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Quorum Sensing Influences *Burkholderia thailandensis* Biofilm Development and Matrix Production

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ABSTRACT

Members of the genus *Burkholderia* are known to be adept at biofilm formation, which presumably assists in the survival of these organisms in the environment and the host. Biofilm formation has been linked to quorum sensing (QS) in several bacterial species. In this study, we characterized *Burkholderia thailandensis* biofilm development under flow conditions and sought to determine whether QS contributes to this process. *B. thailandensis* biofilm formation exhibited an unusual pattern: the cells formed small aggregates and then proceeded to produce mature biofilms characterized by "dome" structures filled with biofilm matrix material. We showed that this process was dependent on QS. *B. thailandensis* has three acyl-homoserine lactone (AHL) QS systems (QS-1, QS-2, and QS-3). An AHL-negative strain produced biofilms consisting of cell aggregates but lacking the matrix-filled dome structures. This phenotype was rescued via exogenous addition of the three AHL signals. Of the three *B. thailandensis* QS systems, we show that QS-1 is required for proper biofilm development, since a *btaR1* mutant, which is defective in QS-1 regulation, forms biofilms without these dome structures. Furthermore, our data show that the wild-type biofilm biomass, as well as the material inside the domes, stains with a fucose-binding lectin. The *btaR1* mutant biofilms, however, are negative for fucose staining. This suggests that the QS-1 system regulates the production of a fucose-containing exopolysaccharide in wild-type biofilms. Finally, we present data showing that QS ability during biofilm development produces a biofilm that is resistant to dispersion under stress conditions.

IMPORTANCE

The saprophyte *Burkholderia thailandensis* is a close relative of the pathogenic bacterium *Burkholderia pseudomallei*, the causative agent of melioidosis, which is contracted from its environmental reservoir. Since most bacteria in the environment reside in biofilms, *B. thailandensis* is an ideal model organism for investigating questions in *Burkholderia* physiology. In this study, we characterized *B. thailandensis* biofilm development and sought to determine if quorum sensing (QS) contributes to this process. Our work shows that *B. thailandensis* produces biofilms with unusual dome structures under flow conditions. Our findings suggest that these dome structures are filled with a QS-regulated, fucose-containing exopolysaccharide that may be involved in the resilience of *B. thailandensis* biofilms against changes in the nutritional environment.

In the environment, many bacteria reside in biofilms. Biofilm growth helps protect the resident bacteria from environmental stresses, such as desiccation, nutrient limitation, and predation (1). Biofilm formation is a coordinated process among community members and can differ from species to species. One key feature of biofilm communities is that they produce an extracellular matrix that serves to hold the community together. This matrix is usually composed of a mixture of extracellular DNA, exopolysaccharides, proteins, and lipid vesicles.

Another type of microbial group behavior is quorum sensing (QS). Many proteobacteria, including *Burkholderia thailandensis*, utilize acyl-homoserine lactone (AHL) quorum-sensing systems. Each AHL QS system consists of a gene pair encoding a LuxI family AHL signal synthase and a LuxR family AHL signal receptor, which also functions as a transcription factor. The AHL signal is diffusible across the bacterial membrane and often reflects bacterial population density. Under conditions of high cell density, the AHL signal reaches a threshold concentration, activating a LuxR family transcriptional regulator. *B. thailandensis* contains three complete AHL signaling systems, termed quorum-sensing circuit 1 (QS-1), QS-2, and QS-3. QS-1 consists of the BtaI1-BtaR1 pair and the signal *N*-octanoyl homoserine lactone (C_8-HSL) (2, 3). QS-2 comprises BtaI2-BtaR2 and *N*-3-hydroxy-decanoyl homoserine lactone ($3OHC_{10}$-HSL) (2, 4), and QS-3 consists of BtaI3-BtaR3 and *N*-3-hydroxy-octanoyl homoserine lactone ($3OHC_8$-HSL) (2, 3). We have shown previously that *B. thailandensis* QS mutants have aggregation defects during planktonic growth and that QS controls a number of factors, including those important for biofilm formation (e.g., exopolysaccharides [capsular polysaccharide I {CPSI},

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TABLE 1 Bacterial strains and plasmids used in this study

