



The Probiotic Bacterium *Phaeobacter inhibens* Downregulates Virulence Factor Transcription in the Shellfish Pathogen *Vibrio coralliilyticus* by *N*-Acyl Homoserine Lactone Production

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ABSTRACT *Phaeobacter inhibens* S4Sm acts as a probiotic bacterium against the oyster pathogen *Vibrio coralliilyticus*. Here, we report that *P. inhibens* S4Sm secretes three molecules that downregulate the transcription of major virulence factors, metalloprotease genes, in *V. coralliilyticus* cultures. The effects of the S4Sm culture supernatant on the transcription of three genes involved in protease activity, namely, *vcpA*, *vcpB*, and *vcpR* (encoding metalloproteases A and B and their transcriptional regulator, respectively), were examined by reverse transcriptase quantitative PCR (qRT-PCR). The expression of *vcpB* and *vcpR* were reduced to 36% and 6.6%, respectively, compared to that in an untreated control. We constructed a *V. coralliilyticus* green fluorescent protein (GFP) reporter strain to detect the activity of inhibitory compounds. Using a bioassay-guided approach, the molecules responsible for *V. coralliilyticus* protease inhibition activity were isolated from S4Sm supernatant and identified as three *N*-acyl homoserine lactones (AHLs). The three AHLs are *N*-(3-hydroxydecanoyl)-L-homoserine lactone, *N*-(dodecanoyl-2,5-diene)-L-homoserine lactone, and *N*-(3-hydroxytetradecanoyl-7-ene)-L-homoserine lactone, and their half maximal inhibitory concentrations (IC₅₀s) against *V. coralliilyticus* protease activity were 0.26 μM, 3.7 μM, and 2.9 μM, respectively. Our qRT-PCR data demonstrated that exposures to the individual AHLs reduced the transcription of *vcpR* and *vcpB*. Combinations of the three AHLs (any two or all three AHLs) on *V. coralliilyticus* produced additive effects on protease inhibition activity. These AHL compounds may contribute to the host protective effects of S4Sm by disrupting the quorum sensing pathway that activates protease transcription of *V. coralliilyticus*.

IMPORTANCE Probiotics represent a promising alternative strategy to control infection and disease caused by marine pathogens of aquaculturally important species. Generally, the beneficial effects of probiotics include improved water quality, control of pathogenic bacteria and their virulence, stimulation of the immune system, and improved animal growth. Previously, we isolated a probiotic bacterium, *Phaeobacter inhibens* S4Sm, which protects oyster larvae from *Vibrio coralliilyticus* RE22Sm infection. We also demonstrated that both antibiotic secretion and biofilm formation play important roles in S4Sm probiotic activity. Here, we report that *P. inhibens* S4Sm, an alphaproteobacterium and member of the *Roseobacter* clade, also secretes secondary metabolites that hijack the quorum sensing ability of *V. coralliilyticus* RE22Sm, suppressing virulence gene expression. This finding demonstrates that probiotic bacteria can exert their host protection by using a multipronged array of behaviors that limit the ability of pathogens to become established and cause infection.

KEYWORDS *N*-acyl homoserine lactones, *Phaeobacter inhibens*, *Vibrio coralliilyticus*, marine pathogens, metalloprotease, oyster disease, probiotic, quorum sensing

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Infections caused by pathogenic marine bacteria are a major problem for both the shellfish and finfish aquaculture industries, causing severe disease and high mortality, which seriously affect aquaculture production and cause significant economic loss (1, 2). Opportunistic pathogens from the *Vibrionaceae* family frequently cause disease in a variety of shellfish (3, 4). For example, *Vibrio coralliilyticus* RE22 (formerly *Vibrio tubiashii* RE22 [5, 6]), a reemerging pathogen of larval bivalve mollusks, causes invasive and toxigenic disease and has been responsible for high mortalities among oysters on the west coast of the United States (3). Vibriosis is characterized by a rapid and large reduction in larval motility, detached vela, and necrotic soft tissue, which result in high mortality within 1 day of infection (7).

While *V. coralliilyticus* virulence certainly involves several factors, the hemolysin and extracellular protease activities are thought to play important roles during pathogenesis in oysters (4, 8). *V. coralliilyticus* RE22 harbors two metalloproteases, VcpA and VcpB (formerly VtpA and VtpB) (4) and contains at least one hemolysin gene locus, *vchBA* (formerly *vthBA*) (8). The extracellular metalloproteases facilitate bacterial invasion and the infection process, acting to enhance tissue permeability and leading to necrotic tissue damage and cytotoxicity in the host (9). Mutations in the two metalloprotease genes resulted in significantly reduced protease activity of the *V. coralliilyticus* RE22 supernatant and toxicity to oyster larvae; thus, these two proteases are considered major virulence factors in *V. coralliilyticus* (4). Although the regulation of the virulence factors in *V. coralliilyticus* RE22 is not fully understood, Hasegawa and Hase (10) reported that VcpR is a member of the TetR family of transcriptional regulators and positively regulates the expression of several virulence genes, including *vcpA*, *vcpB*, and *vchBA* in *V. coralliilyticus* RE22. VcpR was also found to exhibit strong homology to several quorum sensing regulators, including orthologs LuxR (*Vibrio harveyi*, identity 84%) and HapR (*Vibrio cholerae*, identity 75%), suggesting that VcpR functions as a quorum sensing regulator in *V. coralliilyticus* RE22. However, the mechanism(s) of VcpR regulation of various virulence factors in *V. coralliilyticus* RE22 has not been fully investigated.

Phaeobacter inhibens is a member of the *Alphaproteobacteria* and of the *Roseobacter* clade (11–13). Previously, we isolated *P. inhibens* S4 from the inner shell surface of a healthy oyster and showed that S4Sm (a spontaneous streptomycin-resistant mutant) functions as a probiotic treatment for oyster larvae. S4Sm exhibited strong antipathogen activity and increased host survival against *V. coralliilyticus* RE22 and *Alliroseovarius* (*Roseovarius*) *crassostreae* challenge (14). We also demonstrated that the production of tropodithietic acid (TDA), a broad-spectrum antibiotic active against many marine pathogens, and biofilm formation play important roles in S4Sm probiotic activity (15). However, the complete mechanisms for the probiotic activities of *P. inhibens* S4Sm are not fully understood.

In addition to antibiotic production, probiotic bacteria are able to secrete various types of secondary metabolites, some of which antagonize pathogenic bacteria. Holmstrøm and Gram (16) reported that *Pseudomonas fluorescens* AH2 secretes siderophores into the culture supernatant, which efficiently chelate iron, resulting in the cessation of growth due to iron deprivation for the pathogen *Vibrio anguillarum*. Li et al. (17) reported that *Lactobacillus reuteri* produces cyclic dipeptides, which are able to quench *agr*-mediated expression of toxic shock syndrome toxin-1 in staphylococci. Bayoumi and Griffiths (18) discovered that bioactive molecules produced by *Bifidobacterium bifidum* possess inhibitory activity against regulatory and virulence genes in the major pathogenicity islands of *Salmonella enterica* serovar Typhimurium and *Escherichia coli* O157:H7.

