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Broad-Spectrum Quorum Quenching and Biocontrol Potential of *Pseudomonas procumbens* sp. nov. and *Pseudomonas polia* sp. nov. via Degradation of Multiple Quorum Sensing Molecules

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ABSTRACT: Bacterial plant diseases present a serious threat to global agriculture, underscoring the need for sustainable biocontrol solutions. Quorum quenching (QQ), which disrupts quorum sensing (QS) to reduce virulence without promoting resistance, offers a promising approach. This study established enrichment cultures with 3-hydroxy palmitic acid methyl ester (3-OH PAME) as the sole carbon source to isolate QQ bacteria against *Ralstonia solanacearum*. Two novel species, *Pseudomonas procumbens* sp. nov. (QE6) and *P. polia* sp. nov. (QL9), exhibited robust and broad-spectrum QQ activity against multiple QS signals, including 3-OH PAME, 3-hydroxy myristic acid methyl ester (3-OH MAME), diffusible signal factor (DSF), and *N*-acyl homoserine lactones (AHLs). Phylogenetic analysis placed both strains within the *P. aeruginosa* group and identified functional genes associated with DSF, AHL, and putative 3-OH PAME/MAME degradation, validated via gene deletion and heterologous expression. Both strains effectively controlled plant bacterial diseases mediated by these QS signals, highlighting their potential as eco-friendly, multifunctional QQ-based biocontrol agents.

KEYWORDS: *quorum sensing, quorum quenching, Pseudomonas, 3-OH PAME, DSF, AHL, biocontrol*

1. INTRODUCTION

Plant pathogenic bacteria pose significant challenges to global agriculture due to their diversity, rapid proliferation, and high infectivity, complicating effective crop protection.[1,2] Among these, bacterial wilt disease caused by *Ralstonia solanacearum* species complex (RSSC), is one of the top ten destructive plant bacterial diseases worldwide.[1] This soil-borne pathogen infects over 450 plant species across 54 families, including tomato, pepper, peanut, potato, and tobacco, causing severe losses due to its high infectivity.[3−5] RSSC infects plants through root wounds, colonizing the xylem and producing abundant exopolysaccharides (EPS) that obstruct vascular tissues, impairing water and nutrient transport and causing wilting and plant death.[6,7] The virulence of RSSC arises from EPS, plant cell wall-degrading enzymes (PCWDEs), and type III effectors etc., rendering it a formidable threat often termed "the cancer of plants".[8−11]

The complexity and rapid dissemination of bacterial plant diseases, along with their robust proliferation, challenge conventional control strategies. Traditional strategies, such as the use of resistant crop varieties, chemical pesticides, and cultural practices, exhibit considerable limitations.[1,12,13] Excessive application of pesticides results in environmental contamination, with nearly 80% of applied chemicals adversely affecting nontarget organisms, soil health, and water systems. This underscores the urgent need for sustainable alternatives.[12,14] Beneficial microorganisms offer eco-friendly solutions for managing plant diseases, acting through the targeted disruption of pathogenic virulence mechanisms.[15−17]

Quorum sensing (QS), a cell-density-dependent communication mechanism, plays a central role in the regulation of bacterial plant diseases by facilitating intercellular signaling among bacteria.[18,19] Through the production, release, and detection of small signaling molecules, bacteria assess their population density and synchronize collective behaviors such as growth, virulence, and metabolic adaptation in response to environmental fluctuations or host invasion.[20] In major plant pathogens such as *R. solanacearum*, *Xanthomonas*, and *Pectobacterium*, QS controls the expression of key virulence determinants, including EPS and PCWDEs.[21] Quorum quenching (QQ), which disrupts QS, attenuates pathogen virulence without promoting resistance development, thereby representing a promising sustainable biocontrol approach.[22,23] QS systems vary across bacterial groups: Gram-positive bacteria typically use small peptide-based autoinducers, while Gram-negative species such as *Pectobacterium* and *Xanthomonas* rely on *N*-acyl homoserine lactones (AHLs) or diffusible signal factors (DSFs).[19,21] More than 100 bacterial species are known to produce AHLs or DSFs, with signal specificity ensuring precise intraspecific communication.[24]

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Apart from the AHL, a unique QS system known as the *phc* (phenotype conversion) system has been identified as a key regulator of virulence, biofilm formation, motility, and microbial interactions in RSSC.[25−28] This system consists of the *phcBSRQ* operon and a LysR family transcriptional regulator PhcA, which together facilitate the synthesis, perception, and signal transduction of the QS autoinducer—either 3-hydroxypalmitic acid methyl ester (3-OH PAME) or 3-hydroxymyristic acid methyl ester (3-OH MAME).[29−32] The methyltransferase PhcB synthesizes these signal molecules, which are detected by the two-component system (TCS) PhcS-PhcRQ, and ultimately activate PhcA transcription.[28,31] Transcriptomic analyses of PhcA mutants in tomato xylem have shown that PhcA regulates over 12% of the genes in *R. solanacearum*, underscoring its role in pathogenicity.[28,33]

Given its critical role in the pathogenicity of *R. solanacearum*, 3-OH PAME represents a key target for controlling bacterial wilt via QQ to disrupt virulence pathways. So far, several bacterial agents capable of degrading 3-OH PAME have been identified. These include the first discovered degradation enzyme, *β*-hydroxypalmitoylmethyl ester hydrolase (*β*-HPMEH), from *Ideonella* sp. 0−0013;[34] several 3-OH PAME hydrolases (ELP86, ELP96, ELP104, and EstDL33) identified via soil metagenomic sequencing;[35] and bacterial species such as *Pseudomonas aeruginosa*, *P. forestsoilum*, *P. tohonis*, *Rhodococcus corynebacterioides*, and *Stenotrophomonas maltophilia*.[36,37] Nevertheless, the currently available QQ bacteria and enzymes remain limited, and none have been successfully applied in practical field settings. This highlights an urgent need for the development of novel broad-spectrum QQ strains.

In this study, 3-OH PAME was used as the sole carbon source to screen for QQ bacteria capable of biocontrolling against bacterial wilt. Two novel species strains, *Pseudomonas procumbens* sp. nov. QE6 and *Pseudomonas polia* sp. nov. QL9, exhibited robust degradation of 3-OH PAME and significantly suppressed virulence factors of *R. solanacearum*. In addition, their capacity to degrade multiple QS signals, including 3-OH MAME, DSF, and AHLs, was investigated to evaluate their potential as multifunctional QQ bacteria for the control of diverse plant bacterial diseases.

Overall, this study highlights the potential of novel QQ-active microbes in the biocontrol of plant bacterial diseases and provides valuable resources for developing green, safe, and efficient agricultural strategies. By targeting key QS signals, these bacteria represent sustainable alternatives to chemical pesticides, supporting eco-friendly agricultural practices and contributing to enhanced global food security.

2. MATERIALS AND METHODS

2.1. Strains, Medium, Cultivation Conditions, and Chemicals

Bacterial strains used in this study are listed in Table S1. *R. solanacearum* NS25 was cultured at 28 °C in CPG medium (casamino acid 1.0g/L, peptone 10.0 g/L, glucose 5.0 g/L; solid medium added with 15.0 g/L agar; pH 7.0). The TTC medium was added with 5% 2,3,5-triphenyltetrazolium chloride (TTC) in a 1:1000 ratio to the melted CPG solid medium. The QQ strains, deletion mutants, and *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*) were grown at 30 °C in Luria−Bertani (LB) medium (tryptone 10.0 g/L, yeast extract 5.0 g/L, and NaCl 10.0 g/L; pH 7.0). LB broth was also used to culture *Escherichia coli* strains at 37 °C. *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) was cultured at 28 °C in nutrient agar (NA) medium (peptone 5.0 g/L, meat extract 3.0 g/L; pH 7.0).

Mineral salt medium [MSM: $(NH_4)_2SO_4$ 2.0 g/L, $Na_2HPO_4{\cdot}12H_2O$ 1.5 g/L, NaH_2PO_4 1.5 g/L, $MgSO_4{\cdot}7H_2O$ 0.2 g/L, $CaCl_2{\cdot}2H_2O$ 0.01 g/L, $FeSO_4{\cdot}7H_2O$, 0.001 g/L; pH 6.5] was used for screening QQ bacteria and testing degradation of QS signals, including 3-OH PAME, 3-OH MAME, DSF, *N*-(3-oxohexanoyl)-*L*-homoserine lactone (3-O-C6-HSL), and *N*-(3-oxooctanoyl)-*L*-homoserine lactone (3-O-C8-HSL).

Pure 3-OH PAME and 3-OH MAME were obtained from Shanghai Zixia Biotechnology Co., Ltd. (China). DSF, 3-O-C6-HSL and 3-O-C8-HSL were purchased from Sigma-Aldrich (USA).

2.2. Preliminary Screening and Isolation of 3-OH PAME Quenching Bacteria

QQ strains were isolated from rhizospheric soils using QS signals as the sole carbon source through enrichment cultures, following established protocols.[34,37−39] Soil samples (20.0 g) from chili pepper and tomato rhizospheres were collected at Qilin North Farm, South China Agricultural University, Guangzhou, China. After homogenization, 5.0 g of mixed soil was inoculated into 200 mL of MSM containing 1 *μ*M 3-OH PAME and incubated at 28 °C and 200 rpm for 7 days. Subsequently, 2 mL of the culture was transferred into fresh MSM containing 1 *μ*M 3-OH PAME (1:100 dilution) and incubated under the same conditions for an additional 7 days. After enrichment, serial dilutions were plated onto LB agar, and colonies exhibiting distinct morphologies (size, color, surface texture) or potential antagonistic activity against *R. solanacearum* NS25 were selected and purified for further analysis.

2.3. Identification of Quorum Quenching Strains

The 16S rDNA of the selected strains was amplified using primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGACTTAACCCCAATCGC-3′) and sequenced by Sanger sequencing. Sequences were analyzed via BLASTn on the NCBI database. Strains identified as belonging to the *Pseudomonas* genus were further characterized using the housekeeping gene *rpoD*, known for its discriminatory power in identifying environmental *Pseudomonas* isolates.[40] Amplification was performed with primers PsEG30F (5′-ATYGAAATCGCCAARCG-3′) and PsEG30R (5′-CGGTTGATKTCCTTGA-3′), followed by sequencing.

2.4. Genome Sequencing of Quenching Bacteria

Genomic DNA of strains QE6 and QL9 was extracted using the EasyPure Bacteria Genomic DNA Kit (TransGen Biotech, Beijing, China). The complete genomes were sequenced by Grandomics Biosciences (Beijing, China) using the Nanopore PromethION platform and Illumina MGISEQ T7 platform. Filtered reads were assembled using Canu v1.5,[41] and genomes were circularized with Circlator v1.5.5.[42] Coding genes were predicted using Prodigal v2.6.3,[43] tRNA genes with tRNAscan-SE v2.0,[44] rRNAs with Infernal v1.1.3, and repetitive sequences with RepeatMasker v4.0.5.[45] Genome sequences of QE6 and QL9 were deposited in the NCBI under accession numbers CP166971.1 (PRJNA1146935) and CP166873.1 (PRJNA1147444), respectively.

