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**Pseudomonas aeruginosa-Derived Rhamnolipids and Other Detergents Modulate Colony Morphotype and Motility in the Burkholderia cepacia Complex**

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**ABSTRACT** Competitive interactions mediated by released chemicals (e.g., toxins) are prominent in multispecies communities, but the effects of these chemicals at subinhibitory concentrations on susceptible bacteria are poorly understood. Although *Pseudomonas aeruginosa* and species of the *Burkholderia cepacia* complex (Bcc) can exist together as a coinfection in cystic fibrosis airways, *P. aeruginosa* toxins can kill Bcc species in vitro. Consequently, these bacteria become an ideal in vitro model system to study the impact of sublethal levels of toxins on the biology of typical susceptible bacteria, such as the Bcc, when exposed to *P. aeruginosa* toxins. Using *P. aeruginosa* spent medium as a source of toxins, we showed that a small window of subinhibitory concentrations modulated the colony morphotype and swarming motility of some but not all tested Bcc strains, for which rhamnolipids were identified as the active molecule. Using a random transposon mutagenesis approach, we identified several genes required by the Bcc to respond to low concentrations of rhamnolipids and consequently affect the ability of this microbe to change its morphotype and swarm over surfaces. Among those genes identified were those coding for type IVb-Tad pili, which are often required for virulence in various bacterial pathogens. Our study demonstrates that manipulating chemical gradients in vitro can lead to the identification of bacterial behaviors relevant to polymicrobial infections.

**IMPORTANCE** Interspecies interactions can have profound effects on the development and outcomes of polymicrobial infections. Consequently, improving the molecular understanding of these interactions could provide us with new insights on the possible long-term consequences of these chronic infections. In this study, we show that *P. aeruginosa*-derived rhamnolipids, which participate in Bcc killing at high concentrations, can also trigger biological responses in *Burkholderia* spp. at low concentrations. The modulation of potential virulence phenotypes in the Bcc by *P. aeruginosa* suggests that these interactions contribute to pathogenesis and disease severity in the context of polymicrobial infections.

**KEYWORDS** *Burkholderia*, *Pseudomonas aeruginosa*, polymicrobial interactions, rhamnolipids, swarming motility

*Microbes can produce a wealth of molecules that mediate many of the positive and negative interactions in polymicrobial communities (1–4). Microbe-derived toxins and antibiotics contribute to negative interactions (e.g., competition) in multispecies...*
communities (5, 6); however, they are likely present in concentration gradients (7). The biological role of sublethal concentrations of microbial toxins in polymicrobial infections is poorly understood but should not be neglected, since subinhibitory concentrations of antibiotics can act as environmental cues on susceptible cells (7–11).

In cystic fibrosis (CF) airways, *Pseudomonas aeruginosa* and species of the *Burkholderia cepacia* complex (Bcc) can coexist (12–14). Interestingly, the Bcc species *Burkholderia cenocepacia* and *Burkholderia multivorans* do not seem to physically interact or form mixed aggregates with *P. aeruginosa* in late disease stage (prior to lung transplantation) (14). Consequently, these in vivo observations strongly suggest that interactions in coinfect ed lungs between *P. aeruginosa* and the Bcc would mainly occur at a distance via diffusible chemicals. Given that *P. aeruginosa* releases small toxic molecules that can kill Bcc species in vitro (15, 16), these bacterial species have become ideal model systems to study the impact of sublethal concentrations of microbe-derived toxins on the biology of susceptible bacteria in the context of polymicrobial communities.

In this study, we took advantage of the toxicity of *P. aeruginosa* (strain PA14) spent medium and the concept of chemical gradients to investigate whether subinhibitory concentrations of *Pseudomonas* spent medium (i.e., the secreted antimicrobial compounds) would modulate the biology of *Burkholderia* species. Our genetic and biochemical results suggest that a small concentration range of rhamnolipids from *P. aeruginosa* modulates Bcc phenotypes, including colony morphotype and swarming motility. Interestingly, these surface-associated phenotypes were not restricted to rhamnolipids but could be recapitulated with other surfactant-like molecules, including surfactin, sodium dodecyl sulfate (SDS), and Triton X-100. Random transposon mutagenesis in two responsive Bcc strains showed that surfactant-associated phenotypes could be blocked or impaired upon exposure to rhamnolipids, demonstrating possible overlapping genetic pathways between colony morphotype and swarming motility. Among the genes identified and required by responsive Bcc strains to exhibit surface-associated phenotypes are those encoding type IV pili belonging to the Flp superfamily and those sharing high genetic homology with type IVb-Tad pili of *P. aeruginosa*, which are different from those involved in twitching motility (type IVa pili) (17). Our findings demonstrate that rhamnolipids, which participate in Bcc killing at high concentrations (15, 16), can also trigger biological responses in *Burkholderia* spp. at low concentrations. The modulation of potential virulence phenotypes of Bcc by *P. aeruginosa* suggests that these interactions contribute to pathogenesis and disease severity in the context of polymicrobial infections.

