Proteomic Analysis of the *Vibrio cholerae* Type II Secretome Reveals New Proteins, Including Three Related Serine Proteases*†‡§

Aleskandra E. Sikora†, Ryszard A. Zielke†, Daniel A. Lawrence‡, Philip C. Andrews‡, and Maria Sandkvist‡

From the Departments of †Microbiology and Immunology, ‡Molecular, Developmental, and Cellular Biology, ‡Internal Medicine, and §Biological Chemistry, University of Michigan, Ann Arbor, Michigan 48109

The type II secretion (T2S) system is responsible for extracellular secretion of a broad range of proteins, including toxins and degradative enzymes that play important roles in the pathogenesis and life cycle of many Gram-negative bacteria. In *Vibrio cholerae*, the etiological agent of cholera, the T2S machinery transports cholera toxin, which induces profuse watery diarrhea, a hallmark of this life-threatening disease. Besides cholera toxin, four other proteins have been shown to be transported by the T2S machinery, including hemagglutinin protease, chitinase, GbpA, and lipase. Here, for the first time, we have applied proteomic approaches, including isotope tagging for relative and absolute quantification coupled with multidimensional liquid chromatography and tandem mass spectrometry, to perform an unbiased and comprehensive analysis of proteins secreted by the T2S apparatus of the *V. cholerae* El Tor strain N16961 under standard laboratory growth conditions. This analysis identified 16 new putative T2S substrates, including sialidase, several proteins participating in chitin utilization, two aminopeptidases, TagA-related protein, cytolysin, RbmC, three hypothetical proteins encoded by VCA0583, VCA0738, and VC2298, and three serine proteases VesA, VesB, and VesC. Focusing on the initial characterization of VesA, VesB, and VesC, we have confirmed enzymatic activities and T2S-dependent transport for each of these proteases. In addition, analysis of single, double, and triple protease knock-out strains indicated that VesA is the primary protease responsible for processing the A subunit of cholera toxin during *in vitro* growth of the *V. cholerae* strain N16961.

Gram-negative bacteria have evolved at least six secretion pathways devoted to the transport of proteins through the cell envelope into either the extracellular environment or directly into host cells (1, 2). The type II secretion (T2S)*2 system was first discovered in *Klebsiella oxytoca* and has been shown to be widely distributed among γ-proteobacteria (3–6). Depending on the bacterial species, the T2S complex consists of 12–16 different constituents that form a multiprotein apparatus spanning the entire cell envelope (7, 8). The conserved components of the T2S machinery include the cytoplasmic ATPase (T2S E), the inner membrane platform (T2S C, F, L, and M), a pilus-like structure (T2S G–K), a protein responsible for the processing of pseudopilins (T2S O), and the secretion pore (T2S D) embedded in the outer membrane (9). The exoprotein precursors are synthesized with N-terminal signal peptides that direct them into the periplasmic space via either the Sec or Tat transport systems (10, 11). After obtaining tertiary conformation, the exoproteins enter the T2S machinery and are subsequently translocated into the extracellular milieu (12, 13). Many key steps in the secretion process are still not well understood, including how the exoproteins are recognized by the T2S system, and a specific secretion signal common to known substrates has not yet been identified.

The T2S system is devoted to secretion of a variety of substrates, including toxins, surface-associated virulence factors, a broad range of enzymes that hydrolyze macromolecules (such as lipids, polysaccharides, and proteins), surfactant(s) important for motility, and certain cytochromes (5, 14–17). The T2S-dependent proteins are of great interest because many of them play important roles in pathogenesis and/or contribute to bacterial fitness in different ecological niches (6, 18–20). Many of the T2S exoproteins were originally identified based on the loss of specific enzymatic activities in culture supernatants of the T2S mutants, and to date only a few comprehensive studies have been undertaken to define a broader array of secreted proteins (21–23). Identification of the T2S substrates from different bacterial species might help to elucidate the mechanism of exoprotein recognition by the T2S system and provide a better understanding of the general role of T2S in pathogenesis and environmental survival.

Our laboratory studies the T2S system in the causative agent of cholera, *Vibrio cholerae*. Cholera is a life-threatening diarrheal disease that predominantly occurs in developing countries of Asia, Africa, and South America (24). The T2S system, extracellular protein secretion (Eps), of *V. cholerae* is responsible for secretion of five known proteins, including cholera tox...
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toxin, chitinase (ChiA-1), chitin-binding protein (GbpA), hemagglutinin protease (HAP), and lipase (25-30).

*V. cholerae* circulates between two very diverse environments, aquatic reservoirs and the gastrointestinal tract of the human body (31). The human host acquires *V. cholerae* through an oral route of infection with contaminated water or food. Following colonization of the small intestine, the bacteria produce and secrete cholera toxin. Although the disease is multifaceted, cholera toxin is the major virulence factor. It stimulates constitutive activation of cellular adenylate cyclase causing severe intestinal fluid loss and watery diarrhea. The persistence of *V. cholerae* in the aquatic niche is likely facilitated by several of the known T2S substrates. GbpA is important for *V. cholerae* attachment to biotic and abiotic chitin surfaces; ChiA1 and HAP play a role in utilization of chitin and insect egg masses as a source of nutrients, respectively (27, 28, 36). Moreover, *gpbA* mutants display defects in adherence to human epithelial cells and in colonization of infant mice, suggesting that GbpA represents a dual colonization factor functioning both in the aquatic environment and the human host (27). Similarly, HAP has been suggested to play a role in infection by facilitating the penetration of *V. cholerae* through the mucus barrier of the gastrointestinal tract and by causing its detachment from the epithelium following the induction of the secretary diarrhea (37, 38).

Despite the significant impact of known T2S substrates on *V. cholerae* pathogenesis and physiology, comprehensive studies have not yet been undertaken to identify additional secreted factors. In pursuit of identifying the T2S secretome of *V. cholerae*, here we have applied global proteomic approaches, including a high throughput technique (iTRAQ) coupled with multidimensional liquid chromatography (LC) and tandem mass spectrometry (MS/MS). We identified 16 new putative T2S-dependent exoproteins, including three related serine proteases VesA, VesB, and VesC. This study focuses on the characterization of these three newly identified proteins.

