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Mutational Analysis of *Burkholderia thailandensis* Quorum Sensing and Self-Aggregation▽†

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Acyl-homoserine lactone (acyl-HSL) quorum-sensing signaling is common to many *Proteobacteria*. Acyl-HSLs are synthesized by the LuxI family of synthases, and the signal response is mediated by members of the LuxR family of transcriptional regulators. *Burkholderia thailandensis* is a member of a closely related cluster of three species, including the animal pathogens *Burkholderia mallei* and *Burkholderia pseudomallei*. Members of this group have similar *luxI* and *luxR* homologs, and these genes contribute to *B. pseudomallei* and *B. mallei* virulence. *B. thailandensis* possesses three pairs of *luxI-luxR* homologs. One of these pairs, BtaI2-BtaR2, has been shown to produce and respond to $3OHC_{10}$-HSL and to control the synthesis of an antibiotic. By using a markerless-exhange method, we constructed an assortment of *B. thailandensis* quorum-sensing mutants, and we used these mutants to show that BtaI1 is responsible for C_8-HSL production and BtaI3 is responsible for $3OHC_8$-HSL production. We also show that a strain incapable of acyl-HSL production is capable of growth on the same assortment of carbon and nitrogen sources as the wild type. Furthermore, this mutant shows no loss of virulence compared to the wild type in mice. However, the wild type self-aggregates in minimal medium, whereas the quorum-sensing mutant does not. The wild-type aggregation phenotype is recovered by addition of the BtaI1-R1 HSL signal C_8-HSL. We propose that the key function of the BtaR1-BtaI1 quorum-sensing system is to cause cells to gather into aggregates once a sufficient population has been established.

Members of the genus *Burkholderia* constitute a large group of metabolically diverse species. We are interested in a subgroup that is closely related at the genomic level and is comprised of the three species *Burkholderia thailandensis*, *Burkholderia pseudomallei*, and *Burkholderia mallei* (63). Of these species, *B. mallei* is the only obligate animal pathogen. *B. mallei* causes glanders in horses, donkeys, and mules and less frequently in humans (61). *B. pseudomallei*, the causative agent of the human disease melioidosis, can be isolated from soil and water in Southeast Asia and northern Australia (7, 34, 43). Both *B. pseudomallei* and *B. mallei* are highly infectious to humans, and both are considered bioterrorism threats (45). Melioidosis is an emerging disease (43, 61). *B. thailandensis* has a limited potential to cause disease (15, 21, 33). Evidence suggests that *B. mallei*, *B. pseudomallei*, and *B. thailandensis* evolved from a common *B. pseudomallei*-like ancestor (37).

We are interested in *N*-acyl-homoserine lactone (acyl-HSL) quorum sensing in *B. mallei*, *B. pseudomallei*, and *B. thailandensis* primarily because quorum sensing has been reported to influence the virulence of *B. mallei* and *B. pseudomallei* and because there are multiple quorum-sensing circuits in each species (53–55). Quorum sensing allows bacteria to sense and respond to their population density (4, 19, 58). In *Proteobacteria*, quorum sensing is often mediated by diffusible acyl-HSL signals. At a critical concentration, acyl-HSLs bind LuxR family transcriptional regulators and affect changes in gene expression (20). Acyl-HSLs are produced by members of the LuxI family of synthases. Different synthases catalyze the production of acyl-HSLs that vary in the nature of the acyl side chain (20, 47). LuxI and LuxR homologs that are adjacently encoded in the genome form cognate pairs. Many bacterial genomes contain multiple cognate pairs and can also contain unpaired orphan LuxR signal receptors that do not have a cognate *luxI* gene nearby in the genome. Relatively little is known about the functions of the orphans (12, 31, 32, 38, 41). Quorum-sensing systems are found in dozens of different *Proteobacteria* species and can affect the transcription of tens or hundreds of different genes in a given species (1, 50, 57, 60). Genes coding for extracellular enzymes and factors are commonly activated by acyl-HSL quorum sensing, and members of the quorum can share these public goods (60).

B. pseudomallei and *B. thailandensis* possess three cognate receptor-synthase pairs (systems 1, 2, and 3) and at least two orphan receptors (53, 55). *B. mallei* possesses the same complement of quorum-sensing genes, except it is missing the system 2 cognate pair (54). It was first reported that the *B. thai-*

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TABLE 1. Bacterial strains and plasmids used

Bacterial strain or plasmid	Genotype or phenotype[a]	Reference or source
Bacterial strain		
E. coli		
DH5α		Invitrogen
DH5α(pECP61.5)	Acyl-HSL bioreporter	40
A. tumefaciens KYC55	Acyl-HSL bioreporter; R10 (pJZ384, pJZ410, pJZ372)	65
P. fluorescens 1855	Acyl-HSL bioreporter; WT (pSF105, pSF107)	30
R. capsulatus ALS1	Acyl-HSL bioreporter; WT (pYP)	49
B. thailandensis		
E264	Soil isolate from central Thailand	6
JBT101	E264 Δ*btaI1*	This study
JBT102	E264 Δ*btaI2*	This study
JBT103	E264 Δ*btaI3*	This study
JBT107	E264 Δ*btaR1*	This study
JBT108	E264 Δ*btaR2*	This study
JBT109	E264 Δ*btaR3*	This study
JBT110	E264 Δ*btaR4*	This study
JBT111	E264 Δ*btaR5*	This study
JBT104	E264 Δ*btaI2* Δ*btaI3*	This study
JBT112	E264 Δ*btaI1* Δ*btaI2* Δ*btaI3*	This study
Plasmids		
pBAD24	Arabinose-inducible expression vector; Amp^r	23
pBTI1	pBAD24 with arabinose-inducible *btaI1*; Amp^r	This study
pBTI3	pBAD24 with arabinose-inducible *btaI3*; Amp^r	This study
pBBR1Tp	Broad-host-range vector; Tp^r	14
pJRC112	pBBR1Tp with *B. thailandensis* mutant *pheS* (304AG); Tp^r	This study
pEX18Tc	*P. aeruginosa* suicide plasmid; Tet^r	25
p34S-Tp	Antibiotic resistance cassette vector; Tp^r	13
pEX18Tp	pEX18Tc with *dhfRII* from p34S-Tp; Tet^r and Tp^r	This study
pJRC115	Mobilizable suicide vector with the *B. thailendensis pheS* counterselectable marker (304AG); Tp^r	This study
pJRC115 *btaI1*	pJRC115 with Δ*btaI1* extending from +13 to +597 with respect to the translational start site; Tp^r	This study
pJRC115 *btaI2*	pJRC115 with Δ*btaI2* extending from +13 to +606 with respect to the translational start site; Tp^r	This study
pJRC115 *btaI3*	pJRC115 with Δ*btaI3* extending from +19 to +594 with respect to the translational start site; Tp^r	This study
pJRC115 *btaR1*	pJRC115 with Δ*btaR1* extending from +13 to +699 with respect to the translational start site; Tp^r	This study
pJRC115 *btaR2*	pJRC115 with Δ*btaR2* extending from +22 to +686 with respect to the translational start site; Tp^r	This study
pJRC115 *btaR3*	pJRC115 with Δ*btaR3* extending from +19 to +675 with respect to the translational start site; Tp^r	This study
pJRC115 *btaR4*	pJRC115 with Δ*btaR4* extending from +16 to +690 with respect to the translational start site; Tp^r	This study
pJRC115 *btaR5*	pJRC115 with Δ*btaR5* extending from +13 to +708 with respect to the translational start site; Tp^r	This study

