A predator-prey interaction between a marine *Pseudoalteromonas* sp. and Gram-positive bacteria

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Predator-prey interactions play important roles in the cycling of marine organic matter. Here we show that a Gram-negative bacterium isolated from marine sediments (*Pseudoalteromonas* sp. strain CF6-2) can kill Gram-positive bacteria of diverse peptidoglycan (PG) chemotypes by secreting the metalloprotease pseudoalterin. Secretion of the enzyme requires a Type II secretion system. Pseudoalterin binds to the glycan strands of Gram positive bacterial PG and degrades the PG peptide chains, leading to cell death. The released nutrients, including PG-derived D-amino acids, can then be utilized by strain CF6-2 for growth. Pseudoalterin synthesis is induced by PG degradation products such as glycine and glycine-rich oligopeptides. Genes encoding putative pseudoalterin-like proteins are found in many other marine bacteria. This study reveals a new microbial interaction in the ocean.
Bacteria are ubiquitous in marine ecosystems and play a vital role in nutrient recycling in the microbial loop\(^1\). Predation on bacteria and the accompanying mortality are important mechanisms for bacterial population control and nutrient recycling. While viruses and protists are known agents responsible for bacterial mortality, predatory bacteria may also contribute substantially to bacterial death, although there are only a few known examples from the ocean\(^2,3\). Arguably, the best studied predators of bacteria are *Bdellovibrio* and *Bdellovibrio*-like organisms (BALOs) that prey on a wide range of Gram-negative bacteria\(^4\).

Like Gram-negative bacteria, Gram-positive bacteria are also widespread in the ocean\(^5-7\), comprising up to 14% and 25% of total bacterial cell counts in seawater and sediments, respectively\(^8\). It is unclear, however, whether Gram-positive marine bacteria can also be preyed on by bacteria.

Predatory bacteria actively hunt and kill their prey and consume their macromolecules as nutrients\(^9\). The strategies used by predatory bacteria to kill their prey bacteria vary from case to case; however, they generally include: (i) epibiotic predation, in which predators consume the prey from the outside\(^9\), (ii) endobiotic predation or direct invasion, in which an individual predatory cell secretes hydrolytic enzymes that perforate and modify the prey cell wall, in order to penetrate either into the periplasmic space or into the cytoplasm\(^2\); or (iii) group attack, in which a quorum of predators produce hydrolytic enzymes/secondary metabolites to degrade the prey cells\(^10,11\). The cell wall is thus usually a key target of attack by hydrolytic enzymes for predatory bacteria to kill their prey. Peptidoglycan (PG) is an important structural component of the bacterial cell wall, accounting for up to 90% of the cell wall in Gram-positive bacteria and 10% in Gram-negative bacteria. Structurally, PG is a network formed by linear glycan strands interconnected by peptide stems that are typically composed of amino acid residues L-Ala, D-Glu, L-Lys/diaminopimelate, and D-Ala, and are linked directly or through a short peptide bridge\(^12\). A certain degree of variation has been found either in the peptide stem, in the glycan strands, or in the position or composition of the peptide bridge\(^12\). Three main classes of PG hydrolases have been described; glycosidases cleave the bonds in the glycan strands, amidases cleave the bond between the glycan strand and the peptide chain, and endopeptidases cleave the bonds within the peptide chains\(^13\).

Here, we show that *Pseudoalteromonas* sp. strain CF6-2, a Gram-negative bacterium from a deep-sea sediment, can kill a wide range of Gram-positive bacteria by secreting a large quantity of the M23 metalloprotease pseudoalterin\(^14,15\), and thus degrading the PG in their cell wall. The released nutrients can then be utilized by strain CF6-2 for growth. Bioinformatics analyses suggest that genes encoding pseudoalterin-like proteins are found in other marine bacteria.

**Results**

**Strain CF6-2 kills Gram-positive bacteria via secreted products.** To analyze whether strain CF6-2 has antagonistic interactions with other marine bacteria, the interaction of strain CF6-2 with a variety of Gram-positive and Gram-negative marine bacteria on agar plates was investigated. Strain CF6-2 had no effect on the growth of several representative Gram-negative marine bacteria (Supplementary Fig. 1) but could inhibit the growth of eight Gram-positive bacterial strains of seven different chemotypes of PG, forming clear zones around its colonies on agar plates (Fig. 1a). We further tested the interaction of strain CF6-2 and a representative Gram-positive bacterium *Staphylococcus warneri* strain MCCC1A00423 (hereafter strain MCCC0423, from South China Sea sediments) in artificial seawater. Co-culturing of the two bacteria led to a strong reduction in the cell numbers of strain MCCC0423 over time and an increase in the cell numbers of strain CF6-2, indicating the ability of strain CF6-2 to prey on strain MCCC0423 cells for nutrients (Fig. 1b).

Because clear zones developed around the strain CF6-2 colonies in Fig. 1a, we hypothesized that strain CF6-2 inhibited the growth of Gram-positive bacteria, likely via its secretion products. To support this, an experiment was designed to examine the nature of non-contact interactions between strains CF6-2 and MCCC0423 in artificial seawater using a Transwell® Permeable Supports device (Fig. 1c). In this device, organic molecules can pass through the permeable membrane (pore size, 0.4 μm) but the cells of strain CF6-2 or MCCC0423 cannot (Supplementary Fig. 2). In the non-contact co-culture experiment, the cell numbers of strain CF6-2 in the upper insert increased over time and the cell numbers of strain MCCC0423 in the lower well decreased over time (Fig. 1d). This result suggests firstly that some compound(s) secreted by strain CF6-2 passed through the permeable membrane into the lower well, leading to the death of strain MCCC0423, and secondly that the resultant nutrients of strain MCCC0423 are capable of supporting the growth of strain CF6-2.

