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Characterization of a *Vibrio fischeri* Aminopeptidase and Evidence for Its Influence on an Early Stage of Squid Colonization

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***Vibrio fischeri* cells are the sole colonists of a specialized light organ in the mantle cavity of the sepiolid squid *Euprymna scolopes*. The process begins when the bacteria aggregate in mucus secretions outside the light organ. The cells eventually leave the aggregate, enter the light organ, and encounter a rich supply of peptides. The need to dissociate from mucus and presumably utilize peptides led us to hypothesize that protease activity is integral to the colonization process. Protease activity associated with whole cells of *Vibrio fischeri* strain ES114 was identified as the product of a putative cell membrane-associated aminopeptidase (PepN). To characterize this activity, the aminopeptidase was cloned, overexpressed, and purified. Initial steady-state kinetic studies revealed that the aminopeptidase has broad activity, with a preference for basic and hydrophobic side chains and k_{cat} and K_m values that are lower and smaller, respectively, than those of *Escherichia coli* PepN. A *V. fischeri* mutant unable to produce PepN is significantly delayed in its ability to colonize squid within the first 12 h, but eventually it establishes a wild-type colonization level. Likewise, in competition with the wild type for colonization, the mutant is outcompeted at 12 h postinoculation but then competes evenly by 24 h. Also, the PepN-deficient strain fails to achieve wild-type levels of cells in aggregates, suggesting an explanation for the initial colonization delay. This study provides a foundation for more studies on PepN expression, localization, and role in the early stages of squid colonization.**

Aminopeptidase N (or PepN) enzymes are metalloaminopeptidases that have been identified in bacteria, archaea, fungi, and mammals, and several have been extensively characterized (1, 2, 7, 8, 14, 15, 23, 30, 38, 42). The primary roles of this class of proteolytic enzymes have long been assumed to be in late-stage processing of intracellular proteins through protein degradation pathways and for the acquisition of essential amino acids (14, 25). However, new functions for aminopeptidases have been identified. These include roles as an antivirulence factor in *Salmonella enterica* serovar Typhimurium (33), a virus receptor (42), and a regulator of cellular stress response (8). Here, our findings suggest that PepN-type enzymes also play a role in beneficial animal-bacterial associations.

Specific strains of the bioluminescent marine bacterium *Vibrio fischeri* (e.g., strain ES114) form a beneficial association with the sepiolid squid, *Euprymna scolopes* (35). As soon as a hatchling squid emerges from its egg, it begins trapping planktonic bacteria, including cells of *V. fischeri*, in mucus secreted from the ciliated epithelial fields of a specialized light organ (32). Although many types of bacteria collect in the mucus secretions, only cells of certain *V. fischeri* strains can dissociate from the mucus, swim into the pores to reach the peptide-rich core of the light organ, and establish a long-term colonization (17, 31, 32). Several *V. fischeri* colonization factors have been described already (16, 40), but no studies to date have described the role of protease activity. We report here the discovery, purification, localization, and activity of an aminopeptidase enzyme (PepN) produced by the squid symbiotic strain *V. fischeri* ES114. Furthermore, we provide evidence that the disruption of *pepN* results in a delay in the initial stages of squid colonization by *V. fischeri.* These findings provide the impetus for additional studies on the role of PepN in the ability of *V. fischeri* to colonize sepiolid squids.

MATERIALS AND METHODS

Identification of aminopeptidase activity. Three-liter cultures of *V. fischeri* strain ES114 were grown overnight in LBS medium (16) at 24°C with shaking to an optical density at 600 nm (OD_{600}) of 0.6. Cells were harvested by centrifugation and the pellets gently washed in room-temperature 20 mM phosphate buffer (pH 7.5) containing 2% NaCl. The washed cells were then removed by centrifugation. The resultant cell-free supernatant was fractionated using a DE52 anion exchange column. The aminopeptidase activity was eluted with 0.3 M NaCl in 20 mM Tris-Cl (pH 7.5). Fractions containing this aminopeptidase activity were identified by monitoring cleavage of the aminopeptidase substrate L-leucine-7-amido-4-methyl coumarin hydrochloride (L-Leu-AMC; Sigma-Aldrich), which fluoresces at 440 nm upon cleavage of the peptide bond (see "Assays for aminopeptidase activity" below). Fractions possessing the aminopeptidase activity were pooled and applied to a Phenyl Sepharose CL-4B column (Amersham Biosciences), and then fractions containing the activity of interest were concentrated using Amicon YM30 Centricon concentrators. The partially purified proteins were then separated on a Tris-glycine gel under nondenaturing conditions. Aminopeptidase activity was visualized by soaking the gel in buffer (20 mM Tris-Cl, pH 7.5) containing 10 μM L-Leu-AMC, followed by illumination with 340-nm UV light. A single fluorescent blue band appeared after about 5 min. The band was excised using a razor blade, and the protein was electroeluted from the gel by placing the gel pieces in a dialysis bag made from 14-kDa-molecular-size-cutoff dialysis tubing. The electroeluted samples were concentrated using Amicon YM10 Centricon concentrators, and the protein was separated by

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Tris-glycine SDS-PAGE. Samples were sent for sequence analysis by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI-TOF MS) (Macromolecular Resources, Colorado State University).

