A Secreted NlpC/P60 Endopeptidase from *Photobacterium damselae* subsp. *piscicida* Cleaves the Peptidoglycan of Potentially Competing Bacteria

**ABSTRACT** Peptidoglycan (PG) is a major component of the bacterial cell wall, forming a mesh-like structure enwrapping the bacteria that is essential for maintaining structural integrity and providing support for anchoring other components of the cell envelope. PG biogenesis is highly dynamic and requires multiple enzymes, including several hydrolases that cleave glycosidic or amide bonds in the PG. This work describes the structural and functional characterization of an NlpC/P60-containing peptidase from *Photobacterium damselae* subsp. *piscicida* (Phdp), a Gram-negative bacterium that causes high mortality of warm-water marine fish with great impact for the aquaculture industry. PnpA (*Photobacterium NlpC-like protein A*) has a four-domain structure with a hydrophobic and narrow access to the catalytic center and specificity for the \(\gamma\)-D-glutamyl-meso-diaminopimelic acid bond. However, PnpA does not cleave the PG of Phdp or PG of several Gram-negative and Gram-positive bacterial species. Interestingly, it is secreted by the Phdp type II secretion system and degrades the PG of *Vibrio anguillarum* and *Vibrio vulnificus*. This suggests that PnpA is used by Phdp to gain an advantage over bacteria that compete for the same resources or to obtain nutrients in nutrient-scarce environments. Comparison of the muropeptide composition of PG susceptible and resistant to the catalytic activity of PnpA showed that the global content of muropeptides is similar, suggesting that susceptibility to PnpA is determined by the three-dimensional organization of the muropeptides in the PG.

**IMPORTANCE** Peptidoglycan (PG) is a major component of the bacterial cell wall formed by long chains of two alternating sugars interconnected by short peptides, generating a mesh-like structure that enwraps the bacterial cell. Although PG provides structural integrity and support for anchoring other components of the cell envelope, it is constantly being remodeled through the action of specific enzymes that cleave or join its components. Here, it is shown that *Photobacterium damselae* subsp. *piscicida*, a bacterium that causes high mortality in warm-water marine fish, produces PnpA, an enzyme that is secreted into the environment and is able to cleave the PG of potentially competing bacteria, either to gain a competitive advantage and/or to

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Citation Lisboa J, Pereira C, Riffllet A, Ayala J, Terceti MS, Barca AV, Rodrigues J, Pereira PIB, Osorio CR, Garcia-del Portillo I, Gomperts Boneca I, do Vale A, dos Santos NMS. 2021. A secreted NlpC/P60 endopeptidase from *Photobacterium damselae* subsp. *piscicida* cleaves the peptidoglycan of potentially competing bacteria. mSphere 6:e00736-20. https://doi.org/10.1128/mSphere.00736-20.

Editor Ana Cristina Gales, Escola Paulista de Medicina/Universidade Federal de São Paulo

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obtain nutrients. The specificity of PnpA for the PG of some bacteria and its inability to cleave others may be explained by differences in the structure of the PG mesh and not by different muropeptide composition.

**KEYWORDS** NlpC/P60, *Vibrio anguillarum*, *Vibrio vulniﬁcus*, X-ray crystallography, cell wall hydrolases, peptidoglycan, *Photobacterium damselae* subsp. *piscicida*, type II secretion system

Peptidoglycan (PG) is a major component of the bacterial cell wall, essential for maintaining structural integrity and internal osmotic pressure, shaping the morphology of bacteria, and providing support for anchoring other components of the cell envelope (1, 2). PG forms a mesh-like structure that enwraps the bacterial cell, referred to as sacculus, which is composed of long chains of two alternating β(1-4) glycosidic-bonded glycans, N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc), cross-linked by short stem peptides, either directly or through bridging peptides (1, 3–5). The stem peptides are usually 4 or 5 amino acids long, contain L- and D-amino acids, and extend from MurNAc (1–4). The most common structure of the stem peptide is L-Ala-γ-D-Glu-mDAP-α-Ala-α-Ala (mDAP stands for meso-diaminopimelic acid) in Gram-negative bacteria and L-Ala-γ-D-Glu-L-Lys-α-Ala-α-Ala in Gram-positive organisms (1, 2, 4).

In spite of its stabilizing function, PG is highly dynamic, with covalent bonds being formed and broken by different enzymes. Multiple hydrolases, capable of cleaving glycosidic (glycosidases) or amide (amidases and peptidases) bonds in the PG sacculus and/or its soluble fragments, play a preponderant role in PG dynamics (1, 2, 6–13). Degradation products resulting from the catalytic activity of PG hydrolases can be recycled for PG de novo biosynthesis and also act as signaling molecules in quorum sensing, triggering antibiotic resistance or regrowth of dormant cells or as effector molecules in immune responses (1, 2, 6, 7, 12, 14, 15). Besides their role in PG dynamics, hydrolases can also be secreted to the environment or injected via type VI secretion systems into the periplasm of other bacteria to confer competitive advantage over competing bacteria that share mixed growth environments or as a way of obtaining nutrients (1, 10, 11, 16–23).

PG peptidases are a widely diverse group of enzymes, with 10 different types of catalytic domains involved in PG hydrolysis described thus far (1, 24). Of these enzymes, cysteine peptidases containing new lipoprotein C/peptide of 60-kDa (NlpC/P60) catalytic domains are present in most bacterial lineages, suggesting that they play an important biological role (1, 24). NlpC/P60-containing peptidases are involved in the catalysis of the N-acetylmuramate-γ-alanine or d-γ-glutamyl-meso-diaminopimelate linkages, with four major groups identified so far: (i) P60-like, (ii) AcmB/LytN-like, (iii) YaeF/poxvirus G6R, and (iv) lecithin retinol acyltransferase (LRAT)-like (24). The NlpC/P60 domain is structurally similar to a primitive papain-like peptidase (24–29) and can be found alone or fused to other domains, with or without catalytic functions, to form multifunctional proteins (1, 2, 24, 26, 30–35). Several of these domains, such as the SH3 (sarcoma homology 3) domain (31, 32, 35), are involved in anchoring hydrolases to cell wall components, allowing their appropriate concentration and positioning for the formation of an efficient enzyme-substrate complex (1).

*Photobacterium damselae* subsp. *piscicida* (*Phdp*) is a Gram-negative, halophilic bacterium that induces an acute infection that rapidly develops into septicemia, resulting in high mortality of warm-water marine fish with devastating consequences for the aquaculture industry (36, 37). Although it has been suggested that *Phdp* remains in a cultivable form in salt water for only 4 or 5 days (38, 39), it was also suggested that it has the ability to enter a dormant, noncultivable but infectious state in salt water and sediment (40). With regard to the mechanisms responsible for the pathogenicity of *Phdp*, it was shown that extracellular products (ECPs) play a fundamental role (41, 42) although among their components, only the toxin AIP56 has been identified and characterized so far (43–47).
The present work reports the structural and functional characterization of a novel NlpC/P60-containing peptidase from Phdp (PnpA). The results show that PnpA is a PG hydrolase with a four-domain structure similar to that of Desulfovibrio vulgaris lysin (DvLysin) and specificity for the γ-glutamyl-meso-diaminopimelic acid bond (26), but with a more hydrophobic and narrower access to the catalytic center. It is also shown that PnpA is secreted into the extracellular medium by the Phdp type II secretion system and acts on the PG of Vibrio anguillarum and Vibrio vulnificus, suggesting that it may provide Phdp an advantage over bacteria competing for the same resources or a way of obtaining nutrients in nutrient-scarce environments, either inside or outside the host. Comparison of the muropeptide compositions of PG, susceptible and resistant to PnpA activity, allowed development of a model suggesting that the susceptibility to PnpA is determined by three-dimensional structural features of the PG and not by their chemical compositions.