In this report, the ability of *P. inhibens* S4Sm to produce secondary metabolites that repress the expression of extracellular protease activity, a major virulence factor, in *V. coralliilyticus* RE22Sm is described. The compounds were identified as *N*-acyl homoserine lactones (AHLs). The active AHLs function to suppress the transcription of *vcpR*, the quorum sensing regulator that positively regulates protease (VcpB) production. These results contribute to a better understanding of interspecies cell-to-cell communication

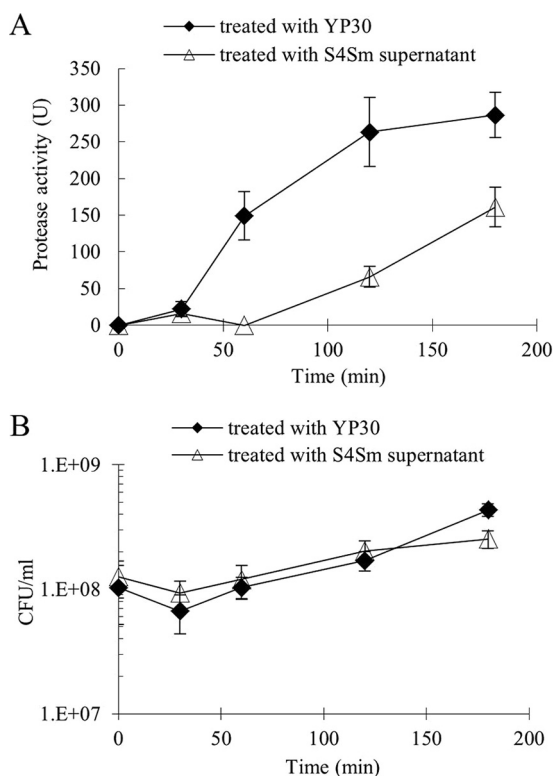


FIG 1 Effects of *P. inhibens* S4Sm culture supernatant on protease activity and growth of *V. coralliilyticus* RE22Sm. (A) Determination of RE22Sm protease activities after addition of S4Sm supernatant or fresh mYP30 medium (control) by measuring OD₄₄₂ of azopeptide from azocasein degradation caused by protease activity. (B) Growth of RE22Sm cells treated with S4Sm supernatant or fresh mYP30 medium (control). Data are averages from two independent experiments and each independent experiment had three replicates. Error bars represent one standard deviation.

between *P. inhibens* and *V. coralliilyticus* and provide an additional mechanism by which probiotic bacteria may attenuate virulence factor production by bacterial pathogens.

RESULTS

***P. inhibens* S4Sm supernatant inhibited protease activity of *V. coralliilyticus* RE22Sm but not growth.** We were curious whether *P. inhibens* S4Sm produced any compounds other than TDA that might affect virulence factors such as protease activity. When a *V. coralliilyticus* RE22Sm ($\sim 1 \times 10^8$ CFU/ml) was incubated in fresh yeast extract-peptone plus marine salts (mYP30) medium containing an equal volume of a sterile-filtered culture supernatant from stationary-phase *P. inhibens* S4Sm culture, the protease activity of *V. coralliilyticus* RE22Sm was suppressed (Fig. 1A). Specifically, at 1 h, no protease activity was detected in the *V. coralliilyticus* RE22Sm culture incubated with the *P. inhibens* S4Sm supernatant, while the *V. coralliilyticus* RE22Sm control culture had 149 ± 33 U of protease activity. At later times, protease activity from the S4Sm-treated sample increased but was still significantly lower than the control. In contrast, the growth rates of *V. coralliilyticus* RE22Sm in mYP30 (control) and in mYP30 supplemented with the *P. inhibens* S4Sm supernatant were nearly identical throughout the 3 h of incubation (Fig. 1B).

***P. inhibens* S4Sm supernatant inhibited transcription of metalloprotease gene *vcpB* and its transcriptional regulator, *vcpR*.** Since the S4Sm culture supernatant suppressed *V. coralliilyticus* RE22Sm protease activity, we examined whether there was a protease enzyme inhibitor in the S4Sm culture supernatant. Our data demonstrated that the mixture of S4Sm and *V. coralliilyticus* RE22Sm cell-free culture supernatants (1:1 mixture) had the same protease activity as the mixture of fresh mYP30 medium and the *V. coralliilyticus* RE22Sm supernatant (1:1 mixture) (see Fig. S1 in the supplemental

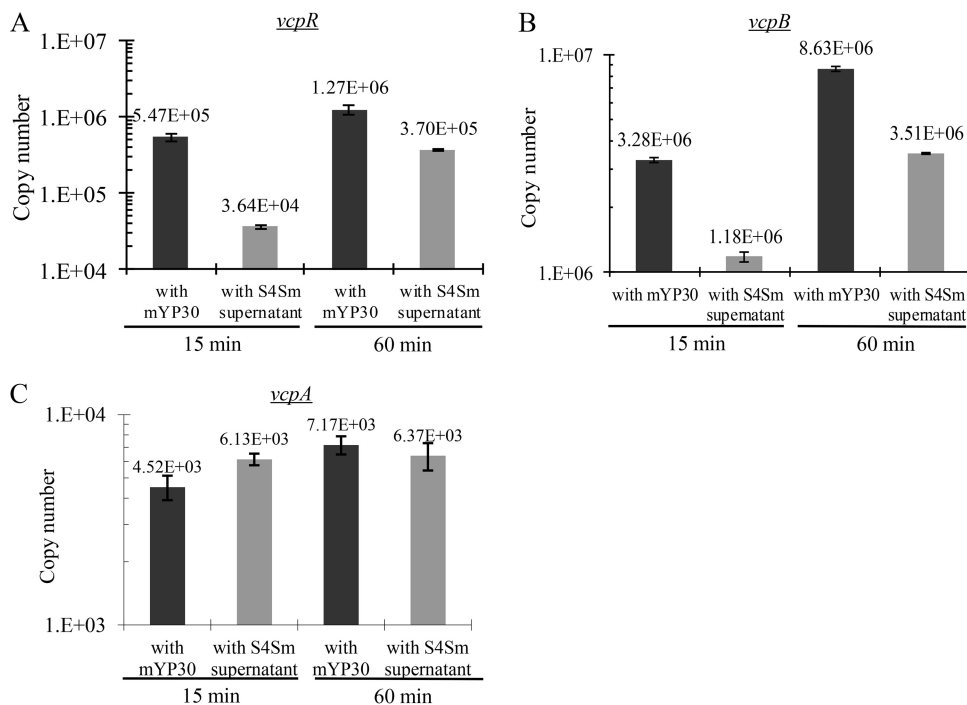


FIG 2 Effects of *P. inhibens* S4Sm stationary-phase culture supernatant on transcription of *vcpA*, *vcpB*, and *vcpR* in *V. coralliilyticus* RE22Sm. Expression of *vcpR* (A), *vcpB* (B), and *vcpA* (C) determined by qRT-PCR analysis of RE22Sm during late-logarithmic-phase growth ($\sim 10^8$ CFU/ml) after the addition of either the *P. inhibens* S4Sm supernatant or sterile mYP30 culture medium. Data are representative of two independent experiments. Each value is the average from three replicates. Error bars represent one standard deviation.

material), indicating that there was no direct protease enzyme inhibitor in the S4Sm supernatant. Next, we used real-time reverse transcriptase quantitative PCR (qRT-PCR) to probe the inhibition of the transcription of the *vcpR*, *vcpB*, and *vcpA* genes. The transcription of *vcpR* and *vcpB* was reduced to 6.6% and 36%, respectively, after 15 min in the treated RE22Sm cultures compared to that in the control (Fig. 2A and B). After 60 min, the transcription of *vcpR* and *vcpB* was 29% and 41%, respectively, of the control. In contrast, there was no significant difference between the transcription levels of *vcpA* in treated and control cultures (Fig. 2C). These data suggested that one or more compounds present in the S4Sm culture supernatant inhibited *V. coralliilyticus* RE22Sm protease activity by suppressing transcription from the *vcpR* transcriptional regulator.

