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Identification and Characterization of a Novel Serine Protease, VvpS, That Contains Two Functional Domains and Is Essential for Autolysis of *Vibrio vulnificus*▽

Moon Sub Lim,[1]‡ Jeong-A Kim,[2]‡ Jong Gyu Lim,[1] Byoung Sik Kim,[1] Kwang Cheol Jeong,[3]† Kyu-Ho Lee,[2] and Sang Ho Choi[1]*

National Research Laboratory of Molecular Microbiology and Toxicology, Department of Agricultural Biotechnology, Center for Food Safety and Toxicology, and Research Institute for Agriculture and Life Sciences, Seoul National University, Seoul 151-921,[1] Department of Environmental Science, Hankuk University of Foreign Studies, Yongin 449-791,[2] and Department of Food Science and Technology, Chonnam National University, Kwang-Ju 500-757,[3] South Korea

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Little is known about the molecular mechanism for autolysis of Gram-negative bacteria. In the present study, we identified the *vvpS* gene encoding a serine protease, VvpS, from *Vibrio vulnificus*, a Gram-negative food-borne pathogen. The amino acid sequence predicted that VvpS consists of two functional domains, an N-terminal protease catalytic domain (PCD) and a C-terminal carbohydrate binding domain (CBD). A null mutation of *vvpS* significantly enhanced viability during stationary phase, as measured by enumerating CFU and differentially staining viable cells. The *vvpS* mutant reduced the release of cytoplasmic β-galactosidase and high-molecular-weight extracellular chromosomal DNA into the culture supernatants, indicating that VvpS contributes to the autolysis of *V. vulnificus* during stationary phase. VvpS is secreted via a type II secretion system (T2SS), and it exerts its effects on autolysis through intracellular accumulation during stationary phase. Consistent with this, a disruption of the T2SS accelerated intracellular accumulation of VvpS and thereby the autolysis of *V. vulnificus*. VvpS also showed peptidoglycan-hydrolyzing activity, indicating that the autolysis of *V. vulnificus* is attributed to the self-digestion of the cell wall by VvpS. The functions of the VvpS domains were assessed by C-terminal deletion analysis and demonstrated that the PCD indeed possesses a proteolytic activity and that the CBD is required for hydrolyzing peptidoglycan effectively. Finally, the *vvpS* mutant exhibited reduced virulence in the infection of mice. In conclusion, VvpS is a serine protease with a modular structure and plays an essential role in the autolysis and pathogenesis of *V. vulnificus*.

Any form of cell death mediated by an intracellular death program has been referred as programmed cell death (8). Although programmed cell death, classically known as apoptosis, is traditionally associated with eukaryotic cells (25), a substantial body of data suggests that bacteria have also evolved programmed cell death to eliminate cells in the course of development and to eradicate defective or damaged cells (24). Autolysis, perhaps the most commonly observed programmed cell death in bacteria, is a self-lysis of the cell wall by intrinsically produced enzymes called autolysins. Bacterial peptidoglycan (PGN) hydrolases are the best-studied autolysins and can be grouped into glucosidases (*N*-acetylglucosaminidases and muramidases), amidases, and peptidases according to the cleavage sites of the enzymes (47). PGN peptidases hydrolyze the various LD and DD bonds in the stem peptides and cross bridges of PGN (47).

Autolysis of defective or damaged cells resulting from exposure to antibiotics and other harmful conditions is frequently observed in bacteria (24). It is becoming increasingly apparent that bacteria exhibit social behavior analogous to that found in higher organisms and form complex biofilms, tightly regulated communities of cells (5). From this point of view, autolysis of damaged cells is also altruistic and beneficial to bacterial communities. Autolysis of cells that are seriously damaged could donate their nutrients to kin cells rather than drain limited resources in a hopeless attempt to repair themselves. Maintenance of a low mutation rate, which is important to keep the integrity of a population, is also a benefit obtainable by elimination of cells with damaged DNA. Recent studies have revealed an additional function of autolysis in bacterial communities, as such extracellular chromosomal DNA (eDNA) resulting from autolysis of bacterial cells is a key component of the biofilm matrix and plays an essential role in maintaining intercellular adhesion and biofilm stability (2).

Although biochemical and molecular biological characteristics of the enzymes and their roles in autolysis of many Gram-positive bacteria have been extensively studied (for a recent review, see reference 47), relatively few studies on the enzymes (and any other cellular components) and their functions that contribute to the autolysis of Gram-negative bacteria have been reported (34). A Gram-negative marine bacterium, *Vibrio vulnificus*, is a causative agent of food-borne diseases, such as life-threatening septicemia and possibly gastroenteritis, in individuals with underlying predisposing conditions (for a recent

* Corresponding author. Mailing address: Department of Agricultural Biotechnology, Seoul National University, Seoul, 151-742, South Korea. Phone: 82-2-880-4857. Fax: 82-2-873-5095. E-mail: choish@snu.ac.kr.

‡ M.S.L. and J.A.K. contributed equally to this work.

† Present address; Department of Animal Sciences, Emerging Pathogens Institute, University of Florida, Gainesville, FL 32611.

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Bacterial strains		
V. vulnificus		
MO6-24/O	Clinical isolate; virulent	47
MO6Δ*lacZ*	MO6-24/O with Δ*lacZ*	1
MS001	MO6-24/O with *vvpS*::*nptI*; Km^r	This study
MS003	MO6-24/O with Δ*lacZ vvpS*::*nptI*; Km^r	This study
MS023	MO6-24/O with Δ*pilD*	This study
MS033	MO6-24/O with Δ*pilD vvpS*::*nptI*; Km^r	This study
MS042	MO6-24/O with Δ*lacZ* Δ*pilD*	This study
MS043	MO6-24/O with Δ*lacZ* Δ*pilD vvpS*::*nptI*; Km^r	This study
E. coli		
DH5α	λ^- ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17*(r_K^- m_K^-) *supE44 thi-1 gyrA relA1*	Laboratory collection
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pir*; Km^r; host for π-requiring plasmids; conjugal donor	27
BL21(DE3)	F^- *ompT hsdS*$_B$(r_B^- m_B^-) *gal dcm* (DE3)	Laboratory collection
Plasmids		
pUC18	Cloning vector; Ap^r	Laboratory collection
pCVD442	R6K γ *ori sacB*; suicide vector; *oriT* of RP4; Ap^r	7
pDS132	R6K γ *ori sacB*; suicide vector; *oriT* of RP4; Cm^r	31
pJH0311	0.3-kb MCS of pUC19 cloned into pCOS5; Ap^r Cm^r	15
pET28a(+)	His_6 tag fusion expression vector; Km^r	Novagen
pMBPparallel1	MBP tag fusion expression vector; Ap^r	40
pRKΩ*lacZ*	pRK415 with promoterless *lacZ* Ω; Sm^r Tc^r	1
pKC990	pUC18 with *vvpS*; Ap^r	This study
pKC9907	pCVD442 with *vvpS*::*nptI*; Ap^r Km^r	This study
pMS0746	pJH0311 with *vvpS*; Ap^r Cm^r	This study
pMS0806	pDS132 with Δ*pilD*; Cm^r	This study
pMS0734	pRKΩ*lacZ*; P_{vvpS}-*lacZ* transcriptional fusion; Sm^r Tc^r	This study
pMS0908	pJH0311 with *pilD*; Ap^r Cm^r	This study
pMS1051	pMBPparallel1 with *vvpS* (for wild-type VvpS); Ap^r	This study
pMS1052	pMBPparallel1 with 3′-truncated *vvpS* (for VvpS389); Ap^r	This study
pMS1053	pMBPparallel1 with 3′-truncated *vvpS* (for VvpS349); Ap^r	This study
pMS1054	pMBPparallel1 with 3′-truncated *vvpS* (for VvpS289); Ap^r	This study
pMS1055	pMBPparallel1 with 3′-truncated *vvpS* (for VvpS209); Ap^r	This study
pMS1061	pJH0311 with *lacZ*; Ap^r Cm^r	This study

[a] Ap^r, ampicillin resistant; Cm^r, chloramphenicol resistant; Km^r, kanamycin resistant; MCS, multicloning sites.

review, see reference 17). However, no studies have yet been reported on the autolysis of *V. vulnificus*. Neither autolysins nor other cellular components involved in the autolysis of the bacterium have been identified. Accordingly, *V. vulnificus vvpS*, encoding a serine protease, VvpS, that appears essential for autolysis of the bacterium was identified and characterized in the present study. It appeared that VvpS, a protein with a highly modular structure, exerts its cell-lytic effects through intracellular accumulation during stationary phase and contains PGN-hydrolyzing activity that is mediated by the C-terminal domain of the protein. Furthermore, the possible role of VvpS in the pathogenesis of *V. vulnificus* was also explored.