RESULTS

Photobacterium damselae subsp. piscicida secretes an NlpC/P60 family protein. Photobacterium damselae subsp. piscicida (Phdp) virulent strains have a relatively simple profile of secreted proteins in mid-exponential-growth-phase cultures (45). Apart from AIP56 toxin, no other proteins have been identified and characterized. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of proteins from Phdp extracellular products (ECPs) precipitated with trichloroacetic acid (TCA) revealed a band of approximately 55 kDa that was excised from the gel and subjected to matrix-assisted laser desorption ionization—time of flight mass spectrometry (MALDI-TOF MS). The obtained MS data were used in a Mascot search against the NCBI database resulting in the identification of a hypothetical protein from Photobacterium damsela subsp. damsela (Phdd) CIP 102761 (VDA_000779; NCBI accession number EEZ39759). The 1,479-nucleotide homologous sequence in the Phdp MT1415 strain (accession number TJZ86030.1) was then amplified using primers designed based on the VDA_000779 sequence. In silico analysis (SignalP 5.0 and NCBI conserved domain search) of its 499-amino-acid translation product predicted a Sec signal peptide (M1 to A19), followed by an N_NLPC_P60 putative stabilizing domain (Pfam PF12912), an SH3b1 (Pfam PF12913/12914), and an NlpC_P60 domain (Pfam PF00877), classifying it as a protein belonging to the NlpC/P60 family, hereafter referred to as PnpA (Photobacterium NlpC-like protein A).

PnpA is encoded in a genetically unstable chromosomal region, and its expression levels are similar at exponential and stationary phases of growth. To investigate the genetic context of pnpA in Phdp MT1415 strain, the draft genome sequence of MT1415 was obtained in this study. Then, homologous DNA sequences of a number of Phdp and Phdd isolates were additionally retrieved from the GenBank database and subjected to comparative sequence analysis (Fig. 1). This revealed that the PnpA-encoding gene is invariably linked to a downstream gene encoding an RNase T and to an upstream gene encoding an α-galactosidase, the latter being a pseudogene in some Phdp isolates. As a whole, the DNA flanking pnpA underwent a massive insertion of transposase genes (IS elements of the IS1 and IS91 families) likely followed by accumulation of inactivating mutations, resulting in a collection of pseudogenes. This process of gene decay not only affected the transposase genes themselves but also flanking genes encoding enzymes putatively involved in sugar metabolism, as α-galactosidases, α-amylases, and pullulanases (Fig. 1). Proliferation of insertion sequences that cause a high frequency of pseudogenes and gene loss is indeed a hallmark of all Phdp genomes studied thus far (48–50). The observation that PnpA- and the RNase T-encoding genes have escaped the inactivation by IS insertions suggests that these two genes may fulfill an important role in Phdp.

Expression levels of pnpA were determined by reverse transcription-PCR (RT-PCR), showing that under the culture conditions used (growth in tryptic soy broth supplemented with NaCl to a final concentration of 1% [wt/vol] [TSB-1] at 25°C), there are no
differences in the level of gene transcription between exponential- and stationary-phase cultures (see Fig. S1 in the supplemental material).

**Overall description of PnpA structure.** For better understanding of the structure-function relationship of PnpA, its three-dimensional structure was solved. The crystal structure of PnpA was determined at 1.4-Å resolution by molecular replacement with DvLysin (PDB entry 3M1U, 26% sequence identity), an endopeptidase from *Desulfovibrio vulgaris* *Hildenborough* (26). The crystal asymmetric unit contains two PnpA molecules, which are essentially identical (root mean square deviation [RMSD] of 0.5 Å for 457 aligned Cα atoms). Table S1 in the supplemental material summarizes the data collection, processing, and refinement statistics.

Analysis of the intermolecular packing interfaces within the crystal lattice suggests that the molecule behaves as a monomer in solution, which is in agreement with the molecular mass estimated by size exclusion chromatography. The PnpA monomer has an overall structure similar to that of DvLysin (26), namely, one N-terminal “c-clip” or “N_NLPC_P60” stabilizing domain (residues N20–N133), two SH3b domains (SH3b1, residues I134–V218; SH3b2, residues D219–T295), and the C-terminal NlpC/P60 catalytic domain (residues P296–K499) (Fig. 2A). The three-dimensional models of DvLysin and PnpA display an RMSD of 2.2 Å (for 405 aligned Cα atoms), suggesting that both proteins may be functionally equivalent. A significant number of structures sharing at least one of the PnpA domains have been identified (Table S2), although so far, PnpA and DvLysin are the only four-domain NlpC/P60-containing peptidases whose structure has been reported.

As in DvLysin (26), the PnpA c-clip domain has an extended helical conformation which surrounds and stabilizes the SH3b1 and NlpC/P60 domains, forming a planar assembly from which the SH3b2 domain protrudes (Fig. S2). Compared to DvLysin, the c-clip domain of PnpA harbors an extension between helices α1 and α2, thereby forming an additional two-stranded antiparallel β-sheet (β2 and β3) and a 3_{10} helix (η4), which protrude into the catalytic groove and close one of its sides (Fig. 2B).

The presence of SH3b domains in prokaryotes has long been documented. These domains have been described as targeting domains, involved in cell wall recognition and binding (1, 24, 35). Despite the lack of amino acid sequence conservation (8% sequence identity), the two SH3b domains in PnpA have a conserved overall fold (RMSD of 3.9 Å for 55 aligned Cα atoms) (Fig. S3). As in DvLysin (26), both PnpA SH3b domains consist of seven conserved strands (βA-βA1-βA2-βB-βC-βD-βE), with the βA-βE strands structurally equivalent to their eukaryotic counterparts (31, 32), while
A1 and A2 form a β-hairpin that corresponds to the RT loops of eukaryotic SH3b domains (Fig. 2A).

As in other NlpC/P60-containing peptidases, the 204-residue-long C-terminal NlpC/P60 catalytic domain of PnpA displays a fold resembling a primitive papain-like cysteine peptidase (24). Its secondary structure elements adopt the topology described for DvLysin, i.e., a six-stranded central β-sheet and five α-helices with αA-αB-αC-βA-αD-βB-αE-αF topology, where αA-αB-αC and αD-αE protect either side of the central β-sheet (Fig. 2A) (26).

PnpA has a narrow and hydrophobic access to the catalytic site. The active site of NlpC/P60 cysteine peptidases consists of a conserved cysteine-histidine dyad and a third polar residue (H, N, or Q) that orients and polarizes the catalytic histidine (24–29). In PnpA, the residues that make up the active site are C324, H395, and N415, the latter similar to the equivalent residue found in the active site of the prototypical papain (51), but differing from the histidine (H408) at the active site of DvLysin (26) (Fig. 3A).

As described for other NlpC/P60-containing peptidases (24–29), the catalytic C324 is

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located at the amino terminus of a helix packing against the central \( \beta \)-sheet that harbors H395 in its second strand and N415 in the third. In the PnpA structure, the thiol group of the catalytic cysteine is oxidized, resulting in the disruption of the characteristic C324 SD-H395 ND1 hydrogen bond and suggesting that the enzyme is in an inactive state (Fig. S4). As advanced for \textit{Bacteroides thetaiotamicron} YkfC (BtYkfC) (26), oxidation of the catalytic cysteine most likely occurred during crystallization or exposure to X-rays (52), since recombinant PnpA from the same purification batch was used in biochemical assays and was catalytically active.