Previously, Hasegawa and Hase (10) identified VcpR as a member of the TetR family of transcriptional regulators and found that it exhibited high homology to several quorum sensing regulators, including LuxR (*V. harveyi*, identity, 84%; E value, $1e-126$) and HapR (*V. cholerae*, identity, 75%; E value, $2e-121$), suggesting that VcpR functions as a quorum sensing regulator in *V. coralliilyticus* RE22 and that elements of the RE22 quorum sensing pathway might be involved in protease production in RE22. It should be noted that genes encoding homologs of the AHL quorum sensing (QS) circuit components, including *luxM*, *luxN*, *luxO*, and *luxU*, have been detected in the draft genome of *V. coralliilyticus* RE22Sm (accession no. [LGLS01000000](https://doi.org/10.1093/aem/0000000000000000) [6]). The *luxM* gene was identified as encoding the acyl-homoserine-lactone synthase, and *luxN* was identified as encoding the AHL receptor protein in the RE22Sm genome. Deletion mutations in *luxM*, *luxN*, or *vcpR* genes resulted in the loss of at least 50% of the protease activity in RE22Sm (see Fig. S2). This indicates that the quorum sensing pathway regulates protease production in RE22.

Isolation of active compounds antagonizing the expression of *V. coralliilyticus* protease. We first confirmed that the pure TDA antibiotic (0.5 to 1.0 $\mu\text{g/ml}$) did not inhibit protease activity but did inhibit *V. coralliilyticus* RE22Sm growth at 1.0 $\mu\text{g/ml}$ (see Fig. S3). This observation strongly suggested that other molecules, not TDA, secreted by

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Resistance	Reference or source
Strains			
<i>P. inhibens</i>			
S4	Previously named <i>Phaeobacter</i> sp. strain S4; wild-type isolate from the inner shell of oysters		14
S4Sm	Spontaneous Sm ^r mutant of S4	Sm ^r	15
<i>V. coralliilyticus</i>			
RE22	Wild-type isolate strain		5, 6
RE22Sm	Spontaneous Sm ^r mutant of RE22	Sm ^r	15
WZ112	Reporter strain, RE22Sm tagged by pSUP203-PvcpB-gfp	Sm ^r Km ^r Ap ^r	This study
CS01	<i>luxN</i> deletion mutant	Sm ^r	This study
CS03	<i>vcpR</i> insertional merodiploid mutant	Sm ^r Km ^r	This study
CS04	<i>luxM</i> deletion mutant	Sm ^r	This study
<i>V. anguillarum</i>			
M93sm	Spontaneous Sm ^r mutant of M93 (serotype J-O-1)	Sm ^r	46
<i>E. coli</i>			
Sm10	<i>thi thr leu tonA lacY supE recA</i> RP4-2 Tc::Mu::Km (λpir)	Km ^r	47
WS203	Sm10(pSUP203)	Cm ^r Km ^r Ap ^r	This study
WS203-vcpB	Sm10(pSUP203-PvcpB-gfp)	Km ^r Ap ^r	This study
JB525	MT102(pJBA132)	Tc ^r	48
JM109L	JM109(pSB1075)	Ap ^r	49
Plasmids			
pSUP203	<i>E. coli</i> - <i>V. anguillarum</i> shuttle vector	Ap ^r Km ^r	This study
pSUP203-PvcpB-gfp	Derivative of pSUP203	Ap ^r Km ^r	This study
pJBA132	<i>luxR</i> -PluxI-RBSII-gfp (ASV) in pME6031 (detects C ₆ - to C ₈ -AHLs)		48
pSB1075	<i>luxCDABE</i> fused to <i>lasRI</i> in pUC18 (detects C ₁₀ - to C ₁₂ -AHLs)		49
pDM5	Suicide vector for construction of deletion mutant; <i>sacBR oriR6K</i>	Cm ^r Km ^r	This study

P. inhibens S4Sm were responsible for the inhibition of protease transcription and activity. Furthermore, the induction of protease activity in *V. coralliilyticus* RE22Sm was delayed for 2 h when the cells were exposed to either the S4Sm supernatant or an ethyl acetate extract of the S4Sm supernatant (see Fig. S4A). The growth of RE22Sm in all treatment groups was unaffected (Fig. S4B). These results suggested that compounds that inhibit *V. coralliilyticus* RE22Sm protease expression were in the ethyl acetate extract and are likely nonpolar molecules. To guide the isolation of the active compounds, a reporter strain, *V. coralliilyticus* WZ112, was constructed (Table 1). WZ112 harbors a plasmid that contains the promoter region of *vcpB* fused to a promoterless *gfp*. WZ112 was cultivated in 96-well plates in the presence of test fractions. The wells containing compounds that inhibit VcpB protease induction had lower fluorescence signals. Further purification of ethyl acetate fractions by using successive reversed-phase medium-pressure chromatography, LH-20 size exclusion chromatography, and C₁₈ high-pressure liquid chromatography (HPLC) resulted in the isolation of three active compounds, herein designated 1, 2 and 3.

Structure determination of the inhibitors. The structures of compounds 1, 2, and 3 were identified as *N*-acyl-homoserine lactones (AHLs) (Fig. 3) using a combination of analyses emphasizing nuclear magnetic resonance (NMR) spectroscopic and mass spectrometric data. Spectral data revealed compound 1 to be the previously reported *Phaeobacter* AHL (3*R*)-*N*-(3-hydroxydecanoyl)-L-homoserine lactone (see Fig. S5A and B) (19, 20). The molecular formula of compound 2 was determined as C₁₆H₂₅NO₃ by high-resolution electrospray ionization-mass spectrometry (HRESI-MS; AB SCIEX TripleTOF 4600) at *m/z* 302.1729 [M+Na]⁺ (calculated for C₁₆H₂₅NO₃Na, 302.1732). Compound 2 showed similar ¹H and ¹³C NMR features as compound 1 (see Fig. S6A and B, respectively); the difference between them resided in the acyl chain. Four olefinic protons signal at δ 6.80 (1 H, dt, *J* = 15.6, 6.3 Hz, H-3), 5.95 (1 H, dt, *J* = 15.6, 1.7 Hz, H-2), 5.55 (1 H, m, H-6), and 5.41 (1 H, m, H-5) were observed in the ¹H NMR spectrum, indicating the presence of two double bonds in the side chain. An analysis of two dimensional (2D) NMR (¹H-¹H correlation spectroscopy [COSY] and heteronuclear multiple bond correlation [HMBC]) (Fig. S6C to E) data enabled the assignment of the

