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Bo R. Park for the degree of Honors Baccalaureate of Science in Microbiology presented on March 25, 2014. Title: Characterization of a Novel Metalloprotease Secreted by the Type II Secretion System in *Vibrio cholerae*.

Abstract approved: _____

Aleksandra E. Sikora

Vibrio cholerae, a Gram-negative bacterium, is the etiological agent of cholera. *V. cholerae* shuttles between the human host and the aquatic reservoir, where it associates with marine organisms such as copepods and vertebrate fish. The bacteria use the Type II Secretion System (T2SS) to release proteins that facilitate *V. cholerae* survival. Here, we describe a novel T2SS-dependent protein, collagenase (Clg). The analysis of the predicted amino acid sequence of Clg revealed a signal peptide, pro-peptide, peptidase M9 domain with a zinc-metalloprotease HEXXH consensus, and two pre-peptidase C-terminal (PPC) domains. Clg was purified to apparent homogeneity from the culture supernatant of *V. cholerae* and its activity was examined using synthetic and putative natural substrates. The protease showed activity in zymogram assays, against DQ gelatin and FALGPA, as well as a purified fish collagen but not against tested human host derived proteins. The enzymatic activity of Clg was blocked in the presence of metalloprotease inhibitors. Additionally, site-directed mutagenesis of the Clg predicted catalytic residues followed by enzymatic assays and SDS-PAGE analyses demonstrated that the zinc-binding motif is crucial for protein activity, but not for protease secretion. The maturation of Clg, which leads to disassembly of the two PPC domains, was also demonstrated.

Key Words: *Vibrio cholerae*, metalloprotease, collagenase, cholera, type II secretion

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Characterization of a Novel Metalloprotease Secreted

by the Type II Secretion System in *Vibrio cholerae*

By

Bo R. Park

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APPROVED:

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Chair, Department of Microbiology

Dean, University Honors College

I understand that my project will become part of the permanent collection of Oregon State University, University Honors College. My signature below authorizes release of my project to any reader upon request.

Bo R. Park, Author

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Characterization of a Novel Metalloprotease Secreted by the Type II Secretion System in *Vibrio cholerae*

Introduction:

Vibrio cholerae

The Gram-negative bacterium *Vibrio cholerae* is the causative agent of cholera, the life-threatening diarrheal disease. Cholera cases are predominant in developing countries that lack clean water supplies (1). According to the World Health Organization (WHO), there are approximately 100,000-120,000 deaths due to cholera each year. One of the worst cholera outbreaks in a recent history was reported in Haiti, following the devastating earthquake in 2010. The Artibonite River, the main river of Haiti that serves as a key water supply for the survivors, was identified as the source of the contamination (1). With the lack of clean sanitation, the disease spread quickly, affecting not only the residents of Haiti, but also neighboring countries. Overall, the outbreak led to more than half a million infections and thousands of deaths. Cholera cases have also been reported in Mexico, India, Bangladesh, as well as countries in Africa and Southeast Asia. Among these countries, cholera deaths have most often been reported in Sierra Leone, Democratic Republic of Congo, Uganda, and Somalia (1). A key element in the survival of *V. cholerae* is its ability to circulate between the aquatic environment and the human host. In the natural reservoir, *V. cholerae* persists freely or in association with various aquatic organisms. Copepods, chironomid egg masses, and vertebrate fish serve as vectors of the bacterium (2, 3). Infection of the human host occurs upon ingestion of food or water contaminated by environmental sources of *V. cholerae*. The bacteria colonize the small intestine and utilize the Type II Secretion System (T2SS) to secrete cholera toxin. Cholera toxin is the major virulence factor of *V. cholerae*. It induces profuse diarrhea in

cholera patients and enable the bacteria to escape back into its natural reservoir. Aside from cholera toxin, the T2SS releases 19 various cargo proteins including chitinases, lipases, serine proteases (VesA, VesB, and VesC), and metalloproteases: hemagglutinin/protease (HapA), LapA, and LapX as well as other classes of proteases capable of degrading extracellular proteins (4). Few of these secreted proteins have been characterized to play a crucial role in *V. cholerae* survival by digesting environmental substrates for nutrition and growth. However, the function of many of the T2S substrates remains to be uncovered.

Metalloproteases

Proteases are enzymes that degrade proteins. Currently, there are six classes of proteases organized based on their active site: serine, threonine, cysteine, aspartate, glutamic acid, and metalloproteases (5). Bacterial extracellular metalloproteases are enzymes that harbor metal ions in the active site for catalysis (6). Metalloproteases are often secreted as inactive pro-enzymes (zymogens) that undergo a maturation process, which depends on another protease or autocleavage, to become active (6). The general function of this class of proteases involves digestion of environmental proteins for bacterial nutrition and pathogenesis. Metalloproteases possess a zinc-dependent active site with a HEXXH consensus sequence (6). Among *Vibrio* spp., metalloproteases have been described to contribute to the pathogenicity and survival of the bacterium. For example, *V. vulnificus* protease (VVP) contributes to skin lesions by destructing the basement membrane and capillary vessels (7, 8). Metalloproteases with similar function have been described in other vibrios including fish pathogens. Overall, these proteases facilitate pathogenicity either directly by digesting host proteins or indirectly by activating another virulence

factor(s) (7). The metalloproteases secreted by *V. cholerae* that act in both environments are HapA and PrtV. In the human host, HapA facilitates the penetration of *V. cholerae* through mucin, the thick lining of the small intestine (9). In addition it has been shown that HapA is capable of processing cholera toxin (10). Meanwhile, the protease degrades chironomid egg masses in the aquatic environment. It has also been shown that HapA degraded GbpA, a chitin binding protein released in vitro (11, 12). The PrtV protease interferes with the host innate immune responses and contributes to persistence of *V. cholerae* in the natural habitat via contact with marine organisms, such as protozoa, copepods, and crustaceans (13, 14). Aside from the human host, PrtV also plays a role in the pathology of *V. cholerae* in *Caenorhabditis elegans* in the aquatic reservoir, as the protease is required to kill the marine organism (15). Another example of a metalloprotease secreted by *V. cholerae* is the ToxR-activated gene A protein, TagA. TagA modifies the cell surface molecules in the human host, likely to facilitate *V. cholerae* infection (16). Overall, while TagA specifically cleaves mucin glycoproteins on human epithelial cells during *V. cholerae* infection, HapA and PrtV exhibit very broad activities towards different proteins including e.g., mucin, lactoferrin, and IgA (9, 14), which makes it difficult to define its precise role in *V. cholerae* pathology.

Collagenase

While elegant studies have described the mechanisms of *V. cholerae* survival in the aquatic environment and human host, there is limited knowledge regarding the factors required for bacterial fitness in both aspects of the life cycle. Among many marine and host nutrient sources, one of the most abundant proteins shared by both environments is collagen (17). Fish collagen in particular resembles mammalian collagen in both amino

acid composition and proportion (18). This carbon source may be used by the bacterium for nutrition. As fish serve as reservoirs of *V. cholerae*, consumption of contaminated fish may facilitate transmission into the human host. Herein, we describe the identification and characterization of a novel metalloprotease, collagenase (Clg), which is encoded by the gene VC1650. The protein consists of 818 amino acids with an approximate molecular weight of 93-kDa. Analysis of the predicted amino acid sequence of VC1650 revealed the following domains: signal peptide (SP), pro-peptide, peptidase M9, and two pre-peptidase C-terminal domains (PPC). Clg possesses a HEXXH motif within peptidase M9 domain, which is a signature of metalloproteases (19, 20).

Statement of Purpose

Vibrio cholerae is a Gram-negative bacterium that causes the life-threatening diarrheal disease, cholera. *V. cholerae* circulates between the human host and the aquatic reservoir, where it can associate with marine organisms including vertebrate fish. The bacteria use the T2SS to release many different proteins that facilitate *V. cholerae* survival in both environments. In the present study, we describe the identification and characterization of a novel T2SS-dependent cargo protein, collagenase (Clg). We hypothesize that Clg is a secreted metalloprotease that contributes to the physiology and pathogenesis of *V. cholerae*. The analysis of the predicted amino acid sequence of Clg revealed the presence of a signal peptide, pro-peptide, peptidase M9 domain with a zinc metalloprotease HEXXH consensus, and two pre-peptidase C-terminal domains. The activity of the recombinant Clg purified from culture supernatants of *V. cholerae* was examined using synthetic and putative natural substrates. These studies showed that Clg displayed enzymatic activity in zymogram assays as well as against DQ gelatin and FALGPA,

which is a substrate specific for bacterial collagenases. Interestingly, Clg also demonstrated proteolytic activity toward purified fish collagen but not against the human host derived proteins such as fibronectin, lactoferrin, and mucin. The proteolytic activity of Clg was blocked in the presence of metalloprotease inhibitors but not serine- or cysteine-protease inhibitors. In addition, site-directed mutagenesis of the Clg predicted catalytic residues H435A, E436A, and H439A followed by enzymatic assays and SDS-PAGE analyses, demonstrated that the catalytic motif is crucial for the protein activity but not for protease secretion. Our investigations also revealed that after secretion, Clg undergoes a maturation process and its two major proteolysis products display similar enzymatic activity.

Materials & Methods:

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. Strains were cultured at 37°C or 25°C in Luria-Bertani (LB) broth, M9 minimal media supplemented with 0.4% glucose (Difco) or 0.4% fish collagen, as indicated in the text. Antibiotics (Teknova, Hollister, CA; Amresco, Solon, OH) were added to solid and liquid culture media at the following concentrations: carbenicillin, 50 μg/mL for plasmid maintenance; chloramphenicol, 4 μg/mL or 30 μg/mL for *V. cholerae* or *E. coli*, respectively; kanamycin, 50 μg/mL; and polymyxin B sulfate, 100 U/mL.