Bacterial strain or plasmid	Genotype or description	Source or reference(s)
Bacterial strains		
DH10B	*E. coli* cloning vehicle	Invitrogen
E264	Wild-type *B. thailandensis*	30
JBT107	E264 Δ*btaR1*	3
JBT108	E264 Δ*btaR2*	3
JBT109	E264 Δ*btaR3*	3
JBT112	E264 Δ*btaI1* Δ*btaI2* Δ*btaI3*	3
JBT124	E264 Δ*bceI* Δ*bceII* ΔCPSIII	This study
CM183	E264 Δ*cdiAIB*::*tmp*	5
CM262	E264 *glmS1 att*Tn*7*::P_{S12}-*yfp ble* Zeo^r	This study
CM265	JBT107 *glmS1 att*Tn*7*::P_{S12}-*yfp ble* Zeo^r	This study
CM269	CM183 *glmS1 att*Tn*7*::P_{S12}-*yfp ble* Zeo^r	This study
CM284	JBT108 *glmS1 att*Tn*7*::P_{S12}-*yfp ble* Zeo^r	This study
CM286	JBT112 *glmS1 att*Tn*7*::P_{S12}-*yfp ble* Zeo^r	This study
CM288	JBT109 *glmS1 att*Tn*7*::P_{S12}-*yfp ble* Zeo^r	This study
CM340	E264 ΔBTH_II1974-II1986::*tmp* ΔCPSII	This study
BTBt21	E264 ΔBTH_I1325-1342::*tmp* ΔCPSI	This study
BTBt27	E264 ΔBTH_I1349-1362::*tmp* ΔCPSIV	This study
Plasmids		
pDONR221 P1-P5r	Gateway-compatible vector with *att*P1 and *att*P5r recombination sites and *ccdB*; Km^r Cm^r	Invitrogen
pEX19EYFP	Source for eYFP; Gm^r	31
pTNS2	R6K replicon; TnsABCD vector; Ap^r	32
pUC18T-mini-Tn7T-Tp	Mini-Tn*7*T-based vector containing a trimethoprim resistance cassette; Tp^r (GenBank accession no. DQ493875)	33
pCM112	pUC18T-mini-Tn*7*T-*ble*-P_{S12} Ap^r Zeo^r	14
pCM249	pUC18T-mini-Tn*7*T-*ble*-P_{S12}-*yfp* Ap^r Zeo^r	This study
pJRC115	Suicide plasmid containing *pheS** counterselectable marker; Tp^r	3, 5
pJRC115 *bceI*	pJRC115 containing the Δ*bceI* deletion fragment; Tp^r	This study
pJRC115 *bceII*	pJRC115 containing the Δ*bceII* deletion fragment; Tp^r	This study
pBT287	Gateway-compatible plasmid containing the T7 gene 10 leader sequence fused with *eyfp*, flanked by *att*R5 and *att*L1 recombination sites; Km^r	This study
pBT409	pUC57-Kan-based plasmid containing the CPSI deletion fragment; Km^r Tp^r	This study
pBT415	pUC57-Kan-based plasmid containing the CPSIV deletion fragment; Km^r Tp^r	This study

CPSII, and CPSIII/cepacian]) and contact-dependent inhibition (CDI) (3, 5).

Many QS-controlled functions, such as the production of secreted factors (e.g., antimicrobials, toxins, virulence factors, and biofilm components) (see reference 6 for a review), are believed to benefit groups of organisms. QS has also long been recognized to contribute to biofilm formation for different species (7). However, the QS-controlled functions that can impact this process are highly variable from species to species. In various species, QS has been shown to play roles in bacterial motility, surface attachment, aggregate formation, biofilm maturation, and biofilm dispersal (8). Within the species *Burkholderia*, while the role of QS in biofilm formation has been most extensively studied for *Burkholderia cepacia* complex members (9, 10), it has also been demonstrated in *Burkholderia pseudomallei* (11, 12).

In this study, we characterized biofilms produced by wild-type and QS mutant strains of *B. thailandensis* E264. Wild-type biofilms produced matrix-filled "dome" structures, a process that was dependent on QS-1. We conducted lectin-staining experiments, which suggested that the dome structures contain a QS-regulated, fucose-containing exopolysaccharide. However, mutational analysis revealed that the four known CPS gene clusters (encoding CPSI to CPSIV) are not required for the production of these structures. Finally, we demonstrated that QS contributes to the ability of *B. thailandensis* biofilms to withstand changes in the nutritional environment.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown in low-salt Luria-Bertani (LB) broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl per liter), and *B. thailandensis* was grown in low-salt LB or FAB medium (13) supplemented with 0.3 mM or 30 mM glucose as indicated. Antibiotics were added to bacteria at the following concentrations as appropriate: for *E. coli*, 25 μg/ml zeocin (Zeo), 100 μg/ml ampicillin (Ap), and 100 μg/ml trimethoprim (Tmp); for *B. thailandensis*, 2 mg/ml Zeo and 100 μg/ml Tmp. Except where indicated otherwise, bacteria were grown at 37°C with shaking. For complementation studies, 2 μM (each) C_8-HSL (Sigma Chemical Co.), $3OHC_8$-HSL (14), and $3OHC_{10}$-HSL (purchased from the School of Molecular Medical Sciences at the University of Nottingham [http://www.nottingham.ac.uk/quorum/compounds.htm]) were added with fresh medium changes at 24 h and 72 h of biofilm growth.

Mutant construction. To generate Zeo-resistant yellow fluorescent protein (YFP)-labeled mutants, we transformed E264, JBT112, JBT107, JBT108, JBT109, and CM183 with pTNS2 and pCM249 as described elsewhere (5) to make CM262 (E264 *glmS1 att*Tn*7*::P_{S12}-*yfp ble*), CM286 (Δ*btaI1* Δ*btaI2* Δ*btaI3 glmS1 att*Tn*7*::P_{S12}-*yfp ble*), CM265 (Δ*btaR1 glmS1 att*Tn*7*::P_{S12}-*yfp ble*), CM284 (Δ*btaR2 glmS1 att*Tn*7*::P_{S12}-*yfp ble*), CM288 (Δ*btaR3 glmS1 att*Tn*7*::P_{S12}-*yfp ble*), and CM269 (Δ*cdiAIB*::*tmp glmS1*

*att*Tn*7*::P_{S12}-*yfp ble*). pCM249 was generated by introducing YFP into pCM112 (pUC18T-mini-Tn7T-*ble*-P_{S12}). The YFP gene insert was amplified with OCM103 and OCM104 from pBT287 as the template DNA. Both the YFP insert and the pCM112 plasmid were cut with HindIII and KpnI and were ligated together using standard cloning methods. pBT287 was constructed by amplifying the *eyfp* gene (encoding enhanced YFP [eYFP]) from pEX19EYFP with OBT335 and OBT342, and the PCR product was then recombined into pDONR221 P1-P5r using BP Clonase II (Gateway technology; Invitrogen).