2.5. Degradation Activity of 3-OH PAME and 3-OH MAME of Quenching Bacteria

The degradation of 3-OH PAME was assessed following modified protocols.[34,37] Bacterial cultures (100 *μ*L, OD_{600} = 1.2) were inoculated into 10 mL of MSM containing 1 *μ*M 3-OH PAME and incubated at 28 °C while being shaken at 200 rpm for 7 days, protected from light. A bacteria-free control was included. After incubation, samples were extracted three times with 10 mL of dichloromethane. The combined organic phases were evaporated to dryness and resuspended in chromatographic-grade methanol. Residual 3-OH PAME was quantified using a Q Exactive Focus Liquid Mass Spectrometer (Thermo Fisher) with a Waters ACQUITY UPLC HSS T3 column (100 Å, 1.8 *μ*m, 2.1 mm × 100 mm; SKU: 186003539). The analytical conditions were as follows: flow rate, 0.3 mL/min; column temperature, 40 °C; mobile phase, methanol:water (80:20, v/v); injection volume, 10 *μ*L. Detection was performed in positive ion mode using the sodium adduct $[M + Na]^+$ at m/z 309.24, with a retention time of 5.5−6.5 min.

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For 3-OH MAME, the same experimental procedure was applied, except that the incubation time was reduced to 1 day, and detection was carried out at m/z 281.20 $[M + Na]^+$ with a retention time window of 3.0−4.0 min. All experiments were conducted in triplicate.

2.6. Esterase Activity Assay

As 3-OH PAME and 3-OH MAME are ester compounds, esterase activity was evaluated using glycerol tributyrate (tributyrin) as a model substrate, which can be enzymatically hydrolyzed to glycerol and butyric acid by esterases or lipases and is commonly used to evaluate microbial ester-bond degradation.[34,35,46] LB agar supplemented with 1% or 2% tributyrin was poured into 90 mm Petri dishes. Bacterial cultures (1 μL, OD_{600} = 1.2) of the tested strains were spotted onto the plates and incubated at 28 °C for 2 and 4 days. Degradation halo diameters were measured. Experiments were conducted in triplicate.

2.7. Phenotypic Characterization of Strains QE6 and QL9

Each strain (QE6 and QL9) was cultured on LB agar plates to observe the colony morphology. The cellular morphology was examined by transmission electron microscopy (TEM) using a JEOL JEM-1400 Plus microscope (JEOL, Japan) at Beijing Zhongkebaice Technology Service Co., Ltd. (Beijing, China).

Carbon source utilization and chemical sensitivity assay profiles of strains QE6 and QL9 were determined using Biolog GEN III microplates (bioMérieux) according to the manufacturer's instructions.

Antagonistic activity against *R. solanacearum* NS25 was assessed using a plate confrontation assay with slight modifications.[47] Strain NS25 was embedded in soft agar, and wells were loaded with cultures of QE6, QL9, or *P. wayambapalatensis* L7 (positive control).[48] Inhibition zones were measured after incubation at 28 °C for 24 h. Additionally, strains QE6 and QL9 were tested for secondary metabolite production or predation on NS25 following the method described previously.[49]

The methods used for determining EPS production, extracellular enzyme (cellulase and polygalacturonase) activity, biofilm formation, and bacterial motility (swimming, swarming, and twitching) were performed according to our previous study.[37]

Antibiotic susceptibility was assessed using antibiotics including ampicillin, kanamycin, streptomycin, gentamicin, tetracycline, and polymyxin B at concentrations ranging from 10 to 640 μg/mL, as previously described.[50] Bacterial cultures (OD_{600} = 1.2) were diluted and incubated in 96-well plates at 28 °C for 48 h.

All experiments were conducted with at least three independent biological replicates.

2.8. Effects of Strains QE6 and QL9 on EPS Production and Extracellular Enzyme Activity of NS25

The impact of strains QE6 and QL9 on EPS production and PCWDE activity of *R. solanacearum* NS25 was evaluated as described previously.[37] Strain NS25 was cocultured with strains QE6 or QL9, and EPS yield and PCWDE activities were measured. Colony morphology was assessed using Rdar phenotyping (Congo red and TTC) plates, while cellulase and polygalacturonase activities were detected using Cel and Peh assay plates. All experiments were performed with at least three replicates.

2.9. RNA Purification and RT-qPCR

Single colonies of strains QE6, QL9, and NS25 were grown until an OD_{600} of 1.0, and NS25+LB (1:1), NS25+QE6 (1:1), or NS25+QL9 (1:1) was added to 10 mL of CPG medium at a ratio of 1:50 for 7 h at 28 °C for RNA extraction. RNA was extracted using the SV Total RNA Isolation System kit (Promega, Madison, WI, USA). For RT-PCR analysis, 0.5 μg of RNA was reverse transcribed using the HiScript III first-strand cDNA Synthesis Kit (Vazyme Biotech Co., Nanjing, China) to generate template cDNA. Expression of the NS25 internal reference gene, *infB,* was selected to balance the cDNA concentrations in the three samples. The primers used for PCR amplification are listed in Table S2. RT-PCR was performed using 2× Rapid Taq Master Mix (Vazyme Biotech Co., Nanjing, China) with the following cycling pattern: 1 cycle at 95 °C for 3 min; followed by 40 cycles at 95 °C for 15 s, 56 °C for 15 s, and 72 °C for 15 s; 1 cycle at 72 °C for 5 min; and kept at 16 °C. Experiments were performed in triplicate.

2.10. Genomic and Phylogenetic Analysis

The *rpoD* gene was amplified from strains QE6 and QL9 genomic DNAs using primers PsEG30F and PsEG790R, and the resulting sequences were compared with those of 213 *Pseudomonas* species obtained from NCBI. Phylogenetic analysis was performed using MEGA X,[51] visualized with iTOL6.0.[52] Average nucleotide identity (ANI) was calculated using fastANI v1.3 (species cutoff: 95%),[53] and the digital DNA−DNA hybridization (dDDH) value was calculated using the Genome-to-Genome Distance Calculator 3.0 (http://ggdc.dsmz.de/). Orthologous gene clusters were inferred using OrthoFinder v2.3.2 based on the proteomes of strains QE6, QL9, and 15 closely related *Pseudomonas* species (21 strians).[54] A genome-based phylogenetic tree was constructed from these orthogroups. The orthogroup data sets generated by OrthoFinder were further used to identify QS genes, and the copy numbers of these genes were mapped onto the tree. AHL- and DSF-degrading enzymes were compared with those of *P. nitroreduces* HS-18 (AigA−C, and DigA−D).[55,56] Candidate 3-OH PAME QQ enzymes (hydrolases ELP86, ELP96, ELP104, and EstDL33), which belong to α/β-hydrolase and β-lactamase domain-containing proteins, were identified based on InterPro entries IPR000073 and IPR001466.[35]

2.11. Construction of QE6 and QL9 Gene Deletion Mutants

To knock out the predicted QQ genes, the upstream and downstream DNA fragments were amplified using primer pairs −UF/−UR and −DF/−DR listed in Table S2 with Phanta Super-Fidelity DNA Polymerase (Vazyme Biotech, Nanjing, China). The fragments were then fused and ligated into *Bam*HI- and *Hind*III-digested suicide vector pK18*mobsacB* using the C112−01 ClonExpress II One Step Cloning Kit (Vazyme Biotech, Nanjing, China). The recombinant vector was transformed into competent *E. coli* DH5α cells and subsequently transferred to wild-type strain cells via triparental mating with the help of plasmid pRK2013. Colonies resistant to 10% sucrose were selected on LB agar plates and verified by PCR using the −F/−R primers (Table S2). All mutants were constructed using similar methods. The deletion of genes was confirmed through sequence analysis.

2.12. Heterologous Expression of Putative Alpha/Beta-Hydrolase Genes

Following a previously described method,[35] the target genes (*abhA*-*abhQ*) encoding IPR000073 domain−containing alpha/beta-hydrolase homologous to ELP96 and EstD33 were amplified from strain QL9 with primers listed in Table S2, cloned into the pUC19 vector using a homologous recombination-based strategy, and transformed into *E. coli* DH5α to evaluate their potentials of esterase activities and their involvement in the degradation of 3-OH PAME/3-OH MAME. *E. coli* DH5α harboring pUC19 was used as a negative control.

2.13. LC-MS Analysis of DSF, 3-O-C6-HSL, and 3-O-C8-HSL Degradation by Strains QE6 and QL9

Strains QE6 and QL9 were precultured overnight in LB medium, harvested, washed, and resuspended in MSM to an OD_{600} of 0.5. The cultures were then inoculated (1:100, v/v) into 5 mL of MSM supplemented with 10 μM DSF, 3-O-C6-HSL or 3-O-C8-HSL, and incubated at 30 °C with shaking at 200 rpm in the dark for 24 h. A sterile control was included. After incubation, the samples were extracted with 20 mL of ethyl acetate, evaporated at 42 °C, and the residue was resuspended in 1 mL of methanol for subsequent LC-MS analysis.

DSF was analyzed using a Q Exactive Focus Liquid Mass Spectrometer (Thermo Fisher) with a Waters ACQUITY UPLC HSS T3 column (100 Å, 1.8 μm, 2.1 × 100 mm; SKU: 186003539). Conditions were set as follows: mobile phase, methanol:water (80:20, v/v); flow rate, 0.3 mL/min; column temperature, 40 °C; injection volume, 10 μL; characteristic ion, $[M-H]^-$, m/z 211.16; retention time, 4.0−6.0 min.

For 3-O-C6-HSL and 3-O-C8-HSL, a gradient elution was applied from 5% to 100% methanol containing 0.01% formic acid over 12 min at a flow rate of 0.4 mL/min. Detection was conducted in positive ion mode at $[M + H]^+$, with m/z 214.10 for 3-O-C6-HSL (retention time

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5.5−6.5 min) and m/z 242.13 for 3-O-C8-HSL (retention time 5.5−7.0 min). All experiments were performed in triplicate.

2.14. Detection of QS Quenching Activity of the Mutants

For the detection of AHL signals, the biosensor strain *Agrobacterium tumefaciens* CF11 (*traR*; *tra*::*lacZ749*) was used.[57] Strains QE6, QL9, and the *aig* mutants were cultured using 3-O-C8-HSL as the sole carbon source, following previously described methods.[56] Both qualitative and quantitative analyses of 3-O-C8-HSL in the cell-free supernatants were performed, and degradation levels in the mutants were compared with their respective wild-type strains.