Assays for aminopeptidase activity. Fluorescence assays were performed using L-Leu-AMC substrate and a fluorimeter capable of detecting fluorescence emission at 440 nm. Reaction mixtures contained 20 mM Tris-Cl (pH 7.5), 10 μM L-Leu-AMC, and 150 μl of washed cells (grown in either LBS or HEPES minimal medium [HMM; see reference 36] with shaking at 24°C to an OD_{600} of between 0.7 and 1.5), 150 μl of cell-free culture supernatant, 150 μl of fractionated cell extract, or 25 μg of purified enzyme. To detect the hydrolysis of mucin, a 5-μl loopful of mid-log-phase cells was streaked onto a basal medium (containing 50% [vol/vol] artificial seawater, 5% Tris-HCl [1 M; pH 7.4], 0.3% [vol/vol] glycerol, 0.006% [wt/vol] K_2HPO_4, 1.5% [wt/vol] agar, and 45% [vol/vol] deionized water) supplemented with 1% (wt/vol) porcine mucin (Sigma Chemical Corp., St. Louis, MO) and incubated at 28°C for between 48 and 72 h. Plates were subsequently stained with 0.1% (wt/vol) amido black in 3.5 M acetic acid for 30 min and destained with 1.2 M acetic acid. Zones of mucin lysis were observed as discolored halos around colonies. To assay for casein hydrolysis, cells were applied (in the same manner as that described for mucinase detection) to LBS agar containing skim milk powder at a final concentration of 4%. A zone of clearing surrounding the resultant colonies indicated casein hydrolysis.

Cloning the gene that encodes the identified aminopeptidase activity. PCR mixtures containing *V. fischeri* genomic DNA were prepared as described previously (12), with the exception that they contained HiFi *Taq* polymerase (1 U/50-μl reaction; Novagen) and primers PepN exp F (5′-CA TATGAGCCAACAACCTCAGGCTA-3′) (forward) and PepN exp R (5′-AAGCTTTTAAGCTAATGCTTTCGTTAC-3′) (reverse), which flank *pepN* (VF_1282), including the putative promoter and terminator. These primers introduced unique NdeI and HindIII sites at the 5′ and 3′ termini, respectively, allowing for directional cloning into an expression vector. The PCR product was gel purified and cloned using the procedure described in the TOPO XL PCR cloning kit (Invitrogen, Carlsbad, CA). Lastly, *pepN* was transferred to pET26b(+) vector and subcloned in *E. coli* BL21(DE3) cells.

Overexpression and purification of the aminopeptidase. Cultures containing the pET26b(+)*PepN* recombinant plasmid were grown at 30°C in LB containing kanamycin (50 μg/ml) and glucose (0.5%) to an OD_{600} of 0.6. Protein production was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM final concentration). Cells were harvested by centrifugation, resuspended in lysis buffer (50 mM Tris-Cl, pH 8.0, 1 mM EDTA, 3 mg/ml lysozyme, and 100 mM NaCl), and disrupted by sonication. Cell debris was removed by centrifugation (1 h at 20,200 × *g*), and the cleared supernatant was dialyzed overnight at 4°C against 16 liters of 20 mM Tris-Cl (pH 7.5) using 50-kDa-molecular-size-cutoff dialysis tubing. The dialyzed lysate was applied to a Whatman DE52 anion exchange column. The column was washed with 20 mM Tris-Cl, pH 7.5, and the aminopeptidase eluted with 20 mM Tris-Cl, pH 7.5, containing 0.2 M NaCl. Fractions containing the aminopeptidase enzyme (as identified using the standard assay) were combined and loaded onto a Whatman QA52 anion exchange column. The protein of interest was eluted with 20 mM Tris-Cl with 0.5 M NaCl, and fractions containing the aminopeptidase were pooled and applied to a Phenyl Sepharose CL-4B column. Fractions containing aminopeptidase activity were identified by A_{280} and the standard assay described previously and then pooled. The purified aminopeptidase was collected and concentrated using an Amicon stirred cell ultrafiltration device fitted with an YM30 membrane. Single-band purity was checked by SDS-PAGE with Coomasie brilliant blue R-250 staining.

Determining the molecular weight and steady-state kinetics of the aminopeptidase. The molecular weight of the aminopeptidase was determined using gel filtration chromatography. A sample of protease was applied to a Sephacryl S-200 column. The flow rate was approximately 0.2 ml/min, and myoglobin (17 kDa), carbonic anhydrase (29 kDa), bovine serum albumin (BSA) (66.4 kDa), and transferrin (78.5 kDa) were used as protein standards. Blue dextran 2000 and DNP-glutamate were used to determine the column void volume and total volume, respectively. Steady-state kinetic assays were carried out in either a fluorimeter or UV-vis spectrophotometer at 24°C in a 3-ml total volume using 1-cm-path-length cuvettes. The buffer was 20 mM Tris-Cl (pH 7.5). The aminopeptidase concentration in the cuvette was held constant at 1.1 nM, and L-Leu-AMC concentrations were varied from 3.33 to 600 μM. Emission and excitation slits were set to 1 nm. The excitation wavelength was set at 365 nm, and emission was monitored at 440 nm. A background spectrum from 380 to 500 nm was taken prior to the addition of enzyme. The reactions of aminopeptidase with AMC substrates were observed at 440 nm, and the fluorescence intensity was converted to concentration changes using a standard curve developed from free 7-amino-4-methyl coumarin (counts per second [cps] = 1.01×10^{11}[product] − 9,923). The reported values are averages from three trials. For analyses using *para*-nitroanilide amino acid (PNA) substrates, the protease concentration in the cuvette was held constant at 11 nM, and PNA concentrations were varied from 3.33 to 500 μM. The reactions of protease with PNA substrates were monitored at 405 nm, and absorbance changes were converted to concentration changes using a standard curve generated with *para*-nitroaniline (calculated ε_{405}, 11,100 M^{-1} cm^{-1}). K_m and k_{cat} values were determined by least-squares fitting of the kinetic data to the Michaelis-Menten equation in SigmaPlot 12.0 software.

