Bacterial quorum sensing quenching activity of Lysobacter leucyl aminopeptidase acts by interacting with autoinducer synthase

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Abstract

Acyl-homoserine lactone (AHL) is the most studied autoinducer in gram-negative bacteria controlling infections of various pathogens. Quenching of AHL signaling by inhibiting AHL synthesis or AHL-receptor binding via small molecular chemicals or enzymatically degrading AHL is commonly used to block bacterial infections. Here, we describe a new quorum-quenching strategy that directly “acquires” bacterial genes/proteins through a defined platform. We artificially expressed a typical AHL synthase gene pcoI from the biocontrol Pseudomonas fluorescens 2P24 in the antifungal bacterium Lysobacter enzymogenes OH11 lacking AHL production. This step led to the discovery of multiple PcoI interacting protein candidates from L. enzymogenes. The individual expression of these candidate genes in 2P24 led to the identification of Le0959, which encodes leucyl aminopeptidase, an effective protein that inhibits AHL synthesis in 2P24. Therefore, we define Le0959 as LqqP (Lysobacter quorum-quenching protein). The expression of pcoI in E. coli could produce AHL, and the introduction of lqqP into E. coli expressing pcoI could prevent the production of AHL. LqqP directly binds to PcoI, and this protein–protein binding reduced the abundance of free PcoI (capable of AHL synthesis) in vivo, thereby blocking PcoI-dependent AHL production. Overall, this study highlights the discovery of LqqP in quenching AHL quorum sensing by binding to AHL synthase via developing a previously-uncharacterized screening technique for bacterial quorum quenching.

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1. Introduction

Quorum sensing (QS) is the behavior of communication within and between bacterial species [1–3]. QS in bacteria produces small signaling molecules, called autoinducers, for cells to communicate with each other. Autoinducers can be released into extracellular environment, with its concentrations increased with cell density. When extracellular autoinducers were accumulated to a certain threshold concentration, they shall activate a number of gene expressions, enabling single-celled bacteria to display group behaviors similar to higher organisms [4,5]. For example, Vibrio fischeri utilizes quorum sensing to regulate the luminescence activity to achieve mutual symbiosis with squid [6]; Pseudomonas aeruginosa communicates through the quorum sensing to change the state of free-living plankton cells to form community-like biofilms and enable them to become resistant to applied antibiotics [7]. Gram-positive and gram-negative bacteria utilize QS communication to regulate a wide variety of physiological activities, such as virulence, symbiosis, conjugation, motility, competence, antibiotic production, sporogenesis, and biofilm formation [2]. Generally, gram-negative bacteria employ acylated homoserine lactone (AHL, Al-1) for communication, while gram-positive bacteria employ processed oligopeptides as autoinducer peptides (AIP). For instance, Staphylococcus aureus produces 8-amino acid cyclic...
short peptides as a QS signal to regulate the expression of virulence genes [14,8,9]. In particular, AHL is an inducer in gram-negative bacteria that has been extensively studied in the past 35 years; they differ in the length and substitution of acyl side chains that confer them the signal specificity [2,10,11]. AHL is synthesized by a family of AHL synthases called Luxl or acyl-carrier protein (acyl-ACP) and S-adenosylmethionine (SAM) mediated by MTAN (S-adenosyl homocysteine nucleosidase) and SRH (S-ribosylhomocysteine) [12]. This catalytic reaction is believed to proceed through a two-step mechanism; the intermediate acyl-SAM is formed from the acyl group transfer of acyl-ACP to the amino group of SAM, followed by the ligation of the methionine moiety and the release of methylthioadenosine [12,13]. The lowest threshold concentration for detection of AHL and its binding to LuxR-type transcription factors result in the regulation of a diverse array of gene expression, cellular processes, and physiological activities that are regulated by AHL [1]. It has been reported that many plant and animal pathogenic bacteria, i.e. Pseudomonas aeruginosa, can use AHL to control the production of virulence factors and promote host infections [14]. These key observations have motivated microbiologists to use various approaches to block bacterial infections by inhibiting AHL-dependent QS signaling, thereby developing effective strategies for crop protection and drug treatment.

Quorum quenching (QQ) refers to all processes involved in the interference of bacterial QS system [15,16]. Scientists have developed several efficient screening methods for discovering QS inhibitors for the treatment of bacterial infections [17–19], and their working mechanisms mainly include (i) inhibition of AHL synthase activity by small-molecule chemicals, such as triclosan (a broad-spectrum antimicrobial agent). Triclosan acts as an enzyme inhibitor of the P. aeruginosa acyl-ACP reductase; it can effectively impair the synthesis of acyl-AHL, resulting in AHL synthase being unable to synthesize N-butyryl-L-homoserine lactone (C4-HSL) in P. aeruginosa [20]; (ii) inhibition of ligand (AHL) receptor interaction to stop AHL signal reception and its transmission. A representative example is the halogenated furanones from the Australian Marine red algae Delisea pulchra, which possess a common structure of a hyperserine lactone ring like AHLs. This structural similarity enables this substance to compete with AHLs for binding to receptors, thereby blocking AHL-induced functional outputs; (iii) disruption of AHL signal molecule by AHL-degrading enzymes. AiiA from Baciulus sp. 240B1, AttM from Agrobacterium tumefaciens CS8 (A6), Mm.l from Muricauoa olearia, and Aii fromRalstonia sp. XJ12B are well-characterized representatives [21–24]. Moreover, inhibition of protective biofilm formation and efflux pump or blocking autoinducing peptides (AIP)-mediated QS has also been addressed as effective QQ strategies [25–27]. In this study, we developed a platform to “fish” genes/proteins from bacteria that do not produce AHL to directly bind to and target AHL synthase, thereby generating a new QQ strategy.

