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RESEARCH ARTICLE

Pseudomonas chlororaphis L5 and *Enterobacter asburiae* L95 biocontrol *Dickeya* soft rot diseases by quenching virulence factor modulating quorum sensing signal

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Abstract

Virulence factor modulating (VFM) is a quorum sensing (QS) signal shared by and specific to *Dickeya* bacteria, regulating the production of plant cell wall degrading enzymes (PCWDEs) and virulence of *Dickeya*. High polarity and trace of VFM signal increase the difficulty of signal separation and structure identification, and thus limit the development of quorum quenching strategy to biocontrol bacterial soft rot diseases caused by *Dickeya*. In order to high-throughput screen VFM quenching bacteria, a *vfmE-gfp* biosensor VR2 (VFM Reporter) sensitive to VFM signal was first constructed. Subsequently, two bacterial strains with high quenching efficiency were screened out by fluorescence intensity measurement and identified as *Pseudomonas chlororaphis* L5 and *Enterobacter asburiae* L95 using multilocus sequence analysis (MLSA). L5 and L95 supernatants reduced the expression of *vfm* genes, and both strains also decreased the production of PCWDEs of *D. zeae* MS2 and significantly reduced the virulence of *D. oryzae* EC1 on rice seedlings, *D. zeae* MS2 on banana seedlings, *D. dadantii* 3937 on potato and *D. fangzhongdai* CL3 on taro. Findings in this study provide a method to high-throughput screen VFM quenching bacteria and characterize novel functions of *P. chlororaphis* and *E. asburiae* in biocontrolling plant diseases through quenching VFM QS signal.

INTRODUCTION

Quorum sensing (QS) is a cell–cell communication mechanism widely present in the microbial community. It enables bacterial populations to monitor their population density and environmental variations, as well as to coordinate and synchronize various behaviors accordingly (Fuqua et al., 1994; Mukherjee & Bossier, 2019). QS plays a primary role in the regulation of virulence factor production and infection processes in a variety of plant pathogenic bacteria (Reverchon et al., 1998; Whiteley et al., 1999). This is integrated by pathogenic

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bacteria into complex regulatory cascades that control the expression of genes involved in a range of functions, such as pigment production, toxin production, extracellular enzyme production, exopolysaccharide synthesis, biofilm formation, motility and plasmid conjugation (Baltenneck et al., 2021; Hawver et al., 2016; Papenfort & Bassler, 2016; Waters & Bassler, 2005). Several critical QS signals have been characterized in Gram-negative pathogenic bacteria, such as *N*-acyl-homoserine lactone (AHL) (Eberhard et al., 1981), *Pseudomonas* quinolone signal (PQS) (Diggle et al., 2003), diffusible signal factor (DSF) (Wang et al., 2004), methyl 3-hydroxypalmitate (3-OH PAME) (Flavier et al., 1997) and the *Dickeya* virulence factor modulating (VFM) system (Nasser et al., 2013). The attempts to identify the signal molecule of this latter system have been unsuccessful. Quorum quenching (QQ), which disrupts QS by degrading QS signals or interfering with the synthesis or perception of signals, accomplishes the purpose of interrupting the signal communication between bacteria (Dong et al., 2007; Sikdar & Elias, 2020; Zhang, 2003). This strategy is different from the way antibiotics control infectious diseases by inhibiting or killing pathogens. It would not affect the pathogen mutation rate, but instead poses a much weaker selective pressure on the bacterial population. Therefore, it is an environment-friendly control strategy against pathogenic bacteria with good prospects for development.

Dickeya, one of the most destructive plant pathogens in the world (Mansfield et al., 2012), is a necrotrophic, Gram-negative pathogen that causes soft-rot and blackleg diseases in a wide range of plants, including economically important crops (e.g. potato, banana, rice, taro, pear, maize, pineapple, sugarcane and chicory) and numerous ornamentals (e.g. *Chrysanthemum*, *Parthenium*, *Dianthus*, *Philodendron*, *Dieffenbachia*, *Pelargonium*, *Saintpaulia*, artichoke, aloes, orchids, hyacinth, iris, *Dahlia*, *Kalanchoe*, *Begonia bertinii*, *Freesia*, *Zantedeschia*, *Clivia* and *Canna edulis* etc.) (Douches et al., 1996.; Hu et al., 2018; Lin et al., 2010; Lv et al., 2019; Tian et al., 2016). It was previously grouped under the genus *Erwinia* encompassing all Enterobacteriaceae pathogenic to plants and was reclassified in 2005 to replace *E. chrysanthemihi* (Samson et al., 2005; Toth et al., 2011). At present, 12 species are recognized in this genus, namely *D. chrysanthemi*, *D. dianthicola*, *D. dadantii*, *D. zeae*, *D. solani*, *D aquatica*, *D. fangzhongdai*, *D. poaceaephila*, *D. lacustris*, *D. undicola*, *D. oryzae* and *D. parazeae* (Brady et al., 2012; Chen et al., 2020; Hugouvieux-Cotte-Pattat et al., 2019; Hugouvieux-Cotte-Pattat & van Gijsegem, 2021; Oulghazi et al., 2019; Samson et al., 2005; Tian et al., 2016; van der Wolf et al., 2014). *Dickeya* spp. secrete a range of plant cell wall degrading enzymes (PCWDEs) (Murdoch et al., 1999). The most destructive one is pectinase, which effectively degrades pectin in the middle lamella and primary wall of plant cells, impregnating plant tissues, causing valuable nutrients to be spilled from the apoplasts and further leading the plant to severe rot (Charkowski, 2018). *Dickeya* spp. are aggressive and destructive. Although it has an asymptomatic phase in the interstitial spaces of plant cells, it progresses rapidly as soon as signs appear. For instance, inoculated tubers or storage roots rot within 2–3 days when conditions are advantageous and infected plants die within a few hours of the initial appearance of wilt. The entire infection process is rapid, so that the plant has little time to initiate its defense responses (Hugouvieux-Cotte-Pattat, 2016). *Dickeya* can survive and multiply in four environmental ecological niches: plants, water, soil and insects (Charkowski, 2018). Moreover, it has a wide survival temperature range from 25 to 39.8°C (Potrykus et al., 2018), which is one of the reasons for the failure to make significant progress in the management of *Dickeya* soft rot in the previous 20 years. A reliable, accurate, and eco-friendly combat strategy for *Dickeya* is very essential as the majority of the species have a wide host range (Hu et al., 2018).

Up till now, three QS systems (AHL, VFM and putrescine) have been characterized to regulate different biological functions in *Dickeya*. Among them, AHL system mainly regulates bacterial motility and biofilm formation (Crépin et al., 2012; Feng et al., 2019; Hussain et al., 2008; Nasser et al., 1998), VFM system modulates PCWDE production, motility and virulence (Lv et al., 2019; Nasser et al., 2013; Potrykus et al., 2014, 2018) and putrescine is involved in cell motility, biofilm formation and plant infection (Shi et al., 2019). Given that the *vfm* gene cluster is uniquely present and conserved in *Dickeya* spp. (Liu et al., 2022), and VFM regulates or even dominates the pathogenic process of *Dickeya*, it has been considered as an ideal target for developing *Dickeya* soft rot control measures. In the *vfm* gene cluster, the two-component system VfmI-VfmH senses extracellular VFM signal and regulates the expression of *vfmE* (Lv et al., 2019; Nasser et al., 2013). The AraC family transcriptional regulator VfmE binds to the *vfmA*, *vfmE*, *vfmF*, *vfmK*, *pelE* and *celZ* promoters to regulate the production of VFM signal and PCWDEs (Nasser et al., 2013), as well as c-di-GMP through its PilZ domain to regulate cell motility (Banerjee et al., 2022; Chen et al., 2022). Meanwhile, extracellular VFM signal promotes the production of VfmE (Nasser et al., 2013), implicating an autoactivation. However, the chemical structure of the VFM QS signal has not been clarified, and there is no information regarding the QQ chemical, posing major obstacles to the development of the QQ strategy in biocontrolling *Dickeya* soft rot.

In this study, a *vfmE-gfp* biosensor was constructed for a high-throughput screening of VFM quenching bacteria that control *Dickeya* virulence. As a result, *Pseudomonas chlororaphis* L5 and *Enterobacter asburiae* L95 were obtained from soil and confirmed to significantly reduce the PCWDE production and virulence of *Dickeya*, and thereafter, alleviate crop soft rot caused by different *Dickeya* spp. by quenching VFM QS signal without affecting the growth of VR2.