Construction of an aminopeptidase-deficient mutant, complementation, and GFP labeling. A Campbell insertion mutation of *pepN* was generated by following standard protocols (6, 19). An internal fragment of *pepN* was amplified using the following primers: PepN int R, 5′ ACTCG GTTACCAGTCCAGTT 3′ (reverse), and PepN int F, 5′ AATCTGGCCG TGATGTTGCA 3′ (forward). The fragment was cloned into pCR-BluntII TOPO and subsequently subcloned into pEVS122 (a suicidal vector in *V. fischeri*). The suicide vector was conjugated into *V. fischeri* using pEVS104 as the helper plasmid, and transconjugates that underwent homologous recombination between *pepN* and the internal *pepN* fragment were selected on LBS supplemented with erythromycin at 5 μg/ml (final concentration). The insertion was confirmed genetically by PCR and phenotypically by the fluorescent aminopeptidase assay described previously. To complement the mutant, the complete *pepN* gene, including promoter and terminator, was amplified using primers PepN orf R (5′ CCCCATA TGGCTGCACCAGAATAATGGCTTAA 3′) and PepN orf F (5′ CCCCA TATG TGAGCAATTTGGAATGTATGCGC 3′). The 5′ region of each primer contains an NdeI site that permitted ligation of *pepN* into the unique NdeI site of the *Vibrio* shuttle vector pVSV105 (11). The resulting recombinant $pepN^+$ plasmid was conjugated into *V. fischeri* cells using the same method as that used for mutant construction. Wild-type and *pepN* mutant strains were then labeled with green fluorescent protein (GFP) as described previously (32).

Subcellular localization of PepN. Fractionation of *V. fischeri* cells was conducted using a combination of the methods of Thein et al. (39), Hoge et al. (18), and Kolibachuk and Greenberg (24). Briefly, 25 ml of stationary-phase culture of each strain grown in HMM with shaking at 24°C was pelleted in a microcentrifuge, the supernatant was discarded, and the cells were resuspended in a sucrose-salt solution, followed by the addition of lysozyme (24). An aliquot of the spheroplasts, when diluted in deionized water, lost turbidity almost immediately. The remaining spheroplasts were collected by centrifugation, and the supernatant was used as the periplasmic fraction (18). Spheroplasts were resuspended in sterile seawater and disrupted by sonication. The lysate was checked by microscopy to verify that complete lysis had occurred. The total membrane fraction was then collected by centrifugation for 1 h at 13,000 × *g*, and the supernatant was used as the cytoplasmic fraction (18). The crude membrane pellet was resuspended in 1 ml of 10 mM Tris (pH 7.5), 15% (wt/wt) sucrose, 5 mM EDTA, and 0.2 mM dithiothreitol (DTT). The solution was then separated into cell and outer membrane fractions by sucrose density gradient

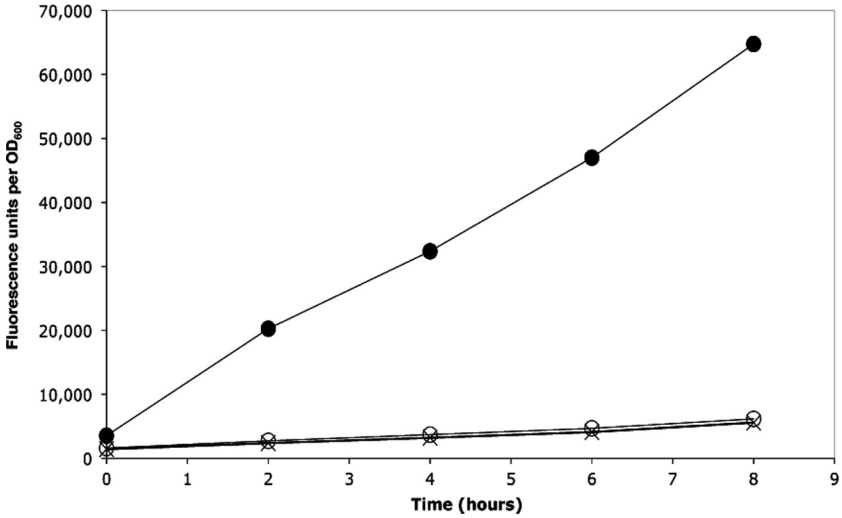

FIG 1 Association of zinc-requiring aminopeptidase activity with cells but not supernatant. An aliquot of turbid cell suspension grown in HMM (see Materials and Methods) was mixed with L-leucine 7-amido-4-methyl-coumarin substrate (●). In parallel, a second aliquot of the turbid cell suspension was mixed with substrate and 500 μM (final concentration) zincov, a zinc-metalloprotease inhibitor (○). Cells were removed by centrifugation from a third aliquot of the turbid culture, and the resulting cell-free supernatant was mixed with substrate (×). Fluorescence emission due to proteolytic cleavage of the substrate was measured in a fluorimeter. These data are typical results from three experiments.

centrifugation as described previously (39). Three 150-μl aliquots of cell membrane, outer membrane, periplasmic fraction, and cytoplasmic fraction were assayed both for aminopeptidase (using L-Leu-AMC substrate) and luciferase activity (13). Luciferase activity served as an indicator of the presence of cytoplasmic proteins in each fraction.

Squid colonization assays. Colonization assays were performed as described previously (12). Briefly, newly hatched, uncolonized squid were placed into separate bowls of symbiont-free seawater (SFS) or seawater containing roughly equal concentrations (between 1,000 and 4,800 cells/ml) of either wild-type *V. fischeri* (strain ES114), the *pepN* mutant, or the complemented *pepN* mutant grown in SWT (12). After 3 h of exposure to the bacteria, squid were rinsed in SFS and then placed into separate vials of SFS. Luminescence emission, which is an indicator of successful colonization, was measured for each squid at 12 and 24 h postexposure to the *V. fischeri* strains. At 12 and 24 h postexposure, squid light organs were homogenized and then homogenates were serially diluted and plated on SWT to determine symbiont concentration per squid. For these single-strain experiments, 200 isolated colonies from squid colonized with the *pepN* mutant were patched onto SWT and SWT supplemented with 5 μg/ml erythromycin (SWT erm5) to verify that the Campbell mutation did not resolve. To determine a mutant's competitiveness with the wild type, juvenile squid were added to seawater containing approximately a 1:1 mix (determined by spectroscopy) of the strains for 3 h and then moved to fresh SFS. The true ratio of the inoculum was determined by spreading dilutions of the inoculated seawater onto SWT and SWT erm5. After 12 and 24 h, the colonized squid were homogenized and dilution plated onto SWT. Two hundred colonies isolated from each squid were then patched onto SWT and SWT erm5 to determine the ratio of mutant to wild type. The relative competitive index was determined by dividing the mutant-to-wild-type ratio in each individual squid by the ratio in the inoculum. Experiments were replicated three times, and the data from one representative experiment are presented.

