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NOTES

Quorum-Sensing Signals and Quorum-Sensing Genes in *Burkholderia vietnamiensis*

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Acyl-homoserine lactone (acyl-HSL) quorum sensing is common to many *Proteobacteria* including a clinical isolate of *Burkholderia cepacia*. The *B. cepacia* isolate produces low levels of octanoyl-HSL. We have examined an environmental isolate of *Burkholderia vietnamiensis*. This isolate produced several acyl-HSLs. The most abundant species was decanoyl-HSL. Decanoyl-HSL in *B. vietnamiensis* cultures reached concentrations in excess of 20 μM. We isolated a *B. vietnamiensis* DNA fragment containing a gene for the synthesis of decanoyl-HSL (*bviI*) and an open reading frame that codes for a putative signal receptor (*bviR*). A *B. vietnamiensis bviI* mutant did not produce detectable levels of decanoyl-HSL.

Many *Proteobacteria* produce *N*-acyl homoserine lactone (acyl-HSL) quorum-sensing signals. Examples include *Pseudomonas aeruginosa*, which uses *N*-butyryl-HSL (C_4-HSL) and *N*-3-oxodocanoyl-HSL ($3OC_{12}$-HSL) as signals to control the expression of dozens of genes, many of which encode virulence factors (24, 28, 47). *Ralstonia solanacearum*, a plant pathogen, uses *N*-octanoyl-HSL (C_8-HSL) in quorum sensing, *Agrobacterium tumefaciens* uses *N*-3-oxooctanoyl-HSL ($3OC_8$-HSL) to control conjugal transfer, and *Erwinia carotovora* uses *N*-3-oxohexanoyl-HSL ($3OC_6$-HSL) to control antibiotic production and other factors (2, 4, 9, 25, 37). Generally, acyl-HSL quorum sensing involves a member of the LuxI family of signal generators and a member of the LuxR family of signal receptor transcription factors (for a recent review, see reference 3).

The genus *Burkholderia* has been a subject of recent attention. Species classifications in this genus remain in flux. Some members of the genus have emerged as serious opportunistic pathogens. For example, some strains of *Burkholderia cepacia* and perhaps other *Burkholderia* species can colonize the lungs of people with cystic fibrosis. In part because *B. cepacia* is resistant to antibiotic therapy, it can be a major health problem in colonized cystic fibrosis patients (10). The fact that *Burkholderia* spp. have multiple chromosomes and very plastic genomes (13) has also brought a research focus to the genus. Furthermore, some members of the genus are capable of degrading environmental pollutants and have been the subject of studies aimed at developing tools for bioremediation (29, 30, 31). For example, the subject of this study, *Burkholderia vietnamiensis* G4, was isolated from a holding pond at an industrial waste treatment facility, and it can degrade trichloroethylene and toluene (18).

A previous report shows that a clinical isolate of *B. cepacia* makes low levels of C_8-HSL in laboratory cultures and that quorum sensing is involved in the regulation of virulence factors including lipases, protease, and siderophores (14). Another strain of *B. cepacia* produces multiple acyl-HSLs, but these signals have not been identified, nor have their concentrations been determined (17). A recent study shows that *B. vietnamiensis* has at least two acyl-HSL generator and receptor gene pairs (16). Here we show that *B. vietnamiensis* G4 produces several acyl-HSLs and we define the genetic element responsible for the production of the most abundant of these acyl-HSLs. This study provides the groundwork for identification of quorum sensing-controlled genes in *B. vietnamiensis* and investigations of the role of quorum sensing in the degradation of environmental pollutants like trichloroethylene.

The bacterial strains and plasmids used in this study are described in Table 1. For acyl-HSL bioassays and for partial purification of *B. vietnamiensis*-produced acyl-HSLs, cultures were grown to an optical density at 600 nm of 1.6 to 2.0 (the late-logarithmic phase of growth) in Difco tryptic soy broth (pH 7.0) at 30°C with shaking (250 rpm). For ^{14}C labeling of acyl-HSLs, cultures were grown as above in basal salts medium (pH 7.0) with 20 mM lactate as a carbon source (8). For conjugation, *B. vietnamiensis* was grown in Luria-Bertani broth (L broth) (6). Antibiotics were included as appropriate. For *Escherichia coli*, the antibiotic concentrations were as follows: ampicillin, 100 μg/ml; chloramphenicol, 35 μg/ml; gentamicin, 10 μg/ml; kanamycin, 35 μg/ml; tetracycline, 10 μg/ml. The antibiotic concentrations for *B. vietnamiensis* G4 were as follows: chloramphenicol, 25 to 35 μg/ml; gentamicin, 10 μg/ml; kanamycin, 35 μg/ml. Plating was carried out on media solidified with 1.5 % agar. *E. coli* was grown in L broth or on Luria-Bertani agar using standard procedures (6).