In DvLysin, access to the catalytic cysteine occurs through a groove between the NlpC/P60 domain on one side and the c-clip helices \( \alpha D \) and \( \alpha E \) plus the SH3b1 domain

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**FIG 3** Structural comparison of the active sites of PnpA and DvLysin. (A) Superposition of the catalytic site of PnpA (colored sticks) and DvLysin (gray sticks). Dashed line indicates the distance between amino acid residues in angstroms. (B) Solid surface representation of PnpA (left) and DvLysin (right). Catalytic grooves are outlined by dashed lines. Residues R414 and R452 from DvLysin are colored cyan and labeled. (C) Comparison of the catalytic cavities of PnpA (left) and DvLysin (right). Hydrophobic and polar residues close to the substrate binding region are colored dark blue (top panel). DvLysin residues involved in substrate binding (27) and conserved in PnpA are colored green (bottom panel). Catalytic residues are colored as in Fig. 2A.
on the other, with the RT loop from the SH3b1 domain closing one end of the groove (Fig. 3B) (26). While this topology is generally maintained in PnpA, the end of the groove opposite to the RT loop is also closed by strands β2 and β3 and the 310 helix h4, creating a narrower access to the catalytic site (Fig. 3B). A minor difference is observed on the "wall" formed by the NlpC/P60 domain, wider in PnpA and closed by R414 and R452 in DvLysin (Fig. 3B). Besides the narrower entrance, two clusters of amino acids confer to the active site cavity of PnpA a more polar and hydrophobic nature than observed for DvLysin (Fig. 3C). However, extensive conservation of substrate-interacting residues between PnpA and DvLysin (Fig. 3C) suggests a similar interaction with meso-diaminopimelic acid (mDAP)-D-Ala from the stem peptide.

**PnpA is secreted by Phdp type II secretion system.** PnpA possesses a typical Sec signal peptide and was identified in the culture supernatants of exponentially growing Phdp cultures, suggesting that it could be actively secreted by the bacteria. Many proteins that are transported via the Sec system into the periplasm are secreted across the outer membrane through a type II secretion system (T2SS) (53, 54). Recently, it was shown that Phdp contains a functional T2SS (44) and that deletion of epsL, which encodes an inner membrane-spanning protein that establishes a critical link between the cytoplasmic and periplasmic parts of that system (55), abolishes the secretion of AIP56 (44). To test the involvement of the T2SS of Phdp in PnpA secretion, the presence of PnpA in total cell lysates and extracellular products of wild-type (WT), ΔepsL and ΔepsL1pEpsL Phdp was analyzed by Western blotting (Fig. 4). PnpA was detected in ECPs, but not in total cell lysates of the WT strain, confirming that it is a secreted protein (Fig. 4A). In contrast, in the ΔepsL strain, PnpA was retained in the cell, likely in the periplasm (Fig. 4B), confirming the involvement of T2SS in PnpA secretion.

**PnpA has specificity for the γ-D-glutamyl-meso-diaminopimelic acid bond.** To investigate the PnpA enzymatic activity toward PG muropeptides and define its substrate specificity, recombinant PnpA was incubated with monomeric trimuropeptides (M3; GlcNAcMurNAc-L-Ala-D-Glu-mDap), tetramuropeptides (M4; GlcNAc-MurNAc-L-Ala-D-Glu-mDap-D-Ala), and pentamuropeptides (M5; GlcNAc-MurNAc-L-Ala-D-Glu-mDap-D-Ala-D-Ala) and the cleavage product(s) analyzed by high-performance liquid chromatography (HPLC) (Fig. 5). PnpA converted all tested muropeptides to dipeptides (M2; GlcNAc-MurNAc-L-Ala-D-Glu), suggesting it cleaves specifically γ-D-glutamyl-meso-diaminopimelic acid bond of monomeric muropeptides.
**PnpA does not hydrolyze Phdp peptidoglycan.** In order to evaluate the involvement of PnpA in Phdp cell wall biogenesis, a Phdp ΔpnpA strain was generated, and the absence of PnpA expression in the mutant strain was confirmed by SDS-PAGE and Western blotting (Fig. 6A and B). Bacterial growth was not affected in the ΔpnpA strain (Fig. 6C). In addition, no differences were detected in the composition of the peptidoglycan from the WT and ΔpnpA strains (Fig. 6D; Table 1). In agreement with this, both WT and ΔpnpA strains showed similar morphology (Fig. 6E). Moreover, PnpA did not display in vitro enzymatic activity against Phdp whole sacculus, since no differences in the muropeptide composition were detected after incubating the PG with active PnpA or inactive PnpA (Fig. 6F and Fig. S5A; Table 2). Altogether, these results suggest that PnpA is not enzymatically active toward intact Phdp PG.

**PnpA has hydrolytic activity toward Vibrio anguillarum and Vibrio vulnificus PG.** The facts that PnpA is actively secreted into the extracellular medium and has no enzymatic activity for Phdp PG raised the possibility that it could cleave PG from other bacteria, functioning as a weapon against competing bacteria or as part of a mechanism to acquire nutrients, e.g., muropeptides from dead bacteria. To address this issue, whole sacculi from several Gram-positive or Gram-negative bacteria were isolated and incubated in vitro with recombinant PnpA or catalytically inactive PnpA (PnpA(C324A)) (Fig. 7 and Fig. S5B to J). Interestingly, only sacculi from *V. anguillarum* and *V. vulnificus* were sensitive to the action of PnpA (Fig. 7 and Fig. S5B and C and S6). Analysis of the insoluble sacculi resulting from digestion with PnpA showed the appearance of novel

![HPLC profiles of each muropeptide](image)
FIG 6 PnpA does not hydrolyze Phdp peptidoglycan. (A) SDS-PAGE of extracellular products (ECPs) and bacterial pellets (Cells) from WT and Δpnpa Phdp. ECPs equivalent to 1.5 ml and cells equivalent to 0.3 ml of early stationary culture were separated by 12% SDS-PAGE (Continued on next page)
muropeptides, not present after incubation with inactive PnPAC324A or vehicle (Fig. 7; Table 2). *V. anguillarum* and *V. vulnificus* PG present a very simple muropeptide composition with three major muropeptides, the monomer GM-tetrapeptide (GM4), the dimer GM4-GM4, and the anhydro-dimer (GM4-GanhM4 and GanhM4-GM4). The high proportion of anhydro-muropeptides indicates that *V. vulnificus* has a PG with short glycan chains (Table 2). PnP treatment led to the appearance of four new muropeptides, GM2, GanhM2, GM4-mDapA, and GanhM4-mDapA. GM2 and GanhM2 products are consistent with the hydrolysis of the γ-D-glutamyl-meso-diaminopimelic acid bond. The presence of GM4-mDapA and GanhM4-mDapA are also consistent with the hydrolysis of a dimer or higher oligomers such as the major dimers GM4-GM4 and GM4-GanhM4 and the trimers GM3-GM4-GM4 and GM4-GM4-GM4 (Table 2) at the γ-D-glutamyl-meso-diaminopimelic acid bond at one of the 4-amino-acid stem peptides.

Analysis of the products released from the *V. vulnificus* PG identified two main tetrasaccharides substituted with the ε-alanine-ε-glutamate dipeptide (GM2-GanhM2) and/or a remain of the dimer cross-link (GM4-GanhM4-mDapA; Fig. 7 and Fig. S6; Table 3). Additionally, the GanhM2 monomer, the remains of the monomer stem peptide mDapA and of dimer cross-link mDapA-mDapA were also released, confirming that PnP is indeed a γ-D-glutamyl-meso-diaminopimelate endopeptidase (Fig. 7 and Fig. S6; Table 3).

In order to assess whether PnP could inhibit the growth of competitor bacteria, the growth of *V. vulnificus* was monitored in the presence of PnP (5 μg ml⁻¹), and no growth inhibition was observed (Fig. S7A). To test the hypothesis that an additional factor secreted by Phdp could assist PnP in reaching the PG, the growth of *V. vulnificus* was monitored in the presence of ECPs from wild-type or ΔpnpA Phdp (Fig. S7B) and in coculture experiments (Fig. S7C). No growth inhibition was observed in any of these experiments. Finally, it was tested whether PnP was able to inhibit the growth of *V. vulnificus* in the presence of EDTA, an external membrane-permeabilizing agent used to mimic conditions that may be encountered in the host, and no effect on growth was observed (Fig. S7D).