Aggregation studies. Newly hatched squid were placed in scintillation vials containing 3 ml of filter-sterilized instant ocean (FSIO) and exposed to 10^6 cells per ml WT *V. fischeri* GFP^+ or the *pepN* GFP^+ mutant in FSIO with 20 μg/ml chloramphenicol for 3 h, at which time they were removed from the inoculum and placed in 3 ml of FSIO containing 0.25 μM Cell Tracker Orange (Invitrogen Corp., Carlsbad, CA) and incubated for 30 min. Squid were then anesthetized in 2% ethanol in FSIO, the mantles and funnels were removed, and the number of bacteria in the aggregates associated within one anterior appendage per animal was quantified. Fluorescently labeled light organs (red) and GFP-producing bacteria (green) were visualized using a Nikon A1R laser-scanning confocal microscope (Nikon Corp., Tokyo, Japan) at the Flow Cytometry and Confocal Microscopy Facility at the University of Connecticut. Images were analyzed using Nikon Elements software (Nikon Corp., Tokyo, Japan).

RESULTS

Cells of *V. fischeri* produce a cell-associated, zinc-requiring aminopeptidase activity. To determine if *V. fischeri* strain ES114 produces aminopeptidase activity, we assayed for activity using several substrates. Strain ES114 hydrolyzed medium containing either porcine mucin or skim milk (data not shown). Also, when whole cells of wild-type strain ES114 grown in HMM were mixed with aminopeptidase substrates such as L-Leu-AMC, proteolytic activity was detected (Fig. 1). The cell-free supernatant contained less than 10% of the aminopeptidase activity of whole cells, suggesting that the observed activity is cell associated and not secreted. Furthermore, the cell-associated aminopeptidase activity was completely inhibited by the addition of 500 μM zincov, a zinc metalloprotease inhibitor.

The aminopeptidase is a PepN homolog. To identify the protein(s) responsible for the observed aminopeptidase activity and to determine if the activity derives from the cytoplasm of lysed cells, proteins were isolated from whole-cell wash fluid, fractionated, and assayed for aminopeptidase and luciferase activity. Only one of the proteins washed from the cells displayed the capacity to cleave fluorescence-emitting aminopeptidase substrates. Seven short peptide sequences (ranging from 13 to 46 amino acids) were derived from this protein by MALDI-TOF MS and identified by a MASCOT search of the *V. fischeri* ES114 Genome Project Database (http://www.ergo-light.com/). The sequences matched the product of a gene annotated VF_1282, aminopeptidase N, or PepN (>99% identity over 17% of the complete PepN protein). The translated protein is 867 amino acids long and lacks a Sec or

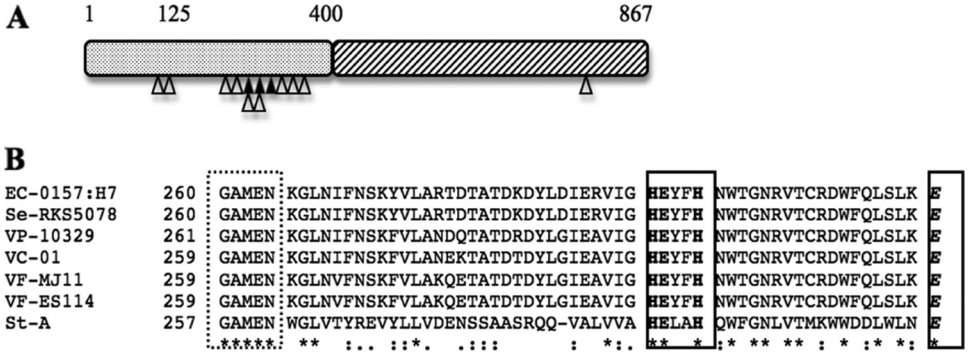

FIG 2 (A) Diagram of PepN showing the location of putative active sites (open triangles) and zinc binding sites (closed symbols). The first approximately 400 amino acids (stippled region) represent the peptidase M1 family domain, which shares 75% identity with *E. coli* PepN. The remainder of the protein (diagonal lines) represents a domain of unknown function that shares 54% identity to *E. coli* PepN. (B) Alignment of PepN homolog catalytic regions. Asterisks indicate amino acid identity at a particular position, whereas a colon or period represents conserved and semiconserved substitutions, respectively. The exopeptidase motif (broken-line box around the amino acids) and the M1 family metalloprotease motif (solid-line boxes around the amino acids) are highlighted. The conserved zinc-binding residues are in boldface, and the catalytic glutamate residues are in boldface italics. Each bacterium is indicated before the sequence and accession numbers (in parentheses) as the following: EC-O157:H7, *E. coli* (BA000007); Se-RKS5078, *Salmonella enterica* (NZ_CM001153); VP-10329, *V. parahaemolyticus* (AFBW01000009); VC-01, *V. cholerae* (AE003852); VF-MJ11, *V. fischeri* (NC_011184); VF-ES114, *V. fischeri* (CP000020); and St-A, *Streptococcus thermophilus* (AJ007700).

TAT signal peptide (3, 34). Furthermore, the gene within *V. fischeri* encoding this enzyme possesses both of the conserved sequences of other *pepN* homologs, namely, the GAMEN sequence beginning at residue 260 and $HEXXHX_{18}E$ sequence at residue 295 (Fig. 2). The *pepN* gene is immediately upstream of VF_1284 (NAD-specific glutamate dehydrogenase) and VF_1285 (dihydroorotate dehydrogenase).