MATERIALS AND METHODS

Strains, plasmids, and culture media. The strains and plasmids used in this study are listed in Table 1. Unless otherwise noted, the *Escherichia coli* and *V. vulnificus* strains were grown in Luria-Bertani (LB) medium at 37°C and LB medium supplemented with 2.0% (wt/vol) NaCl (LBS) at 30°C, respectively.

Cloning of *V. vulnificus vvpS* and generation of the *vvpS* and *lacZ vvpS* mutants. *V. vulnificus* genomic DNA was prepared and digested with Sau3AI, ligated with pUC18 (Table 1), and introduced into *E. coli* DH5α. A positive clone that exhibited proteolytic activity on the LB plate supplemented with skim milk (1.5% [wt/vol]) was obtained. The plasmid, named pKC990, in that clone harbored a 2.4-kb insert with an open reading frame (ORF) of 1,599 nucleotides. Since a database search for homology to the amino acid sequence deduced from the ORF singled out a putative serine protease (NCBI; http://www.ncbi.nlm.nih.gov), the ORF was named *vvpS* (*V. vulnificus* serine protease gene).

To inactivate *vvpS* in pKC990 *in vitro*, a 1.2-kb *nptI* DNA conferring resistance to kanamycin (28) was inserted into an EcoRV site present within the coding region of *vvpS*, and the resulting 3.6-kb *vvpS*::*nptI* was subcloned into pCVD442 (7) to generate pKC9907 (Table 1). To generate a *vvpS* mutant by homologous recombination, *E. coli* SM10 λ*pir tra* (containing pKC9907) (27) was used as a conjugal donor to *V. vulnificus* MO6-24/O (47). For the construction of the *lacZ vvpS* double mutant, MO6Δ*lacZ*, an isogenic mutant of MO6-24/O that lacks *lacZ*, was used as the recipient (1) (Table 1). The conjugation and isolation of the transconjugants were conducted using methods previously described (16), and the resulting *vvpS* and *lacZ vvpS* mutants chosen for further analysis were named MS001 and MS003, respectively (Table 1). pMS0746 was constructed by subcloning *vvpS* amplified by PCR using the primers VVPSCF01 and VVPSCR01 into the broad-host-range vector pJH0311 and used for complementation of the *vvpS* mutation (Tables 1 and 2) (15).

Purification of the truncated VvpSC protein and Western blot analysis. The DNA encoding the C-terminal 143 amino acids of VvpS, VvpSC, was amplified by PCR using the primers HISVVPSF01 and HISVVPSR01 (Table 2). The PCR product was subcloned into a His_6 tag expression vector, pET28a(+) (Novagen, Madison, WI). The resulting His-tagged VvpSC was expressed in *E. coli* BL21(DE3), and the protein was purified by affinity chromatography according to the manufacturer's procedure (Ni-nitrilotriacetic acid [NTA] agarose; Qiagen, Valencia, CA). The purified VvpSC was used to raise anti-VvpS polyclonal antibodies. Polyclonal antibodies specific for the protein were made by immunizing New Zealand White rabbits on 3 occasions at 3-week intervals with 500 μg of the protein for each immunization (AbFrontier, Seoul, South Korea).

The bacterial cells grown in LBS were harvested, washed, broken by sonication

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′→3′)[a]	Use
VVPSCF01	CGAGCTCATAAGCGCCCGATTAAACAA	Complementation of *vvpS*
VVPSCR01	CCCCGGGAAACGAGGCTTGTGGGTAAG	Complementation of *vvpS*
VVPSPM00	ATCGGATCCCTGTGAAAGAACCTCTGC	*vvpS-lacZ* fusion
VVPSPM18	AGGCTGCAGAAACTTTCCGTGATTATGGT	*vvpS-lacZ* fusion
PILDF01	TGCATGCTGACCACTGGTGTCCCTATT	Construction of *pilD* mutant
PILDF02	TTTGCGGCCGCAAAAGCAAGGTATTGAGATGG	Construction of *pilD* mutant
PILDR01	TTTGCGGCCGCAAACAGGGAAGCTAAACCAAT	Construction of *pilD* mutant
PILDR02	ATCTAGAGTAGGCGCTGTTTCTCAATG	Construction of *pilD* mutant
PILDCF01	CGAGCTCGATAACCCTCGCAGTTTACC	Complementation of *pilD*
PILDCR01	CCCCGGGATCCAATCACTAAAGCCATA	Complementation of *pilD*
HISVVPSF01	CGGATCCACCACCACACCACCAGATGG	Construction of His_6 VvpSC
HISVVPSR01	CCTCGAGGTTAAAGTTTTGTCCAAGCT	Construction of His_6 VvpSC
MBPVVPSF01	CATGCCATGGCAGAGGTTCTTTCACAGACG	Construction of MBP-VvpS
MBPVVPSR01	AACTGCAGAAACGAGGCTTGTGGGTAAG	Construction of MBP-VvpS
MBPVVPSR02	AACTGCAGTTAATCTTTCGCGCTTGCCGAGAC	Construction of MBP-VvpS389
MBPVVPSR03	AACTGCAGTTAGGTTTGACCATTAGAAAGCGT	Construction of MBP-VvpS349
MBPVVPSR04	AACTGCAGTTAAGGCGTATAGCCAACAGAAAC	Construction of MBP-VvpS289
MBPVVPSR05	AACTGCAGTTATGCTACGTCTCCCACATTGGC	Construction of MBP-VvpS209

[a] Regions of oligonucleotides not complementary to the corresponding genes are underlined.

(Ultrasonic processor; Sonics & Materials, Inc., CT), and clarified by centrifugation to generate cell lysates (22). Cellular fractions were prepared by using the PeriPreps Periplasting Kit (Epicentre Biotechnologies, Madison, WI) as previously described (10). The resulting cytoplasm and periplasm fractions, equivalent to 10 μg of total proteins, were subjected to Western blot analyses. Proteins (10 μg) from the cell lysates, cellular fractions, or culture supernatant (10 μl) were resolved by SDS-PAGE (37), and immunoblotting was performed according to the procedure previously described by Lee et al. (22). The protein concentrations were determined by the Bradford method (3), with bovine serum albumin used as the standard.

Construction of MBP-VvpS and MBP-VvpS with C-terminal deletions. To create a set of mutant VvpS with C-terminal deletions, the primer MBPVVPSF01 (Table 2) was used in conjunction with one of the following primers for PCR amplification of the coding regions of *vvpS*: MBPVVPSR01 (for amino acids 24 to 532, wild-type VvpS), MBPVVPSR02 (for amino acids 24 to 389, VvpS389), MBPVVPSR03 (for amino acids 24 to 349, VvpS349), MBPVVPSR04 (for amino acids 24 to 289, VvpS289), or MBPVVPSR05 (for amino acids 24 to 209, VvpS209) (Table 2). Since overexpressed VvpS proteins were insoluble (data not shown), the PCR product was used to make an in-frame gene fusion with the 3′ end of the *malE* gene encoding maltose-binding protein (MBP) in pMBPparallel1 (40) and resulted in pMS1051, pMS1052, pMS1053, pMS1054, and pMS1055, respectively (Table 1). The *malE-vvpS* (or truncated *vvpS*) junctions of the plasmids were sequenced to confirm that the two coding regions were in identical reading frames. The resulting MBP-VvpS and MBP-VvpS with C-terminal deletions were expressed and purified by affinity chromatography according to the manufacturer's procedures (Amylose resin; New England BioLabs, Ipswich, MA).