**Pseudoalterin is involved in killing Gram-positive bacteria.** The killing activity of the compound(s) secreted by strain CF6-2 in the non-contact co-culture of strains CF6-2 and MCCC0423 was abolished by heating (Supplementary Fig. 3a) or by an addition of 2 mM Zn\(^{2+}\) (Supplementary Fig. 3b). Since strain CF6-2 can secrete a large amount of the thermolabile protease pseudoalterin\(^14\) and because additional Zn\(^{2+}\) can inhibit the activity of pseudoalterin\(^15\), we therefore postulated that strain CF6-2 secreted pseudoalterin in the non-contact co-culture experiment. Indeed, this was supported by protease activity assays and western blot assays. Both the pseudoalterin protein and its activity were detected in the non-contact co-culture, none of which, however, was detectable in the culture of strain CF6-2 or strain MCCC0423 when cultured alone (Fig. 2a), suggesting that pseudoalterin production of strain CF6-2 was induced in the co-culture. In order to demonstrate the involvement of pseudoalterin in killing Gram-positive bacteria, we constructed a mutant of strain CF6-2, Δpse, in which the pseudoalterin gene was knocked out. As expected, this mutant did not secrete pseudoalterin any more (Fig. 2b) and lost the ability to inhibit the growth of strain MCCC0423 cells (Fig. 2c), indicating the involvement of pseudoalterin in killing MCCC0423 cells. Moreover, in vitro experiments showed that recombinant pseudoalterin could kill the cells of strain MCCC0423, leading to a strong dose-dependent reduction of cell numbers over time (Fig. 2d). Recombinant pseudoalterin is also capable of killing 14 other Gram-positive marine bacteria representing 10 different chemotypes of PG (Fig. 2e).

**Pseudoalterin is active against Gram-positive bacterial PG.** Next, we set out to uncover the mechanisms by which pseudoalterin kills the Gram-positive bacteria. Scanning electron microscopy (SEM) images shown in Fig. 2f demonstrated significant alteration of cell surface structure of strain MCCC0423 after pseudoalterin treatment. The dynamics of this interaction was observed using time-lapse atomic force microscope (AFM) and the movie presented in the Supplementary Video file (Supplementary Movie 1) showed gradual alteration and subsequent collapse of cell structure of strain MCCC0423 during pseudoalterin treatment. Indeed, we observed debris of individual cells of strain MCCC0423 (Fig. 2g) using high-resolution transmission electron microscopy (TEM).
Because PG is a major cell wall component of Gram-positive bacteria, we hypothesized that pseudoalterin likely kills these Gram-positive bacteria by degrading PG. Thus, the activity of pseudoalterin against the PG from strain MCCC0423 was investigated. Indeed, pseudoalterin showed significant activity towards the degradation of purified PG from strain MCCC0423 (Fig. 3a, b). Moreover, wild-type cells of strain CF6-2 were capable of degrading PG of strain MCCC0423, whereas the Δpsn mutant strain had lost this ability, and this ability could be restored by complementing the psn gene but could not by complementing a mutant psn gene that encodes an inactive pseudoalterin protein (Fig. 3c). Our data therefore suggest that pseudoalterin secreted by strain CF6-2 is responsible for killing Gram-positive bacteria, likely through the degradation of PG in the cell wall although an indirect cause of cell death due to pseudoalterin treatment cannot be completely ruled out.

**Pseudoalterin production is induced by specific fragments of PG.** When PG of strain MCCC0423 was used as the sole carbon and nitrogen source for strain CF6-2, pseudoalterin was expressed and secreted during the growth of strain CF6-2 cells (Fig. 4a), indicating that pseudoalterin was inducible by PG of strain MCCC0423. In the non-contact co-culture experiment, pseudoalterin production was also induced (Fig. 2a). Because PG of strain MCCC0423 is unlikely to be able to pass through the membrane, owing to its high molecular weight and low solubility, we reasoned that a soluble fragment derived from the degradation of PG of strain MCCC0423 would serve as an inducing signal molecule for pseudoalterin synthesis in strain CF6-2. To identify this signal molecule, various amino acids and peptides derived from the peptide chain of Gram-positive bacterial PG were tested for their ability for pseudoalterin induction. Both free glycine and its oligopeptides, as well as several glycine-enriching peptides including AGGGGG, AGGGG, and AGGG, could induce the transcription of psn, whereas other amino acids or peptides had little inducing effect (Fig. 4b, c). Of all the molecules tested, glycine had the strongest inducing effect (Fig. 4b, c). Transcription of psn in strain CF6-2 could be significantly induced by glycine at concentrations of 250 μM–20 mM (Fig. 4d). Moreover,
2 mM glycine in the medium induced the secretion of active pseudoalterin and the further addition of glycine after 6 and 12 h could significantly increase the production of active pseudoalterin (Fig. 4e). This indicates that the induction of pseudoalterin synthesis in strain CF6-2 needed a constant stimulation by extracellular glycine in appropriate concentrations. Glycine exists in the peptide bridges of PG of a variety of Gram-positive bacteria but absent from PG from Gram-negative bacteria and certain other Gram-positive bacteria. Pseudoalterin synthesis is thus specifically induced by glycine and glycine-enriching oligopeptides derived from Gram-positive bacterial PG.