EXPERIMENTAL PROCEDURES

Strains and growth conditions

All strains were stored at −80°C in 20% glycerol. *D. zeae* MS2, *D. oryzae* EC1, *D. dadantii* 3937, *D. fangzhongdai* CL3 and its derived strains were grown in Lysogeny Broth (LB) medium (Lv et al., 2019) at 28°C with shaking (200 rpm). *P. chlororaphis* L5 and *E. asburiae* L95 were grown in LB or in minimal medium (MM) with mannitol and glycerol (both 2 g/L) as carbon sources (Hussain et al., 2008) at 28°C with 200 rpm shaking. *Escherichia coli* strains were grown in LB medium at 37°C with shaking. Antibiotics kanamycin (Km) and polymyxin B sulfate (Pm) at a final concentration of 50 μg/mL were added when necessary. Plating was conducted on LB or MM medium solidified with 1.5% agar. Bacterial strains and plasmids used in this study are listed in Table 1.

Construction of GFP recombinant plasmids and VFM biosensors VR, VR1 and VR2

To construct the VFM signalling biosensor, *D. oryzae* EC1 genomic DNA was used as the template to obtain the VfmEpro amplificon using primer pairs of pvfmE-F/pvfmE-R (Table 2). The product was purified using NucleoSpin gel and PCR clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Düren Neumann Neander) and then ligated with the *Hind*III/*Bam*HI-digested pPROBE-NT plasmid using the ClonExpress MultiS kit (Vazyme Biotech Co.). The recombinant plasmid pVfmEpro-*gfp* or the original pPROBE-NT plasmid was further transformed into *E. coli* DH5α competent cells. The positive transformants were confirmed by sequencing using pNT-F/R primers (Table 2). Subsequently, DH5α (pVfmEpro-*gfp*), HB101(pRK2013) and *D. oryzae* Δ*zmsA* were co-cultured on LB agar plate, and then diluted and grown on MM plates containing Km. Single colonies were selected for PCR amplification to obtain strain Δ*zmsA*(pVfmEpro-*gfp*) (named as VR2). Meanwhile, Δ*zmsA*Δ*vfmE*(pVfmEpro-*gfp*) (VR1) and Δ*zmsA*(pPROBE-NT) (VR) were also generated using triparental conjugation method.

TABLE 1 Bacterial strains and plasmids.

Strain or plasmid	Relevant characteristic(s)	Source
Dickeya		
D. zeae MS2	Wildtype, isolated from infected banana	Hu et al. (2018)
D. oryzae EC1	Wildtype, isolated from infected rice	Zhou et al. (2011)
Δ*zmsA*	*zmsA* deletion mutant of *D. oryzae* EC1	Zhou et al. (2011)
Δ*zmsA*Δ*vfmE*	*zmsA* and *vfmE* deletion mutant of *D. oryzae* EC1	Lab collection
ΔlacZ + vfmE(lacZ)	VFM biosensor for *D. oryzae* EC1	Lv et al. (2019)
VR	Δ*zmsA* haboring pPROBE-NT	This work
VR1	Δ*zmsA*Δ*vfmE* haboring pVfmEpro-*gfp*	This work
VR2	Δ*zmsA* haboring pVfmEpro-*gfp*	This work
D. dadantii 3937	Pathogen isolated from infected *Saintpaulia ionantha*	Lv et al. (2019)
A5243	VFM biosensor for *D. dadantii* 3937 with a *vfmE::uidA* reporter fusion	Nasser et al. (2013)
D. fangzhongdai CL3	Pathogen isolated from infected taro	Lab collection
Pseudomonas chlororaphis L5	Strain isolated from rice fields in Xiaogan, Hubei, China	This work
Enterobacter asburiae L95	Strain isolated from rice fields in Xiaogan, Hubei, China	This work
E. coli		
DH5α	*supE44ΔlacU169 (ϕ80lacZΔM15), hsdR17 recA1 endA1 gyrA96 thi-1 relA1 λpir*	Lab collection
HB101(pRK2013)	Strain haboring pRK2013	Lab collection
Plasmids		
pPROBE-NT	Promoter-probe vector, Km^R	Hu et al. (2022)
pVfmEpro-*gfp*	pPROBE-NT derivative with PCR fragment containing *vfmE* promoter region, Km^R	This work

TABLE 2 Primers used in this study.

Name	Primer sequence (5′–3′)	Amplicon
Primers for plasmid detection		
pNT-F	TTGGGGATCGGAAGCTT	Sequencing primer of pPROBE-NT
pNT-R	CACCTTCACCCTCTCCACTG	
Primers for amplification of *vfmE* promoter		
pvfmE-F	ggaattggggatcggaagcttCCGCAAAACTGACTTCACCTTT	Construction of pVfmEpro-*gfp*
pvfmE-R	gagctcggtacccggggatccGGTTATTGTTAAAAATATCGTTATCTACGG	
Primers for bacterial molecular identification		
27f	AGAGTTTGATCCTGGCTCAG	Amplification of bacterial 16S rRNA fragment
1492r	GGTTACCTTGTTACGACTT	
$rpoB_P$-F	TGGCCGAGAACCAGTTCCGCGT	Amplification of *rpoB* fragment of L5
$rpoB_P$-R	CGGCTTCGTCCAGCTTGTTCAG	
$rpoD_P$-F	CGGTTGATGTCCTTGA	Amplification of *rpoD* fragment of L5
$rpoD_P$-R	ATCGAAATCGCCAAGCG	
$gyrB_E$-F	ACGTCGTGGCCTCGCGATCC	Amplification of *gyrB* fragment of L95
$gyrB_E$-R	TTAAAGGACTCTTTCACCAG	
$rpoD_E$-F	ATGACGGAACACGACAAATCCT	Amplification of *rpoD* fragment of L95
$rpoD_E$-R	CGGCTTCGTCCAGCTTGTTCAG	
Primers for RT-qPCR		
rpoB-F	GGCGTGCTGAAGATCGTTAAGG	Reference gene of RT-qPCR analysis
rpoB-R	GCGTGCCGTTCTCATCGTAAG	
vfmE-F	GGCGGGAATTATTGACTA	RT-qPCR analysis
vfmE-R	GTTGAAGAGACGACAGATA	
vfmA-F	GGTCGGTGTTCAAGGGGAAT	RT-qPCR analysis
vfmA-R	CGTCCAGCAGACAGCCATTA	
celZ-F	CACTCGCATTCAGCAGAAAACA	RT-qPCR analysis
celZ-R	AGTGCAATACCGTTATTTATCGC	
pelD-F	GATCGGCCACAGCGACAG	RT-qPCR analysis
pelD-R	CGTCGCCCAGATACACGTT	
prtC-F	TGCTCTGCATTAAATTTCACAA	RT-qPCR analysis
prtC-R	GTCAAGATGACGCTCAACAC	
pehK-F	CCATCTGGTGAGTTGCCGT	RT-qPCR analysis
pehK-R	TTTGGCGGTGGTTTTGATGC	

Visualization of the VFM biosensors

To visualize the constructed strains, 5 μL of VR, VR1 and VR2 bacterial cultures at OD_{600} of 1.0 were taken to glass slides, and the fluorescence was observed on the fluorescence inverted microscope (Carl Zeiss) and photographed. Furthermore, the mean fluorescence intensity (MFI) values of the above three bacterial cultures were detected using the CytoFLEX flow cytometer (FCM) (CytoFLEX).

Sensitivity detection of the VFM biosensors

The Δ*zmsA* strain was grown on a LB plate containing Pm and single colonies were inoculated in MM medium at 28°C and shaken at 200 rpm until the OD_{600} value reached 1.0. The supernatant was separated by centrifugation at 4°C, 8000 rpm and filtered through a 0.22 μm bacterial filter to obtain the crude extract of the VFM QS signal, which was kept at −20°C for storage. The VFM signal crude extract was added to LB medium at the final concentrations of 0%, 10%, 20% and 40% (v/v). VR2 bacterial culture (OD_{600} of 1.0) was added into LB medium containing different concentrations of crude signal extract at a ratio of 1:100. A 96-well tissue culture plate (Jet Bio-Filtration Co.) was taken and 100 μL of the above bacterial cultures in each concentration was added to the wells in 3 replicates, and the bacteria were incubated at 28°C with shaking. The MFI and OD_{600} values were measured every 3 h using multifunctional mcroplate reader (MMR) (Microplate reader, BioTek).

The excitation and emission wavelength of MFI was set at 485 and 528 nm, respectively. The VR and VR1 were used as blank controls.

Screening of VFM quenching bacteria

Field soil samples collected from rice fields in Xiaogan, Hubei, China, were mixed thoroughly, weighed 5.0 g and placed in a sterilized centrifuge tube with 15 mL of fresh LB medium, incubated at 28°C with shaking at 200 rpm for 15 min, and left until the soil particles settled at the bottom of the centrifuge tube. The supernatant was diluted in a gradient with sterile water, 100 μL of 10^{-5}, 10^{-6} and 10^{-7} dilutions were applied to LB plates and incubated for 24 h at 28°C, and the resulting single colonies were picked, labeled, cultured in LB medium overnight at 28°C with shaking at 200 rpm.