Determination of protease and PGN-hydrolyzing activities. The protease activity of MBP-VvpS was determined as a casein-hydrolyzing activity, as previously described (45). Briefly, 100 μM MBP-VvpS was added to 500 μl of 0.25% (wt/vol) azocasein (Sigma, St. Louis, MO) solution in 100 mM sodium citrate buffer, pH 6.0. The resulting reaction mixture was incubated for 2 h at 30°C, and the A_{366} was measured. One unit of enzyme activity is defined as an increase in A_{366} of 0.01 per hour for protease activity. For PGN-hydrolyzing activity, *V. vulnificus* PGN was isolated by the method of Glauner (14), and *Staphylococcus aureus* PGN was purchased (Sigma). The reaction was initiated by addition of 100 μM MBP-VvpS to 500 μl of 0.25 mg/ml PGN solution in 100 mM potassium phosphate buffer, pH 6.0. After incubation for 2 h at 30°C, the A_{450} was determined (49). One unit of enzyme activity is defined as a decrease in the A_{450} of 0.01 per hour for PGN-hydrolyzing activity.

Measurement of viability and autolysis. For determination of viability, cultures in LBS were inoculated with an initial cell density at an A_{600} of approximately 0.005 and incubated at 30°C with shaking. Samples were removed at the indicated times, and CFU and membrane integrity were measured. The membrane integrity was analyzed using a Live/Dead *Bac*Light viability kit (Molecular Probes, Eugene, OR) as described elsewhere (41). After staining, randomly selected areas were imaged using an epifluorescence microscope (×1,000 magnification; Olympus BX51, Tokyo, Japan) to differentiate viable (with an intact cell membrane; green) and nonviable (lacking an intact cell membrane; red) cells.

To measure autolysis as a function of β-galactosidase release into culture supernatants (42), pMS1061 that constitutively expresses β-galactosidase was constructed by subcloning the *lacZ* gene from pUC18 into pJH0311 (Table 1) and was introduced into *V. vulnificus* by conjugation. β-Galactosidase activity in the cell-free supernatants was determined as described previously (42) and reported in Miller units (26). The release of eDNA was measured for determination of autolysis of the cultures as previously reported (46). Briefly, supernatants of the samples were filtered and concentrated 10-fold using a 10-kDa cutoff membrane (Sartorius Stedim Biotech, Gottingen, Germany). The concentrated samples were loaded on a 0.5% agarose gel, and high-molecular-weight eDNAs were visualized by staining with ethidium bromide.

Construction of a P_{vvpS}-*lacZ* transcriptional fusion. A P_{vvpS}-*lacZ* transcriptional fusion reporter was created by subcloning the 477-bp upstream promoter region of *vvpS* (P_{vvpS}), amplified by PCR using the two primers VVPSPM00 and VVPSPM18, into pRKΩ*lacZ* (Tables 1 and 2). The plasmid carries a promoterless *lacZ* gene (1). The resulting P_{vvpS}-*lacZ* reporter, pMS0734, was transferred into MO6Δ*lacZ* (1) by conjugation, and the β-galactosidase activity was determined by the chloroform-sodium dodecyl sulfate method described previously (26).

Construction of the *pilD*, *pilD vvpS*, *lacZ pilD*, and *lacZ pilD vvpS* mutants. The *V. vulnificus pilD* gene encoding the type IV leader peptidase of the type II secretion system (T2SS) (29) was inactivated *in vitro* by deletion of about four-fifths (696 bp out of 870 bp) of the *pilD* ORF using the PCR-mediated linker-scanning mutation method (23). Pairs of primers—PILDF01 and PILDR01 (for amplification of the 5′ amplicon) or PILDF02 and PILDR02 (for amplification of the 3′ amplicon)—were designed and used as listed in Table 2. The 2.1-kb DNA fragment containing Δ*pilD* was amplified by PCR using the mixture of both amplicons as the template and PILDF01 and PILDR02 as primers and then ligated with SphI-XbaI-digested pDS132 (31), forming pMS0806. *E. coli* SM10 λ*pir tra* (containing pMS0806) was used as a conjugal donor to MO6-24/O, MS001, MO6Δ*lacZ*, and MS003 for the construction of the *pilD*, *pilD vvpS* double, *lacZ pilD* double, and *lacZ pilD vvpS* triple mutants, respectively. The resulting four mutants were named MS023, MS033, MS042, and MS043, respectively (Table 1).

Determination of mouse mortality. Mouse mortalities from the wild type and *vvpS* mutant were compared using ICR mice (specific pathogen free; Seoul National University), as described elsewhere (19). The *V. vulnificus* strains grown in LBS broth at 30°C were harvested and suspended in phosphate-buffered saline (PBS) to the appropriate concentrations. Groups ($n = 10$) of 7-week-old normal female mice, without an iron-dextran pretreatment, were injected intraperitoneally with 0.1 ml of the bacterial suspensions, and mouse mortalities were recorded for 24 h. All manipulations of mice were approved by the Institute of Laboratory Animal Resources of Seoul National University. To examine the functions of the secreted extracellular VvpS in the pathogenesis of *V. vulnificus*,

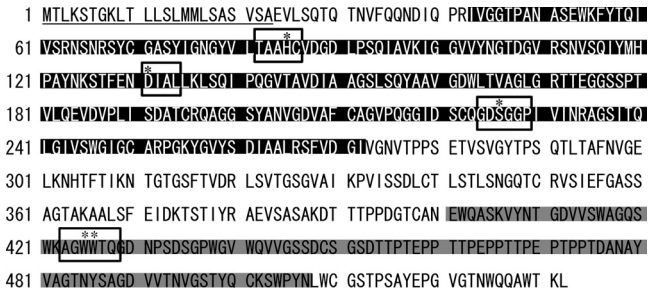

FIG. 1. Sequence analysis of *V. vulnificus* VvpS. The amino acid sequence of VvpS deduced from the *vvpS* nucleotide sequence of MO6/24-O (GenBank CP002469) is shown with the PCD (black) and CBD (gray) predicted using the InterProScan program (European Bioinformatics Institute; http://www.ebi.ac.uk/Tools/InterProScan). The putative signal peptide is underlined. The conserved regions of the PCD (TAAHC, DIAL, and GDSGGP) and CBD (stWWst) are indicated by open boxes. The asterisks (*) indicate the conserved catalytic triad (H, D, and S) in the PCD and two solvent-exposed aromatic residues in the CBD, respectively. s, small residue; t, turn-like (4).

the purified VvpS protein (at a concentration of 15 mg kg of body weight^{-1} per mouse) was injected into the mice, and the mortality of the mice was observed as described above.

Data analyses. Averages and standard errors of the mean (SEM) were calculated from at least three independent trials. Mouse mortality was evaluated using the log rank test program (http://bioinf.wehi.edu.au/software/russell/logrank/). All other data were analyzed by Student's *t* test with the SAS program (SAS software; SAS Institute Inc., Cary, NC). The significance of differences between experimental groups was accepted at a P value of <0.05.

RESULTS

Sequence analysis of *V. vulnificus* VvpS. The 532-amino-acid sequence of pre-VvpS was deduced from the *vvpS* nucleotide sequence *V. vulnificus* MO6-24/O (48) (GenBank, accession number CP002469) and analyzed using the InterProScan program (European Bioinformatics Institute; http://www.ebi.ac.uk/Tools/InterProScan) (Fig. 1). The pre-VvpS protein contains an N-terminal signal peptide with a putative cleavage site located between the 23rd amino acid (Ala) and the 24th (Glu), suggesting that VvpS is a secreted protein. The deduced mature VvpS was comprised of 509 amino acids with a theoretical mass of 52.97 kDa and a pI of 4.69. Further searches for amino acid sequences similar to those of *V. vulnificus* VvpS identified three coding regions from the *V. harveyi*, *V. parahaemolyticus*, and *V. alginolyticus* genome sequence database (GenBank accession numbers EEZ85352, BAC59905, and EAS77110, respectively), with about 67 to 70% identity in the deduced amino acid sequences (data not shown). However, the functions of the gene products of these three coding regions have not yet been addressed.

