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 diffusable 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 (C₈-HSL) (2, 3). QS-2 comprises BtaI2-BtaR2 and N-3-hydroxy-decanoyl homoserine lactone (3OH-C₁₀-HSL) (2, 4), and QS-3 consists of BtaI3-BtaR3 and N-3-hydroxy-octanoyl homoserine lactone (3OH-C₈-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], capsule [CPSII], and CPSIII] [2, 3]).
CPSI, 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/mlampicillin (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) C6-HSL (Sigma Chemical Co.), 30HC6-HSL (14), and 30HC10-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 attTn7::P347-yfp ble), CM286 (∆btaI1 ∆btaI2 ∆btaI3 glmS1 attTn7::P347-yfp ble), CM265 (∆btaI1 glmS1 attTn7::P347-yfp ble), CM269 (∆btaI1 glmS1 attTn7::P347-yfp ble), CM284 (∆btaI1 glmS1 attTn7::P347-yfp ble), CM286 (∆btaI1 glmS1 attTn7::P347-yfp ble), CM288 (∆btaI1 glmS1 attTn7::P347-yfp ble), and CM269 (∆cdiAIB::tmp glmS1)

TABLE 1 Bacterial strains and plasmids used in this study

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<th>Bacterial strain or plasmid</th>
<th>Genotype or description</th>
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<tr>
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Plasmids

| pDONR221 P1-P5r         | Gateway-compatible vector with attP1 and attP5r recombination sites and ccdB; Km' Cm' | Invitrogen |
| pEX19EYFP               | Source for eYFP; Gm'                                                                 | 31         |
| pTNS2                   | RedI replicon; TnsABCD vector; Ap'                                                | 32         |
| pUC18T-mini-Tn7::Tp    | Mini-Tn7-based vector containing a trimethoprim resistance cassette; Tp' (GenBank accession no. DQ493875) | 33         |
| pCM112                  | pUC18T-mini-Tn7::ble-P54, Ap' Zeo*                                               | 14         |
| pCM249                  | pUC18T-mini-Tn7::ble-P54, Ap' Zeo*                                               | This study |
| pJRC115                 | Suicide plasmid containing pheS* counterselectable marker; Tp'                     | 3, 5       |
| pJRC115 bceI           | pJRC115 containing the ΔbceI deletion fragment; Tp'                               | This study |
| pJRC115 bceII           | pJRC115 containing the ΔbceII deletion fragment; Tp'                              | This study |
| pBT287                  | Gateway-compatible plasmid containing the T7 gene 10 leader sequence fused with eYFP, flanked by attR5 and attL recombination sites; Km' | This study |
| pBT409                  | pUC57-Kan-based plasmid containing the CPSI deletion fragment; Km' Tp'            | This study |
| pBT415                  | pUC57-Kan-based plasmid containing the CPSIV deletion fragment; Km' Tp'           | This study |
attN7::PcM249-yfp bcl1. PM249 was generated by introducing YFP into PCM112 (pUC18T-mini-Tn7T-bcl1). The YFP gene 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 ligated together using standard cloning methods. pBT287 was constructed by amplifying the yfp gene (encoding enhanced YFP [eYFP]) from pEX919EYFP with OBT335 and OBT342, and the PCR product was then recombined into pDONR221 P1-P5r by using BP Clonase II (Gateway technology; Invitrogen).

The ΔCPSI mutant (BTB21 [ΔBTH_I1325-1334::mpy]) and the ΔCPSIV mutant (BTB27 [ΔBTH_I1349-1362::mpy]) 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 (Geneviz) to create pBT409 and pBT415. The CPSI construct contains the first ~850 bp of BTH_I1324, followed by a tri-methoprim resistance cassette and then the last ~550 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_I11974-I11986::mpy]) 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 Bio-Labs). The assembled fragment was then amplified in a final PCR with DNA molecules together by Gibson product ligation (New England Bio-Labs). The assembled fragment was then recombined into pDONR221 P1-P5r using BP Clonase II (Gateway technology; Invitrogen).

The ΔCPSII mutant was constructed by delivering modified DNA to the *B. thailandensis* genome using homologous recombination with the pJRCl15 suicide delivery plasmid as described previously (3). First, we constructed suicide delivery plasmids to knock out each of the bce gene clusters. For bcel, we made a deletion from base +40 of BTH_I10543 to base +394 of BTH_I10552, with respect to the translational start site. For bcelI, we made a deletion spanning BTH_I10691 to BTH_I10695, including all but the last 42 bases of BTH_I10691 and all but the last 6 bases of BTH_I10695 (BTH_I10691 and BTH_I10695 face in opposite directions). Briefly, PCR-amplified fragments flanking the bcel (oligonucleotide bcel d1–4) and bcelI (oligonucleotide bcelI d1–4) gene clusters were generated using overlap extension PCR and were then cloned into the suicide delivery plasmid (3) using PCR-generated XbaI and HindIII restriction sites, creating pJRCl15 bcel and pJRCl15 bcelI. The deletion constructs were sequentially used to make a bcel bcelI 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; 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 bcel d5-7 and bcelI 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 (OD600) 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 using 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 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 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 1 (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 (SIA).

**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 decreasing 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 non-fluorescent zones within the aggregates. At later time points, these regions became more defined and increased in height, creating a
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 (H9004 btaI1 H9004 btaI2 H9004 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-
Quorum Sensing in *B. thailandensis* Biofilm Development

Quorum sensing (QS) plays a pivotal role in the development of *B. thailandensis* biofilms, as it can alter the initial interactions of the bacterium with the surface. At 24 h, small aggregates of the btaR1 mutant were present on the surface. These aggregates showed a more diffuse structure compared to 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, with these larger aggregates being 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 in later 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), 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 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 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 in later biofilm development.

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 bio-synthetic 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 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), 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 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.
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.

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 UEA (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...
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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