**DISCUSSION**

In this work, the structural and functional characterization of PnP, an NlpC/P60 family peptidase secreted by *Photobacterium damselae* subsp. *piscicida* (Phdp) is reported. PnP is not essential for Phdp cell wall biogenesis and does not cleave Phdp PG, but it degrades the PG of *V. anguillarum* and *V. vulnificus*, two bacterial species that share the same hosts and/or environment as Phdp. On the basis of these observations, it is proposed that PnP may allow Phdp to fight competitors or to acquire nutrients from dead coinhabitants.

Many cysteine peptidases containing the NlpC/P60 domain were characterized thus far (1, 2, 24, 26, 30, 33–35), several of which display a four-domain organization similar to PnP. However, until now, only the three-dimensional structure of DvLysin from *Desulfovibrio vulgaris* was reported, with a N-terminal "c-clip" or "N_NLPC_P60" stabilizing domain, two SH3b domains, and a C-terminal NlpC/P60 cysteine peptidase domain (26). Furthermore, among the known DvLysin and PnP orthologs, only EcgA from

**FIG 6 Legend (Continued)**

and stained with Coomassie blue. Recombinant PnP (rPnP; 2 μg) was used as a reference. The gel shown is representative of two independent experiments. (B) Western blotting detection of PnP in WT and ΔpnpA strains (top panel; ECPs and cells equivalent to 0.3 ml of culture) and stained with Coomassie blue. Recombinant PnP (rPnP; 2 μg) was used as a control. The gel shown is representative of three independent experiments. (C) Deletion of pnpA does not affect bacterial growth. Phdp MT1415 and MT1415ΔpnpA strains were grown in TSB-1 at 25°C. Growth curves were generated from three replicates for each strain. The results shown are representative of two independent experiments. (D) Total ion current (TIC) of digested and reduced PG from wild-type Phdp MT1415 (top) and MT1415ΔpnpA (middle and bottom; (i) and (ii) correspond to two independent cultures of Phdp MT1415ΔpnpA). (E) Deletion of pnpA does not affect bacterial morphology. Bacteria labeled with wheat germ agglutinin (WGA)-Alexa Fluor 488 (top panel; bars, 2 μm). The lengths of at least 150 bacteria from two independent experiments were measured and graphed (bottom panel, mean length ± standard deviation [SD] [error bar]). Statistical significance was tested by Student’s t test, and no differences were observed. (F) Total ion current (TIC) of digested and reduced PG of Phdp previously incubated with vehicle, PnP, or catalytically inactive PnPAC324A; the corresponding reduced supernatants are shown in Fig. S6 in the supplemental material.
| Category and target name | ReTi (min) | Formula | ReTi | Error (ppm) | MT1415 (WT) | ΔpnpA (1) | ΔpnpA (2) |
|--------------------------|------------|---------|------|-------------|-------------|-----------|-----------|
| Monomers                 |            |         |      |             |             |           |           |
| GMDi peptide             | 9.11       | C₂₀H₃₈N₅O₁₇ | 698.2588 | 699.2936   | 699.2931   | 0.74      | 4.71      |
| GMTripeptide             | 6.00       | C₁₇H₃₈N₆O₁₅ | 870.3706 | 436.1929   | 436.1926   | 0.69      | 13.42     |
| GMTripeptide + Gly       | 7.35       | C₁₇H₃₈N₆O₁₅ | 927.3921 | 464.7040   | 464.7035   | 1.08      | 3.23      |
| GMTripeptide-K           | 8.00       | C₁₇H₃₈N₆O₁₅ | 998.4656 | 500.2406   | 500.2401   | 1.10      | 4.00      |
| GMTetrapeptide-R         | 8.67       | C₁₇H₃₈N₆O₁₅ | 1,097.5163 | 549.7629 | 549.7617   | 2.18      | 0.01      |
| GMTripeptide-Mipa-mDap   | 10.58      | C₁₇H₃₈N₆O₁₅ | 1,099.5132 | 550.7639 | 550.7635   | 1.09      | 4.96      |
| GMTetrapeptide           | 9.66       | C₁₇H₃₈N₆O₁₅ | 941.4077 | 471.7115   | 471.7111   | 0.85      | 27.21     |
| GMNMDipeptide            | 17.10      | C₁₇H₃₈N₆O₁₅ | 678.2596 | 679.2683   | 679.2669   | 2.12      | 0.13      |
| GMNMTripeptide           | 13.73      | C₁₇H₃₈N₆O₁₅ | 850.3444 | 851.3517   | 851.3517   | 0.06      | 0.56      |
| GMNMTetrapeptide         | 16.27      | C₁₇H₃₈N₆O₁₅ | 921.38149 | 922.3888   | 922.3888   | 1.89      | 0.25      |
| Dimers                   |            |         |      |             |             |           |           |
| GMTripeptide-GMTripeptide| 14.03      | C₂₃H₄₄N₆O₂₃ | 1,722.7306 | 862.3735   | 862.3726   | 1.03      | 3.55      |
| GMTripeptide-GMTripeptide| 14.75      | C₂₃H₄₄N₆O₂₃ | 1,793.7677 | 897.8941   | 897.8911   | 3.30      | 3.55      |
| GMTripeptide-GMTripeptide| 15.03      | C₂₃H₄₄N₆O₂₃ | 1,793.7677 | 897.8923   | 897.8911   | 1.26      | 8.67      |
| GMTripeptide-GMTripeptide| 13.72      | C₂₃H₄₄N₆O₂₃ | 1,779.7521 | 890.8842   | 890.8833   | 1.01      | 1.08      |
| GMTetrapeptide-GMTripeptide| 15.81 | C₂₃H₄₄N₆O₂₃ | 1,864.8048 | 933.4097   | 933.4097   | 0.86      | 15.10     |
| GMTetrapeptide-GMTripeptide| 14.47      | C₂₃H₄₄N₆O₂₃ | 1,850.7892 | 926.4035   | 926.4019   | 1.76      | 1.41      |
| GMTetrapeptide-GMTripeptide| 16.34      | C₂₃H₄₄N₆O₂₃ | 1,935.842 | 968.9301   | 968.9283   | 1.86      | 0.13      |
| GMNMTripeptide-GMTripeptide| 11.00      | C₂₃H₄₄N₆O₂₃ | 1,313.5722 | 657.7948   | 657.7934   | 2.16      | 0.02      |
| GMNMTripeptide-GMTripeptide| 11.40      | C₂₃H₄₄N₆O₂₃ | 1,313.5722 | 657.7948   | 657.7934   | 2.16      | 0.04      |
| GMNMTripeptide-GMTripeptide| 11.96      | C₂₃H₄₄N₆O₂₃ | 1,313.5722 | 657.7948   | 657.7934   | 2.16      | 0.08      |
| GMNMTetrapeptide-GMTripeptide| 10.48 | C₂₃H₄₄N₆O₂₃ | 1,184.5296 | 593.2721   | 593.2723   | 0.86      | 2.02      |
| GMNMTetrapeptide-GMTripeptide| 11.04      | C₂₃H₄₄N₆O₂₃ | 1,184.5296 | 593.2721   | 593.2723   | 0.37      | 0.26      |
| GMNMTripeptide-GMPentapeptide| 12.25 | C₂₃H₄₄N₆O₂₃ | 1,384.6093 | 693.3133   | 693.3119   | 1.96      | 0.13      |
| GMNMTripeptide-GMTripeptide| 18.70      | C₂₃H₄₄N₆O₂₃ | 1,702.7044 | 852.3603   | 852.3595   | 0.96      | 1.46      |
| GMNMTripeptide-GMTripeptide| 19.38      | C₂₃H₄₄N₆O₂₃ | 1,773.7415 | 887.8795   | 887.8780   | 1.69      | 0.57      |
| GMNMTripeptide-GMTripeptide| 19.58      | C₂₃H₄₄N₆O₂₃ | 1,773.7415 | 887.8795   | 887.8780   | 1.69      | 1.20      |
| GMNMTripeptide-GMTripeptide| 19.75      | C₂₃H₄₄N₆O₂₃ | 1,773.7415 | 887.8795   | 887.8780   | 1.69      | 0.58      |
| GMNMTripeptide-GMPentapeptide| 20.41 | C₂₃H₄₄N₆O₂₃ | 1,844.7786 | 923.3986   | 923.3966   | 2.18      | 1.03      |
| GMNMTripeptide-GMTripeptide| 23.52      | C₂₃H₄₄N₆O₂₃ | 1,753.7153 | 877.8675   | 877.8649   | 2.96      | 0.01      |
| GMNMTripeptide-GMTripeptide| 23.81      | C₂₃H₄₄N₆O₂₃ | 1,753.7153 | 877.8675   | 877.8649   | 2.96      | 0.01      |
| GMNMTripeptide-GMTripeptide| 24.40      | C₂₃H₄₄N₆O₂₃ | 1,824.7524 | 913.3866   | 913.3835   | 3.39      | 0.01      |
| GMNMTripeptide-GMTripeptide| 17.31      | C₂₃H₄₄N₆O₂₃ | 2,342.9847 | 782.0025   | 782.0022   | 0.38      | 0.27      |