The amino acid sequence of VvpS exhibited a modular structure consisting of an N-proximal protease catalytic domain (PCD), a linker domain, and a C-terminal carbohydrate binding domain (CBD), as depicted in Fig. 1. The PCD contains TAAHC, DIAL, and GDSGGP regions that are highly conserved in most serine proteases (35). The three amino acids His85, Asp131, and Ser226 of VvpS, embedded in the TAAHC, DIAL, and GDSGGP regions, respectively, could compose the catalytic triad of VvpS, as observed in most serine proteases (32). The VvpS CBD is grouped into CBD family III based on the sequence homology (data not shown) (4). The primary structure of the VvpS CBD contains the highly conserved stWWst (s, small residue; t, turn-like) motif essential for interaction with carbohydrate substrates (Fig. 1) (4). Common features of bacterial CBDs that include conserved Trp and Gly residues, high contents of hydroxyl amino acids, and low contents of charged amino acids are also found in the VvpS CBD (12). The results indicated that VvpS is a serine protease hydrolyzing specific molecules possessing carbohydrate residues, such as PGN.

Construction of the *vvpS* mutant and protease activity of VvpS. The *vvpS* isogenic mutant MS001 of *V. vulnificus* (Table 1) was constructed by an allelic exchange, and the insertional disruption of the *vvpS* gene in MS001 was confirmed by PCR as previously described (reference 16 and data not shown). By Western blot analysis, a protein of the predicted size of VvpS, about 53 kDa, was detected in the culture supernatant of the wild type harvested at 12 h, supporting the idea that VvpS is secreted to the cell exterior (Fig. 2A). The VvpS protein in the culture supernatant of the *vvpS* mutant, however, was not detected by Western blotting, indicating that the disruption of *vvpS* in the mutant MS001 resulted in complete loss of production of VvpS. We examined whether reintroduction of a recombinant *vvpS* could complement the loss of VvpS production of MS001. The VvpS production of MS001(pMS0746) was restored to the wild-type level (Fig. 2A).

To characterize the enzymatic properties of VvpS, the protease activity of the purified MBP-VvpS was determined in the presence of different protease inhibitors. The protease activity of MBP-VvpS was apparent and reached 3 units, whereas the protease activity of MBP, as a negative control, was not detectable (Fig. 2B). The addition of 1 mM phenylmethylsulfonyl fluoride (PMSF) into the reaction mixture resulted in a substantial reduction of the protease activity of MBP-VvpS, and the residual level of protease activity corresponded to approximately 1/10 of that observed in the absence of the inhibitor (Fig. 2B). In contrast, the protease activity of MBP-VvpS was not affected significantly in the presence of 10 mM EDTA, indicating that VvpS is a serine protease rather than a metalloprotease (44). Therefore, we have designated the gene *vvpS* to represent *V. vulnificus* serine protease to differentiate it from the other genes encoding other potential proteases of *V. vulnificus*.

Effects of VvpS on the viability of *V. vulnificus*. When compared by measuring viable cell numbers as CFU ml^{-1}, the *vvpS* mutant and parental wild type grew equally well in LBS during exponential phase and reached about 10^9 to 10^{10} cells per ml at 24 h (Fig. 3A). Between 48 and 72 h, the viability of the wild type began to decrease, and no viable cells were detectable after 96 h of culture, whereas the viability of the *vvpS* mutant was well maintained until 96 h of culture (Fig. 3A). The result indicated that the viability of the *vvpS* mutant is significantly higher than that of the wild type. Again, the enhanced viability of the *vvpS* mutant was attenuated to a level comparable to that of the wild type by the reintroduction of *vvpS* on pMS0746 (Fig. 3A).

Differential staining of the viable and dead cells using a Live/Dead *Bac*Light viability kit demonstrated that the red dead cells were detected in the wild-type culture at 24 h, whereas none appeared in the *vvpS* mutant culture until 48 h

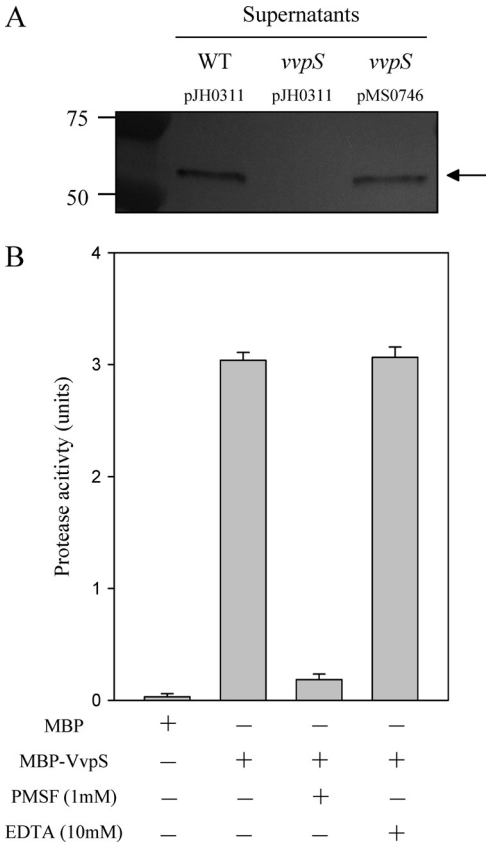

FIG. 2. VvpS in the supernatants of *V. vulnificus* and the effects of the protease inhibitors on VvpS activity. (A) Cultures of the wild type and *vvpS* mutant harboring each plasmid as indicated were grown in LBS for 12 h at 30°C, and the VvpS proteins in the supernatants were determined by Western blot analysis. Complementation of the mutant with a functional *vvpS* (pMS0746) is also presented, as indicated. Molecular mass markers (Precision Plus Protein standards; Bio-Rad Laboratories) and VvpS proteins (arrow) are shown in kilodaltons. (B) MBP-VvpS protein (100 μM) was used for the determination of protease activity, defined as an azocasein-hydrolyzing activity in the presence of either 1 mM PMSF or 10 mM EDTA. The same amount of MBP protein was used as a negative control. The error bars represent the SEM.

(Fig. 3B). In addition, the *vvpS* mutant culture maintained more viable cells than that of the wild type. While a substantial portion of the *vvpS* mutant cells remained viable, no viable cells were detectable in the wild-type culture at 96 h. Complementation of the *vvpS* mutation in MS001 with a functional *vvpS* (pMS0746) reduced the enhanced viability to a level comparable to that of the wild type (Fig. 3B). These results suggested that VvpS could play an essential role in the death of *V. vulnificus* during stationary phase in batch laboratory culture.

Autolysis and eDNA release. To examine whether VvpS contributes to the death of *V. vulnificus* by inducing cell lysis, the release of β-galactosidase and eDNA into the culture supernatants was measured at the indicated time points (Fig. 4A and B). For the parental *lacZ* strain (MO6Δ*lacZ*) harboring pMS1061 expressing a *lacZ* gene constitutively, β-galactosidase release increased during stationary phase and reached a maximum at 96 h (Fig. 4A). The disruption of *vvpS* in the *lacZ vvpS* mutant (MO6Δ*lacZ vvpS*) resulted in a significant reduction of β-galactosidase release into the culture supernatant, suggesting that VvpS contributes to the lysis of *V. vulnificus* cells during stationary phase.