**EXPERIMENTAL PROCEDURES**

**Bacterial Growth Conditions**—Bacterial cultures were propagated at 37 °C in Luria-Bertani (LB) medium (Fisher) or under AKI conditions (39) with supplements as specified in the text. Antibiotics (Sigma) were used at the following concentrations: carbenicillin, 50 or 100 μg/ml for chromosomal or plasmid expression, respectively; kanamycin, 50 μg/ml; streptomycin, 100 μg/ml; polymyxin B sulfate, 100 units/ml.

**Proteomic Analysis of Proteins in the Culture Supernatants**—To detect proteins that are present in culture supernatants, a method utilizing pyrogallol red-molybdate-methanol was used as described previously (40). Briefly, supernatants were isolated by centrifugation from stationary cultures (16 h, A600 about 4) of NΔeps and parental wild-type strain of *V. cholerae* N16961 grown in LB medium, filtered through 0.2-μm nylon filters (Fisher), followed by high speed centrifugation (170,000 × g, 4 °C, 3 h) to remove outer membrane vesicles. Culture supernatants (0.95 ml) were mixed with equal volume of pyrogallol red-molybdate-methanol solution (0.05 mM pyrogallol red, 0.16 mM sodium molybdate, 1.0 mM sodium oxalate, 50 mM succinic acid, 20% methanol, adjusted to pH 2), and the pH of the mixture was immediately adjusted to 2.8 for optimal precipitation. Samples were incubated at room temperature for 1 h followed by overnight incubation at 4 °C. Proteins were pelleted by centrifugation at 10,000 × g for 1 h, washed twice with ice-cold acetone, and reconstituted in SDS loading buffer. Samples were matched by equivalent A600 loaded onto the gel, and resolved for a short length, ~2 cm, by SDS-PAGE. Gel fragments containing proteins were excised, cut into smaller pieces, and incubated in 100 μl of 100 mM ammonium bicarbonate followed by incubation in 100 μl of acetonitrile. Proteins were reduced in 10 mM dithiothreitol, and the cysteines were blocked in 55 mM iodoacetamide. The gel plugs were dehydrated in acetonitrile, and proteins were digested overnight at 37 °C in the presence of trypsin (10 μg). Tryptic fragments were extracted by addition of formic acid and acetonitrile to final concentrations of 1 and 2%, respectively, and subjected to one-dimensional LC-MS/MS as described below. Data analysis was performed using ProteinPilot as described below for the quantitative proteomic analysis approach.

Quantitative comparison of proteins present in the culture supernatants of wild-type and isogenic T2S mutants (NΔeps and NΔepsD) of *V. cholerae* N16961 (29) was performed by iTRAQ technology coupled with multidimensional LC and MS/MS analysis as follows. Stationary cultures (A600 of ~4) of wild-type and NΔeps and NΔepsD of *V. cholerae* N16961 were back-diluted 1:100 in 1000 ml of prewarmed LB, grown with agitation to the late stationary phase of growth (16 h, A600 about 4). Culture supernatants were separated from the cells by centrifugation (4000 × g for 30 min) followed by filtration through 0.2-μm filters (Nalgene). Protease inhibitor mixture (Complete Mini; Roche Applied Science) was added to the supernatants. For iTRAQ labeling culture supernatants were subsequently treated as described previously (22), and the total protein concentration in the supernatants was measured before and after precipitation using the 2-D Quant kit (GE Healthcare) according to the manufacturer’s instructions. A total of 100 μg of supernatant proteins from each sample was precipitated overnight in 80% acetone following two washes in 100% acetone. Proteins were labeled with iTRAQ tags according to manufacturer’s instructions (Applied Biosystems). Reactions products were combined and stored at −20 °C before two-dimensional LC. Wild-type and the T2S mutants samples were treated in parallel through the labeling procedure. The following labels were used in each experiment: 114 and 116 for wild-type and 115 and 117 to label proteins derived from T2S mutants (NΔeps and NΔepsD) in each technical duplicate and biological replica experiments.

**Two-dimensional Liquid Chromatography and Mass Spectrometry**—Differentially labeled protein samples were mixed and fractionated by strong cation exchange chromatography using a Paradigm MG4 HPLC system (Michrom BioResources) on a packed Polystrylyl Aspartamide™ column; 200 × 2.1 mm; 5 μm; 200 Å (poly-LC). For the second dimension, LC and
mass spectra acquisition samples were treated as described previously (41).

**Data Analysis**—MS/MS spectra were analyzed using the Paradigm search algorithm, part of the ProteinPilot software (version 3.0) (Applied Biosystems) (42) against the SwissProt target-decoy *V. cholerae* data base with the following settings: sample type, iTRAQ 4plex; cysteine alkylation, methyl methanethiosulfonate; digestion, trypsin; ID focus, biological modifications. Thorough ID search and false discovery rate calculations were used. The ProGroup algorithm built within ProteinPilot software was used to perform the statistical analysis on the identified peptides to determine the minimal set of identifications. Only proteins with *p* values <0.05 associated with a ratio were included for further analysis. Proteins with TS2 mutant/wild-type iTRAQ ratios of <0.5 and >2.0 were considered as down- or up-regulated, respectively. We utilized bioinformatics tools, such as PsortB (43, 44) and SignalP (45), to predict the presence of a signal peptide for proteins that were present in lower quantities in the culture supernatants of the TS2 mutants. The MS/MS data obtained were deposited into TRANCHE with the Tranche Hash Aev4cSaBMXqFXX1f6x2y2PhyChlV/Um7HekfZDuip85t/o6-InhpB80q5crSk4m4h3l3aOyOzu4bkYiNFaZmyHGlccAAAAA-AJRrWw.

**Genetic Techniques**—Chromosomal DNA isolated from *V. cholerae* N16961 was used as templates for PCR. PCRs were carried out with PfuUltra™ DNA polymerase (Stratagene). Primers were synthesized by IDT Technologies, Inc. (Coralville, IA). The resulting PCR amplification products were purified, subcloned into pPCR-Script™ampicillin resistance SK(+) (Stratagene), and then cloned into the appropriate vector as indicated. Introduction of plasmids and suicide vectors into *V. cholerae* strains was performed by conjugation with *Escherichia coli* s17.1 or triparental conjugation as described previously (29, 47, 48).