**Pseudoalterin is secreted via a type II secretion system.** The type II secretion system (T2SS) of strain CF6-2 is encoded by a set of 12 gsp (general secretion pathway) genes, including a large
Mechanism of PG degradation by pseudoalterin. Next, we set out to characterize the mechanism by which pseudoalterin degrades PG of Gram-positive bacteria using purified PG of strain MCCCC0423. During the purification process of pseudoalterin, we noticed that pseudoalterin could bind to Sephadex gel matrix. This suggests that pseudoalterin may have affinity to carbohydrate chains because Sephadex gel is primarily composed of carbohydrate polymers. To test this hypothesis, the binding ability of pseudoalterin to three representative insoluble natural polysaccharides (chitosan, cellulose, and chitin) and PG was tested. Pseudoalterin could bind to all three natural polymers tested, with the highest binding ability to PG (Fig. 5a), suggesting that pseudoalterin may bind on the glycan strand of PG.

Because pseudoalterin is a member of the M23 metalloprotease family, we hypothesized that it degrades PG by attacking the peptide chain rather than the glycan strand. To test this hypothesis, the products released from the degradation of PG derived from strain MCCCC0423 by pseudoalterin hydrolysis were analyzed. Chiral derivatization-HPLC analysis showed that amino acids derived from strain MCCC0423 by pseudoalterin hydrolysis were released from the degradation of PG because Sephadex gel is primarily composed of carbohydrate polymers. To test this hypothesis, the binding ability of pseudoalterin to three representative insoluble natural polysaccharides (chitosan, cellulose, and chitin) and PG was tested. Pseudoalterin could bind to all three natural polymers tested, with the highest binding ability to PG (Fig. 5a), suggesting that pseudoalterin may bind on the glycan strand of PG.

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Fig. 4 Induction and secretion of pseudoalterin in strain CF6-2. 

a. Induction of psn expression in strain CF6-2 by PG of strain MCCC0423. Control, strain CF6-2 was cultured in the medium without PG.

b. Inducing effects of peptides derived from PG on psn expression. O, L-Orn; iq, D-iso-Gln.

c. Inducing effects of amino acids from PG on psn expression.

d. Inducing effects of different concentrations of glycine on psn expression.

The extracellular pseudoalterin production of strain CF6-2 induced by 2 mM glycine added at 0, 6, and/or 12 h. Control, strain CF6-2 was cultured in the medium without glycine. The pseudoalterin production was represented by the extracellular elastinolytic activity.

f. Non-contact co-culture of strain ΔgspE and strain MCCC0423. The upper panel shows the growth of the strains. The lower panel shows pseudoalterin production detected by western blot. The graphs show data from triplicate experiments (mean ± SD). Source data are provided as a Source Data file.
Fig. 5 PG binding and degradation by pseudoalterin. a SDS-PAGE analysis of the binding ability of pseudoalterin on three insoluble polysaccharides and PG. The experiments were performed with a fixed amount of pseudoalterin (0.2 mg ml\(^{-1}\)) and increasing concentrations of substrates. “S” and “P” refer to the amount of protein present in the supernatant and the pellet after centrifugation, respectively. BSA in place of pseudoalterin was used as a negative control. Each graph is a representative of at least three repeats. b Chiral derivatization-HPLC analysis of the D/L-amino acids and the peptides released from PG of strain MCCC0423 by pseudoalterin hydrolysis with an Automatic Amino Acid Analyzer (Hitachi L8900, Japan). Data are from triplicate experiments (mean ± SD). c Analysis of the amino acids released from PG of strain MCCC0423 by pseudoalterin hydrolysis with an Automatic Amino Acid Analyzer (Hitachi L8900, Japan). Data are from triplicate experiments (mean ± SD). d The cleavage sites of pseudoalterin on strain MCCC0423 PG. Arrows indicate the cleavage sites determined according to the products released by pseudoalterin from two synthetic PG peptides, AaKAGGGGGA and Lactic acid-AekAGG. Red and black arrows indicate the preferred and less preferred bonds by pseudoalterin, respectively, which are deduced based on the relative production of the released products in MS spectra (Supplementary Figs. 4 and 5). M, NAM; G, NAG. Source data are provided as a Source Data file.

that experiment. The hydrolysis of the peptide chain of PG by pseudoalterin will lead to the collapse of the cell wall and the death of the Gram-positive bacteria. The substances released from the predated cells, together with the free amino acids and oligopeptides released from PG, provide nutrients for strain CF6-2 to thrive, as observed in the co-culture of strains CF6-2 and MCCC0423 (Fig. 1b, d).