High-throughput screening of VFM quenching bacteria was performed using the MMR. Briefly, fresh bacterial suspension of *vfmE-gfp* biosensor VR2 at OD_{600} of 1.0 was prepared, added to the LB medium at a ratio of 1:100, mixed well and added to the 96-well tissue culture plate with 200 μL (2×10^6 CFU) per well and 1 μL of the above pure soil bacterial culture (2×10^6 CFU) was added into the well. Each bacterial sample was repeated three times, with sterile LB medium as the blank control and 1 μL of *E. coli* DH5α (2×10^6 CFU) as the negative control. After the inoculation was completed, the plate was sealed by a sealing film, covered and put into the MMR for 24 h. The values of OD_{600} and OD_{485}/OD_{528} were measured every 3 h.

Dual culture plate antagonism assay

To prepare the antagonistic assay plate, 15 mL of melted LB agar medium was poured into a 10 cm × 10 cm square Petri dish. After solidification, 15 mL of melted (40–50°C) 1% agarose mixed with 150 μL of fresh VR2 bacterial cultures were added onto the LB plates. Wells 5 mm in diameter were punched and filled with 20 μL of L5 or L95 bacterial culture or supernatant concentrate. The dish was incubated at 28°C for 16–18 h and photographed. The assay was performed in triplicate and repeated three times independently.

Validation of the VFM QQ bacteria using CytoFLEX flow cytometer (FCM)

In order to validate the screened candidate VFM QQ bacteria L5 and L95, FCM was further used to detect the *vfmE* promoter activity of the VR2 in the co-cultures at different time periods. Specifically, overnight cultures of VR2, L5 and L95 were prepared and their OD_{600} values were adjusted to 1.0. Next, 500 μL of L5 or L95 together with 500 μL of VR2 suspension were added to 100 mL of LB medium. LB medium containing only 500 μL of VR2 was set as a blank control. The samples were taken every 4 h until 12 h. The MFI values of all samples were measured using MMR and FCM. All samples were coated on LB plates containing Km and Pm (neither L5 nor L95 was Km or Pm resistant) for counting the colony forming unit (CFU) of VR2 in each plate. L5 and L95 CFU were also counted on the co-culture coated plates based on the different morphology between the L5 and VR2 colonies, or the L95 and VR2 colonies.

Determination of the growth curves of EC1, VR2, L5 and L95

To compare the growth rates of EC1, VR2, L5 and L95, the OD_{600} values of the overnight bacterial cultures were adjusted to 1.0 and 500 μL of which was added into 100 mL of the MM with manitol and glycerol and LB liquid media, grown at 28°C by shaking at 200 rpm for 48 h, and the OD_{600} values were measured every 4 h. The experiment was performed in triplicate and repeated three times independently.

Concentration of the L5 and L95 supernatants

After resurrection on LB plates, single colonies of L5 and L95 were inoculated into 1 L of MM medium and shaken at 200 rpm at 28°C until the OD_{600} value reached 1.5. The bacterial solutions were centrifuged at 4500 rpm, 4°C for 30 min. The precipitates were discarded and the supernatants were frozen at −80°C. The water was removed from the samples using the vacuum freeze dryer (Telstar), and each of the dried samples was then dissolved in 5 mL of phosphate buffer saline (PBS) solution and centrifuged at 8000 rpm, 4°C for 30 min. The precipitates were removed and the supernatants were filtered through a 0.22 μm bacterial filter to obtain the 1000-fold concentrated supernatants of the two QQ strains.

Molecular identification of L5 and L95

Individual colonies of L5 and L95 were picked and cultured. The genomic DNAs were extracted using the EasyPure Bacteria Genomic DNA Kit (TransGen Biotech Co.) and used as the templates to amplify the 16S rRNA gene sequences using the primer pair of 27f and 1492r (Table 2). The PCR products were sequenced,

and a homology search was carried out using BLASTn (Basic Local Alignment Search Tool) at the National Center for Biotechnology Information (NCBI) website (https://www.ncbi.nlm.nih.gov/). In order to clarify the taxonomic status of the QQ bacteria, the multilocus sequence analysis (MLSA) was performed, including housekeeping genes of RNA polymerase β subunit (*rpoB*) and RNA polymerase subunit D (*rpoD*) for strain L5, and DNA gyrase subunit B (*gyrB*) and *rpoD* for strain L95. The GenBank accession Nos. of strain L5 are OP964057.1 for 16S rRNA, OQ053243.1 for *rpoB* and OQ053242.1 for *rpoD*, and those of strain L95 are OP964363.1 for 16S rRNA, OQ053245.1 for *gyrB* and OQ053244.1 for *rpoD*. Joint sequence phylogenetic trees were constructed using the neighbor-joining (NJ) method with MEGA 6.0 software with 1000 bootstrap replicates.

Measurement of PCWDE activities

Fresh L5 and L95 bacterial cultures at OD_{600} of 1.5 were centrifuged at 8000 rpm, 4°C for 30 min. The supernatants were filtered through a 0.22 μm bacterial filter, resuspended with PBS solution and the precipitates were crushed at low temperature for 40 min using an ultrasonic crusher and stored at −20°C. Cellulase (Cel), pectate lyase (Pel), polygalacturonase (Peh) and protease (Prt) assay media were prepared as described previously (Zhou et al., 2016), 40 mL of each medium was poured into a 13 cm × 13 cm square Petri dish, after solidification, wells in a diameter of 5 mm were punched, and 30 μL of *D. zeae* MS2 bacterial culture equally mixed with L5 or L95 supernatant or precipitate was added to the wells. Equal volumes of *D. zeae* MS2 bacterial cultures and PBS were mixed to serve as the control. The plates were placed in an incubator at 28°C. The Peh and Pel plates were treated with 1 M HCl for 15 min after 12–14 h. The Cel plates were stained with 0.1% (w/v) Congo red solution for 15 min after 12–14 h, followed by 2 washes with 1 M NaCl, and the Prt plates were placed for 24 h for direct observation. Each treatment was repeated three times. The halo radius was measured and collated using Image J v. 1.52a software. For testing the action of the concentrated supernatants of L5 and L95, the supernatant concentrates were mixed with *D. zeae* MS2 bacterial cultures in proportions of 20%, 40% and 60%, respectively.

Evaluation of the biocontrol potential of the VFM QQ bacteria

The pathogenic bacteria *D. zeae* MS2, *D. oryzae* EC1, *D. dadantii* 3937 and *D. fangzhongdai* CL3 and the VFM QQ bacteria L5 and L95 were grown on LB agar plates overnight at 28°C. *E. coli* DH5α was grown on LB plate at 37°C. All the bacteria were resuspended with PBS solution to OD_{600} of 0.5. Each of the pathogenic bacteria was mixed thoroughly with equal volume of PBS (positive control), DH5α, L5 or L95. Equal amount of L5 or L95 suspension was set as the negative control and PBS solution and DH5α used as blank controls. The experiment was repeated three times independently.

For inoculation on rice seedlings, 10 mL of DH5α, EC1, L5 and L95 and 20 mL of EC1 + DH5α, EC1 + L5 and EC1 + L95 mixtures were diluted to 100 mL with PBS and then evenly poured onto the rice seedling (with roots pierced with sterile needles) and kept at 28°C. The results were recorded at regular intervals, and the disease severity of rice was recorded as described previously (Xue et al., 2021). Briefly, the virulence scores were calculated as follows: 0, no visible infection symptoms; (1) discoloration only at the inoculation point on the pseudo-stem; (2) discoloration and soft rot spread throughout the stem; (3) discoloration and soft rot spread from stem to leaves or growing point, and/or the plant can no longer support leaf weight.

For inoculation on banana seedlings, 200 μL of the PBS, DH5α + PBS, L5 + PBS, L95 + PBS, MS2 + PBS, MS2 + DH5α, MS2 + L5 or MS2 + L95 was injected into the pseudostems and kept at 28°C for 7 days. The disease severity was calculated as described above and previously (Feng et al., 2019).

For inoculation on potato, 2 μL of the PBS, DH5α + PBS, L5 + PBS, L95 + PBS, 3937 + PBS, 3937 + DH5α, 3937 + L5 or 3937 + L95 was injected onto the center of the potato slices; and 2 μL of PBS, DH5α + PBS, L5 + PBS, L95 + PBS, CL3 + PBS, CL3 + DH5α, CL3 + L5 or CL3 + L95 was injected onto the center of the taro slices. The inoculated materials were kept at 28°C. The results were recorded after 24 h, and the lesion area was measured by Image J software.