Genetic manipulations. The *V. cholerae* N16961 genome sequence was used to design oligonucleotides (Table 2). 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, Sugar Land, TX), as indicated in the text, were utilized as templates in PCR reactions. The PCR reactions were performed using Q5 High-Fidelity DNA Polymerase (New England BioLabs) and primers synthesized by Integrated DNA Technologies. All obtained gene constructs were sequenced at the Center for Genome Research and Biocomputing at Oregon State University (OSU). Restriction enzymes as well as T4 DNA ligase were purchased from New England BioLabs.

Construction of expression plasmids. To place the *clg* gene under the control of P_{TAC} promoter, the VC1650 with a native *rbs* and a signal peptide was amplified with oligonucleotides ClgF and ClgR (Table 2). The PCR product was sub-cloned into the pCR-Script-Amp cloning vector (Stratagene) to generate pCR-Script-Clg. Subsequently, the EcoRI-PstI fragment containing *clg* was cloned into likewise digested broad-host expression vector, pMMB67EH, to yield pClg (Table 1).

To engineer the recombinant C-terminally 6×His-tagged Clg, the sequence encoding six histidine residues was incorporated within the primer rClgR. The fragment of N16961 chromosomal DNA containing the VC1650 gene with its native rbs and signal peptide was amplified in PCR reaction using primers rClgF and rClgR (Table 2). The resulting PCR product was digested with EcoRI and XbaI and cloned into pMMB67-EH to yield pClg-His.

Site-directed mutagenesis of the predicted catalytic residues of Clg. Mutagenesis of the predicted catalytic residues H435A, E436A, and H439A was performed using pCRScript-Clg as a template, appropriate primers (Table 2), and the QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA) as described in the instructions provided by the manufacturer. The presence of desired mutations was verified by DNA sequencing. Subsequently, the individual mutated variants of the 90-kDa form of Clg derived from early stationary phase of growth (ClgV) were cloned into EcoRI-PstI digested pMMB67-EH to generate pClgH435A, pClgE436A, and pClgH439A, respectively, (Table 1) and introduced into wild-type *V. cholerae* by a tri-parental conjugation following the scheme described below.

Construction of the Δclg knockout strain. The Δ*clg* knockout strain was constructed in the following steps. First, the cassette encoding chloramphenicol resistance (Cm^R) was amplified with primers CmF and CmR using plasmid pKD3 as a template (Tables 1-2). The PCR product was digested with NcoI and cloned into the NcoI site within the *clg* gene carried in pCR-Script-Clg to generate pCR-Script-Δ*clg*. Consequently, the plasmid was digested with SalI and SacI, and the digested 3.5–kb fragment containing the insertionally inactivated *clg* gene was cloned into a suicide vector pCVD442, to yield

pCVDΔ*clg* (Table 1). The pCVDΔ*clg* was introduced into N16961 strain of *V. cholerae* via tri-parental conjugation (21) using a donor strain, *E. coli* SY327λpir carrying pCVDΔ*clg*, and a helper strain, *E. coli* MM294 (Table 1). After conjugation, bacteria were spread onto a selective medium for Vibrio spp., thiosulfate-citrate-bile salts-sucrose (TCBS) agar (22), which was supplemented with chloramphenicol. To select for the second recombination event all procedures were performed as described previously (21). Chloramphenicol resistant colonies, that were sensitive to β-lactam antibiotics, were selected for PCR reactions and DNA sequencing to verify the presence of the CmR cassette within the VC1650 gene on the *V. cholerae* chromosome.

Purification of the C-terminally 6×His-tagged Clg. The overnight culture of *V. cholerae* N16961 harboring pClg-His was diluted 1:100 into 1 L of fresh LB media supplemented with carbenicillin, and overproduction of Clg-His was induced with 100 μM IPTG. Bacteria were grown at 37°C for 4 h until they reached early stationary phase of growth (OD_{600} of about 4). The culture supernatants were separated from cells by centrifugation, passaged through 0.2 μM filters (VWR), and precipitated with ammonium sulfate at 40% saturation. The pellet was solubilized in buffer A (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 1 mM imidazole). The soluble fraction was subjected to dialysis against buffer A overnight. Subsequently, the sample was centrifuged and the supernatant was applied to a 10-mL purification column (Thermo Scientific) containing Ni-NTA resin equilibrated in buffer A. The washing buffer containing 20 mM Tris-HCl pH 8.0, 300 mM NaCl, and 10 mM imidazole was applied, and the protein was finally eluted with 250 mM imidazole in 20 mM Tris-HCl pH 8.0 and 500 mM NaCl. The purified recombinant Clg-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 weight cut-off of 3 kDa (PALL Life Sciences). The amount of purified metalloprotease was determined using the DC Protein Assay (BioRad) according to the manufacturer's recommendation.

Purification of trout fish collagen. Extraction of fish collagen was performed using a modified method described previously (23). Briefly, a 1 lb. rainbow trout was rinsed with ddH_2O and skinned. All subsequent steps were performed at 4°C. The skin was deproteinized by incubation in 0.1 N NaOH at the sample/alkali solution ratio of 1:10 (w/v) for 24 h with changing the solution every 6 h. Then, the skin was washed with ice-cold water until the mixture reached basic pH. The fatty acids were removed from skin by stirring in 10% butyl alcohol at sample/alkaline solution ratio 1:10 (w/v) for 24 h. The solution was exchanged with a 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 with solid/solvent ratio of 1:15. The sample was centrifuged at 8000 × *g* for 30 min. The supernatant was stored at 4°C, while the precipitate was subjected to re-extraction for 16 h with gentle stirring in 0.5 M acetic acid with a sample/alkaline solution ratio of 1:30 (w/v). To obtain the soluble acid fraction, the dissolved precipitate was centrifuged at 8000 × *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 8000 × *g* for 35 min and dissolved 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 weight cut-off of 10 kDa (Thermo Scientific) for 24 h with a change of a buffer every 4-8 h. The solution was further

dialyzed against 15 volumes of ddH_2O with frequent changes of water until a neutral pH was obtained (7 days).

Enzymatic assays of Clg activity. Proteolytic activity of Clg was assessed using either purified recombinant Clg-His or culture supernatants isolated from *V. cholerae*. The Clg-His was purified as described above. To obtain culture supernatants, the overnight cultures of *V. cholerae* N16961 carrying either empty vector pMMB67-EH (p), pClg, pClgH435A, pClgE436A, or pClgH439A were diluted 1:100 into fresh LB supplemented with 100 µM IPTG and the samples were withdrawn at different stages of bacterial growth as indicated in the text. The supernatants were separated from bacterial cells as described previously (21). The enzymatic activity of Clg against a fluorescein conjugate gelatin, DQ-gelatin from pig skin (EnzCheck, Molecular Probes, Eugene, OR), was measured using either 50 µL of culture supernatants or the purified Clg-His at 5 nM concentration following the instructions provided by the manufacturer. The assays were conducted in a 96-well black microtiter plate format (Greiner BioOne) using the Synergy HT Multi-Microplate Reader (BioTek). The increase in fluorescence, which corresponds to the protease activity, was monitored at 37ºC during 10 min after addition of the substrate and normalized by either optical density of the cultures or Clg concentration. The effect of different inhibitors including 1,10-phenanothroline (Phe, 5 mM), ethylenediaminetetraacetic acid (EDTA, 5 mM), ethylene glycol tetraacetic acid (EGTA, 5 mM), leupeptin (1 mM), phenylmethylsulfonyl fluoride (PMSF, 5 mM), N-methylmaleimide (5 mM), and benzamidine (5 mM) on Clg proteolytic activity was studied by examining the ability of the enzyme to process DQ-gelatin after incubation of

either culture supernatants isolated from wild-type *V. cholerae* carrying pClg or purified Clg, as specified in the text, with individual chemicals at 37ºC for 1 h.

Subfactionation procedures. Overnight cultures of the strains N16961 pClg-His and Δ*eps* pClg-His were back diluted into fresh LB supplemented with 50 μM IPTG and grown for 4 h at 37˚C. The supernatants were separated from bacterial cells by centrifugation at 1500 × *g* for 10 min at RT. The periplasmic fractions were isolated using previously described methods (21) with the exception that the time of incubation of bacterial cell pellets with polymyxin B sulfate was increased to 1 h.

Substrate specificity assays. The ability of the purified Clg-His to degrade specific collagenase substrate, N-(3-(2-furyl)acryloyl)-Leu-Gly-Pro-Ala (FALGPA, Bachem), was performed as described previously (24), with the following modifications. All examined proteins were loaded as 22.5 μL of 0.4 mg/mL protein in 150 μL volume reactions. The negative control for the assays included modified trypsin (NEB) while Collagenase D from *Clostridium histolyticum* (Roche) was utilized as a positive control. The purified ClgV protein (645 nM) and FALGPA (at concentration range from 2.0 to 8.0 mM) were mixed in 50 mM Tricine pH 7.5 buffer containing 400 mM NaCl and 10 mM $CaCl_2$. The reactions were incubated at 37ºC for 5 min, followed by enzymatic activity measurement at 30ºC for 1 h. The change in absorbance was examined at 345 nm using the Synergy HT Multi-Microplate Reader. The one unit of Clg activity is defined as the amount processing 1 μmol of FALGPA at 30ºC per min. These experiments were conducted in biological triplicates. In addition, purified fish collagen and commercially available (Sigma) human fibronectin, lactoferrin, and immunoglobin A (IgA), as well as porcine stomach mucin, and bovine albumin serum (BSA) were examined as the

candidate substrate proteins of the Clg. In these assays, 12 μg of fish collagen and commercially available substrates were incubated for 1 h at 37ºC in the presence of 5 nM and 100 nM Clg-His, respectively, in 20 mM Tris-HCl pH 8.0 supplemented with 1 mM $CaCl_2$. The biological substrates were also incubated with culture supernatants isolated from *V. cholerae* carrying pClg. All reactions were subsequently examined by SDS-PAGE and Colloidal Coomassie staining. Experiments were performed at least on three separate occasions and representative results are shown.