**RESULTS**

**Exposure to low concentrations of *P. aeruginosa* spent medium leads to a colony morphotype change in *B. cenocepacia* strain K56-2.** To investigate whether *P. aeruginosa*-secreted molecules could trigger biological responses by *Burkholderia* spp., we monitored morphological changes of *B. cenocepacia* strain K56-2 upon growth on agar containing small amounts (0 to 10% [vol/vol]) of *P. aeruginosa* PA14 spent medium. Growth of *B. cenocepacia* on medium containing *P. aeruginosa* spent medium at 1% (vol/vol) consistently led to a morphological change characterized by colonies that were larger in size, flat, and spread out compared to the wild type (WT) (Fig. 1). However, these changes were not observed with supernatant at 5 or 10% (Fig. 1). Growth inhibition of *B. cenocepacia* K56-2 as an explanation for the lack of morphological change is unlikely, as demonstrated by the sizes of the colonies with spent medium (5 or 10%) being similar to those in agar without *P. aeruginosa* spent medium (Fig. 1) and by the minimal growth effect in liquid cultures (see Fig. S1 in the supplemental material) (15). Together, these data strongly suggest that the response is dose dependent and that growth inhibition alone is not sufficient to explain the lack of colony differentiation at higher concentrations of *Pseudomonas* spent medium.

Change in morphotype was not due to inheritable genetic mutations, since regrowth of differentiated colonies on agar lacking *Pseudomonas* spent medium led to
WT-looking colonies (data not shown). Furthermore, nonresponding colonies initially exposed to concentrations of supernatant not impacting colony size (i.e., ≥5%) could subsequently exhibit the described morphological change when grown on agar containing inducing concentrations of *Pseudomonas* spent medium (0.5 to 1%). Together, these data demonstrate that the colony morphology change of *B. cenocepacia* K56-2 is due to *P. aeruginosa* supernatant and not to genetic changes.

The described response by *B. cenocepacia* K56-2 was specific to *P. aeruginosa* metabolites, since the addition of supernatants to agar medium (1% [vol/vol]) from other bacterial species, like *Staphylococcus aureus* (strain RN6390), *Stenotrophomonas maltophilia* (strain K279a), *Escherichia coli* K-12 (strain MG1655), or *B. cenocepacia* K56-2, failed to produce larger colonies (data not shown). Altogether, these results demonstrate that *P. aeruginosa* metabolites modulate a reversible colony morphotype in *B. cenocepacia* K56-2, demonstrating the proof-of-concept that new biological functions or cryptic phenotypes can be uncovered when studying interspecies interactions.

**Rhamnolipids Modulate Burkholderia Phenotypes**

*Rhamnolipids from P. aeruginosa induce a colony morphological change in some strains of B. multivorans, B. cenocepacia, and Burkholderia dolosa.* All three *P. aeruginosa* quorum-sensing (QS) systems (i.e., Las, Rhl, and PQS) were individually shown to be required for effective killing of Bcc species (15, 16). To determine whether these regulatory networks could also be involved in the colony morphology change of *B. cenocepacia* K56-2, spent medium from single isogenic mutants with disruption in each QS system (*lasR*, *rhlR*, and *pqsA* mutants) were tested for their ability to induce morphological changes in *B. cenocepacia* K56-2. Supernatants at 1% from both the Δ*lasR* and Δ*rhlR* mutants were unable to induce a colony morphology change (Fig. 2A), while the Δ*pqsA* mutant spent medium behaved like its WT *P. aeruginosa* PA14 parent (data not shown), suggesting that the active molecule was positively regulated by both LasR and RhlR but not PqsA.

We next evaluated the bioactivity of spent medium lacking QS-regulated toxins active against the Bcc (15, 16). Supernatants were extracted from 24-h cultures of isogenic mutants with disruption in biosynthesis pathways for the production of phenazines (Δ*phz* mutant), rhamnolipids (Δ*rhlA* mutant), or hydrogen cyanide (Δ*hcnABC* mutant). Spent medium from the Δ*rhlA* mutant was unable to induce the described morphotype (Fig. 2B), while supernatants from the Δ*hcnABC* or Δ*phz* mutant still impacted colony morphology (data not shown). These genetic results strongly suggested that rhamnolipids could be the metabolite modulating the morphological change in colonies of *B. cenocepacia* K56-2. To confirm our genetic studies, we tested purified di-rhamnolipids obtained from planktonic cultures of *P. aeruginosa* PA14, and they recapitulated the morphological change of *B. cenocepacia* K56-2 observed with spent medium (Fig. 2B). Although the *rhlR* and Δ*rhlA* mutants had no detectable rhamnolipids (Fig. 2C) and consequently no effect on the morphology of *B. cenocepacia* colonies (Fig. 2A), the Δ*lasR* mutant spent medium was inactive (Fig. 2A) but had detectable levels of rhamnolipids (Fig. 2C). The production of intermediate levels of rhamnolipids by the Δ*lasR* mutant suggested that higher concentrations of spent medium were required to modulate the morphological change that would normally be
inhibitory with WT spent medium. In fact, the addition of spent medium at 5% from the ΔlasR mutant was sufficient to induce the colony morphology change (Fig. 2D). These results corroborated findings from a previous report showing that loss-of-function rhlR and lasR mutants from host-adapted CF isolates produced small and intermediate amounts of rhamnolipids, respectively (18). In agreement with our genetic and biochemical results, active spent media from the ΔpqsA, Δphz, and ΔhcnABC mutants correlated with the presence of rhamnolipids (Fig. 2C).