To detect acyl-HSLs and to determine their relative abundance, we used a radiotracer procedure as described elsewhere

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or characteristics	Source or reference
B. (*cepacia*) *vietnamiensis*		
G4	Typed as *B. vietnamiensis* by J. LiPuma (personal communication)	15
FMT-05	*fabF* mutant derived from G4	This study
IMT-61	*bviI* mutant derived from G4	This study
RMT-14	*bviR* mutant derived from G4	This study
E. coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	27
MG4	Δ(*argF-lac*)*U169 zah-735*::Tn *10 recA56 srl*::Tn *10*	26
S17-1 (λ*pir*)	*recA thi pro hsdR*	32
VJS533	*rec*Δ*A56 ara* Δ(*lac-proAB*)X111 *rpsL* (ϕ80 *lacZ*ΔM15)	35
XL-1 Blue	*recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac*[F′ *proAB lacI*^{q}ZΔM15 Tn *10* (Tet^r)]	Stratagene
R. solanacearum		
AW1-AI8	*sol18*::SP	2
Plasmids		
p395B	*sol* reporter; *aidA*::*lacZ*, Tc^r	2
pBBR1MCS-2	Mobilizable broad-host-range cloning vector, Km^r	11
pBBR1MCS-5	Mobilizable broad-host-range cloning vector, Gm^r	11
pBCL5-2	8.5-kb *Hin*dIII fragment containing the *bviI*, *bviR*, and *fabF*-like gene from *B. vietnamiensis* G4 in pUC19	This study
pBCSFM	A 952-bp *Rsr* II fragment of the *fabF*-like gene from pBCL-2 was replaced with the pBBR1MCS-5 Gm^r marker; the resulting DNA was digested with *Pst*I, treated with the Klenow fragment, and cloned into the *Eco*RV site in pSUP102	This study
pBCSIM	*bviI*, *fabF*-like gene *Pst*I fragment from pBCL5-2 treated with Klenow fragment and blunt-end cloned into *Eco*RV-digested pSUP102; a 570-bp *Bsi*W *bviI* fragment replaced with a Gm^r fragment PCR amplified from pBBR1MCS-5	This study
pBCSRM	4.4-kb *bviR bviI Eco*RV fragment from pBCL5-2 cloned into the *Eco*RV site of pSUP102, 1-kb Gm^r marker from pBBR1MCS-5 cloned into unique *Sfi*I site	This study
pECP61.5	*rhl* reporter; *rhlR rhlA*::*lacZ* Ap^r	24
pHV200I$^-$	*lux* reporter; *luxR luxI′CDABE* Ap^r	22
pHV300I$^-$	*lux* reporter; *luxR luxI′CDABE* Cm^r	7
pKDT17	*las* reporter; *lasB*::*lacZ plac-lasR* Ap^r	21
pSUP102	*E. coli*-specific mobilizable vector Cm^r Tc^r	32

(33). Unless otherwise specified, *B. vietnamiensis* was grown in 5 ml of basal salts plus lactate medium. When the culture reached late logarithmic phase, 5.0 μCi of L-[1-^{14}C]methionine was added. After a 10-min incubation, the culture was extracted twice with equal volumes of acidified ethyl acetate and the extract was evaporated to dryness. The radiolabeled acyl-HSLs were dissolved in 20% methanol and fractionated by C_{18} reverse-phase high-performance liquid chromatography (HPLC). The amount of radiolabel in each fraction was determined by standard scintillation counting procedures. The retention times of the radioactive peaks corresponded to the retention times of synthetic acyl-HSL standards (Quorum Sciences Inc, Coralville, Iowa).