Swarming motility assays. Soft agar plates for swarming assays were prepared as previously described (25). The media were supplemented with carbenicillin (100 μg/mL) and 100μM IPTG. Overnight cultures of *V. cholerae* were back diluted (1:100) into fresh LB media, cultured to early stationary phase of growth (until OD_{600} of 4), and the cells were stabbed into semi-solid LB agar. Subsequently, the plates were incubated in a humid chamber at either 37˚C or 25˚C for 5 h or 20 h, respectively, and the swarm diameters were measured. Experiments were performed in biological triplicates and means and SEMs are presented.

SDS-PAGE, Zymography and Immunoblotting. To the samples prepared as described above LDS Loading buffer (Invitrogen) supplemented with dithiotreitol (50 mM) was added. Subsequently, proteins were separated in 4-12% Bis-Tris polyacrylamide gels (NuPAGE, Invitrogen), and stained with Silver staining kit (Invitrogen) or Colloidal coomassie, as indicated in the text. Samples of periplasmic fractions and supernatants were matched by equivalent OD_{600} units. In immunoblotting analysis the proteins were transferred to nitrocellulose membrane (Pall Life Tech) using Turbo blot (BioRad). The membranes were blocked in 5% milk in phosphate buffer saline (PBS at pH 7.0, Li-Core)

supplemented with 0.1% Tween 20, probed with monoclonal anti-His antisera (Thermo; 1:1000 dilution), followed by incubation with Immun-Star Goat Anti-Rabbit (GAR)-HRP Conjugate (BioRad; 1:10,000 dilution). Immunoblots were developed using Clarity Western ECL-Substrate (BioRad) on Chemi-Doc^{TM} MP System (BioRad).

In zymography assays, culture supernatants separated from *V. cholerae* cells at different time points of bacterial growth, as specified in the text, were normalized by OD_{600}, and loaded into wells of either 10% zymogram (gelatin) or 12% (casein) gels (Novex, Invitrogen) according to the instructions provided by the manufacturer. Following electrophoresis, the gels were incubated in renaturing buffer for 30 min at RT with gentle agitation. The renaturing buffer was decanted and the gel was equilibrated with the developing buffer for 30 min at RT with gentle agitation. The buffer was decanted and fresh developing buffer was added for incubation at 37ºC overnight. Finally, the gels were stained with Colloidal Coomassie.

Statistical analyses. The statistical analyses were performed using GraphPad Prism software (GraphPad Software Version 6.0, San Diego, California). An unpaired Student's t-test was used to analyze the data and the statistical significance (p <0.05).

Table 1. Strains and plasmids used in this study

Strain/plasmid	Description/relevant genotype[1]	Reference/source
V. cholerae strains		
N16961	Wild-type El Tor O1 biotype, Sm^r	Laboratory collection
NΔ*eps*	N16961 Δ*epsC-N* Cm^r	(21)
NΔ*clg*	N16961 Δ*clg* Cm^r	This study
E. coli strains		
MC1061	F− araD139 Δ(ara-leu)7697 Δ(lac)X74 rpsL hsdR2 mcrA mcrB1	(26)
MM294 (pRK2013)	Donor of transfer function for triparental conjugation	(27)
Plasmids		
pMMB67-EH	Expression vector, Ptac promoter, Amp^r	(28)
pBBRlux	ori pBBR1, promoterless luxCDABE, Cm^r	(29)
pKD3	Cloning vector, Amp^r and Cm^r	(30)
pCRScript	Cloning vector, Amp^r	Stratagene
pCRScript-Clg	*clg* cloned into pCRScript, Amp^r	This study
pCR-clg::cm	Insertionally inactivated *clg* cloned into pCRScript, Amp^r and Cm^r	This study
pCVD442	Suicide vector containing *sacB*, Amp^r	(31)
pΔclg	Insertional mutation of *clg* cloned into	This study

	pCVD442	
pClg	pMMB67-EH carrying Ptac-Clg	This study
pClg-His	pMMB67-EH carrying Ptac-Clg with a C-terminal His_6 tag	This study
pClgH435A	pMMB67-EH carrying Ptac-ClgH435A	This study
pClgE436A	pMMB67-EH carrying Ptac-ClgE436A	This study
pClgH439A	pMMB67-EH carrying Ptac-ClgE439A	This study

[1]Sm^r, streptomycin resistant; Amp^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant

Table 2. Oligonucleotide primers designed and used in this study

Name	Sequence (5' to 3')
Primers for cloning wild-type alleles[1]	
ClgF	GAATTCCATCATAAATAGGTTTTGCAGTGGTC
ClgR	CTGCAGTCAGTCGAAATAGGCCACCAT
rClgF	GAGCTCGAATTCCATCATAAATAGGTTTTGCAGTGTC
rClgR	CTGCAGTCTAGACACCACCACCACCACCACTAAGAAGTCGAAATAGGCCACCATTT
Primers for creating NΔclg knockout strain	
CmF	CATATGCCATGGTGTGTAGGCTGGAGCTGCTT
CmR	CATATGCCATGGCATATGAATATCCTCCTTAG
Primers for site-directed mutagenesis	
H435F	ATTTGTCGATTCTCAATTTAGAGGCTGAGTACACTCATTATCTGG

	ACG
H435R	CGTCCAGATAATGAGTGTACTCAGCCTCTAAATTGAGAATCGAC AAAT
E436F	GATTCTCAATTTAGAGCATGCGTACACTCATTATCTGGACG
E436R	CGTCCAGATAATGAGTGTACGCATGCTCTAAATTGAGAATC
H439F	TTTAGAGCATGAGTACACTGCTTATCTGGACGCGCGCTTC
H439R	GAAGCGCGCGTCCAGATAAGCAGTGTACTCATGCTCTAAA

[1]F, forward; R, reverse

Results:

Protein Identification.

Our laboratory studies the mechanisms underlying an evolutionarily conserved virulence factor, the Type II Secretion (T2S) pathway. In particular, we are interested in determining the function and structure of T2S-dependent cargo proteins, regulation of T2S, and recognition of cargo proteins by the T2S complex. In our investigations, we utilized a model organism, *V. cholerae*. In recent proteomic analysis of the *V. cholerae* secretome, we identified 14 novel T2S substrates including three serine proteases VesA, VesB, and VesC (32). While inspecting the chromosomal location of the gene encoding VesC (VC1649), we found that an adjacent gene, VC1650, was annotated in the KEGG database as a putative metalloprotease. This gene is located in an opposite orientation to VC1649 and encodes a putative protein of 818 amino acids with a predicted molecular weight of 93-kDa. Analysis of the deduced amino acid sequence of VC1650 revealed the presence of a signal peptide (residues 1-29), a propeptide (residues 30-267), and a peptidase M9 domain at amino acids 268–558. This domain contains three residues (underlined) that are characteristic for the catalytic site of metalloproteases, HEYTH, located at amino acids 435, 436, and 439 (Fig. 1 A). In addition, two PPC domains were also identified at amino acids 559 – 818. These domains are often absent in active proteases (33).