In addition, the described morphological response by B. cenocepacia was not unique to strain K56-2, as 12 of 21 other strains, representing three of the most prevalent Bcc species in the CF population (i.e., Burkholderia multivorans, B. cenocepacia, and Burkholderia dolosa) (19), responded to P. aeruginosa spent medium or purified rhamnolipids (extracted from planktonic cultures of P. aeruginosa PA14, as described in Materials and Methods) with a similar change in morphology (Table 1). The 13 Bcc strains responding to P. aeruginosa-derived rhamnolipids had subtle differences in terms of morphotypes, and those exhibited by B. dolosa (Fig. 3) were visually different than those of B. cenocepacia K56-2 (Fig. 1 and 2). In addition, high concentrations of rhamnolipids had an inhibitory effect on the change in colony morphology for B. dolosa (Fig. 3B), which was consistent with the effects of greater amounts of spent medium on the morphology of B. cenocepacia K56-2 (Fig. 1). Together, our genetic and biochemical results demonstrate that P. aeruginosa-derived rhamnolipids specifically modulate similar morphotypes in some but not all tested Bcc strains.

Pseudomonas-derived rhamnolipids induce motility in responsive Burkholderia strains. A key function of rhamnolipids in the biology of P. aeruginosa is the reduction of surface tension, allowing surface motility, commonly referred to as swarming motility (20–22). B. cenocepacia K56-2 can swarm over semiliquid surfaces (23–25), but previous observations suggested that B. cenocepacia K56-2 could not swarm on minimal medium (S. P. Bernier and P. A. Sokol, unpublished data).

Since rhamnolipids and supernatant-containing rhamnolipids had a profound effect on Bcc morphotypes (Fig. 1 and 3 and Table 1), we tested whether rhamnolipids could also impact the ability of Burkholderia spp. to swarm in a similar manner. We first evaluated the ability of a few representative Bcc strains to swarm under typical permissive (nutrient broth plus 0.5% glucose [NBG]) and nonpermissive (M9 plus 0.5% Casamino Acids [M9cas]) swarming conditions in the presence or absence of WT
In the absence of *P. aeruginosa* spent medium, all tested Bcc strains, with the exception of *B. cenocepacia* strain H111 (26), failed to move out from the point of inoculation on M9CAA, while all strains exhibited swarming motility with the addition of 1% *P. aeruginosa* spent medium, albeit to different degrees (Table S3). Under permissive swarming conditions (i.e., with NBG), most strains had a greater zone of swarming when exposed to *P. aeruginosa* spent medium, suggesting

**TABLE 1** Morphotype change in *B. multivorans*, *B. cenocepacia*, and *B. dolosa*

<table>
<thead>
<tr>
<th>Bcc species</th>
<th>Strain</th>
<th>Colony morphotype change&lt;sup&gt;a&lt;/sup&gt; on:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT medium</td>
</tr>
<tr>
<td><em>B. multivorans</em></td>
<td>C1576</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C5393</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CF-A1-1</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>LMG 13010</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>ATCC 17616</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>249-2</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>JTC</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>C1962</td>
<td>+</td>
</tr>
<tr>
<td><em>B. cenocepacia</em></td>
<td>K56-2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>J2315</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>BC7</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>C5424</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>PC184</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>H111</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>HI2424</td>
<td>−</td>
</tr>
<tr>
<td></td>
<td>ATCC 17765</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>CEP511</td>
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<td>−</td>
</tr>
<tr>
<td></td>
<td>C6433</td>
<td>−</td>
</tr>
<tr>
<td><em>B. dolosa</em></td>
<td>LO6</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>LMG 18943</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>PC543</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup>Colony morphology change upon growth on LB agar containing 1% (vol/vol) spent medium from *P. aeruginosa* PA14 (WT) or the ΔrhlA mutant or 2 μg·mL<sup>−1</sup> purified di-rhamnolipids (RLs). +, change in morphotype; −, no colony morphology change; ND, not determined.

*P. aeruginosa* PA14 spent medium. In the absence of *P. aeruginosa* spent medium, all tested Bcc strains, with the exception of *B. cenocepacia* strain H111 (26), failed to move out from the point of inoculation on M9CAA, while all strains exhibited swarming motility with the addition of 1% *P. aeruginosa* spent medium, albeit to different degrees (Table S3). Under permissive swarming conditions (i.e., with NBG), most strains had a greater zone of swarming when exposed to *P. aeruginosa* spent medium, suggesting

**FIG 3** Modulation of *B. dolosa* colony morphotype by *P. aeruginosa*-derived rhamnolipids. Colony morphotypes of different *B. dolosa* strains on LB agar medium containing 1% spent medium extracted from planktonic cultures of *P. aeruginosa* PA14 (A) or increasing concentrations of purified di-rhamnolipids (B). Insets in panel A show the entire agar plates, for which close-up pictures are provided to better distinguish changes in colony morphotype.
that the activity was maintained under both conditions (Table S3). The ability to swarm on M9CAA agar was due to the presence of rhamnolipids, since ΔrhlA mutant spent medium did not allow Bcc strains to swarm (Fig. 4A and S2A), while purified di-rhamnolipids recapitulated the swarming phenotype observed with supernatant-containing rhamnolipids (Fig. 4A). As was observed for the colony morphotypes (Fig. 1 and 3), high concentrations of spent medium or purified rhamnolipids had an inhibitory effect on swarming motility (Fig. 4A, S2B, and S3). Although swarming motility on NBG occurred independently of P. aeruginosa spent medium for some Bcc strains (Table S3), the addition of supernatant from the ΔrhlA mutant did not have any impact on swarming, reconfirming that rhamnolipid is the active compound in WT PA14 spent medium (Fig. S3). Interestingly, swimming motility was also affected in a fashion similar to that of swarming by the presence of rhamnolipids (Fig. 4B and S4). Together, these results demonstrate that Pseudomonas-derived rhamnolipids modulate the motility of Bcc species in a manner similar to the described morphological change.