The ΔCPSI mutant (BTBt21 [ΔBTH_I1325-1342::*tmp*]) and the ΔCPSIV mutant (BTBt27 [ΔBTH_I1349-1362::*tmp*]) were created via natural transformation of *B. thailandensis* with a deletion fragment as described previously (15). The CPSI and CPSIV deletion fragments were synthesized into pUC57-Kan (Genewiz) to create pBT409 and pBT415. The CPSI construct contains the first ~850 bp of BTH_I1324, followed by a trimethoprim resistance cassette and then the first ~850 bp of BTH_I1343. EcoRI sites flank the deletion fragment in pBT409. The CPSIV construct contains the first ~800 bp of BTH_I1348, followed by a trimethoprim resistance cassette and then the last ~550 bp of BTH_I1363. PvuII sites flank the deletion fragment in pBT415. Integration of the deletion alleles was verified by PCR and sequencing with two sets of primers: for CPSI, OBT675 with OBT676 and OBT677 with OBT678; for CPSIV, OBT668 with OBT676 and OBT677 with OBT669.

The ΔCPSII mutant (CM340 [ΔBTH_II1974-II1986::*tmp*]) was constructed by first generating a deletion fragment with PCR and then introducing the DNA fragment into *B. thailandensis* by natural transformation as described previously (15). To generate the CPSII deletion fragment, we first used PCR to generate three DNA molecules. The first consisted of approximately 1,000 bp of sequence upstream of the CPSII genes and was generated with primers OCM134 and OCM133. This fragment contained a 3′ primer-encoded sequence complementary to the fragment carrying trimethoprim resistance. The second fragment contained the trimethoprim resistance cassette from pUC18T-mini-Tn7T-Tp and was made with primers OCM131 and OCM132. The third fragment contained approximately 900 bp of sequence downstream of the CPSII genes and was made with primers OCM114 and OCM113. The 5′ end of this fragment contained a primer-encoded sequence complementary to the 3′ end of the fragment carrying trimethoprim resistance. We next stitched the DNA molecules together by Gibson product ligation (New England BioLabs). The assembled fragment was then amplified in a final PCR with primers OCM134 and OCM113, purified, and used to transform *B. thailandensis* to yield the trimethoprim-resistant mutant strain CM340.

The ΔCPSIII mutant was constructed by delivering modified DNA to the *B. thailandensis* genome using homologous recombination with the pJRC115 suicide delivery plasmid as described previously (3). First, we constructed suicide delivery plasmids to knock out each of the *bce* gene clusters. For *bceI*, we made a deletion from base +40 of BTH_II0543 to base +394 of BTH_II0552, with respect to the translational start site. For *bceII*, we made a deletion spanning BTH_II0691 to BTH_II0695, including all but the last 42 bases of BTH_II0691 and all but the last 6 bases of BTH_II0695 (BTH_II0691 and BTH_II0695 face in opposite directions). Briefly, PCR-amplified fragments flanking the *bceI* (oligonucleotide bceI d1-4) and *bceII* (oligonucleotide bceII d1-4) gene clusters were generated using overlap extension PCR and were each cloned into the suicide delivery plasmid (3) using PCR-generated XbaI and HindIII restriction sites, creating pJRC115 *bceI* and pJRC115 *bceII*. The deletion constructs were sequentially used to make a *bceI bceII* double mutant in *B. thailandensis* so as to create strain JBT124. Each deletion plasmid was introduced into *B. thailandensis* by electroporation; transformants were selected with trimethoprim; and deletion mutants in which the integrated plasmid was excised were selected using minimal glucose agar with 1% *p*-Cl-phenylalanine supplement as described previously (3). The mutant constructs and final deletion mutant were confirmed by PCR and sequencing using oligonucleotides bceI d5-7 and bceII d5-7.

Biofilm growth and imaging. Continuous flow cell biofilm reactors were prepared and assembled as described previously (16). Log-phase cultures, grown in FAB with 30 mM glucose, were diluted to a final optical density at 600 nm (OD_{600}) of 0.01 in FAB with 0.3 mM glucose. Flow cell chambers were then inoculated with these diluted cultures and were incubated inverted for 1 h before the initiation of flow. Biofilms, which were continuously supplied with fresh FAB with 0.3 mM glucose at 10 ml/h, were grown for 96 h at room temperature. A Zeiss LSM 510 confocal laser-scanning microscope was used to image the biofilms, and Volocity software (Improvision) was used for compiling image series. For most experiments, the biofilms were visualized via the YFP expressed by the cells. Biofilms of the CPS mutants, which lacked the YFP biomarker, were stained with 5 μm Syto9 (Life Technologies) for 15 min and were then rinsed for 5 min before imaging. For the attachment assays, cells attached to the glass surface were imaged by bright-field microscopy 30 min after the initiation of flow, with a minimum of 60 fields imaged per sample.

Biomarker staining. Ninety-six-hour biofilms were stained with biomarkers for 15 min and were then rinsed for 5 min before imaging. To stain for DNA, Syto62 (Life Technologies) at 5 μM was used; to stain for proteins, NanoOrange (Life Technologies) at a 1/20 dilution was used; and to stain for lipids, FM4-64 (Life Technologies) at 5 μg/ml was used. The following rhodamine-conjugated lectins (lectin kits I and II; Vector Laboratories) were tested at 100 μg/ml: *Hippeastrum* hybrid agglutinin (HHA), concanavalin A (ConA), *Dolichos biflorus* agglutinin (DBA), peanut agglutinin (PNA), *Ricinus communis* agglutinin I (RCA I), soybean agglutinin (SBA), *Ulex europaeus* agglutinin I (UEA), wheat germ agglutinin (WGA), *Griffonia simplicifolia* lectin I (GSL I), *Lens culinaris* agglutinin (LCA), *Phaseolus vulgaris* erythroagglutinin (PHA-E), *Phaseolus vulgaris* leucoagglutinin (PHA-L), *Pisum sativum* agglutinin (PSA), succinylated WGA, and *Sophora japonica* agglutinin (SJA).

