protease(s) (see Fig. S1 to S3).

The time course experiments determined that VchC undergoes a maturation process in which the 90-kDa protein is converted to the C-terminally truncated 68-kDa protease lacking the PPC domains (Fig. 5A to D). Supporting these findings, the N-terminal amino acid sequencing analysis showed that the corresponding two forms of proteases observed in the supernatants isolated from early-stationary-phase cultures of *V. parahaemolyticus* were constituents of the VppC collagenase (63). Further, the processing of VchC was stimulated by a yet-to-be identified extracellular factor(s) (Fig. 5E). Incubation of the purified, unprocessed form of VchC with the supernatants isolated from *V. cholerae* lacking T2SS yielded the truncated version of VchC (Fig. 5E), which precluded involvement in this process of another T2S-dependent protease. Although maturation often is required in the transformation of the inactive proenzyme into its active form (2, 44), both the full-length and the truncated VchC were found to display similar collagenolytic activities (Fig. 5C). The two forms of VchC contain the collagenase unit:activator (M9N) and peptidase M9 domains, which have been shown in collagenase G from *C. histolyticum* as the only modules indispensable for collagen degradation (23, 64, 65). Very limited information is available regarding the tertiary structure of the *Vibrio* collagenases belonging to the M9A subfamily, and the function and biochemical properties of their domains, as well as the physiological importance of the C-terminal processing, remain to be addressed. It can be speculated that the removal of both PPC domains exposes the repeated FAXWXXT motif, which has been implicated in binding to insoluble type I collagen (32), and provides a better interaction with the substrate.

Finally, our studies demonstrate that VchC is a true collagenase which degrades a synthetic substrate specific for collagenases, FALGPA, and biologically relevant macromolecules, fish and human type I collagens (Fig. 6B and C). Fish collagens and gelatins contain proportions and compositions of amino acids similar to

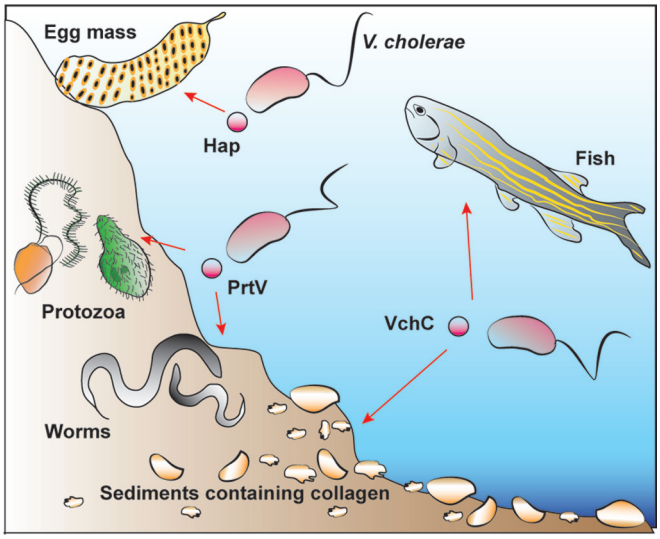

FIG 7 Extracellular metalloproteases may contribute to the fitness of *V. cholerae* in the aquatic niche. During the aquatic stage of the life cycle, *V. cholerae* persists freely and in association with insects' egg masses, fish, and sediment-inhabiting meiofauna, where organic matter, including collagens, accumulates. The secreted metalloproteases HapA, PrtV, and VchC may function as important contributors to *V. cholerae* persistence in the natural environment by generating nutrients for growth (HapA degrades the gelatinous matrix covering the eggs, and VchC digests collagen) or, like PrtV, by providing protection against grazing by marine bacterivorous predators (protozoa and nematodes).

those of mammalian collagen, with decreased amounts of proline and hydroxyproline but increased amounts of serine, threonine, methionine, and hydroxylysine (52). Type I collagen belongs to the group of fibrillar collagens that are distributed from sponges to humans and constitutes over 95% of total collagens in many animal tissues (66, 67). Collagens provide a rich nutrient source for bacteria that possess enzymes enabling their degradation. Thus, we propose that VchC facilitates *V. cholerae* survival in the aquatic niches and contributes to the recycling of marine nitrogen (Fig. 7). Among other metalloproteases implicated in environmental adaptation, *V. cholerae* produces the broad-specificity proteases HapA, belonging to the MEROPS family M4 (thermolysin family), and PrtV (M6 family) (1). HapA protease has been shown to cause degradation of the gelatinous matrix of the chironomid egg masses, one of the reservoirs of *V. cholerae*, whereas PrtV protected the bacteria from grazing by the predators worms and protozoa (Fig. 7) (5, 68, 69).