When determined by agarose gel electrophoresis, high-molecular-weight eDNAs released from lysed cells were more abundant in the supernatant of the wild type than in the supernatant of the *vvpS* mutant (Fig. 4B). The eDNAs were detected in the supernatant of the wild type as early as 24 h. For the *vvpS* mutant, the eDNA initially appeared in the supernatant of the *vvpS* mutant at 48 h, and its amount was significantly smaller than that of the wild type. When pairs of primers designed to hybridize specifically to the *V. vulnificus* genomic DNA were used, DNA fragments of identical size were amplified from the eDNAs and *V. vulnificus* chromosomal DNA by PCR (data not shown). This result indicated that the eDNAs are indeed *V. vulnificus* chromosomal DNA and confirmed that VvpS contributes to the lysis of the bacteria. Therefore, since VvpS is an endogenously produced enzyme, it is reasonable to assume that VvpS is an autolysin of *V. vulnificus* and that lysis by VvpS is autolysis.

Intracellular localization of VvpS. When measured using pMS0734, a P_{vvpS}-*lacZ* reporter (Table 1), the *vvpS* expression appeared at the beginning of growth, increased during exponential phase, and then decreased after 24 h (data not shown). Interestingly, when analyzed by Western blotting, the cellular location of the VvpS protein varied at different growth stages. The VvpS protein was found primarily in the culture supernatant harvested at 12 h (Fig. 5A). However, the extracellular level of VvpS was not detectable after 24 h, and most of the VvpS accumulated in the cells (Fig. 5B). Since autolysis of *V. vulnificus* became evident after 24 h, this result suggests that autolysis of the bacteria relies, not on the expression level of VvpS, but rather on the accumulation of VvpS in the cells. In addition, when the exact intracellular location of VvpS in cells grown for 24 h was monitored by Western blot analysis, greater amounts of VvpS were detected in the periplasmic space than in the cytoplasmic space (Fig. 6). The periplasmic location of VvpS indicated that the protein might be able to degrade the cell wall as a step in the autolysis of *V. vulnificus*.

Effect of *pilD* mutation on the secretion of VvpS and autolysis of *V. vulnificus*. *V. vulnificus* has been shown to possess a T2SS responsible for the extracellular secretion of at least three degradative enzymes (29). In order to determine whether the T2SS is involved in the secretion of VvpS, a mutant that lacks PilD, a component of the T2SS, was constructed and the VvpS secretion of the mutant was compared with that of the parental wild type (Fig. 7A). A Western blot analysis revealed that the VvpS protein was not detected in the culture supernatant of the *pilD* mutant at 12 h, indicating that VvpS of *V. vulnificus* is also secreted via the T2SS. The lack of VvpS secretion in the *pilD* mutant was restored by the introduction of pMS0908 (Table 1) carrying a recombinant *pilD*. In contrast, when the intracellular levels of VvpS in the *pilD* mutant and wild-type cells grown for 12 h were compared, more VvpS (and its degradation products) was accumulated in the *pilD* mutant cells (Fig. 7B). These results indicated that disruption of the T2SS by the *pilD* mutation is able to accelerate the accumulation of VvpS in the cells.

To examine whether this accelerated accumulation of VvpS can affect autolysis, the lysis of *V. vulnificus* strains was com-

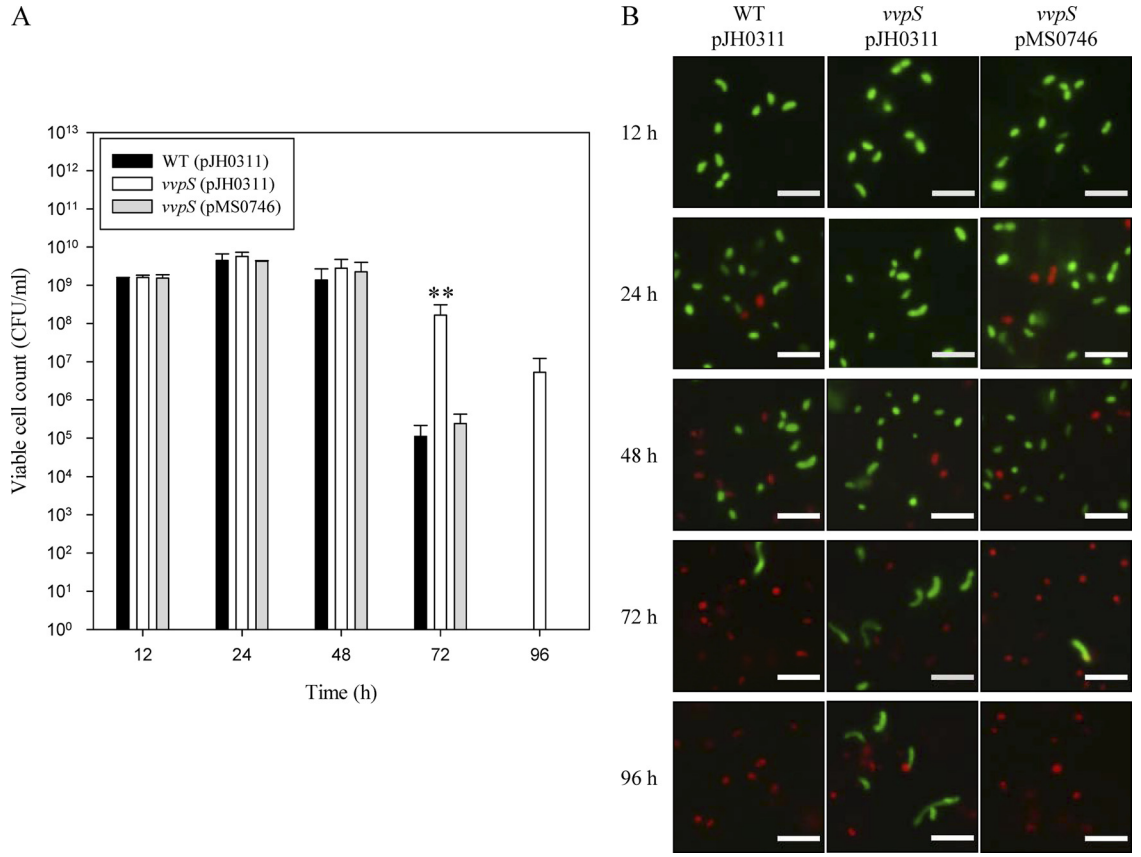

FIG. 3. Effects of the *vvpS* mutation on the viability of *V. vulnificus*. Cultures of the wild type and *vvpS* mutant harboring each plasmid, as indicated, were grown in LBS at 30°C, and samples were removed at the indicated times for enumeration of viable cells as CFU ml^{-1} (A) and differential staining of viable (green) or dead (red) cells (B). A functional *vvpS* (pMS0746) was used for complementation of the *vvpS* mutant. The error bars represent the SEM. **, $P < 0.005$ relative to the wild type at the indicated time. The *V. vulnificus* cells were stained with the Live/Dead *Bac*Light viability kit. Bars = 5 μm.

pared by measuring the release of β-galactosidase as shown in Fig. 4A. Based on the β-galactosidase activity in the culture supernatants, the lysis of the *pilD* mutant was significantly higher and faster than that of the parental wild type (Fig. 7C). This observation indicated that the accelerated accumulation of VvpS in the cells contributed to the enhanced lysis of the *pilD* mutant. Consistent with this, the increased level of β-galactosidase activity in the supernatant of the *pilD* mutant was reduced to the level of the parental wild type by additional disruption of *vvpS* (Fig. 7C).

PGN-hydrolyzing activity of VvpS. All of these observations suggested that VvpS might be able to cleave the cell wall of *V. vulnificus*. Therefore, PGN was prepared from the *V. vulnificus* cell wall and used for the determination of the PGN-hydrolyzing activity of MBP-VvpS. As shown in Fig. 8, MBP-VvpS digested the *V. vulnificus* PGN, indicating that VvpS possesses cell wall-hydrolyzing activity. The presence of 1 mM PMSF, but not 10 mM EDTA, inhibited the PGN-hydrolyzing activity of MBP-VvpS. The patterns of inhibition were similar to the inhibition of protease activities, which were determined by measuring the casein-hydrolyzing activity (Fig. 2B), suggesting that the PGN hydrolysis is attributable to the proteolytic activity of MBP-VvpS (Fig. 8). Since no PGN-hydrolyzing activity of serine proteases with a conserved catalytic His-Asp-Ser triad has been reported, a staphylococcal PGN was purchased and used to reexamine the PGN-hydrolyzing activity of MBP-VvpS. MBP-VvpS hydrolyzed the staphylococcal PGN as well, confirming the PGN-hydrolyzing activity of MBP-VvpS (Fig. 8). Taken together, the data demonstrated that VvpS is responsible for lysis of the cell wall and thus autolysis of *V. vulnificus*.