(Continued on next page)
| Category and target name | ReTi (min)<sup>a</sup> | Formula | Molecular mass<sup>b</sup> | Quantity of muropeptides (%) from Phdp strain<sup>c</sup> | Error (ppm) | MT1415 (WT) | ΔpnpA (1) | ΔpnpA (2) |
|--------------------------|------------------------|---------|-----------------------------|-------------------------------------------------|-------------|-------------|----------|----------|
| Trimers                  |                        |         |                             |                                                  |             |             |          |          |
| GMTriptide-GMTetrapeptide-GMTetrapeptide | 18.25                 | C₁₀₀H₁₈₀N₁₂₀O₆₀ | 2,717.1649 | [M+nH]<sup>++</sup> | 906.7307 | 906.7289 | 1.93     | 0.91 | 0.83 | 1.15 |
| GMTetrapeptide-GMTetrapeptide-GMTetrapeptide | 18.74                 | C₁₁₁H₁₈₅O₆₁N₂₁ | 2,788.202 | [M+nH]<sup>++</sup> | 930.4092 | 930.4079 | 1.40     | 1.39 | 1.42 | 1.51 |
| Cross-linking (%)        |                        |         |                             |                                                  |             |             |          |          |
| Avg glycan chain length  |                        |         |                             |                                                  |             |             |          |          |

<sup>a</sup> ReTi, retention time.  
<sup>b</sup> M, molecular mass.  
<sup>c</sup> Data from two independent cultures of Phdp MT1415 ΔpnpA are shown by (1) and (2).
| Category and target name | ReTi (min) | Formula | Molecular mass | Quantity of muropeptides (%) |
|--------------------------|------------|---------|----------------|-------------------------------|
|                          |            |         | M (neutral mass) | [M+nH]^+ Th. | [M+nH]^+ Error (ppm) | Phdp | PnpA | PnpA C324A | Vibrio vulnificus |
| Monomers                 |            |         |                 |                  |                      |      |      |              |                  |
| GM Dipeptide             |            |         |                 |                  |                      |      |      |              |                  |
| GM Tripeptide            | 9.12       | C$_6$H$_{12}$N$_4$O$_7$ | 698.2858 | 699.2929 | 699.2931 | -0.29 | 5.22 | 6.87 | 5.20 | 0.03 | 22.58 | 0.93 |
| GM Tetrapeptide          | 6.12       | C$_8$H$_{16}$N$_4$O$_9$ | 870.3706 | 846.1925 | 436.1926 | -0.23 | 10.99 | 10.05 | 10.65 | 0.82 | 0.81 | 1.24 |
| GM Tripeptide +Gly       | 7.41       | C$_6$H$_{12}$N$_4$O$_7$ | 927.3921 | 464.7035 | 464.7033 | 0.43 | 3.69 | 3.42 | 3.59 | 0.97 | 0.57 | 0.95 |
| GM Tripeptide-K          | 8.10       | C$_8$H$_{16}$N$_4$O$_9$ | 998.4656 | 500.2406 | 500.2401 | 1.10 | 0.50 | 0.47 | 0.49 | 0.33 | 0.19 | 0.32 |
| GM Tetrapeptide-R        | 8.75       | C$_8$H$_{16}$N$_4$O$_9$ | 1079.5163 | 549.7629 | 549.7617 | 2.18 | 0.01 | 0.01 | 0.01 | 5.77 | 3.48 | 5.70 |
| GM Tripeptide-Mipa-mDap  | 10.62      | C$_8$H$_{16}$N$_4$O$_9$ | 1099.5132 | 550.7645 | 550.7639 | 1.09 | 9.29 | 9.08 | 9.20 | 0.34 | 0.21 | 0.33 |
| GM Tetrapeptide          | 9.69       | C$_8$H$_{16}$N$_4$O$_9$ | 941.4077 | 471.7109 | 471.7111 | -0.42 | 27.25 | 27.26 | 27.58 | 44.64 | 30.82 | 42.12 |
| GanhM Dipeptide          | 17.10      | C$_6$H$_{12}$N$_4$O$_7$ | 678.2596 | 679.2674 | 679.2669 | 0.79 | 0.14 | 1.24 | 0.20 | 0.03 | 11.50 | 0.56 |
| GanhM Tripeptide         | 13.74      | C$_8$H$_{16}$N$_4$O$_9$ | 850.3444 | 851.3513 | 851.3517 | -0.41 | 0.44 | 0.03 | 0.46 | 0.13 | 0.00 | 0.18 |
| GanhM Tetrapeptide       | 16.30      | C$_8$H$_{16}$N$_4$O$_9$ | 921.3819 | 922.3893 | 922.3889 | 0.59 | 0.13 | 0.01 | 0.12 | 2.21 | 0.03 | 1.93 |

(Continued on next page)
| Category and target name | ReTi (min) | Formula | Molecular mass<sup>c</sup> | Quantity of muropeptides (%) |  |  |  |
|--------------------------|------------|---------|---------------------------|-----------------------------|---|---|---|
|                          |            |         | M (neutral mass) | [M+nH]<sup>++</sup> | Error (ppm) | Phdp | PnpA | PnpA<sub>C324A</sub> | Vibrio vulnificus | Vehicle | PnpA | PnpA<sub>C324A</sub> |
| Trimmers                 |            |         |                       |                             |              |     |     |     |                             |           |     |     |     |
| GMTripeptide-GMTetrapeptide-GMTetrapeptide | 18.21 | C<sub>108</sub>H<sub>180</sub>N<sub>20</sub>O<sub>60</sub> | 2,717.1649 | 906.7293 | 906.7289 | 0.45 | 1.22 | 1.23 | 1.23 | 0.15 | 0.06 | 0.16 |
| GMTetrapeptide-GMTetrapeptide-GMTetrapeptide | 18.69 | C<sub>111</sub>H<sub>185</sub>O<sub>60</sub>N<sub>21</sub> | 2,788.202 | 930.4081 | 930.4079 | 0.21 | 1.23 | 1.24 | 1.25 | 0.81 | 0.25 | 0.88 |
| Cross-linking (%)        |            |         |                       |                             |              |     |     |     |                             |           |     |     |     |
| Avg glycan chain length  |            |         |                       |                             |              |     |     |     |                             |           |     |     |     |

<sup>a</sup>PnpA degradation products absent from native PG are shown in boldface type.