To better understand the molecular interaction between pseudoalterin and PG, the crystal structure of pseudoalterin was determined to 1.9 Å (Table 1). The sequence identity of pseudoalterin to the M23 peptidases LasA and lysostaphin is 54% and 15%, respectively. Like LasA\(^\text{18}\) and lysostaphin\(^\text{19}\), pseudoalterin contains a catalytic domain and a C-terminal domain (Fig. 6a). The catalytic domain of pseudoalterin, comprising five β-strands and four connecting loops (loop 1–loop 4), is quite similar to those of LasA and lysostaphin, with a root mean square deviation (RMSD) of 0.64 and 4.0 Å, respectively (Fig. 6a, b). Similar to that of LasA, the C-terminal domain of pseudoalterin consists of four antiparallel β-strands, and is different from that of lysostaphin that consists of eight β-strands\(^\text{19}\) (Fig. 6b). While the function of the C-terminal domain of lysostaphin interacts directly with the peptide bridge present in PG\(^\text{20}\).

As shown in Fig. 5a, pseudoalterin may bind on the glycan strand of PG. Since the catalytic domain is responsible for the hydrolysis of the PG peptide chain, we reasoned that the C-terminal domain is responsible for binding to the PG glycan strands. To support this hypothesis, attempts were made to model pseudoalterin with a PG fragment containing both the glycan strand and the peptide chain. Unfortunately, we failed to obtain a model with good quality. Alternatively, we modeled pseudoalterin with the glycan strand and with the peptide chain, respectively, by molecular docking simulations (MDS). In the model of the pseudoalterin: (NAG–NAM)\(_2\) binary complex (NAG, N-acetylmuramic acid; NAM, N-acetylmuramic acid), the glycan strand (NAG–NAM)\(_2\) interacts with the C-terminal domain (Fig. 6c). This was supported by an analysis of the binding ability of the C-terminal domain-deleted mutant (AC, N134-R173) to chitin and PG. The deletion mutant lost almost all chitin-binding ability and a large portion of the PG-binding ability (Fig. 6d). Analysis of the model indicated that (NAG–NAM)\(_2\) interacts with the C-terminal domain mainly by forming H-bonds with the residues H133, N134, Y136, R141, D143, N154, Y136, E159, R163, and R164 (Fig. 6c). Site-directed mutations on these residues showed that the PG-binding ability of the eight mutants, H133A, N134A, R141A, N154A, Y157A, E159A, R163A, and R164A, was reduced (Fig. 6e), but the circular dichroism spectra of these mutants had
no detectable differences from that of pseudoalterin (Supplementary Fig. 6). These data indicate that these residues in the C-terminal domain are involved in the binding of pseudoalterin to the PG glycan strand.

In the modeled pseudoalterin:AeKaA/GGGGGA binary complexes, AeKaA/GGGGGA is bound in the catalytic cavity of pseudoalterin with a similar mode to the tetraglycine phosphate in LytM21 (Fig. 6f, g), a member of the M23 proteases that shows 19.1% sequence identity to pseudoalterin. H21, D34, and H120 chelating the zinc ion in pseudoalterin are equivalent to H210, D214, and H293 that chelate the zinc ion in LytM21 (Fig. 6f, g). Y204, H260, H291, N303 in LytM are involved in substrate recognition and catalysis21. The equivalents of these residues in pseudoalterin are Y149, H79, H118, and N23 (Fig. 6f, g). Among them, either H79 or H118 can act as a general base/acid in PG degradation as shown in Fig. 4b, c. In addition, the complexes, AeKaA/GGGGGA interacts with the residues N23, Q113, and Y141, mainly through H-bond formation (Fig. 6f, g), and mutation of these residues (N23A, Q113A, and Y149F) caused significant reduction in both the activity and the binding ability of the enzyme to PG (Fig. 6h). This suggests that these residues are involved in binding the peptide substrate. The circular dichroism spectra of all of the aforementioned mutants were indistinguishable from that of the wild-type pseudoalterin (Supplementary Fig. 8), indicating that changes in the PG binding and hydrolysis of these mutants are caused by amino acid replacement rather than structural alteration of the enzyme.

To probe the structural basis for the specificity of pseudoalterin on PG (Fig. 5c), we compared the catalytic cavity of pseudoalterin with those of LasA (PDB code: 3IT7), LytM (PDB code: 4YZB), and lysostaphin (PDB code: 4QP5) (Supplementary Fig. 9a). The catalytic cavity of pseudoalterin is similar to those of LasA and LytM, and more open than that of lysostaphin (Supplementary Fig. 9a, b), which suggests that, like these homologs, pseudoalterin also prefers to bind Gly/Ala. This is supported by our biochemical analysis presented in Fig. 5d. In addition, compared to LasA, the side chain of Q113 of loop 3 of pseudoalterin makes the S1 pocket much deeper (Supplementary Fig. 9c, d), and may be capable of orienting the long side chain of amino acids. Thus, in addition to Gly and Ala, the deeper and more open catalytic cavity of pseudoalterin may accommodate residues with a longer side chain such as Lys and Glu in PG. This makes it possible for pseudoalterin to repeatedly digest the heterogeneous PG from strain MCCC04243, generating a variety of products from PG degradation as shown in Fig. 4b, c.

Taken together, our biochemical results and structural analyses suggest that, compared to other M23 proteases, pseudoalterin has a specific conformation to bind and degrade Gram-positive bacterial PG efficiently.