Effects of the CBD on the activity of VvpS. To examine the role of the CBD in the enzymatic activity of VvpS, we constructed *vvpS* 3′-deletion mutants and compared the abilities of their products to act as proteases and PGN hydrolases (Fig. 9). MBP-VvpS289 with C-terminal deletions of up to 243 amino acid residues still showed protease activities comparable to those of wild-type MBP-VvpS. The results indicated that the N-terminal amino acid residues prior to position 289 of VvpS constitute an independently folded domain that possesses proteolytic activity. These results were consistent with the prediction that the N-terminal region contains a serine protease catalytic domain based on the VvpS sequence analysis (Fig. 1). Interestingly, the MBP-VvpS with C-terminal deletions of 143 or more amino acid residues revealed significantly reduced PGN-hydrolyzing activity (Fig. 9). These results demonstrated that the C-terminal 143 amino acid residues, predicted to be a CBD, are indeed required for PGN-hydrolyzing activity. As anticipated, MBP-VvpS209 with a deletion of 323 C-terminal

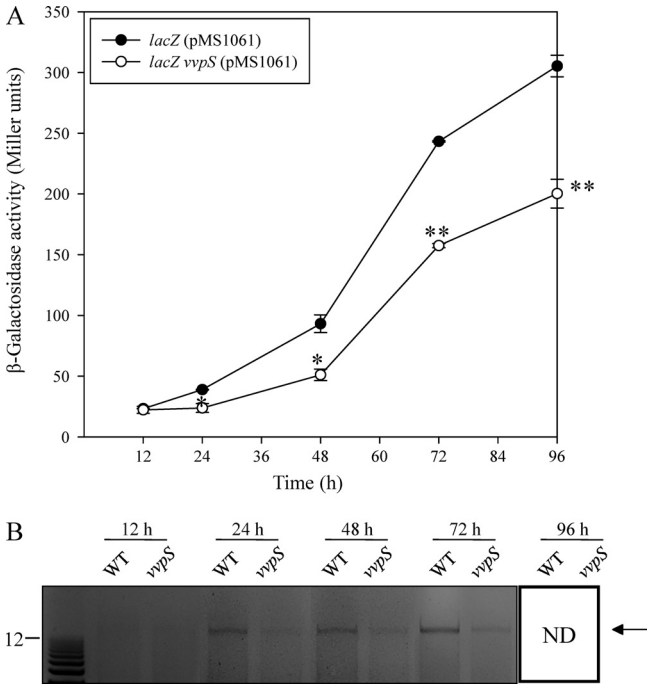

FIG. 4. Effects of the *vvpS* mutation on the autolysis of *V. vulnificus*. (A) Cultures of the parental *lacZ* mutant and *lacZ vvpS* double mutant harboring plasmid pMS1061, which constitutively expresses β-galactosidase, were grown in LBS at 30°C. Samples were removed at the indicated times, and β-galactosidase activities in the supernatant were measured (reported in Miller units). The error bars represent the SEM. ∗, $P < 0.05$, and ∗∗, $P < 0.005$ relative to the parental *lacZ* mutant at the indicated times. (B) Cultures of the wild type and *vvpS* mutant were grown in LBS at 30°C, and samples were removed at the indicated times for detection of eDNAs released into the supernatant. The eDNAs were stained with ethidium bromide. Molecular size markers (1-kb Plus DNA Ladder; Invitrogen) and eDNAs (arrow) are shown in kilobases. ND, not detected.

amino acid residues, which lacks a part of the PCD, showed no evident protease activity and PGN degradation.

Virulence in mice is dependent on *vvpS*. The role of the *vvpS* gene in virulence was also examined using a mouse model, and

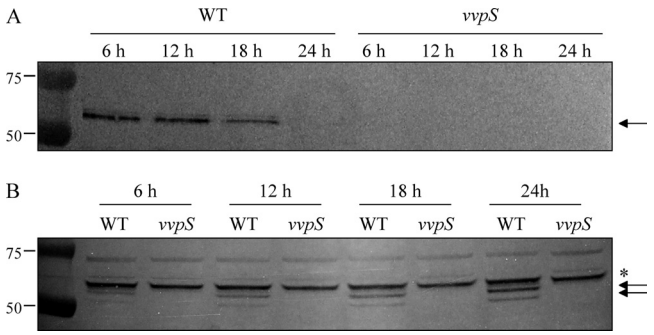

FIG. 5. Growth phase-dependent localization of VvpS. (A and B) Supernatants (A) and cell lysates (B) of the wild type grown in LBS at 30°C were prepared at the indicated times and examined for the presence of VvpS protein by Western blot analyses. The Western blots are presented as described in the legend to Fig. 2. Molecular mass markers (Precision Plus Protein standards; Bio-Rad Laboratories) for VvpS proteins (arrows) and a cross-reacting protein (asterisk) are shown in kilodaltons.

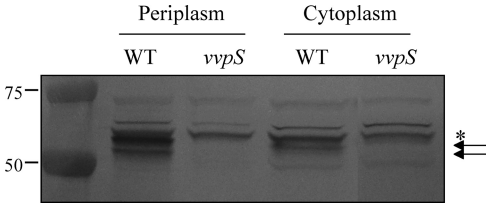

FIG. 6. VvpS in the cytoplasmic and periplasmic spaces. Cytoplasm and periplasm fractions of the wild type and *vvpS* mutant grown in LBS for 24 h were prepared by using the PeriPreps Periplasting Kit (Epicentre Biotechnologies, Madison, WI). The resulting fractions, equivalent to 10 μg of total proteins, were loaded in each lane and examined for the presence of VvpS protein by Western blot analyses. The Western blots are presented as described in the legend to Fig. 2. Molecular mass markers (Precision Plus Protein standards; Bio-Rad Laboratories) for VvpS proteins (arrows) and a cross-reacting protein (asterisk) are shown in kilodaltons.

survival curves of mice injected intraperitoneally with either the *vvpS* mutant or the parental wild type at doses of 10^6 or 10^5 CFU were monitored for 24 h (Fig. 10A and B). The results showed that the deaths of mice injected with the *vvpS* mutant were consistently and significantly delayed ($P < 0.05$; log rank test) compared to those of mice injected with the parental wild type. Therefore, for the mouse model of intraperitoneal infection, the *vvpS* mutant appeared to be significantly less virulent than its parental wild type. As such, these results indicated that the *V. vulnificus* VvpS is apparently important for the pathogenesis of the bacterium.

The functions of the secreted extracellular VvpS in the pathogenesis of *V. vulnificus* were also assessed by injecting the MBP or MBP-VvpS protein into mice and by evaluating the mortality of the mice. There was no death in both groups of mice injected with either protein within 24 h (data not shown), indicating that the secreted VvpS is not an important virulence factor. From the result, it seemed unlikely that the increased virulence observed in the wild type resulted from the activity of the extracellular VvpS secreted into the host environment during infection. Thus, when taken together, the results of the present study proposed that the accumulated intracellular VvpS, rather than the secreted extracellular protein, contributes to autolysis and plays an important role in the pathogenesis of *V. vulnificus*.