RNA extraction and quantitative reverse transcription PCR (RT-qPCR)

The fresh *D. zeae* MS2 bacterial culture was transferred to LB medium containing 10% of L5 or L95 supernatant concentrate for growth until OD_{600} reached 0.8. Pure *D. zeae* MS2 bacterial culture at OD_{600} of 0.8 was set as the control. Bacterial total RNA was extracted from three samples using the Eastep Super Total Extraction Kit (Promega Biotech Co.). Then 1 μg of RNA was reversely transcribed using HiScriptII Q RT SuperMix kit (Vazyme Biotech Co.). qPCR solution was prepared using ChamQ SYBR qPCR Master Mix (Vazyme Biotech Co.). The reaction was performed in a QuantStudio 12 K Flex real-time PCR instrument (Life Technologies). To calculate the relative expression levels of the target genes *vfmA*, *vfmE*, *celZ*, *pelD*, *prtC* and *pehX*, the expression level of a housekeeping

gene *rpoB* was used as an internal control. The primers used are listed in Table 2. The relative gene expression level was determined by the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Verification of the quenching effect of L5 and L95 on VFM signal

For measurement of the quenching effect of L5 and L95 on the VFM signal produced by *D. oryzae* EC1 and *D. zeae* MS2, *ΔlacZ+vfmE(lacZ)* biosensor constructed previously was used (Lv et al., 2019). Under dark condition, 20 mL of MM agar medium supplemented with 40 μg/mL of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) was poured into a 9 cm diameter Petri dish. After solidification, the medium was cut into separate strips in 0.8 cm width, and 1 μL of L5+LB, L95+LB, EC1+LB, EC1+L5, EC1+L95, MS2+LB, MS2+L5 or MS2+L95 was spotted at the top of the strips, followed by 1 μL of consecutive *ΔlacZ+vfmE(lacZ)* culture (OD_{600} of 1.0) spots. The blueness of the biosensor indicated the production of the VFM signal. The quenching effect of L5 and L95 on the EC1 and MS2 VFM signal was determined by the shortening of the blue distance, compared with the EC1 and MS2 strips.

A5243 biosensor was used to report the VFM signal produced by *D. dadantii* 3937 (Nasser et al., 2013). For measurement of the quenching effect of L5 and L95 on the 3937 VFM signal, fresh L5, L95, 3937, and A5243 suspensions at OD_{600} of 1.0 were prepared. X-Glu (β-D-glucuronide) in a final concentration of 40 μg/mL was added to the MM medium, which was inoculated with A5243 at a ratio of 1:100, and 1 mL was added into each well of a 24-well tissue culture plate. Subsequently, 10 μL of L5 or L95 bacterial suspension mixed with 10 μL of 3937 was added into the wells. The well inoculated with 10 μL of *D. dadantii* 3937 alone was used as a positive control. Each treatment was repeated three times. The plate was sealed with a sealing film and covered, grown by shaking at 200 rpm, 28°C for 24 h, and the results were observed and photographed periodically.

For quantitation of the β-Gal activity, 20 μL of A5243 culture (OD_{600} of 1.8 in LB medium supplemented with Km) was added into 20 mL of MM medium supplemented with Km and mixed thoroughly, 1 mL of which was transferred into each well of the 24-well tissue culture plate. To analyze the β-Gal activity of the bacterial supernatants, 100 μL of MM medium (negative control), L5+MM, L95+MM, L5+3937, L95+3937 or 3937+MM (positive control) 12-h culture supernatants were added into the wells, grown at 28°C by shaking at 200 rpm for 12 h and the OD_{600} values were measured. Next, 100 μL of the culture was transferred into a 1.5-mL tube and centrifuged at 4°C, 4000 rpm for 25 min and 10 μL of the supernatant was added into a fresh 1.5-mL tube, mixed with 10 μL of extraction buffer [1 L contains 50 mL of 0.1 M PBS at pH 7.0, 1 mL of 10% SDS, 2 mL of 0.5 M EDTA at pH 8.0, 100 μL of TritonX-100, 100 mL of β-mercaptoethanol, 88 mg of 4-methylumbellifery -β-D-glucuronide (4-MUG)] and 80 μL of assay buffer (extraction buffer without 4-MUG), incubated at 37°C for 10 min and cooled at room temperature for 20 min. The reaction was terminated by adding 200 μL of 0.2 M Na_2CO_3 solution. Finally, the solution was serially diluted and 200 μL of which was transferred into the well of the 96 well-tissue culture plate and the MFI was measured by MMR with excitation and emission wavelength at 485 and 528 nm, respectively. The β-Gal activity was calculated based on $MFI \times 0.1/(\beta \times 30 \times 0.01 \times OD_{600})$, where 0.1 is the total reaction volume (mL), 30 is the reaction time (min), 0.01 is the volume (mL) of bacterial reaction culture and β is the formula coefficient of β-Gal activity standard curve.

RESULTS AND DISCUSSION

Construction and verification of *vfmE-gfp* biosensor VR2

Since its first discovery by Nasser et al. (2013), the VFM QS system has been recognized as the highly conserved primary pathogenic QS system out of three extant QS systems in the genus *Dickeya*. Previous studies revealed that an AraC-type family transcriptional regulator VfmE can respond to the VFM signal produced by *D. dadantii, D. oryzae* and *D. zeae* via the VfmH response regulator (Lv et al., 2019; Nasser et al., 2013). Thus, we used the promoter of *vfmE* from *D. oryzae* EC1 to construct a VFM QS signal reporting biosensor.

Considering that some strains of *Dickeya* produce zeamine toxins, which may inhibit the growth of VFM quenching bacteria, a *zmsA* deletion mutant of *D. oryzae* EC1 (*D. oryzae* EC1 Δ*zmsA*) that lost its ability to produce zeamines was selected as the host strain of the VFM signal biosensor in this study (Zhou et al., 2011). Initially, a fragment of the *vfmE* promoter was cloned upstream of a promoterless *gfp* gene in the pPROBE-NT vector, and the recombinant plasmid pVfmEpro-*gfp* was then transferred into the Δ*zmsA* strain to obtain the *vfmE-gfp* reporter VR2 (Table 1). Our previous study has demonstrated that deletion of *vfmE* in *D. oryzae* impaired the production of the VFM signal (Lv, 2018). Thus, the recombinant plasmid pVfmEpro-*gfp* was also transferred into the signal deficient mutant Δ*zmsA*Δ*vfmE* to obtain the VR1 strain, and the pPROBE-NT plasmid was transferred into Δ*zmsA* to obtain the VR strain (Table 1), both of which serve as blank controls for signal response. As shown in Figure S1, the VR2 biosensor can be

easily visualized using fluorescence inverted microscopy, whereas the fluorescence of VR and VR1 strains were dim (Figure S1A). Specifically, the mean fluorescence intensity (MFI) values measured by FCM were 283.6 ± 15.1, 2149.8 ± 275.9 and 63190.8 ± 668.7 for VR, VR1 and VR2, respectively (Figure S1B,C).

To verify the sensitivity of the VFM biosensor VR2 in response to the VFM signal, Δ*zmsA* crude extracts containing different concentrations of VFM signal were added into the LB medium inoculated with VR, VR1 and VR2 strains, respectively. Cell densities of the cultures at 3 h intervals showed that all the three biosensors were able to grow normally in LB or the medium containing VFM signal (Figure 1A). The MFI values measured by MMR revealed that even with the addition of crude extracts, VR and VR1 strains still failed to emit fluorescence. However, the VR2 strain had a higher MFI value than VR and VR1 strains after 6-h culture, and with the addition of signal extract, the enhancement of VR2 fluorescence was proportional to the extract concentrations (Figure 1B). These results suggest that VR2, but not VR and VR1, could respond sensitively to the signal and reflect the cell or medium signal concentrations with fluorescence. Previous studies indicated that the VFM signal sharply decreased when OD_{600} value is over 1.2 (Banerjee et al., 2022; Liu et al., 2022; Lv et al., 2018), probably due to the quenching enzymes produced by *Dickeya* itself. Moreover, incubation times of 12–15 h showed higher resolution in responding to VFM signal (Figure 1B). Thus, we used the VR2 biosensor for screening VFM quenching bacteria under the condition of co-culture time at 12 h.