[a] Tp^r, trimethoprim resistant; Amp^r, ampicillin resistant; Tet^r, tetracycline resistant; WT, wild type.

landensis quorum-sensing signals were hexanoyl-HSL (C_6-HSL), octanoyl-HSL (C_8-HSL), and decanoyl-HSL (C_{10}-HSL) (55); however, a careful analysis of *B. thailandensis* system 2 (encoded by *btaR2* and *btaI2*) revealed that the primary signal was *N*-3-hydroxy-decanoyl-HSL ($3OHC_{10}$-HSL), and the system also produced significant amounts of, and responded to, *N*-3-hydroxy-octanoyl-HSL ($3OHC_8$-HSL) (18). The studies of the BtaR2-BtaI2 quorum-sensing system raise questions about the signals that are specific to the other two *B. thailandensis* systems. We can speculate about the signals based on findings with the conserved systems 1 and 3 in *B. mallei* (16, 17) and system 1 in *B. pseudomallei* (51). The *B. mallei* and *B. pseudomallei* systems 1 both involve C_8-HSL (17, 51), and system 3 in *B. mallei* involves $3OHC_8$-HSL (16).

In addition to the signals produced by quorum-sensing system 2, our group has also shown that system 2 controls a number of genes required for synthesis of an antibiotic (18). However, it is unclear what the signals are for systems 1 and 3 and what behaviors other than antibiotic synthesis are controlled by quorum sensing in *B. thailandensis*. Here, we describe a method for the construction of *B. thailandensis* quorum-sensing mutants. We describe studies with various mutants that establish the principal acyl-HSLs for systems 1 and 3, and we show that a primary function of system 1 in *B. thailandensis* is to induce self-aggregation. Quorum sensing did not appear to contribute to the virulence of *B. thailandensis* in our mouse infection model.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and chemicals. The bacterial strains we used are described in Table 1. *Escherichia coli* and *B. thailandensis* were grown in Luria-Bertani (LB) broth, 20% LB broth, M9 minimal medium (35) with either 0.4% glucose or 20 mM succinate as a carbon and energy source and additional supplements as indicated, or Biolog IF-0 inoculation medium. Agar plates contained 1.5% Bacto agar (BD). Antibiotics were at the following concentrations (per ml): 30 μg trimethoprim (Tp), 30 μg kanamycin, 100 μg ampicillin, and 30 μg gentamicin for *E. coli*, and 100 μg Tp for *B. thailandensis*. For counterselection during mutant construction, we used M9 minimal agar plates with glucose and 10 mM *para*-chloro-phenylalanine (*p*-Cl-Phe). For arabinose

promoter activation in *E. coli*, we used 0.2% (wt/vol) L-arabinose. To assess the aggregation of *B. thailandensis*, we used M9 minimal medium with glucose and MOPS (morpholinepropanesulfonic acid) buffer (50 mM; pH 7.0). The starting optical density at 600 nm (OD_{600}) for aggregation experiments was 0.01, and the inocula were from LB broth cultures grown with shaking for 24 h at 37°C. Acyl-HSLs were added to media where indicated at the following concentrations: 2 μM of D,L-C_8-HSL and 1 μM L-3OHC_8-HSL.

DNA manipulations and transformation procedures. Routine procedures were used for DNA manipulations (46). Plasmid DNA was isolated using Qiaprep mini-spin kits (Qiagen). PCR and plasmid fragments were isolated by using Qiaquick PCR purification or gel extraction kits (Qiagen). Chromosomal DNA was prepared by using Gentra Puregene Cell kits (Qiagen). PCR products were generated using an Epicentre Failsafe PCR System or a Roche ExpandLong Template PCR system.

Transformation of *B. thailandensis* was by electroporation in a 0.2-cm electroporation cuvette with 100 μl of competent cells at a voltage of 2.5 kV and a time constant of approximately 5 ms. This was followed by a 2-h recovery in 500 μl LB broth with shaking at 37°C before the cells were plated on LB agar plates containing appropriate antibiotics. We used at least 2 μg nonreplicative plasmid DNA for allelic recombination. Preparation of competent *B. thailandensis* cells was as follows. Overnight cultures were diluted 1:500 in 100 ml of LB broth. When the OD_{600} reached 0.5, the cells were pelleted by centrifugation, washed twice with equal volumes of cold water, and then washed once and suspended in 0.2 ml of cold 10% glycerol. These cell suspensions constituted our competent cell preparations.