The gammaproteobacterial genus Lysobacter represents a group of antimicrobial biocontrol agents. Bacteria from this genus are common environmental inhabitants; they prey on other microorganisms by using secreted antimicrobial compounds and growth-inhibiting enzymes [28,29]. Except for L. brunescens OH21 that contains a typical AHL synthase, almost all other reported members of Lysobacter do not produce AHL [30]. This observation led us to hypothesize that the genome of those Lysobacter species that do not produce AHL may carry previously uncharacterized genes/proteins to quench its QS signaling pathway. To discover such naturally occurring, new QQ mechanisms, we aim to identify Lysobacter proteins that directly bind and target AHL synthase by developing a platform involving heterogeneous gene expression and protein binding. To achieve this goal, we selected L. enzymogenes OH11, which does not produce AHL and is the most studied species in the Lysobacter genus, as the working model. A representative AHL synthase gene known as pcol from the antifungal Pseudomonas fluorescens 2P24 was fused with the FLAG tag and artificially expressed in L. enzymogenes OH11. The Pcol-FLAG protein was isolated by co-immunoprecipitation (co-IP) and further expressed in P. fluorescens 2P24 alone to screen for target candidate(s) that can inhibit the production of AHL in P. fluorescens (Fig. 1). Via such screening, we discovered that Le0959 from L. enzymogenes OH11, which encodes an active leucyl aminopeptidase, can function as a new QQ protein. In terms of mechanism, Le0959 failed to directly degrade AHL, but bind with Pcol to reduce the abundance of Pcol (capable of AHL synthesis) as evidenced in E. coli. This study highlights the development of a previously-uncharacterized screen technique for bacterial quorum quenching and the discovery of LqqP in quenching AHL quorum sensing by binding to AHL synthase.

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

The strains and plasmids used in this study are shown in Table S1. Escherichia coli strains were grown in Lysogenic broth (LB) medium at 37 °C with appropriate antibiotics. Unless otherwise specified, Lysobacter enzymogenes and Pseudomonas fluorescens 2P24 were grown in LB medium at 28 °C using the following antibiotics: kanamycin (Km), 30 µg/mL, gentamicin (Gm), 30 µg/mL, tetracycline (Tc), 30 µg/mL for plasmid maintenance. For growth curve analysis, Pseudomonas fluorescens 2P24 and its derivatives were grown in liquid LB medium at 28 °C, and their growth was monitored by measuring the optical density at 600 nm (OD600) every 2 h. Three replicates were performed for each strain and the bacterial growth curves were drawn based on the average OD600 values.

2.2. Heterogeneous expression of pcol in L. enzymogenes or E. coli

The coding region of Pcol from P. fluorescens 2P24 was amplified by PCR using the primers listed in Table S2. The PCR product was cloned into the broad-host vector pBBR1-MCS5 and pUCP26 by restriction enzyme digestion (Table S1). The resulting plasmid was transformed into L. enzymogenes OH11 or E. coli strains (DH5α and BL21) by electroporation, and verified by PCR and western blotting.

2.3. Identification of Pcol-binding proteins from L. enzymogenes

The precipitation of the Pcol-FLAG binding proteins and their identification by mass spectrometry were performed as previously described [31]. The plasmid carrying Pcol-FLAG was introduced into L. enzymogenes OH11 by electroporation. The transformant was grown in LB until OD600 reached 1.0. The cells from the 40-mL culture were then harvested and the culture supernatant was saved. The supernatant was re-suspended in 2 mL 0.1 M PBS (pH, 7.4), followed by sonication (Sonifier 250; Branson Digital Sonifier, Danbury, USA). The insoluble material was removed by centrifugation at 6000 rpm at 4 °C for 20 min. Then, according to the manufacturer’s instructions, 2 mL of soluble protein was mixed with 50 µL of anti-FLAG magnetic beads (Bimake, Shanghai, China) to capture the Pcol-binding proteins. After incubating overnight at 4 °C, the beads were washed three times with 500 µL of 0.01 M PBS (pH, 7.4) containing 0.5% Tween 20. Proteins bound to the beads were eluted with 50 µL elution buffer (0.2 M glycine, pH 2.5), followed by eluent neutralization with 10 µL neutralization buffer (1.5 M Tris, pH 9.0). The eluted protein samples
were identified by mass spectrometry at Beijing Protein Innovation Co., Ltd (Beijing, China).

2.4. AHL bioassays

The AHL plate bioassay was performed as previously described [32]. In brief, the production of AHL was detected by the effective AHL bioassay strain, *Agrobacterium tumefaciens* JZA1 [32]. The JZA1 strain was inoculated into 100 mL liquid LB medium, with the final concentration of antibiotics being 30 g/mL for gentamicin, 30 g/mL for kanamycin, 30 g/mL for spectinomycin and 12 g/mL for tetracycline. It was incubated at 28°C for 24 h until the OD 600 reached 1.5. 10 mL JZA1 culture was then added into 50 mL LB solid medium at a temperature of 50°C and 40 g/mL for 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal) was added at the same time. After mixing, plates containing JZA1 were prepared, followed by inoculation of 3 μL of AHL samples on the plate surfaces. These prepared plates were incubated at 28°C for 18 h to observe the size of the blue circles.

JZA1-based AHL quantification was carried out by the β-galactosidase method in a previous work [32]. In short, JZA1 and gentamicin (30 g/mL), grammycin (30 g/mL) and tetracycline (12 g/mL) were shaken at 28°C and 220 rpm for 24 h in 100 mL liquid LB. When OD 600 reached 1.5, 1 mL JZA1 culture was then added into 50 mL LB solid medium at a temperature of 50°C and a final concentration of 40 g/mL for 5-bromo-4-chloro-3-indolyl-D-galactoside (X-gal) was added at the same time. After mixing, plates containing JZA1 were prepared, followed by inoculation of 3 μL of AHL samples on the plate surfaces. These prepared plates were incubated at 28°C for 18 h to observe the size of the blue circles.