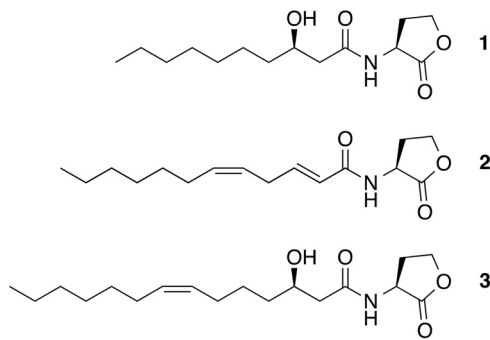


FIG 3 Chemical structures of *N*-acyl homoserine lactones produced by *P. inhibens* S45m. The compounds are 1, (3*R*)-*N*-(3-hydroxydecanoyl)-*L*-homoserine lactone; 2, (2*E*,5*Z*)-*N*-(dodecanoyl-2,5-diene)-*L*-homoserine lactone; and 3, (3*R*,7*Z*)-*N*-(3-hydroxytetradecanoyl-7-ene)-*L*-homoserine lactone.

positions of the double bonds. The 2*E*,5*Z* configurations were determined by the coupling constant ($J_{2,3} = 15.6$ Hz) and the nuclear Overhauser effect spectroscopy (NOESY) correlation between H-5 and H-6. Thus, compound 2 was elucidated as the new AHL (2*E*,5*Z*)-*N*-(dodecanoyl-2,5-diene)-*L*-homoserine lactone. Compound 3 was identified as (3*R*,7*Z*)-*N*-(3-hydroxytetradecanoyl-7-ene)-*L*-homoserine lactone by comparing the NMR and MS data with those previously reported for this molecule (Fig. S5C and D) (21).

Exposure to the individual AHL reduced transcription of *vcpR* and *vcpB*. All three AHLs showed concentration-dependent inhibition of protease activity on late exponential-phase *V. coralliilyticus* RE22Sm (Fig. 4). The half maximal inhibitory concentrations (IC_{50} s) of 1, 2, and 3 were determined to be 0.26 μ M, 3.7 μ M, and 2.9 μ M, respectively. None of the molecules demonstrated growth effects against *V. coralliilyticus* RE22Sm at the highest concentration tested (5 μ M) (see Fig. S7).

We then asked if the AHLs also altered the transcription of genes involved in *V. coralliilyticus* RE22Sm protease production. The data presented in Fig. 5 demonstrate that when compounds 1, 2, and 3 were added to cultures at their calculated IC_{50} concentrations, the transcription levels of *vcpR* and *vcpB* were reduced to ~14% to 15% and ~42% to 51%, respectively, of the untreated control. In contrast, the transcription of *vcpA* was unaffected by AHL exposure (98% to 110% of the untreated control). Furthermore, while treatment with any single AHL compound at its IC_{50} suppressed *V. coralliilyticus* RE22Sm protease activity by ~50% of the untreated control (Fig. 6), the effects of combinations of these AHLs were additive. The treatment of *V. coralliilyticus* RE22Sm cells with a combination of any two AHLs (each at IC_{50}) decreased protease activities further: 32% for compounds 1 plus 2, 27% for compounds 1 plus 3, and

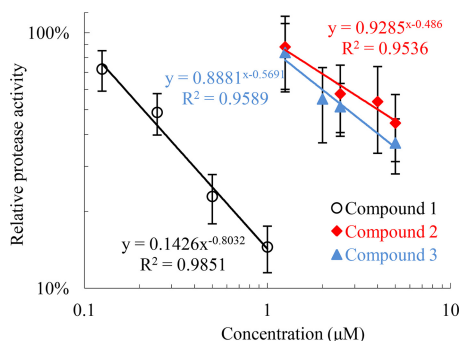


FIG 4 Concentration-response analyses of the three AHLs. Series of different concentrations of each AHL were used to treat *V. coralliilyticus* RE22Sm and the protease activities were measured. Concentration-response curves (trend lines) of each AHL were obtained. Data are the averages from two independent experiments and each independent experiment had three replicates. Error bars represent one standard deviation.

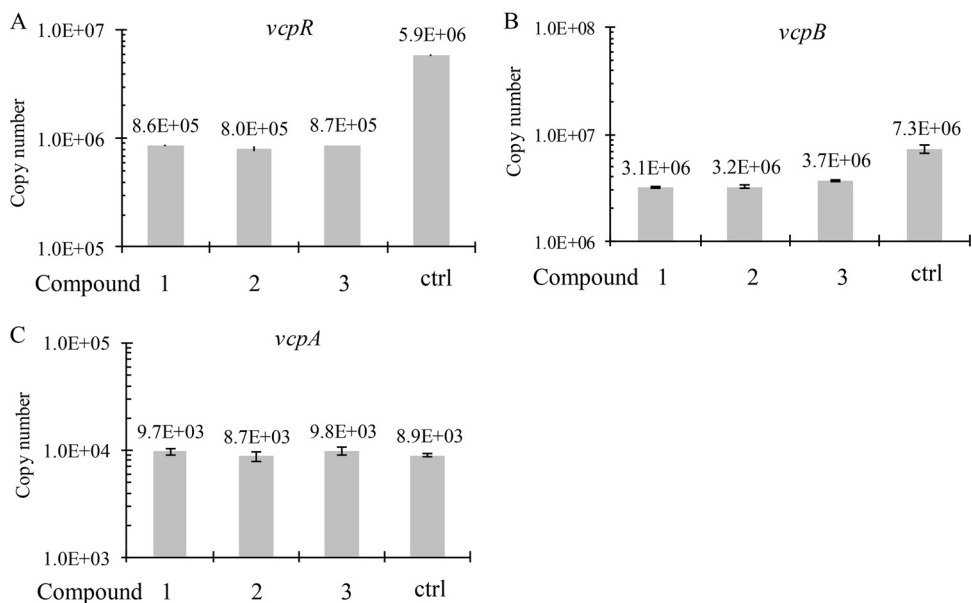


FIG 5 Effects of single AHL treatment on transcription of *vcpA*, *vcpB*, and *vcpR* in *V. coralliilyticus* RE22Sm. Expression of *vcpR* (A), *vcpB* (B), and *vcpA* (C) determined by qRT-PCR analysis of *V. coralliilyticus* RE22Sm treated with individual AHLs (at their own IC₅₀) for 15 min during late-logarithmic-phase growth (~10⁸ CFU/ml). Data are representative of two independent experiments. Each value is the average from three replicates. Error bars represent one standard deviation.

34% for compounds 2 plus 3. The treatment of *V. coralliilyticus* RE22Sm cells with all three AHLs (each at IC₅₀) resulted in the greatest inhibition of protease activity, with the treated cells having only 18% of the control cell activity (Fig. 6). These data suggest that the three AHLs produced by *P. inhibens* S4Sm inhibit the transcription of the transcriptional regulator, VcpR, to downregulate transcription of the metalloprotease VcpB.