Plasmid and strain construction. The plasmids we used are described in Table 1. The sequences of oligonucleotide primers are available upon request. To construct the BtaI1 and BtaI3 expression vectors pBTI1 and pBTI3, the open reading frames of *btaI1* (BTH_II1512) and *btaI3* (BTH_II0804) were PCR amplified from *B. thailandensis* genomic DNA. The primers were designed to incorporate EcoRI and XbaI restriction sites at the ends of the PCR products. The PCR products were digested with EcoRI and XbaI and ligated to EcoRI-XbaI-digested pBAD24 (23) to generate pBTI1 and pBTI3, which contained *btaI1* and *btaI3*, respectively. The plasmids were constructed so that *btaI1* and *btaI3* expression was driven by the L-arabinose-inducible promoter (P_{BAD}) present in the parent plasmid pBAD24.

We developed a method for making *B. thailandensis* quorum-sensing mutants that is similar to a recently developed method for making unmarked chromosomal mutations in a variety of *Burkholderia* species (3). Mutations were introduced by markerless allelic exchange. Unmarked mutations were made during two homologous recombination steps. In the first recombination step, a nonreplicative plasmid with a modified gene was introduced into the recipient strain, where it could integrate into the chromosome at the target gene. For this step, we selected for Tp resistance encoded by the plasmid-borne dihydrofolate reductase gene, *dhfrIIa*. In the second recombination step, the integrated plasmid was excised from the chromosome, leaving behind either the original wild-type allele or the desired modified allele. The plasmid contained a counterselectable *pheS* allele with an A304G mutation, which conferred sensitivity to *p*-Cl-Phe (3, 27, 29).

The suicide plasmid pJRC115 was used to deliver quorum-sensing-gene deletions to the *B. thailandensis* chromosome. This plasmid has *dhfRIIa* and the *B. thailandensis* mutant *pheS* allele. To construct pJRC115, we excised the *dhfRII* cassette from p34S-Tp (13) by KpnI digestion, treated the cassette with Klenow fragment (New England Biolabs) to create blunt ends, and ligated this DNA to EcoRV-BstZ171-digested pEX18Tc (25), thereby creating pEX18Tp. The mutant *pheS* allele was introduced into pEX18Tp to make pJRC115. To construct the mutant *pheS* allele, the *B. thailandensis pheS* (BTH_I2591) open reading frame and upstream promoter were PCR amplified to make two products from bp −406 to +906 and from bp +885 to +1081 relative to the predicted translational start site. During amplification, a primer-incorporated missense mutation in codon 304 (A304G) was created. We used a second PCR to anneal and amplify the two products. Primer-incorporated XbaI and BamHI sites were created at the ends of this product during amplification. The product was digested with XbaI and BamHI and ligated to XbaI-BamHI-digested pBBRTp (14) to make pJRC112, a replicative vector with the mutant *pheS* allele. pJRC112 was digested with XbaI and SmaI to release the mutant *pheS* allele, and this was ligated to NheI-NruI-digested pEX18Tp to make pJRC115 (Table 1).

To construct gene deletion vectors, we used overlap extension PCR to generate PCR products with about 1 kb of DNA homologous to DNA flanking the intended sites of deletion. We annealed and amplified the products by PCR. Primer-encoded restriction enzyme sites were introduced into the ends of the PCR products during amplification. The *btaR4* deletion allele was digested with NheI and HindIII. The other alleles were digested with XbaI and HindIII. The digested PCR products were ligated to XbaI-HindIII-digested pJRC115. To construct the single-mutant strains JBT101-103 and 107-111, the relevant pJRC115-derived suicide vector was used to transform *B. thailandensis* by electroporation, and Tp-resistant transconjugants were identified on selective LB agar plates. The transformants were grown in LB broth for 10 h in the absence of selection to allow excision of the plasmid from the chromosome and then counterselected on minimal medium-plus-glucose agar plates supplemented with *p*-Cl-Phe. Mutations were confirmed by sequencing PCR products generated with primers targeting chromosomal DNA flanking the recombination sites. To make strains with multiple gene deletions, alleles were sequentially introduced by the method described above. The double mutant JBT104 (Δ*btaI2* Δ*btaI3*) was constructed from JBT103 (Δ*btaI3*) as an intermediate step to make JBT112 (Δ*btaI1* Δ*btaI2* Δ*btaI3*).

Acyl-HSL identification. We used three different approaches to analyze acyl-HSL production; liquid chromatography/mass spectrometry/mass spectrometry (LC/MS/MS), radioisotope analysis, and measurements of biological activity as described below.

For LC/MS/MS, acyl-HSLs were extracted from 5-ml cultures grown with shaking in LB broth to stationary phase (OD_{600} = 4.0). The cells were removed from the culture fluid by centrifugation, and the culture fluid was extracted with two equal volumes of acidified ethyl acetate (0.1 ml/liter glacial acetic acid). The extracts were dried under a stream of nitrogen gas, suspended in 100 μl of 100% methanol, and subjected to LC/MS/MS analysis as described previously (22). We used deuterated C_6-HSL and deuterated C_{12}-HSL as internal standards.

We used a radiotracer assay similar to that described previously by Schaefer et al. (48) to monitor acyl-HSLs produced by recombinant *E. coli*. Briefly, 5-ml cultures of *E. coli* DH5α containing pBTI1 or pBTI3 were grown in LB broth plus 0.2% L-arabinose (starting OD_{600}, 0.025). When the OD_{600} reached 0.5, we pelleted the cells by centrifugation at 2,750 × *g* for 10 min and suspended the cells in the pellet in 5 ml of M9 minimal medium containing L-arabinose and succinate. After 10 min at 37°C with shaking, 5 μCi of L-[1-^{14}C]methionine (American Radiolabeled Chemicals) was added to the cell suspension. After a further 3 h of incubation at 37°C, the cell suspension was extracted with two equal volumes of acidified ethyl acetate (glacial acetic acid; 0.1 ml/liter). The ethyl acetate fraction was evaporated to dryness under a stream of nitrogen gas, and the residue was dissolved in 50% methanol. The dissolved acyl-HSLs were separated by C_{18} reverse-phase high-performance liquid chromatography (HPLC) in a 10 to 100% methanol gradient with a flow rate of 1 ml/min over 70 fractions.

