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Host Adaptation Predisposes *Pseudomonas aeruginosa* to Type VI Secretion System-Mediated Predation by the *Burkholderia cepacia* Complex

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SUMMARY

Pseudomonas aeruginosa and *Burkholderia cepacia* complex (Bcc) species are opportunistic lung pathogens of cystic fibrosis (CF) patients. While *P. aeruginosa* can initiate long-term infections in younger CF patients, Bcc infections only arise in teenagers and adults. Both *P. aeruginosa* and Bcc use type VI secretion systems (T6SSs) to mediate interbacterial competition. Here, we show *P. aeruginosa* isolates from teenage/adult CF patients, but not those from young CF patients, are outcompeted by the epidemic Bcc isolate *Burkholderia cenocepacia* strain AU1054 in a T6SS-dependent manner. The genomes of susceptible *P. aeruginosa* isolates harbor T6SS-abrogating mutations, the repair of which, in some cases, rendered the isolates resistant. Moreover, seven of eight Bcc strains outcompeted *P. aeruginosa* strains isolated from the same patients. Our findings suggest certain mutations that arise as *P. aeruginosa* adapts to the CF lung abrogate T6SS activity, making *P. aeruginosa* and its human host susceptible to potentially fatal Bcc superinfection.

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AUTHOR CONTRIBUTIONS

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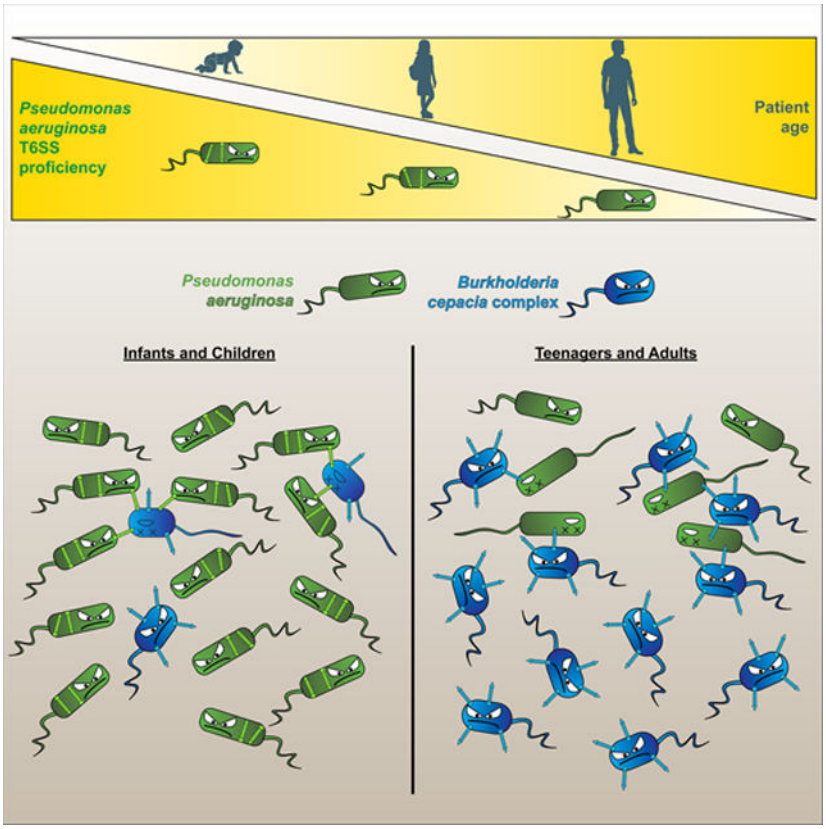
DECLARATION OF INTERESTS

The authors declare no competing interests.

Graphical Abstract



eTOC BLURB

Pseudomonas aeruginosa infects cystic fibrosis (CF) patients of all ages. *Burkholderia cepacia* complex (Bcc) infections are restricted to teenage/adult CF patients. Perault *et al.* demonstrate Bcc pathogens, using the type VI secretion system (T6SS), only outcompete host-adapted *P. aeruginosa* isolates, which become T6SS-deficient during evolution within the CF respiratory tract.