**Cloning and Overexpression of VC1649, VC1200, and VCA0803 Loci**—The VC1649 and VCA0803 loci were amplified with the following primers pairs: Fwd, 5′-GAGCTCCTGGGCATGACCAGGTCAGATCGATAGATC-3′, and Rev, 5′-GATGCGAACTTTATGATCAAGTGTTT-3′. The PCR products were digested with SphI and XbaI-HindIII and cloned into likewise cut pBAD33 (54). The obtained plasmids were transformed into *E. coli* H9262 and then selected with ampicillin and chloramphenicol. The resulting plasmids carried the cloned loci and were named pBAD33-VC1649, pBAD33-VC1200, and pBAD33-VCA0803.

**Deletion Mutants**

Construction of Isogenic Single, Double, and Triple Protease Deletion Mutants—All mutants were constructed by in-frame deletions of the individual genes in *V. cholerae* N16961 using allelic exchange and homologous recombination procedures as described previously (29). To create single, double, and triple protease deletion mutants in VCA0803, VC1200, and VC1649 genes, plasmids pΔvesA, pΔvesB, and pΔvesC, respectively, were used. These plasmids were constructed as follows. The upstream (500 bp) and downstream (500 bp) regions of the DNA flap VCA0803 locus were amplified with oligonucleotide pairs Fwd, 5′-GTCGACACACATCGACCAGAGCTTCC-3′, and Rev, 5′-GATGCGAACTTTATGATCAAGTGTTT-3′, and cloned into pCVD442 (53). The chloramphenicol cassette was amplified from pBAD33 (54) vector using primer pair Fwd, 5′-GATGCGAACTTTATGATCAAGTGTTT-3′, and Rev, 5′-GATGCGAACTTTATGATCAAGTGTTT-3′, and cloned into SacI-Smals digested pCVD442 containing 0.5-kb flanking regions of VCA0803 to yield pΔvesA. To construct pΔvesB, upstream (490 bp) and downstream (495 bp) fragments of VC1200 locus were amplified with primer pairs Fwd, 5′-GATGCGAACTTTATGATCAAGTGTTT-3′, and Rev, 5′-GATGCGAACTTTATGATCAAGTGTTT-3′, and cloned into pCVD442 (53). The PCR products were digested with SacI-KpnI and cloned into like wise cut pUC18K (55). Subsequently, the fragment containing upstream region of VC1200, kanamycin cassette (apha3 cassette), and the downstream region of VC1200 was cut out with restriction enzymes SacI-Smals and cloned into likewise digested pCVD442 to yield pΔvesB. To delete the VC1649 locus, suicide plasmid pΔvesC was constructed as follows. The 1-kb regions located upstream and downstream of VC1649 were amplified using oligonucleotide pairs Fwd, 5′-GATGCGAACTTTATGATCAAGTGTTT-3′, and Rev, 5′-GATGCGAACTTTATGATCAAGTGTTT-3′. The PCR products were digested with SacI-KpnI and cloned into like wise cut pUC18K (55). Subsequently, the fragment containing upstream region of VC1200, kanamycin cassette (apha3 cassette), and the downstream region of VC1200 was cut out with restriction enzymes SacI-Smals and cloned into likewise digested pCVD442 (53) to yield pΔvesC. To delete the VC1649 locus, suicide plasmid pΔvesC was constructed as follows. The 1-kb regions located upstream and downstream of VC1649 were amplified using oligonucleotide pairs Fwd, 5′-GATGCGAACTTTATGATCAAGTGTTT-3′, and Rev, 5′-GATGCGAACTTTATGATCAAGTGTTT-3′. The PCR products were digested with SacI-KpnI and cloned into likewise cut pUC18K (55). Subsequently, the fragment containing upstream region of VC1200, kanamycin cassette (apha3 cassette), and the downstream region of VC1200 was cut out with restriction enzymes SacI-Smals and cloned into likewise digested pCVD442 (53) to yield pΔvesC.

**Processing of Cholera Toxin Assays**—Enzymatic activities of the serine proteases VesA, VesB, and VesC were assessed by analyzing their effect on the intact cholera toxin A subunit (CT A) as follows. Cell-free culture supernatants containing uncleaved CT A isolated from *V. cholerae* strain NΔvesABC grown under AKI conditions (39) were mixed with equal volumes of cell-free supernatants isolated from NΔvesABC carry-
ing either pMMB67, pVesA, pVesB, or pVesC cultured in LB medium supplemented with either 100 μM IPTG (pVesA and pVesB) or 25 μM IPTG (pVesC). Immunoblot analysis with anti-CT showed that wild-type and isogenic mutants of V. cholerae N16961 did not produce detectable levels of cholera toxin while cultured in LB medium (data not shown). Next, the reactions were incubated at 37 °C for 2 h without or with the addition of either leupeptin (10 μM), EGTA, or EDTA (10 mM). Finally, samples were subjected to precipitation with pyrogallol red-molybdate-methanol and processed further as described below.

**SDS-PAGE, Silver Staining, and Western Blotting**—Protein contents in the culture supernatants were analyzed by SDS-PAGE and silver staining as described previously (29). For all experiments, samples were matched by equivalent A₀₆₀₀. CT A and B subunits were separated under denaturating and reducing conditions as indicated. Immunoblots were primarily blocked in 3% BSA in TBS buffer (50 mM Tris, 200 mM NaCl, pH 8), followed by incubation with polyclonal anti-CT antibodies (1:5000; Sigma) using as a positive control purified cholera toxin (Sigma) and finally developed (50). Immunoblots were developed using typhoon Trio (Amersham Biosciences). The semi-quantification of the amounts of processed and unprocessed cholera toxin A subunit was performed with ImageQuant TL (GE Healthcare) software using the rubber band method for background subtraction. The data are presented as percent of processed cholera toxin A subunit and represent averages and S.E. obtained from six independent experiments.