DSF degradation was assessed using MSM plates containing DSF as the sole carbon source, following previously described methods.[55] Wild-type strains and *dig* mutants were inoculated on the plates, and DSF degradation was evaluated by measuring the clear zones around the colonies. The degradation ability of mutants was compared to that of the wild-type strains. All experiments were performed in triplicate.

2.15. Evaluation of the Biocontrol Efficacy of Strains QE6 and QL9

To assess the biocontrol efficacy of strains QE6 and QL9 against *R. solanacearum* NS25, peanut, tomato, and *Casuarina equisetifolia* seedlings were inoculated using previously reported methods.[37,48] Briefly, strain NS25 (OD_{600} = 1.2) was mixed 1:1 with LB (positive control), QE6, QL9 or *E. coli* DH5α carrying pUC19, pUC19-ELP96, pUC19-EstD33, pUC19-*abhA1*−*3*, pUC19-*abhG*, or pUC19-*abhI* cultures. QE6, QL9, or LB was each mixed 1:1 with CPG medium as negative controls. For *C. equisetifolia*, 10 mL of each mixture was irrigated onto seedlings with one-third of the fibrous roots trimmed. For peanuts and tomatoes, 200 μL of each mixture was injected into the stems. Disease symptoms were recorded every 24 h.

To determine the control of rice bacterial leaf blight caused by *Xoo*, strains QE6 and QL9, their *dig* mutants, and *Xoo* were cultured overnight in LB or NA medium and adjusted to OD_{600} = 1.0. The QQ strains or *dig* mutants were mixed with a *Xoo* suspension (1:1, v/v). QQ strain with PBS, *Xoo* with PBS, and sterile PBS (negative control) were also prepared. The mixtures were centrifuged at 3000 rpm for 10 min, resuspended in PBS, and then inoculated onto rice seedlings using the leaf-clipping method. The plants were maintained under greenhouse conditions, and disease severity was evaluated based on lesion length measurements.

To evaluate the control of potato soft rot caused by *Pcc*, strains *Pcc*, QE6, QL9, and their *aig* mutants were cultured overnight in LB medium and adjusted to OD_{600} = 1.0. Potato tubers were surface sterilized, sliced, and inoculated at the center of each slice with 2.5 μL of bacterial suspensions (*Pcc* + QQ strain or *Pcc* + PBS). After incubating at room temperature for 24 h, the lesion areas were measured. All experiments were performed in triplicate.

2.16. Statistical Analyses

The statistical analysis was performed using GraphPad Prism 10.1.2 software (San Diego, CA, USA). Ordinary one-way ANOVA and Student's *t*-test were used to analyze the data, where * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$, and **** indicates $p < 0.0001$.

3. RESULTS

3.1. Screening and Identification of 3-OH PAME-Degrading Strains from the Rhizospheres of Diseased Tomato and Chili Pepper Plants

In this study, 7 bacterial strains capable of utilizing 3-OH PAME as the sole carbon source were isolated from rhizosphere soils of diseased tomato and chili pepper plants collected at Qilin North Farm, South China Agricultural University, Guangzhou, Guangdong, China. Among them, strains QE1, QE6, and QE9 originated from tomato soils, while QL2, QL4, QL5, and QL9 were isolated from chili soils (Table S3).

The 16S rDNA sequences of these strains were analyzed by using BLASTn to query the NCBI database. Strains QE1 and QL5 were identified as *Pseudomonas knackmussii*, whereas strains QE9, QL2, and QL4 were assigned to *Acinetobacter junii*, *Cytophaga* sp., and *Stenotrophomonas maltophilia*, respectively. Strains QE6 and QL9 were classified as *Pseudomonas* sp. (Table S3), suggesting that the ability to degrade 3-OH PAME may be a common characteristic within the *Pseudomonas* genus. To achieve a higher taxonomic resolution, the *rpoD* gene was sequenced. Strains QE1 and QL5 showed 99.32% and 99.05% identity, respectively, to *P. knackmussii* B13. In contrast, QE6 and QL9 exhibited 95.54% and 96.65% identity to *P. nitritireducens* WZBFD3−5A2 and *P. nicosulfuronedens* LAM1902, respectively, both below the 98% species delineation threshold.[40] Therefore, QE6 and QL9 were selected for further analysis.

The 3-OH PAME degradation efficiencies of the selected strains were evaluated by culturing them in MSM supplemented with 1 μM 3-OH PAME as the sole carbon source for 7 days. Residual 3-OH PAME was extracted with dichloromethane, quantified by LC−MS, and compared with a blank control to determine the degradation rates. QE6 and QL9 degraded 95.52% and 97.29% of the substrate, respectively (Figure 1A).

3.2. High-Efficiency Degradation of 3-OH MAME by Strains QE6 and QL9

Early studies revealed that RSSC phylotype IIA strains, such as K60 and AW1, produce 3-OH PAME,[30] whereas the model strain GMI1000 synthesizes 3-OH MAME.[31] Sequence comparisons of PhcB proteins from these two groups revealed significant differences within the methyltransferase domain, suggesting that these structural differences may be essential for substrate specificity.[31] In this study, RSSC strains EP1[58] (from eggplant) and NS25[37] (from *C. equisetifolia*) were analyzed, and their PhcB proteins were identical to those of GMI1000 and OE1−1 (Figure S1A,B), implying that these strains are also likely producers of 3-OH MAME (Figure S1B). Subsequent LC-MS analysis confirmed that both EP1 and NS25 indeed synthesize 3-OH MAME (Figure S1C).

Given that 3-OH MAME serves as another key signaling molecule in the *phc* QS system of many RSSC strains,[28,59] it was important to evaluate the degradation capacity of strains QE6 and QL9 toward this compound. When cultured with 1 μM 3-OH MAME as the sole carbon source for 24 h, strains QE6 and QL9 degraded 70.96% and 77.51% of the substrate, respectively—significantly more than the uninoculated control (Figure 1B). These results demonstrate that both QE6 and QL9 efficiently degrade the *phc* QS signals produced by RSSC.

Previous structural analog studies indicated that the (3R)-hydroxy group, methyl ester moiety, and acyl chain length are critical for eliciting QS responses in *phcB* mutants.[31] Since both 3-OH PAME and 3-OH MAME are ester compounds, we further assessed the esterase activity of QE6 and QL9 using glycerol tributyrate (tributyrin) as a model substrate.[34,46] The results showed that both strains exhibit comparable tributyrin-degrading activities, with esterolytic efficiency increasing over time (Figure 1C).

3.3. Genome Sequencing and Phylogenetic Analysis Identify QE6 and QL9 as Novel *Pseudomonas* Species

To further elucidate the molecular basis of the 3-OH PAME and 3-OH MAME quenching activities in strains QE6 and QL9, their genomes were sequenced. Assembly of both QE6 and QL9 genomes generated complete circular chromosomes with sizes of 6,159,158 and 6,478,654 bp, encoding 5491 and 5989 coding sequences (CDSs), respectively (Figure 2, Table 1).

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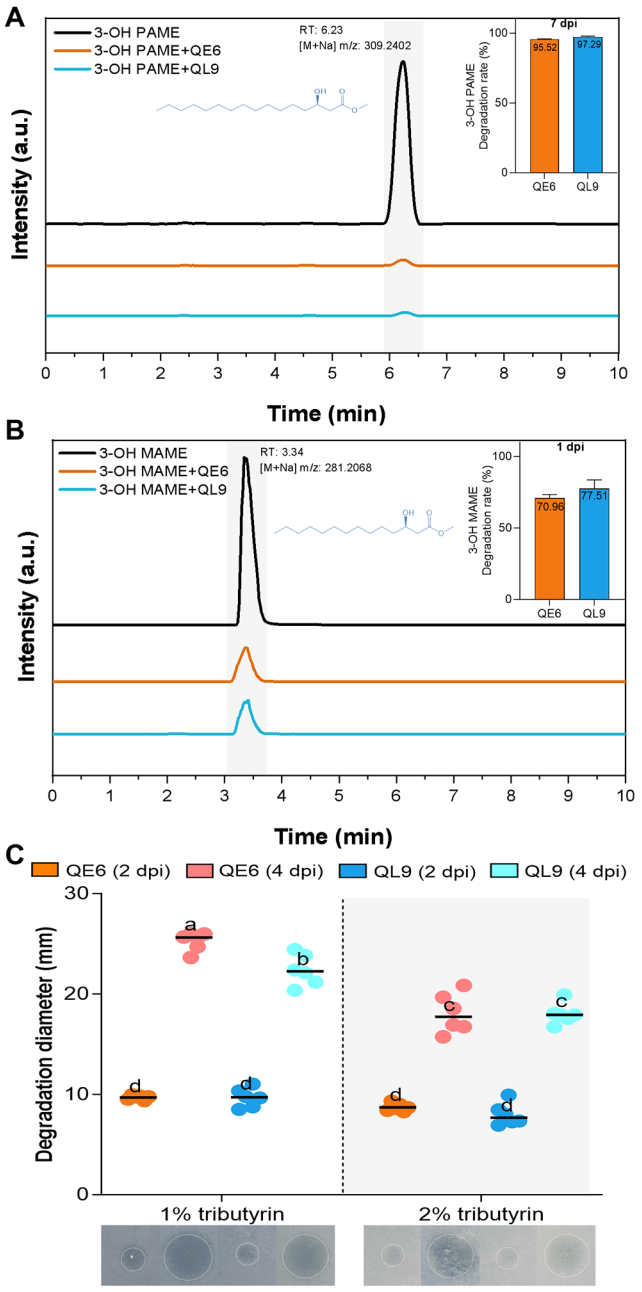

Figure 1. Degradation of 3-OH PAME and 3-OH MAME, and esterase activity of strains QE6 and QL9. (A) LC-MS chromatograms showing the degradation of 3-OH PAME, monitored at the characteristic ion $[M + Na]^+$ (m/z = 309.24) within the retention time window of 5.5−6.5 min. (B) LC−MS chromatograms illustrating the degradation of 3-OH MAME, detected via the characteristic ion $[M + Na]^+$ (m/z = 281.20) within the retention time window of 3.0−4.0 min. (C) Esterase activity assay of strains QE6 and QL9. Bacterial cultures (1 μL, OD_{600} = 1.2) were spotted onto LB agar containing 1% or 2% tributyrin and incubated at 28 °C. Halo diameters indicating degradation were measured at 2 and 4 days post inoculation (dpi). Statistical significance was determined by one-way ANOVA; different letters denote significant differences ($p < 0.05$).