**Structurally different surfactants and detergents modulate behaviors of Burkholderia in a manner similar to Pseudomonas-derived rhamnolipids.** To determine whether surfactants structurally different from P. aeruginosa-derived rhamnolipids would have similar effects on swarming motility and colony morphotype of Burkholderia, we evaluated a number of other detergents; these were the commercial detergents Triton X-100 and SDS and the biologically derived surfactant surfactin from *B. dolosa*.
Bacillus subtilis (27), as well as a supernatant from A549 lung epithelial cells known to produce surfactant (28).

The addition of Triton X-100, SDS, surfactin, and A549 supernatant to M9CAA swarming agar allowed B. dolosa PC453 to move across surfaces in a manner similar to P. aeruginosa-derived rhamnolipids (Fig. 5). Furthermore, as with rhamnolipids, increased motility was observed only in a small window of concentrations, which was particularly true for both Triton X-100 and SDS, with maximal swarming motility occurring between 0.001 and 0.0025% (Fig. 5A). In addition, changes in colony morphology upon exposure to SDS, Triton X-100, and surfactin at concentrations modulating swarming motility were similar to those observed with rhamnolipids or P. aeruginosa spent medium (Fig. 5C and S5). Altogether, these results demonstrate that surfactants of different structures can modulate biological responses, such as swarming motility and colony morphology change in responsive Burkholderia strains in a fashion similar to P. aeruginosa-derived rhamnolipids.

**Colony morphotype change does not alter antibiotic resistance.** Several studies have demonstrated that bacterial cells exposed to molecules, such as ammonia, trimethylamine, indole, hydrogen sulfide, polyamines, and even rhamnolipids, exhibited an altered resistance profile to antibiotics compared to nonexposed bacteria (7, 29–34). To determine whether surfactants at concentrations modulating surface-associated phenotypes could also modulate antibiotic resistance in the Bcc, we assessed the resistance profiles of seven strains responsive to rhamnolipids (Table 1 and Fig. S2) representing B. dolosa (PC543, LMG 18943, and LO6), B. multivorans (C1576 and CF-A1-1), and B. cenocepacia (K56-2 and H111) to 24 antibiotics on agar with or without surfactant using the disk diffusion assay.

The addition of surfactants at concentrations modulating colony morphotype and motility via supernatant-containing rhamnolipids (1% [vol/vol] WT PA14 spent medium) or surfactin (0.25 μg · ml⁻¹) led to small but not significant sensitivity changes to all tested antibiotics compared to those in agar without surfactant for all seven Bcc strains (Table S4). Consequently, these results suggest that rhamnolipids in the presence of...
other P. aeruginosa metabolites (i.e., spent medium) or purified surfactin do not modify antibiotic resistance of the Bcc in vitro at concentrations affecting morphotype and motility.

**Identification of Burkholderia genes required for responding to Pseudomonas-derived rhamnolipids.** To determine whether morphotype change and modulation of swarming motility in response to rhamnolipids shared overlapping pathways, we performed random transposon mutagenesis. Our genetic screen was designed to select for Burkholderia mutants that could no longer differentiate morphologically on agar containing 1% spent medium from WT P. aeruginosa PA14. We initially mutagenized the responsive B. dolosa PC543 strain and screened approximately 16,000 transposon mutants, of which a total of 34 mutants were consistently unable to differentiate morphologically like their WT parent (Table 2).

The insertion of multiple transposons into the same gene(s) and/or within the same genetic clusters indicates that the transposon screen was close to saturating. Our

### Table 2: Identified genes required for colony morphology change in B. dolosa PC543

<table>
<thead>
<tr>
<th>Strain or mutant no.*</th>
<th>Morphotype change with other surfactantsb</th>
<th>Swarmingc</th>
<th>Tn insertion site (BDSB locus tag)‡</th>
<th>Gene and/or function(s)</th>
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</thead>
<tbody>
<tr>
<td>WT PC543</td>
<td>+</td>
<td>++</td>
<td></td>
<td>aapZ, aquaporin Z</td>
</tr>
<tr>
<td>1 (1C6)</td>
<td>–</td>
<td>–</td>
<td>RSO0420</td>
<td>Two-component response regulator</td>
</tr>
<tr>
<td>2 (1E2)</td>
<td>–</td>
<td>–</td>
<td>RSO0420</td>
<td>Two-component response regulator</td>
</tr>
<tr>
<td>3 (1G12)</td>
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<td>+</td>
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<td>Two-component response regulator</td>
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<td>4 (2C4)</td>
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<td>Two-component response regulator</td>
</tr>
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<td>8 (2B12)*</td>
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<td>RSO0420</td>
<td>Two-component response regulator</td>
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</tr>
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<td>30 (1D2)</td>
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<td>+</td>
<td>RSO0420</td>
<td>Two-component response regulator</td>
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<td>–</td>
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<td>+</td>
<td>RSO0420</td>
<td>Two-component response regulator</td>
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</table>

*The original transposon number is given in parentheses.