Strain CF6-2 can utilize D-Ala and D-Glu for growth. As shown in Fig. 4a, wild-type strain CF6-2 can grow with PG as the sole carbon and nitrogen source, suggesting that it may be able to utilize the amino acids released from PG degradation for growth. The released amino acids include both L- and D-amino acids (Fig. 5b, c). So far, only a few bacteria are known to be able to utilize D-amino acids for growth22 and the recycling of D-amino acids in the marine microbial loop is not well understood. Here, the ability of strain CF6-2 to utilize the two D-amino acids, D-Ala and D-Glu, released from PG degradation was investigated. The results showed that strain CF6-2 could indeed grow on D-Ala and D-Glu as the sole nitrogen source (Fig. 7a, b). When both D- and L-Ala/Glu were present in the medium, L-amino acids were preferentially used, particularly for L-Ala (Fig. 7c, d).

Pseudoalterinin-like proteases are found in other marine bacteria. In order to better understand the ecological significance of pseudoalterin-mediated killing of Gram-positive bacteria in the environment, the distribution of pseudoalterin-like protease sequences in public databases, such as the non-redundant protein database in NCBI, was investigated. Pseudoalterin-like sequences were found in 160 marine bacterial strains which were isolated from a variety of environmental samples, including seawater (70), marine sediments (72), and hydrothermal vents (18) (Supplementary Data 1). Most of these strains are Gram-negative bacteria (134/160), particularly from the genera Pseudoalteromonas (30), Vibrio (17), Shewanella (13), and Aeromonas (5) (Supplementary Fig. 10a). The predominance of pseudoalterin-like proteases in Pseudoalteromonas was also confirmed by probing its distribution in the Tara Oceans Microbiome database using the Ocean Gene Atlas tool23. Around 86% of the pseudoalterin-like proteases from the Tara Oceans metagenomes was classified as Pseudoalteromonas (Supplementary Fig. 10b). We also found expression of genes encoding putative pseudoalterin-like proteases in published metatranscriptomics datasets from coastal and marine sediments, using the Integrated Microbial Genomes and Microbiomes (IMG/M)24 database. Although only partial sequences were retrieved from these metatranscriptomes due to the length of short-reads, multiple sequence alignment confirmed that the key residues involved in the co-ordination of metals and the substrate are highly conserved (Supplementary Fig. 11). Overall, our analyses suggest that pseudoalterin-like proteases are expressed by bacteria in the ocean.

Discussion

In addition to viral lysis and protist grazing, bacterial predation also plays an important role in marine bacterial
Fig. 6 Structural basis of PG binding and degradation by pseudoalterin. **a** The overall structure of pseudoalterin. The C-terminal domain is marked in a black dotted box. **b** Structural superposition of pseudoalterin with LasA and lysostaphin. On the left is the front view and on the right is the side view. Pseudoalterin structure is colored in green, LasA in pink and lysostaphin in gray. **c** Model of the pseudoalterin:(NAG–NAM)$_2$ binary complex constructed by MSD. The interface of pseudoalterin with (NAG–NAM)$_2$ in the black dotted box is amplified. **d** SDS-PAGE analysis of the binding ability of GST-pseudoalterin and its C-terminal domain-deleted mutant (ΔC, N134–R173) to PG and chitin. **e** Model of the pseudoalterin:AeKaA binary complex constructed by MSD. The interface of pseudoalterin with AeKaA in the black dotted box is amplified. **f** Model of the pseudoalterin:GGGGGA binary complex constructed by MSD. The interface of pseudoalterin with GGGGGA in the black dotted box is amplified. **g** Activities of pseudoalterin and its mutants to PG. Values are normalized against the activity of WT pseudoalterin. Data are from triplicate experiments (mean ± SD). **h** SDS-PAGE analysis of the binding ability of pseudoalterin and its mutants to PG. In **d**, **e**, and **i**, the experiments were performed with a fixed amount of protein (0.1 mg ml$^{-1}$ in **d** and **e** and 0.05 mg ml$^{-1}$ in **i**) and substrates (2.0 mg). “C” represents the total amount of protein, “S” and “P” refer to the amount of protein present in the supernatant and the pellet after centrifugation, respectively. Each graph is a representative of at least three repeats. Source data are provided as a Source Data file.
activity was found against Gram-positive bacteria. The Gram-positive bacterium
strain CF6-2 cultured in the medium containing 2 mM D-Glu. Mycobacterium
genus (Fig. 2e). The Gram-positive bacteria of the genus (Fig. 2e).

Mortality of a variety of Gram-positive bacteria 11,23,26. Halobacteriovorax species, belonging to BALOs, have been reported to be marine bacterial predators. They prey on Gram-negative bacteria by entering and residing within the periplasmic space of a host bacterium where they utilize cytoplasmic nutrients of the host to support growth and replication 27,28. Other reported marine predatory bacteria include Pseudoalteromonas piscicida, which preys on strains of Vibrio and Shewanella 29, and Saprospiraceae strains, which prey on strains of Photobacterium and Vibrio, as well as diatoms and cyanobacteria 30.

In this study, we describe a new predator–prey interaction between Gram-negative and Gram-positive bacteria in the ocean. We show that the Gram-negative bacterium strain CF6-2 isolated from deep-sea sediment can kill a variety of Gram-positive bacteria with different PG chemotypes. Unlike Halobacteriovorax species that are intracellular predators 27,28, strain CF6-2 is an extracellular predator, which kills Gram-positive bacteria by secreting an extracellular protease, pseudoalterin, that degrades the PG in the prey’s cell wall (Fig. 8). Interestingly, pseudoalterin has no killing activity against Gram-negative bacteria or against Gram-positive bacteria of the Mycobacterium genus (Fig. 2e).