To determine the taxonomic classifications of QE6 and QL9, we compared their genomes with 318 *Pseudomonas* type strain genomes available in the NCBI RefSeq database, which altogether represent all of the known *Pseudomonas* species (Table S4). Genome-based homology analysis revealed that strain QE6 is most closely related to *P. nitritireducens* WZBFD3−5A2^T, with an average nucleotide identity (ANI) of 89.7201%, a genome coverage of 78.81%, and a digital DNA−DNA hybridization (dDDH) value of 35.2%. Strain QL9 exhibited the highest identity to *P. nicosulfuronedens* LAM1902^T, with an ANI of 92.8145%, a genome coverage of 83.05%, and a dDDH value of 46.5% (Tables S4 and S5). All ANI and dDDH values were well below the commonly accepted thresholds for species delineation (ANI ≥ 95% and dDDH ≥ 70%), supporting the classification of strains QE6 and QL9 as novel *Pseudomonas* species.

In addition, the BIOLOG GEN III MicroPlate analysis showed that strains QE6 and QL9 exhibited highly similar phenotypic profiles. Both strains utilized α-D-glucose, D-fructose-6-phosphate, D-serine, L-arginine, L-glutamic acid, glucuronamide, quinic acid, L-lactic acid, citric acid, α-ketoglutaric acid, L-malic acid, γ-aminobutyric acid, propionic acid, and acetic acid as carbon sources. Minor differences were observed, with strain QL9 able to utilize mucic acid and D-gluconic acid, whereas strain QE6 was not. In chemical sensitivity assays, both strains grew at pH 6.0 and tolerated 1% NaCl, but showed reduced growth at pH 5.0 and in the presence of 4−8% NaCl. Both strains were resistant to D-serine, rifamycin SV, minocycline, lincomycin, vancomycin, nalidixic acid, potassium tellurite, and aztreonam (Table S6). Comparison with the BIOLOG database indicated that the phenotypic fingerprints of strains QE6 and QL9 were most similar to those of *P. nitroreduces*, with moderate confidence [QE6: PROB (Probability) = 0.969, SIM (Similarity Index) = 0.667, DIST (Distance) = 4.734; QL9: PROB = 0.912, SIM = 0.667, DIST = 4.972].

Based on their colony characterization (described below), we propose the names of *Pseudomonas procumbens* sp. nov. for strain QE6 and *Pseudomonas polia* sp. nov. for strain QL9.

3.4. Transmission Electron Microscopy Phenotypic Characterization of Strains QE6 and QL9

TEM revealed that cells of strains QE6 and QL9 were rod-shaped with rounded ends, measuring approximately 1.6−2.0 μm in length and 0.5−0.7 μm in width. A single polar flagellum was observed in both strains (Figure S2A).

3.4.1. Colony Morphology. After 48 h of incubation on LB agar at 28 °C, single colonies of strain QE6 measured 2−4 mm in diameter, appeared white, nearly round, flat with slightly central convex surfaces, and displayed smooth surfaces with irregular margins. In contrast, single colonies of strain QL9 were 2−3 mm in diameter, moist, grayish-white, round, smooth, and developed dispersed edges in later incubation stages (Figure S2B).

3.4.2. Antagonism against the NS25 Pathogen. The antagonistic activities of QE6 and QL9 against *R. solanacearum* NS25 were assessed by using a plate confrontation assay. While the positive control, *P. wayambapalatensis* strain L7,[48] formed a clear inhibition zone around the well on NS25-lawn plates, neither QE6 nor QL9 produced any visible hyaline ring (Figure S2C), indicating a lack of direct antagonistic effect against NS25.

3.4.3. Predation toward the NS25 Pathogen. Predation assays further confirmed that neither QE6 nor QL9 produced secondary metabolites or exhibited predatory behavior against NS25 (Figure S2D).

3.4.4. Cell Motility. To evaluate motility, swimming and swarming motilities (mediated by flagella) and twitching motility (mediated by type IV pili, T4P) were examined. Both strains exhibited active movement on solid surfaces and at

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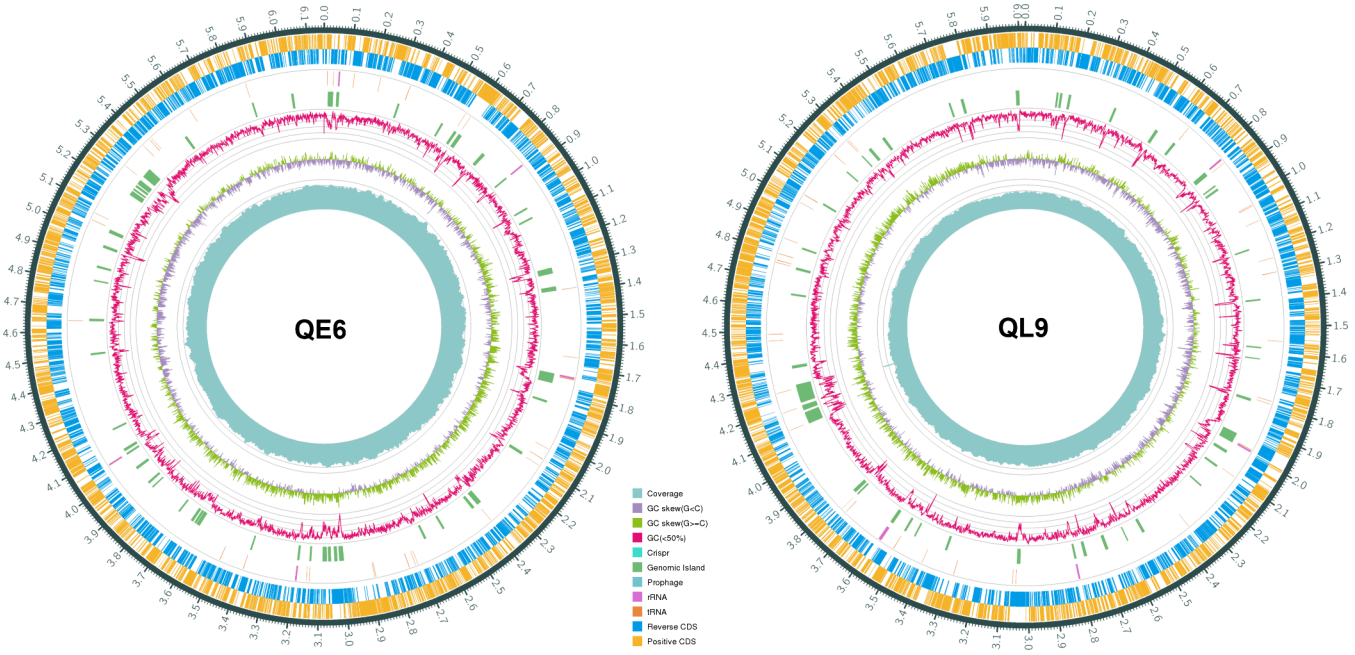

Figure 2. Circular genome maps of *Pseudomonas procumbens* sp. nov. QE6 and *P. polia* sp. nov. QL9. The outer to the inner rings indicate genes on the direct strand, genes on the complementary strand, tRNAs (orange), rRNAs (purple), CRISPR sequences (blue), genomic islands (green), and GC skew.

Table 1. Genomic Features of Strains QE6 and QL9

feature	QE6	QL9
size (bp)	6,159,158	6,478,654
GC content (%)	65.23	65.07
CDS	5,491	5,989
rRNA	16	16
tRNA	66	70
CRISPR	0	4
genomic island	50	49
prophage region	1	2

solid−liquid interfaces. Notably, QL9 showed stronger swimming but weaker twitching motility compared to QE6 (Figure S2E), which may reflect adaptations to their respective environmental origins.

3.4.5. Biofilm Formation. Biofilms, protective membrane-like structures formed during bacterial surface attachment and proliferation, contribute to stress resistance.[60] When they were cultured on SOBG medium, both QE6 and QL9 were capable of forming biofilms. QE6 produced more substantial biofilms than QL9 (Figure S2F), implying a stronger potential for plant colonization.

3.4.6. Antibiotics Sensitivity. The antibiotic susceptibility profiles of QE6 and QL9 were tested against six antibiotics. Both strains were sensitive to kanamycin, streptomycin, gentamicin, and polymyxin B, but resistant to ampicillin. Additionally, QL9 showed a higher resistance to tetracycline (Table S7).

3.5. Strains QE6 and QL9 Reduced Both EPS Production and Cell Wall Degrading Enzyme Activity in *R. solanacearum* NS25

To evaluate the impact of QE6 and QL9 on EPS production, we compared the EPS phenotypes of NS25, the two quenching strains, and their cocultures. Radar phenotyping revealed that NS25 colonies produced smooth and viscous EPS, whereas QE6 and QL9 formed dry, wrinkled, and irregular colonies. In mixture cultures, the EPS surface morphology resembled that of QE6 or QL9 rather than NS25 (Figure 3A), indicating that both strains inhibit EPS production of NS25.

On TTC medium, NS25 colonies appeared contiguous and mucoid, with thick, white EPS margins. In contrast, QE6 and QL9 formed flat colonies with dry, diffuse edges. In mixed inoculations, no contiguous NS25-like colonies were observed, and EPS accumulation was markedly reduced, similar to the morphology of QE6 and QL9 colonies (Figure 3B). These results further support the inhibitory effects of QE6 and QL9 on EPS production in NS25.

On Cel and Peh assay plates, NS25 mixed with QE6 or QL9 exhibited significantly reduced enzymatic activity compared to NS25 alone (Figure 3C), indicating that both strains also impaired the PCWDE production of NS25.

To investigate the consequence of the 3-OH MAME quenching caused by strains QE6 and QL9, we measured the expression of the *phc* QS-regulating downstream genes. The results showed that coculture of NS25 with QE6 or QL9 resulted in slightly downregulated expression of the *phc* system (*phcA* and *phcS*) and significantly downregulated expression of genes related to EPS and cellulase production (Figure 3D). This indicates that quenching of 3-OH MAME caused by strains QE6 and QL9 could reduce the level of expression of *phc*-regulated genes.