We used four different bioassays to monitor acyl-HSLs produced by *B. thailandensis* and *B. thailandensis* quorum-sensing mutants. To prepare samples for analysis, 5-ml cultures were grown to an OD_{600} of 4.0 in LB broth with MOPS (50 mM; pH 7.0), the cells were removed from the culture fluid by centrifugation, the culture fluid was extracted twice with acidified ethyl acetate, and the ethyl acetate fraction was evaporated to dryness under a stream of nitrogen gas. The residue was dissolved in 1 ml acidified ethyl acetate, and the ethyl acetate solutions were used in bioassays. For the bioassays, we used *Agrobacterium tumefaciens* KYC55(pJZ372)(pJZ384)(pJZ410) with *traI*::*lacZ* and *traR* on separate plasmids (65), *Pseudomonas fluorescens* 1855(pSF105)(pSF107) with *phzA*::*lacZ* and *phzR* on separate plasmids (30), *E. coli* DH5α(pECP61.5) with *rhlA*::*lacZ* and *ptac-rhlR* on the same plasmid (39, 40), and *Rhodobacter capsulatus* ALS1(pYP) with *orfg2*::*lacZ* on the plasmid and *gtaR* on the chromosome (49). Details of the bioassay procedures have been described elsewhere (17, 30, 40, 49).

PM analysis. We used a Phenotype MicroArray (PM) system (Biolog, Hayward, CA) according to the manufacturer's instructions. We screened carbon, nitrogen, and phosphorus sources by using PM-1, PM-2, and PM-3 microtiter plates. Inocula were prepared by scraping colonies from 20% LB agar plates, suspending the cells in Biolog IF-0 inoculating fluid, and adjusting the transmittance to 85%. The cell suspensions were then diluted 1:200 in IF-0 containing Biolog Redox Dye Mix A, which contains a redox dye used to measure cell respiration. These cell suspensions were used as inocula for PM plates, which were incubated without shaking at 37°C in an OmniLog instrument. The respiration-dependent color changes in each well were measured at 15-min intervals for 3 days.

Mouse infection experiments. Specific-pathogen-free BALB/c mice were obtained from Jackson Laboratories (Bar Harbor, ME). All animals were housed in laminar flow cages and were permitted ad lib access to sterile food and water. Euthanasia was accomplished with intraperitoneal pentobarbital, followed by exsanguination by cardiac puncture. The Institutional Animal Care and Use Committee of the University of Washington approved all experimental procedures. For infection, 20 ml LB broth was inoculated with a single colony of *B. thailandensis* E264, JBT101 (Δ*btaI1*), or JBT112 (Δ*btaI1* Δ*btaI2* Δ*btaI3*). After 18 h with shaking at 37°C, the bacteria were washed twice and suspended in

TABLE 2. Relative amounts of acyl-HSLs produced by *B. thailandensis* wild type and quorum-sensing mutants

Acyl-HSL	Relative acyl-HSL abundance in culture extracts[a]			
	E264 (wild type)	JBT101 (Δ*btaI1*)	JBT103 (Δ*btaI3*)	JBT112 (Δ*btaI1* Δ*btaI2* Δ*btaI3*)
$3OHC_4$-HSL	4 (1)	ND[b]	ND	ND
C_8-HSL	30 (2)	ND	39 (1)	ND
$3OHC_8$-HSL	21 (0.5)	22 (1)	7 (1)	ND
$3OHC_9$-HSL	1	ND	ND	ND
$3OHC_{10}$-HSL	43 (0.3)	78 (1)	55 (2)	ND

[a] The values are the average relative abundances (percent) of acyl-HSLs from two independent cultures, and the range is indicated in parentheses.
[b] ND, none detected.

phosphate-buffered saline to the desired density. The mice were exposed to aerosolized bacteria by using a snout-only inhalation system (In-Tox Products) (59). Aerosols were generated from a MiniHeart hi-flo nebulizer (Westmed) driven at 40 lb/in^2. Airflow through the system was maintained for 10 min at 24 liters per min, followed by a 5-min air purge. Bacterial deposition in each experiment was determined from quantitative culture of the left lungs from four sentinel mice sacrificed immediately after infection with each strain. Lung tissue was homogenized in 1 ml phosphate-buffered saline, and serial dilutions were plated on LB agar. Colonies were counted after 2 to 4 days at 37°C under 5% CO_2. For the number of recovered CFU per lung from sentinel mice sacrificed immediately after infection, see the legend to Fig. 5. Infected mice (four female and four male for each strain) were monitored for 14 days postexposure. The animals were examined daily for illness or death, and abdominal surface temperatures were measured using a Ranger MX4P digital infrared thermometer (Raytek). Ill animals with temperatures of <23°C, ruffled fur, eye crusting, hunched posture, and lack of resistance to handling were deemed terminal and were euthanized.

RESULTS

***btaI1*- and *btaI3*-dependent production of acyl-HSLs.** We used LC/MS/MS (22) and a [^{14}C]methionine incorporation assay (48) to determine what acyl-HSLs are produced by *B. thailandensis*. Both of these methods detect acyl-HSLs in a complex mixture without bias toward acyl side chain length or substitution and provide information on the relative abundances of the acyl-HSLs in the sample (22, 48). LC/MS/MS was used to analyze acyl-HSLs in ethyl acetate extracts of stationary-phase culture fluid from wild-type *B. thailandensis*. We found that extracts had C_8-HSL, $3OHC_8$-HSL, $3OHC_{10}$-HSL, and a relatively small amount of *N*-3-(hydroxybutanoyl)-HSL ($3OHC_4$-HSL) and *N*-3-(hydroxynonenoyl)-HSL ($3OHC_9$-HSL) (Table 2). The levels of $3OHC_4$-HSL and $3OHC_9$-HSL were about 4% and 1% of the total fatty acyl-HSL content, respectively (Table 2).