Properties of recombinant PepN. To further characterize the PepN protein, we purified it to greater than 95% purity. Size-exclusion chromatography revealed that the recombinant PepN is a monomeric protein of approximately 96,200 Da, in close agreement with the predicted value of 97,964 Da determined from its 867-amino-acid sequence and a molecular mass of 97,904 Da calculated from mass spectral data. Steady-state kinetic experiments revealed that PepN hydrolyzes a variety of *para*-nitroanilide and 7-amido-4-methyl coumarin (AMC) amino acid substrates (Table 1). However, PepN does not hydrolyze L-Pro-AMC to a significant degree and is inhibited by the aminopeptidase inhibitor bestatin (reference 27 and data not shown). The aminopeptidase has broad activity, with a preference for basic and hydrophobic side chains (Arg > Lys > Phe > Met > Leu > Val) as indicated by the specificity constant (k_{cat}/K_m), K_m, and k_{cat}, values.

***pepN* encodes the major aminopeptidase activity.** To test whether the *pepN* gene is responsible for the observed aminopeptidase activity, we created a null mutation by Campbell insertion into the *pepN* open reading frame. Inactivation of *pepN* did not result in any decrease in the mucinolytic or caseinolytic activity displayed by strain ES114, nor did it result in pyrimidine auxotrophy (suggestive of a polar effect on VF_1285) or any defects in growth, luminescence, or motility (data not shown). However, whole mutant cells produced less than 10% of the aminopeptidase activity of the wild-type strain (Table 2). In the wild type, the majority (≥85%) of subcellular aminopeptidase activity was detected in the cytoplasmic fraction. Interestingly, a significant amount (≥14%) of activity was detectable in the purified cell membrane fraction. A small amount (≤0.03%) of cytoplasmic luciferase activity was also detectable in this fraction. The periplasmic fraction contained low levels of both aminopeptidase (≤0.3%) and luciferase (≤0.04%) activity. Expression of *pepN* from its native promoter on pVSV105 completely restored aminopeptidase activity to the mutant cells, albeit to a higher level than that of the wild-type strain (Table 2). This is likely due to multiple copies of pVSV105 being maintained in these cells (11). These data indicate that *pepN* encodes the major cell-associated aminopeptidase activity.

PepN-deficient cells of *V. fischeri* are delayed in colonizing squid. The *pepN* mutant varies from the wild type by no more than 2% in its growth rate in various media (including the afore-

TABLE 1 Kinetic characterization of purified PepN substrate hydrolysis[a]

Substrate	K_m (M)	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} M^{-1})
Leu-AMC	$17 \times 10^{-6} \pm 3 \times 10^{-6}$	5.7 ± 0.8	3.4×10^5
Phe-AMC	$60 \times 10^{-6} \pm 13 \times 10^{-6}$	6.5 ± 1.6	1.1×10^5
Met-AMC	$25 \times 10^{-6} \pm 2 \times 10^{-6}$	11 ± 1.5	4.4×10^5
Arg-AMC	$14 \times 10^{-6} \pm 4 \times 10^{-6}$	33 ± 2	2.4×10^6
Leu-PNA	$43 \times 10^{-6} \pm 11 \times 10^{-6}$	5.9 ± 0.9	1.4×10^5
Lys-PNA	$19 \times 10^{-7} \pm 3.2 \times 10^{-7}$	27 ± 3	1.4×10^7
Arg-PNA	$39 \times 10^{-7} \pm 6 \times 10^{-7}$	39 ± 5.4	1.0×10^7

[a] K_m and k_{cat} values are averages from three trials ± standard errors.

TABLE 2 Distribution of PepN activity

Location	Activity[a] of:		
	ES114 (wild type)	*pepN* mutant	*pepN*$^+$ mutant
Cytoplasm	6,760	22	8,121
Cell membrane	1,150	31	3,213
Periplasm	29	21	621
Outer membrane	36	32	54
Whole cells	10,150	995	14,323
Cell-free supernatant	328	291	970

[a] Results are given as fluorescence units per OD_{600} unit (5×10^8 cells) above background. Values are representative of three separate experiments.

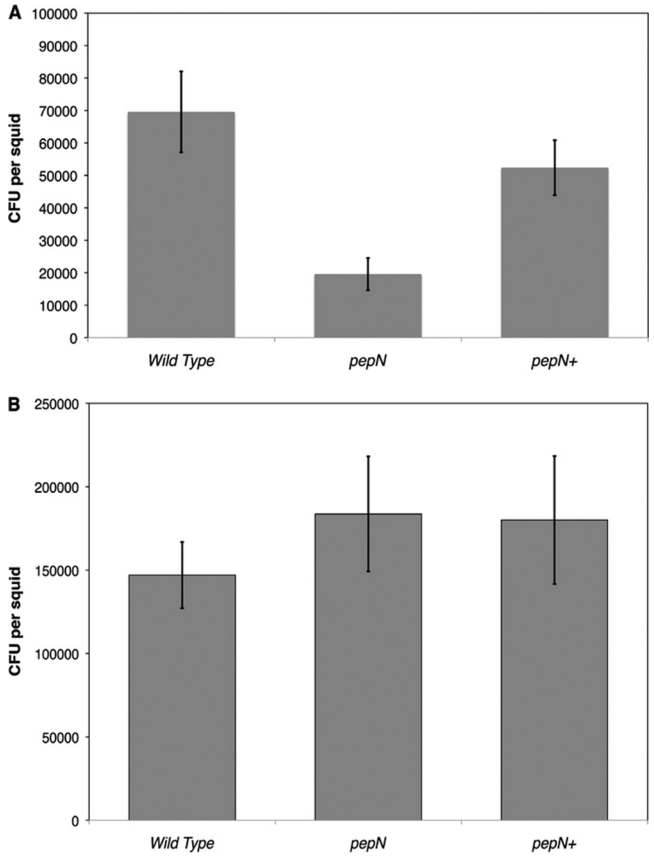

FIG 3 Colonization of squid by wild-type *V. fischeri*, *pepN* mutant, and *pepN* mutant expressing *pepN* on plasmid pVSV105. Squid were homogenized and plated at 12 (A) and 24 (B) h postinoculation (see Materials and Methods). Each bar represents the averages from 16 to 20 squids (with standard errors) and are representative of three separate experiments.