3.6. Multiple Quorum-Quenching Genes Were Predicted in QE6 and QL9 Genomes

Phylogenetic analysis based on the *rpoD* gene, conducted with 213 *Pseudomonas* strains, placed QE6 and QL9 within the *P. aeruginosa* group, showing close relatedness to the known DSF- and AHL-degrading strain *P. nitroreduces* HS-18 (Figure 4A).[55,56] Whole-genome comparisons with 21 strains (19 type strains, QL5 and HS-18) from the *P. aeruginosa* group using ANI values confirmed that QE6 and QL9 represent novel species, forming a cluster adjacent to HS-18 (Figure 4B). OrthoFinder analysis identified orthologous genes encoding DSF-degrading

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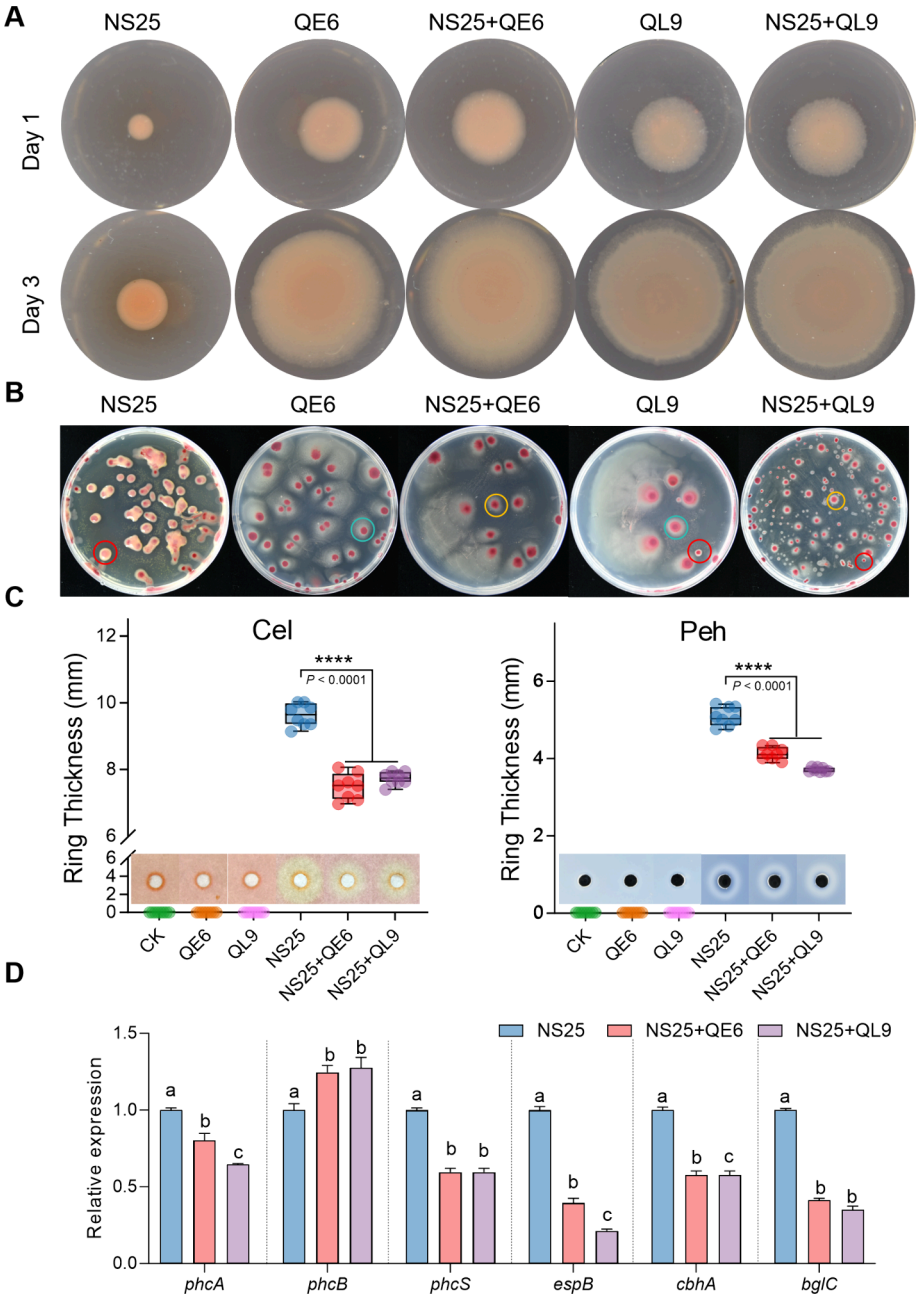

Figure 3. Strains QE6 and QL9 suppressed EPS production and reduced cellulase (Cel) and polygalacturonase (Peh) activities in *R. solanacearum* NS25. (A) EPS production by NS25 was inhibited by QE6 and QL9 on YEB medium. (B) EPS production of NS25 was reduced in the presence of QE6 and QL9 on TTC plates. NS25, QE6, and QL9 colonies were circled in red, blue, and yellow, respectively. (C) Both QE6 and QL9 significantly decreased the enzymatic activities of Cel and Peh in NS25. (D) Expression of 3-OH PAME/MAME-regulated genes in *R. solanacearum* NS25 was analyzed by RT-qPCR in three groups: NS25, NS25 + QE6, and NS25 + QL9. Statistical significance was determined by one-way ANOVA; **** indicates $p < 0.0001$ and significantly different values (ANOVA $p < 0.05$) are indicated by different letters.

(DigA−D) and AHL-degrading enzymes (AigA−C). Notably, both genomes harbor multiple α/β-hydrolase (IPR000073) domain−containing proteins, a conserved feature of experimentally validated 3-OH PAME-quenching enzymes such as ELP96 and EstD33,[35] indicating a putative role of these homologues in 3-OH PAME signal degradation. We named them AbhA−AbhQ (α/β-hydrolase, *abh*) (Figure 4B, Table S8). The copy numbers of these genes were mapped onto the phylogenetic tree (Figure 4B). The results showed that all species within the *P. aeruginosa* group probably possessed known 3-OH PAME, DSF, and AHL-degrading enzymes.

3.7. Strains QE6 and QL9 Are Capable of Degrading DSF and AHL QS Signals

To experimentally validate the DSF and AHL quenching capabilities of QE6 and QL9, both strains were cultured with DSF, 3-O-C6-HSL, or 3-O-C8-HSL as the sole carbon source. After 24 h, LC−MS analysis revealed the complete degradation of DSF by both strains (Figure 4C). Under the same conditions, 3-O-C6-HSL was degraded by 79.30% and 79.81% in cultures of QE6 and QL9, respectively, compared to the uninoculated control (Figure 4D). In addition, 3-O-C8-HSL was degraded by 83.98% and 85.95% by QE6 and QL9, respectively (Figure 4E).

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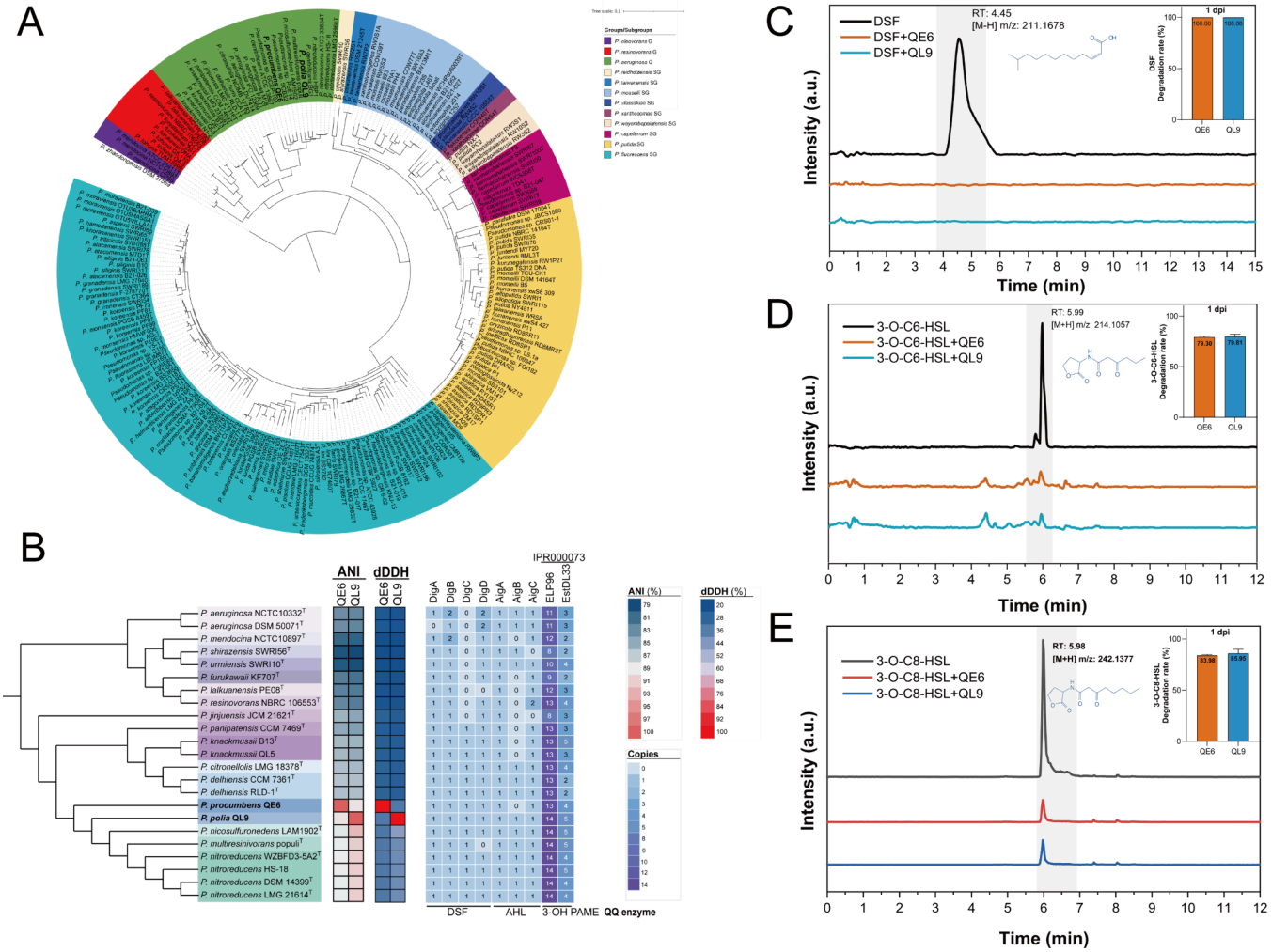

Figure 4. Phylogenetic and quorum-quenching enzyme analyses of strains QE6 and QL9. (A) Phylogenetic tree constructed from the *rpoD* gene sequences of *Pseudomonas* strains QE6, QL9, and other *Pseudomonas* strains retrieved from the NCBI database. The tree was built using the Maximum Likelihood method with GTR+G+I model in MEGA X. Bootstrap values were calculated from 1,000 replications; only values higher than 50% are shown. (B) Genome-based phylogenetic tree of QE6, QL9, and 15 closely related *Pseudomonas* species (21 strains), generated with OrthoFinder. Average Nucleotide Identity (ANI) values were calculated using fastANI v1.3 with a 95.0% threshold and digital DNA−DNA hybridization (dDDH) values were calculated using GGDC. Orthogroup data sets from OrthoFinder were used to identify quorum-quenching (QQ) genes, and their copy numbers are illustrated on the tree. AHL- and DSF-degrading enzymes (AigA−C, DigA−D) were compared with those of *P. nitroreduces* HS-18. Candidate quorum-quenching α/β-hydrolase domain-containing proteins, homologous to ELP96 and EstD33, were identified based on the conserved InterPro domain IPR000073. (C) Degradation of DSF by strains QE6 and QL9. LC-MS chromatogram showing the degradation of DSF, monitored at the characteristic ion $[M-H]^-$ with m/z 211.16 and a retention time range of 4.0−6.0 min. (D) LC−MS chromatogram illustrating the degradation of 3-O-C6-HSL, detected via the characteristic ion $[M + H]^+$ with m/z 214.10 and a retention time range of 5.5−6.5 min. (E) LC−MS chromatogram illustrating the degradation of 3-O-C8-HSL, detected via the characteristic ion $[M + H]^+$ with m/z 242.13 and a retention time range of 5.5−7.0 min.