The *B. thailandensis* signal synthase BtaI2 was characterized in a separate study and shown to catalyze $3OHC_8$-HSL and $3OHC_{10}$-HSL synthesis (18). To determine which acyl-HSLs were produced by each of the other two *B. thailandensis* acyl-HSL synthases, we constructed strains with individual in-frame deletions of the signal synthase genes (*btaI1* and *btaI3*) and analyzed the acyl-HSL production of these strains by LC/MS/MS. The *btaI1* mutant (JBT101) produced $3OHC_8$-HSL and $3OHC_{10}$-HSL but did not produce detectable levels of C_8-HSL (Table 2), supporting the conclusion that BtaI1 is a C_8-HSL synthase. The *btaI3* mutant (JBT103) produced detectable levels of C_8-HSL, $3OHC_8$-HSL, and $3OHC_{10}$-HSL; however, the relative abundance of $3OHC_8$-HSL was threefold less than that of the wild type. It is important to note that Table 2 shows relative abundances of acyl-HSLs and not absolute values. Based on these results, we hypothesized that BtaI3 is a $3OHC_8$-HSL synthase and that the $3OHC_8$-HSL detected in the *btaI3* mutant strain is produced by BtaI2.

To verify that BtaI1 is a C_8-HSL synthase and that BtaI3 is a $3OHC_8$-HSL synthase, the proteins were each expressed in *E. coli*, and acyl-HSL production was monitored by using a radiotracer assay, which incorporates one ^{14}C atom into each acyl-HSL molecule regardless of side chain length or substitution (48). Recombinant *E. coli* expressing BtaI1 incorporated ^{14}C into one major product that coeluted with C_8-HSL by C_{18} reverse-phase HPLC (Fig. 1). Recombinant *E. coli* expressing BtaI3 had one major product that coeluted with $3OHC_8$-HSL (Fig. 1). These results are consistent with the conclusion that BtaI1 is a C_8-HSL synthase and BtaI3 is a $3OHC_8$-HSL synthase.

Quorum sensing and metabolic capabilities. In other bacteria, such as *Pseudomonas aeruginosa* and *Vibrio fischeri*, quorum sensing affects the range of utilizable carbon and energy sources. In particular, proteases are often under quorum control (1, 40, 50, 52). To test whether quorum sensing controls metabolism in *B. thailandensis*, we used Biolog phenotype microarrays to compare the abilities of the wild type and a quorum-sensing mutant to utilize 344 different compounds (5). We constructed a quorum-sensing mutant with unmarked, in-frame deletions of all three of the acyl-HSL synthase genes (*btaI1*, *btaI2*, and *btaI3*) by using a *pheS* counterselection method (see Materials and Methods). This strain did not produce any acyl-HSLs that were detectable by LC/MS/MS (Table 2) or by any of four bioassays that in combination should detect the majority of described fatty acyl-HSLs (Table 3). The wild-type strain produced acyl-HSLs that were detectable by LC/MS/MS and by three of the four bioassays (Tables 2 and 3). The Biolog phenotypic-array method measures the respiration rate as an indication of growth. Both the wild type and the mutant metabolized a wide range of nutrients (detailed in Table S1 in the supplemental material). Interestingly, the mutant showed greater respiration rates on most substrates tested. This is

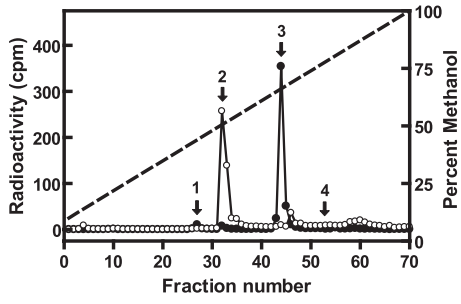

FIG. 1. Methanol gradient HPLC separation of radioactive acyl-HSLs produced by recombinant *E. coli* expressing *B. thailandensis* signal synthases. Shown is *E. coli* containing the *btaI1* vector pBTI1 (●) or the *btaI3* vector pBTI3 (○). The *y*-axis values are radioactive counts per minute (cpm) divided by 10^3 for pBTI1 or 10^2 for pBTI3. The radiotracer assay provides a measure of the relative abundances of all acyl-HSLs produced, regardless of side chain length or substitution. The arrows indicate the fractions in which synthetic C_6-HSL (1), $3OHC_8$-HSL (2), C_8-HSL (3), or C_{10}-HSL (4) was eluted.

TABLE 3. Activation of acyl-HSL reporters by culture extracts of *B. thailandensis* wild type and an acyl-HSL synthesis mutant

Bioassay strain[a]	Range of acyl-HSLs detected	β-Galactosidase activity (fold induction) of:	
		E264 (wild type)	JBT112 (Δ*btaI1* Δ*btaI2* Δ*btaI3*)
A. tumefaciens KYC55(pJZ372)(pJZ384)(pJZ410)	Broad range	2×10^5 (±9%)	1 (±11%)
P. fluorescens 1855(pSF105)(pSF107)	$3OHC_6$-HSL, $3OHC_8$-HSL	4×10^2 (±7%)	1 (±5%)
E. coli DH5α(pECP61.5)	C_4-HSL, C_6-HSL	13 (±12%)	1 (±11%)
R. capsulatas ALS1(pYP)	C_{14}-HSL, C_{16}-HSL	1 (±8%)	1 (±9%)

[a] Bioassays are described in Materials and Methods. The induction was in comparison to controls with no culture extracts added. The values are the averages from at least two independent cultures, and the range is indicated in parentheses.

exemplified by the examples shown in Fig. 2. We tested the abilities of the parent and the quorum-sensing mutant to degrade proteins. Both strains were capable of degrading gelatin and casein (data not shown). Our results led us to suggest that *B. thailandensis* quorum sensing does not affect the metabolic diversity of *B. thailandensis* as it does in *P. aeruginosa* (40, 50), for example. However, quorum sensing does have a negative influence on the rate of respiration on most carbon, nitrogen, or phosphorus substrates under the conditions of our experiments.