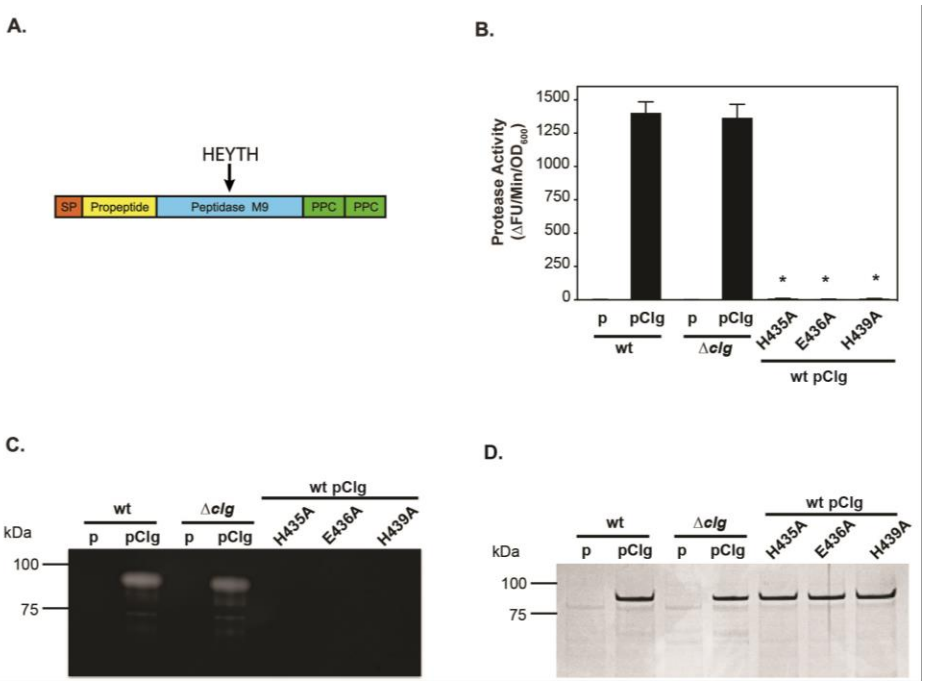

Figure 1. Clg is a secreted protease that exhibits metalloprotease activity. (A) Domain architecture of VC1650. SP, signal peptide; pro-peptide, peptidase M9 (HEXXH conserved motif); PPC, pre-peptidase C-terminal domain. (B) Quantitative analysis of metalloprotease activity. Protease activity against DQ gelatin was measured in supernatants isolated from *V. cholerae* N16961 wt p, Δ*clg* p, wt pClg, Δ*clg* pClg, and wt ectopically expressing mutated variants of Clg: H435A, E436A, and H439A. The protease activity is expressed as a change in fluorescence (ΔFU) normalized by optical density (OD_{600}) of bacterial cultures. All experiments were performed at least on three separate occasions in technical triplicates and mean ± standard error of the mean (SEM) are presented. The statistically significant differences ($p<0.05$) are indicated by *. (C) Qualitative analysis of metalloprotease activity. Enzymatic activity of Clg was examined by gelatin zymography. Supernatants isolated from wt pClg, and Δ*clg* pClg strains were diluted 10-fold. Samples were normalized by the same OD_{600} units and separated in 10% Tris-glycine gel containing 0,1% gelatin as a substrate. The gel was stained with Colloidal Coomassie. (D). Analysis of protein profiles in the culture supernatants isolated from different *V. cholerae* strains (as indicated). Samples were normalized by the same OD_{600} units, separated by 10% SDS-PAGE, and the proteins were visualized following staining with Colloidal Coomassie.

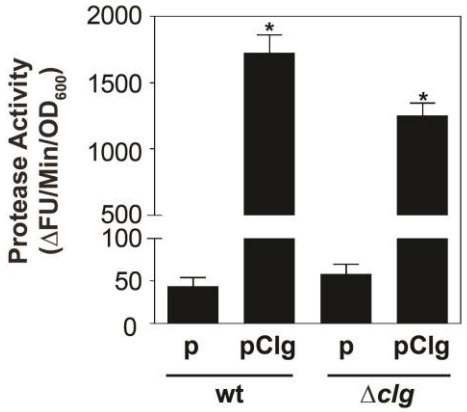

Figure 2. Genetic inactivation of *clg* does not affect proteolytic activity against DQ gelatin. Protease activity against DQ gelatin was measured in supernatants of *V. cholerae* N16961 wt p, Δ*clg* p, wt pClg, and Δ*clg* pClg cultured until late stationary phase of growth (16 h). The protease activity is presented as a change in fluorescence (ΔFU) and normalized by optical density (OD_{600}) of bacterial cultures. All experiments were performed at least on three separate occasions in technical triplicates and the bars represent mean ± SEMs. The statistically significant differences ($p<0.05$) are indicated by *.

Clg is a secreted protein that displays proteolytic activity.

To investigate the function of VC1650, we verified that the gene encodes an active metalloprotease. The Δ*clg* deletion mutant was constructed in *V. cholerae* N16961 via homologous recombination as described in Materials and Methods. Subsequently, the protease activity was examined in culture supernatants of wt and isogenic Δ*clg V. cholerae* strains cultured in LB medium at 37˚C at early and late stationary phase of growth (OD_{600} of 4) (Fig. 1 B and Fig. 2). Harvesting the supernatants at early stationary phase allowed preservation of the full-length form of Clg (90-kDa) (Fig. 1 D and 7 B). While a processed form of Clg (68-kDa) accumulated in supernatants isolated at the late stationary phase of growth. The enzymatic activity of Clg was examined in culture supernatants isolated from wild type and Δ*clg* using DQ gelatin, a fluorescent substrate that is commonly used to assess metalloprotease activity (Fig. 1 B). The Clg activity was

measured as the change in fluorescence, normalized by the time of the reaction (10 min) and the density of the bacterial culture. The protease activity at the early stationary phase of *V. cholerae* growth was close to the background level (wt p and Δ*clg* p*;* Fig. 1 B), while at the late stationary stage (16 h) the activity was on average 44 ± 9.8 ΔFU and 59 ± 10 ΔFU (mean ± SEM) in culture supernatants isolated from the wt and Δ*clg* strain, respectively (Fig. 2). These experiments indicated that lack of Clg did not affect the detected metalloprotease activity and suggested that Clg is not produced under these growth conditions. To further examine whether VC1650 encodes an active protease, the gene was cloned under an IPTG inducible promoter located on a broad-host cloning vector, pMMB67EH (28). The resulting pClg was introduced via conjugation into the wild type and isogenic Δ*clg* strains of *V. cholerae*. Subsequently, the production of Clg was induced with 100 μM IPTG and the supernatants were separated from *V. cholerae* cells by centrifugation at early stationary phase (4 h from back dilution, at OD_{600} of 4). The proteolytic activity measured in culture supernatants of wt carrying pClg and Δ*clg* pClg was 1545 ± 46 ΔFU and 1364 ± 101 ΔFU (mean ± SEM), respectively (Fig. 1 B). Similar to the activity of wt pClg and Δ*clg* pClg in the late stationary phase, metalloprotease activity was 1727 ± 134 ΔFU and 1253 ± 92 ΔFU (mean ± SEM), respectively (Fig. 2).

In addition, culture supernatants isolated from wt p, Δ*clg* p, wt pClg, and Δ*clg* pClg were resolved by SDS-PAGE and the proteins were stained with Colloidal Coomassie to assess Clg secretion (Fig. 1 C). These studies revealed the presence of an apparent 90-kDa protein band in the supernatants derived from *V. cholerae* overproducing Clg (Fig. 1 D). This protein band migrated according to the predicted molecular weight of Clg lacking

the signal peptide and as expected, it was absent in the wt p and Δ*clg* p supernatants (Fig. 1 D). Corroborating these findings, the zymography analysis revealed Clg activity (Fig. 1 C). The white areas indicate clearing or digestion of gelatin, which demonstrates protease activity. The dark areas, which lack clearing of gelatin, indicate a lack of protease activity. Clearing was observed only in wt pClg and Δ*clg* pClg strains. All subsequent experiments were performed at early stationary phase of *V. cholerae* growth unless stated otherwise.

The catalytic residues of Peptidase M9 are crucial for Clg activity.

The predicted catalytic site of Clg possesses three amino acids characteristic for metalloproteases, HEXXH, and lies within the peptidase M9 domain (Fig. 1 A). To determine the role of the individual residues, a site-directed mutagenesis approach was utilized. Subsequently, the mutated forms of Clg (H435A, E436A, and H439A) were cloned into pMMB67EH, introduced into *V. cholerae,* overexpressed, and the effect of these point mutations on Clg activity was examined (Fig. 1 B). The activity of wild type Clg reached 1545 ± 46 ΔFU (mean ± SEM), whereas the mutated variants Clg H435A, Clg E436A, and Clg H439A had completely abolished protease activity. These experiments indicated that Clg requires the zinc-binding motif for enzymatic activity. In addition, the proteolytic activity of mutated versions of Clg was verified qualitatively by zymography (Fig. 1 C). Culture supernatants collected from wt pClg and Δ*clg* pClg strains of *V. cholerae* displayed clearing on the zymogram gel. However, there was a lack of clearing by proteins in culture supernatants isolated from mutated variants of Clg. To verify whether the alteration of the catalytic site affects Clg secretion, we analyzed the protein profiles of the isolated supernatants by SDS-PAGE and Coomassie staining (Fig.

1 D). A 90-kDa Clg band was present in all samples, indicating that while the conserved residues are essential for enzymatic activity, they do not affect protease secretion.

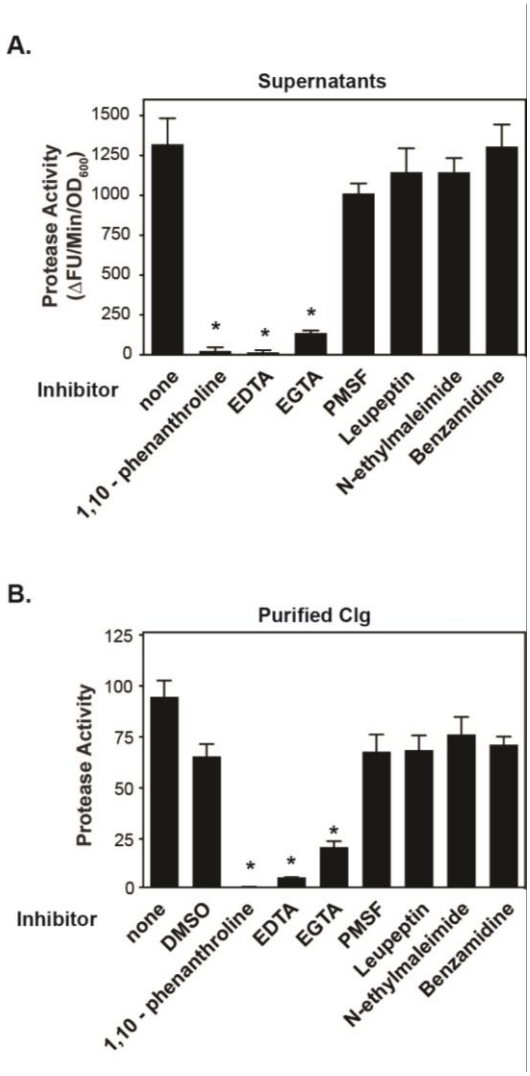

Figure 3. The proteolytic Clg activity is abolished by metalloprotease inhibitors. (A) Analysis of metalloprotease activity in the presence of protease inhibitors. Supernatants isolated from *V. cholerae* N16961 wt p, wt pClg, and wt pClg were incubated with either metallo-, serine- or cysteine protease inhibitors. Subsequently, Clg activity was examined using DQ gelatin. The protease activity is presented as a change in fluorescence (ΔFU) and normalized by optical density (OD_{600}) of bacterial cultures. (B) Activity of purified Clg incubated with and without protease inhibitors. The purified, recombinant variant of Clg was incubated in the presence and absence of various protease inhibitors (as indicated) and its proteolytic activity was assessed against DQ gelatin. All experiments were performed in technical triplicates on three separate occasions and mean ± SEMs are presented. The statistically significant differences ($p<0.05$) are indicated by *.