Biofilms under stress conditions. Continuous flow cell biofilm reactors were grown as described above for 96 h and were gradually exposed to sterile phosphate-buffered saline (PBS), pH 7, in a step gradient where the flow was kept constant while an increasing concentration of PBS was introduced into the system. The step gradient was performed by increasing the flow of PBS by 1.25 ml/h every 30 min while decreasing the amount of FAB medium pumped at the same rate until only PBS was being fed to the system. Forty-eight hours after the initiation of PBS exposure, biofilms were stained with Syto62 (Life Technologies) at 5 μM for 15 min; they were then rinsed for 5 min at 10 ml/h before imaging. Imaging was performed using a confocal microscope as described above. To determine total biomass, 20 images per sample were acquired, and the total number of voxels in the image above a threshold fluorescence level (1,000 arbitrary units [AU]) were counted using Volocity software (Improvision). The heights of the dome structures in the wild-type biofilms were measured as the distance from the tallest part of the dome straight down to the base of the biofilm, using ZEN software (Zeiss). Due to experiment-to-experiment variation, the data for each experiment were normalized to those for an internal wild-type sample.

RESULTS AND DISCUSSION

***B. thailandensis* biofilm formation is complex and is characterized by QS-dependent, cell-free, matrix-rich structures.** We investigated *B. thailandensis* biofilm formation using a flow system and confocal laser-scanning microscopy. Using this approach, we assayed the ability of a YFP-expressing wild-type *B. thailandensis* E264 strain to form biofilms in a defined medium supplemented with glucose as the sole carbon source, as described previously (17). Small, surface-associated bacterial aggregates appeared 24 h postinoculation (Fig. 1A). At 48 h, the relative amount of biofilm biomass had increased, with cells present in larger 3-dimensional aggregates (Fig. 1A). Given time, we observed the presence of nonfluorescent zones within the aggregates. At later time points, these regions became more defined and increased in height, creating a

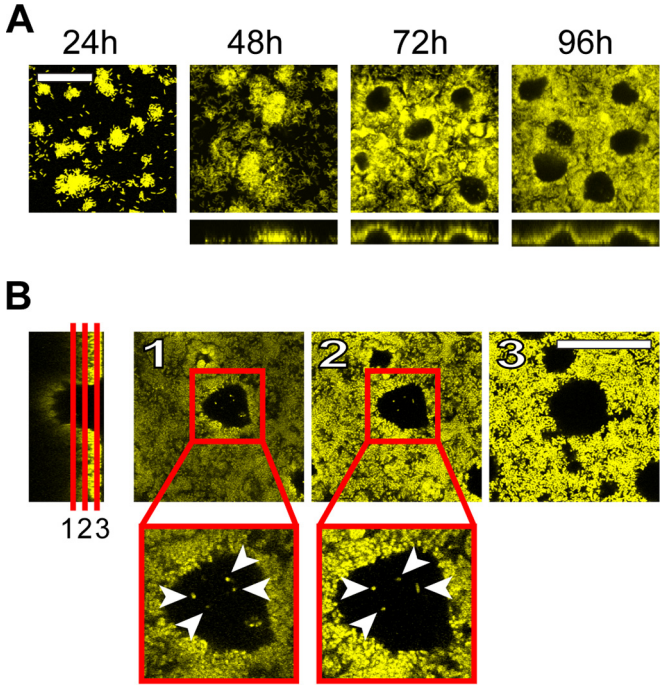

FIG 1 *B. thailandensis* forms biofilms with dome structures. (A) Biofilms of YFP-expressing wild-type *B. thailandensis* were grown in a flow cell system and were imaged at the times indicated. Representative images are shown with the cross-sectional view on top and a side view on the bottom. Bar, 50 μm. (B) Close-up of the dome structure in a 96-h wild-type biofilm. On the far left is a side view. Cross-sectional views are shown on the right. Insets for two cross-sections are shown below with arrowheads marking the cells inside the domes. Bar, 50 μm.