2.5. Bacterial two–hybrid assay

The BacterioMatch II two-hybrid (B2H) system (Agilent Technologies, USA) was used to detect protein–protein interactions in a previous work [33]. In short, the coding regions of target proteins (LqqP and PcoI) were cloned into pBT and pTGR plasmids and transformed into *E. coli* blue MRF´ Kan. Plasmids pBT-GacS and pTRG-GacS (Table S1) were used as positive controls [33], while transformants containing empty pTRG and pBT vectors were used as negative controls. All co-transformants were spotted onto the selective medium and grown at 28°C for 2 days. If there is a direct physical interaction between PcoI and LqqP, the transformed *E. coli* strain containing the two vectors are expected to grow well on the reference medium (+3AT + Str r) that is based on the minimal medium (M9) supplemented with 5 mM 3-AT, 2 μg/mL Str, 12.5 μg/mL tetracycline, 34 μg/mL chloramphenicol, and 30 μg/mL kanamycin, as described in a previous work [33]. The LB agar is a non-selective medium (-3AT-Str r) containing 12.5 μg/mL tetracycline, 34 μg/mL chloramphenicol, and 30 μg/mL kanamycin, as described previously [33]. The purpose of this medium is to ensure that both vectors can be successfully transformed into *E. coli* blue MRF´ Kan.

![Fig. 1. A platform for identifying the Pcol-binding proteins from Lysobacter enzymogenes OH11 that inhibit the production of AHL by Pseudomonas fluorescens 2P24. Step 2–3 are proposed by results shown in Fig. S2, while the results of the step 4–7 are provided in Fig. 2A-B.](image-url)
2.6. Protein expression and purification

The LqqP and PcoI proteins were expressed as His6-fusions and purified by affinity chromatography. The coding region of pcol was cloned into plasmid pET30a (Table S1) using primers listed in Table S2. The coding region of lqqP was cloned into plasmid pClod (Table S1). The genes were then expressed in E. coli BL21(DE3) (Table S1). The His6-fusion proteins were purified from 400 mL E. coli BL21(DE3) carrying the pET30a or pClod TF plasmid derivatives using Ni-NTA resin (GE Healthcare, Shanghai, China). The strains were grown to OD600 0.4 at 37 °C, and then 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Sigma, USA) was used to induce gene expression at 28 °C for 4 h to obtain Pcol or at 15 °C for 24 h to obtain LqqP. The concentration of purified proteins was determined by BCA protein assay kit (Sangon Biotech, Shanghai, China). Protein purity was assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.7. Pull-down and Co-IP assays

The pull-down assays were performed as previously described [34]. The reaction mixture contains 5 μM Pcol-His and LqqP-GST or GST in 1.5 mL PBS buffer. Then 50 μL GST resin was added and samples incubated overnight at 4 °C. The resin was collected by centrifugation at 4 °C (500 g, 5 min) and washed 3 times with PBS containing 1% Triton X-100 to remove nonspecifically bound proteins. The proteins captured on the GST-beads were eluted by boiling in 4 × SDS loading dye for 6 min, after which the samples were subjected to SDS-PAGE and Western blotting. Protein detection involved the use of GST- (ab19256), His- (ab18184) and RNA polymerase (ab191598) specific antibodies (Abcam, UK).

The Co-IP assays were performed in a previous work [34]. In brief, the coding regions of the target proteins (LqqP, LqqP C78Yand Pcol) were cloned into pBRR1-MCS5 and pUCP26 plasmids, and co-transformed into E. coli DH5α or BL21(DE3). The transformant was grown in LB until OD600 reached 1.0. The cells from the 40-mL culture were then harvested, then resuspend in 2 mL 0.1 M PBS (pH, 7.4), followed by sonication (Sonifier 250; Branson Digital Sonifier, Danbury, USA). The insoluble material was removed by centrifugation at 6000 rpm for 20 min at 4 °C. Then 50 μL FLAG (Bimake, Shanghai, China) resin was added and samples were incubated overnight at 4 °C. The beads were then washed three times with 500 μL of 0.01 M PBS (pH, 7.4) containing 0.5% Tween 20. Proteins bound to the beads were eluted with 90 μL elution buffer (0.2 M glycine, pH 2.5), followed by neutralization with 10 μL neutralization buffer (1.5 M Tris, pH 9.0). Afterwards, the samples were subjected to SDS-PAGE and western blotting. Protein detection involved the use of FLAG- (M20008S) and His- (ab18184) specific antibodies (Abmart, Shanghai, China).

2.8. Microscale thermophoresis assay, MST

As previously described [33], the protein–protein binding affinities were determined by MST using Monolith NT.115 (NanoTemper Technologies, Germany). In brief, the LqqP-His protein was labeled with the fluorescent dye RED-Tris-NTA (NanoTemper Technologies GmbH, Germany) by amine conjugation. The labeled LqqP protein at a constant concentration (100 nM) in MST buffer was titrated against Pcol-His (concentration range, 0.229 nM-7.5 μM). The MST premium-coated capillaries (Monolith NT.115 MO-K005, Germany) were used to load the samples into the MST instrument at 25 °C with high MST power and 60% LED power. The laser on and off times were set to 30 and 5 s, respectively. All experiments were conducted in triplicate. Data were analyzed using Nanotemper Analysis software v.1.2.101 (NanoTemper Technologies, Germany).

2.9. Bioinformatics analyses

To identify LqqP homologues, LqqP was used as a query to run local BLASTp to identify the corresponding homologues in the selected bacterial genomes. A similar protein was considered present when the E-value was lower than 10−5 with a similarity percentage with the corresponding homologous protein higher than 40%.

2.10. Biofilm formation test

The biofilm formation of P. fluorescens 2P24 was performed as previously described [35]. In short, P. fluorescens 2P24 and its derivative strains were grown in liquid LB until the OD600 reached 1.0. Then 500 μL of bacterial culture was taken and added into a 2-mL tube containing 1 mL of fresh LB. The inoculated culture was cultivated at 28 °C for 36 h. The biofilm was washed three times with sterilized double distilled H2O (ddH2O), and then treated with 0.3% crystal violet (CV) for 15 min. Afterwards, CV-stained biofilm was washed three times with ddH2O and resuspended in 95% ethanol (1 mL). The biomass was quantified at 570 nm by a spectrophotometer (Biotek, USA).

2.11. LqqP enzymatic activity assay

The leucine aminopeptidase activity was assayed by incubating 3 μg of purified LqqP-His and its derivatives with 100 mM of L-Leucine p-nitroanilide hydrochloride (Aladdin, Shanghai, China) in 1 mL reaction buffer (50 mM Tris-HCl, pH 7.5 and 5 mM NiCl2). The samples were incubated at 28 °C for 9 h, and the reaction was stopped by cooling the mixture on ice for 10 min before the optical density at 405 nm (OD405) was measured using a microplate reader to indicate the enzyme activity as previously described [36].