*Colony morphotype change on agar containing other surfactants, such as SDS (0.002%), Triton X-100 (0.0025%), or surfactin (0.5 µg · ml⁻¹). +, change in morphotype; –, no colony morphology change.

**Swarming motility after 48 h on M9CAA plus 1% spent medium from WT B. dolosa PC543 are reported as follows. Largest zones of swarming, such as that for the WT, are represented by +++. Intermediate zone sizes relative to the WT are represented by +, +, and +/–. No zone of swarming is represented by –.**

**Transposon (Tn) insertion mapping was analyzed using the genome sequences and annotations from B. dolosa PC543 (59) available in the Burkholderia Genome Database (58). Genes in parentheses represent the open reading frames likely affected by the transposon inserted in the intergenic region.**

**Mutants 7 and 8 and mutants 24 and 25 had the same Tn insertion site and were obtained from the same mutagenesis reaction (i.e., they were clonal).**

**A homologous gene was also found to be essential for morphotype change with B. multivorans ATCC 17616 (see Table S2).**

**SAM, 6-adenosylmethionine.
screen identified genes involved in different bacterial functions and systems, such as signaling (a two-component regulator), gene regulation (sigma-54 regulator, mucB1, and rseI), transport (aqpZ and vacJ), and type IV pili (Table 2). A large number of transposon mutations affected genes coding for proteins or structures that are associated with the bacterial surface, such as the type VI secretion system, type IV pili (T4P), a porin (AqpZ), and the outer membrane components of an ABC transporter (VacJ). Among those identified genes, two putative T4P biosynthesis clusters were selected with some homology to T4aP (pilD mutant) and T4bP-Tad of P. aeruginosa (17). The latter cluster, T4bP-Tad, was highly represented, with 18 mutants selected within an 18.5-kb genetic locus containing 16 genes (17 mutants with unique insertions and 1 clonal mutant) (Fig. 6 and Table 2). Twelve of the 16 genes within that region show high homology to those coding for T4bP-Tad of P. aeruginosa (Fig. S6) (35) belonging to the Flp superfamily (36, 37). Supplemental evidence for the role of this genetic cluster in the colony morphotype is the selection of 8 transposon mutants (7 with unique insertions and 1 clonal) out of 13 in the same T4P cluster in the rhamnolipid-responsive B. multivorans ATCC 17616 strain (Fig. 6) through a second mutagenesis screen (~5,200 mutants) (Table S5). In addition, a mutant with an insertion in BDSB_RS09495 of B. dolosa PC543 failed to differentiate morphologically upon exposure to the P. aeruginosa supernatant, reconfirming the phenotype of the transposon mutants (mutants 22 and 23) (Fig. 6).

Although the transposon mutants were selected for their ability to respond to rhamnolipids, none of the 34 mutants of B. dolosa PC543 displayed an altered morphology upon exposure to SDS, Triton X-100, or surfactin (Table 2) as was observed for the WT parent. These data demonstrate the commonality among these genetic pathways involved in responding to a broad range of surfactants. In terms of motility, 18 mutants were unable to swarm on M9CAA containing 1% P. aeruginosa PA14 spent medium, while two mutants were not affected and 14 mutants had reduced zones of swarming motility compared to their WT parent (Table 2). Although not all mutants were negative for swarming, 32 out of 34 mutants were impaired in their ability to move away from the point of inoculation compared to the WT. Among the selected mutants, two distinct groups were unable to swarm in the presence of rhamnolipids: those with an insertion in the aquaporin gene aqpZ and those with an insertion in genes coding for type IV pili (T4P), with the exception of the mutant with an insertion in cpaE, also part of the T4P cluster (Table 2). This demonstrates the common genetic pathways shared between swarming motility and colony morphotype in response to rhamnolipids or other surfactants (surfactin, SDS, and Triton X-100).

Altogether, these results strongly suggest a central role for T4bP-Tad in the biology of Burkholderia in response to P. aeruginosa-derived rhamnolipids with respect to swarming motility and colony morphology.

Host-adapted P. aeruginosa CF isolates are diverse in their ability to modulate B. dolosa morphotype. It is well documented that P. aeruginosa establishes phenotypically and genetically diverse populations in CF airways (38–41). In LB cocultures, P. aeruginosa strains PA14 and PAO1 typically kill Bcc species; however, we recently showed that phenotypically diverse CF isolates from a single patient obtained over a period of 10 months were severely impaired in their ability to kill B. cenocepacia K56-2 (15).
Using the same 26 *P. aeruginosa* CF isolates previously tested for competition in cocultures (15), we determined whether diversity among those isolates could also be demonstrated by their ability to modulate morphotype changes in *B. dolosa* PC543. Spent medium from 24-h LB cultures was extracted from each of the *P. aeruginosa* CF isolates and added to agar medium at 1 and 5% (vol/vol) final concentrations. In comparison to PA14, where the optimal morphotype change of *B. dolosa* PC543 occurred at 1%, all possible outcomes were observed with the different tested *P. aeruginosa* CF isolates, with some leading to no response from *B. dolosa* PC543 at both concentrations, while others triggered optimal responses at either the 1 or 5% concentration (Fig. 7). At the population level, our data demonstrate the heterogeneity of *P. aeruginosa* and that subinhibitory levels of the spent medium allow *Burkholderia* spp. to respond as a function of rhamnolipid concentrations.