To confirm the results of the radiotracer analysis and to gain quantitative data about each of the acyl-HSLs produced by *B. vietnamiensis*, we measured the levels of acyl-HSLs in HPLC-fractionated culture fluid extracts by using bioassays. Four different bioassays were employed, each being selective for different acyl-HSLs. The *E. coli*(pHV200I$^-$) assay shows greatest sensitivity to 3OC$_6$-HSL (22). The *E. coli* MG4(pKDT17) assay shows greatest sensitivity to 3OC$_{12}$-HSL (21, 22). The *E. coli*(pECP61.5) assay shows greatest sensitivity to C$_4$-HSL (20). The *Ralstonia solanacearum* AW1-AI8(p395B) assay shows greatest sensitivity to C$_8$-HSL (2). The material analyzed by the bioassays was obtained as follows: cells were removed from the fluid of a 3-liter culture by centrifugation, and the culture fluid was extracted twice with equal volumes of acidified ethyl acetate. The extract was concentrated by rotary evaporation at 40 to 45°C and fractionated by C_{18} HPLC. Samples from each fraction eluted in HPLC were analyzed by the bioassays. Standard curves with synthetic acyl-HSLs were generated, and the amounts of acyl-HSLs in HPLC fractions were determined by comparison to the standard curves.

To confirm assignments based on HPLC retention times, fractions constituting peaks were pooled, concentrated by rotary evaporation, and subjected to further separation by HPLC in water containing methanol at a percentage of 15% lower than that in which they were eluted in the gradient. Active fractions were concentrated and analyzed by gas chromatography-mass spectrometry as described previously (23).

DNA manipulations were done by standard procedures (1, 27). For cloning the *B. vietnamiensis* quorum-sensing genes, an unsized library of *Hin*dIII chromosomal DNA fragments was constructed in pUC19 (38). The library was used to transform *E. coli* DH5α(pHV300I$^-$). Ampicillin-resistant, luminescent colonies were selected for further study. DNA sequencing was performed at the University of Iowa DNA Facility.

For insertional mutagenesis, genes were cloned in pSUP102

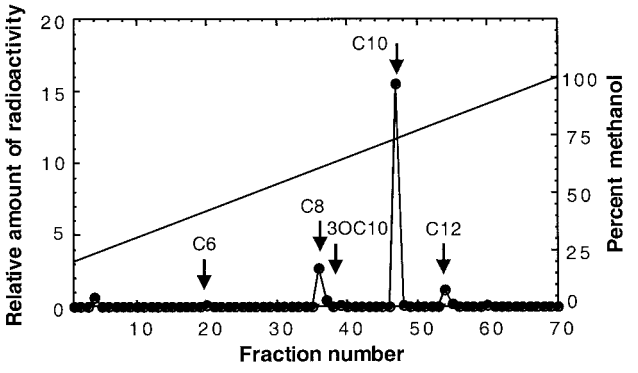

FIG. 1. HPLC analysis of acyl-HSLs produced by *B. vietnamiensis* G4. Acyl-HSLs were labeled with ^{14}C, extracted from culture fluid, and fractionated by HPLC. Radioactivity was measured to identify peaks containing ^{14}C-labeled acyl-HSLs. As indicated by the arrows, synthetic acyl-HSL standards were eluted as follows: C_6-HSL, fraction 20; C_8-HSL, fraction 36; C_{10}-HSL, fraction 47: $3OC_{10}$-HSL, fraction 39; C_{12}-HSL, fraction 54. The methanol concentration of the gradient is shown by the solid line.

and a gentamicin cassette from pBBR1MCS-5 was inserted in the cloned gene (Table 1). The pSUP102 derivatives were transferred from *E. coli* S17-1 (λ*pir*) into *B. vietnamiensis* by conjugation using the following procedure: donors (mid-log phase) and recipients (stationary phase) were grown in L broth with appropriate antibiotics. Cells were mixed at a donor-to-recipient ratio of 4:1, washed in phosphate-buffered saline (pH 7.4), and suspended in a small volume of phosphate-buffered saline. Mating mixtures were spotted on Luria-Bertani (LB) agar and grown at 30°C overnight. Transconjugants were obtained by selection on Simmons citrate agar (SCA) (6) containing gentamicin. Screening transconjugants for chloramphenicol sensitivity allowed us to obtain insertion mutants. The constructs were confirmed by PCR and Southern blotting.