These findings confirm that QE6 and QL9 are proficient in degrading multiple QS signals, including DSF and AHLs.

3.8. QE6 and QL9 Effectively Control Bacterial Wilt Caused by *R. solanacearum*

To determine the roles of strains QE6 and QL9 in controlling bacterial wilt caused by *R. solanacearum*, greenhouse pot experiments were conducted. Seedlings of peanut, tomato, and *C. equisetifolia* were inoculated via root drenching or stem injection according to plant-specific methods. In peanut plants inoculated with NS25 alone, wilting symptoms emerged within 2 days post inoculation (dpi), and all seedlings succumbed by 8 dpi. In contrast, co-inoculation with NS25+QE6 or NS25+QL9 resulted in only mild wilting in a single seedling, with a final mortality rate of 10% at 8 dpi; the majority of plants remained healthy (Figure 5A, B). In tomato, wilting began at 2 dpi in the NS25-treated group, leading to complete death by 9 dpi. Seedlings treated with NS25+QE6 or NS25+QL9 exhibited significantly reduced disease incidence, with final mortality rates of 20% and 10%, respectively, at 9 dpi. No symptoms were observed in the other treatment groups (Figure 5A,C). In *C. equisetifolia*, wilting first appeared at 7 days post inoculation (dpi) in the NS25-inoculated seedlings. By 46 days post incubation, severe wilt symptoms were evident in the NS25 group, whereas plants treated with NS25+QE6 or NS25+QL9 remained markedly healthier and comparable to the uninoculated controls (Figure 5A, D).

In addition, tomato seedling growth was monitored for 1 week after inoculation. Seedlings inoculated with *R. solanacearum* NS25 exhibited typical wilting symptoms, whereas seedlings treated with QE6 or QL9 displayed healthy growth with no visible disease symptoms (Figure 5E). Measurements of root

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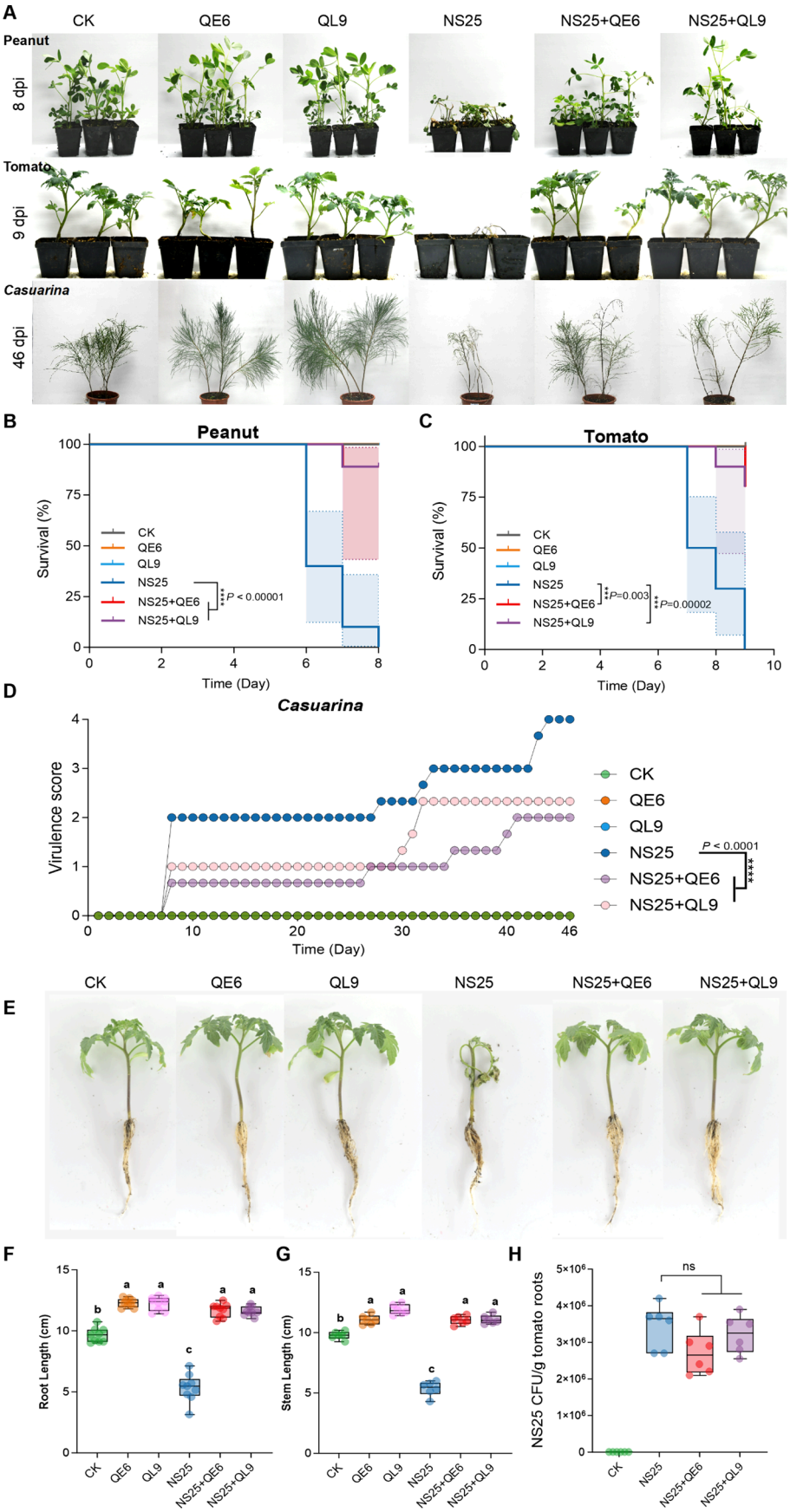

Figure 5. Symptoms of bacterial wilt in peanut, tomato, and *Casuarina equisetifolia* seedlings inoculated with strains NS25, QE6, and QL9. (A) Growth status of plants recorded at the indicated time points. (B, C) Survival curves of plants inoculated with NS25 and the quenching bacteria. (D) Disease index curves of *C. equisetifolia* plants inoculated with NS25 and the quenching bacteria from 1 to 46 dpi. Peanut and tomato seedlings were inoculated

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Figure 5. continued

via stem injection with 200 μL of bacterial mixtures (n = 10). *C. equisetifolia* seedlings were inoculated by root irrigation with 10 mL of the following mixtures (OD_{600} = 1.2) after root wounding: LB+CPG, QE6+CPG, QL9+CPG, NS25+LB, NS25+QE6, and NS25+QL9. (E) Morphology of tomato seedlings 1 week post inoculation washed with sterile water. (F) Root length of tomato seedlings. (G) Stem length of tomato seedlings. (H) Number of *R. solanacearum* NS25 colony-forming units (CFU) in tomato roots. Statistical analysis was performed using one-way ANOVA; **** indicates p < 0.0001; ns indicates not significant; and significantly different values (ANOVA p < 0.05) are indicated by different letters.

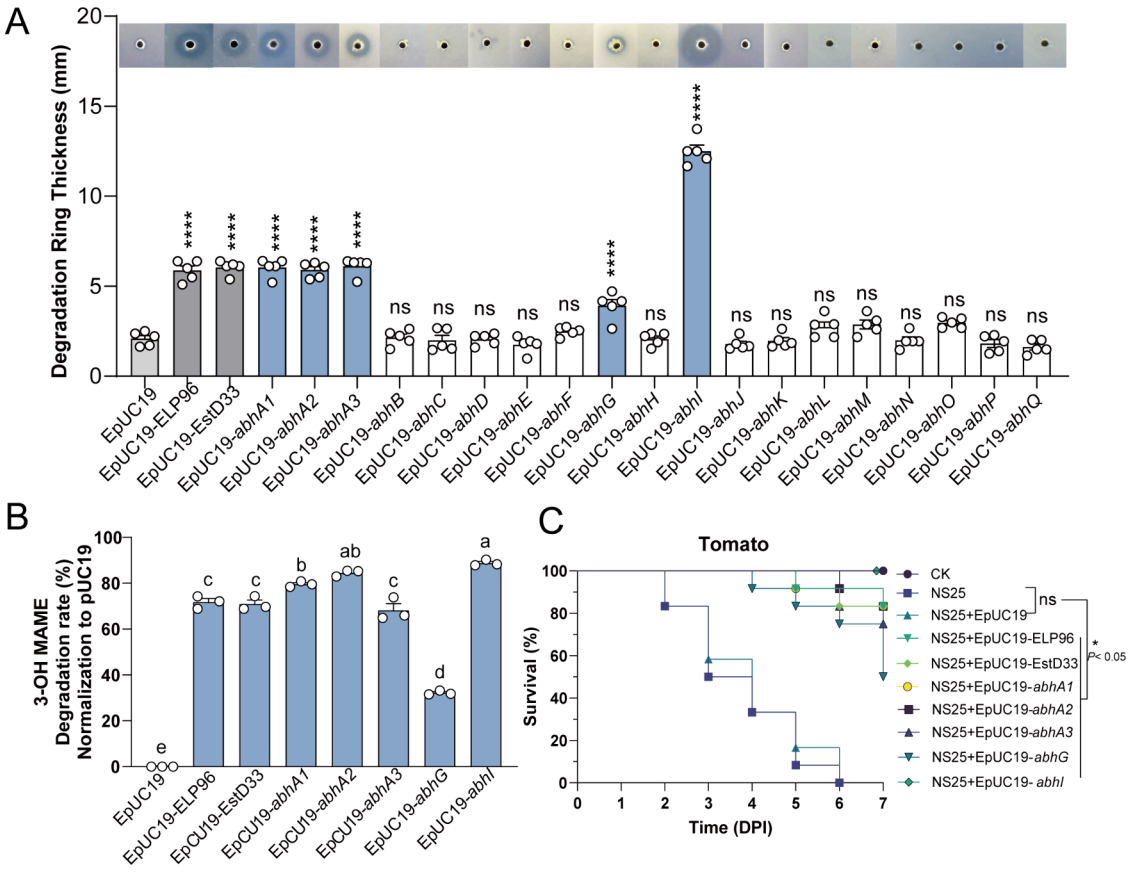

Figure 6. Heterologous expression and functional validation of candidate 3-OH PAME/MAME-degrading α/β-hydrolase proteins from QL9. (A) Preliminary esterase activity of *E. coli* DH5α expressing candidate Abh proteins (AbhA1−AbhQ) on tributyrin agar plates. ELP96 and EstD33 were used as positive controls, and *E. coli* carrying empty pUC19 served as the negative control. Clear halos indicate hydrolytic activity. Statistical significance was determined by Student's t-test; **** indicates p < 0.0001; ns indicates not significant. (B) LC-MS analysis of 3-OH MAME degradation by *E. coli* strains expressing AbhA1−3, AbhG, and AbhI. Degradation efficiencies were calculated after 24 h. Data represent mean ± SEM of three independent experiments. Statistical significance was determined by one-way ANOVA; different letters denote significant differences (p < 0.05). (C) In planta biocontrol activity of recombinant *E. coli* strains co-inoculated with *R. solanacearum* NS25 in tomato seedlings. Statistical significance was determined by one-way ANOVA; * indicates p < 0.05; ns indicates not significant.