**Suckling Mice Experiments**—The mice were performed as described previously (57) with the following modifications. Briefly, the overnight cultures of wild-type and the isogenic NΔvesABC mutant strain of V. cholerae N16961 cultured in LB medium were back-diluted and grown for about 4 h until reaching a stationary phase of growth (A₀₆₀₀ about 4). The cultures were washed twice in PBS, and 50 μl of suspensions containing either wild-type and the NΔvesABC mutant mixed at a 1:1 ratio (competition assays) or individual strains at ~10⁷ CFUs (single infections) were orogastrically delivered to infant mice. In each experiment 12 CD-1 mice (Harlan Laboratories) were infected. To assess the colonization dynamics in the competition experiments, 7 and 5 animals were euthanized after 3 and 24 h post-infection, respectively. Their small intestines were homogenized in PBS buffer, and bacterial CFUs were determined on LB agar plates supplemented with streptomycin. To enumerate the fraction of mutant CFUs, all colonies from the streptomycin agar plates were patched on LB agar containing kanamycin as a selective marker. In single infection studies, wild-type and triple protease knock-out strains were inoculated separately to 5 and 7 mice, respectively. The mice were euthanized after 24 h post-infection, and the bacterial CFUs were scored as described above on LB agar plates supplemented with streptomycin. The animal experiments were performed in accordance with University of Michigan Committee on Use and Care of Animals guidelines.

**RESULTS**

*V. cholerae Secretes a Variety of Proteins in Rich Medium*—Our previous studies showed that inactivation of the T2S pathway has a broad impact on *V. cholerae* physiology, such that not only is the secretion process inhibited, but the integrity of the cell envelope is also compromised. This results in induction of extracytoplasmic and oxidative stress responses and in alterations in iron homeostasis (29, 58). As a consequence, culture supernatants isolated from the T2S mutants display radically different protein profiles in comparison with wild-type strains with both the number of protein species present as well as their levels being elevated (29). Because of these physiological changes, it is impossible, by traditional techniques of protein separation such as one- or two-dimensional gel electrophoresis, to distinguish proteins in wild-type supernatant that are specifically secreted by the T2S system. Instead, to quantitatively compare the supernatants of the wild-type and T2S mutants, and to identify specific T2S substrates, we have analyzed the culture supernatants by two alternative and independent proteomic approaches as follows: a qualitative secretome analysis and a quantitative high throughput technique, iTRAQ, coupled with multidimensional LC-MS/MS analysis. In the qualitative proteomic analysis, cell-free supernatants from wild-type *V. cholerae* N16961 and the isogenic NΔeps strain (in which the entire epsC-epsN gene cluster has been removed (29)) were ultracentrifuged to remove outer membrane vesicles, precipitated, and analyzed by MS/MS.

Overall, this qualitative experimental approach showed that *V. cholerae* releases a variety of proteins while cultured under standard laboratory growth conditions. These experiments identified a total of 187 proteins that were validated based on at least two independent peptides with a 1% false discovery rate. Approximately half (84) of the proteins were common to both strains, whereas 19 proteins were identified solely in the supernatant isolated from wild-type *V. cholerae*, and 84 protein species were unique to the NΔeps strain (supplemental Tables S1–S3). The greater number of protein species detected in the culture supernatant derived from the T2S mutant was consistent with our previous observations that inactivation of the T2S pathway results in a leaky cell envelope where periplasmic and cytoplasmic proteins are nonspecifically released to the culture medium (supplemental Table S3) (29, 58).

We considered the following as putative T2S substrates: 1) those that were only present in the wild-type supernatant; 2) those that contained predicted N-terminal signal peptides and/or were predicted to be extracellularly secreted, and 3) those whose gene expression was not reduced in the T2S mutants (58). Proteins that met these criteria are detailed in Table 1. Importantly, our experimental approach identified three proteins, HAP, ChiA-1, and GbpA, that have previously been recognized as T2S substrates by other means (26–28, 30). Moreover, using this proteomic method, we identified new potential T2S substrates, including cytolysin, RbmC, four proteins involved in chitin degradation (chitin oligosaccharide deacetylase and proteins encoded by ORFs VCA0140, VC0769, and VCA0027), two proteases (LapX and Lap) and sialidase (neuraminidase) (Table 1) (27, 28, 59–62). In addition, two
putative serine proteases (VC1200 and VC1649), a protein designated as TagA-related protein (VCA0148), and a hypothetical protein encoded by VCA0583 were also specifically detected in the wild-type supernatant, suggesting that they are secreted by the T2S system as well (Table 1). A few additional proteins, including outer membrane porins, were also unique to the wild-type culture supernatant (supplemental Table S1); however, they were not believed to be T2S substrates. These proteins have been shown to use other pathways for transport across or insertion into the outer membrane and are likely differentially expressed in the NΔeps strain due to the alteration in cell envelope stability and induction of RpoE and oxidative stress responses (supplemental Table S1) (29, 58).

High Throughput Proteomic Analysis of the V. cholerae Secretome—To better quantitate the difference between the secreted proteome of the wild-type and T2S mutant strains and to identify additional putative T2-secreted proteins, we employed a high throughput iTRAQ-based LC-MS/MS technique. This proteomic approach allows for simultaneous comparison of complex protein mixtures and was recently applied to profile bacterial, fungal, and eukaryotic secretomes (22, 63–65). To provide additional controls for these experiments, we used two isogenic eps mutants (NΔeps and NΔepsD) in addition to the wild-type strain (58). As shown in Fig. 1, in each iTRAQ experiment, proteins precipitated from supernatants of wild-type, NΔeps, and NΔepsD cultures were subjected to trypsin digestion, and the obtained peptides were labeled at their free amine group with different isobaric mass tag labels as follows: 114 and 116 for the technical replica of wild-type samples and 115 and 117 for the technical replica of T2S mutant samples. The double duplex labeling provided internal controls for uniform isobaric tagging in each experiment. Comparison of the intensity of these reporter labels permitted the relative quantification of identical peptides in each digest and thus the proteins from which they originate (66). Comparison of the abundance ratios for all identified proteins showed a tight distribution within the technical duplicate samples of wild-type (114/116) and both T2S mutants (115/117) with mean values close to 1.0 (supplemental Figs. S1 and S2). The biological replica experiments revealed a total of 386 and 206 different protein species for the NΔeps/WT samples and 362 and 324 proteins for the NΔepsD/WT samples; however, only proteins with p values below 0.05 for the calculated iTRAQ ratios were analyzed further. This reduced the total number of proteins to 144 for the NΔeps/WT samples and 152 for the NΔepsD/WT samples. Complete lists of these proteins are provided in the supplemental Tables S4 and S5.