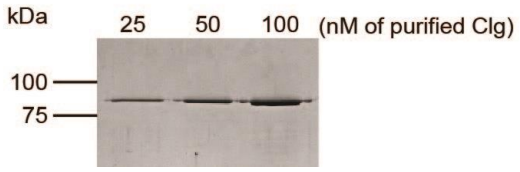

Figure 4. SDS-PAGE analysis of the purified recombinant Clg-6xHis. The engineered recombinant Clg-6xHis was cloned into pMMB67EH to create pClg and introduced into wt *V. cholerae* N16961 by conjugation. The bacteria were grown until early stationary phase of growth in LB media supplemented with 100 µM IPTG. The supernatant was separated from bacterial cells by centrifugation and the proteins were precipitated using ammonium sulfate. The precipitate was solubilized and the soluble fraction was used to purify Clg-6xHis by affinity chromatography. The eluted and concentrated samples containing 25, 50, and 100 nM of Clg-His were assessed for purity by SDS-PAGE and Colloidal Coomassie staining.

Clg is a metalloprotease.

To further verify that Clg belongs to metalloproteases, different protease inhibitors including 1, 10-phenanthroline, EDTA, EGTA, PMSF, N-ethylmaleimide, benzamidine, and leupeptin were utilized. Culture supernatants collected from wt *V. cholerae* carrying pClg were incubated with individual protease inhibitors and assessed for enzymatic activity against DQ gelatin (Fig. 3 A). The level of Clg activity was significantly reduced in the presence of metalloprotease inhibitors (1, 10-phenanthroline, EDTA, and EGTA), revealing Clg activity at 23 ± 23 ΔFU, 15 ± 15 ΔFU, and 136 ± 17 ΔFU (mean ± SEM), respectively. While in contrast, in the presence of serine and cysteine protease inhibitors (PMSF, N-ethylmaleimide, benzamidine, and leupeptin) there was no significant effect on Clg activity.

To perform careful biochemical analysis of Clg activity, we aimed to purify Clg. First, the *clg* gene was fused in frame with six histidine residues at the C-terminus and the PCR product was cloned into pET28 and introduced into *E. coli* BL21 (DE3). However, this heterologous host was not optimal for protein purification as the protein was expressed

poorly and partitioned into the insoluble fraction. In an alternative approach, the C-terminally 6xHis-tagged Clg was engineered, cloned into pMMB67EH, and introduced into *V. cholerae* via triparental conjugation. *V. cholerae* expressing pClg-6xHis was cultured to early stationary phase, culture supernatants were harvested, and the proteins were precipitated with ammonium sulfate. The precipitate was suspended in a buffer (as described in Materials and Methods) and the soluble fraction was dialyzed overnight, followed by purification via Ni-NTA affinity chromatography. Examination of eluted fractions by SDS-PAGE and Colloidal Coomassie staining revealed that Clg was purified to 99% homogeneity (Fig. 4). The activity of purified Clg (5 nM) was subsequently examined against DQ gelatin in the presence and absence of various inhibitors (Fig. 3 B). Corroborating our studies with culture supernatants, the activity of the purified Clg was only inhibited in the presence of 1,10-phenanthroline, EDTA, and EGTA. Collectively, our analysis confirmed that Clg belongs to metalloproteases.

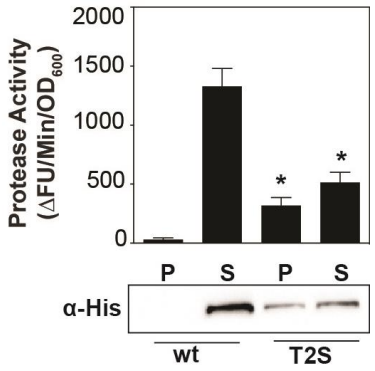

Figure 5. Secretion of Clg is affected in the T2S-knockout strain. The engineered recombinant C-terminally 6xHis-tagged Clg was cloned under the control of an inducible promoter located in pMMB67EH. The plasmid was introduced into wt and isogenic T2S-knockout of *V. cholerae* N16961. The bacteria were grown in LB media at 37°C. The cultures were harvested at early stationary stage of growth and the supernatants (S) and periplasmic fractions (P) were isolated. Data are shown as metalloprotease activity measured in isolated fractions against DQ gelatin. All assays were performed at least on three separate occasions in technical triplicates and mean ± SEMs are presented, *$p<0.05$. The samples containing periplasmic and supernatant fractions were normalized

by OD_{600}, resolved by SDS-PAGE, and the presence of Clg was analyzed by Western blotting with anti-His antibodies. A representative immunoblot is shown.

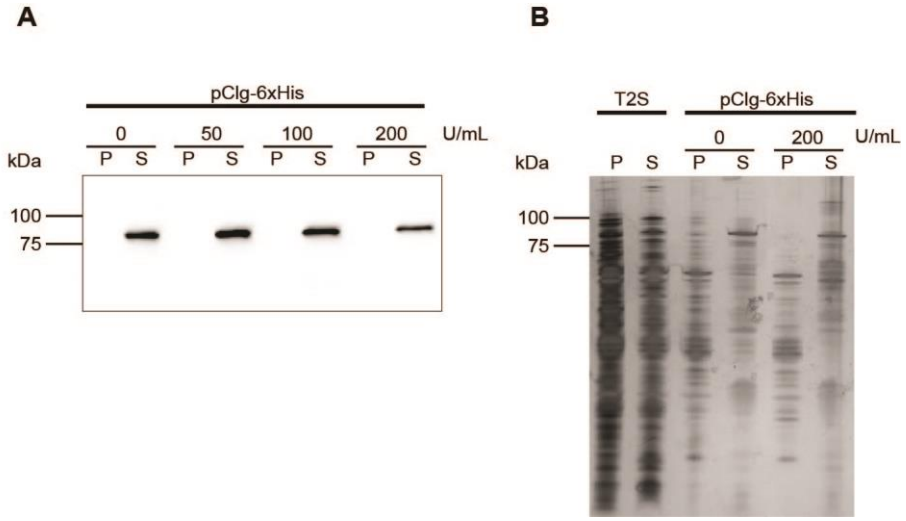

Figure 6. Extracytoplasmic stress response does not affect Clg secretion. To induce the cell envelope stress, the wt *V. cholerae* N16961 carrying pClg-6xHis was treated with different concentrations of a membrane-integrity perturbing agent, polymyxin B sulfate (U/ml). Subsequently, supernatants and periplasmic fractions were harvested and examined by SDS-PAGE followed by either immunoblotting analysis with anti-His antisera (A) or silver staining (B). Samples were normalized by OD_{600}.

Clg is a T2S-dependent protein.

We hypothesized that the translocation of Clg across *V. cholerae* outer membrane to the extracellular space depends on the functional T2SS. To test this hypothesis, the plasmid containing recombinant Clg-His, pClg-His, was introduced into *V. cholerae* lacking the entire T2SS gene cluster (Δ*eps*). Next, periplasmic and supernatant fractions were collected from both strains of *V. cholerae*, and examined for the Clg localization using immunoblotting analysis with anti-His antibodies (Fig. 5). In the Δ*eps* strain, an intense band corresponding to Clg-His was present at similar levels in both the periplasmic and supernatant fractions, whereas in wt *V. cholerae*, the protein was only detected in the extracellular milieu. The proteolytic activity of Clg against DQ gelatin was also examined in these subcellular fractions (Fig. 5). Corresponding to the immunoblotting

analysis, there was very little detectable Clg activity in the periplasm isolated from wt *V. cholerae* (28.79 ± 15.96), while in the Δ*eps* strain the Clg activity was 317.6 ± 68.58 ΔFU (mean ± SEM) (Fig. 5). The enzymatic activity in the wt supernatants reached 1329 ± 151.8 ΔFU, which was 2.6-fold higher than that in the T2S-knockout strain, indicating that secretion of Clg is T2S-dependent (Fig. 5). The presence of Clg in the supernatants of Δ*eps* strain could be explained by the compromised outer membrane integrity, which is associated with a lack of a functional T2S system (21). On the other hand, transport of Clg may not rely on the T2S pathway. Additionally, the stress on the cell envelope may cause the accumulation of the protein in the periplasm. In an attempt to dissect these possibilities, we utilized a chemical probe, polymyxin B sulfate. This antimicrobial peptide causes alterations in the bacterial membrane integrity and induces cell envelope stress guarded by the alternative sigma factor RpoE, thus partially mimicking the phenotype observed in the T2S knockout strains (21, 35). The localization of Clg-His in the periplasmic and supernatant fractions of wt *V. cholerae* treated with increasing concentrations of polymyxin B sulfate was examined by SDS-PAGE and immunoblotting analysis (Fig. 6 A). There was no change in localization of Clg upon induction of extracytoplasmic stress and Clg was only present in the extracellular milieu. In addition, membrane perturbation of wt *V. cholerae* treated with polymyxin B sulfate was verified by SDS-PAGE and silver staining (Fig. 6 B). Silver staining analysis revealed that there were more proteins present in the extracellular space in cultures grown in the presence of 200 U/ml polymyxin B sulfate, indicating that the membrane was perturbed by this antimicrobial agent. Together, these studies suggest that Clg requires the T2SS to be translocated from the periplasmic space to the extracellular environment.