Inhibitory effects of *P. inhibens* S4Sm AHLs against *V. coralliilyticus* RE22Sm protease production were specific. Previously, Ke et al. (22) showed that in *Vibrio harveyi* long-chain AHLs acted as antagonists of *N*-[(*R*)-3-hydroxybutanoyl]-L-

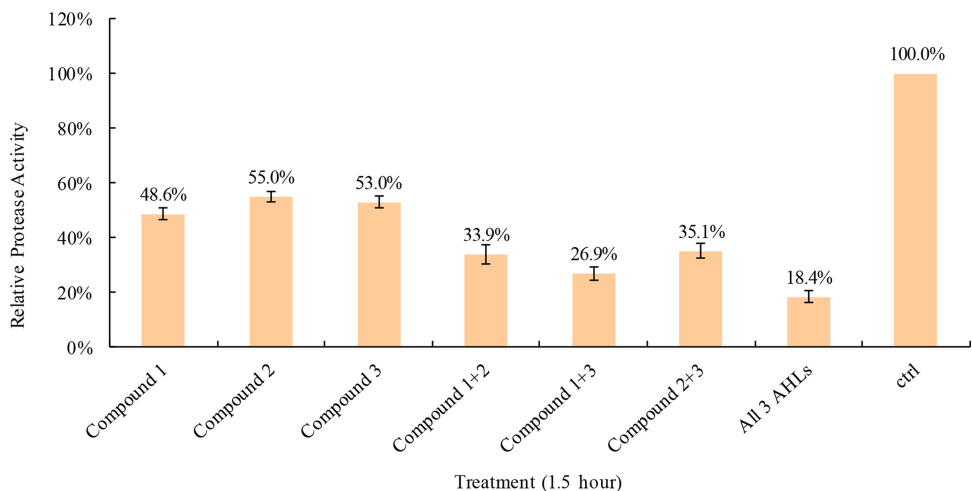


FIG 6 Effects of different combinations of three AHLs on protease activity of *V. coralliilyticus* RE22Sm. Determination of protease activities of *V. coralliilyticus* RE22Sm strain treated with a single AHL (at the IC₅₀), various combinations of AHLs (each at the calculated IC₅₀), or an appropriate amount of methanol (ctrl) by measuring OD₄₄₂ of azopeptide from azocasein degradation caused by protease activity. Data are representative of two independent experiments. Each value is the average from three replicates. Error bars represent one standard deviation.

homoserine lactone (3OH-C₄-HSL) binding to LuxN, causing a suppression of bioluminescence. We tested whether other AHL molecules, which share the same acyl-chain lengths as the *P. inhibens* S4Sm AHLs but with different modifications, would show similar inhibitory activity against *V. coralliilyticus* protease transcription. *V. coralliilyticus* RE22Sm cells were treated with the synthetic AHLs *N*-decanoyl-DL-homoserine lactone, *N*-dodecanoyl-DL-homoserine lactone, or *N*-tetradecanoyl-DL-homoserine lactone at concentrations of 0.26 μ M, 3.7 μ M, or 2.9 μ M (the IC₅₀s of compounds 1, 2, and 3), respectively, to determine if the inhibition of the transcription of *vcpR* and *vcpB* was specific to AHLs produced by *P. inhibens* S4Sm. The data presented in Fig. S8 show that the transcription levels of *vcpB* and *vcpR* were reduced to 60% to 100% and 44% to 88%, respectively, of the untreated control and were not statistically significant. Additionally, these AHLs did not affect the growth of *V. coralliilyticus* RE22Sm (Fig. S8C).

Our data suggest that AHLs from *P. inhibens* S4Sm act as antagonists to quorum sensing in *V. coralliilyticus*. We were interested to determine whether the *P. inhibens* S4Sm AHLs act to inhibit the transcription of *vanT* (a *vcpR* homolog; the two proteins, VanT and VcpR, are 83% identical, 90% similar, E value = 3e-130) in *V. anguillarum* M93Sm. Using two AHL indicator strains, *E. coli* JB525 (detects C₆- to C₈-HSLs) and *E. coli* JM109L (detects C₁₀- to C₁₂-HSLs) (23), we found that *V. anguillarum* M93Sm secreted both C₆- to C₈-HSLs and C₁₀- to C₁₂-HSLs (corresponding to 3-OH-C₆-HSL and 3-oxo-C₁₀-HSL reported by Croxatto et al. [24] in *V. anguillarum* NB10), while *V. coralliilyticus* RE22Sm and *P. inhibens* S4Sm secrete C₁₀- to C₁₂-HSLs but not C₆- to C₈-HSLs. When the effect of the *P. inhibens* S4Sm culture supernatant on *V. anguillarum* M93Sm *vanT* expression was examined, we found that the expression of *vanT* was not affected (see Fig. S9). These data further support the idea that the *P. inhibens* S4Sm AHL antagonism to quorum sensing is highly specific.

DISCUSSION

In this study, we present data that *P. inhibens* S4Sm produces AHLs, which block virulence factor production and may contribute to *P. inhibens* probiotic activity against the marine pathogen *V. coralliilyticus*. Specifically, we found that the stationary-phase culture supernatant of *P. inhibens* S4Sm repressed protease activity, but not growth, of *V. coralliilyticus* RE22. Our data suggest, but do not conclusively demonstrate, that the repression of protease activity resulted from a repression of the transcriptional activator *vcpR* and the subsequent repression of the protease gene *vcpB*. A *vcpB-gfp* reporter strain of *V. coralliilyticus* was constructed to help identify three AHLs as the repressors of *vcpR* and *vcpB*. Since the active compounds are AHLs, this suggests that they function as quorum sensing (QS) compounds for *P. inhibens* S4Sm and as quorum quenching (QQ) compounds against *V. coralliilyticus* RE22.

Quorum sensing has been demonstrated to regulate diverse physiological activities in Gram-positive and Gram-negative bacteria, including virulence, symbiosis, competence, conjugation, antibiotic production, motility, and biofilm formation (25). Gram-negative bacteria generally use AHLs as autoinducers for QS. Quorum quenching is a disruption of QS pathways by any of several mechanisms, including antagonist binding to the native AHL receptor, degradation of native AHL signals, suppression of AHL synthetase, and receptor activities (25). Thus, QQ can interfere with QS-regulated bacterial physiological functions (23, 26). For example, synthetic antagonists are able to block pathogenic bacterial quorum sensing in *Pseudomonas aeruginosa* and *Agrobacterium tumefaciens* (27). Additionally, McClean et al. (28) found that in *Chromobacterium violaceum*, exogenously added AHLs (C₁₀- to C₁₄-HSLs) inhibited the production of violacein in the presence of inducing concentrations of the native QS AHL (C₆-HSL) and may represent QQ by competitive inhibition. Hemolytic activity (downregulation of streptolysin S) and viability of *Streptococcus pyogenes* were inhibited by oxo-C₁₂-HSL and oxo-C₁₄-HSL and by AHLs secreted by *Pseudomonas aeruginosa* (29). This inhibition appears to be an example of interspecies signaling or eavesdropping. In contrast, some bacteria, such as *Bacillus* and *Streptomyces* strains, are able to produce AHL-degrading

enzymes, such as lactonase (30) and acylase (31). Other bacteria have been shown to secrete non-AHL secondary metabolites that interfere with QS-controlled processes (32). The interference with QS-regulated virulence has been proposed as a new direction for the development of novel antimicrobial chemotherapies (32–34).