**DISCUSSION**

We have shown a new type of interspecies interaction between Bcc species and *P. aeruginosa* involving rhamnolipids. The functions of rhamnolipids in the biology of *P. aeruginosa* are pleiotropic and have been associated with virulence, immune modulation, antimicrobial activity, biofilm development, and surface motility (42). Herein, we show that some strains of *B. cenocepacia*, *B. dolosa*, and *B. multivorans* exhibit a colony morphotype change and increased motility (surfactant-associated phenotypes) upon exposure to small amounts of rhamnolipids or other surfactants (i.e., surfactin, SDS, and Triton X-100).

Whether surfactant-associated phenotypes occur *in vivo* remains an unanswered question, but the required concentrations of rhamnolipids to induce these traits in responsive Bcc strains (~1 to 5 μg·ml⁻¹) (Fig. 2 to 4) are physiologically relevant. In fact, Read et al. reported rhamnolipid concentrations in the CF lung reaching 65 μg·ml⁻¹ (43), while Kownatzki et al. noted much smaller amounts (up to 8 μg·ml⁻¹) (44). These differences could be reflective of the phenotypic heterogeneity of *P. aeruginosa* between patients (38, 40, 41) and/or the regional diversification of *P. aeruginosa* (38). The regional diversification possibility is of interest since rhamnolipid-mediated inter-
actions in coinfected areas would be dependent on two factors: (i) the ability of *Pseudomonas* isolates to produce rhamnolipids at the appropriate concentrations and (ii) the ability of *Burkholderia* strains/isolates to respond accordingly. Our data are consistent with these putative *in vivo* scenarios. More specifically, we showed that *P. aeruginosa* CF isolates from a single patient differently modulated the morphotype of *B. dolosa* PCS43 (Fig. 7) and that not all tested Bcc strains could respond to *P. aeruginosa*-derived rhamnolipids (Table 1). The selection of nonresponding mutants in *B. dolosa* PCS43 and *B. multivorans* ATCC 17616 demonstrated that genetic alterations could directly impair the ability of Bcc strains to differentiate morphologically in the presence of rhamnolipids (Table 2; see also Table S5 in the supplemental material). However, nonsynonymous mutations targeting genes identified by our genetics (Tables 2 and S5) were not enriched in populations of *B. dolosa* and *B. multivorans* in CF airways (45–47), suggesting that their biological functions are likely maintained in the lungs.

A determination of how rhamnolipids trigger surfactant-associated phenotypes in the Bcc was beyond the scope of this study. Rhamnolipids could induce an active biological response (e.g., transcriptional), establish physicochemical interactions with the bacterial membrane, or a combination of both. Previous studies showed that rhamnolipids reduced both lipopolysaccharides (LPS) and outer membrane proteins (OMPs) of *P. aeruginosa* in a concentration-dependent manner (48, 49). Removal of LPS occurred more specifically at concentrations above the critical micelle concentration (CMC) (48, 49), while OMPs, such as OprF, OprD, OprJ, and OprM, decreased only below the CMC (49). The typical CMC values for rhamnolipids are generally between 40 and 100 µg·ml⁻¹ (30, 50). Although these physicochemical interaction studies have not been performed on Bcc strains, surfactant-associated phenotypes occur at concentrations of ~1 to 5 µg·ml⁻¹, which are well below the typical CMC values for rhamnolipids. For example, a reduction in *P. aeruginosa* OMPs occurred at approximately 50 µg·ml⁻¹, which is 10 to 50 times more than the concentration range required to get surfactant-associated phenotypes. Whether rhamnolipids have a similar effect on OMPs of *Burkholderia* remains unknown. However, our genetics (Tables 2 and S5) strongly suggest that surface proteins or appendages may play a key role for *Burkholderia* spp. to exhibit surfactant-associated phenotypes upon exposure to low concentrations of rhamnolipids.