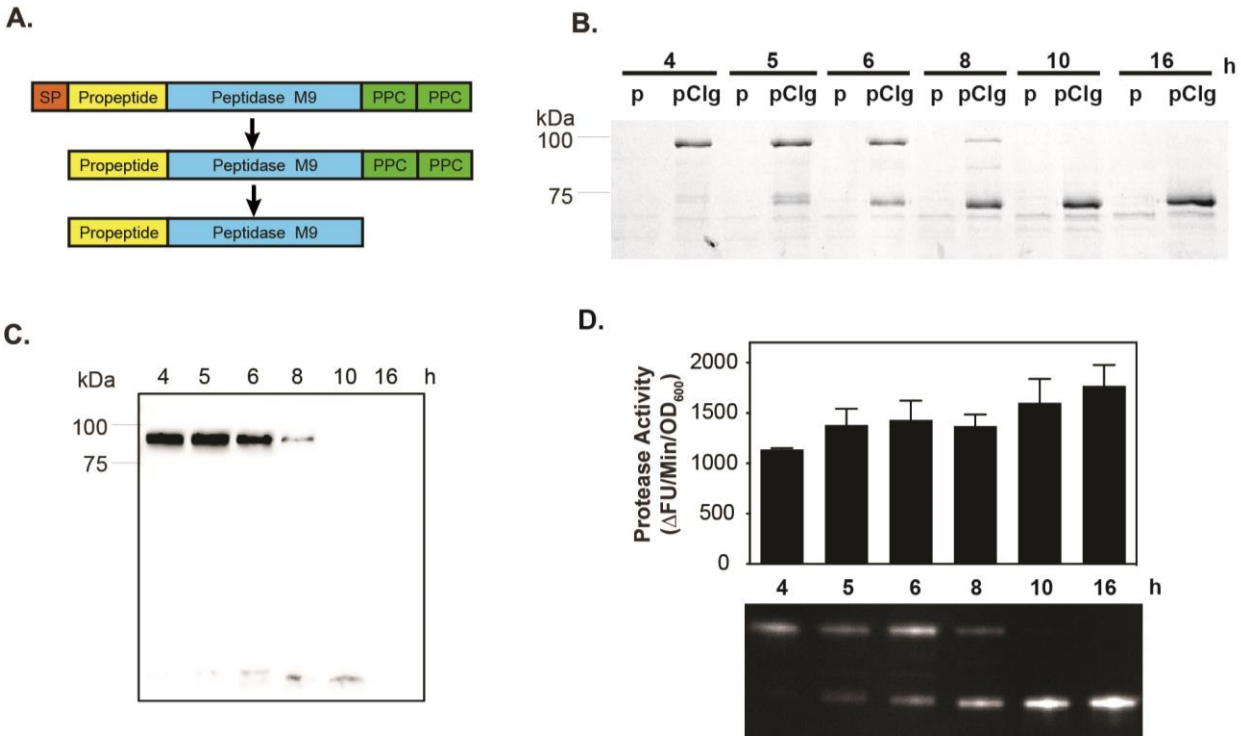

Figure 7. Clg undergoes processing. (A) A predicted scenario of Clg maturation. Signal peptide (SP) is cleaved upon translocation of the protein to the periplasm. The two pre-peptidase C-terminal domains (PPC) are cleaved off from the protein after secretion. (B) Clg processing. *V. cholerae* N16961 carrying the engineered recombinant C-terminally 6xHis-tagged Clg cloned under the control of an inducible promoter located in pMMB67EH, pClg-6xHis, was grown in LB medium at 37ºC and at different time points of bacterial growth (4, 5, 6, 8, 10, and 16 h) samples were withdrawn and the culture supernatants were isolated. The harvested supernatants were examined by SDS-PAGE and Colloidal Coomassie staining. (C) Immunoblotting analysis of isolated supernatants with anti-His antibodies. (D) Clg activity against either DQ gelatin (bar graph) or using gelatin zymography was assessed in culture supernatants collected at distinct time points of bacterial growth (as indicated). There was no statistically significant difference in the measured protease activity as determined by student's *t-test*.

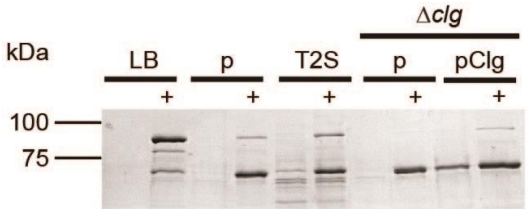

Figure 8. Clg is capable of autocleavage, but requires other factor(s) for complete maturation. To study the mode of maturation of Clg, culture supernatants isolated from early stationary cultures of different *V. cholerae* N16961 strains (as indicated) were incubated with purified Clg-6xHis for 16 h (+) LB media alone and a purified Clg incubated in LB were used as controls.

Clg undergoes a maturation process.

Proteases are known to undergo maturation to become active enzymes (6). Previously, a secreted metalloprotease from *Aeromonas sobria* was found to undergo a conversion from its inactive to active form through the aid of a serine protease also secreted by the bacterium (36). Additionally, some proteases display autoproteolytic properties, such as LasA secreted by *Pseudomonas aeruginosa* (37). LasA possesses a propeptide at the N-terminal end of the protein. This propeptide acts as an intramolecular chaperone to drive the conversion of LasA to its active conformation. To assess the maturation process of Clg, culture supernatants of wt *V. cholerae* carrying either empty vector or pClg were collected at 4, 5, 6, 8, 10, and 16 h from back dilution and examined by SDS-PAGE and Colloidal Coomassie staining (Fig. 7 B). After 4, 5, 6, and 8 h of culturing, 90-kDa and 68-kDa protein bands were present. From 4 to 6 h of growth, the 90-kDa band began to fade. At 8 h of growth the 90-kDa appeared less prominent, and a faint band at approximately 80-kDa was observed, along with an intense 68-kDa band. After 10 and 16 h of growth, only the 68-kDa band was detected under the tested conditions. Based on the analysis of the molecular weight of the mature protease, we hypothesized that the two PPC domains were cleaved. PPC domains have often been reported to be absent in active proteases (20). Corroborating these findings, immunoblotting analyses of culture supernatants of *V. cholerae* expressing pClg-His revealed the presence of the 90-kDa form of Clg at 4, 5, and 6 h of growth (Fig. 7 C). At 8 h of growth, the full length Clg began to fade and was undetectable by 10 h. Because the His epitope has been engineered to the C-terminus of Clg we concluded that the PPC domains were cleaved. This observation was further supported by zymography studies. After 6 h of growth, both 90-

kDa and 68-kDa forms of Clg digested the substrate, with the full length Clg producing more clearing on the gel. At 8 h, the 90-kDa band faded while the 68-kDa band was observed. After 10 and 16 h of growth, only the activity of the mature form of the protease was detected. The two different forms of Clg, 90-kDa and 68-kDa, displayed similar levels of protease activity as revealed by a quantitative DQ gelatin assay (Fig. 7 D). Together, our analysis showed that Clg undergoes a maturation process, but does not necessarily become more active upon maturation, rather, Clg remains active in the periplasmic space and in all its forms following secretion (Fig. 5 and Fig. 7).

We also examined the mechanism(s) underlying Clg maturation. We hypothesized that another protein(s) secreted by *V. cholerae* participated in this process. To test this hypothesis, the purified Clg was incubated with culture supernatants isolated from different strains of *V. cholerae* (Fig. 8). As serine proteases have been shown to contribute to the maturation of metalloproteases, the culture supernatant of the triple serine-protease knockout was used. In addition, we employed the T2S knockout strain to examine whether maturation of Clg depends on the other cargo protein(s) secreted by the T2SS. The mature form of Clg was detected upon incubation with supernatants derived from wt p, *Δclg* p, *Δclg* pClg, and *ΔvesABC* p (data not shown). As a control for the experiments, the purified Clg was also incubated with LB broth. Three distinct bands with different intensities (abundance) were reproducibly present in this reaction, indicating that Clg might also undergo autocleavage. In the presence of *V. cholerae* culture supernatants, the full length Clg was almost completely processed to its mature form, indicating that Clg is aided by other proteases for complete maturation. In the culture supernatant of the T2S mutant, however, the band representing the full form of

Clg was present at a greater intensity. This implied that a T2S-dependent protein contributed to Clg maturation. These studies suggested that while Clg may undergo some autocleavage, the extracellular metalloprotease requires other secreted factor(s) for complete maturation.