Our results provide the first evidence of a *V. cholerae* connection with collagen as well as molecular and functional insights into the *Vibrio* M9A collagenases. Because *Vibrio* species produce collagenases and are ubiquitous inhabitants of various aquatic niches, their ecological impact also should be considered in terms of the decomposition of insoluble collagen sediments and further contribution to global nitrogen and carbon circulation.

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REFERENCES

1. **Shinoda S, Miyoshi S.** 2011. Proteases produced by *Vibrios*. Biocontrol Sci **16:**1–11. http://dx.doi.org/10.4265/bio.16.1.
2. **Miyoshi S.** 2013. Extracellular proteolytic enzymes produced by human pathogenic *Vibrio* species. Front Microbiol **4:**339. http://dx.doi.org/10.3389/fmicb.2013.00339.
3. **World Health Organization.** 2012. Cholera annual report 2011. Wkly Epidemiol Rec **87:**289–304.
4. **Sikora AE.** 2013. Proteins secreted via the type II secretion system: smart strategies of *Vibrio cholerae* to maintain fitness in different ecological niches. PLoS Pathog **9:**e1003126. http://dx.doi.org/10.1371/journal.ppat.1003126.
5. **Broza M, Halpern M.** 2001. Pathogen reservoirs. Chironomid egg masses and *Vibrio cholerae*. Nature **412:**40. http://dx.doi.org/10.1038/35083691.
6. **Vezzulli L, Guzman CA, Colwell RR, Pruzzo C.** 2008. Dual role colonization factors connecting *Vibrio cholerae*'s lifestyles in human and aquatic environments open new perspectives for combating infectious diseases. Curr Opin Biotechnol **19:**254–259. http://dx.doi.org/10.1016/j.copbio.2008.04.002.
7. **Senderovich Y, Izhaki I, Halpern M.** 2010. Fish as reservoirs and vectors of *Vibrio cholerae*. PLoS One **5:**e8607. http://dx.doi.org/10.1371/journal.pone.0008607.
8. **Pruzzo C, Vezzulli L, Colwell RR.** 2008. Global impact of *Vibrio cholerae* interactions with chitin. Environ Microbiol **10:**1400–1410. http://dx.doi.org/10.1111/j.1462-2920.2007.01559.x.
9. **Yu C, Lee AM, Bassler BL, Roseman S.** 1991. Chitin utilization by marine bacteria. A physiological function for bacterial adhesion to immobilized carbohydrates. J Biol Chem **266:**24260–24267.
10. **Chen XL, Xie BB, Bian F, Zhao GY, Zhao HL, He HL, Zhou BC, Zhang YZ.** 2009. Ecological function of myroilysin, a novel bacterial M12 metalloprotease with elastinolytic activity and a synergistic role in collagen hydrolysis, in biodegradation of deep-sea high-molecular-weight organic nitrogen. Appl Environ Microbiol **75:**1838–1844. http://dx.doi.org/10.1128/AEM.02285-08.
11. **Ran LY, Su HN, Zhou MY, Wang L, Chen XL, Xie BB, Song XY, Shi M, Qin QL, Pang X, Zhou BC, Zhang YZ, Zhang XY.** 2014. Characterization of a novel subtilisin-like protease myroicolsin from deep sea bacterium *Myroides profundi* D25 and molecular insight into its collagenolytic mechanism. J Biol Chem **289:**6041–6053. http://dx.doi.org/10.1074/jbc.M113.513861.
12. **Ran LY, Su HN, Zhao GY, Gao X, Zhou MY, Wang P, Zhao HL, Xie BB, Zhang XY, Chen XL, Zhou BC, Zhang YZ.** 2013. Structural and mechanistic insights into collagen degradation by a bacterial collagenolytic serine protease in the subtilisin family. Mol Microbiol **90:**997–1010. http://dx.doi.org/10.1111/mmi.12412.
13. **Bassler BL, Gibbons PJ, Yu C, Roseman S.** 1991. Chitin utilization by marine bacteria. Chemotaxis to chitin oligosaccharides by *Vibrio furnissii*. J Biol Chem **266:**24268–24275.
14. **Li X, Roseman S.** 2004. The chitinolytic cascade in *Vibrios* is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor/kinase. Proc Natl Acad Sci U S A **101:**627–631. http://dx.doi.org/10.1073/pnas.0307645100.
15. **Meibom KL, Li XB, Nielsen AT, Wu CY, Roseman S, Schoolnik GK.** 2004. The *Vibrio cholerae* chitin utilization program. Proc Natl Acad Sci U S A **101:**2524–2529. http://dx.doi.org/10.1073/pnas.0308707101.
16. **Lipp EK, Huq A, Colwell RR.** 2002. Effects of global climate on infectious disease: the cholera model. Clin Microbiol Rev **15:**757–770. http://dx.doi.org/10.1128/CMR.15.4.757-770.2002.
17. **Vezzulli L, Pezzati E, Moreno M, Fabiano M, Pane L, Pruzzo C, VibrioSea Consortium.** 2009. Benthic ecology of *Vibrio* spp. and pathogenic *Vibrio* species in a coastal Mediterranean environment (La Spezia Gulf, Italy). Microb Ecol **58:**808–818. http://dx.doi.org/10.1007/s00248-009-9542-8.
18. **Collin B, Rehnstam-Holm AS, Lindmark B, Pal A, Wai SN, Hernroth B.** 2012. The origin of *Vibrio cholerae* influences uptake and persistence in the blue mussel *Mytilus Edulis*. J Shellfish Res **31:**87–92. http://dx.doi.org/10.2983/035.031.0111.
19. **Lutz C, Erken M, Noorian P, Sun S, McDougald D.** 2013. Environmen-