2.12. Homology modeling of LqqP

Modeling of LqqQ was carried out by using the PHYRE2程序 (ref?). The DALI program [37] was then used to further search the homologues of the modeled structures in the PDB. Seven structures with a Z score larger than 40 were obtained, and they all have the function of leucine aminopeptidase. We then superimposed the model structure on the highest-scoring analog (PDB: 2EWB) to find its active site residues. We found that five active site residues (K225, D230, D248, D307, and E309) are conserved between the two structures, and they coordinate well with the two zinc ions required for activity [38].

2.13. Data and material availability statement

The sequence data of this study have been submitted to the NCBI GenBank and included in Table S3. All other data required to evaluate the conclusions in the paper are provided in the paper or supporting information.

3. Results

3.1. L. enzymogenes LqqP acts as a new type of quorum-quenching proteins that block production of Pseudomonas AHL

Pseudomonas fluorescens 2P24 encodes a typical AHL synthase called Pcol, which is necessary for AHL production [35], while L. enzymogenes OH11 cannot produce AHL according to an earlier study [39]. To discover whether the L. enzymogenes OH11 genome encodes any genes that can directly target Pcol in P. fluorescens 2P24 to inhibit its AHL production, we established a platform that
involves heterogeneous gene expression and protein binding. In this platform (Fig. 1 and Fig. S1), the AHL synthase gene pcoI was fused with the FLAG tag and cloned into a broad-host vector pBBR1-MCS5. The recombinant vector was then transformed into L. enzymogenes OH11 that does not produce AHL, in which the transcription of the pcoI-FLAG fusion gene is driven by a constitutive promoter from the plasmid. This step generated a recombinant strain named OH11-PcoI. The AHL produced by the OH11-PcoI strain was determined and validated by a well-known AHL biosensor strain JZA1 and LC-MS/MS technology (Fig. S1). In the context of OH11-PcoI, proteins that interact with PcoI-FLAG can be captured by Co-IP using anti-FLAG antibody (Fig. S2), and subsequently identified by LC-MS/MS. The identified candidate genes were further expressed separately in the AHL-producing P. fluorescens 2P24 to screen out target candidate(s) that inhibit the production of AHL in P. fluorescens 2P24 (Fig. 1).

As shown in Fig. 2A, eleven PcoI interaction candidates identified by LC-MS/MS from L. enzymogenes OH11 were randomly selected for further study. Each of the eleven candidate genes was individually cloned into the broad-host vector pBBR1-MCS5 and introduced into 2P24 respectively. The quantitative analysis of AHL recognized by JZA1 revealed that the expression of Le0959 in 2P24 significantly reduced the amount of AHL, while the expression of the remaining ten genes in 2P24 did not show this effect (Fig. 2B & 2C). The pcoI mutant (ΔpcoI) unable to produce AHL was served as a negative control (Fig. 2B & 2C). In the following sections, we define Le0959 as the Lysobacter quorum-quenching protein, LqqP. Since AHL is reported to positively regulate biofilm formation in P. fluorescens 2P24 [35], we investigated whether the expression of lqqP in 2P24 could impair this AHL-controlled phenotype by blocking the AHL production, and results in Fig. 2D supported this hypothesis. We also observed that the expression of lqqP in 2P24 did not affect bacterial growth (Fig. S3). In the following sections, we define Le0959 as the L. enzymogenes OH11-PcoI strain.

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3.2. LqqP encodes an active leucyl aminopeptidase, and its ability to inhibit the production of P. fluorescens AHL does not depend on this enzymatic activity.

Since all reported QQ proteins discovered to date function as AHL-degradation enzymes, we tested whether LqqP has any ability to degrade AHL. For this purpose, 100-nM commercial AHL [N-(3-oxodecanoyl)-L-homoserine lactone, 3-oxo-C10-HSL] was added to the culture of the AHL-deficient P. fluorescens mutant Δpcol expressing lqqP. The results of Fig. S4 showed that expression of lqqP or the negative control gfp in Δpcol failed to degrade the supplemented AHL that is determined by the AHL biosensor strain JZA1. Under similar conditions, expression of the positive control moml encoding a known AHL-degrading enzyme [21] effectively degraded the supplemented AHL in a time-dependent manner (Fig. S4). These results indicate that LqqP does not directly degrade AHL. Moreover, BLASTP searches in the NCBI database reveal that LqqP had no sequence similarity/identify to any reported AHL-degradation enzymes, such as AiiA, AttM, MomL and AiiD.

Since LqqP encodes a predicted leucyl aminopeptidase, we are interested to understand whether the enzymatic activity of LqqP is related to its function in P. fluorescens 2P24. A homolog-modeling reveals that LqqP possess three key amino residues – D230, E248, E309 that are expected to be essential for LqqP enzyme activity (Fig. 3A). As a random attempt, we replaced D230 with alanine by site-direct mutagenesis to produce the D230A variant. Expression of the LqqP(D230A) mutant in the wild-type 2P24 still effectively impaired AHL production (Fig. 3B & 3C). We thus generated a double variant LqqP(D230A-E248A) by replacing D230, D248 and E309 with alanine. Again, the LqqP(D230A-E248A-E309A) variant was still active in inhibiting AHL production when expressed in 2P24 (Fig. 3B & 3C). Finally, we generated a triple variant LqqP(D230A-E248A-E309A) by replacing D230, D248 and E309 with alanine simultaneously. As the result, introduction of this variant gene into 2P24 still inhibited the production of AHL (Fig. 3B & 3C). These results indicate that the enzyme activity of LqqP does not seem to contribute to its function in blocking AHL production. To validate this finding, we used the pCold-TF vector to express the native LqqP as well as the LqqP(D230A-E248A-E309A) triple variant, in which both target genes were fused with a His tag and a prokaryotic ribosomal binding partner protein (trigger factor, TF) that can induce co-translational folding of new peptide chains in E. coli BL21. Enzyme activity test showed that the His-TF-LqqP fusion does exhibit aminopeptidase activity (Fig. 3D), while His-TF-LqqP(D230A-E248A-E309A) triple variant gave no detectable enzyme activity like that of native control, the His-TF fusion protein (Fig. 3D). These results indicate that LqqP encodes an active leucyl aminopeptidase, and its function in blocking AHL production seems to be independent of its enzymatic activity. To support this conclusion, we isolated a variant of LqqP (LqqP(C78A)) by site muta-
genesis and found that this variant LqqP showed higher aminopeptidase activity compared to the native LqqP, but still failed to inhibit the production of the Pcol-dependent AHL in 2P24 (see below).