Furthermore, pseudoalterin had killing activity against the Gram-positive bacterium Pontibacillus sp. MCCCIA04056, whereas no activity was found against Gram-positive bacteria Mycobacterium spp., although they all have the same PG chemotype 12,16,31,32. The PG of the aforementioned Mycobacterium strains is covered by a large number of unusual lipids in the cell wall 31 and that of Gram-negative bacteria is sandwiched between two membranes. Thus, inaccessibility of pseudoalterin to the bacterial PG may be the main reason why pseudoalterin cannot kill these bacteria. A bioinformatics investigation showed that other Gram-negative bacteria possess genes encoding putative pseudoalterin-like proteases. Further metagenome and metatranscriptome analyses showed that similar genes are found and expressed in marine environments.

There are several lines of evidence to support the conclusion that strain CF6-2 preys on some Gram-positive marine bacteria for nutrients using the secreted pseudoalterin as a weapon. (i) Pseudoalterin synthesis is only induced by characteristic fragments from Gram-positive bacterial PG, e.g. glycine and glycine-rich oligopeptides. This can explain why strain CF6-2 cannot kill Gram-negative bacteria or some Gram-positive bacteria on agar plates, because the PG of these bacteria contains no glycine and thus is unable to induce pseudoalterin synthesis. Although it has been speculated that specific fragments from PG are signaling molecules for PG hydrolase induction in predatory bacteria 13, neither the identity of the fragments nor the bacterial receptors for these fragments have been identified. (ii) Pseudoalterin can bind to the glycan strand of Gram-positive bacterial PG with its C-terminal domain and degrade the PG peptide with its catalytic domain. Unlike other reported PG hydrolases, which usually have one or limited cleavage sites on PG 18,19,33 pseudoalterin seems to be able to cleave all the peptide bonds in the Gram-positive bacterial PG peptide chain, although it shows preference for bonds with Gly/Ala at P1′/P1′ positions. In addition, although pseudoalterin is only induced by glycine and glycine-enriching oligopeptides, it can also degrade bacterial PG without glycine.

Glycine resulting from PG degradation is a strong inducer for pseudoalterin expression in strain CF6-2 (Fig. 4). Indeed, qRT-PCR results showed that pseudoalterin is induced above background at 100 μM glycine in the medium. However, glycine concentration rarely reaches μM in surface seawater, hence the significance of pseudoalterin-mediated predation is uncertain in marine water columns. Yet, our metagenome analyses have confirmed that bacteria in seawater do have the genetic potential and Pseudoalteromonas represents the major clade encoding...
pseudoalterin-like proteins. In coastal and marine sediments, however, several reports have shown that glycine concentration in sediment pore water as well as sediment traps can reach hundreds of μM34,35. Coincidently, Pseudoalteromonas is frequently detected as an important member of the microbial community in marine sediments36,37. It is therefore likely that pseudoalterin-mediated predation may occur in marine sediments where the Gly concentration for inducing pseudoalterin expression can be achieved locally. Indeed, this view is further supported by the analyses of metatranscriptomics datasets, showing expression of genes encoding pseudoalterin-like proteases in sediment samples. Taken together, the data presented in this study suggest that Pseudoalteromonas can prey on Gram-positive bacteria by secreting pseudoalterin. Whether this strategy is adopted by Pseudoalteromonas sp. CF6-2 and Bacillus sp. CF12-9 were originally isolated from a marine sediment sample from the South China Sea40. Other bacterial strains used in this study (Supplementary Table 1) were obtained from MCCC (Marine Culture Collection of China). All strains were cultivated at 20 °C on a plate containing marine Luria-Bertani (LB) medium composed of (w/v) 1% peptone, 0.5% yeast extract, 1.5% agar (Sigma-Aldrich, USA), and Transwell® Permeable Supports (Diameter, 24 mm; pore size, 0.4 μm) from Corning (USA). PG was extracted from strain MCCC0423 cells using the method of Takano et al.38.

**Methods**

**Bacterial strains, growth conditions, and materials.** Pseudoalteromonas sp. CF6-2 and Bacillus sp. CF12-9 were originally isolated from a marine sediment sample from the South China Sea40. Other bacterial strains used in this study (Supplementary Table 1) were obtained from MCCC (Marine Culture Collection of China). All strains were cultivated at 20 °C on a plate containing marine Luria-Bertani (LB) medium composed of (w/v) 1% peptone, 0.5% yeast extract, 1.5% agar and 3% sea salt (pH 8.0), and then stored at 4 °C for short-term use, or mixed with 15% glycerin (v/v) and stored at −80 °C for long-term use. Sea salt was purchased from Sigma-Aldrich (USA), and Transwell® Permeable Supports (Diameter, 24 mm; pore size, 0.4 μm) from Corning (USA). PG was extracted from strain MCCC0423 cells using the method of Takano et al.38.