<sup>b</sup>ReTi, retention time.

<sup>c</sup>M, molecular mass.
Salmonella enterica serovar Typhimurium was functionally characterized (56). Although the three molecules are very similar (25 to 27% amino acid sequence identity) (see Fig. S8A in the supplemental material), DvLysin does not have the insertion found in PnpA and EcgA and that in PnpA closes the side of the catalytic groove opposed to the RT loop (Fig. 2 and 3 and Fig. S8B). Despite these differences, residues involved in substrate binding in DvLysin (26) are conserved in PnpA and EcgA (Fig. 3C and Fig. S8B), in agreement with their specificity for the γ-D-glutamyl-meso-diaminopimelic acid bond (Fig. 5) (26, 56). However, unlike DvLysin (26) and EcgA (56), which were more active toward tetra- and trimuropeptides, respectively, PnpA showed activity toward penta-, tetra-, and tripeptides (Fig. 5).

So far, the cellular localization of DvLysin and its function in D. vulgaris cell wall biogenesis remain unknown (26). Regarding EcgA, its expression is induced when S. Typhimurium is inside eukaryotic cells, localizing in the inner and outer membranes where it plays a role in PG remodeling and contributes to S. Typhimurium virulence (56). In contrast, PnpA is secreted by the T2SS into the extracellular medium (Fig. 4), and deletion of pnpA does not affect Phdp growth, PG composition, and morphology (Fig. 6C to E). Accordingly, PnpA has no in vitro hydrolytic activity toward Phdp sacculi (Fig. 6F and Fig. S5A). Altogether, these results suggest that PnpA is not involved in Phdp cell wall biogenesis.

The resistance of Phdp PG to the activity of PnpA is in sharp contrast with the ability of PnpA to hydrolyze penta-, tetra-, and trimuropeptides, since the chemical composition

**FIG 7** Total ion current (TIC) of digested and reduced PG of V. vulnificus previously incubated with vehicle, PnpA, or catalytically inactive PnpA(C324A); the corresponding reduced supernatants are shown in Fig. S6.
of Phdp PG suggested that it would be a target of PnpA. This unexpected resistance to PnpA was not exclusively observed with PG from Phdp, as it also occurred when using sacculi from multiple bacterial species (Fig. S5). In fact, PGs from V. anguillarum and V. vulnificus were sensitive to the activity of PnpA, despite having a PG composition characteristic of Gram-negative bacteria and similar to the composition of some PG shown to be resistant to PnpA hydrolysis. Hence, PnpA specificity for V. anguillarum and V. vulnificus PG cannot be explained by their muropeptide composition and may be related to specific three-dimensional features of the PG mesh. Accordingly, the analysis of the V. anguillarum and V. vulnificus PG composition shows that these two species have a high proportion of anhydro-muropeptides, a trademark of the end of glycans, indicating that their glycan chains are rather short compared to other Gram-negative bacteria. Consequently, structural analysis of the products released upon incubation of the sacculi of V. anguillarum and V. vulnificus with PnpA identified a high proportion of the tetrasaccharide GM2-GanhM2. This suggests that the PG of V. anguillarum and V. vulnificus is enriched in tetrasaccharides. The simultaneous release of mDapA-mDapA suggests that these tetrasaccharides are linked to the rest of the PG by one or even two cross-links. These results combined with the rather simple muropeptide composition of V. anguillarum and V. vulnificus suggest that the vulnerability of V. anguillarum and V. vulnificus to PnpA might arise from the fact that their PGs rely on very short, highly cross-linked glycans. Hence, hydrolysis of the stem peptides by PnpA leads to a rapid destruction of the PG layer while in other Gram-negative species, because they have much longer glycans, PG integrity can be maintained by multiple dimers along the same glycan chain (Fig. 8).

Expression levels of pnpA in standard culture conditions do not vary between the logarithmic and stationary growth phases (Fig. S1) but increase under iron-limited conditions or in response to oxidative stress (57). However, in vivo, no changes in pnpA expression were detected after intraperitoneal infection of sole (Solea senegalensis) with Phdp (57), and deletion of pnpA did not affect Phdp virulence in a sea bass (Dicentrarchus labrax) intraperitoneal infection model (Fig. S7E). This suggests that PnpA is likely dispensable at late systemic phases of Phdp infection but does not exclude a role of PnpA in earlier stages of the infection. It is known that, during the systemic phase of Phdp-induced disease, the exotoxin AIP56 plays a major role by neutralizing host phagocytic defenses (43–45, 47, 58). However, little is known about the early stages of the infection. Here, it is shown that PnpA specifically hydrolyzes the sacculi of V. anguillarum and V. vulnificus (Fig. 7, Fig. S5B and C, and Fig. S6), two other

### TABLE 3 Analysis of PG reduced supernatants from Phdp and Vibrio vulnificus after incubation with PnpA

| Target name | ReTi (min) | Formula | Molecular mass | Error (ppm) | Supernatant<sup>a</sup> | Phdp PG + PnpA | V. vulnificus + PnpA |
|-------------|------------|---------|----------------|-------------|---------------------|----------------|---------------------|
| mDap-Alanine + H₂O | 2.11 | C₁₀₂H₁₅₅N₃₈O₃₇ | 261.1325 | 262.1398 | 262.1398 | 1.39 | 8.84E + 06 | 1.41E + 09 |
| mDap-Alanine-mDap-Alanine + H₂O | 2.27 | C₂₀₅H₂₇₅N₇₉O₇₉ | 504.2544 | 505.2610 | 505.2617 | 1.39 | 8.84E + 06 | 1.41E + 09 |

<sup>a</sup>Values indicate the intensity of the corresponding muropeptide by mass spectrometry analysis in arbitrary units.
enterobacteria present in the marine environment (59–61) and, at least in the case of V. anguillarum, reported as infecting the same hosts as Phdp (37, 61). This suggests that before reaching the systemic phase, Phdp may secrete PnpA to gain competitive growth advantage over bacteria sharing a complex community environment, such as the gastrointestinal tract, or to obtain nutrients in an environment where nutrient scarcity can compromise its survival, either inside the host or in water or sediment (40). These strategies have been first described for Gram-positive bacteria (21, 23), which have their PG exposed on the cell surface, accessible to secreted PG hydrolases (10, 11, 22). Gram-negative bacteria, despite having their PG protected by the outer membrane, can inject PG hydrolases, including NlpC/P60 family peptidases, into the periplasm of neighboring bacteria through type VI secretion systems (10, 17, 19, 20). The examples of using bacterial exohydrolases to target Gram-negative competitors are restricted to predatory bacteria such as myxobacteria (11) and Bdellovibrio bacteriovorus (18). Another example where PG hydrolases are secreted to eliminate competing