3.3. C78 is required for LqqP function

To explore how LqqP inhibits the production of AHL by P. fluorescens, we set out to identify the key amino residue(s) required for LqqP functioning. Bioinformatics analyses reveal that LqqP is conservatively distributed in many AHL- and non-AHL-producing bacterial species (Fig. 4A). To test whether the LqqP homologs have the ability to block the production of P. fluorescens AHL, we first selected PF4660 and PA3831 from the AHL-producing P. fluorescens 2P24 and P. aeruginosa PAO1 respectively. The introduction of plasmid-borne PF4660 or PA3831 into 2P24 could not inhibit AHL production (Fig. 4B & 4C). We then chose XC3992 from Xanthomonas campestris 8004, which is a phylogenetically-related species of Lysobacter that cannot produce AHL. Similarly, the expression of XC3992 carried by the plasmid in 2P24 failed to impair AHL production (Fig. 4B & 4C). Finally, we chose five LqqP homologous genes from other Lysobacter species, including Lg5105 from L. gummosus OH17, Lc4609 from L. capsici NF87, La3159 from L. antibioticus OH13, GLE4458 from L. enzymogenes C3, and Lb3180 from L. brunescens OH21. The individual expression of these LqqP homologous genes in 2P24 led to the discovery that two of them (Lc4609 and Lg5105), like LqqP itself, prevented the production of AHL, but not for the remaining three (La3159, Lb3180 and GLE4458) (Fig. 4B & 4C). The successful expression of all LqqP homologous genes in 2P24 was confirmed by Western blotting (Fig. S5A). Together, these results indicate that some
Lysobacter LqqP homologues are functionally divergent to block the AHL production in \textit{P. fluorescens} 2P24. Notably, GLE4458 from \textit{L. enzymogenes} C3 only differs from LqqP with nine residue substitutions (Fig. 4D), but it fails to inhibit AHL production when it is artificially expressed in 2P24 (Fig. 4B & 4C). While there are 9 different residues carried by LqqP compared to GLE4458, only 7 single or combination mutations were made in this study. Briefly, we randomly generated seven LqqP variants by single substitution or combination, which share the same residue(s) of GLE4458, LqqP Q172E-Q189R, and LqqP G313V-T319A (Fig. 4E & 4F). The expression of each LqqP variant in 2P24 by plasmid led to the discovery that only the C78 residue is the key to the LqqP function. The expression of the LqqP \textit{pc78y} gene in 2P24 could not inhibit the production of AHL (Fig. 4E & 4F). Western blotting revealed that the above single substitution or combination did not affect the abundance of LqqP in 2P24 (Fig. 5B). These results support the conclusion that LqqP seems to require C78 to prevent the production of \textit{P. fluorescens} AHL.

3.4. LqqP directly binds to Pcol and this binding weakens the abundance of free Pcol in vivo

Why LqqP needs residue C78 to prevent the production of \textit{P. fluorescens} AHL? We first tested whether LqqP directly binds to Pcol, because LqqP is a potent interacting protein of Pcol, as shown in Fig. 2A. To achieve this goal, we first chose the BacterioMatch II two-hybrid (B2H) system, which is a reliable method for evaluating protein–protein interactions based on the transcriptional activation of the HIS3 reporter gene, allowing transformed \textit{E. coli} strain carrying two target genes to grow in a given media that is supplemented with 3-amino-1, 2, 4-triazole (3-AT), a competitive inhibitor of His3 enzyme [40]. Using this tool, we did detect the LqqP-Pcol interaction (Fig. 5A). To verify this observation, we tested the ability of Pco-His to pull down LqqP-GST, and we did observe a positive signal (Fig. 5B). To further quantify the LqqP-Pcol binding affinity, we adopted the microscale thermophoresis (MST) method, which is a powerful technique for quantifying biomolecular interactions[41]. Through MST, we found that Pcol-His bind to LqqP-TF-His with a moderately strong affinity ($K_a$ = 0.646 μM), while the binding of Pcol-His with TF-His fusion could not be observed by MST (Fig. 5C). These results strongly supported the specific binding of LqqP to Pcol.

Is C78 essential for the LqqP-Pcol binding? To facilitate the investigation, we used the genetic background based on \textit{E. coli} for subsequent experiments. We co-transformed the pUCP-Pcol and pBBR-LqqP in \textit{E. coli} DH5α, and drove the expression of pcol and lqqP by a constitutive promoter from the corresponding plasmid. The results of the \textit{in vivo} Co-IP assays clearly reveal that the substitution of C78 by tyrosine (Tyr) prevents the binding of LqqP to Pcol (Fig. 6A), suggesting that C78 is indeed required for the LqqP-Pcol interaction.

To understand whether the LqqP-Pcol binding contributes to the function of LqqP by blocking the production of Pcol-dependent AHL, we carried out corresponding experiments. We found that compared with the control \textit{E. coli} DH5α containing pUCP-Pcol and the GFP vector control (pBBR-GFP), the \textit{E. coli} DH5α carrying pUCP-Pcol and pBBR-LqqP had significantly lower AHL production (Fig. 6B), indicating that binding of LqqP appears to block the Pcol-dependent AHL production. To support this observation, we further found that the co-expression of Pcol and LqqP\textit{pc78y} variant in \textit{E. coli} DH5α failed to inhibit Pcol-mediated AHL production (Fig. 6B). These results together indicate that the association of LqqP with Pcol is essential for LqqP to inhibit the production of Pcol-dependent AHL, and C78 of LqqP seems to play an important role.
How does the binding of LqqP to PcoI inhibit the production of PcoI-dependent AHL? As one of the possibilities, we tested whether the PcoI expression profile would be altered after LqqP binding. To test this hypothesis, the native LqqP and its variant LqppC78Y were co-expressed with pcoI in E. coli DH5α. We found that, compared to the gfp-pcoI co-expression control, the co-expression of LqqP-pcoI reduced the PcoI expression profile at an OD600 of 1.0 or 1.5 (Fig. 6C), while the co-expression of LqqP or its derivative LqppC78Y gene on the abundance of PcoI in E. coli DH5α. The average data from three experiments is presented, ± SD. "*" P < 0.01. The band intensities were quantified and analyzed using ImageJ [https://imagej.nih.gov/ij/], with numbers representing the relative intensities of the corresponding bands. The intensity levels of the bands in lane “PcoI-GFP” were set to 1.00. “ns” stands for not statistically significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussions

In the past decades, thousands of bacterial genomes have been deposited in public database, however, most of them are empha-
sized for functional genomics study [42]. To expand the usage of bacterial genomes, we have developed a platform (Fig. 1) that involves heterogeneous gene expression and protein interactions to discover bacterial genes for quorum quenching by directly tar-
getting AHL synthase. In this study, we found that LqqP from L. enzymogenes OH11 is a new type of quorum-quenching proteins. LqqP cannot directly degrade AHL, which is a common function of all reported quorum-quenching enzymes, such as AiiA and Momp [21,23]. The main difference is that LqqP targets PcoI, the AHL synthase of P. fluorescens, by binding to and reducing its free protein abundance, thereby inhibiting the production of PcoI-dependent AHL. Our results thus highlight the discovery of the Lysobacter LqqP in quenching quorum sensing through binding to PcoI and the screening technique developed in this study (Fig. 7).

In fact, LqqP encodes an active leucyl aminopeptidase whose function in bacteria is usually to catalyze the release of N-terminal amino acids, preferentially leucine, but not glutamic acid or aspartic acid [43,44]. In Escherichia coli and Salmonella typhimurium, leucyl aminopeptidase catalyzes the removal of unsubstituted N-terminal amino acids from various peptides, and is involved in the processing and regular turnover of intracellular proteins [45]. Yet, this enzyme also functions as a DNA-binding protein, there-
fore, as a transcriptional repressor or activator, it controls the expression of virulence-related genes in the operon of pathogenic Escherichia coli, Vibrio cholerae and Pseudomonas aeruginosa [46–48]. In this study, we further expanded the role of this enzyme family by detecting the ability of LqqP to block the synthesis of PcoI-dependent AHL. Interestingly, although LqqP encodes an active aminopeptidase, its function of blocking the synthesis of PcoI-dependent AHL does not seem to be related to its enzyme activity (Fig. 3B-3D). This mode of action independent of enzyme activity may endow LqqP with unique features as a quorum-quenching protein. This conclusion is partly supported by the following observation: the expression of pcoI in E. coli enables it to produce AHL, in which there is a well-characterized active leucyl aminopeptidase PepA [43]. The failure of multiple LqqP homologues to prevent the production of PcoI-dependent AHL tested in this study can serve as additional evidence. It is also noteworthy that LqqP is discovered in L. enzymogenes OH11 that does not produce AHL, but it is difficult to directly link its function with its genomic distribution in non-AHL-producing bacteria, because we found several LqqP homologues such as XC3992, La3159, and CLE4458 from the phylogenetically-related and non-AHL-produc-
ing X. campestris 8004, L. antibioticus OH13 and L. enzymogenes C3, respectively, were all unable to block the PcoI-dependent AHL production (Fig. 4B & 4C). Therefore, how LqqP can function independent of its enzyme activity and specifically bind to PcoI remains unclear. The structural characterization of the LqqP-PcoI complex will allow people to address these interesting questions in the future.
A notable finding of this study is the moderately strong binding affinity between LqqP and PcoI. This specific protein–protein binding appears to be required for LqqP to quench quorum-sensing by targeting PcoI. Although the complex structure of LqqP-PcoI have yet to be determined, we have identified residue C78 of LqqP as an important site for the LqqP-PcoI interaction (Fig. 6 A-6C). Interestingly, C78 is not located in the 145–458 region (the C-terminal LqqP truncation) that can efficiently block the PcoI-mediated AHL production (Fig. 2 E & 2F). This suggests that the C78 residue may be essential for LqqP to adopt a correct conformation required for PcoI binding or it may be located at the binding interface between LqqP and PcoI. Moreover, C78 did not appear to be a conserved residue signature for LqqP function as a novel AHL quorum-quenching protein, because the LqqP homologues (Lg5105 and Lc4609) that are capable of blocking PcoI-dependent AHL production in 2P24 have a different residue (Y78) at the same position (Fig. S7).

Another notable finding of this study is the demonstration that the co-expression of LqqP and PcoI and their binding seems to significantly reduce the free PcoI abundance as demonstrated in E. coli DH5α (Fig. 6C). Although the underlying mechanism of this observation remains obscure, the targeting of Pcol by LqqP may be associated with the Lon protease, which is an ATP-dependent serine protease that can mediate selective degradation of protein variants or abnormal proteins [49]. According to previous reports, the loss of Lon in Pseudomonas chlororaphis HT66 and Pseudomonas aeruginosa PA01 significantly increased the abundance of AHL synthase. In vitro degradation assay suggested that the Lon protease can directly degrade AHL synthase [50,51]. However, other unidentified factors are also possibly present to help the targeting process of PcoI by LqqP in E. coli. Moreover, quenching AHL QS by directly targeting AHL synthase by proteins as presented by the PcoI-LqqP complex is mechanistically different to that of small-molecule inhibitors of AHL synthases, such as the well-studied J8-C8 [52]. It is documented that the small molecule J8-C8 acts as an artificial ligand of the AHL synthase TofI of Burkholderia glumae strain BGR1; it inhibits AHL production by binding to TofI and occupying the binding site of acyl-ACP acyl chain, as reported earlier [52].

The discovery that LqqP can bind to and target PcoI also provides a clue to explain why the PcoI homologues are lost but LqqP remains in L. enzymogenes OH11 during genome evolution. L. enzymogenes OH11 secretes a major antifungal metabolite called heat-stable antifungal factor (HSAF), which enters into the surrounding environment to inhibit the growth of fungal pathogens [53].