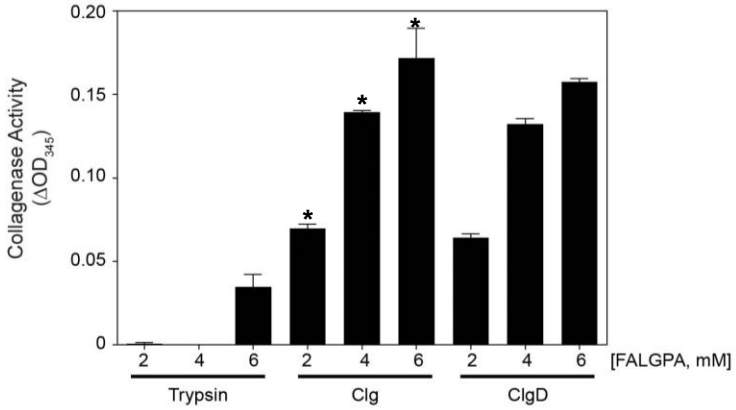

Figure 9. Clg displays collagenolytic activity. FALGPA, a collagenase specific substrate, was used to determine collagenolytic activity of Clg. The purified proteases Clg-6xHis, Trypsin, or the *Clostridium histolyticum* collagenase D, ClgD, were incubated with FALGPA for 5 min at 37˚C. The proteolytic activity was measured for 1.5 h as change in the absorbance at OD_{345} at 30˚C. Trypsin and 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. *$p<0.05$.

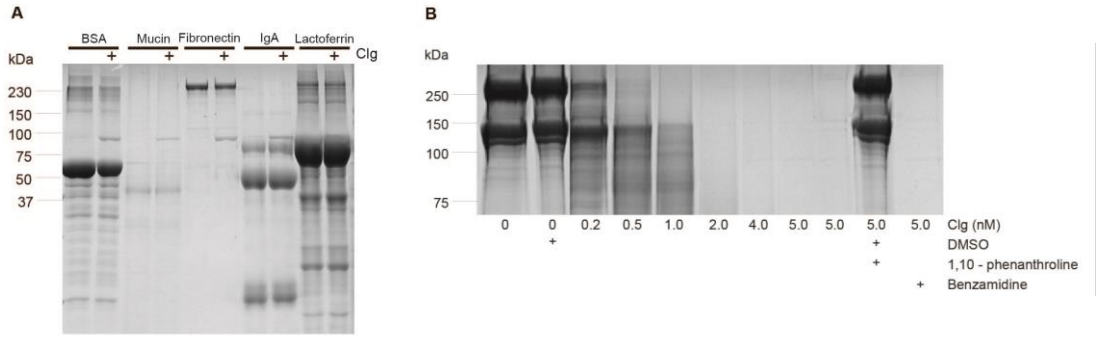

Figure 10. Clg specifically degrades fish collagen. (A) To determine substrate-specificity of Clg, the purified protein was incubated for 1 h at 37 ˚C with the following substrates: bovine serum albumin (BSA, control), mucin, fibronectin, Immunoglobulin A (IgA), and lactoferrin. The reactions were subsequently separated by SDS-PAGE and stained with Colloidal Coomassie. (B) Purified fish collagen was incubated for 1 h at 37˚C with increasing concentrations of Clg-6xHis. There was greater digestion of fish collagen with increasing concentrations of Clg. The ability of Clg to proteolytically

cleave fish collagen was also tested in the presence of metalloprotease inhibitor (1, 10-phenanthroline) and serine protease inhibitor (benzamidine). DMSO was added to the reaction as a vehicle control for 1, 10-phenanthroline.

The novel *V. cholerae* protease specifically digests fish collagen.

To further verify that Clg displays collagenase activity, proteolytic activity was measured against FALGPA, a substrate specific for bacterial collagenases (24). Clg activity was compared to trypsin (negative control) and Collagenase D (ClgD) from *C. histolyticum* (positive control) (Fig. 9). The activity of Clg was similar to ClgD, while trypsin displayed significantly lower activity against FALGPA. This indicated that Clg displays collagenolytic activity.

In an attempt to identify natural substrates of Clg, the following biologically relevant substrates present in the mammalian host were tested: mucin, fibronectin, lactoferrin, and IgA (Fig. 10 A). In addition, bovine serum albumin (BSA) was included as a control. The purified, recombinant Clg-His was incubated with the substrates for 1 h at 37ºC. Subsequently, the samples were loaded on SDS-PAGE and the proteins were visualized by staining with Colloidal Coomassie. None of the tested protein substrates was digested by Clg during the time of the reaction.

Previously, researchers also discovered that *V. cholerae* may reside in the intestinal tract of fish or on fish skin (3). The collagen of fish skin was extracted from trout skin to investigate the Clg putative biological function. The purified fish collagen was incubated with increasing concentrations of recombinant Clg and the reaction was examined by SDS-PAGE and staining with Colloidal Coomassie (Fig. 10 B). In contrast to the lack of Clg activity against mammalian substrates, with increasing concentrations of the purified protein, there was greater digestion of fish collagen. The protease completely digested

this biological substrate (at a concentration as low as 5 nM, Fig. 10 B). In addition, the presence of a metalloprotease inhibitor, 1, 10-phenanthroline, completely abolished digestion of fish collagen by Clg while the serine protease inhibitor (benzamidine) did not block the protease activity. These studies further supported that VC1650 encodes a metalloprotease with collagenolytic activity.

V. cholerae N16961	Swarm diameter (mm)	
	25°C	37°C
p	11.6 ± 0.8	11.2 ± 0.4
Δ*clg* p	10.8 ± 0.7	11.1 ± 0.6
Δ*clg* pClg	12.3 ± 0.6	11.2 ± 0.3

Table 3. Clg does not play a role in swarming motility of *V. cholerae*. Isogenic strains of *V. cholerae* N16961 including wt p, Δ*clg* p, and Δ*clg* pClg were grown in liquid media and at early stationary phase of growth the cells were stabbed into swarming agar plates. The plates were incubated in a humid chamber at either 25ºC or 37ºC. Swarming diameter was recorded for both growth conditions after 16 and 6 h of incubation, respectively. All experiments were performed on at least three independent occasions. Mean values and SEMs are reported in millimeters (mm).

Clg does not affect motility of *V. cholerae*. Several extracellular proteases have been implicated in facilitating motility of bacteria by reducing surface tension (38). To determine whether Clg influences *V. cholerae* motility, the wt p, Δ*clg* p, and Δ*clg* pClg strains were examined on swarming agar plates at 25ºC and 37ºC, two temperatures encountered by *V. cholerae* in the aquatic reservoir and the human host, respectively (Table 3). In the absence of Clg, motility of *V. cholerae* was unaffected at both temperatures. This suggested that under the tested conditions, Clg does not contribute to the *V. cholerae* motility.

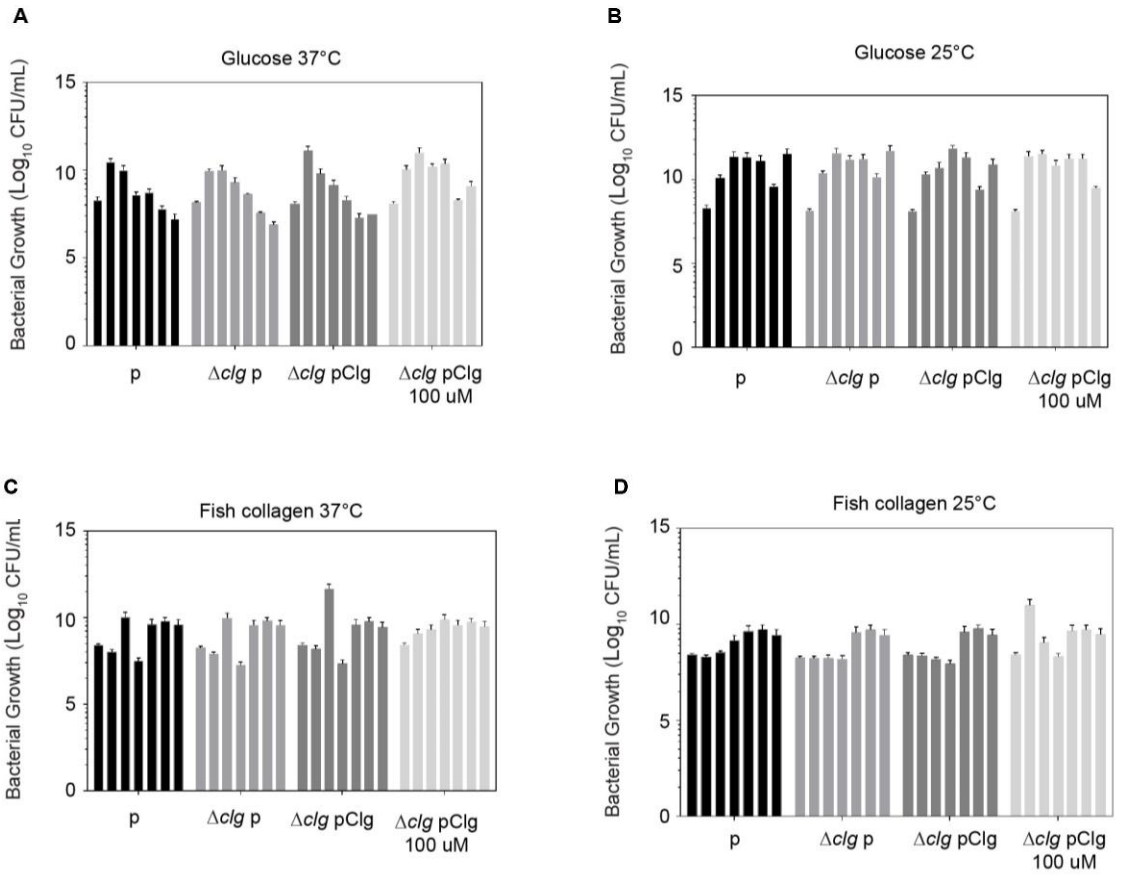

Figure 11. Clg is not necessary for survival of *V. cholerae* in mimimal media supplemented with fish collagen as the sole carbon source. *V. cholerae* N16961 strains wt p, Δ*clg* p, and Δ*clg* carrying pClg were grown in minimal media supplemented with either fish collagen or glucose as a sole carbon source. The expression of *clg* was induced with 100 µM IPTG (as indicated). The samples were withdrawn every 24 h of bacterial growth during the period of 96 h. Serial dilutions of cultures were spotted onto LB agar to examine the colony forming units (CFU/mL). The graphs represent bacterial growth in minimal media supplemented with glucose at 37 ºC (A) and at 25 ºC (B), and in minimal media supplemented with fish collagen at 37 ºC (C) and 25 ºC (D).