tal reservoirs and mechanisms of persistence of *Vibrio cholerae*. Front Microbiol **4:**375. http://dx.doi.org/10.3389/fmicb.2013.00375.
20. **Martin WR, Sayles FL.** 2003. The recycling of biogenic material at the seafloor, p 37–65. *In* Holland HD, Turekian KK (ed), Treatise on geochemistry, vol 7. Pergamon, Oxford, United Kingdom.
21. **Watanabe K.** 2004. Collagenolytic proteases from bacteria. Appl Microbiol Biotechnol **63:**520–526. http://dx.doi.org/10.1007/s00253-003-1442-0.
22. **Rawlings ND, Barrett AJ, Bateman A.** 2012. MEROPS: the database of proteolytic enzymes, their substrates and inhibitors. Nucleic Acids Res **40:**D343–D350. http://dx.doi.org/10.1093/nar/gkr987.
23. **Duarte AS, Correia A, Esteves AC.** 22 April 2014. Bacterial collagenases–a review. Crit Rev Microbiol http://dx.doi.org/10.3109/1040841X.2014.904270.
24. **Park BR, Zielke RA, Wierzbicki IH, Mitchell KC, Withey JH, Sikora AE.** 2014. Abstr 114th Gen Meet Am Soc Microbiol, poster B-1544.
25. **Sikora AE, Lybarger SR, Sandkvist M.** 2007. Compromised outer membrane integrity in *Vibrio cholerae* type II secretion mutants. J Bacteriol **189:**8484–8495. http://dx.doi.org/10.1128/JB.00583-07.
26. **Kobayashi T, Enomoto S, Sakazaki R, Kuwahara S.** 1963. A new selective isolation medium for the *Vibrio* group; on a modified Nakanishi's medium (TCBS agar medium). Jpn J Bacteriol **18:**387–392.
27. **Suphatharaprateep W, Cheirsilp B, Jongjareonrak A.** 2011. Production and properties of two collagenases from bacteria and their application for collagen extraction. New Biotechnol **28:**649–655. http://dx.doi.org/10.1016/j.nbt.2011.04.003.
28. **Zielke RA, Simmons RS, Park BR, Nonogaki M, Emerson S, Sikora AE.** 2014. The type II secretion pathway in *Vibrio cholerae* is characterized by growth phase-dependent expression of exoprotein genes and is positively regulated by sigmaE. Infect Immun **82:**2788–2801. http://dx.doi.org/10.1128/IAI.01292-13.
29. **Van Wart HE, Steinbrink DR.** 1981. A continuous spectrophotometric assay for *Clostridium histolyticum* collagenase. Anal Biochem **113:**356–365. http://dx.doi.org/10.1016/0003-2697(81)90089-0.
30. **Sikora AE, Zielke RA, Lawrence DA, Andrews PC, Sandkvist M.** 2011. Proteomic analysis of the *Vibrio cholerae* type II secretome reveals new proteins, including three related serine proteases. J Biol Chem **286:**16555–16566. http://dx.doi.org/10.1074/jbc.M110.211078.
31. **Kim SK, Yang JY, Cha J.** 2002. Cloning and sequence analysis of a novel metalloprotease gene from *Vibrio parahaemolyticus* 04. Gene **283:**277–286. http://dx.doi.org/10.1016/S0378-1119(01)00882-4.
32. **Lee JH, Ahn SH, Lee EM, Jeong SH, Kim YO, Lee SJ, Kong IS.** 2005. The FAXWXXT motif in the carboxyl terminus of *Vibrio mimicus* metalloprotease is involved in binding to collagen. FEBS Lett **579:**2507–2513. http://dx.doi.org/10.1016/j.febslet.2005.03.062.
33. **Lee CY, Su SC, Liaw RB.** 1995. Molecular analysis of an extracellular protease gene from *Vibrio parahaemolyticus*. Microbiology **141**(Part 10)**:** 2569–2576. http://dx.doi.org/10.1099/13500872-141-10-2569.