mentioned mucin- and skim milk-containing media), rate of motility on soft agar, and luminescence emission per cell in culture during log phase (data not shown). The latter two phenotypes have been demonstrated to be important in colonization (16, 40). Despite these similarities, after 12 h from the initial exposure to squid, the *pepN* insertion mutant was impaired in its ability to colonize squid relative to the wild type (Fig. 3). However, this colonization deficiency by the mutant was not apparent after 24 h. These data indicate that the impairment was due to a delay in the initiation of colonization. Notably, per-cell luminescence by the mutant and wild-type strains in the light organ differed by less than 5% (data not shown). Because initiation defects are typically exacerbated during head-to-head competition with the wild type, we also examined the competitiveness of the *pepN* mutant. At 12 h after initial infection, the wild type significantly outcompeted the mutant in roughly 75% of the squid (Fig. 4). However, after 24 h, the competitive edge for the wild type was no longer apparent (Fig. 4).

PepN-deficient cells of *V. fischeri* fail to form normal aggregates. The timing of the decrease in colonization efficiency by the *pepN* mutant suggests impairment at the earliest stage of colonization, initiation, which includes aggregation, migration to the pores, and early growth in the light organ prior to the first venting (32). The first of these events, aggregation, was observed by incubating newly hatched squid with GFP-labeled wild-type *V. fischeri* or *pepN* mutant bacteria, identifying the presence of aggregates, and quantifying the number of bacteria that form in aggregates. The *pepN* mutant produced aggregates, but they were consistently smaller in size than those of the wild-type strain (Fig. 5), possibly explaining the delay in light organ colonization.

DISCUSSION

Here, we report the identification, purification, activity, localization, and role in symbiosis of a previously uncharacterized aminopeptidase from cells of the squid symbiont *V. fischeri* strain ES114. There are several reports of activities that mediate the establishment and persistence of *V. fischeri* cells in the squid light organ (16, 35, 40). However, none have addressed the possible role of aminopeptidase. The squid light organ is rich in host-derived mucus, amino acids, and peptides capable of nourishing populations of up to 10^{11} cells per ml in adult squid (17, 31, 32). Thus, we reasoned that aminopeptidase activity could be important in this association.

E. coli possesses PepA, PepB, PepC, and PepN; however, PepN is responsible for the majority of the aminopeptidase activity (8). *V. fischeri* also possesses homologs of these aminopeptidases, and as with *E. coli*, a PepN-type enzyme is responsible for the majority of the aminopeptidase activity detectable by cleavage of L-Leu-AMC substrate (Fig. 1 and Table 2). Sequence comparisons reveal significant identity between *V. fischeri* PepN and several known aminopeptidase N genes (Fig. 2), strongly supporting its classification as an aminopeptidase N family peptidase. The PepN aminopeptidases (EC 3.4.11.2) belong to the M1 family of metalloproteases, are zinc dependent, and are classified as gluzincins based on the conserved $HEXXHX_{18}E$ zinc-binding motif (1, 2, 20). Studies of the *E. coli* and *S.* Typhimurium PepN enzymes have demonstrated that they possess both exopeptidase and endopeptidase activities (7, 8), and that a conserved GXMEN peptide sequence is associated with the exopeptidase activity (1, 2). The *V. fischeri* enzyme possesses both of these conserved sequences: the GAMEN sequence begins at residue 260 and the $HEXXHX_{18}E$ sequence at residue 295 (Fig. 2).

Steady-state kinetic studies revealed that PepN has a broad affinity for its N-terminal substrates (Table 1), and a preference for basic and hydrophobic residues, while exhibiting little to no reactivity toward proline substrates. These results are similar to those reported for the *E. coli* PepN enzyme (7, 8, 14). However, unlike *E. coli* PepN, *V. fischeri* PepN demonstrates an even wider range of substrate specificity with its ability to cleave substrates with N-terminal Val and Met residues. Furthermore, while *V. fischeri* PepN k_{cat} values for all substrates are approximately an order of magnitude lower than those reported for *E. coli* PepN (8, 14), it is interesting that the K_m values are approximately two orders of magnitude smaller than those of *E. coli* PepN.

PepN enzymes, including that of *V. fischeri*, do not possess an N-terminal signal sequence and are generally accepted to be cytosolic (14, 15), often in association with the cell membrane (4, 21, 29). For example, PepN activity in *Escherichia coli* and *Pseudomonas aeruginosa* was localized to the cell membrane using electron microscopy and other approaches to detect artificial aminopeptidase substrate hydrolysis by whole and fractionated cells (4, 21, 29). More specifically, the *E. coli* PepN appeared to be concentrated at the cell poles, presumably bound to the periplasmic side of the cell membrane by strong ionic interactions that might require an integral membrane protein (29). In a similar study, re-