**Construction of mutants.** The knockout mutants of strain CF6-2, Δpsn and ΔgspE, as well as their complementary strains, Δpsn ΔpEVpsn and ΔgspE ΔpEVgspE, were constructed as described previously39–41. The plasmids, bacterial strains, and primers used in this study are listed in Supplementary Tables 2 and 3 and Supplementary Data 2. Site-directed mutagenesis in pseudoalterin was introduced by PCR and the mutations were verified by DNA sequencing. For recombinant protein expression, the plasmid pGEX-4T-1 containing the appropriate insert was expressed in Escherichia coli strain BL21 (DE3) at 15 °C, 100 r.p.m. for 10 h, with 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) as an inducer. The proteins were purified with glutathione-Sepharose 4B matrix (GE Healthcare, Sweden). For the expression of active pseudoalterin and its mutants, the plasmid pEV containing the appropriate insert was expressed in strain Δpsn, the pin-deleted mutant of strain CF6-2, and the expressed proteins were purified as described previously42. The binding ability of the wild-type pseudoalterin and its mutants to Gram-positive bacterial PG and other polysaccharides was analyzed using the method of Valenza et al.42. The circular dichroism spectra of pseudoalterin and its mutants were monitored between 200 and 240 nm at 25 °C on a Jasco J-810 spectropolarimeter (Japan).

**Predation activity of strain CF6-2 and its mutants on bacteria.** Strain CF6-2 (or its mutants) and the target bacterial strains were pre-cultured in liquid marine LB medium overnight. Cells were collected by centrifugation and re-suspended in artificial seawater. The OD600 of the cell suspensions was adjusted with artificial seawater according to the needs of the following experiments. To detect the killing activities of strain CF6-2 and its mutants against other bacteria on a plate, Burkholder agar diffusion assays were performed as described previously43. Briefly, the target bacterium layer was prepared by mixing 1% (v/v) bacterial suspension (OD600 = 1.0) with 0.6% (w/v) molten ZoBell agar in artificial seawater. Then, 2 μl of strain CF6-2 (or its mutants) (OD600 = 0.1) was spotted on to the agar surface. The plates were incubated face-up at 20 °C for 3 days. To test the predation activity of strain CF6-2 on strain MCCC0423 in liquid culture, 1% (v/v) cell suspensions of strain CF6-2 and strain MCCC0423 (OD600 = 1.0) were mixed in artificial seawater and were cultivated at 20 °C with shaking. Then, the colony formation units (CFU) for each of the bacteria were calculated at regular intervals for 20 h. Individual cultures of strain CF6-2 and strain MCCC0423 were used as controls. Experiments were performed in three biological replicates.

The non-contact interaction between strain CF6-2 (or the ΔgspE mutant) and strain MCCC0423 was performed in a Transwell® permeable support device. Strain CF6-2 or the mutant ΔgspE cells (OD600 = 0.1, 1.5 ml) were added into the upper insert. Strain MCCC0423 cells (OD600 = 6.0, 2.6 ml) were added into the lower well. The device was then incubated at 20 °C, and the OD600 of the cultures in both the lower well and the upper insert was measured every 20 h. In addition, the supernatant from the non-contact co-culture was boiled for 10 min to determine the effect of heat-treatment on the lysis activity of the supernatant to the cells of strain MCCC0423. Furthermore, 2 mM of Zn2+ was added in the Transwell experiment to determine its impact on the non-contact interaction between strains CF6-2 and MCCC0423. All experiments were performed in three biological replicates.

**Western blot analysis.** After the non-contact co-culture of strain CF6-2/ΔgspE and strain MCCC0423 were incubated at 20 °C for 60 h, proteins in 1 ml of the
culture supernatant in the lower well was precipitated by adding 100 μl 1.25 M trichloroacetic acid. The precipitated proteins were dissolved in 10 μl of 50 mM Tris-HCl (pH 9.0), 2% SDS, 0.1 M EDTA, 10% glycerol, and 0.1% SDS. After separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis in 15% (w/v) polyacrylamide gels, proteins were visualized by Coomassie blue staining or silver staining.

**Pseudoaerolactus activity assays.** The activity assays of pseudoaerolactus towards elastin and PG were carried out according to Zhao et al.15 and Iversen et al.45, respectively. A 50 μl reaction mixture (10 μM elastin or α1-PI, 10 mM Bis-Tris (pH 7.1), and 0.6 M magnesium formate dehydrate for 20 s and then frozen in liquid nitrogen before X-ray diffraction. X-ray diffraction data were collected from single crystals using a Rigaku BL17U beamline at the Shanghai Synchrotron Radiation Facility (SSRF, China). The collection wavelength is 0.9791 Å. The crystal structure of pseudoaerolactus was solved using the structure of LasA (PDB code 3IT5) as a model. Automated model building was performed with Auto Build in PHENIX. Several rounds of refinement and manual building were then performed with CNS, respectively. Data refinement statistics are given in Table 1. Ramachandran Plot analysis suggests that 97.04% of the residues are in the favored region and 2.96% of the residues are in the allowed region.