Two VFM quenching bacteria were screened out

Single bacteria were isolated from rice rhizosphere soil and cultured in LB medium at 28°C overnight, and 2×10^6 CFU of which were added into the wells of 96-well tissue culture plate containing LB medium supplemented with VR2 culture (2×10^6 CFU). The MFI values were measured first by MMR. Among the 300 randomly tested rhizosphere bacteria, 32 decreased the MFI value of the VR2 (Table S1), which were then measured for their antagonistic activities against VR2. As a result, 22 strains with no VR2 inhibition activity were confirmed, 13 of which had M/O reduction rate over 70% (Table S1) and were then evaluated for their potentials on reducing the soft rot symptoms on potato slices. Among them, two strains designated as L5 and L95 were screened out due to their ability to significantly reduce the occurrence of potato soft rot (Figure S2). L5 and L95 were also confirmed by FCM to have significantly lower MFI values in the co-culture samples than in the VR2 blank control samples. Figure 2A,B indicate a decreasing trend in MFI values when QQ bacteria were added compared with the control; both L5 and L95 significantly reduced the MFI values of VR2 at 8 and 4 h of co-culturing in the MMR and FCM measurement, respectively. Compared with the results in the MMR measurement (Figure 2A), the reduction of the VFM biosensor MFI values in the presence of the QQ bacteria seemed more significant in the FCM measurement. In addition, during the FCM measurement, the MFI value of VR2 slightly decreased at 12 h compared to 8 h (Figure 2B), possibly due to its self-quenching activity. Flow cytometry is more sensitive

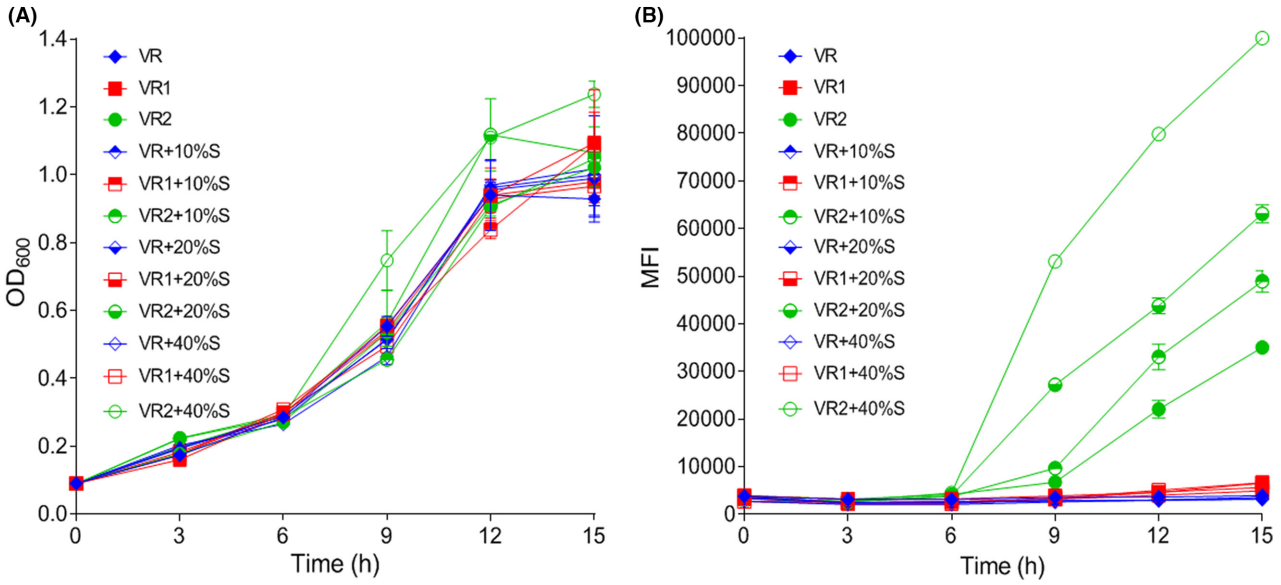


FIGURE 1 The growth curves of VFM biosensors (A) and their responses to the VFM signal (B). VR, VR1 and VR2 indicate Δ*zmsA*(pPROBE-NT), Δ*zmsA*Δ*vfmE*(pVfmEpro-*gfp*) and Δ*zmsA*(pVfmEpro-*gfp*), respectively. The MFI values of VR, VR1 and VR2 biosensors after adding with different concentrations of signal crude extracts (%) were measured by multifunctional microplate reader every 3 h.

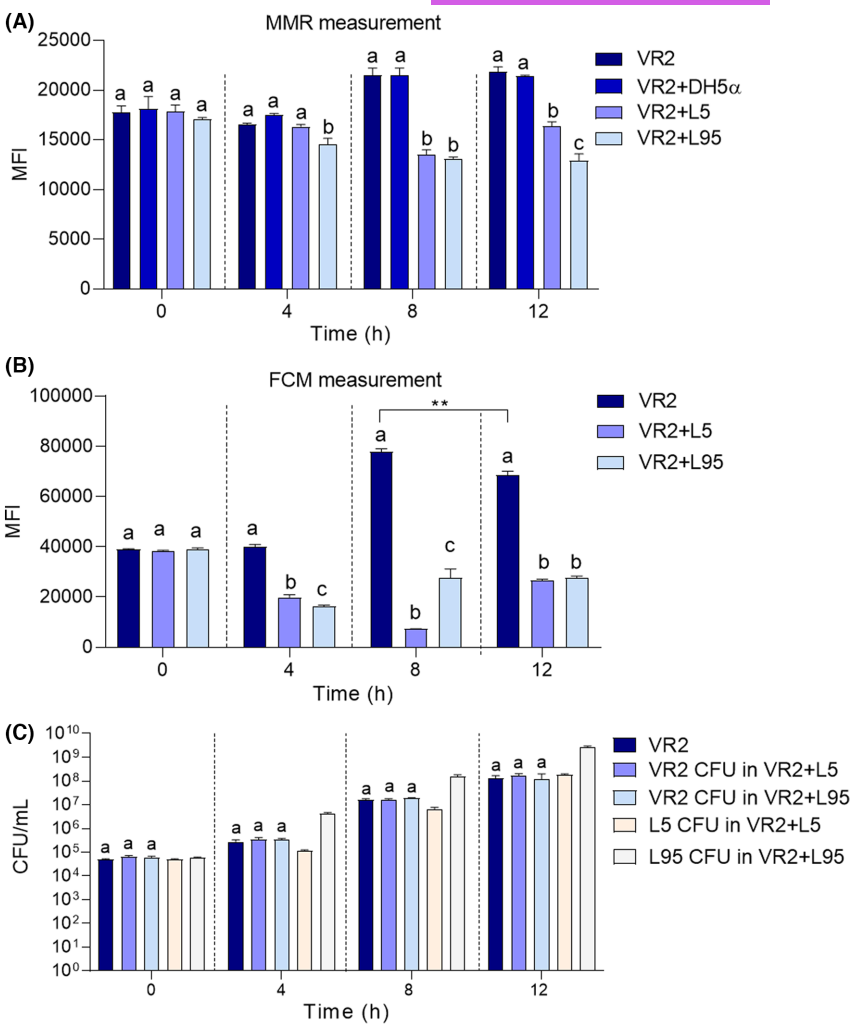


FIGURE 2 The MFI values of the VFM biosensor VR2 co-cultured with the screened VFM QQ bacteria L5 and L95, measured by multifunctional microplate reader (MMR) (A) and CytoFLEX flow cytometer (FCM) (B), and the CFU values of VR2 and the QQ bacteria in the co-cultures (C) every 4 h. The experiments were performed in triplicate and repeated three times independently. The data were subjected to one-way ANOVA analysis by Graphpad Prism 9. Significantly different values ($p < 0.05$) are indicated by different letters. The data of VR2 at 8 h and 12 h were also subjected to Student's *t*-test analysis by Graphpad Prism 9 (**$p < 0.01$).

as fluorescence intensity can be measured on the level of single cells instead of integrating a signal across a population. Thus, with an appropriate gating strategy, it could be even possible to determine the proportion of cells that have lost or maintained fluorescence.

In order to explore whether the reduction of MFI was caused by the decrease of VR2 strain number due to nutrition uptake competition between VR2 and the quenching strains, we periodically measured the CFU of VR2 in each sample. The results showed that the VR2 cells in the samples were steadily increasing and the amount of VR2 in the samples co-cultured with QQ bacteria was not significantly different from that of the control group (Figure 2C), excluding the possibility that the MFI reduction is due to the growth reduction of VR2. L5 and L95 bacteria also grew well in the co-cultures (Figure 2C).

Growth of VR2 was not affected by L5 and L95

In order to further verify the biological control effect of L5 and L95 strains is not achieved by inhibiting the growth of VR2, we performed an antagonistic test of L5 and L95 versus VR2, and vice versa. The results showed that there were no clearly visible growth inhibition halos between the QQ bacteria and VR2 (Figure S3A,B), indicating that both strains of QQ bacteria had no antagonistic activity against VR2, consistent with the abovementioned co-culturing experiment (Figure 2C). We also tested the antagonistic activity of the concentrated QQ bacterial supernatants against VR2, and the results also indicated no growth inhibitory activity of them (Figure S3A), agreeable with our further experiments in which the concentrated QQ bacterial supernatants had no effect on the growth of *D. zeae* MS2 (Figure S4A). In order to have more information about the potential of L5 and L95 strains as biocontrol agents, we compared the growth rates of EC1, VR2, L5 and L95 strains in LB and MM media. Results showed that these four strains have similar growth patterns in the minimal medium, while strains L5 and L95 grew better than EC1 and VR2 in the LB medium (Figure S3C).