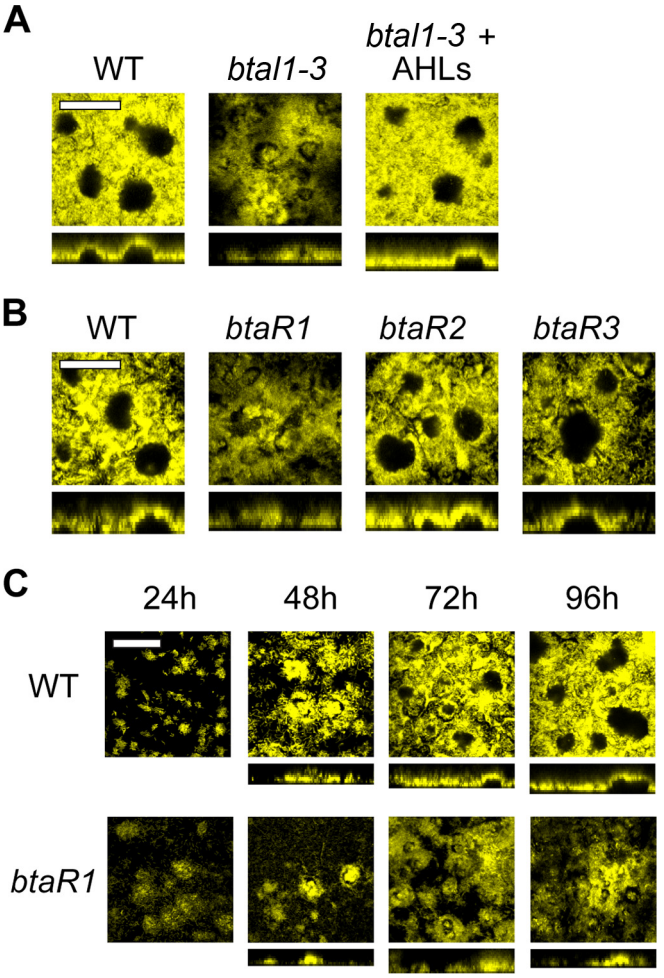

FIG 2 QS-1 is required for proper biofilm formation. (A and B) Representative images of 96-h YFP-expressing *B. thailandensis* biofilms. WT, Wild type; + AHLs, exogenous addition of AHLs. The genotypes of isogenic mutants are indicated. The *btaI1 btaI2 btaI3* mutant (*btaI1-3*) cannot produce AHLs; the *btaR1*, *btaR2*, and *btaR3* mutants lack QS-1, QS-2, and QS-3, respectively. (C) Time course of biofilm development for YFP-expressing WT and *btaR1* mutant strains. For the images of the 72-h and 96-h *btaR1* biofilms and of the 96-h *btaI1 btaI2 btaI3* biofilms but not for earlier time points, the contrast has been enhanced to increase the visibility of the biomass. Bars, 50 μm.

dome structure (Fig. 1A; see also Fig. S1 in the supplemental material). Bacterial cells were observed around the dome structures, but the interior was largely free of bacteria. Close inspection showed that these regions contained a few bacterial cells (Fig. 1B). Interestingly, the bacteria within these domes were immobilized, suggesting that the domes contain a viscous matrix material.

QS has been shown to influence biofilm formation for multiple bacterial species (7, 18–21). Previous studies showed that for *B. thailandensis* E264, QS promotes aggregate formation in liquid broth (3, 5), suggesting that QS might also be important for biofilm formation by this bacterium. We compared biofilms formed by a wild-type strain and an isogenic mutant strain that cannot produce AHLs (Δ*btaI1* Δ*btaI2* Δ*btaI3*). The biofilms of the AHL-negative strain lacked domes (Fig. 2A). When synthetic AHLs were exogenously added back to the growth medium 24 h postinoculation, after initial attachment had occurred, the AHL-negative strain formed biofilms with domes similar to those of the wild-type strain (Fig. 2A).

Following the observation that the AHL-negative strain was defective at wild-type biofilm formation, we asked which of the QS systems was required for wild-type *B. thailandensis* biofilm development. Since our previous data showed that addition of the individual AHLs leads to activation of multiple QS systems (5), we chose to analyze the individual *btaR* mutants instead of the *btaI* mutants to limit the effects of cross talk on our analysis. Biofilms of each individual *btaR* mutant (the *btaR1*, *btaR2*, and *btaR3* mutants) were compared with that of the wild type (Fig. 2B). The *btaR1* mutant biofilms were essentially identical to those of the AHL-negative strain (Fig. 2A and B; see also Fig. S1 in the supplemental material), while the biofilms formed by the *btaR2* and *btaR3* mutants closely resembled those of the wild-type strain, which were characterized by dome structures (Fig. 2B). Like the AHL-negative strain, the *btaR1* mutant produced a thick biofilm that lacked dome structures. In contrast to the reduced biomass observed in the biofilms of *B. cepacia* QS mutants (20, 22), *B. thailandensis btaR1* biofilms did not differ significantly from the wild type in total biomass ($n = 3$; $P > 0.1$). Together, these results show that QS-1 is required for proper biofilm formation in *B. thailandensis*.

Next, we conducted a time course of biofilm formation by the wild type and the *btaR1* mutant strain to gain insight into the timing of the QS-dependent biofilm phenotype. At 30-min postinoculation, there was no difference in surface attachment between the wild type and the *btaR1* mutant (see Fig. S2 in the sup-

plemental material), suggesting that QS does not play a critical role in the initial interactions of *B. thailandensis* E264 with the surface. At 24 h, small aggregates of the *btaR1* mutant were present on the surface. They appeared more diffuse than those observed in wild-type biofilms at the same time (Fig. 2C). By 48 h, the *btaR1* mutant strain produced larger aggregates than the wild type, yet these larger aggregates were less densely distributed on the surface (Fig. 2C). At 72 h and 96 h, the *btaR1* mutant biofilm, while increasing in biomass, did not produce any of the dome structures seen in the wild-type biofilm (Fig. 2C). In summary, while the *btaR1* mutant, like the AHL-negative strain, can produce a biofilm, biofilm development is altered in the *btaR1* mutant relative to that in the wild type, with the most pronounced effects occurring later in biofilm development.

Similar time course analyses were performed with the *btaR2* and *btaR3* mutants. As expected, unlike the *btaR1* mutant biofilm, the *btaR2* and *btaR3* mutant biofilms resembled wild-type biofilms at all time points (see Fig. S3 in the supplemental material). Our results, therefore, show that QS-2 and QS-3 are not required under the conditions of these experiments. However, the possibility that they play a subtle role in biofilm development that is not observable in these experiments cannot be completely ruled out.

To address the possibility that the *btaR1* mutant has an intrinsic growth or viability defect in the biofilm growth medium (FAB with 0.3 mM glucose), we measured planktonic bacterial growth in a similar medium (FAB with 30 mM glucose) for 72 h. In this assay, the *btaR1* mutant had no viability defect. In fact, the *btaR1* mutant accumulated to a higher cell density than the wild type at all time points tested (see Fig. S4 in the supplemental material). These results demonstrate that the *btaR1* mutant strain does not have an intrinsic viability or growth defect in the medium used in this study.