The protease inhibition activity of the *P. inhibens* S4Sm supernatant was transient, as it gradually diminished during the experiment (Fig. 1A; see also Fig. S3A in the supplemental material). One possibility for this observation is the competitive inhibition of *V. coralliilyticus* RE22-AHL-mediated QS regulation of *vcpR* gene expression by *P. inhibens* S4Sm AHLs. Specifically, as *V. coralliilyticus* RE22Sm cells grow, increasing concentrations of native AHLs are produced, diluting the exogenous *P. inhibens* S4Sm-AHLs and resulting in decreased antagonizing effects from *P. inhibens* S4Sm-AHL molecules toward *V. coralliilyticus* RE22Sm protease activity. Additionally, the inhibitory effects of the three *P. inhibens* S4Sm AHLs on *V. coralliilyticus* RE22Sm protease activity were additive. This suggests that each of the three S4Sm AHLs is able to competitively bind the *V. coralliilyticus* RE22Sm LuxN to interfere with its function as a LuxU-PO₄ phosphatase and cause QQ effects. It should be noted that a search of the *V. coralliilyticus* RE22Sm genome (accession no. [LGLS01000000](https://www.ncbi.nlm.nih.gov/nucl/100000000)) revealed only one *luxN* gene. Additional studies comparing the AHLs of *P. inhibens* S4Sm and *V. coralliilyticus* RE22, as well as AHL binding to LuxN, need to be carried out in order to better define this QQ mechanism.

Previously, Berger et al. (19) showed that *P. inhibens* produces *N*-3-hydroxy-decanoylhomoerine lactone, which is involved in the QS induction of the TDA biosynthesis genes in *P. inhibens*. Similarly, *N*-(3-hydroxytetradecanoyl-7-ene)-L-homoerine lactone is produced by *Rhizobium leguminosarum*, where it regulates several QS-mediated pathways, including growth inhibition, adaptation to stationary phase, and shorter-chain AHL production (35). To our knowledge, the production of *N*-(dodecanoyl-2,5-diene)-L-homoerine lactone has not been previously reported and is a newly discovered AHL. No previous studies have described any biological functions of either *N*-(dodecanoyl-2,5-diene)-L-homoerine lactone or *N*-(3-hydroxytetradecanoyl-7-ene)-L-homoerine lactone in *P. inhibens*. The synthetic AHLs did not have an inhibitory effect on *V. coralliilyticus* RE22Sm *vcpR* and *vcpB* transcription, suggesting that the inhibition from *P. inhibens* S4Sm AHLs on *V. coralliilyticus* RE22Sm protease production is specific.

Generally, AHL molecules contain common homoerine lactone moieties and a specific acyl side chains. The side chains vary among different species, and the specificity is determined by such factors as length and sites of oxidation (36). It was previously demonstrated that LuxN of *V. harveyi*, which detects *N*-[(*R*)-3-hydroxybutanoyl]-L-homoerine lactone (3OH-C₄-HSL), can be antagonized by AHLs that lack the hydroxyl group on C-3 if the AHLs have a long acyl chain (*N*-hexanoyl-L-homoerine lactone [C₆-HSL], *N*-octanoyl-L-homoerine lactone [C₈-HSL], *N*-decanoyl-L-homoerine lactone [C₁₀-HSL], and *N*-dodecanoyl-L-homoerine lactone [C₁₂-HSL]) (22). In contrast, our data demonstrate that the expression levels of *vcpR* and *vcpB* were much lower in *V. coralliilyticus* RE22Sm cells exposed to *P. inhibens* S4Sm AHLs than in *V. coralliilyticus* RE22Sm cells exposed to saturated long-chained AHLs. These observations suggest that the three AHLs produced by *P. inhibens* S4Sm are specific inhibitors of the QS system of the oyster pathogen *V. coralliilyticus* RE22, which regulates the transcription of the master regulator gene *vcpR* and, in turn, the transcription of the metalloprotease gene *vcpB*.

Previously, we demonstrated that *P. inhibens* S4Sm is an effective probiotic organism because of its ability to form a biofilm and secrete an antibiotic (TDA) (14, 15). In this study, we showed that S4Sm produces antivirulence agents, AHLs, that appear to cause QQ in a specific pathogen, *V. coralliilyticus* RE22, which shares the same environmental niche. This finding further demonstrates that probiotic bacteria can exert their host protection by using a multipronged array of behaviors that limit the ability of pathogens to become established and cause harm.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All bacterial strains and plasmids used in this study are listed in Table 1. *P. inhibens* S4 was isolated from the inner surface of an oyster shell (14). *P. inhibens* strains and *V. coralliilyticus* strains were routinely grown in yeast extract (0.5%)-peptone (0.1%) broth plus 3% marine salts (mYP30) (14), supplemented with the appropriate antibiotic, in a shaking water bath at 27°C. Cell densities were estimated by the optical density at 600 nm (OD_{600}) and more accurately determined by serial dilution and spot plating. The specific conditions for each experiment are described in the text. *Escherichia coli* strains were routinely grown in Luria-Bertani broth plus 1% NaCl (LB10) (37). Antibiotics were used at the following concentrations: streptomycin 200 $\mu\text{g/ml}$ (Sm^{200}) and chloramphenicol 5 $\mu\text{g/ml}$ (Sm^5) for *P. inhibens*, Sm^{200} , ampicillin 100 $\mu\text{g/ml}$ (Ap^{100}), Sm^5 , and kanamycin 50 $\mu\text{g/ml}$ (Km^{50}) for *Vibrio* strains, and Ap^{100} , Cm^{20} , Km^{50} , and tetracycline 15 $\mu\text{g/ml}$ (Tc^{15}) for *E. coli*. The genes were renamed to reflect the reclassification of *V. tubiashii* RE22 to *V. coralliilyticus* RE22 (e.g., *vtpA* to *vcpA*, *vtpB* to *vcpB*, and *vtpR* to *vcpR*) (38).

***P. inhibens* supernatant challenge assay.** The *P. inhibens* supernatant challenge assay was a modification of the method described previously by Holmström and Gram (16). *P. inhibens* S4Sm supernatant challenge experiments were initially conducted by using exponentially growing *V. coralliilyticus* cultures (OD_{600} of 0.6 to 0.8) and transferring 1 ml of a culture to 4 ml of mYP30 medium containing 2.5 ml of S4Sm supernatant and 1.5 ml of fresh mYP30. For the control, 1 ml of an exponentially growing *V. coralliilyticus* culture was added to 4 ml of fresh mYP30. Cell densities (CFU/ml) were determined at different times after challenge. Also, 1 ml *V. coralliilyticus* RE22Sm supernatant and mRNA were collected at each time point and stored at -20°C and -74°C , respectively, for future use. All experiments were repeated at least twice.