To identify proteins that were differentially secreted in the T2S mutants compared with the wild-type strain, we first established a cutoff of ≥2.0-fold change in the iTRAQ ratios of T2S mutant/wild-type (iTRAQ ratios below 0.5) for proteins considered likely T2S substrates. Similarly to our qualitative anal-

TABLE 1
Putative T2S dependent proteins identified by MS/MS analysis

| Accession no. | Gene       | Name                                      | Wild type | NΔeps |
|---------------|------------|-------------------------------------------|-----------|-------|
| P09545        | VCA0219    | Cytolysin (VCC)                           | 23*       | 14    |
| P0C8E9        | VC1784     | Sialidase (neuraminidase)                 | 21        | 36    |
| Q9KN26        | VCA0140    | Spundolin-related protein                 | 13        | 1     |
| Q9KLD4        | VCA0812    | Leucine aminopeptidase (Lap)              | 12        | 6     |
| Q9KTH2        | VC0930     | RbmC                                      | 9          | 13    |
| Q9KRI1        | VC1649     | Trypsin-putative (VesC)                   | 9          | 12    |
| Q9KLD3        | VCA0813    | Aminopeptidase (LapX)                     | 4          | 3     |
| Q9KN18        | VCA0148    | TagA-related protein                      | 4          | 8     |
| Q9KQ6P        | VC1952     | Chitinase (ChIA-1)                        | 4          | 1     |
| Q9KSH6        | VC1280     | Chitin oligosaccharide deacetylase (COD)  | 3          | 3     |
| Q9KMW1        | VC0769     | Chitinase, putative                       | 3          | 7     |
| Q24153        | VCA0865    | Hamagglutinin protease                    | 2          | 8     |
| Q9KND8        | VCA0027    | Chitinase                                 | 2          | 7     |
| Q9KSO6        | VC1200     | Trypsin-putative (VesB)                   | 2          | 2     |
| Q9KLD5        | VCA0811    | Glc-Nag-binding protein (GbpA)            | 2          | 2     |
| Q9KMO6        | VCA0583    | Putative uncharacterized protein          | 2          | 2     |

* This table includes selected proteins that were identified in wild type supernatants, and the number of unique peptides is reported for each experiment.

FIGURE 1. Experimental work flow of V. cholerae T2 secretome analysis by iTRAQ coupled with LC/MS/MS. In each iTRAQ experiment, supernatants obtained from stationary cultures of wild-type and isogenic T2S mutants (NΔeps and NΔepsD) were filtered, precipitated, and subjected to trypsin digestion. Subsequently, the tryptic peptides were labeled at their free amines using four different isobaric mass tag labels (114, 116, 115, and 117 for wild-type and for each of eps mutant samples, respectively). Next, the samples were pooled and subjected to a strong cation exchange and reverse phase fractions followed by MS/MS analysis.
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**TABLE 2**
Putative T2S-dependent proteins identified by iTRAQ analysis

| Accession no. | Gene     | Name                          | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
|--------------|----------|-------------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|--------|
|              |          | NΔeps/WT Score | NΔeps/WT Score | NΔepsD/WT Score | NΔepsD/WT Score | SignalP | PostB |
| Q9KN26       | VCA0140  | Spindolin-related protein     | 0.11   | 14     | 0.15   | 14.7   | 0.1   | 14     | 0.1   | 1.16  |
| P09545       | VCA0219  | Cytolysin (VCC)               | 0.19   | 69.3   | 0.23   | 19.6   | 0.2   | 63.8   | 0.26  | 22.64 |
| P09669       | VCA1789  | Sialidase (neuraminidase)     | 0.27   | 37.9   | ND*    | 0.22   | 30.37 | ND*   | 0.28  | Yes   |
| Q9KL3E3      | VCA0803  | Serine protease, putative     | 0.21   | 2.85   | ND     | ND     | ND    | ND     | ND    | Yes   |
| Q9KN18       | VCA0148  | TagA-related protein          | 0.23   | 8      | NP     | NP     | NP    | 0.39  | 6.1   | No    |
| Q9KRJ1       | VCA1649  | Trypsin-putative (VesC)       | 0.23   | 12.7   | 0.23   | 10     | 0.3   | 11.7   | 0.28  | 10    |
| Q9KLK3       | VCA0813  | Aminopeptidase (LapX)         | ND     | ND     | 0.16   | 4.46   | ND    | ND     | NP    | Yes   |
| Q9KNK8       | VCA0027  | Chitinase                     | 0.46   | 12     | ND     | NP     | ND    | NP     | ND    | Yes   |
| Q9KWK1       | VCO769   | Chitinase, putative           | NP     | NP     | NP     | 0.39  | 11.7  | NP    | Yes   |
| Q9KPR8       | VCA2298  | Putative, uncharacterized     | ND     | ND     | ND     | 0.18  | 7     | ND    | Yes   |

58. For example, the eps/WT iTRAQ ratios indicated lower abundance of OmpU, LamB, and several other putative outer membrane proteins likely reflecting a general inhibition of synthesis or degradation of outer membrane proteins that is linked to the activation of rpoE stress response in the T2S mutants of V. cholerae (29, 68). The presence of outer membrane proteins in the supernatants was likely due to a contamination with membrane vesicles as the culture media were not subjected to ultracentrifugation prior to precipitation (see under “Experimental Procedures”).