**Hydrolytic production and cleavage sites of pseudoaerolactus towards PG**. PG (OD600 = 1.0) was digested with 20 μg/ml pseudoaerolactus in 20 mM Tris-HCl (pH 9.0) at 25 °C for 24 h. The released amino acids in the hydrolysate were analyzed by an Automatic Amino Acid Analyzer (Hitachi L8900, Japan). The D/L-amino acids and peptides in the hydrolysate were also analyzed by HPLC. The D/L-amino acids in the hydrolysate were derivatized with FDA (N-(12,14-dinitro-5-fluorophenyl)-l-alanine, Marfey’s reagent; Sigma) as described by Hess et al.46. Derivatized amino acids were separated with a linear gradient of formic acid (30 mL, pH 2/6) on a l-cysteine on an HPLC with an Inertsil ODS-3 column (250 × 4.6 mm; 5 μm particle size) (Shimadzu, Japan) at a flow rate of 1.5 ml min⁻¹ and detected at 340 nm. Glycine, ε-alanine, l-alanine, l-lysine, γ-glutamate, AG, AGG, AGGG, GGG, GGGG, AGGGG, A, AeK, AeKa, AeKaA, eK, eKa, eKaA, Ka, KaA, and aA concentrations at 0.5–4.0 mg ml⁻¹ served as standards.

Peptides AeKAGGGGG and lactic acid AeKAGG were synthesized by ChinaPeptides Co., Ltd (China). Each of these peptides (50 μg) was hydrolyzed in 100 μl 20% (v/v) HCl at 121 °C for 24 h. The hydrolysates were subjected to LC-MS analysis to determine the molecular masses of the released peptides. The sequences of the released peptides were determined by using ExPasy tools.

**Expression and production of pseudoaerolactus strain CF6-2**. The expression level of pseudoaerolactus in strain CF6-2 was determined by real-time quantitative PCR (qPCR). Strain CF6-2 was incubated in artificial seawater containing 0.8% (w/v) PG of strain MCCC4032, 1% (w/v) mannitol and 2 mM peptides, 1% (w/v) mannitol and 2 mM amino acid, or 1% (w/v) mannitol and different concentrations of glycine. Artificial seawater containing 1% (w/v) mannitol and 0.5% (w/v) casamino acid served as the negative control. All of these media contained 0.5 mM CaCl₂ and 0.5 mM Na₂HPO₄. Total RNA was extracted with the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using the PrimeScript™ RT reagent kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Japan). The qPCR reaction was performed on the LightCycler® 480 (Roche, Switzerland). The relative expression level was indicated as fold change which was calculated using the LightCycler® 480 software. Each sample for qPCR was performed in triplicate and a mean value and standard deviation were calculated. Primers used for qPCR are listed in Supplementary Data 2. Pseudoaerolactus production of strain CF6-2 induced by glycine was determined by measuring the extracellular elastolinolytic activity as described previously23. Strain CF6-2 was incubated at 20 °C in artificial seawater containing 0.8% (w/v) PG, 0.5% yeast extract, 0.5% casein, 0.5 mM CaCl₂, and 0.5 mM Na₂HPO₄. As an inducer, 2 mM glycine was added into the medium at 0, 6, and 12 h. The extracellular elastolinolytic activity was measured every 12 h. Strain CF6-2 was incubated in the medium without glycine served as the negative control.

**Utilization of α-Ala and D-Glu by strain CF6-2**. Strain CF6-2 was incubated at 20 °C in the media with amino acids as the sole nitrogen source. The media containing 0.8% (w/v) PG, 0.5% yeast extract, 0.5% casein, 0.2 M phosphate buffer (pH 8.0), and 2 mM D-amino acid (or a mixture of 2 mM D-amino acid and 2 mM l-amino acid). The OD600 of the cultures was measured at

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different time points to detect the growth of strain CF6-2. The concentrations of D- or L-amino acid in the cultures were determined by using circular dichroism (Jasco J-810 Spectropolarimeter, Japan) or by using an Aromatic Amino Acid Analyzer (Hitachi L8900, Japan).

Bioinformatics. The NCBI non-redundant protein database was used to search for the distribution of pseudoalterin-like proteases in genome-sequenced bacterial isolates (Supplementary Data 1). Pseudoalterin amino acid sequence of strain CF6-2 (accession number ADU33224) was used as the query with an e-value cut-off of e^{-30}. The Tara Oceana Microbiome (Reference Gene Catalog V1, prokaryotes) was probed for the distribution of pseudoalterin-like proteases in marine metagenomes using ADU33224 as the query sequence with an e-value cut-off of e^{-20}, which resulted in 165 hits. Phylogenetic distribution of these homologs retrieved from the Tara Oceana metagenome was analyzed using the Krona toolset60 and the data are presented in Supplementary Fig. 10. The IMG/M metatranscriptome database was used to search for the transcription of pseudoalterin-like proteases in natural coastal marine and sediment samples (Supplementary Data 3). The Pseudoalterin sequence of strain CF6-2 (ADU33224) was used as the query with an e-value cut-off of ≤ 20.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The structure of pseudoalterin has been deposited in the Protein Data Bank (PDB) under the accession code 6K4K.

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Author contributions

B.-L.T. and J.Y. performed the majority of the experiments and data interpretation. X.-L.C. directed the study. P.W. and H.L.Z. purified and crystallized pseudoalterin, P.W. and C.-Y.L. solved the structure. H.-N.S. performed AFM observation. J.Y. and I.L. performed bioinformatic analyses. H.D. helped in modeling. Y.Y., S.Z., L.W., and X.-Y.Z. helped in experiments. X.-L.C., B.-L.T., J.Y., and P.W. wrote the manuscript. M.W., A.M., and Y.C. did critical revision of the manuscript for important intellectual content. Y.-Z.Z. designed the study.

Competing interests

The authors declare no competing interests.

Additional information

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