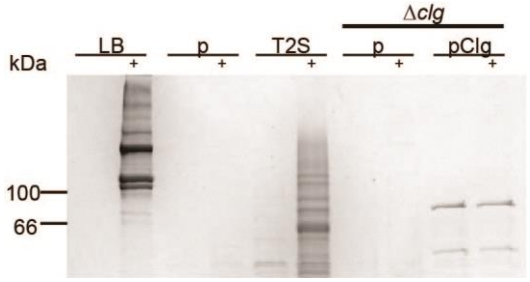

Figure 12. *V. cholerae* secretes other proteases that digest fish collagen. *V. cholerae* N16961 strains (as indicated) were grown in LB medium to early stationary phase of growth. The supernatants were isolated from bacterial cells, incubated with fish collagen at 37ºC for 1 h, separated by SDS-PAGE, and stained with Colloidal Coomassie. Fish collagen incubated in LB alone was used as a negative control. The (+) represents addition of fish collagen.

Other proteases contribute to the survival of *V. cholerae* in minimal growth media supplemented with fish collagen.

To determine if Clg plays a role in utilization of fish collagen as a sole carbon source for *V. cholerae*, the survival of wt p, Δ*clg* p, and Δ*clg* pClg strains was examined as colony forming units (CFU/mL) in minimal media supplemented with fish collagen during growth at either 37ºC or 25ºC (Fig. 11 C and D). Minimal media supplemented with glucose was used as a control (Fig. 11 A and B). Surprisingly, there was no significant difference between p, Δ*clg* p, and Δ*clg* pClg strains (under the tested growth conditions). This suggested that other proteases might also be responsible for degrading fish collagen and therefore aid in *V. cholerae* survival. To determine whether other proteins secreted by *V. cholerae* are involved in collagen degradation, supernatants isolated from different strains of *V. cholerae* were incubated with fish collagen (Fig. 12). Interestingly, fish collagen was not completely digested when incubated with supernatants isolated from the isogenic Δ*eps* p strain. Together, these experiments suggested that in addition to Clg, other protease(s) secreted by the T2SS contribute to the digestion of fish collagen, thus facilitating the survival of the bacteria in minimal media supplemented with this substrate.

Discussion:

Clg belongs to the class II *Vibrio* metalloproteases that possess a HEXXH consensus and display activity to collagen but no other biological substrates (39). Identification and characterization of a novel metalloprotease contributes to the importance of *Vibrio* metalloproteases that display collagenase activity as a factor in bacterial survival and/or pathology.

V. cholerae utilizes the T2SS to secrete multiple proteins that facilitate bacterial survival and pathogenesis during the dual life cycle. However, scarce information is available regarding the contribution of individual proteins secreted by the T2SS for *V. cholerae* fitness. Here, we identified and characterized a novel T2S-dependent metalloprotease that specifically digests fish collagen. Our data suggest that Clg uses the T2S machinery for extracellular transport. While many studies describe the structure and function of this secretion system, there is still a lack of information regarding the mechanism underlying the secretion process of individual proteins. By characterizing the structure of secreted proteins, we may gain more insights about how the T2SS recognizes and secretes its cargo proteins across the outer membrane. The predicted amino acid sequence of Clg revealed the characteristic residues within the catalytic site of Clg in the peptidase M9 domain (Fig. 1 A). Using site-directed mutagenesis, we altered each catalytic residue to alanine and examined the protease activity of Clg lacking individual amino acids. Protease activity against DQ gelatin showed that these residues were essential for Clg activity. Site-directed mutations may result in changes in protein structure (40). Therefore, we verified whether introduction of the point mutations affected recognition of the protein by the T2SS by SDS-PAGE and Colloidal Coomassie staining. This analysis

showed that while individual residues within the Clg catalytic site are essential for protease activity, they do not affect protease secretion (Fig. 1 D).

The analysis of Clg maturation revealed that Clg undergoes autocleavage, but requires other proteases for complete maturation by removal of the C-terminal domains. Previously, it was described that proteases undergo cleavage to become active (6). However, Clg displayed metalloprotease activity in all its forms. *V. mimicus* secretes a metalloprotease also capable of digesting collagen, VMC. Upon cleavage of the C-terminal domains of VMC, the protease displayed binding to type I collagen (39). The analysis of VMC revealed that the cleavage of the C-terminal domains contains a collagen-binding motif FAXWXXT, which is also present in Clg. This suggests that Clg undergoes processing, possibly to expose the collagen-binding motif for higher affinity to bind collagen.

Following biochemical analysis of the protein, we analyzed the putative function of Clg. First, human host derived proteins were incubated with purified Clg and examined on SDS-PAGE. Clg was unable to digest these substrates. Therefore, we hypothesized that Clg may function in the marine environment to digest fish collagen. While *V. cholerae* resides in the marine environment, the bacteria encounter vertebrate fish and may utilize this organism to facilitate bacterial survival (3). Incubation of the purified protein with fish collagen revealed that Clg specifically digests fish collagen. As the composition and proportion of amino acids of fish collagen is similar to that of mammalian collagen, it is possible that Clg may also play a role at some point during infection. The bacteria may secrete proteases to help shed the cells located at the upper intestinal epithelium in order to access the buried collagen. In the host, HapA and TagA play a role in nutrient

acquisition for colonization of the gut (9, 13). HapA displays mucinase activity, while TagA cleaves mucin glycoproteins to provide a carbon and nitrogen energy source for *V. cholerae* growth in the intestine. The digestion of the proteins on the surface of the intestinal epithelium, as well as the diarrhea that induces shedding of the epithelial cells, may reveal deeper layers of the intestine to induce activity of other proteases. Previously, bacterial collagenases have been shown to cause tissue destruction, allowing the bacteria to gain access to more anaerobic sites buried within host tissue (41). VppC, a collagenoltyic metalloprotease secreted by *V. parahaemolyticus*, a bacterium that causes gastroenteritis upon infection or wound-infection upon exposure (42). It was previously shown that VppC contributes to wound-infection by facilitating bacterial spread by degrading extracellular components to cause skin damage. Clg may act to break down collagen for nutrient acquisition to contribute to *V. cholerae* survival in this way.

Additionally, in contrast to metalloproteases such as HapA and PrtV, Clg displays very specific activity towards collagen, as it failed to degrade other substrates encountered by *V. cholerae* in the human host (Fig. 10 A). While Clg specifically digests collagen, our data showed that our protease is not the only protease produced by *V. cholerae* for this purpose. The analysis of bacterial growth in minimal media supplemented with fish collagen revealed that the lack of Clg did not inhibit *V. cholerae* survival. Subsequent analysis of the ability of other proteins present in various strains of *V. cholerae* to digest fish collagen revealed that other proteases were capable of collagen digestion. This indicated functional redundancy of collagen-degrading enzymes. It is possible that under particular conditions, collagen may be required for *V. cholerae* survival. Additionally, collagen present in dead fish and other collagen-rich aquatic organisms may be utilized

by free-living *V. cholerae* in a similar fashion. Deseasin MCP-01 is an extracellular protease from a deep-sea bacterium, *Pseudoalteromonas* sp. SM9913 (17). These proteases degrade marine collagen to contribute to recycling of marine nitrogen. This indicates that many bacterial collagenases are present in the marine environment, facilitating bacterial survival. Therefore, some of these proteases may play redundant functions.

Conclusion:

Our investigations focused on the characterization of Clg, a novel protein secreted by *V. cholerae*. We took several different approaches to investigate the structure and function of this protein. Our findings revealed that Clg is a T2S-dependent metalloprotease that undergoes a maturation process following secretion, producing two forms of the protease (90-kDa and 68-kDa). Clg remains an active protease in all its forms. All subsequent studies investigating Clg activity were performed using the 90-kDa form of the protein. We demonstrated that Clg is able to digest synthetic peptides by zymography and enzymatic assays against DQ gelatin and FALGPA. Clg also specifically digests fish collagen, but does not display activity towards other tested substrates that *V. cholerae* encounters in human host. Our analyses also revealed that while Clg is capable of digesting fish collagen, other proteases also contribute to this activity.

For further Clg characterization, the X-ray crystallography may be used to determine the structure of the protein. Determining the structure of this protein is important to understand how the T2SS works in recognition and secretion of specific cargo proteins. We still have to identify the conditions that allow production of native Clg. The next step in studying the maturation of Clg would be to investigate which protease contributes to its maturation. Lastly, we determined that Clg is capable of degrading fish collagen. However, the precise function of Clg is still unknown. Therefore, more substrates may be examined for substrate-specificity, such as human collagen. Understanding more about its role in the host and environment may reveal more information about its potential function in the *V. cholerae* dual life cycle.

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