Our data show that for *B. thailandensis*, QS-1 plays an important role in normal biofilm development. While an AHL-negative strain and a QS-1 regulatory mutant formed biofilms with aggregates, these biofilms failed to develop wild-type biofilm morphology. Since QS represses motility-associated genes in *B. thailandensis* (3, 5, 23), and differences in motility affect biofilm architecture in other organisms (21), we considered the possibility that motility differences between the wild-type and QS mutant strains contribute to the altered biofilm phenotype of the QS mutants. To address this possibility for *B. thailandensis*, we looked to our chemical complementation experiments, where synthetic AHL signals were added back to the growth medium of the AHL synthesis mutant. We saw restoration of wild-type biofilm formation to the AHL synthesis mutant only when AHLs were added 24 h postinoculation, which is after the attachment phase of biofilm formation. Furthermore, not only did we not see a difference in attachment between the wild type and the *btaR1* mutant (Fig. S2 in the supplemental material), but the *btaR1* mutant biofilms also contained aggregates similar to those in the wild-type biofilms early in biofilm development (Fig. 2C, 24 and 48 h). Together, these data suggest that the defect of the *btaR1* mutant in mature biofilm formation is not due to differences in motility during early biofilm formation. It remains possible, however, that motility differences between the wild type and QS mutants play a role in later stages of biofilm development.

Furthermore, our data suggest that QS may not be active during the initial stages of biofilm formation and may actually be detrimental during the attachment phase of biofilm formation in *B. thailandensis*. While the addition of AHLs 24 h postinoculation rescued the dome-forming biofilm phenotype of a QS mutant (Fig. 2A), we failed to rescue the biofilm phenotype of the QS mutant when AHLs were added at the time of inoculation (data not shown). Furthermore, while wild-type cells did not exhibit decreased YFP fluorescence during biofilm formation, *btaR1* cells had lowered levels of YFP fluorescence after 48 h of biofilm growth (Fig. 2C). While we do not understand the regulation leading to this decrease in fluorescence, the time scale suggests that QS does not influence this phenotype until after the initial stages of biofilm formation.

A QS-controlled exopolysaccharide appears to contribute to biofilm formation. To gain insight into which QS-controlled factor or factors influence biofilm development in *B. thailandensis*, we considered known QS-1-controlled factors that have been suggested to play a role in biofilm formation (5). We showed previously that BtaR1 activates the genes for contact-dependent inhibition (CDI) (5). Since recent studies have shown that CDI genes contribute to cell aggregation (24) and static biofilm formation (25) in *B. thailandensis*, we tested the role of CDI in biofilm formation under flow conditions. In contrast to the role of CDI in static biofilm formation (25), we observed that a CDI mutant (*cdiAIB*) formed biofilms similar to those of the wild type under flow conditions (see Fig. S5 in the supplemental material).

Because biofilm formation is closely linked to exopolysaccharide production in many species, we examined the roles of four gene clusters suspected to contribute to exopolysaccharide production: CPSI, CPSII, CPSIII, and CPSIV. While CPSI, CPSII, and CPSIII mutants formed biofilms similar to those of the wild type, CPSIV biofilms were phenotypically distinct (Fig. 3). The mat of biomass at the biofilm base was thicker in the CPSIV mutant biofilm than in that of the wild type. Furthermore, CPSIV mutant biofilms contained cell-free dome structures that were smaller than those of the wild type and occurred less frequently. These results show that dome formation is impaired but not absent in the CPSIV mutant strain (Fig. 3). In conclusion, none of the four previously described CPS clusters were required for dome formation.

We showed previously that BtaR1 regulates genes in the biosynthetic operons of CPSI, CPSII, and CPSIII (5). While the previous results show that BtaR1 does not regulate genes involved in CPSIV biosynthesis (5), it is possible that BtaR1 does regulate these genes during biofilm formation under our growth conditions. Ultimately, each of the CPS mutants did form biofilms, suggesting that the exopolysaccharides encoded by these individual clusters are not essential for the production of biofilms. In other species, when one exopolysaccharide is absent from the biofilm matrix, other exopolysaccharides have been shown to compensate for it (26); thus, it is possible that multiple exopolysaccharides are involved in dome formation in *B. thailandensis* biofilm development and that mutation of multiple CPS loci would be necessary to abrogate the formation of these structures.

The QS-1 system regulates the production of a fucose-containing biofilm exopolysaccharide. In some cases, individual bacteria were found within the dome, but they did not swim freely (Fig. 1B). This led us to hypothesize that the domes contain a bacterially produced matrix. To investigate the material inside the domes, we probed the chemical nature of this matrix material, as a complementary approach to determining the BtaR1-controlled factor involved in biofilm formation. In many species, the biofilm matrix is composed of extracellular DNA, proteins, lipid vesicles, and exopolysaccharides. Therefore, we stained mature biofilms