**FIG 8** Structural model of Phdp (A) and V. vulnificus (B) PG suggesting that the higher degree of cross-linking of very short glycans in the V. vulnificus PG may explain its vulnerability to the enzymatic activity of PnpA.
bacteria is that reported for the urogenital pathogenic protozoan *Trichomonas vaginalis* (16), which has acquired by lateral genetic transfer two genes of bacterial origin encoding NlpC/P60 endopeptidases that the parasite secretes to degrade bacterial PG and thus outcompete bacteria from mixed cultures (16). However, it remains unclear how these exohydrolases reach the PG of the Gram-negative targets. Here, it was also not clarified how PnpA reaches the PG in *V. vulnificus* and *V. anguillarum* cell wall, since no growth inhibition was detected in several *in vitro* tests with *V. vulnificus* (Fig. S7), suggesting that the access of PnpA to the periplasm of competing bacteria may depend on conditions present at specific stages of the Phdp life cycle, when Phdp and competitors meet.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions.** *Photobacterium damselae* subsp. *piscicida* (Phdp) virulent strain MT1415 isolated from sea bass in Italy (45) was cultured at 25°C in tryptic soy broth (TSB) or tryptic soy agar (TSA) supplemented with NaCl to a final concentration of 1% (wt/vol) (TSB-1 and TSA-1, respectively). The Δepsl and ΔpnpA strains were cultured under the same conditions as the wild type. Δepsl + pEpsl and ΔpnpA + pPnpA complemented strains were cultured in TSB-1 or TSA-1 supplemented with 10 μg ml⁻¹ of gentamicin (TSB-1_gm and TSA-1_gm, respectively). Stocks of bacteria were maintained at –80°C in TSB-1 supplemented with 15% (vol/vol) glycerol. To obtain growth curves, bacteria grown on agar plates for 48 h were suspended in TSB-1 or TSB-1_gm at an optical density at 600 nm (OD₆₀₀) of 0.3 to 0.6. These suspensions were inoculated in 20 ml TSB-1 (1:100 dilution). One-milliliter aliquots were removed (in triplicate) and transferred to 24-well culture plate, and the OD₆₀₀ was determined kinetically (1 point/h) using a BioTek Synergy 2 spectrofluorometer (BioTek U.S., Winooski, VT, USA) at 25°C with continuous slow agitation, for 60 to 70 h. Growth curves were constructed using GraphPad Prism software (La Jolla, CA, USA).

**Construction of ΔpnpA strain.** An in-frame (nonpol) deletion of the almost complete pnpA coding sequence was constructed following an allelic exchange procedure as previously described (62). In brief, the 3’ and 5’ flanking sequences were PCR amplified using suitable primers (Mut_NlpC_1Eco [5’-GGGATATTTCGAGTTCCGATTGTAATCT-3’], Mut_NlpC_2Bam [5’-GGGATCCGAGTTCTAGGATGATTAAT-3’], Mut_NlpC_3Bam [5’-GGGATCCGAGTTCTAGGATGATTAATGCTA-3’], and Mut_NlpC_4Bam [5’-GGGATCCGAGTTCTAGGATGATTAATGCTA-3’] [restriction sites are underlined]). The PCR products were ligated to obtain an in-frame deletion of ca. 90% of the PnpA coding sequence. The deleted allele construction was cloned into the suicide vector pNidKan containing the pnpA gene, which confers sucrose sensitivity, and R6K ori, which requires the pir gene product for replication. The plasmid containing the deleted allele was transferred from *Escherichia coli* S17-1-pir into the rifampin-resistant derivative of Phdp MT1415 by drop mating for 24 h on TSA plates prepared with seawater. Cells were then scrapped off the plate and selected on TSA supplemented with kanamycin (Kan) (50 μg ml⁻¹) for plasmid integration. A selected Kan’ clone was further selected for sucrose resistance (15% [wt/vol]) for a second recombination event. This led to Phdp ΔpnpA mutant strain, which was tested by PCR to verify the correct allelic exchange.

**Bacterial cell extracts and extracellular products.** Phdp was grown in TSB-1 at 25°C with shaking (160 rpm) and centrifuged (6,000 × g, 5 min, 4°C), and the pellets (total cell extracts) and culture supernatants were collected. Supernatants were filtered (0.22 μm) to obtain extracellular products (ECPs). For SDS-PAGE, proteins in the ECPs were precipitated with trichloroacetic acid (TCA) as previously described (45).

**PnpA identification.** ECPs from Phdp strain MT1415 were subjected to SDS-PAGE followed by Coomassie blue staining. A protein band of approximately 55 kDa was analyzed by MALDI-TOF MS in a 4800 Proteomics Analyzer (Applied Biosystems) at TOPLAB GmbH. The MS data were used for a Mascot search against the NCBInr sequence database.

**Draft genome sequence of Phdp MT1415 and genomic context of pnpA locus.** To delete the PnpA-encoding gene in Phdp MT1415, it was necessary to obtain at least 2 kb of upstream and downstream sequences free of repetitive insertion sequence elements that would compromise the specific recombination steps during allelic exchange. Therefore, the draft genome sequence of strain MT1415 was obtained, using an Illumina platform as previously described (48) and deposited in the GenBank database under accession number SUMH00000000. A comparative analysis was conducted by retrieving the genomic contexts of pnpA genes in different Phdp and *Photobacterium damselae* subsp. *damsela* (Phdd) isolates whose draft or complete genomes are available in the GenBank database. The GenBank locus tag numbers of the pnpA homologues used in this analysis are VDA_000779 (Phdd type strain CIP 102761), PDPUS_2_00834 (Phdp 91–197), PDPJ_2_00460 (Phdp OT-S1443), BEI67_17705 (Phdp L091106-03H), and BDMQ10000002 (Phdp Di21). For the pnpA negative Phdd strain RM-71, the draft genome sequence as a source of homologous flanking DNA sequences was used (accession number NZ_LYBT00000000.1). The DNA sequences were handled with Vector NTI 10.3.0 sequence editor (Invivogen).

**Recombinant PnpA.** The pnpA open reading frame (ORF) (GenBank accession number T1Z86030.1) was amplified from Phdp MT1415 genomic DNA using Pfu DNA polymerase (Thermo Scientific) and primers 5’-gccggcATGATATAAATTAAATTTATGCGCT-3’ and 5’-gctgctgagTTTTCAATAGATTTTTC-3’ (target sequences are in uppercase letters) and cloned into pET28a(+) using the NcoI and XhoI restriction sites, in frame with a C-terminal 6×His tag. Mutation of C⁵²⁴ to alanine was achieved by site-directed
mutagenesis by inverse PCR using Q5 high fidelity DNA polymerase (New England BioLabs), pET28-PnpA as the template, and primers (5'-GGCTCGTGGATTTAAAGATTATCCG-3' and 5'-ATCATTATGAAAATCCATCCCCCAT-3'). Proteins were expressed in E. coli BL21(DE3) CodonPlus-RIL (Stratagene). Four liters of LB medium with 50 μg ml$^{-1}$ kanamycin and 25 μg ml$^{-1}$ chloramphenicol were inoculated with pET28-PnpA or pET28-PnpA$^{C274A}$ transformed bacteria and incubated at 37°C until an OD$_{600}$ of 0.6 to 0.8 was reached. Cultures were cooled at 17°C for 30 min, followed by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to induce protein expression. After 20 h, cells were harvested by centrifugation, resuspended in 50 mM Bis-Tris (pH 6.5) and 500 mM NaCl, and sonicated. Lysates were centrifuged (34,957 × g, 30 min, 4°C), and the soluble fraction was applied to a nickel-nitrilotriacetic acid (Ni-NTA) column (ABT), followed by anion-exchange chromatography (Bio-Scale Mini Macro-Prep High Q; Bio-Rad). Fractions containing the recombinant proteins were pooled and injected into a size exclusion chromatography column (Superose 12 10/300 GL; GE Healthcare) equilibrated with 50 mM Bis-Tris (pH 6.5) and 500 mM NaCl. Fractions containing the desired protein were pooled, concentrated to 6 to 7 mg ml$^{-1}$, frozen in liquid nitrogen, and stored at -80°C. Protein concentration was determined in a NanoDrop ND-1000 UV-visible (UV-Vis) spectrophotometer (Thermo Fisher Scientific) considering the extinction coefficient and the molecular weight calculated with the ProtParam tool (https://web.expasy.org/protparam/).