L5 and L95 were identified as *Pseudomonas chlororaphis* and *Enterobacter asburiae*, respectively

To determine the taxonomic identifications of strains L5 and L95, we first sequenced their 16S rRNA fragments.

The results showed that the 16S rRNA sequence of strain L5 is most similar to that of many *P. chlororaphis* strains (sequence similarity: 99.93%), while the rRNA sequence of strain L95 is most similar to that of *E. asburiae* strains 1808-013 and FDAARGOS 1432 (sequence similarity: 99.87%). Subsequently, fragments of *rpoB* and *rpoD Pseudomonas* housekeeping genes of strain L5 were also amplified, and the phylogenetic tree based on the joint sequences revealed that strain L5 gathers in the cluster of *P. chlororaphis* (Figure S5A). Similarly, strain L95 is located in the same cluster of *E. asburiae* in the *gyrB* and *rpoD* (*Enterobacter* housekeeping genes) joint phylogenetic tree (Figure S5B). In summary, we classified strains L5 and L95 as *P. chlororaphis* and *E. asburiae*, respectively.

L5 and L95 decreased the production of PCWDEs generated by *D. zeae* MS2

Previous studies have indicated the important role of VFM signal in regulating the production of PCWDEs (Liu et al., 2022; Lv et al., 2019; Nasser et al., 2013; Potrykus et al., 2014, 2018). To test whether the two VFM quenching bacteria L5 and L95 might impact the ability of *D. zeae* MS2 to generate PCWDEs, we added equal volume of their supernatants (filtered with a 0.22 μm syringe filter) and precipitates (homogenized with an ultrasonic crusher) into the MS2 bacterial cultures. Compared with the MS2 culture alone, the supernatants of both strains slightly reduced the production of all the tested PCWDEs, while the precipitates remarkably decreased the PCWDE production of *D. zeae* MS2 (Figure 3A). The PCWDE reduction extent suggests that the quenching activities of L5 and L95 were mainly reserved in bacterial cells and were detected in trace amounts extracellularly. In consideration of this aspect, we concentrated the supernatants of L5 and L95 in 1000 folds and added to MM medium at different proportions to culture *D. zeae* MS2. The results indicated that the decrease in PCWDE activities was proportional to the proportion of the supernatant concentrates added (Figure 3B). Simultaneously, we also determined the CFU number of *D. zeae* MS2 in the wells added with the highest proportion (60%) of concentrated supernatants in the Pel assay plates (Figure S4B). The results showed that the CFU numbers of MS2 in the MS2 + 60%L5c and MS2 + 60%L95c samples are similar to that in the MS2 + MM control sample (Figure S4A), suggesting that the concentrated QQ bacterial supernatants had no inhibitory activity on the growth of *D. zeae* MS2. In summary, we concluded that L5 and L95 could reduce the production of PCWDEs generated by *D. zeae* MS2 through quenching VFM signaling.

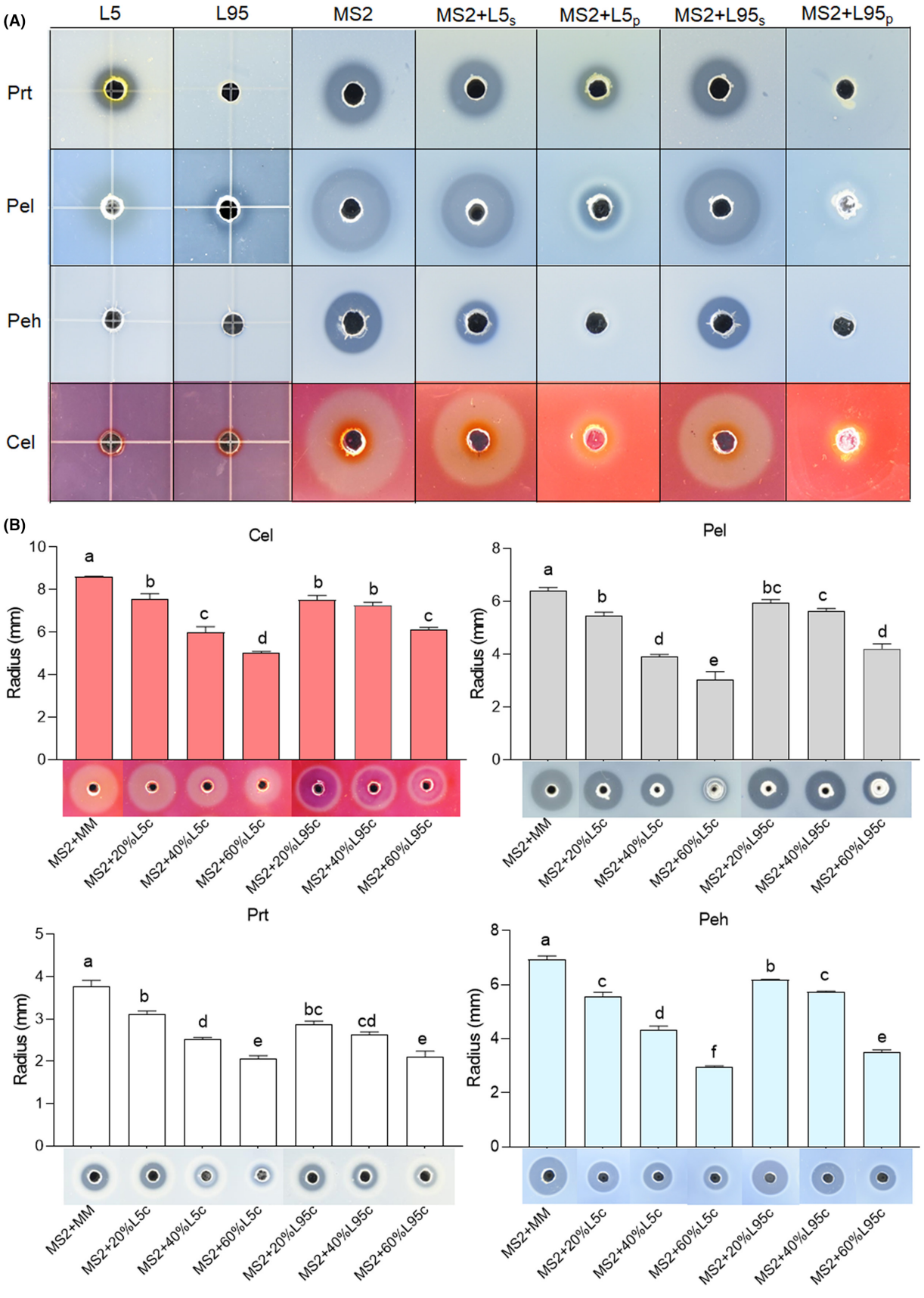
L5 and L95 have promising biocontrol potentials

Since the VFM QQ strains L5 and L95 attenuate the production of PCWDEs, to comprehensively verify whether they can reduce the virulence of *Dickeya* spp. in practical application, we performed pathogenicity tests on different plant materials inoculated with their original soft rot *Dickeya* pathogens. According to the results, rice seedlings treated with *D. oryzae* EC1 (Figure 4A), banana seedlings treated with *D. zeae* MS2 (Figure 4B), potato slices treated with *D. dadantii* 3937 (Figure 4C), and taro slices treated with *D. fangzhongdai* CL3 (Figure 4D) all developed serious soft rot symptoms. Nevertheless, plant materials inoculated with pathogens mixed with equal amounts of L5 or L95 significantly attenuated or even lost necrosis symptoms (Figure 4). Moreover, maceration was not observed in the plant materials treated with L5 and L95 suspensions (Figure 4), indicating that L5 and L95 are safe for further use as biocontrol agents. This finding demonstrated the efficacy of VFM quenching bacteria in biocontrolling soft rot diseases caused by *Dickeya* spp.