Chrome azurol S assays were used to measure siderophore activity (28). Egg yolk agar assays were used to estimate bacterial production of lecithinase (6). Difco brain heart infusion agar assays were used to measure extracellular protease activity (34).

To gain information on whether *B. vietnamiensis* produced acyl-HSLs and, if so, in what sort of relative abundance, we incubated cultures with carboxy-labeled [^{14}C]methionine. The cell-free culture fluid was extracted with ethyl acetate and fractionated by HPLC. As described previously, the amount of radiolabel in fractions that comigrate with known acyl-HSLs corresponds to the relative abundance of that acyl-HSL (33). There were three main peaks of radioactivity (Fig. 1). The largest peak corresponded to C_{10}-HSL, the next largest peak corresponded to C_8-HSL, and the third peak comigrated with C_{12}-HSL.

To confirm that the radioactive peaks in the experiments described above represented acyl-HSLs and to gain information about the concentrations of acyl-HSLs in culture fluid, we performed quantitative bioassays on HPLC fractions of an ethyl acetate extract of 3 liters of fluid from an early-stationary-phase culture. We identified C_6-, C_8-, C_{10}-, and C_{12}-HSLs, and we also identified $3OC_{10}$-HSL. The concentrations of these molecules in the culture fluid were 0.12 μM C_6-HSL, 2.2 μM C_8-HSL, 22.4 μM C_{10}-HSL, 0.003 μM $3OC_{10}$-HSL, and 0.65 μM C_{12}-HSL. As expected, gas chromatography-mass spectrometry of the three most abundant peaks showed spectra identical to the spectra of synthetic C_{10}-, C_8-, and C_{12}-HSLs (data not shown).

We screened a library of *B. vietnamiensis* genes in *E. coli* containing a reporter plasmid, pHV300I$^-$. This plasmid contains the acyl-HSL-responsive *Vibrio fischeri lux* gene cluster and does not contain a functional acyl-HSL synthase gene. We obtained a luminous strain of *E. coli* that contained a plasmid (pBCL5-2) with an 8.5-kb insert of *B. vietnamiensis* DNA. In *E. coli*, pBCL5-2 directed the synthesis of C_6-, C_8-, C_{10}-, and C_{12}-HSL. As in *B. vietnamiensis*, C_{10}-HSL was the most abundant of the acyl-HSLs. The *B. vietnamiensis* DNA in pBCL5-2 was sequenced, and an analysis revealed a gene that coded for a LuxI homolog and a divergently transcribed gene coding for a LuxR homolog with a rather large 1.2-kb intergenic region.

The gene coding for the LuxI homolog was 660 bp, and it was identical to the recently described *bviI* (16). The gene coding for the LuxR homolog was 714 bp and was identical to *bviR* (16). There was a gene coding for a putative transport protein downstream of *bviR* and a gene showing greatest similarity to *fabF*-like genes from *P. aeruginosa* and *A. tumefacians* (BLAST scores of 10^{-145}) downstream of *bviI*. The intergenic region contained an assortment of small open reading frames, the longest of which was 384 bp. None of these open reading frames showed significant similarity to known gene products. It is possible that the intergenic region codes for a small regulatory RNA or protein.

We analyzed acyl-HSL production in the *B. vietnamiensis* *bviR*, *bviI*, and *fabF*-like gene mutants. As monitored by the radiotracer assay, the *bviI* mutant did not make detectable levels of any acyl-HSL except C_8-HSL, which was present in trace amounts [<10 nM, compared to 2,200 nM C_8-HSL in the parent as measured by the *E. coli*(pHV200I$^-$) bioassay]. This, together with the analysis of acyl-HSL production by *bviI*-containing *E. coli* (see above), indicates that *bviI* codes for an acyl-HSL synthase that is responsible for the production of all of the abundant acyl-HSLs. The presence of traces of octanoyl-HSL in cultures of the *bviI* mutant suggests that there is another poorly expressed or poorly active acyl-HSL synthase, one that directs the synthesis of C_8-HSL, the same signal as that produced by CepI in *B. cepacia*. This is consistent with a recent report by Lutter et al. (16). The parent *B. vietnamiensis* produces much more C_8-HSL than the mutant. One explanation is that BviI is responsible for the majority of the C_8-HSL produced in the parent and the hypothetical second acyl-HSL synthase contributes very little to the total C_8-HSL level.