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**Fig. 7. Summary of AHL quorum quenching strategies.** (A) Three well-known AHL quorum-quenching strategies. Quenching of AHL signaling by inhibiting AHL synthesis (middle green arrow) or AHL-receptor binding (bottom green arrow) by small molecular chemicals or enzymatically degrading AHL (top green arrow) are indicated. (B) A proposed model of LqqP-mediated quenching of Pcol-dependent AHL signaling, representing a new quorum-quenching mechanism through protein–protein interaction. The direct binding of LqqP and Pcol reduces the abundance of free Pcol in E. coli through an unknown mechanism. Lux and Pcol: AHL synthase, LuxR and PcoR: AHL receptor transcriptional regulator. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
found that the production of AHL in OH11 conferred by the heterogenous expression of Pcol did not affect HSAF production, while in-frame deletion of *lqqP* in OH11 caused the strain to overproduce HSAF (Fig. S9). This indicates that *LqqP* seems to play a vital role in maintaining the HSAF homeostasis, which may help *L. enzymogenes* to fine-tune the amount of HSAF as an antifungal weapon, because the overproduction of HSAF is more than just an energy-consuming process but also impairs the adaptability of bacteria in nature.

The discovery that *LqqP* targets Pcol also led us to test whether *LqqP* also targets other AHL synthases. The result shows that *LqqP* can also effectively interfere with the Rhl-mediated AHL system in the pathogenic *P. aeruginosa* PA01. RhlII in *P. aeruginosa* is a well-characterized AHL synthase responsible for the synthesis of C4-HSL, which binds to the transcription factor RhlRI to activate expression of diverse downstream genes associating with the production of elastase, pyocyanin, hemolysin and rhamnolipid [54,55]. We found that the expression of *lqqP* in PA01 significantly impaired the amount of RhlII-mediated butyl-homoserine lactone (N-butyl homoserine lactones, C4-HSL) and several AHL-controlled functions (Fig. S10A-10E). Furthermore, *LqqP* directly binds to RhlII (Fig. S11A & 11B). These results indicate that *LqqP* can target AHL synthases from beneficial and pathogenic *Pseudomonas* species.

As reported earlier, the AHL-mediated quorum sensing plays key roles not only for the full virulence of plant pathogenic bacteria, but also for promoting antimicrobial activity or colonization capacity of plant beneficial bacteria [35]. Since most reported quorum-quenching enzymes (i.e. AiiA and MomoI) have broad-spectrum capacity in degrading AHLs, it seems to be very difficult for them to selectively degrade AHLs produced only by plant pathogenic bacteria but not by plant beneficial bacteria after filing for crop protection. As a unique quorum-quenching protein, *LqqP* fails to degrade AHLs, but quenches AHL signaling by binding and targeting AHL synthases, as evidenced by the finding of *LqqP* bound to Pcol and induced Pcol degradation in vivo. This capacity may enable us to design the wild-type *LqqP* or its engineered versions to recognize only the AHL synthases of plant pathogenic bacteria, but not those of plant beneficial bacteria, by selectively targeting on the AHL signaling of plant pathogenic bacteria to reduce their virulence on hosts without affecting the AHL signaling of plant beneficial bacteria.

Finally, it is also noteworthy that although the heterogenous expression of *LqqP* in *P. fluorescens* 2P24 shows a strong ability in inhibiting the production of Pcol-dependent AHL, our results indicate that *LqqP* does not seem to have the opportunity to directly enter the *P. fluorescens* cells because we observed that supplementing the purified *LqqP* proteins to the culture of *P. fluorescens* 2P24 did not inhibit AHL production (Fig. S12). Therefore, the delivery of *LqqP* into AHL-producing bacteria by biotechnology approaches, i.e. nanotechnology may be a promising approach in future.

5. Conclusions

This study highlights a novel quorum-quenching gene through the defined quorum-quenching platform developed in this study. This platform may be universal after minor modifications to uncover genes from bacterial, fungal or plant genomes of interest, to bind and target component(s) of bacterial virulence-associated protein secretion systems and/or signaling pathway(s), by which scientists can develop new strategies to hinder the infection of numerous pathogenic bacteria for crop protection and medical therapy.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.csbj.2021.11.017.

References

[1] Fuqua C, Greenberg EP. Listening in on bacteria: acyl-homoserine lactone signalling. Nat Rev Mol Cell Biol 2002;3(9):685–95. https://doi.org/10.1038/ nrm942.
[2] Miller MB, Bassler BL. Quorum sensing in bacteria. Annu Rev Microbiol 2001;55(1):165–99. https://doi.org/10.1146/annurev.micro.55.1.165.
[3] Ng W-L, Bassler BL. Bacterial Quorum-Sensing network architectures. Annu Rev Genet 2009;43(1):197–222. https://doi.org/10.1146/annurev-genet- 102008-113430.
[4] Papenfort K, Bassler BL. Quorum sensing signal response systems in Gram-negative bacteria. Nat Rev Microbiol 2016;14(9):576–88. https://doi.org/ 10.1038/nrmicro20168.
[5] Whiteley M, Diggle SP, Greenberg EP. Progress in and promise of bacterial quorum sensing research. Nature 2017;551(7680):313–20. https:// doi.org/10.1038/nature24624.
[6] Waters CM, Bassler BL. Quorum sensing: Cell-to-cell communication in bacteria. Annu Rev Cell Dev Biol 2005;21(1):319–46. https://doi.org/10.1146/ annurev.cellbio.21.070204.102105.
[7] Thi MTT, Wibowo D, Rehm BHA, Pseudomonas aeruginosa biofilms. Int J Mol Sci 2020;21(22):8671. https://doi.org/10.3390/ijms21228671.
[8] Fuqua WC, Winani SC, Greenberg EP. Quorum sensing in bacteria: the LuxR-Lux box family of cell-density-responsive transcriptional regulators. J Bacteriol 1994;176(2):269–75. https://doi.org/10.1128/ jb.176.2.269-275.1994.
[9] Ji G, Beavis RC, Novick RP. Cell density control of staphylococcal virulence mediated by an octapeptide pheromone. Proc Natl Acad Sci 1995;92(26):12055–9. https://doi.org/10.1073/pnas.92.26.12055.
[10] Yi Li, Dong X, Grenier D, Wang K, Wang Y. Research progress of bacterial quorum sensing receptors: Classification, structure, function and characteristics. Sci Total Environ 2021;763:140301. https://doi.org/10.1016/j. scientotenv.2020.143031.
[11] Lee J, Wu J, Deng Y, Wang J, Wang C, Wang J et al. A cell-cell communication signal integrates quorum sensing and stress response. Nat Chem Biol 2013;9(5):339–43. https://doi.org/10.1038/nchembio.1275.
[12] Parsk Ser, Vl Dl, Hanzela BL, Cronan JE, Greenberg EP. Acyl-homoserine-lactone quorum-sensing signal generation. Proc Natl Acad Sci 1999;96(8):4360–5. https://doi.org/10.1073/pnas.96.8.4360.
[13] Watson WT, Minogue TD, Vl Dl, van Bodman SB, Churchill MEA. Structural basis and specificity of acyl-homoserine lactone signal production in bacterial quorum sensing. Mol Cell 2002;9(3):685–94. https://doi.org/10.1016/S1097- 2765(02)00480-X.