34. **Lee JH, Kim GT, Lee JY, Jun HK, Yu JH, Kong IS.** 1998. Isolation and sequence analysis of metalloprotease gene from *Vibrio mimicus*. Biochim Biophys Acta **1384:**1–6. http://dx.doi.org/10.1016/S0167-4838(97)00205-7.
35. **Danin-Poleg Y, Cohen LA, Gancz H, Broza YY, Goldshmidt H, Malul E, Valinsky L, Lerner L, Broza M, Kashi Y.** 2007. *Vibrio cholerae* strain typing and phylogeny study based on simple sequence repeats. J Clin Microbiol **45:**736–746. http://dx.doi.org/10.1128/JCM.01895-06.
36. **Stine OC, Alam M, Tang L, Nair GB, Siddique AK, Faruque SM, Huq A, Colwell R, Sack RB, Morris JG, Jr.** 2008. Seasonal cholera from multiple small outbreaks, rural Bangladesh. Emerg Infect Dis **14:**831–833. http://dx.doi.org/10.3201/eid1405.071116.
37. **Rashed SM, Azman AS, Alam M, Li S, Sack DA, Morris JG, Jr, Longini I, Siddique AK, Iqbal A, Huq A, Colwell RR, Sack RB, Stine OC.** 2014. Genetic variation of *Vibrio cholerae* during outbreaks, Bangladesh, 2010-2011. Emerg Infect Dis **20:**54–60. http://dx.doi.org/10.3201/eid2001.130796.
38. **Alm RA, Stroeher UH, Manning PA.** 1988. Extracellular proteins of *Vibrio cholerae*: nucleotide sequence of the structural gene (*hlyA*) for the haemolysin of the haemolytic El Tor strain 017 and characterization of the *hlyA* mutation in the non-haemolytic classical strain 569B. Mol Microbiol **2:**481–488. http://dx.doi.org/10.1111/j.1365-2958.1988.tb00054.x.
39. **Yamamoto K, Ichinose Y, Shinagawa H, Makino K, Nakata A, Iwanaga M, Honda T, Miwatani T.** 1990. Two-step processing for activation of the cytolysin/hemolysin of *Vibrio cholerae* O1 biotype El Tor: nucleotide sequence of the structural gene (*hlyA*) and characterization of the processed products. Infect Immun **58:**4106–4116.
40. **Vandooren J, Geurts N, Martens E, Van den Steen PE, Opdenakker G.** 2013. Zymography methods for visualizing hydrolytic enzymes. Nat Methods **10:**211–220. http://dx.doi.org/10.1038/nmeth.2371.
41. **Jung CM, Matsushita O, Katayama S, Minami J, Sakurai J, Okabe A.** 1999. Identification of metal ligands in the *Clostridium histolyticum* ColH collagenase. J Bacteriol **181:**2816–2822.
42. **Connell TD, Metzger DJ, Wang M, Jobling MG, Holmes RK.** 1995. Initial studies of the structural signal for extracellular transport of cholera toxin and other proteins recognized by *Vibrio cholerae*. Infect Immun **63:**4091–4098.
43. **Sikora AE, Beyhan S, Bagdasarian M, Yildiz FH, Sandkvist M.** 2009. Cell envelope perturbation induces oxidative stress and changes in iron homeostasis in *Vibrio cholerae*. J Bacteriol **191:**5398–5408. http://dx.doi.org/10.1128/JB.00092-09.
44. **Wandersman C.** 1989. Secretion, processing and activation of bacterial extracellular proteases. Mol Microbiol **3:**1825–1831. http://dx.doi.org/10.1111/j.1365-2958.1989.tb00169.x.
45. **Hase CC, Finkelstein RA.** 1991. Cloning and nucleotide sequence of the *Vibrio cholerae* hemagglutinin/protease (HA/protease) gene and construction of an HA/protease-negative strain. J Bacteriol **173:**3311–3317.
46. **Vaitkevicius K, Rompikuntal PK, Lindmark B, Vaitkevicius R, Song T, Wai SN.** 2008. The metalloprotease PrtV from *Vibrio cholerae*. The FEBS J **275:**3167–3177. http://dx.doi.org/10.1111/j.1742-4658.2008.06470.x.