In agreement with our qualitative proteomic experiment (supplemental Table S1), the iTRAQ analysis also demonstrated that the majority of proteins present in the supernatants of the NΔeps and NΔepsD mutants were present at elevated levels in comparison with the wild-type strain (supplemental Tables S4 and S5). Most of these proteins are normally located in the periplasm or cytoplasm, and their presence in the culture media of the eps mutants indicates increased cell lysis as observed previously (29). Within this group, proteins that participate in iron metabolism and different stress responses, including oxidative stress, were highly represented (up to 10-fold increased levels) (supplemental Tables S4 and S5). These findings are in agreement with the data from our previous biochemical and microarray experiments, which indicated that inactivation of the T2S system results in induction of rpoE and oxidative stress responses and misregulation of iron metabolism (58).

**Enzymatic Activities of VesA, VesB, and VesC**—Our proteomic analysis revealed that V. cholerae secretes among other proteins three putative serine proteases (VCA0803, VC1200, and VC1649; Tables 1 and 2). We decided to focus on the initial characterization of these three proteins for the remainder of this study and designated these proteins as Ves (for Vibrio extracellular serine protease). Although the genes encoding
these proteases do not belong to the same operon, for simplicity we named them as VesA (VCA0803), VesB (VC1200), and VesC (VC1649). NCBI Blast homology searches of the predicted amino acid sequences of VesA, VesB, and VesC indicated that they are ~30% identical to each other and that homologous proteins are widely distributed among Vibionales but are otherwise scarce among Gram-negative bacteria. All three protein sequences revealed a predicted signal peptide and a putative protease domain with a catalytic triad characteristic for serine-type proteases as follows: histidine, aspartic acid, and serine (Fig. 2A) (71). The extensive homology between the protease domains of the three putative proteases and trypsinozen as well as the linear arrangement of their catalytic residues, HDS, suggest that they belong to the chymotrypsin family, which includes proteases such as chymotrypsin, trypsin, and elastase (supplemental Fig. S3). As we have previously described, culture supernatants of wild-type but not the T2S mutants contain an enzyme(s) that is capable of cleaving the fluorogenic peptide Boc-Gln-Ala-Arg-AMC (Boc-QAR-AMC), a substrate commonly used for trypsin and trypsin-like proteases, it is possible that these proteases contribute to the activity observed in wild-type supernatants (29, 52, 68, 72). To determine whether these putative proteases contribute to the measured activity, we constructed single, double, and triple isoegenic protease knock-outs of V. cholerae N16961 and assessed their ability to hydrolyze Boc-QAR-AMC. There was an 80% decrease in the proteolytic activity in the supernatants isolated from the double (N∆vesA, N∆vesB, and N∆vesC), double (N∆vesAB, N∆vesAC, and N∆vesBC), and triple (N∆vesABC) protease mutant strains. Strains were grown in LB media to the stationary phase of growth (16 h). The protease activity assays were performed in technical triplicates. Bars represent mean ± S.E. (n = 10).

**FIGURE 2. VesB primarily contributes to the protease activity in wild-type cultures.** A, three newly identified proteases possess putative signal peptides (SP) and predicted trypsin-like domains. Fragment of sequence alignment with underlined catalytic triad characteristic for serine proteases is shown. B, enzymatic activities against Boc-QAR-AMC were examined in culture supernatants isolated from cultures of wild-type (wt), single (N∆vesA, N∆vesB, and N∆vesC), double (N∆vesAB, N∆vesAC, and N∆vesBC), and triple (N∆vesABC) protease mutant strains. Strains were grown in LB media to the stationary phase of growth (16 h). The protease activity assays were performed in technical triplicates. Bars represent mean ± S.E. (n = 10).
Finally, to verify the proteomic data that secretion of VesA, VesB, and VesC relies on the T2S machinery, each of these proteases were overproduced in the wild-type and T2S mutants (N

/H9004

/eps

, and N

/H9004

/epsD

). There was a statistically significant 2-, 16-, and 15-fold increase in the protease activity measured in the supernatants isolated from wild-type V. cholerae carrying either pVesA, pVesB, or pVesC in comparison with the activity detected in the wild-type supernatant isolated from the control cultures carrying the empty vector (Fig. 4). In contrast, undetectable or close to background levels of activity against Boc-QAR-AMC were detected in the supernatants isolated from both T2S mutants overproducing these proteases (Fig. 4). These experiments further supported the proteomic results suggesting that VesA, VesB, and VesC require the T2S system for extracellular secretion.

Assessing the Role of VesA, VesB, and VesC in the Survival and Colonization of V. cholerae in Infant Mice—Extracellular proteases play important roles in bacterial pathogenesis by generating nutrients, creating a niche for colonization, suppressing host defense mechanisms, and supporting host dissemination (74–76). Because all these processes can, in turn, have a positive impact on the fitness of the bacteria in the host, we decided to assess whether the proteases contribute to V. cholerae fitness in the infant mouse model as a first attempt to determine their roles. We reasoned that if the proteases have an impact on V. cholerae survival in the intestinal environment, the N

/vesABC

strain lacking all three proteases should display a more severe defect than individual knockouts. Hence, to test the coloniza-
tion dynamics, competitive colonization assays were performed with the wild-type and NΔvesABC strains. A group of 12 animals was inoculated orogastrically with a mixture containing equal numbers of wild-type and NΔvesABC bacteria, and five and seven animals were euthanized at 3 and 24 h post-infection, respectively. Their small intestines were excised and homogenized, and bacterial CFUs were recovered on selective LB agar plates. The competitive indices of the NΔvesABC strain versus the wild-type V. cholerae corrected by the number of CFUs in the inoculum were 0.85 ± 0.13 and 0.67 ± 0.13 at 3 and 24 h post-infection (median and standard deviation reported), respectively, indicating that the triple mutant displayed neither significant growth nor colonization defects when co-inoculated with the wild-type strain. We also performed single infection assays because the NΔvesABC strain could potentially benefit from the proteases secreted by the wild-type V. cholerae strain when co-inoculated. Groups of five and seven infant mice were inoculated orogastrically with the wild-type and the NΔvesABC strain, respectively. The average numbers of CFUs recovered from the isolated intestines after 24 h post-infection were 8.07 × 10^7 and 5.12 × 10^7 for wild-type and mutant strain, respectively. Taken together with the competition assay, these experiments suggest that VesA, VesB, and VesC do not contribute to the intestinal survival and colonization capability of V. cholerae in infant mice.