https://doi.org/10.1016/j.csbj.2021.11.017.
[14] Pearson JP, Gray KM, Passador L, Tucker KD, Eberhard A, Iglewski BH, et al. Structure of the autinducer required for expression of Pseudomonas aeruginosa virulence genes. Proc Natl Acad Sci USA 1994;91(1):197–201. https://doi.org/10.1073/pnas.91.1.197.

[15] Grandclément C, Tannières M, Moréra S, Dessaux Y, Faure D, Camara M. Quorum quenching: role in nature and applied developments. Fems Microbiol Rev 2019;43(1):51–80. https://doi.org/10.1093/femsre/fuy072.

[16] Murugaiyah SA, Gerth ML. Engineering quorum quenching enzymes: progress and perspectives. Biochem Soc T 2019;47(3):793–800. https://doi.org/10.1042/BST20181625.

[17] Christensen QH, Grove TL, Booker SJ, Greenberg EP. A high-throughput screen for quorum-sensing inhibitors that target acyl-homoserine lactone synthases. Nat Biotechnol 2013;31(4):1381–20. https://doi.org/10.1038/nbt.2671.

[18] Nandi S. Recent Advances in ligand and structure based screening of potent quorum sensing inhibitors against antibiotic resistance induced bacterial virulence. Recent Pat Biotechnol 2016;10(2):195–216. https://doi.org/10.2174/18783275166662014045.

[19] Kumar M, Saxena M, Saxena AK, Nandi S. Recent Breakthroughs in various antimicrobial resistance induced quorum sensing biosynthetic pathway mediated targets and design of their inhibitors. Comb Chem High T Scr 2020;23(6):458–76. https://doi.org/10.4155/ccc.20.316.

[20] Wang J, Jiao H, Meng J, Qiao M, Du H, He M, et al. Baicalin inhibits biofilm formation and the quorum-sensing system by regulating the msra drug efflux pump. J Antimicrob Chemother 2015;69(6):1253–61. https://doi.org/10.1093/jac/dku328.

[21] Chen H, Qian G, Xie Y, Hang J, Shen H, et al. Quorum-sensing inhibitors against antibiotic resistance induced bacterial infection. Microb Biotechnol 2020;13(10):2326–34. https://doi.org/10.1111/1751-7915.13744.

[22] Jiang J, Ramamurthy S, Zhang J. Engineered quorum quenching enzymes: progress and perspectives. Microb Biotechnol 2019;12(9):2258–82. https://doi.org/10.1111/1751-7915.13796.

[23] Jeong HK, Stagon L, Calderwood SB. pepA, a gene mediating pH regulation of virulence genes in Vibrio cholerae. J Bacteriol 2001;183(1):178–88. https://doi.org/10.1128/JB.183.1.178-188.2001.

[24] Woolwine SC, Wozniak DJ. Identification of an Escherichia coli pepQ homolog and its involvement in suppression of the algB phenotype in mucoid Pseudomonas aeruginosa. J Bacteriol 1999;181(1):107–16. https://doi.org/10.1128/JB.181.1.107-116.1999.

[25] Leeuwenhoek 2006;89(2):267–80. https://doi.org/10.1007/s10482-005-9028-5.

[26] Cordeiro AL, Hirose M, Tani Y. Lysobacter aeruginosa Pseudomonas aeruginosa structure of the autoinducer required for expression of Pseudomonas aeruginosa quorum sensing system. J Bacteriol 1999;181(1):107–16. https://doi.org/10.1128/JB.181.1.178-188.2001.

[27] Appt I, Chaikins JS, Belov KE, Chang J, Cheng AL, et al. The HSAF, a tetramic acid-containing macrolactam from Magnaporthe oryzae, inhibits a leucyl aminopeptidase from rice blast fungus Magnaporthe oryzae. Environ Microbiol 2018;20(10):3226–34. https://doi.org/10.1002/ijmm.10573.

[28] Yang N, Ding S, Chen F, Zhang X, Xia Y, Di H, et al. The Crc protein participates in the quorum sensing response. J Bacteriol 2001;183(1):178–88. https://doi.org/10.1128/JB.183.1.178-188.2001.

[29] Lou L, Qian G, Xie Y, Hang J, Chen H, Zaleta-Rivera K, et al. Biosynthesis of architecture of the c-di-GMP-dependent network regulating antibiotic synthesis. Proc Natl Acad Sci USA 2012;109(24):9453–9. https://doi.org/10.1073/pnas.1119952109.

[30] Ling J, Zhou L, Wu G, Zhao Y, Jiang T, Liu F. The AHL quorum-sensing system of Pseudomonas aeruginosa is under the control of RhlR-RhlI, another set of regulators in strain REL606 and BL21(DE3). J Mol Biol 2009;394(2):223–32. https://doi.org/10.1016/j.jmb.2009.03.035.