One of these identified surface structures were type IV pili (Fig. 6), more specifically, the T4bP-Tad belonging to the Flp superfamily (36, 37). Tad pili were genetically (flp-rcp-tad locus) discovered in *Agregatibacter* (previously *Actinobacillus*) *actinomyces-temcomitans* for their role in Flp pilus production, rough morphotype, autoaggregation, and biofilm formation (36, 37). Here, we show that the *flp-rcp-tad* locus is present in both *B. dolosa* and *B. multivorans* and required for surface-associated phenotypes in the presence of rhamnolipids. In agreement with our current findings, a *cpaC* deletion mutant of *B. cenocepacia* K56-2 was also impaired in swarming motility (24). In the host, the *flp-rcp-tad* locus of *B. cenocepacia* K56-2 was expressed in the lungs using a rat chronic airway infection model but at lower levels than at high-density *in vitro* cultures (51). Together, these studies demonstrate that the *flp-rcp-tad* locus is expressed in the host and functionally required for certain bacterial traits, such as motility, in at least three Bcc species. In the Bcc, the *tad* locus (*flp-rcp-tad*) has 12 conserved genes with *P. aeruginosa* (Fig. S5). Although the positive two-component regulatory PrpA-PrpB system of the *tad* locus in *P. aeruginosa* (52) is absent in the Bcc locus (Fig. S5), a BLASTP search of these proteins in *B. dolosa* PCS43 identified the BDSB_RS01490 (identity, 33.5%; E value, 4e⁻¹⁶) and BDSB_RS01495 (identity, 31.2%; E value, 3e⁻¹²) pair as possible homologues of PrpA and PrpB, respectively. BDSB_RS01495 was the best hit for PrpB, while BDSB_RS01490 was the eighth best hit for PrpA. Interestingly, our transposon screen in *B. dolosa* PCS43 selected BDSB_RS01490 (Table 2). Although the BDSB_RS01490 mutant was not completely negative for swarming motility compared to most T4bP-Tad mutants (Table 2), this does suggest based on homology with *P. aeruginosa* that this two-component system may positively regulate T4bP-Tad in the
Bcc. However, multiple layers of regulation are probably involved in the biogenesis of T4bp-Tad in the Bcc, since an alternative sigma-54 transcriptional regulator gene downstream of tadG is present in the Bcc, which was likely affected in mutant 6 of B. dolosa PCS43 (Table 2 and Fig. 6).

In conclusion, our study demonstrates that manipulating chemical gradients (7) can lead to the identification of in vitro bacterial behaviors of possible relevance in polymicrobial infections. Gradient concentrations of microbial toxins in CF airways could be the result of dilutions in tissue and mucus due to the spatial organization of each species within the community and/or the reduced toxicity of host-adapted P. aeruginosa (15). Rhamnolipids have antibiotic properties (30), but they are likely acting synergistically with other toxins rather than alone to kill the Bcc (15, 16). Consequently, dilution of other toxins from P. aeruginosa spent medium was essential for identifying the described interaction. This dilution approach was also true for another interaction that we recently described in which nonlethal concentrations of P. aeruginosa spent medium were protective for B. cenocepacia against toxic levels of cyanide (15). In the context of polymicrobial infections, surfactant-associated phenotypes would represent short-term bacterial traits exhibited in response to the surrounding environment rather than long-term phenotypes often associated with pathoadaptation. These interactions mediated by rhamnolipids affecting Bcc morphotype and motility could directly affect the overall virulence of the community. Interestingly, colony morphology change has previously been associated with Bcc pathogenesis (23, 53). Although the virulence aspect of surface-associated phenotypes was not evaluated in this study, hyperpiliated isolates of B. cenocepacia associated with modified colony phenotype were also more persistent in the lungs of mice than were less-piliated isolates (53). In addition to pilus modification in the production of exopolysaccharides may also impact morphotype in a fashion similar to alginate production in P. aeruginosa leading mucoid colonies. In fact, based on homology to the negative regulators (MucA and MucB) of alginate biosynthesis (54), the selection of mucA and mucB mutants strongly suggests that modification in the production of a possibly unknown exopolysaccharide may also impact morphotype upon, at least, exposure to rhamnolipids (Table 2). The mucoidy morphotype, mediated by alginate production, plays a role in the protection of P. aeruginosa cells against host defense mechanisms and therefore persistence (54).

Since rhamnolipid concentrations affecting colony morphotype did not modify antibiotic resistance (Table S4), it would be interesting to determine whether exposure to surfactants would increase the tolerance of the Bcc to different host defense mechanisms. Understanding these possibilities would help elucidate mechanisms used by the Bcc to persist in the host within polymicrobial communities.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains and plasmids used in this study are described in Table S1 in the supplemental material. Bacterial strains were routinely grown in LB-Miller broth or on 1.5% LB-Miller agar (EMD Chemicals, Inc., Gibbstown, NJ) supplemented with antibiotics, when appropriate, and incubated at 37°C. Nutrient broth (NB; Becton, Dickinson and Company [BD], Sparks, MD) and M9 minimal medium (BD) containing 0.5% Casamino Acids (M9CAA; BD) were used for the motility assays. Antibiotics were added to the culture medium of E. coli, 80°C in 10% skim milk (BD). All chemicals used in this study were purchased from Sigma-Aldrich (St. Louis, MO).

Extraction of bacterial spent medium and rhamnolipids. Bacterial cells from overnight cultures (4 ml of LB medium in borosilicate glass test tubes) were spun down (2 min, 8,000 rpm) and washed once in phosphate-buffered saline (PBS). Flasks containing 25 ml of culture medium were inoculated with 80 µl of washed cells (1:300 dilution) and incubated for 24 h at 37°C with shaking (175 rpm). Bacterial cells were subsequently spun down, and the resulting supernatant was filtered using 0.2-µm-pore-size filters (Millipore) and stored at 4°C. Rhamnolipids were extracted as previously described (15) from 2 liters of P. aeruginosa PA14 grown in LB medium at 37°C with constant shaking (250 rpm) for 16 to 18 h. Di-rhamnolipids were resuspended in dimethyl sulfoxide (DMSO).

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Colony morphology assay. Bacterial cells from overnight cultures were pelleted for 2 min at 8,000 rpm and washed with PBS, and a final 10−6 dilution of plated-agar agar contained spent medium at a final concentration of 1, 5, or 10% (vol/vol). The plates were incubated at 37°C for 24 to 48 h.