***B. thailandensis* quorum sensing and intercellular aggregation.** Our phenotype microarray results suggested that quorum sensing has a global affect on the rate of cellular growth and metabolism. We propose that this could result from control of processes that affect cellular respiration or by the imposition of a metabolic burden on dividing cells. A possible explanation arose from our observation that wild-type *B. thailandensis* aggregated and formed macroscopic clumps when grown in a minimal medium similar to that used for phenotype microarray experiments, whereas the acyl-HSL synthase-deficient mutant JBT112 did not form clumps (Fig. 3A). Aggregation of the mutant strain was restored when we added C_8-HSL and $3OHC_8$-HSL to the minimal medium (Fig. 3A). Although there was no obvious aggregation of the wild type or mutant in LB broth, wild-type cultures spotted on LB agar plates and grown for 4 days exhibited a wrinkly appearance commonly associated with the production of an exopolysaccharide (EPS), whereas the mutant colonies were not wrinkly (Fig. 3B). We suspect that in our phenotype microarray experiments aggre-

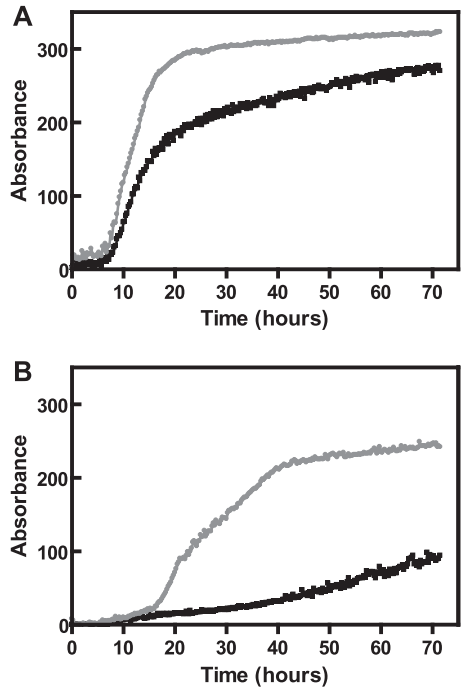

FIG. 2. Metabolism of α-D-glucose (A) and L-isoleucine (B) by *B. thailandensis* wild-type and JBT112 (Δ*btaI1* Δ*btaI2* Δ*btaI3*) strains. Metabolism was monitored as respiration with glucose or L-isoleucine as the sole source of carbon in Biolog phenotype microarray experiments. Respiration was measured at 15-min intervals for 72 h. Shown are the *B. thailandensis* wild type E264 (black) and the quorum-sensing signal synthase-deficient mutant JBT112 (gray).

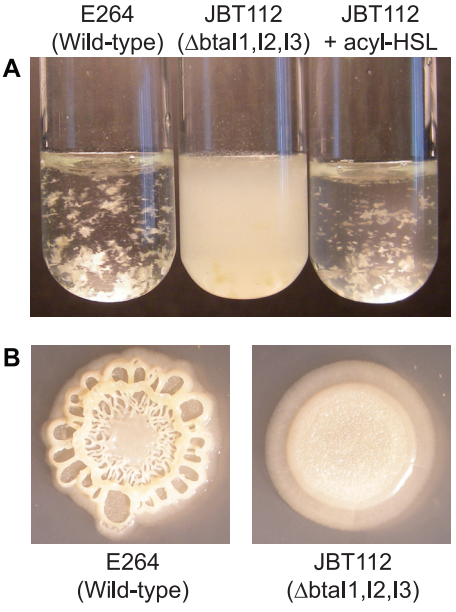

FIG. 3. Phenotype of a quorum-sensing signal synthase-deficient mutant. (A) Aggregation in shaken liquid cultures. When grown in minimal medium, the wild-type E264 cells aggregate and form clumps. The quorum-sensing mutant JBT112 (Δ*btaI1* Δ*btaI2* Δ*btaI3*) does not show visible aggregation. The cultures were agitated just prior to image acquisition. Ethyl acetate solutions of C_8-HSL and $3OHC_8$-HSL were added to a sterile glass culture tube and dried to completion just prior to addition of the culture medium and inoculum. (B) Growth of wild-type E264 and the mutant JBT112 on solid medium. The inoculum was 5 μl from stationary-phase cultures grown for 24 h in LB broth. The photographs were taken with a digital camera mounted on an Olympus SZX12 dissecting microscope after incubation for 24 h at 37°C and an additional 72 h at room temperature. The colonies measured approximately 1 cm in diameter.

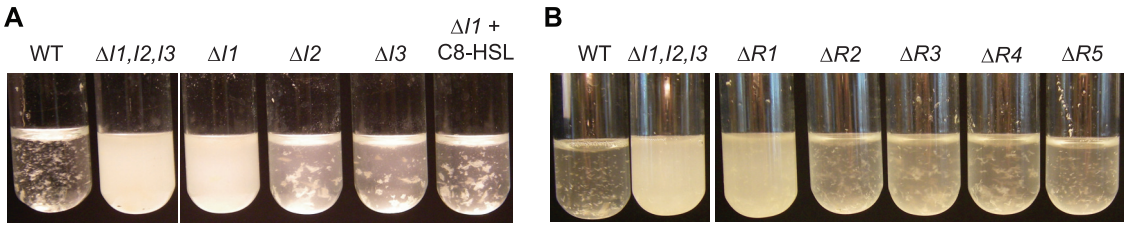

FIG. 4. *B. thailandensis* BtaR1 and BtaI1 are required for cell aggregation. *B. thailandensis* wild-type (WT) and quorum-sensing mutant strains were grown in minimal medium to assess aggregation, as for Fig. 3. (A) The following strains were studied: JBT101 (Δ*btaI1*), JBT102 (Δ*btaI2*), JBT103 (Δ*btaI3*), and JBT101 with C_8-HSL (added as described for acyl-HSL additions in the legend to Fig. 3). (B) Aggregation of *B. thailandensis* signal receptor mutant strains JBT107 (Δ*btaR1*), JBT108 (Δ*btaR2*), JBT109 (Δ*btaR3*), JBT110 (Δ*btaR4*), and JBT111 (Δ*btaR5*). Strain JBT112 (Δ*btaI1* Δ*btaI2* Δ*btaI3*) is also shown.