and stem length showed that seedlings treated with QE6 or QL9 maintained significantly greater root and stem lengths compared with those of the NS25-inoculated and CK groups (Figure 5F, G). Quantification of NS25 populations in tomato roots showed no significant differences in colony-forming unit (CFU) counts among the NS25, NS25 + QE6, and NS25 + QL9 treatments (Figure 5H), suggesting that the quenching bacteria do not reduce the occurrence of bacterial wilt by inhibiting the growth of NS25.

These results indicate that strains QE6 and QL9 are effective in preventing *R. solanacearum*-induced bacterial wilt and may also promote plant growth. The strong biocontrol performance highlights the potential of both strains as promising agents for preventing *R. solanacearum* infections.

3.9. 3-OH PAME/MAME Degrading Genes in QL9 Were Functionally Verified

To further verify that the biocontrol efficacy of QE6 and QL9 against bacterial wilt is mediated through a QQ mechanism, the previously characterized 3-OH PAME-quenching enzymes ELP96 and EstD33, together with the putative α/β-hydrolase proteins AbhA1−AbhQ, were heterologously expressed in *E. coli* DH5α using the plasmid pUC19. Following previously established methods,[35] tributyrin agar plates were first used for preliminary esterase activity assays. As expected, the positive controls of *E. coli* harboring ELP96 or EstD33 produced prominent clearance zones. Compared with the negative control (*E. coli* carrying pUC19), strains expressing AbhA1−3, AbhG, and AbhI also exhibited detectable hydrolysis halos, among which AbhI generated the largest clearance zone (Figure 6A). Subsequently, strains expressing AbhA1−3, AbhG, and AbhI

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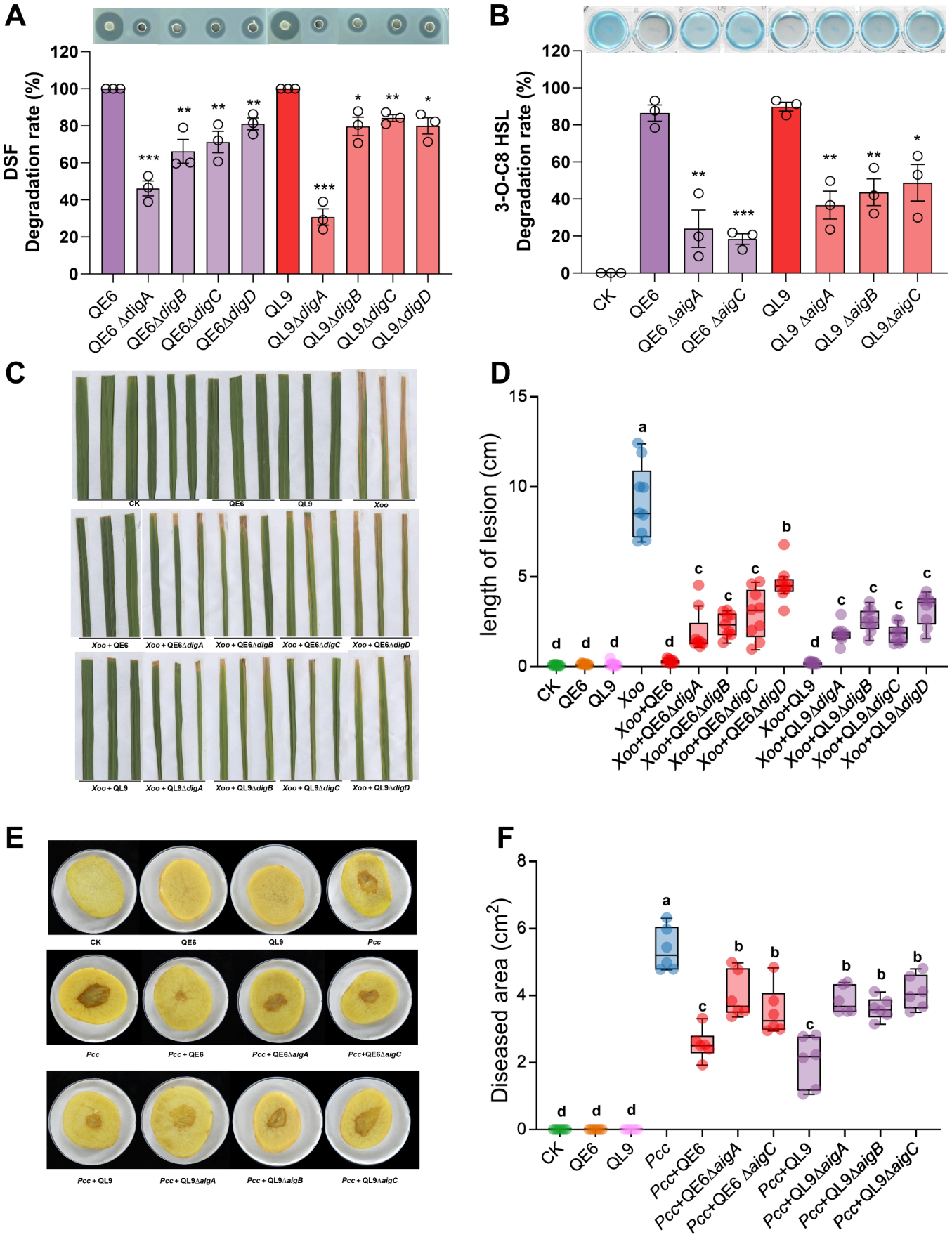

Figure 7. Biocontrol efficacy of strains QE6 and QL9 against rice bacterial blight caused by *Xoo* and potato soft rot caused by *Pcc* via QQ mechanism. (A) AHL degradation: Detected using the biosensor strain *Agrobacterium tumefaciens* CF11. Strains were cultured with 3-O-C8-HSL as the sole carbon

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Figure 7. continued

source, and supernatant degradation was analyzed. Mutants were compared with wild-type strains. (B) DSF degradation: Assessed on MSM plates containing DSF as the sole carbon source. Clear zones around colonies indicate DSF degradation; mutants were compared with wild-type strains. (C) Representative symptoms observed on rice leaves at 14 dpi using leaf-clip inoculation method. The experiment was repeated three times with similar results. (D) Measurements of lesion length on infected rice leaves. (E) Disease symptoms developed on potato tuber slices at 24 h post inoculation (hpi) using slice-injection method. (F) Quantification of lesion area on potato tubers. Statistical analysis was performed using one-way ANOVA; * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$; and significantly different values (ANOVA $p < 0.05$) are indicated by different letters.

were subjected to quantitative degradation assays using 3-OH MAME as the substrate. Compared with the negative control, strains expressing AbhA1 and AbhA3 showed degradation efficiencies comparable to those of the positive controls (ELP96 and EstD33),[35] achieving >60% degradation within 24 h. Notably, strains expressing AbhA2 and AbhI displayed stronger activity, with degradation efficiencies exceeding 80%, whereas AbhG exhibited relatively weaker activity (~30%) (Figure 6B). To assess the biological relevance of these enzymes to disease suppression in planta, the above *E. coli* strains were co-inoculated with *R. solanacearum* NS25 into tomato seedlings. Seedlings inoculated with NS25 alone or co-inoculated with NS25 and *E. coli* carrying pUC19 showed 100% mortality. In contrast, seedlings co-inoculated with NS25 and strains expressing ELP96, EstD33, or most Abh proteins exhibited significantly improved survival. Except for the AbhG treatment, which showed approximately 50% mortality, all other treatments resulted in mortality rates below 20%. Notably, seedlings treated with AbhI showed no mortality and maintained healthy growth (Figure 6C). All plants in the negative control group without pathogen inoculation remained healthy.

Collectively, these results provide functional evidence that the biocontrol activity of QE6 and QL9 against bacterial wilt is associated with the quenching of the *R. solanacearum* QS signal 3-OH MAME.

3.10. QE6 and QL9 Effectively Control Rice Bacterial Leaf Blight Caused by *Xoo* and Potato Soft Rot Caused by *Pcc* via QQ Mechanism

The biocontrol potential of the QQ strains QE6 and QL9 was evaluated using inoculation assays on host plants infected with *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), which primarily employs DSF molecules such as DSF, BDSF, and CDSF for QS,[61] and *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*), which utilizes 3-O-C6-HSL and 3-O-C8-HSL as QS signals.[62]

To verify whether the biocontrol efficacy of QE6 and QL9 against *Xoo* and *Pcc* is associated with *dig* and *aig* genes (Table S8), we individually deleted the WT strains and their relative mutants for DSF and AHL degrading activities. The results showed that mutants lacking *digA*−*digD* showed a pronounced reduction in DSF degradation efficiency compared with that of WT strains, with *digA* deletion causing the strongest effect in both strains (Figure 7A). Using the biosensor reporter strain CF11, we first confirmed that strains QE6 and QL9 did not produce 3-O-C6-HSL or 3-O-C8-HSL (Figure S3). Subsequently, 3-O-C8-HSL degradation assays showed a similar pattern, where disruption of the *aig* genes in these strains significantly reduced their ability to degrade AHL compared with the wild-type strains (Figure 7B).

Consistent with these biochemical assays, the loss of *dig* or *aig* genes substantially weakened the biocontrol efficacy of QE6 and QL9 in planta. In rice leaf inoculation assays, *Xoo*-induced lesion lengths were strongly suppressed by wild-type QE6 and QL9, whereas deletion mutants displayed significantly increased lesion development (Figure 7C, D). Among the mutants, Δ*digA* strain showed the greatest loss of protective capacity, indicating a central role of *digA* in disease suppression. A similar trend was observed in potato tuber assays challenged with *Pcc*. While wild-type QE6 and QL9 markedly reduced maceration symptoms, mutants lacking *aig* genes exhibited significantly larger diseased areas (Figure 7E, F). Notably, deletion of *aig* genes led to a pronounced increase in the level of tissue maceration.