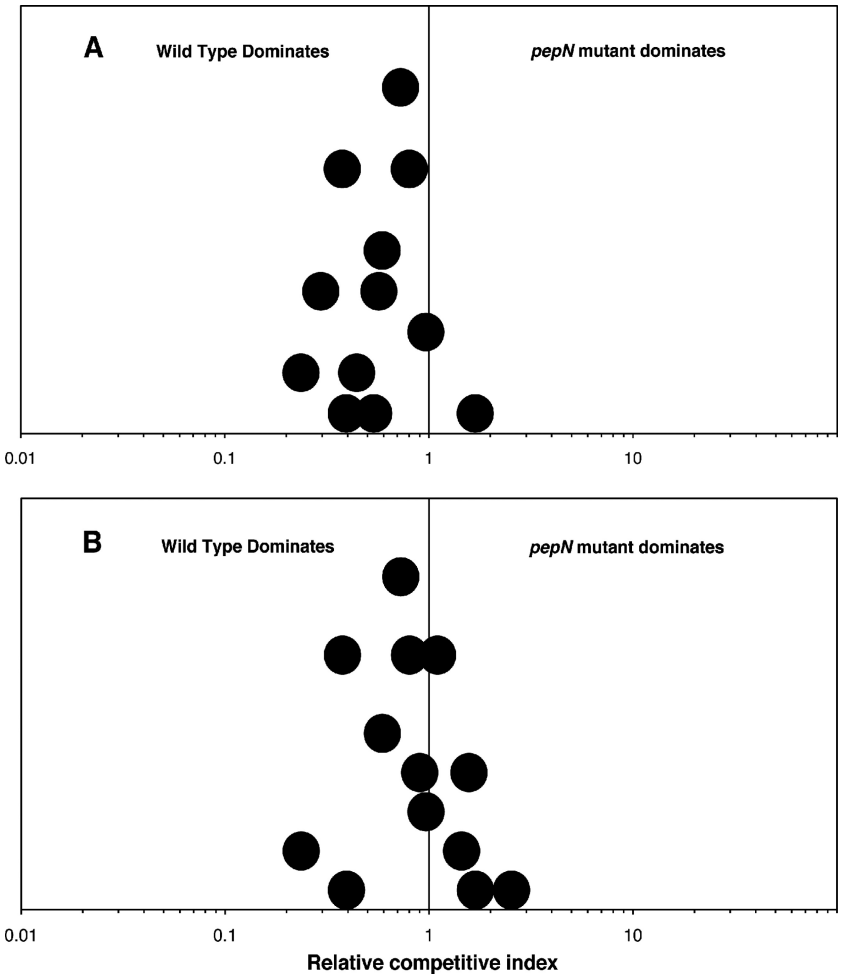

FIG 4 Relative competitiveness of wild-type *V. fischeri* and *pepN* mutant in squid. Newly hatched squid were exposed to a mixed inoculum containing equal cell numbers of each strain. Squid were homogenized and plated at 12 (A) and 24 (B) h postinoculation (see Materials and Methods). The ratio of mutant to parental bacterial strain in each light organ is expressed as the relative competitive index (RCI) and is indicated by a circle. An RCI above 1.0 indicated that the mutant was dominant. These are representative data from three separate experiments.

searchers fractionated cells of *P. aeruginosa* and then used artificial aminopeptidase substrates to localize PepN activity to the cell membrane and cytoplasmic fractions (4). *V. fischeri* PepN activity is also cell associated and readily accessible to exogenously supplied substrates (e.g., L-Leu-AMC) and inhibitors (Fig. 1). In *V. fischeri*, as with *P. aeruginosa* and *E. coli*, the vast majority of PepN activity was associated with the cytoplasm, with much of the remaining activity found in the cell membrane fraction (Table 2). However, some of the membrane-associated aminopeptidase reported in this study might be due to cross-contamination with cytoplasmic contents, because the membrane fraction also contained a small amount of cytoplasmic luciferase activity. Our data, combined with evidence that L-Leu-AMC did not cross the cell membrane in an unidentified Gram-negative marine bacterium (26), led us to hypothesize that PepN is closely associated with the cell membrane. However, the methods used in this study do not allow us to say on which side of the membrane PepN actually resides or whether it might be localized to the cell poles, as in *E. coli* (29). The lack of PepN activity in the periplasmic and outer membrane fractions is consistent with the absence of a signal peptide or other evidence for PepN secretion across the cell membrane. Combined, our findings are not unexpected, given what is already known about the location of other PepN enzymes. The apparent close proximity of PepN to the cell membrane probably reflects its role in the transport and degradation of extracellular peptides (4, 20).

Euprymna scolopes is a nocturnal predator that presumably is aided by bacterium-derived light (22), and in exchange for this light it provides its symbiont with nutrients for growth (17, 31). Analysis of light organ contents in adult squid suggested that host-derived mucin, amino acids, and peptides fuel both initial colonization and daily bacterial repopulation after venting 95% of the contents of its light organ each day at dawn (5, 17, 31). Both the wild-type and *pepN* mutant strains hydrolyzed casein and porcine mucin (data not shown), but the impaired ability of the mutant to metabolize an aminopeptidase substrate (Table 2) supports the possibility that it would have a growth disadvantage in the peptide-rich squid light organ (17). Our findings do support an initial delay in colonization for the PepN mutant (Fig. 3, 4, and 5), but beyond 12 h after initial infection, the mutant appears to achieve wild-type colonization levels in squid (Fig. 3 and 4). Similarly, the mutant was outcompeted by the wild type in the first 12 h after initial