DISCUSSION

Over one-third of all known proteases, commonly called peptidases or proteolytic enzymes, are serine proteases. Serine proteases, named after the catalytic serine residue, are usually endopeptidases and catalyze bond hydrolysis in the middle of polypeptide chains (32). Serine proteases are subdivided based on catalytic mechanisms and common ancestry, and trypsin-like proteases are the most abundant among serine proteases (32). VvpS belongs to the trypsin-like proteases and contains the conserved catalytic His-Asp-Ser triad in the PCD (Fig. 1). VvpS is capable of hydrolyzing PGN, as well as casein (Fig. 2B and 8). Although the catalytic mechanism of VvpS is not yet clear, it is reasonable to assume that VvpS is also most likely to cleave a bond (or bonds) in the stem or cross bridge peptides of PGN. Consistent with this, structural analysis reported previously that Ser is implicated in the catalysis of a certain num-

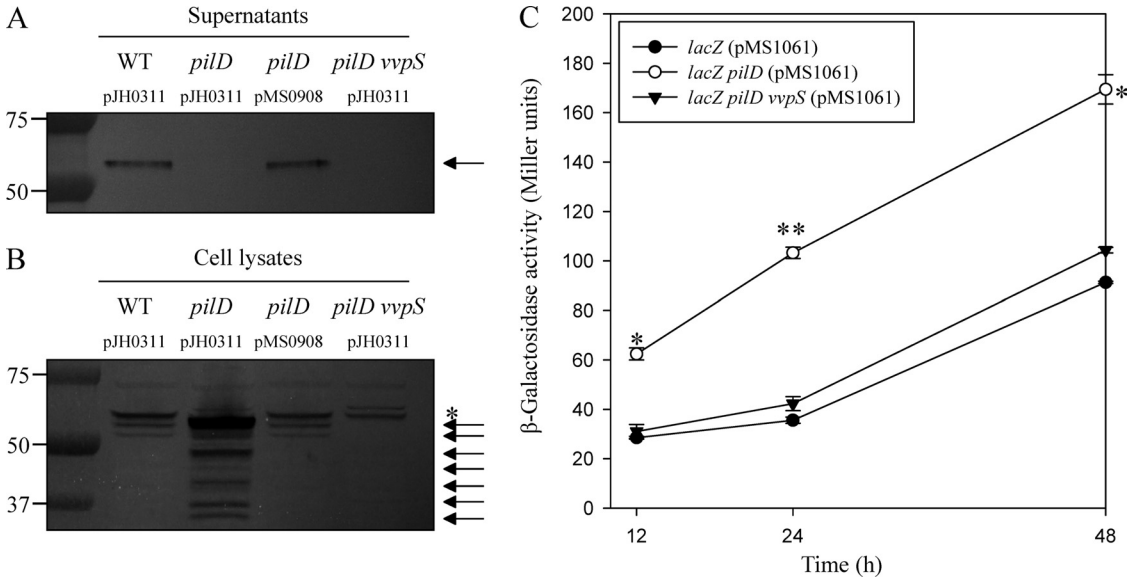

FIG. 7. Secretion and accumulation of VvpS mediated by the T2SS and autolysis of *V. vulnificus*. (A and B) The VvpS proteins in the supernatants (A) and cell lysates (B) of the wild type, *pilD* mutant, complemented strain, and *pilD vvpS* double mutant grown in LBS for 12 h at 30°C were examined by Western blot analyses. The Western blots are presented as described in the legend to Fig. 2. Molecular mass markers (Precision Plus Protein standards; Bio-Rad Laboratories) for VvpS proteins (arrows) and a cross-reacting protein (asterisk) are shown in kilodaltons. (C) Cultures of the parental *lacZ* and *lacZ pilD* double and *lacZ pilD vvpS* triple mutants harboring plasmid pMS1061, which constitutively expresses β-galactosidase, were grown in LBS at 30°C. Samples were removed at the indicated times, and β-galactosidase activities in the supernatant were measured (reported in Miller units). The error bars represent the SEM. *, $P < 0.05$, and **, $P < 0.005$ relative to the parental *lacZ* mutant at the indicated times.

ber of PGN peptidases (19, 20, 21, 38, 39). However, these serine PGN peptidases do not contain the catalytic His-Asp-Ser triad and thus do not belong to the trypsin-like proteases. Therefore, VvpS, a trypsin-like protease, seems to be a unique and rare PGN peptidase that is evolutionarily unrelated to previously reported serine PGN peptidases.

Interestingly, VvpS was able to hydrolyze the PGNs of *V. vulnificus* and *S. aureus* with comparable efficiencies (Fig. 8). Although information on the molecular features of the *V. vulnificus* PGN is not yet available, the amino acid constituents of the peptides in the PGNs of the two bacteria would be different from each other. Therefore, it is most likely that VvpS could hydrolyze a bond (or bonds) at residues such as L- and D-Ala that are shared in the stem peptides of both Gram-

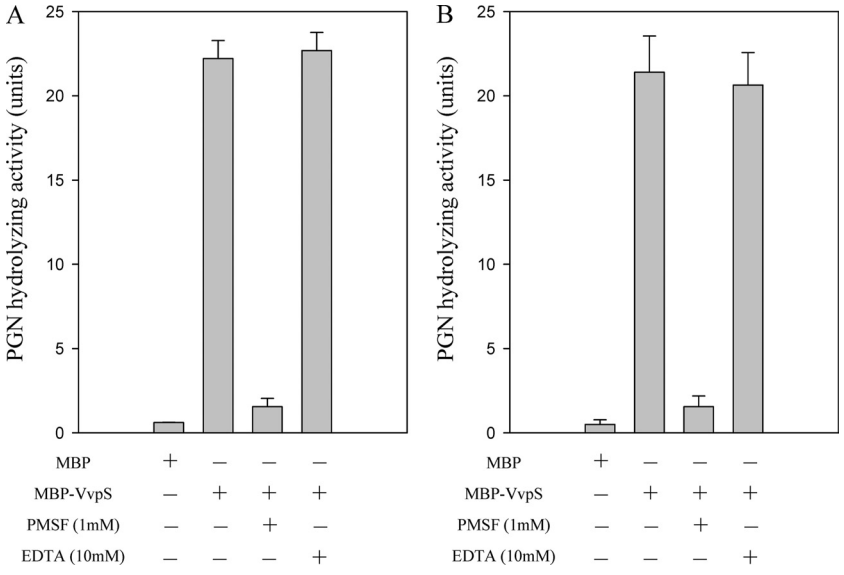

FIG. 8. Peptidoglycan-hydrolyzing activity of MBP-VvpS. MBP-VvpS protein (100 μM) was used for determination of PGN-hydrolyzing activity. PGN-hydrolyzing activity was determined using insoluble PGNs of *V. vulnificus* (A) and *S. aureus* (B) as substrates, defined as an activity of hydrolytic solubilization of the PGNs, and measured as a decrease in the A_{450} in the presence of either 1 mM PMSF or 10 mM EDTA. The same amount of MBP protein was used as a negative control. The error bars represent the SEM.