Role of the Serine Proteases in the Processing of Cholera Toxin— Many bacterial toxins are activated through proteolytic cleavage, which results in the separation of the catalytic domain from the remainder of the toxin (77). Cholera toxin is an example of a toxin that requires activation and consists of a single A subunit (CT A) and five identical B subunits. An exposed loop in CT A is the target of proteases, and cleavage at this site results in the generation of two polypeptide chains CT A1 and CT A2 that remain connected by a disulfide bridge until reduced (78). Several lines of evidence suggest that V. cholerae secretes its own protease(s) that is responsible for cleaving and thus activating choler toxin (79). CT A obtained from culture supernatants of V. cholerae is usually found in the processed form; however, nonprocessed CT A can be isolated when the culture media are supplemented with either serine or metalloprotease inhibitors (79–81). It has also been shown that purified proteases such as the V. cholerae metalloprotease HAP or human trypsin and elastase are each able to cleave and activate CT A (79, 81). Moreover, unprocessed CT A has been isolated from a protease-deficient strain of V. cholerae; however, the molecular identity of the protease remains unknown (81). We hypothesized that the newly identified proteases may play a role in the proteolytic cleavage of CT A. To test this hypothesis, supernatants isolated from wild-type, single, double, and triple protease mutants grown under conditions that induce the production of cholera toxin (39) were examined by SDS-PAGE in the presence of reducing agent followed by immunoblotting with polyclonal anti-CT antibodies (Fig. 5A). In the wild-type strain, the majority (65.4%) of the CT A migrated as a 23-kDa peptide, corresponding to the migration of the A1 polypeptide of the purified and reduced cholera toxin (Fig. 5A, compare lanes 2 and 3). In contrast, supernatants isolated from the NΔvesA mutant contained mostly the intact form of CT A (69%), which migrated as 29-kDa protein corresponding to the mobility of purified and nonreduced CT A (Fig. 5A, compare lane 1 with 4). Deletion of either vesB or vesC, however, did not result in statistically significant reduction in the processing of CT A subunit (Fig. 5A, lanes 5 and 6). In accordance with these data, supernatants isolated from the double and triple knock-out strains NΔvesAB, NΔvesAC, and NΔvesABC, in which VesA was absent, contained up to 80% of unprocessed CT A (Fig. 5A, lanes 7, 8, and 10).

The cleavage of CT A could be restored by providing ectopically expressed vesA in both the NΔvesA and NΔvesABC strains (Fig. 5B, lanes 2 and 4, respectively). Although removal of vesB had only a minimal effect on the CT A processing, overproduction of VesB in the NΔvesABC strain also led to complete processing of CT A (Fig. 5B, lane 5). Variable results were obtained when VesC was overproduced, either there was no effect on the CT A processing or it resulted in the general degradation of cholera toxin (data not shown). Collectively, these results suggest that VesA and to a lesser extent VesB are

![Graph](image)
Type II Secretion-dependent Proteins of Vibrio cholerae

causedef some processing of CT A (Fig. 6, lane 10), and the action of this protease could also be blocked by the addition of leupeptin but not by metal chelators (Fig. 6, lanes 11–13). The proteolytic activity of VesC using CT A as a substrate was also tested; however, addition of VesC had no effect on processing of CT A (Fig. 6, lanes 14–17). Finally, there was also some processing of CT A visible as a very light band corresponding to the 23-kDa CT A1 in the absence of VesA and VesB, and when the samples were treated with leupeptin, which might be due to the action of HAP (Fig. 6, lanes 2–5, 7, 11, and 14–17). In summary, these results indicate that under laboratory growth conditions CT A is processed by the endogenous proteases of V. cholerae once secreted to the extracellular milieu. The processing occurs primarily by the newly identified VesA protease with a moderate contribution from VesB and HAP.

**DISCUSSION**

This is the first study describing a comprehensive analysis of the V. cholerae T2 secreton. We have used a combination of proteomic approaches to compare the protein profiles of wild-type and T2S-deficient strains, and we expanded the list of T2S substrates from five to 21 (Tables 1 and 2). Our study shows that applying different proteomic approaches is beneficial and complementary, especially when complex protein samples are analyzed. Although further verification of the results may be necessary for a subset of proteins, together the results from our proteomic experiments indicate that V. cholerae secretes a variety of hydrolytic enzymes, including sialidase, cytolysin, two aminopeptidases, four chitinases, three trypsin-like serine proteases, and a TagA-related protein in addition to cholera toxin, HAP, ChiA, GbpA, and lipase (Tables 1 and 2). Similarly to other T2S systems, in V. cholerae the T2S machinery is primarily engaged in transport of hydrolytic enzymes that likely play a role in modifying the surroundings and generating nutrients and thus might support bacterial fitness in different ecological niches, including the human host. The T2S machinery is capable of secreting as many as 25 different substrates, as demonstrated for the T2S system in L. pneumophila (18, 21). Our proteomic studies focused on the analysis of the T2 secreton of V. cholerae cultured under a single growth condition, and responsible for cleaving CT A under laboratory growth conditions.

It has been shown previously that purified HAP is able to cleave CT A, and we show here that overproduction of HAP in the ΔvesA or ΔvesABC strains results in processing of the CT A subunit (Fig. 5b, lanes 7 and 9); however, very little HAP is normally made in V. cholerae N16961 due to a frameshift mutation in the positive regulator HapR (82), and therefore, we believe that VesA is the primary protease responsible for processing of CT A in laboratory-grown N16961. Further support for this suggestion comes from the finding that addition of leupeptin to the culture media resulted in complete blocking of CT A processing in the wild-type V. cholerae strain (supplemental Fig. S3).