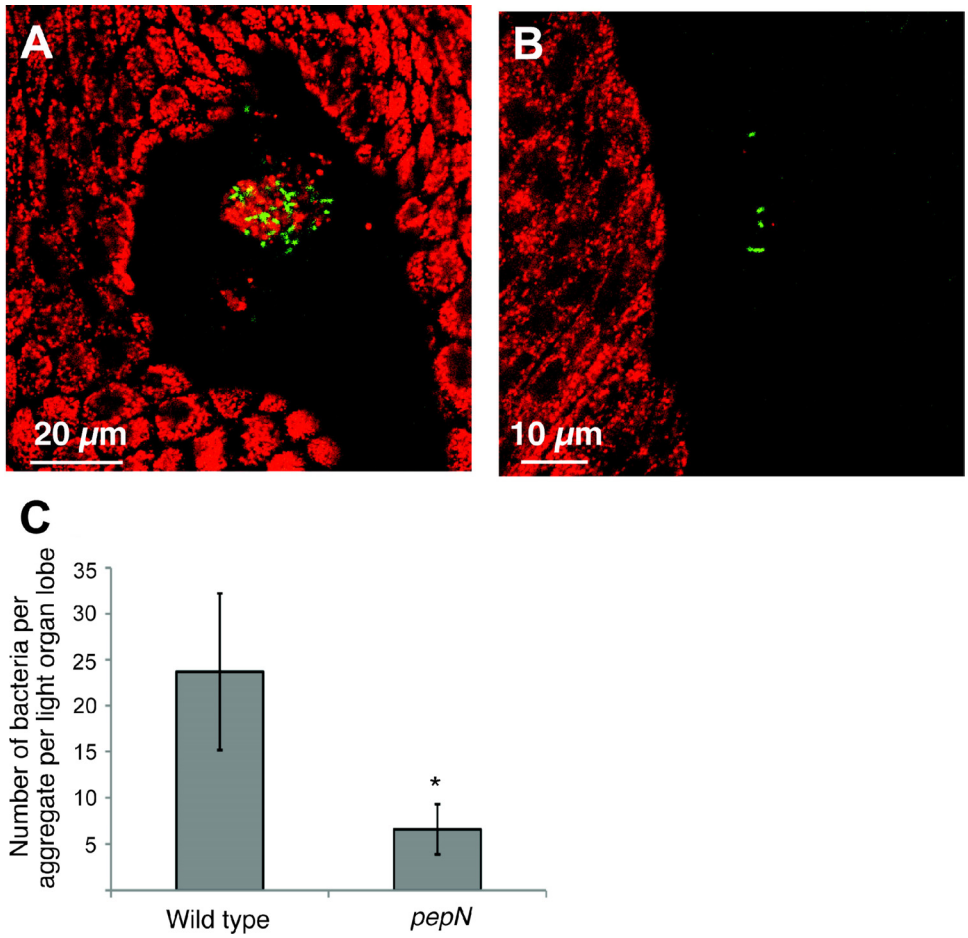

FIG 5 Aggregation defect of the *pepN* mutant. Newly hatched squid were incubated with 10^6 GFP-labeled wild-type (A) or *pepN* mutant (B) *V. fischeri* for 3 h, counterstained with Cell Tracker Orange (red) to visualize the light organ surface, and then examined by confocal microscopy. (C) The number of bacteria per aggregate per light organ lobe was determined by direct counting, and averages ± standard deviations are shown ($n = 10$ per treatment). An asterisk indicates significant difference from the wild type (Student's *t* test at $P < 0.001$). The figure and graph are representative of three separate experiments.

infection; however, the competition defect was no longer apparent after 24 h (Fig. 4). Such findings in the competitions could be due to a gradual complementation of the defect by the presence of the wild-type bacterium. However, this could not explain the early colonization defect by the mutant in single-strain inoculations. Transcriptome analysis of established populations of *V. fischeri* cells within adult squid light organs might provide an explanation for the eventual recovery of the mutant in squid between 12 and 24 h after initial infection (41). These studies revealed a dynamic nutritional interaction in the light organ environment in which *V. fischeri* genes regulating chitin/*N*-acetylglucosamine fermentation are upregulated at night but are then suppressed in favor of genes enabling anaerobic respiration of glycerol during daylight hours (41). Along these lines, efficient regulation of *N*-acetylglucosamine and chitin utilization is required for normal, early colonization of squid (28). Squid mucin appears to contain N-acetyl galactosamine, among other components (32), which also could serve as a sole carbon and nitrogen source for the bacteria. Perhaps the residual aminopeptidase activity of the mutant (Table 2) is sufficient to utilize the abundant peptides, and/or the ability to take advantage of other nutrient sources in the light organ could account for its eventual recovery.

Aside from their role in peptide metabolism, aminopeptidases such as PepN may be involved in bacterial susceptibility to the host stress response during colonization. For example, *E. coli* cells lacking *pepN* are able to grow better than wild-type cells when exposed to sodium salicylate-induced stress, and overexpressing PepN reverses this phenotype (8). Similarly, the ability of *Salmonella* Typhimurium cells to proliferate during systemic infection of mice is modulated by PepN (33). In that study, the loss of PepN allowed cells of *Salmonella* to achieve a much higher cell density in mice relative to the wild type. Perhaps PepN reduced the density of *Salmonella* cells in mice by modulating the host stress response (33) or through its ability to cleave proteins responsible for combating the stress (8). Conversely, in our study, the loss of PepN resulted in a diminished capacity of mutant cells to aggregate in squid mucus secretions outside the light organ and achieve normal cell numbers within the light organ early in the colonization process (Fig. 3, 4, and 5). Squid mucus and the light organ interior are protected from nonspecific colonization by a variety of host defenses, including nitric oxide (NO) (10, 31). In fact, the presence of NO scavengers allows *V. fischeri* cells to form much larger aggregates in squid mucus compared to the aggregates they form in the absence of scavengers (31). Just as *Mycobacterium tuberculosis* relies on protease activity to suppress NO stress in mice (9), the pos-

sibility that PepN influences NO (or another) stress response in squid, thereby modulating density of *V. fischeri* cells early in the colonization process, is an intriguing area of future research.

If PepN activity is important in *V. fischeri* beyond its putative role in the initial encounter with squid mucus and the early stages of light organ infiltration, its absence appears to be compensated for beyond 12 h postinfection. Notably, PepN was detected by liquid chromatography/MS in the light organ of adult squid (37). Thus, the bacteria are expressing the enzyme in squid well beyond 24 h, but its role is unclear. Taken together, these findings lay the foundation for more detailed studies on PepN expression, localization, and role in the early stages of the association between *V. fischeri* and its squid host, *E. scolopes*.

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