L5 and L95 supernatants inhibited VFM-related gene transcription

In order to further confirm that L5 and L95 reduce the production of PCWDEs and the pathogenicity of *Dickeya* by quenching the VFM signaling pathway, we measured the impacts of L5 and L95 concentrated supernatants on the expression of the VFM-related genes in *D. zeae* MS2. The *D. zeae* MS2 genome contains the conserved VFM-encoding gene cluster which has four transcriptional units, including *vfmAZBCD*, *vfmE*, *vfmFGHIJ* and *vfmKLMNOPQRSTUVW*, among which *vfmAZBCD* and *vfmKLMNOPQRSTUVW* are responsible for the synthesis of VFM signal (Nasser et al., 2013). In this study, *vfmA* and *vfmE* were selected to represent the expression levels of the *vfm* gene cluster in *D. zeae* MS2. The expression of *pelE*

FIGURE 3 The impacts of L5 and L95 quorum quenching bacteria on the production of PCWDEs derived from *D. zeae* MS2. L5 and L95 precipitates (L5p and L95p) significantly reduced the production of PCWDEs in MS2, while their supernatants (L5s and L95s) slightly decreased the PCWDE production (A). Addition of 1000 fold-L5 and L95 supernatant concentrates (L5c and L95c) to MS2 bacterial broth in different proportions significantly attenuated the production of PCWDEs in MS2, and the reduction level was positively correlated with the proportions of the supernatants added (B). Cel, cellulase; Pel, pectate lyase; Prt, protease; Peh, polygalacturonase. The assay was performed in triplicate and repeated three times independently. The data were subjected to one-way ANOVA analysis by Graphpad Prism 9. Significantly different values ($p < 0.05$) are indicated by different letters.



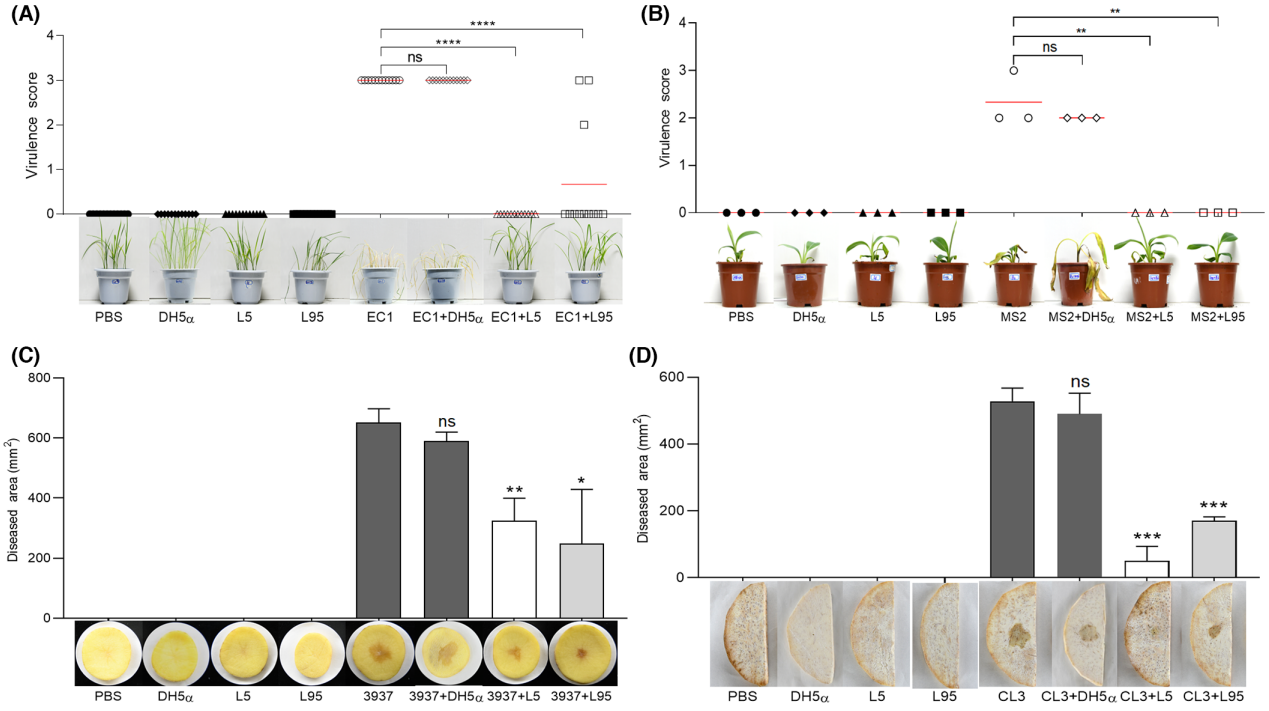


FIGURE 4 Control efficacy of VFM quenching bacteria L5 and L95 against crop soft rot diseases on rice caused by *D. oryzae* EC1 (A), banana caused by *D. zeae* MS2 (B), potato caused by *D. dadantii* 3937 (C) and taro caused by *D. fangzhongdai* CL3 (D). Twelve rice seedling, three banana seedlings, three potato and taro slices were inoculated each time, and three independent tests were performed with similar results. The data were subjected to Student's *t*-test analysis by Graphpad Prism 9 (ns, no statistical significance; ns, no significance; $*p<0.05$; $**p<0.01$; $***p<0.001$; $****p<0.0001$).

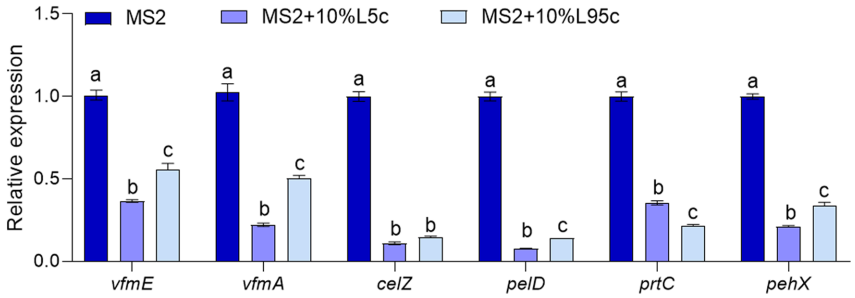


FIGURE 5 Effect of L5 and L95 concentrated supernatants on *vfmE*, *vfmA*, *celZ*, *pelD*, *prtC* and *pehX* gene expression in MS2. The MS2 bacterial culture was collected at an OD_{600} of 0.8 and RNA was extracted. A housekeeping gene *rpoB* was used as an endogenous control for data analysis. The experiment was performed in triplicate and repeated three times independently. The gene expression was compared between the MS2 cultured with VFM QQ bacterial L5 or L95 concentrated supernatant and the pure MS2 without quenching bacterial supernatants using one-way ANOVA analysis by Graphpad Prism 9. Significantly different values ($p<0.05$) are indicated by different letters.

and *celZ* genes directly regulated by VfmE was also tested (Nasser et al., 2013). The resulting data indicated that the concentrated supernatants of both L5 and L95 significantly decreased the mRNA levels of *vfmE*, *vfmA*, *celZ*, *pelD*, *prtC* and *pehX* (Figure 5). In particular, the concentrated L5 supernatant reduced the mRNA level of *vfmE* by more than 65% and *vfmA* by about 75% (Figure 5). Comparatively, the concentrated L95 supernatant had a slightly lower ability to repress the expression of the *vfm* gene cluster, causing 45% and 50% reductions in the mRNA levels of *vfmE* and *vfmA*, respectively (Figure 5). Previous studies have demonstrated that deletion of *vfmA* and *vfmE* impaired the production of the VFM signal in *D. oryzae* EC1 (Lv, 2018), and transcription of *vfmE* was activated by the VFM signal (Lv et al., 2019; Nasser et al., 2013). Therefore, we propose that L5 and L95 suppressed the expression of key genes of the VFM gene cluster probably due to the quenching of VFM QS signal, which caused a significant reduction in the ability of *Dickeya* spp. soft rot bacteria to produce PCWDEs and achieve the effect of biological control.