gation has a negative influence on respiration, because cells deep within aggregates have limited access to oxygen. The conclusion that aggregation affects cellular respiration is supported by the following result. We monitored the growth of both the wild type and the quorum-sensing mutant in LB broth, a medium in which neither strain forms aggregates, and we found that the growth rates were identical (both strains had a doubling time of 36 min at 37°C with shaking) (data not shown).

The *btaI1-btaR1* quorum-sensing system is required for cell aggregation. Next, we sought to determine which of the multiple *B. thailandensis* quorum-sensing systems is required for cell aggregation. To address this issue, we constructed strains with mutations in individual signal synthase genes or signal receptor genes. Our *btaI1* mutant did not aggregate, whereas the *btaI2* and *btaI3* mutants formed aggregates indistinguishable from wild-type aggregates (Fig. 4A). Aggregation was restored in the *btaI1* mutant strain by the addition of C_8-HSL (the acyl-HSL product of BtaI1 activity) to the growth medium (Fig. 4A). This leads to the conclusion that clumping is dependent on the BtaI1-BtaR1 system. In support of this conclusion, we found that a *btaR1* mutant lost the clumping phenotype but *btaR2*, *-R3*, *-R4*, and *-R5* mutants all formed aggregates (Fig. 4B).

Quorum sensing and virulence of *B. thailandensis*. The published literature shows that the virulence of a variety of *B. pseudomallei* and *B. mallei* quorum-sensing mutants is attenuated in animal models, including airborne mouse infection models (53, 54, 56). Like *B. pseudomallei*, *B. thailandensis* infects, causes a melioidosis-like disease, and can kill mice when administered by aerosol, as described elsewhere (28, 59). Thus, we compared the virulence of *B. thailandensis* quorum-sensing mutants to that of the wild-type strain by administering about 5×10^4 bacteria per lung (a dose sufficient for the wild type to kill about 75% of infected mice) by aerosol (28). As expected, seven out of eight wild-type-infected mice died within a week (Fig. 5). The triple acyl-HSL synthase mutant JBT112 and the *btaI1* mutant JBT101 were also tested and were as virulent as the wild type. At least in our model, quorum sensing does not appear to be a *B. thailandensis* virulence determinant.

DISCUSSION

We have been interested in the conserved quorum-sensing circuits of *B. thailandensis*, *B. pseudomallei*, and *B. mallei* because these bacteria are closely related at the genomic level but have divergent lifestyles. Here, we report a characterization of quorum-sensing signals, and we report that quorum sensing activates a self-aggregation system in *B. thailandensis*. To perform our analysis, we developed an allelic replacement strategy that allowed the construction of strains with multiple unmarked, in-frame deletions in an otherwise genetically unmodified strain of *B. thailandensis*. During the course of our studies, a somewhat similar technique was developed by Barrett et al. (3). Because *B. thailandensis* is naturally resistant to many antibiotics as a result of antibiotic efflux, genetic manipulations prior to this study were commonly done in a strain with an efflux pump mutation that confers sensitivity to a wider range of antibiotics (8, 36). However, efflux pumps have been reported to influence quorum-sensing signaling in *B. pseudomallei* (9, 10).

An earlier publication on quorum-sensing signal production by *B. thailandensis* indicated that *btaI1* directed synthesis of C_8-HSL, *btaI2* directed synthesis of C_{10}-HSL, and *btaI3* encoded a C_6-HSL synthase (55). We recently found that *B. thailandensis* produces primarily C_8-HSL, $3OHC_8$-HSL, and $3OHC_{10}$-HSL and only very small amounts of other acyl-HSLs (18). Furthermore, we found that BtaI2 is not a C_{10}-HSL synthase but is a $3OHC_{10}$-HSL synthase that also produces some $3OHC_8$-HSL (18). Thus, a first step in the study de-

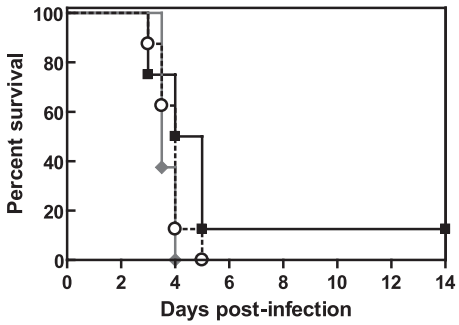

FIG. 5. Virulence of *B. thailandensis* quorum-sensing mutants in a BALB/c mouse respiratory-infection model. Shown is survival following infection with aerosolized suspensions of wild-type *B. thailandensis* strain E264 (■), strain JBT112 (◆), or strain JBT101 (○). The target dose was 5×10^4 CFU/lung, and the actual number of CFU per lung recovered from sentinel mice sacrificed immediately after infection was 3.7 (±standard error of the mean [SEM] of 0.3) $\times 10^4$ for E264, 3.8 (±SEM of 0.4) $\times 10^4$ for JBT112, and 5.0 (±SEM of 0.4) $\times 10^4$ for JBT101. Twelve mice were infected with each strain, and four sentinel mice were harvested immediately after infection.