Antagonism assays confirmed that QE6 and QL9 did not directly inhibit pathogen growth (Figure S4), indicating that their biocontrol effects are mediated specifically through QQ. Collectively, these findings demonstrate that QE6 and QL9 effectively attenuate the virulence of both *Xoo* and *Pcc* via QQ mechanisms, highlighting their potential as versatile biocontrol agents against bacterial wilt, rice bacterial leaf blight, and potato soft rot.

4. DISCUSSION

The identification of microbial enzymes capable of degrading QS signals to achieve the QQ effect has emerged as a prominent research direction in infectious disease prevention and control.[21−23] A large number of Gram-negative bacteria utilize AHLs as QS signaling molecules. Consequently, QQ has been proposed as a novel biocontrol strategy against AHL-dependent bacterial pathogens.[63] Current studies on the QQ-mediated resistance to bacterial wilt have primarily focused on AHL-quenching enzymes,[64] with limited attention given to enzymes targeting 3-OH PAME or 3-OH MAME—signaling molecules that play a critical role in regulating virulence in *R. solanacearum*.[59] Recent efforts have involved the chemical synthesis of QS inhibitors that competitively block the binding of 3-OH MAME to PhcS, attenuating the virulence of *R. solanacearum* OE1−1 in tomato.[65] Additionally, four 3-OH PAME hydrolases identified from soil macrogenomes were shown to inhibit the virulence factor production in *R. solanacearum*.[35] Earlier, a β-HPMEH enzyme from *Ideonella* sp. demonstrated 100% degradation efficiency toward 3-OH PAME.[34] However, the biocontrol efficacy of these enzymes against plant bacterial wilt remains unvalidated.

This study aimed to identify QQ-active strains capable of degrading 3-OH PAME to mitigate *R. solanacearum*-induced bacterial wilt. Notably, two novel species, *P. procumbens* sp. nov. (QE6) and *P. polia* sp. nov. (QL9), were isolated from tomato and chili pepper rhizospheres and showed promising wilt-suppressive effects in peanut, tomato, and *C. equisetifolia* (Figure 4). Both strains exhibited robust esterase activity and efficiently degraded 3-OH PAME and 3-OH MAME (Figure 1A, part B). These findings suggest that QE6 and QL9 hydrolyze the ester bond of QS signals, releasing long-chain fatty acids that are further metabolized via β-oxidation to support bacterial growth and provide biosynthetic precursors.[34,66,67] This underscores the potential of rhizospheric bacteria as sustainable QQ agents for managing plant bacterial diseases.

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The genus *Pseudomonas*, comprising diverse Gram-negative bacteria, is widely recognized for its biocontrol potential against plant pathogens.[68] Many *Pseudomonas* species serve as effective biological control agents (BCAs), capable of root colonization and broad-spectrum antagonism through the production of bioactive secondary metabolites (SMs), such as antibiotics, siderophores, and volatile organic compounds (VOCs).[17,68] Additionally, QQ-active *Pseudomonas* strains from rhizospheric and phyllospheric environments exhibit extracellular enzymatic activity that enhances biocontrol utility.[69] For instance, *Pseudomonas* sp. strain G synthesizes carbamoylphosphate to degrade DSF signals and reduce cabbage black rot,[70] while *P. aeruginosa* SJ01 degrades DSF to mitigate citrus canker.[71] Similarly, *P. segetis* P6 degrades AHLs to attenuate potato soft rot,[72] and a 3-OH PAME-degrading *P. aeruginosa* isolate inhibits EPS production in *R. solanacearum*, controlling eggplant wilt under greenhouse conditions.[36]

In this study, strains QE6 and QL9 were phylogenetically assigned to the *P. aeruginosa* group (Figure 4A) and showed high degradation activity toward 3-OH PAME and 3-OH MAME, along with strong esterolytic activity (Figure 1). Unlike typical *P. aeruginosa* group members that rely on bacteriostatic secondary metabolites for pathogen inhibition,[73,74] QE6 and QL9 showed no detectable direct antagonism against *R. solanacearum* NS25, *Xoo,* and *Pcc* (Figures S2C,D and S4). Instead, they attenuated wilt symptoms primarily by quenching 3-OH PAME and 3-OH MAME signaling, thereby reducing the production of Cel, Peh, and EPS in *R. solanacearum* (Figure 3).

Although fluids from *P. aeruginosa* strains have been reported to degrade 3-OH PAME,[36] it is noteworthy that QE6 and QL9 belong to this group. Furthermore, *P. forestsoilum* sp. Q1−7 and *P. tohonis* Q4−3—isolated from *C. equisetifolia* branches and displaying high 3-OH PAME quenching and biocontrol potential—fall within the closely related *P. resinovorans* group.[37] This group also includes *P. nitroreduces* HS-18, which degrades DSF and AHL signals.[55,56] Genomic analyses further indicated that members of the *P. aeruginosa* group—particularly those closely related to *P. nitroreducens*—encode enzymes for degrading DSF, AHL, and 3-OH PAME/MAME, including those containing the IPR000073 domain (Figure 4B). LC-MS confirmed that QE6 and QL9 degrade both DSF and AHL (Figure 4C−E). Therefore, we hypothesize that *Pseudomonas* strains with 3-OH PAME/MAME-quenching activity are predominantly enriched within the *P. aeruginosa* group, which may possess a combined capacity to degrade DSF, AHL, and 3-OH PAME/MAME signals. This evolutionary proximity suggests conserved genetic mechanisms for QQ in QE6 and QL9, enhancing their ecological and biocontrol relevance.

In contrast to prior QQ studies targeting single QS signals (e.g., specific AHL variants[38] or mixed inhibitors and enzymes[75]), QE6 and QL9 exhibit broad-spectrum QQ activity. Genomic analysis identified genes encoding known DSF-degrading (DigA-D) and AHL-degrading (AigA-C) enzymes as well as putative 3-OH PAME-degrading enzymes (IPR000073), providing a genetic basis for their ability to concurrently degrade multiple QS signals (Figure 4B). The heterologous expression and deletion of the predicted genes validated the degradative functions of the corresponding QS signals (Figures 6 and 7). Functionally, both strains reduced virulence factor production (EPS, Cellulases, Polygalacturonases) in *R. solanacearum* and effectively controlled bacterial wilt in multiple hosts (Figures 3 and 5). They also showed efficacy against rice bacterial leaf blight (caused by *Xoo*) and potato soft rot (caused by *Pcc*) (Figure 7), highlighting their potential as multifunctional QQ agents. These findings underscore the promise of combining diverse QQ mechanisms for the sustainable control of bacterial infections.

Environmentally, QE6 and QL9 represent eco-friendly alternatives to chemical pesticides, supporting global sustainable agriculture initiatives. Chemical pesticides, while effective, lead to environmental contamination, with up to 80% of applications affecting nontarget organisms and ecosystems.[14] In contrast, the QQ-based biocontrol selectively disrupts pathogen virulence without promoting resistance. The rhizosphere origin of QE6 and QL9 further enhances their practical applicability, as these strains are naturally adapted to soil environments and can be integrated into existing agricultural systems.[76] Although they were clustered within the *P. aeruginosa* group, they exhibited no phytotoxicity but promoted plant growth. Given the opportunistic pathogenicity of *P. aeruginosa* sensu stricto, future efforts will focus on enzyme-based QQ applications to improve biosafety. Building on these findings, previous studies demonstrated that QQ bacteria can improve disease management when combined with other strategies; for example, a T3SS inhibitor enhanced control of rice bacterial leaf blight when applied alongside QQ bacteria.[77] These observations suggest that QE6 and QL9 could be integrated with conventional methods—such as reduced chemical doses or additional biocontrol agents—for synergistic disease control.

Despite these promising findings, several limitations remain. The biocontrol efficacy of QE6 and QL9 was validated under controlled conditions, necessitating field trials to confirm their performance. Additionally, although genomic analysis suggests the presence of multiple QS signal-degrading enzymes, their functional characterization and mechanistic details require further investigation. Future work should employ synthetic biology to optimize key QQ enzymes and improve strain performance in diverse agricultural contexts. Efforts should also be directed toward elucidating the molecular basis of their broad-spectrum QQ activity and evaluating their efficacy against other QS-mediated pathogens.

In conclusion, *P. procumbens* sp. nov. QE6 and *P. polia* sp. nov. QL9 represent a paradigm shift in bacterial disease management. As novel, broad-spectrum QQ agents, they offer transformative potential for sustainable agriculture. By providing an environmentally safe alternative to chemical pesticides, these strains pave the way for innovative biocontrol strategies that enhance crop protection and global food security.

■ ASSOCIATED CONTENT

Data Availability Statement

All relevant data are within the manuscript and the Supporting Information.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.5c18073.

Production of 3-OH MAME by *R. solanacearum* strains NS25 and EP1 (Figure S1); phenotypic characterization of strains QE6 and QL9 (Figure S2); detection of 3-O-C6-HSL and 3-O-C8-HSL production by strains QE6 and QL9 (Figure S3); antagonistic activity of strains QE6 and QL9 against *Xoo* and *Pcc* (Figure S4); bacterial strains and plasmids used in this study (Table S1); PCR primers used in this study (Table S2); sequence identity of isolated strains with their closest relatives based on 16S rDNA and

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rpoD gene sequences (Table S3); digital DNA−DNA hybridization values between QE6 and QL9 and representative genomes of *Pseudomonas* (Table S5); biochemical characteristics of strains QE6 and QL9 (Table S6); and antibiotics sensitivity of strains QE6 and QL9 (Table S7) (PDF)

Average nucleotide identity (ANI) values between QE6, QL9, and the *Pseudomonas* genomes in the NCBI database (Table S4) (XLSX)

Comparative genomic analysis of putative QQ enzymes involved in DSF-, AHL-, and 3-OH PAME-mediated signaling in QE6 and QL9 and representative *Pseudomonas* type strains (Table S8) (XLSX)

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Author Contributions

[‖]X.X., Y.Y., and Y.Q. contributed equally to this work. J.Z. and M.H. conceived and designed the experiments. X.X., Y.Y., Y.Q., Z.L., Y.X., Y.O., S.Z., and M.H. isolated and identified the QQ strains and performed the corresponding functional assays, including QQ tests and phenotypic analyses of gene knockout mutants. Y.X. and M.H. constructed the phylogenetic tree. M.H. and X.Z. analyzed the genome data. Y.Q., J.Z., and M.H. wrote and revised the manuscript. X.C. and L.Z. revised the manuscript. All authors contributed to the article and approved the submitted version.

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Notes

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