Detection and quantification of protease activity. The protease activity of the culture supernatants was quantified using the azocasein method of Windle and Kelleher (39) as modified by Denkin and Nelson (40). *V. coralliilyticus* filtered supernatant (100 μl) was incubated for 30 min at 27°C with 100 μl of azocasein solution. The reactions were terminated by the addition of trichloroacetic acid (TCA; 10% [wt/vol]) to a final concentration of 6.7% (wt/vol). The mixture was allowed to stand for 2 min and centrifuged (12,000 $\times g$ for 8 min) to remove unreacted azocasein, and the supernatant containing azopeptides was suspended in 700 μl of 525 mM NaOH. The absorbance of the azopeptide supernatant was measured at 442 nm. Protease activity units (U) were calculated with the following equation: $U = [1,000 (OD_{442})/CFU] \times 10^9$, where OD_{442} is the optical density at 442 nm.

mRNA extraction. mRNA extraction was performed using the protocol previously described by Li et al. (41). *V. coralliilyticus* cells under different treatments were treated with RNA Protect bacterial reagent (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was isolated using an RNeasy kit and QIAcube (Qiagen) according to the manufacturer's instructions and stored at -74°C for future use.

Real-time qRT-PCR. Real-time reverse transcriptase quantitative PCR (qRT-PCR) was performed using the protocol previously described by Li et al. (41). qRT-PCR was used to quantify various mRNAs by use of a Roche480 Multiplex quantitative PCR system and Brilliant II SYBR green single-step qRT-PCR master mix (Agilent Technologies, Wilmington, DE) with 10 ng of total RNA in 20- μl reaction mixtures. The thermal profile was 50°C for 30 min, 95°C for 15 min, and then 40 cycles of 95°C for 30 s and 60°C for 30 s. The qRT-PCR primers are listed in Table 2. Fluorescence was measured at the end of the 60°C step during every cycle. The PCR products for each gene were obtained and purified according to a PCR cleanup kit protocol (Monarch PCR & DNA cleanup kit, T1030S; NEB). A standard curve using the purified PCR products was generated for each gene as described by the qRT-PCR protocol from the Genomics and Sequencing Center of the University of Rhode Island (42). A suspension of $\sim 1 \times 10^8$ copies of a gene template was obtained and then diluted in a 10-fold dilution series (in nuclease-free water containing 100 ng/ μl yeast tRNA; Sigma-Aldrich, St. Louis, MO) to obtain $\sim 1 \times 10^1$ copies of the template. The standard curve generated for each gene was used to ascertain primer efficiency and calculate absolute copy numbers (42). Samples were run in triplicates along with no-RT and no-template controls. All experiments were repeated at least twice.

Challenge of *V. coralliilyticus* cells with ethyl acetate extract of *P. inhibens* S4Sm supernatant. The *P. inhibens* cell-free supernatant was extracted twice with ethyl acetate (1:1 volume ratio), and the combined organic layers were concentrated using a rotary evaporator and stored at -20°C until use. The extract was redissolved in fresh mYP30 medium and added to *V. coralliilyticus* RE22Sm cells ($\sim 10^8$ CFU/ml). The CFU/ml of RE22Sm cells was determined at 1-, 2-, and 3-h time points. The *V. coralliilyticus* RE22Sm supernatant was collected at each time point and stored at -20°C .

Construction of reporter strain *V. coralliilyticus* WZ112 and *gfp* reporter assay. *V. coralliilyticus* RE22Sm was tagged by pSUP203-PvcpB-*gfp*. The kanamycin resistance gene, cloned from pCR2.1 TOPO, replaced the tetracycline region in pSUP202-*gfp*. The resulting plasmid was designated pSUP203-*gfp*. The promoter region of *vcpB* was cloned and fused with the *gfp* region in pSUP203-*gfp*. The primers for the amplification of clones are described in Table 2. *E. coli* Sm10 harboring plasmid pSUP203-PvcpB-*gfp* was conjugated with *V. coralliilyticus* RE22Sm by using procedures described previously (43). The transconjugants were confirmed by antibiotic selection and fluorescence microscopy and designated strain WZ112. Aliquots (50 μl) of test compounds from the *P. inhibens* S4Sm supernatant (see below) were transferred into a 96-well microtiter plate, dried *in vacuo*, and then resuspended in 200 μl of *V. coralliilyticus* WZ112 culture (OD_{600} of 0.3). After 1.5 h of incubation at 27°C, OD_{600} and fluorescence signals were measured (Mx3005 multiplex quantitative PCR system, plate read function). The relative fluorescence (RF) was calculated as fluorescence/ OD_{600} .

TABLE 2 Primers used in this study

Primer	Sequence (5'→3') ^a	Description
pw200	GCGGTAAGCTTGAACACGTAGAAAGCCAGTCC	Amplification of <i>kan</i> ORF, forward, with HindIII site
pw201	CTATATGGATCCCGTTTCTGGGTGAGCAAAAAC	Amplification of <i>kan</i> ORF, reverse, with BamHI site
pw204	GATGTTGGTACCTGTGGATCTCTGCCATTGGCT	Amplification of <i>vcpB</i> promoter region, forward, with KpnI site
pw205	CTGCATGGTACCTATGATTTCTCTTATCGTGCC	Amplification of <i>vcpB</i> promoter region, reverse, with KpnI site
pw178 ^b	GACATCTTTAAATCTGAAGGTGGTCTAC	qRT-PCR amplicon of partial <i>vcpA</i> , forward
pw179 ^b	GTAGTATTGAGAAGCATGGTCGATAGAG	qRT-PCR amplicon of partial <i>vcpA</i> , reverse
pw184 ^b	GGTTTCTGTTGCTACAGTTTTAACTACTTC	qRT-PCR amplicon of partial <i>vcpR</i> , forward
pw185 ^b	ATATTATCTGATAGGAAGTTTGAGAACTGAC	qRT-PCR amplicon of partial <i>vcpR</i> , reverse
pw190 ^b	CTAAAAGCATCGTTTGATTTGGTAGCACTAA	qRT-PCR amplicon of partial <i>vcpB</i> , forward
pw191 ^b	GTAAGAAACAATGTCACCATCGTATCACTAC	qRT-PCR amplicon of partial <i>vcpB</i> , reverse
<i>luxN</i> 5'fp	tgtggaatcccgaggagactAACAGTTATTTTCATACAGATCTTC	Amplification of <i>luxN</i> 5' fragment, forward
<i>luxN</i> 5'rp	ctcttattgAAATCATGTAAATACACGAGTTC	Amplification of <i>luxN</i> 5' fragment, reverse
<i>luxN</i> 3'fp	tacatgattACAATAAGAGTGGCGGCAG	Amplification of <i>luxN</i> 3' fragment, forward
<i>luxN</i> 3'rp	gcatgcggtaacctgagctTCTCCCGATTACCTGATAG	Amplification of <i>luxN</i> 3' fragment, reverse
<i>vcpR</i> 5'fp	tgtggaatcccgaggagactTATACAACTCAATTGGCAAGG	Amplification of <i>vcpR</i> 5' fragment, forward
<i>vcpR</i> 5'rp	gattgctttgATGCGATTTCCATCAGTTG	Amplification of <i>vcpR</i> 5' fragment, reverse
<i>vcpR</i> 3'fp	gaaatcgcatCAAAGCAATCGAGCGTGG	Amplification of <i>vcpR</i> 3' fragment, forward
<i>vcpR</i> 3'rp	gcatgcggtaacctgagctTCTAGGTAGCTTTGTGTCAG	Amplification of <i>vcpR</i> 3' fragment, reverse
<i>luxM</i> fp	GCCGAGCTCCACAACATGCAGAAATACTC	Amplification of <i>luxM</i> ORF, forward
<i>luxM</i> rp	GCCTCTAGAAACTCAATTTATGGCGTTCT	Amplification of <i>luxM</i> ORF, reverse
pr25cs	CCTCCTGTTCACTACTGACGGGGTG	For sequencing of the DNA fragment inserted in pDM4 multicloning site

^aPrimer design according to the Gibson assembly protocol (44). Lowercase nucleotides for 5'fp and 3'rp primers are homologous to the pDM4 vector backbone and to each other for proper Gibson assembly. Underlined sequences are engineered restriction sites.