The acyl-HSL production pattern of the *bviR* mutant was essentially identical to that of the *bviI* mutant. We interpret this to indicate that, like many other acyl-HSL synthesis and response systems, *bviI* is positively autoregulated by the acyl-HSL signal it produces and the cognate R protein (5). Because of its proximity to *bviR-bviI*, we wondered if the *fabF*-like gene was involved in generation of acyl-ACPs for *bviI* specifically. To investigate this, we generated a null mutation in the *fabF*-like gene. The strain with the mutation in the *fabF*-like gene grew somewhat slower than the parent. That it could grow at al indicates that there are other fatty acid synthesis genes in *B. vietnamiensis* that can fulfill the role played by this gene. Al-

though the absolute levels were somewhat reduced (two- to threefold lower), the mutant produced acyl-HSLs in the same relative abundance as the parent did. Therefore, we conclude that this *fabF*-like gene is not involved in the specificity of quorum-sensing signal generation.

BviR and BviI did not appear to regulate factors controlled by quorum sensing in *B. cepacia*. Previous reports showed that quorum sensing in *B. cepacia* controlled siderophore, protease, and lipase production (14, 17). Therefore, we examined the influence of the BviR-BviI system on these factors in *B. vietnamiensis*. Neither the parent, *B. vietnamiensis* G4, nor the *bviR* or *bviI* mutants produced detectable levels of protease or lipase. All three strains produced siderophores, as measured by the chrome azurol S assay, and the levels of the siderophores produced were indistingushable. Presumably, the genes regulated by quorum sensing in *B. vietnamiensis* are different from those regulated by quorum sensing in *B. cepacia*. In fact, *B. vietnamiensis* produces an antibiotic, which appears to be under quorum-sensing control (K. Lee, personal communication).

In summary we have shown that *B. vietnamiensis* produces detectable levels of several acyl-HSLs, the most abundant of which is C_{10}-HSL. This is present in 10-fold excess over the second most abundant acyl-HSL, C_8-HSL. Other bacteria make acyl-HSLs with acyl side chains ranging from 4 to 14 carbons. To our knowledge, this is the first report of a bacterium that makes predominantly C_{10}-HSL. Other investigators have used gas-chromatography-mass spectrometry and have obtained similar results with respect to acyl-HSL production by *B. vietnamiensis* G4 (Lee, personal communication). The *B. cepacia* acyl-HSL is C_8-HSL, and cultures of the one studied strain of *B. cepacia* make $<0.01\%$ of the total acyl-HSLs made by *B. vietnamiensis* G4 (14). Most acyl-HSL-producing bacteria make micromolar quantities of acyl-HSLs, as does *B. vietnamiensis* G_4. The *bviI* gene directs *E. coli* to produce *B. vietnamiensis* acyl-HSLs, and a *B. vietnamiensis bviI* mutation results in the loss of C_{10}-HSL production. The *bviI* mutant produced trace amounts of C8-HSLs. This is consistent with the finding of a *bviI* homolog in *B. vietnamiensis* (16).

Work by other investigators suggests that extracellular lipase, protease, and siderophore production may be controlled by quorum sensing in some *B. cepacia* clinical isolates (14, 17). We found that *B. vietnamiensis* G4 did not produce extracellular proteases or lipases that were comparable to the *B. cepacia* enzymes and that siderophore production did not seem to be controlled by quorum sensing. Our results are consistent with those obtained by K. Lee and colleagues (personal communication). Further studies are required to elucidate the targets of quorum-sensing control in *B. vietnamiensis* G4. There is a suggestion that antibiotic synthesis in *B. vietnamiensis* G4 may be controlled by quorum sensing (Lee, personal communication). The availability of a *bviI* mutant strain and the knowledge that C_{10}-HSL is the main acyl-HSL should allow the development of screens of quorum-sensing-controlled genes in *B. vietnamiensis* G4.

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