47. **Edwin A, Rompikuntal P, Bjorn E, Stier G, Wai SN, Sauer-Eriksson AE.** 2013. Calcium binding by the PKD1 domain regulates interdomain flexibility in *Vibrio cholerae* metalloprotease PrtV. FEBS Open Bio **3:**263–270. http://dx.doi.org/10.1016/j.fob.2013.06.003.
48. **Bassler BL, Yu C, Lee YC, Roseman S.** 1991. Chitin utilization by marine bacteria. Degradation and catabolism of chitin oligosaccharides by *Vibrio furnissii*. J Biol Chem **266:**24276–24286.
49. **Li X, Wang LX, Wang X, Roseman S.** 2007. The chitin catabolic cascade in the marine bacterium *Vibrio cholerae*: characterization of a unique chitin oligosaccharide deacetylase. Glycobiology **17:**1377–1387. http://dx.doi.org/10.1093/glycob/cwm096.
50. **Vezzulli L, Pezzati E, Repetto B, Stauder M, Giusto G, Pruzzo C.** 2008. A general role for surface membrane proteins in attachment to chitin particles and copepods of environmental and clinical vibrios. Lett Appl Microbiol **46:**119–125. http://dx.doi.org/10.1111/j.1472-765X.2007.02269.x.
51. **Leys SP, Riesgo A.** 2012. Epithelia, an evolutionary novelty of metazoans. J Exp Zool B Mol Dev Evol **318:**438–447. http://dx.doi.org/10.1002/jez.b.21442.
52. **Eastoe JE.** 1955. The amino acid composition of mammalian collagen and gelatin. Biochem J **61:**589–600.
53. **Yung PY, Kjelleberg S, Thomas T.** 2011. A polyphasic approach to the exploration of collagenolytic activity in the bacterial community associated with the marine sponge *Cymbastela concentrica*. FEMS Microbiol Lett **321:**24–29. http://dx.doi.org/10.1111/j.1574-6968.2011.02306.x.
54. **Harrington DJ.** 1996. Bacterial collagenases and collagen-degrading enzymes and their potential role in human disease. Infect Immun **64:**1885–1891.
55. **Mookhtiar KA, Van Wart HE.** 1992. *Clostridium histolyticum* collagenases: a new look at some old enzymes. Matrix **1:**116–126.
56. **Sandkvist M.** 2001. Type II secretion and pathogenesis. Infect Immun **69:**3523–3535. http://dx.doi.org/10.1128/IAI.69.6.3523-3535.2001.
57. **Kazemi-Pour N, Condemine G, Hugouvieux-Cotte-Pattat N.** 2004. The secretome of the plant pathogenic bacterium *Erwinia chrysanthemi*. Proteomics **4:**3177–3186. http://dx.doi.org/10.1002/pmic.200300814.
58. **Jha G, Rajeshwari R, Sonti RV.** 2005. Bacterial type two secretion system secreted proteins: double-edged swords for plant pathogens. Mol Plant Microbe Interact **18:**891–898. http://dx.doi.org/10.1094/MPMI-18-0891.
59. **Rondelet A, Condemine G.** 2013. Type II secretion: the substrates that won't go away. Res Microbiol **164:**556–561. http://dx.doi.org/10.1016/j.resmic.2013.03.005.
60. **Ma J, Campbell A, Karlin S.** 2002. Correlations between Shine-Dalgarno sequences and gene features such as predicted expression levels and operon structures. J Bacteriol **184:**5733–5745. http://dx.doi.org/10.1128/JB.184.20.5733-5745.2002.
61. **van Belkum A, Scherer S, van Alphen L, Verbrugh H.** 1998. Short-sequence DNA repeats in prokaryotic genomes. Microbiol Mol Biol Rev **62:**275–293.
62. **Gemayel R, Cho J, Boeynaems S, Verstrepen KJ.** 2012. Beyond junk-variable tandem repeats as facilitators of rapid evolution of reg-