^bAnnealing temperature is 60°C.

Identification of compounds that repress transcription of *vcpR* and *vcpB*. *P. inhibens* S45m (50 × 1 liter) was cultured in mYP30 medium. The cultures were grown to stationary phase (shaking at 175 rpm for 96 h at 28°C). The cells were pelleted by centrifugation (10,000 × *g* for 10 min), and the resulting supernatants were acidified to pH 3 with formic acid. The acidified supernatants were twice extracted with equal volumes of acidified ethyl acetate (0.1% formic acid), and the organic phases were concentrated *in vacuo* at 27°C. The resulting extract of *P. inhibens* S45m supernatant was separated by Sephadex LH-20 column chromatography (3 cm by 80 cm) using chloroform-methanol (1:1 [vol/vol]) to provide 60 fractions. Fractions (13 to 27) active in the WZ112 *gfp* reporter assay were pooled (fraction A, 123 mg) and further separated by medium-pressure liquid chromatography (prepacked C₁₈ column, 2 cm by 30 cm; Star Instruments, Manassas, VA, USA), eluting with a linear gradient of 10% to 100% methanol in water at 3 ml/min. The active fractions (21 to 29 of 29) were combined (fraction B, 48.5 mg) and further purified by HPLC (Hitachi Elite LaChrom system consisting of an L2130 pump, L-2200 autosampler, L-2455 diode array detector, and a Phenomenex Luna C₁₈ column [250 mm by 10 mm, 5-5 μm], operated by EZChrom Elite software) and eluting with methanol-water (40% to 100% methanol in H₂O over 25 min, 3 ml/min) to provide compounds 1 (2.0 mg), 2 (600 μg), and 3 (500 μg).

Concentration-response analyses of three AHL compounds. Exponentially growing *V. coralliilyticus* cultures (OD₆₀₀ of 0.6 to 0.8) were treated with serial dilutions of the purified AHL molecules (0.125 to 5.0 μM) from *P. inhibens*. One volume of *V. coralliilyticus* culture was added to 4 volumes of fresh mYP30 medium containing a specific concentration of AHL (dissolved in methanol, 0.4% final concentration). Medium containing only 0.4% methanol (final concentration) was used as a control. *V. coralliilyticus* RE225m supernatants were collected at 1.5 h and used to examine the protease activity. A series of concentration-response (protease activity) data were obtained and plotted. According to the concentration-response curve, the half maximal inhibitory concentration (IC₅₀) was calculated for each purified AHL.

Treatment of *V. coralliilyticus* cells with purified AHLs. *V. coralliilyticus* cultures (OD₆₀₀ of 0.6 to 0.8) were treated with compounds 1 to 3 at their corresponding IC₅₀s, both individually and in combination with the other AHL molecules, as described above in "Concentration-response analyses of three AHL compounds." Cell densities (CFU/ml) were determined at 15 min and 90 min after the addition of the AHL(s), and *V. coralliilyticus* RE225m culture supernatants and mRNA were collected at each time point and stored at -20°C and -74°C, respectively.

Treatment of *V. coralliilyticus* cells with synthetic AHLs. *V. coralliilyticus* cultures (OD₆₀₀ of 0.6) were individually treated with *N*-decanoyl-DL-homoserine lactone, *N*-dodecanoyl-DL-homoserine lactone, or *N*-tetradecanoyl-DL-homoserine lactone (Sigma-Aldrich, St. Louis, MO) at a concentration of 0.26 μM, 3.7 μM, or 2.9 μM, respectively, as described above in "Treatment of *V. coralliilyticus* cells with purified AHLs." Cell densities (CFU/ml) were determined at 0 min and 90 min after the addition of the AHL(s). *V. coralliilyticus* RE225m culture supernatants and mRNA were collected after the 90-min challenge and stored at -20°C and -74°C, respectively.

Construction of *V. coralliilyticus* RE225m merodiploid and allelic exchange mutants. The modified pDM4 plasmid containing a kanamycin (Km) resistance gene, pDM5, was used to construct the allelic exchange mutants (Table 1) as described by Gibson et al. (44). The quorum sensing genes

luxN, *vcpR*, and *luxM* were amplified by PCR from *V. coralliilyticus* RE22Sm genomic DNA, PCR purified, and inserted into pDM5 via the Gibson assembly reaction. The plasmid, pDM5, was linearized at the *SacI* restriction enzyme site within the multicloning region (MCR) for all mutation-destined Gibson assemblies. The Gibson assembly ligation mixture was introduced into *E. coli* Sm10 by electroporation with the Bio-Rad Gene Pulser II. Transformants were selected for on LB20 Cm²⁰ agar plates, and confirmed by PCR. The mobilizable suicide vector, pDM5, was transferred from *E. coli* Sm10 into *V. coralliilyticus* RE22Sm by conjugation (45). Transconjugates were selected by utilizing the kanamycin resistance gene located on the suicide plasmid. The incorporation of the target gene fragments into the suicide vector was confirmed by PCR analysis using the appropriate 5' forward primer and a check primer, pr25cs. The subsequent recombination resulted in insertional, single-crossover deletion merodiploid mutants. Next, double-crossover resolution of the merodiploids through sucrose counterselection yielded stable allelic exchange mutants. The double-crossover transconjugates were selected for by growth on mYP10 Sm²⁰⁰ plus 10% sucrose agar plates for a second crossover event. Sucrose was used as the counterselective agent, because pDM5 contains the *sacBR* gene cassette, which encodes levansucrase, ultimately converting sucrose to toxic levan (45). Putative allelic exchange mutants were screened for kanamycin sensitivity. Kanamycin-sensitive recombinants were then screened by PCR using the appropriate Gibson assembly primers and gel electrophoresis for the deletion mutation.

Statistical analysis. Two-tailed Student's *t* tests assuming unequal variances were used for statistical analyses for all experiments. *P* values of <0.05 were considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01545-18>.

SUPPLEMENTAL FILE 1, PDF file, 2.1 MB.

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W.Z. and D.R.N. designed the study. W.Z. created the strains used in this study. W.Z. performed all experiments under the supervision of D.R.N. with the following exceptions: C.P. and W.Z. performed extraction of *P. inhibens* S4Sm supernatant, C.P. and T.Y. performed the fractionation of *P. inhibens* S4Sm supernatant and structuring of active compounds under the supervision of D.C.R., and E.J.S. performed the experiments with the synthetic AHLs. W.Z. and D.R.N. wrote the paper with contributions and editing by C.P., T.Y., E.J.S., C.W.S., and D.C.R. The formatting of the paper was performed by W.Z. and D.R.N. All authors read and approved the final version of the manuscript.

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