Next, we aimed to verify that the processing of CT A takes place in the extracellular milieu after the proteins are secreted. Culture supernatant isolated from ΔvesABC grown under CT-inducing conditions, which resulted in the accumulation of uncleaved CT A, was harvested and mixed with supernatants isolated from the same mutant strain carrying either empty vector (p), pVesA, pVesB, or pVesC that were cultivated in LB medium, which does not support cholera toxin production (83). Following incubation to allow for cleavage of CT A by the exogenously added proteases, the samples were examined by SDS-PAGE and immunoblotting. Additionally, to test the effect of different protease inhibitors on the ability of the proteases to process CT A, the supernatants isolated from the strains grown in LB were preincubated with either leupeptin, EGTA, or EDTA, prior to mixing with the supernatant containing the unprocessed toxin. As shown in Fig. 6, CT A in the control sample (supernatant from ΔvesABC strain treated with supernatant from ΔvesABC carrying vector only) remained mostly unprocessed throughout the experiment (lanes 2–5). In contrast, addition of supernatant containing VesA led to full cleavage of CT A (Fig. 6, lane 6) indicating that processing of CT A by VesA likely occurs once both proteins have been secreted to the extracellular milieu. The enzymatic activity of VesA was inhibited by leupeptin (Fig. 6, lane 7) but not by EDTA or EGTA (Fig. 6, lanes 8 and 9, respectively). In agreement with our genetic analysis, exogenously provided VesB

**FIGURE 6. Processing of the A subunit of cholera toxin occurs following secretion and is inhibitable by the serine protease inhibitor leupeptin.** Culture supernatants from the ΔvesABC strain of V. cholerae N16961 carrying either vector alone (p) or overproducing proteases (pVesA, pVesB, or pVesC) grown in LB medium (no CT production, CT−) with the addition of 100 μM IPTG. These supernatants were precultivated at 37 °C for 15 min with 10 μM leupeptin (lanes 3, 7, 11, and 15), 10 mM EGTA (lanes 4, 8, 12, and 17), 10 mM EDTA (lanes 5, 9, 13, and 17), or no protease inhibitor (lanes 2, 6, 10, and 14). Subsequently, culture filtrates obtained from the ΔvesABC strain grown under AKI conditions to produce CT (CT+) were added, and the samples were further incubated at 37 °C for 2 h. At that time, the reaction was immediately blocked by precipitation (see “Experimental Procedures”), and CT A processing was analyzed by SDS-PAGE and immunoblotting with anti-CT antibodies. Purified CT with reducing agent (Fig. S3) is included to show the position of processed A subunit. The mobility of the intact A subunit, A1 polypeptide, and B subunit are indicated on the left side of the figure. Data shown are representative of three independent studies.
therefore the complete repertoire of the T2-secreted proteins in *V. cholerae* was not revealed. For example, cholera toxin and lipase, two previously identified T2S substrates, were not detected in this study because the expression of their respective genes are under strict regulatory controls and require growth conditions that differ from the one used here (29, 39). On the other hand, we did establish that *V. cholerae* produces and secretes several proteins predicted to play a role in different steps of chitin degradation and utilization even though the cultures were grown in the absence of chitin. This was not surprising, however, because it has been shown previously that these genes remain expressed in *V. cholerae* grown in LB medium (84). Besides degrading chitin, these proteins may play an additional function in *V. cholerae* physiology by participating in processing of other GlcNAc containing carbohydrates or proteins.

The proteins discovered in our study expand the tools used to investigate the molecular mechanism of substrate recognition by the T2S machinery and provide a new reference for elucidating the function of secreted proteins in *V. cholerae* physiology. With the exception of cholera toxin, HAP, and GbpA, the impact of individual secreted proteins on *V. cholerae* pathogenesis and metabolism is understudied. Hence, we focused on the initial characterization of the three newly identified proteases, VesA, VesB, and VesC, and investigated their hydrolytic activities against a fluorogenic peptide commonly used for trypsin-like proteases. We also established several lines of evidence that VesA plays a pivotal role in the processing of the A subunit of cholera toxin *in vitro*. Because it has been well documented that growth of *V. cholerae* in the presence of serine protease inhibitors markedly reduced the processing and activation of CT A, we assume that the activity inhibited was primarily that of VesA (81).

Biochemical assays with purified proteins have shown that proteases such as HAP, trypsin, and elastase are capable of processing CT A (79, 81). Moreover, studies with human intestinal T84 cell lines have demonstrated that the presence of an additional factor(s) is also capable of CT A cleavage (Fig. 5A). In light of these findings, it is very likely that host proteases are responsible for CT A activation during intestinal colonization of *V. cholerae*; however, there may be conditions, yet to be determined, during which *Vibrio* proteases such as VesA and HapA play significant roles in the processing and activation of CT A. Nevertheless, in this study for the first time a genetic analysis demonstrated that *V. cholerae* N16961 lacking vesA secretes mostly unprocessed CT A, suggesting that VesA is the primary protease responsible for CT A processing *in vitro*. Based on the immunoblot analysis, about 30% of the CT A was processed in the vesA knock-out strain, suggesting that additional factor(s) is also capable of CT A cleavage (Fig. 5A). Based on observations made by us and others, the most likely candidate(s) for additional endogenous proteins that are responsible for the processing of CT A is VesB and HAP (79). Because of the very low level of HAP in *V. cholerae* strain N16961, VesB is likely the primary protease responsible for the residual processing of CT A. This conclusion is supported by the finding that *V. cholerae* N16961 grown in the presence of the serine protease inhibitor leupeptin, which inhibits both VesA and VesB, secretes intact CT A (supplemental Fig. 4).

We did not find that VesC protease is capable of processing cholera toxin under tested conditions (Figs. 5 and 6). However, a very recent study has shown that VesC induces a hemorrhagic response when injected into rabbit ileal loops suggesting that it may play a role in pathogenesis (73). As we have showed here; however, this activity does not seem to be required for intestinal colonization of mice.

Importantly, our study revealed that the T2S system of *V. cholerae* secretes proteins that are functionally associated with cholera toxin, including VesA, Hap, and sialidase. Sialidase contains a sialic acid binding domain that is responsible for localizing the enzyme to higher organized sialic gangliosides in the intestinal mucus and cleavage of sialic acid groups revealing GM1, the receptor for cholera toxin (86, 87). VesA, Hap, and sialidase likely work synergistically, ensuring the proper localization and activation of the toxin immediately after its secretion, thus further underscoring the importance of the T2S system in *V. cholerae* pathogenesis.

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