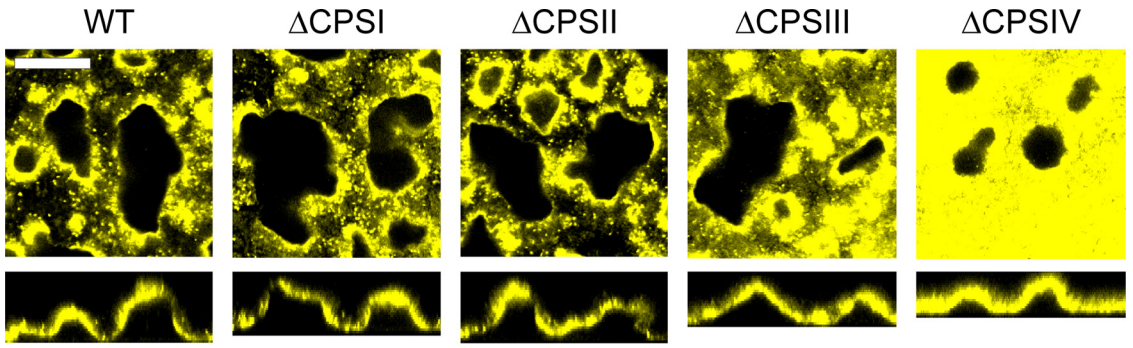

FIG 3 Dome formation is impaired but not absent in the ΔCPSIV mutant strain. Representative images of Syto9-stained 96-h biofilms of the wild type (WT) and of strains that cannot produce specific capsular polysaccharides (ΔCPSI, ΔCPSII, ΔCPSIII, and ΔCPSIV) are shown. Bar, 50 μm.

with a variety of probes that bind these components. First, we used Syto62, NanoOrange, or FM4-64 to test whether the matrix material inside the dome structures contained nucleic acids, proteins, or lipids, respectively. While the wild-type biofilm biomass surrounding the domes stained positive with these probes, the material inside the domes did not (Fig. 4).

We then tested whether the domes contain exopolysaccharides by using a panel of fluorescently labeled lectins, which are known to bind to specific sugar moieties. After testing a panel of 14 different lectins, we observed a few lectins that stained the biofilm biomass but did not stain the dome interior (Fig. 5). These were HHA (which binds to mannose), PNA (which binds to galactose), and RCA I (which binds to galactose and *N*-acetylgalactosamine). Interestingly, we found that one lectin, UEA (which binds to fucose), stained both the biomass and the space within the domes of wild-type biofilms (Fig. 5). While UEA failed to stain 24-h wild-type biofilms, this lectin brightly stained the aggregates in 48-h wild-type biofilms (see Fig. S6 in the supplemental material), suggesting that the production of this fucose-containing polysaccharide starts as larger aggregates form. Together, our results suggest that the *B. thailandensis* biofilm matrix contains exopolysaccharides with mannose, galactose, *N*-acetylgalactosamine, and fucose moieties and that the matrix material inside the domes consists of a fucose-containing exopolysaccharide.

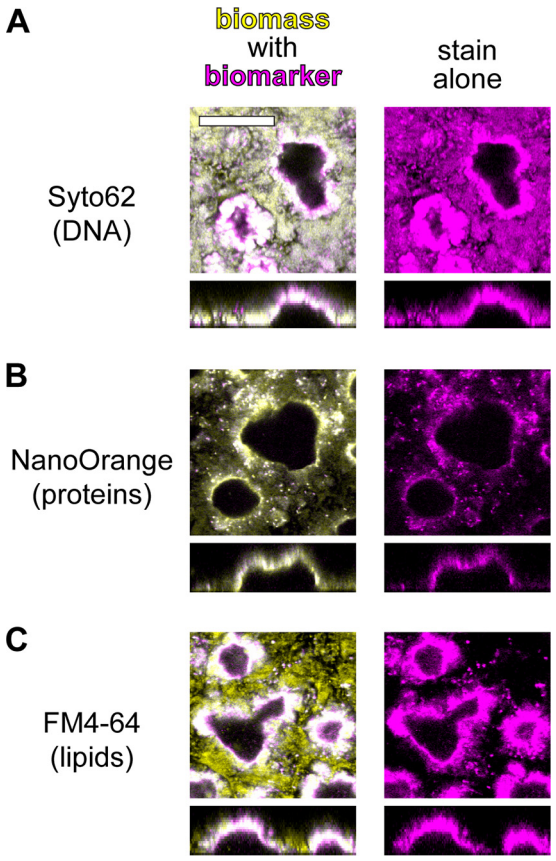

FIG 4 Dome structures of wild-type biofilms do not contain DNA, proteins, or lipids. Shown are representative images of 96-h YFP-expressing wild-type biofilms stained with various biomarkers: Syto62, for the visualization of DNA (A), NanoOrange, for the visualization of proteins (B), and FM4-64, for the visualization of lipids (C). Within each panel, a merged image of the biomass (yellow) and the biomarker (magenta) is shown on the left, and the biomarker alone is shown on the right. Bar, 50 μm.

Interestingly, we observe this fucose-containing exopolysaccharide at the same time that we start to observe the deviation in biofilm development for QS mutants. To further examine this correlation, we stained *btaR1* biofilms with the four lectins identified above. We found that *btaR1* mutant biofilms stained positively with three of the lectins: HHA, PNA, and RCA (Fig. 5). While the lectin-staining patterns of the wild type and the *btaR1* mutant differed, this difference is most likely due to the change in biofilm structure. In agreement with the role of the fucose-containing exopolysaccharide in dome production, the UEA lectin did not stain the 96-h *btaR1* mutant biofilm (Fig. 5). This result corroborates our results above showing that the UEA lectin stained inside the domes (Fig. 5) and that BtaR1 is required for the production and/or accumulation of this putative fucose-containing exopolysaccharide during biofilm development (Fig. 2B). Together, our results suggest that the QS-1 system regulates the production of a fucose-containing exopolysaccharide that plays a role in wild-type biofilm formation.