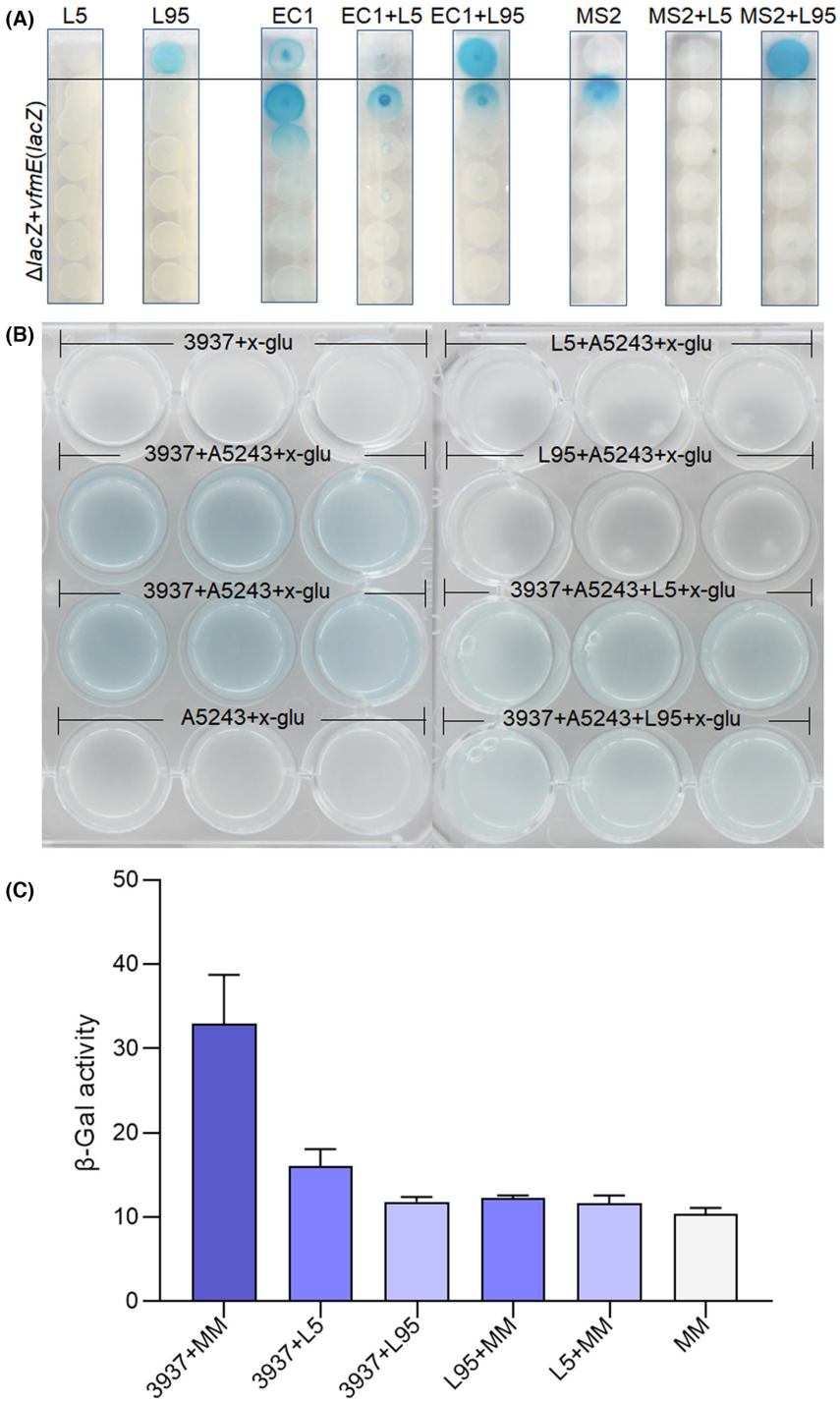


FIGURE 6 L5 and L95 decreased the VFM signal produced by *D. oryzae* EC1, *D. zeae* MS2 and *D. dadantii* 3937. (A) For detection of the VFM signal produced by EC1 and MS2, 1 μL of the tested strains were spotted at the top of the MM agar strips supplemented with 40 μg/mL of X-Gal, followed by 1 μL of consecutive *ΔlacZ+vfmE(lacZ)* VFM biosensor culture (OD_{600} of 1.0) spots. The blue distance indicates the signal intensity. (B) For detection of the VFM signal produced by *D. dadantii* 3937, 1 mL of MM medium containing 40 μg/mL x-Glu and 1% A5243 bacterial culture (OD_{600} = 1.0) was added into each well of a 24 well-tissue culture plate. 10 μL of 3937, L5 and L95 bacterial cultures (OD_{600} = 1.0) were then added when required and incubated at 28°C for 12 h. (C) For quantitation of the β-Gal activity, 100 μL of 12-h culture supernatants of the tested strains were added into the wells containing 1 mL of MM medium and 1 μL of A5243 culture (OD_{600} = 1.8), grown at 28°C by shaking at 200 rpm for 12 h and centrifuged at 4°C, 4000 rpm for 25 min. 10 μL of the supernatant was mixed with 10 μL of extraction buffer and 80 μL of assay buffer, incubated at 37°C for 10 min and cooled at room temperature for 20 min. The reaction was terminated by adding 200 μL of 0.2 M Na_2CO_3 solution, serially diluted and measured the MFI values by MMR. The β-Gal activity was calculated based on $MFI \times 0.1/(\beta \times 30 \times 0.01 \times OD_{600})$, where β is the formula coefficient of β-Gal activity standard curve.

L5 and L95 quenched the VFM signal produced by *Dickeya*

To verify the quenching ability of both strains on the VFM QS signal, we used the previously constructed biosensor *ΔlacZ + vfmE(lacZ)* in detecting the VFM signal produced by *D. oryzae* EC1 and *D. zeae* MS2 (Lv et al., 2019), using the methods described (Hussain et al., 2008). Briefly, the tested strains were spotted at the top of the assay strips, and the biosensor *ΔlacZ + vfmE(lacZ)* was then spotted below. The VFM signal produced by EC1 and MS2 strains could diffuse and be sensed by the biosensor, thus the diffusive distance of blue reflects the signal intensity. The results showed that after incubation for 12 h, L95 turned blue by itself due to its *lacZ* gene, whereas L5 did not; the addition of both quenching strains reduced the VFM signal produced by both EC1 and MS2 (Figure 6A).

Our previous study has shown that although the *vfm* gene cluster is conserved and shares high sequence identities in different *Dickeya* spp. (Liu et al., 2022), the chemical structures of the VFM signal may differ between species, since the VFM signal biosensor of *D. dadantii* 3937 A5243 that senses the VFM signal produced by 3937 (Table 1) could not sense the VFM signal produced by *D. oryzae* EC1 and *D. zeae* MS2, and vice visa (Lv et al., 2019). Given that these two quenching bacteria have a good performance on biocontrolling potato soft rot caused by *D. dadantii* 3937 (Figure 4C), we believed that they could probably quench the VFM signal produced by 3937. To verify this, we used the 3937 VFM signal biosensor A5243 to measure the quenching ability of L5 and L95. The results showed that the addition of L5 and L95 bacterial cultures could quench the VFM QS signal produced by *D. dadantii* 3937 (Figure 6B). Quantification of the β-Gal activity also indicated the quenching effect of L5 and L95 on the VFM signal produced by *D. dadantii* 3937 (Figure 6C).

CONCLUSION

Bacterial pathogens commonly employ more than one set of QS systems to regulate the expression of virulence genes. *Dickeya* are important soft rot bacterial pathogens worldwide. In *Dickeya* genus, VFM has been demonstrated as the primary QS system contributing to pathogen virulence. However, the chemical structure of the signal has not been identified yet, and the signal can be self-quenched by *Dickeya* after completing infection process, which has become a barrier to the development of the QQ technology for *Dickeya* soft rot. This study is dedicated to finding QQ bacteria that achieve biocontrol effects by quenching the VFM QS signal in *Dickeya* bacteria. By using a sensitive VFM biosensor VR2 constructed by us, two QQ strains with a significant effect on quenching the VFM signal and reducing the PCWDE production were screened out and identified. Both strains were not harmful to the host plants and were able to attenuate the virulence of various *Dickeya* pathogens to the corresponding host plants. Finally, we demonstrated that both strains can decrease the transcript levels of the VfmE regulated genes in *D. zeae* MS2. The above findings reveal the potential of *P. chlororaphis* L5 and *E. asburiae* L95 as biocontrol QQ agents in controlling infectious diseases caused by VFM-dependent bacterial pathogens. Our work also lays a solid foundation for the identification of VFM quenching genes.

AUTHOR CONTRIBUTIONS

Fan Liu: Data curation (lead); formal analysis (lead); writing – original draft (lead); writing – review and editing (equal). **Ming Hu:** Data curation (equal); formal analysis (equal); project administration (equal). **Xu Tan:** Data curation (equal); formal analysis (equal). **Yang Xue:** Formal analysis (supporting). **Chuhao Li:** Data curation (supporting). **Si Wang:** Resources (equal). **Mingfa Lv:** Resources (equal). **Xiaoyuan Chen:** Resources (equal). **Xiaofan Zhou:** Data curation (supporting). **Lian-Hui Zhang:** Funding acquisition (lead). **Jianuan Zhou:** Conceptualization (lead); funding acquisition (lead); investigation (lead); methodology (lead); supervision (lead); writing – review and editing (lead).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The 16S rRNA, *gyrB, rpoB* and *rpoD* gene sequencing data used in this study have been deposited to the GenBank, accession numbers OP964057.1, OQ053243.1 and OQ053242.1 for 16S rRNA, *rpoB* and *rpoD* of strain L5, and OP964363.1, OQ053245.1 and OQ053244.1 for 16S rRNA, *gyrB* and *rpoD* of strain L95, respectively.

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SUPPORTING INFORMATION

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