ulatory and coding sequences. Genes **3:**461–480. http://dx.doi.org/10.3390/genes3030461.

63. **Miyoshi S, Nitanda Y, Fujii K, Kawahara K, Li T, Maehara Y, Ramamurthy T, Takeda Y, Shinoda S.** 2008. Differential gene expression and extracellular secretion of the collagenolytic enzymes by the pathogen *Vibrio parahaemolyticus*. FEMS Microbiol Lett **283:**176–181. http://dx.doi.org/10.1111/j.1574-6968.2008.01159.x.
64. **Eckhard U, Schonauer E, Ducka P, Briza P, Nuss D, Brandstetter H.** 2009. Biochemical characterization of the catalytic domains of three different *Clostridial collagenases*. Biol Chem **390:**11–18. http://dx.doi.org/10.1515/BC.2009.004.
65. **Eckhard U, Brandstetter H.** 2011. Polycystic kidney disease-like domains of clostridial collagenases and their role in collagen recruitment. Biol Chem **392:**1039–1045. http://dx.doi.org/10.1515/BC.2011.099.
66. **Miller EJ, Gay S.** 1987. The collagens: an overview and update. Methods Enzymol **144:**3–41. http://dx.doi.org/10.1016/0076-6879(87)44170-0.
67. **Exposito JY, Valcourt U, Cluzel C, Lethias C.** 2010. The fibrillar collagen family. Int J Mol Sci **11:**407–426. http://dx.doi.org/10.3390/ijms11020407.
68. **Halpern M, Gancz H, Broza M, Kashi Y.** 2003. *Vibrio cholerae* hemagglutinin/protease degrades chironomid egg masses. Appl Environ Microbiol **69:**4200–4204. http://dx.doi.org/10.1128/AEM.69.7.4200-4204.2003.
69. **Vaitkevicius K, Lindmark B, Ou G, Song T, Toma C, Iwanaga M, Zhu J, Andersson A, Hammarstrom ML, Tuck S, Wai SN.** 2006. A *Vibrio cholerae* protease needed for killing of *Caenorhabditis elegans* has a role in protection from natural predator grazing. Proc Natl Acad Sci U S A **103:**9280–9285. http://dx.doi.org/10.1073/pnas.0601754103.