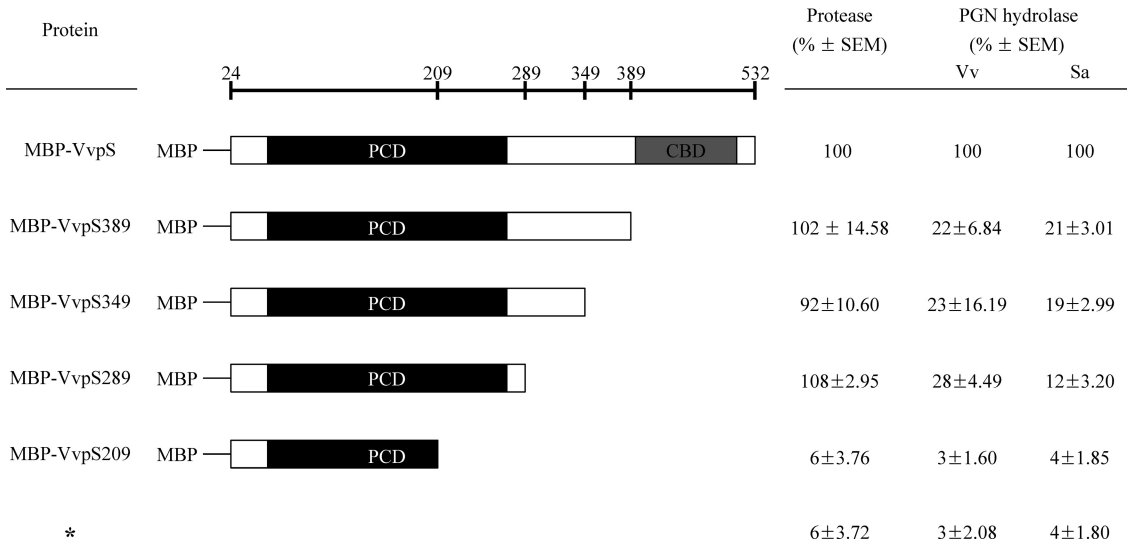

FIG. 9. C-terminal deletions of VvpS and activities of protease and PGN hydrolase. Shown is a schematic representation of the mutant MBP-VvpS with C-terminal deletions. The amino acid residues up to which each deletion extended are indicated by the numbers at the top. The wild-type MBP-VvpS and its C-terminal deletion mutants are shown below, with the PCD and CBD predicted by the amino acid sequence analysis. The relative activities of the protease and PGN hydrolase are shown on the right. The protease and PGN hydrolase activities were assessed as described in the legends to Fig. 2 and Fig. 8, respectively. Vv, *V. vulnificus*; Sa, *S. aureus*; *, MBP protein as a negative control.

negative and Gram-positive PGNs (36). Many PGN hydrolases often have one or several domains that bind to PGN (or the cell wall) and greatly affect the specificity and thus activity of the enzymes (47). Consistent with this, VvpS is a modular structure with a C-terminal CBD, and deletion of the CBD significantly impaired PGN-hydrolyzing activity without destroying the casein-hydrolyzing activity (Fig. 9). This indicates that VvpS also needs to bind to the PGN molecules through the CBD to hydrolyze them effectively.

The T2SS allows the secretion of a wide range of proteins from the cytoplasm to the extracellular space via a two-step process (for a recent review, see reference 11). The first step is the transport of unfolded and folded proteins across the cytoplasmic membrane into the periplasmic space by the Sec pathway and the twin-arginine transport (Tat) pathway, respectively (9, 11). Proteins targeted for the Sec or Tat pathway possess an N-terminal signal peptide, and once the preprotein reaches the periplasm, the signal peptide is cleaved off by specific leader peptidases, resulting in a mature protein. The second step mediates the subsequent translocation of the mature protein across the outer membrane and employs a complex machinery consisting of multiple components, including PilD (9). A null mutation in *pilD* would disconnect this two-step process of *V. vulnificus* and result in intracellular accumulation of VvpS. Therefore, it is most likely that VvpS is trapped in the periplasmic space, rather than the cytosol, in the *pilD* mutant, as proposed in Fig. 7. In a similar way, a T2SS that requires ATP for its activity would not be optimally functional

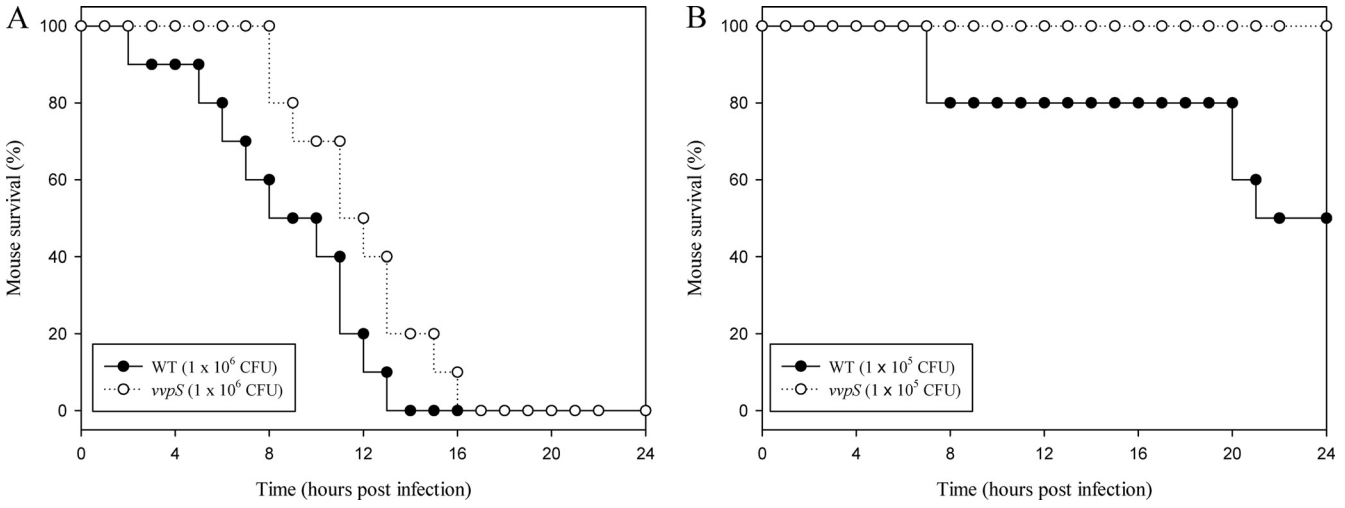

FIG. 10. Mortality of the *vvpS* mutant for mice. (A and B) Seven-week-old specific-pathogen-free female ICR mice were intraperitoneally infected using different concentrations of the wild type or *vvpS* mutant as indicated. Mouse survival was monitored for 24 h.

during stationary phase, during which the cellular ATP concentration is decreased significantly (9, 30). In addition, the *pilD* expression was measured using a $P_{pilABCD}$-*lacZ* transcriptional fusion reporter and appeared to decrease during stationary phase (data not shown). This growth phase-dependent variation of T2SS activity could explain the stationary-phase-specific accumulation of VvpS in cells and autolysis, as observed in the present study (Fig. 3 and 5).

It is noteworthy that *V. vulnificus* proliferates for several weeks in sterile seawater (18). Consistent with this, cell death of *V. vulnificus* was observed in LBS but not in a nutrient-limited minimal medium (data not shown). This indicated that autolysis of *V. vulnificus* occurs only where nutrients such as amino acids and peptides are present and is more likely to occur in host environments rather than in seawater. If autolysis of *V. vulnificus* occurs in the host, cell wall- and membrane-associated polysaccharides, such as lipopolysaccharide (LPS), capsular polysaccharide (CPS), and PGN, would be released. The contribution of the cell surface polysaccharides to bacterial pathogenicity has already been extensively studied in terms of the host-pathogen interaction (for a review, see reference 13). It has also been demonstrated that the cell surface polysaccharides protect *V. vulnificus* by assisting with evasion of phagocytosis by host defense cells and resistance to the bactericidal effects of serum (33, 43). As observed in other pathogens (2), the release of eDNA from lysed *V. vulnificus* cells could also be used for the development of biofilms, which are known to be resistant to host immune systems (6). Although the *vvpS* mutant was apparently less virulent in a mouse model than its parental wild type (Fig. 10), it is still unclear whether autolysis of a subpopulation is indeed beneficial for the rest of the bacterial community to survive the host immune system. Therefore, more work is necessary to illuminate the exact function of autolysis in *V. vulnificus* pathogenesis.

In summary, *V. vulnificus vvpS* encoding a serine protease with a modular structure was identified in the present study. A null mutation of *vvpS* significantly reduced autolysis of *V. vulnificus*, indicating that VvpS is an autolysin responsible for autolysis of the bacteria. VvpS was secreted via the T2SS and accumulated in the periplasmic space in stationary-phase cells to contribute to the autolysis of *V. vulnificus*. Therefore, a disruption of the T2SS accelerated intracellular accumulation of VvpS and thereby autolysis of *V. vulnificus*. VvpS also showed PGN-hydrolyzing activity, which is mediated by the presence of a CBD. Finally, the *vvpS* mutant showed reduced virulence in the infection of mice, indicating that VvpS is essential for pathogenesis, as well as autolysis, of *V. vulnificus*.

ACKNOWLEDGMENTS

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