scribed here was to determine the relevant signals produced by the other two *B. thailandensis* acyl-HSL synthases, BtaI1 and BtaI3. Our evidence with unmarked synthase deletion mutants and with recombinant *E. coli* indicates that, in agreement with the initial report, BtaI1 is a C_8-HSL synthase but BtaI3 is a $3OHC_8$-HSL synthase. It is not clear why the initial report presented a different picture for BtaI2 and BtaI3, but we believe there were two potential explanations. First, an efflux pump mutant was used in the study, and efflux pumps have since been shown to affect acyl-HSL production by members of the *B. thailandensis-B. pseudomallei-B. mallei* group (9, 10). Second, the acyl-HSLs were detected by a single bioassay, the *A. tumefaciens* assay. Because this assay is particularly sensitive to aliphatic acyl-HSLs in the C_6-C_{10} chain length range, the investigation could have easily missed the most abundant hydroxy-acyl-HSLs and focused on minor acyl-HSL species. It now seems clear from the results reported here and elsewhere (16, 17, 51) that in *B. thailandensis*, as well as *B. mallei* and *B. pseudomallei*, quorum-sensing system 1 involves a C_8-HSL signal and system 3 a $3OHC_8$-HSL signal.

Previously, we reported that the *B. thailandensis* BtaI2-BtaR2 quorum-sensing circuit induces the production of an antibiotic predicted to be a nonribosomal peptide-polyketide hybrid molecule (18); but what else might be regulated by quorum sensing in *B. thailandensis*? The initial report on *B. thailandensis* quorum sensing showed that signal synthase and receptor mutants generated from the efflux pump-defective strain showed motility defects and minor differences in hemolysin production and utilization of some carbon sources compared to the parent, but these phenotypes were not complemented with plasmid-borne copies of the relevant genes or by addition of the appropriate signals in the case of the signal synthase mutants (55). We used our quorum-sensing signal-deficient strain JBT112 to reexamine these phenotypes and found a motility phenotype similar to that reported but no discernible quorum-sensing-dependent differences in hemolysin production (data not shown). In addition, our extensive screen of carbon, nitrogen, and phosphorus sources did not reveal any substrates for which *B. thailandensis* quorum sensing was required. Our results suggest that the hemolysin and carbon utilization defects previously reported (55) might have been strain specific and perhaps unrelated to quorum sensing.

We also found that the virulence of *B. thailandensis* in a mouse infection model was unaffected by quorum-sensing mutations. Our model and our dosing were similar to those used in *B. pseudomallei* experiments that suggested a role for quorum sensing in virulence (53). Perhaps there is a fundamental difference in the roles of the highly conserved *B. thailandensis*, *B. pseudomallei*, and *B. mallei* quorum-sensing systems that is related to the divergent niches these species are thought to occupy. However, it will be important to revisit the issue of quorum-sensing control of virulence in *B. pseudomallei* and *B. mallei* now that it is possible to generate unmarked deletions in wild-type strains. Due to past difficulties in working with *B. mallei* and *B. pseudomallei*, the virulence phenotypes of quorum-sensing mutants were not complemented.

Although virulence and metabolic diversity were not affected in our quorum-sensing mutant, we did find that the strain lost a characteristic clumping phenotype exhibited by the wild-type strain when grown in minimal medium and exhibited a smooth colony morphology on LB agar compared to the rough or wrinkly colony morphology of the parent (Fig. 3). We were able to demonstrate that the clumping or self-aggregation phenotype was dependent on BtaR1 and BtaI1 and could be restored in a *btaI1* mutant by addition of the BtaI1-generated signal C_8-HSL (Fig. 4). The molecular basis for self-aggregation in *B. thailandensis* remains unknown; however, two lines of evidence support the view that it is at least in part mediated by EPS production: (i) the rough or wrinkly colony morphology reported in this study (Fig. 3) has been associated with EPS production in other bacteria, such as *Vibrio cholerae* (62) and *P. aeruginosa* (24), and (ii) we used microarrays to identify quorum-sensing-controlled genes, and a number of these are predicted to be involved in EPS production. Mutations in two of these quorum-sensing-controlled putative EPS synthesis genes, BTH_II0551 and BTH_II0695, caused aggregation defects (J. R. Chandler and E. P. Greenberg, unpublished data). Quorum-sensing control of cell aggregation is a common theme that has been observed in other organisms, including *Yersinia pseudotuberculosis* (2), *Pseudomonas aerofaciens* (64), *Erwinia chrysanthemi* (26) *Rhodobacter sphaeroides* (42), and many clinical isolates of *P. aeruginosa* (L. Hoffman and S. I. Miller, personal communication). In some cases, quorum sensing induces aggregation (26) and, in other cases, dispersion (2, 42, 64). One controversy that has arisen in the quorum-sensing field is whether acyl-HSL signaling is in fact a system that enables cooperativity among members of a group or whether it might serve a different purpose. Others have proposed that such systems are not quorum-sensing systems but systems for diffusion sensing (44) that benefit individual cells by enabling them to monitor the diffusion of small molecules (acyl-HSLs) before inducing expression of more metabolically expensive secreted protein products. The recurring theme of acyl-HSL induction of cell-cell aggregation is another argument that signaling is involved in cooperative behavior. The aggregation response requires multiple cells, and it is not likely to benefit a solitary individual.

It is not yet clear what the survival advantage is for a cell density-dependent switch from dispersed cells to aggregation in *B. thailandensis*. Among the possibilities, aggregation may enable the establishment of microcolonies in the natural habitat of *B. thailandensis*, such as in mixed microbial communities in Southeast Asian rice paddies or soils. Aggregates consist of *B. thailandensis* cells at very high density, and because the BtaR2-BtaI2 system activates antibiotic synthesis, one might expect the local antibiotic concentration to be high and to serve to protect the aggregate from competing bacteria. As we learn more about quorum sensing in *B. thailandensis*, we hope to gain an understanding of why clumping and antibiotic synthesis are controlled by two separate quorum-sensing systems. Finally, it is interesting that rough colony morphology has been described for the great majority of *B. pseudomallei* clinical isolates, with about 10% of isolates showing a smooth colony morphology (11). It will be interesting to determine whether at least some *B. pseudomallei* smooth-colony isolates have defects in quorum-sensing system 1.

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