Swarming and swimming motility assays. Assays were performed as previously described (23, 24), with a few modifications. Briefly, 2 μl of an overnight culture was spotted in the middle of swarm and swim plates, and plates were allowed to dry for 1 h at room temperature and incubated at 37°C for 24 to 48 h for the swarming assays and 24 to 48 h for the swimming assays. Motility plates were made of NB with 0.5% glucose (NBG) or M9CAA supplemented with agar at 0.5% (wt/vol) for swarm plates and 0.25% (wt/vol) for swim plates. Spent medium (0.5, 1, or 5%) or purified di-rhamnolipids (0.5, 0.75, 5, or 10 μg ml−1) were added to motility agar.

Detection of rhamnolipids. Synthesis of rhamnolipids by P. aeruginosa strains was visualized as previously described (15). Briefly, 3 μl of an overnight culture was spotted onto agar plates containing methylene blue and cetathrimethylammonium bromide (CTAB) and incubated for 24 h at 37°C and subsequently at 4°C for 24 h. The zone and size around the colony were indicative of rhamnolipids.

Bacterial growth with bacterial spent medium. Culture of B. cenocepacia K56-2 with P. aeruginosa spent medium was performed as previously described (15). Briefly, overnight cultures of B. cenocepacia K56-2 were diluted to an optical density at 600 nm (OD600) of 0.1 and added to 96-well plates at a final dilution of 1:200 (vol/vol). The final volume per well was 100 μl, including the amount of spent medium extracted from P. aeruginosa PA14. The inoculated plate was incubated at 37°C for 24 h with constant shaking in a plate reader (Synergy H1; BioTek Instruments, Inc., Winooski, VT), and OD600 measurements were taken every 30 min. To avoid evaporation, 65 μl of filtered mineral oil was added on top of the bacterial culture in each well.

Antibiotic resistance assay. Antibiotic sensitivity was performed using the disk diffusion assay. Briefly, the entire surface of LB agar plates was streaked with cotton swabs previously immersed into LB medium containing test bacteria that were diluted to an OD600 of 0.1 from an overnight culture.

Genetic manipulations. Plasmids constructed in this study were made using the homologous recombination in Saccharomyces cerevisiae, as previously described (55). Restriction enzymes and Phusion high-fidelity DNA polymerase were obtained from New England BioLabs (Ipswich, MA).

(i) Single-crossover insertional mutant in B. dolosa. The creation of an insertional mutant in B. dolosa was performed using the vector pMQ87Tp (Table S1). This vector was constructed from pMQ87 (55) by inserting the trimethoprim resistance cassette (dhfr) at the XhoI restriction site by homologous recombination in S. cerevisiae that was previously amplified from pSChraBout (56) using primers dhfr5’ and dhfr3’ (Table S2). For insertional mutants, an internal DNA fragment (~500 to 700 bp) from the gene to be mutated was PCR amplified from B. dolosa (strain PCS43) and subsequently cloned into pMQ87Tp (linearized with SmaI) via homologous recombination in S. cerevisiae. The resulting plasmid in yeast was isolated and transformed into E. coli DH5α cells, for which newly extracted plasmid was sequenced (MOBIG Lab, McMaster University, Hamilton, Ontario, Canada) using the universal primers M13F and M13R (Table S2) for verification.

(ii) Transposon mutant libraries in B. dolosa and B. multivorans. Random transposon mutagenesis was performed as previously described (23, 56). Briefly, plasmid pSChraBout (56) was conjugated into B. dolosa (strain PCS43) and B. multivorans (strain ATCC 17616) by triparental mating using pRK2013 as a mobilizing plasmid (57). Transconjugants were selected on Pseudomonas Isolation agar (PIA; BD) containing 100 μg · ml−1 Tp. The mutation of BDSB_RS09405 was carried out using the suicide vector pMQ87Tp-BDSB_RS09405 (Table S1) that was created using primers BDSB_RS09405-5L and BDSB_RS09405-3L (Table S2).

(iii) Screening of the transposon mutant libraries. Transposon mutant colonies isolated on selective agar plates were scraped off with 2 ml of LB broth (EMD Chemicals, Inc.) and diluted to OD600 of 1.0, and a final 10−7 dilution was plated on LB agar plates containing Tp and 1% (vol/vol) P. aeruginosa PA14 supernatant. Transposon mutants that were unable to display the morphological change characterized by larger and spread-out colonies were selected for further analyses.

(iv) Mapping of transposon insertion sites. Transposon insertion mapping was performed as previously described (23, 56). Briefly, genomic DNA was extracted from overnight cultures of selected transposon mutants by using the Promega Wizard genomic DNA purification kit (Promega, Madison, WI), digested with Nott or Xhol restriction enzymes (NEB), self-ligated using T4 DNA ligase (Rapid DNA ligation kit; Roche, Germany), and transformed into E. coli DH5α competent cells. Transformants were selected on LB agar plates containing 100 μg · ml−1 Tp. Subsequently, plasmids from these respective transformants were extracted and sequenced (MOBIG lab) using primer 824 (56). The sequences obtained were then compared using BLAST to sequences from Bcc genomes available at the Burkhoderia Genome Database (58).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00171-17.

SUPPLEMENTAL FILE 1, PDF file, 4.1 MB.
REFERENCES