Reverse transcription and quantitative PCR (qRT-PCR). Total RNA was isolated from exponential (OD$_{600}$ of 0.4) and stationary (OD$_{600}$ of 1.2) cultures of Phdp strain MT1415. Bacterial pellets were resuspended in 25 mM Tris buffer supplemented with 20% (wt/vol) glucose and 0.5 M EDTA (pH 8.0) and lysed with phenol acid and glass beads by vortexing (4°C, 20 min). Lysates were centrifuged at 16,000 × g, 5 min, and the top liquid phase was collected. RNA was extracted using the TripleXtractor reagent (Grisp) and treated with DNase I (Turbio DNA-free; Ambion) following the manufacturer’s recommendations. RNA purity and integrity were verified by 1% (wt/vol) agarose gel electrophoresis in an Experion automated electrophoresis system (Bio-Rad). One microgram of RNA was reverse transcribed into cDNA (Script; Bio-Rad). Quantitative real-time PCR was performed in 20 μl reaction mixtures containing 1 μl cDNA, 10 μl Taq Universal SYBR green Supermix (Bio-Rad Laboratories), and 0.25 μM primers (PnpA forward primer [5'-GGATTGGGCTACCTCCTGTTCA-3'], PnpA reverse primer [5'-CCACCGAGG-CATTAAACATT-3'], 16S forward primer [5'-AUCTGAGGCTAGAGTCTT-3'], and 16S reverse primer [5'-CACAACTCCAAAGTAGACAT-3']), using the following protocol: 1 cycle at 95°C (3 min) and 40 cycles with 0.2° rotation, 0.2-s exposure) and indexed and integrated with XDS (65). Space group determination, data scaling, and merging were performed with POINTLESS and AIMLESS from the CCP4 program suite (66). The structure of PnpA was solved by molecular replacement with Phaser MR as implemented in the CCP4 program suite (66, 67) using the coordinates of a putative gamma-D-glutamyl-L-diamino acid endopeptidase from Desulfovibrio vulgaris Hildenborough (DvLysin, PDB entry 3M1U, 26% sequence identity) as the search model. Phase refinement and initial model building were performed using ARP/wARP (68). Model completion and refinement were done iteratively with COOT (69) and Phenix.refine (70, 71), respectively. Refinement and structure validation statistics are summarized in Table S1 in the supplemental material. All illustrations of macromolecular models were produced with PyMOL (72). The experimental data were deposited with the Structural Biology Data Grid (73) under accession number https://doi.org/10.15785/SGBGRID/736.

In vitro muropeptide cleavage assays. To investigate the PnpA enzymatic activity toward PG muropeptides, isolated M3 (GlcNAc-MurNAc-L-Ala-D-Glu-mDAP), M4 (GlcNAc-MurNAc-L-Ala-D-Glu-mDAP-D-Ala), and M5 (GlcNAc-MurNAc-L-Ala-D-Glu-mDAP-D-Ala-o-Ala) muropeptides from Salmonella enterica were incubated with 50 μg of PnpA in 50 mM Tris (pH 8.0) and 300 mM NaCl for 3 h at 37°C. The

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products of the reaction were analyzed by reverse-phase HPLC (Waters 1525 system) as previously described (56).

**Peptidoglycan (PG) purification.** Bacteria were grown in TSB-1 at 25°C with shaking (160 rpm) to exponential (OD600 of 0.4 to 0.5) or stationary (OD600 of 1.2 to 1.4) phases. Bacterial cells (~10^11) were centrifuged (4,200 × g, 10 min, room temperature [rt]), washed twice and resuspended in phosphate-buffered saline (PBS), and immediately mixed 1:1 (vol/vol) with a boiling solution of 8% SDS, drop by drop. Boiling was maintained for 8 h with stirring, followed by overnight incubation at rt. Samples were centrifuged (150,000 × g, 40 min, 4°C), the pellets were washed three times with ultrapure water (150,000 × g, 40 min, 4°C), resuspended in 10 mM Tris (pH 7.6) and 0.06% (wt/vol) NaCl with or without 100 μg ml⁻¹ α-amylase, and incubated at 37°C for 90 min. Samples were treated for 2 h at 60°C with 100 μg ml⁻¹ pronase E preactivated by incubation in the same buffer for 60 min at 60°C. Pronase E digestion was stopped by adding SDS (5.3% [wt/vol] final concentration) and heating at 100°C for 20 min. PG was recovered by centrifugation (300,000 × g, 10 min) and washed with ultrapure water.

**Analysis of Phdp PG composition and PG cleavage assays.** To analyze the PG composition of the Phdp MT1415 and MT1415ΔpnpA strains, PGs were purified as described above, digested overnight at 37°C in sodium phosphate buffer supplemented with 100 IU of mutanolysin from Streptomyces globisporus (ATCC 21553; Sigma), and reduced with NaH4B. After 30 min at rt and centrifugation, the reduced muropeptides were diluted in acidified water with formic acid (FA) and analyzed by high-performance liquid chromatography (HPLC) or HPLC/high-resolution mass spectrometry (HRMS). HPLC/HRMS was performed on an Ultimate 3000 UHPLC system coupled to a quadrupole orbitrap mass spectrometer (qExactive Focus; Thermo Fisher Scientific). Reduced muropeptides were eluted on an C18 analytical column (Hypersil gold aQ; 1.9 μm, 2.1 × 150 mm) held at 50°C under a 200 μl min⁻¹ flow rate. A binary solvent system composed of acidified water (H2O + 0.1% FA; mobile phase A) and acidified acetonitrile (CH3CN + 0.1% FA; mobile phase B) was used for chromatographic separation. The composition was linearly increased to 12.5% mobile phase B over 25 min, increased to 20% mobile phase B for 5 min, and held for an additional 5 min. It was then stepped down to 0% over and held for 10 min to return initial conditions.

Exactive Focus was operated under electrospray ionization in positive mode and data-dependent acquisition mode (ddMS2) control by Xcalibur 4.0. For structural confirmation of muropeptides, higher-energy collisional dissociation (HCD) fragmentation was set up with a normalized collision energy at 35%. Data were processed both with the software TraceFinder 3.3 (Thermo Fisher Scientific) and Xcalibur 4.0 for peak area determination.

For testing PnpA activity against macromolecular PG, PGs from Phdp and several bacterial species, purified as described above, were incubated with 100 μg PnpA or inactive PnpAC324A at 37°C overnight in 50 mM Tris (pH 8.0) and 300 mM NaCl. PGs incubated with vehicle were used as controls. After digestion, PGs were analyzed by HPLC or HPLC/HRMS as described above.

**Accession number(s).** The draft genome sequence of strain MT1415 was obtained and deposited in the GenBank database under accession number SUMH00000000. The experimental data were deposited with the Structural Biology Data Grid (73) under accession number https://doi.org/10.15785/SBGGRID/736. The structure factors and atomic coordinates of PnpA are deposited in the RCSB Protein Data Bank with accession number 6SQX.

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, TIF file, 0.1 MB.

**FIG S2**, TIF file, 1.3 MB.

**FIG S3**, TIF file, 0.6 MB.

**FIG S4**, TIF file, 1.1 MB.

**FIG S5**, TIF file, 0.5 MB.

**FIG S6**, TIF file, 0.7 MB.

**FIG S7**, TIF file, 0.5 MB.

**FIG S8**, TIF file, 2.5 MB.

**TABLE S1**, PDF file, 0.1 MB.

**TABLE S2**, PDF file, 0.2 MB.

**ACKNOWLEDGMENTS**

We are grateful for access to the HTX crystallization facility (Proposal ID: BIOSTRUCTX_8167). The support of the X-ray Crystallography Scientific Platform of i3S (Porto, Portugal) is also acknowledged.

This work was financed by Fundo Europeu de Desenvolvimento Regional (FEDER) funds through the COMPETE 2020 Operacional Program for Competitiveness and Internationalization (POCI), Portugal 2020, and by Portuguese funds through Fundação para a Ciência e a Tecnologia/Ministério da Ciência, Tecnologia e Ensino Superior (FCT) in the framework of the project POCI-01-0145-FEDER-030018 (PTDC/CVT-CVT/30018/
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