To determine which CPS cluster encodes this fucose-containing exopolysaccharide, we stained 96-h biofilms of the CPSI, CPSII, CPSIII, and CPSIV mutants with UEA. All four CPS mutants produced biofilms that stained positively with the UEA lectin (see Fig. S7 in the supplemental material). These results are consistent with the ability of the CPS mutants to form biofilms with dome structures. There are two possible explanations for why the deletion of the individual CPS clusters did not abrogate the UEA staining. First, it is possible that there is an additional CPS cluster

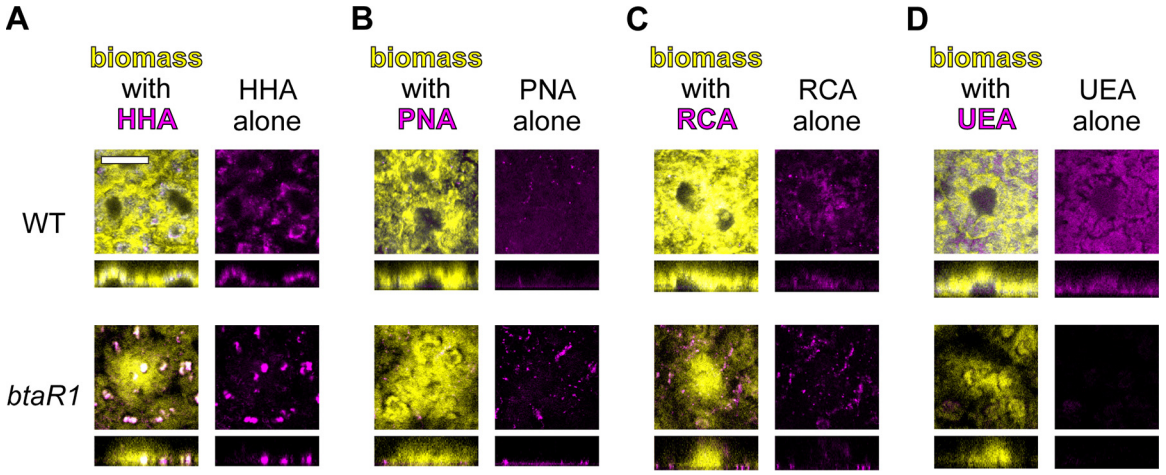

FIG 5 QS-1-deficient biofilms are not stained with a fucose-binding lectin. Shown are representative images of 96-h YFP-expressing biofilms stained with the following lectins to visualize matrix polysaccharides: HHA, which stains for mannose (A); PNA, which stains for galactose (B); RCA, which stains for galactose and *N*-acetylgalactosamine (C); and UEA, which stains for fucose (D). Within each panel, the wild-type (WT) biofilm is shown at the top and the *btaR1* mutant biofilm at the bottom. A merged image of the biomass (yellow) and the lectin (magenta) is shown on the left, and an image of the lectin alone is shown on the right. In the images of *btaR1* biofilms, the contrast has been enhanced for the biomass but not the lectin to increase visibility. Bar, 50 μm.

in *B. thailandensis* that has not been annotated and is responsible for the biosynthesis of the fucose-containing exopolysaccharide. Second, it is possible that the different exopolysaccharides share the same precursors, such that one CPS would integrate an activated sugar precursor that, under wild-type conditions, would be used in the biosynthesis of another CPS. In such a case, mutation of multiple CPS loci would be necessary to abrogate UEA staining.

QS promotes biofilm resilience under stress conditions. Following the observation that biofilm structure was impacted by QS, we hypothesized that QS provides additional benefits to the biofilm community. To test this idea, we asked whether there were differences between wild-type and *btaR1* biofilms after a shift in the nutritional environment. Specifically, we examined the effect of replacing the growth medium with phosphate-buffered saline (PBS) on the dispersal of mature biofilms. PBS was added to the system as a step gradient while a constant flow rate was maintained. After 48 h of PBS exposure, we observed less biomass in both wild-type and *btaR1* mutant biofilms than in biofilms that were not exposed to PBS. However, the shift in conditions had a more severe impact on *btaR1* biofilms, which lost 20.0% ± 3.9% more biomass than wild-type biofilms ($n = 3$; $P = 0.01$). These results suggest that QS-regulated functions contribute to the ability of biofilms to withstand a shift in the nutritional environment, which may not be too surprising, since QS has been linked to nutrient acquisition in a variety of species (27).

Although it is currently unclear which QS-regulated function(s) of *B. thailandensis* is responsible for the phenotype observed, there is one enticing possibility. Among other roles, matrix components have been suggested to serve as a source of nutrients under starvation conditions (28, 29). This led us to hypothesize that the material in the BtaR1-regulated domes may promote survival during adverse environmental conditions, such as starvation. To initially address this possibility, we examined changes in dome height as a proxy for a reduction in the matrix exopolysaccharide during PBS exposure. We observed a 30.4% ± 12.4% reduction in the dome heights of wild-type biofilms after the 48-h exposure period ($n = 3$; $P = 0.05$). While it is difficult at this point to determine whether the wild-type biofilms used the exopolysaccharide inside the dome structures as a source of nutrients, we speculate that the biofilm cells may use the material inside the dome structures during starvation. Of course, further studies are necessary to draw specific conclusions on the roles of QS and the dome structures in the adaptation of *B. thailandensis* biofilms to changes in the nutritional environment.

B. thailandensis is closely related to the highly pathogenic species *Burkholderia pseudomallei*. *B. thailandensis* and *B. pseudomallei* share conserved physiology, including nearly identical QS systems. In agreement with our results for *B. thailandensis*, QS-1 has been linked to biofilm formation in static biofilm assays of *B. pseudomallei* (11, 12). While the hypothesis still needs to be tested, we predict that QS-1 control of biofilm formation in *B. thailandensis* E264 has many elements that are conserved in other *B. thailandensis* strains and in *B. pseudomallei*. This work contributes to a better understanding of the role of QS in biofilm formation in this group of closely related *Burkholderia* species.

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