INTRODUCTION

The respiratory tracts of individuals suffering from cystic fibrosis (CF) are hospitable environments for microorganisms, and thus CF patients harbor complex, dynamic microbial communities in their airways that can include opportunistic pathogens (Carmody et al., 2015; Filkins and O'Toole, 2015; Lipuma, 2010; J. Zhao et al., 2012). *Pseudomonas aeruginosa* and certain members of the *Burkholderia cepacia* complex (Bcc), a taxonomic group containing at least 17 *Burkholderia* spp. (Salsgiver et al., 2016), cause devastating infections in CF patients, with Bcc pathogens often causing superinfections in *P. aeruginosa*-infected patients (Govan and Deretic, 1996; Lipuma, 2010; Mahenthiralingam et al., 2005). While *P. aeruginosa* infects young CF patients and is the most common opportunistic CF pathogen by early adulthood, Bcc infections are less common and, for unknown reasons, limited to older CF patients, typically teenagers and adults (Cystic Fibrosis Foundation, 2019). Unlike other CF pathogens, Bcc strains are more frequently associated with person-

to-person spread (Biddick et al., 2003; Chen et al., 2001; Govan et al., 1993) and can progress to a fatal necrotizing pneumonia and bacteremia termed “cepacia syndrome” (Isles et al., 1984; Lipuma, 2010). While *P. aeruginosa* and Bcc do not colocalize within the lungs of CF patients (*P. aeruginosa* is predominantly located in the airway lumen and Bcc within phagocytes), the *P. aeruginosa* burden in co-infected patients tends to be lower than in patients infected by *P. aeruginosa* alone (Schwab et al., 2014).

Interbacterial interactions likely occur within the polymicrobial CF respiratory tract and may influence disease progression (Bisht et al., 2020; Filkins and O’Toole, 2015; O’Brien and Fothergill, 2017; Peters et al., 2012). Interbacterial competition is hypothesized to be one of the strongest determinants of ecology and evolution within polymicrobial communities (Foster and Bell, 2012). A prevalent and well-understood mechanism of interbacterial competition is that mediated by type VI secretion systems (T6SSs) (Alteri and Mobley, 2016; Russell et al., 2014), which are predicted to be present in ~25% of Gram-negative bacteria (Boyer et al., 2009). T6SSs use a bacteriophage-like mechanism to deliver effector proteins directly into target bacterial or eukaryotic cells (Basler et al., 2012; Hachani et al., 2016; Hood et al., 2010; Pukatzki et al., 2007). Antibacterial T6SS effectors disrupt diverse biological processes within target cells, and cognate immunity proteins protect T6SS-producing cells from autotoxicity (Ahmad et al., 2019; Russell et al., 2014; Ting et al., 2018). Type VI secretion (T6S) has been studied in the Bcc pathogen *Burkholderia cenocepacia* strain J2315 (*Bc*J2315), which produces a T6SS that is important for infection of macrophages and influences the host immune response (Aubert et al., 2015; 2016; Hunt et al., 2004; Rosales-Reyes et al., 2012). Recent bioinformatic analysis has revealed T6SS-encoding genes throughout the Bcc, with one system (referred to as T6SS-1) predominating; however, several species encode multiple T6SSs (Spiewak et al., 2019). The *B. cenocepacia* strain H111 T6SS was shown to have modest antibacterial activity (Spiewak et al., 2019). Whether T6SSs in other Bcc pathogens have antibacterial activity is unknown.

P. aeruginosa produces three separate T6SSs (the H1-, H2-, and H3-T6SSs), and while both the H1- and H2-T6SSs are antibacterial weapons, the H1-T6SS is the stronger mediator of interbacterial competition (Allsopp et al., 2017; Hood et al., 2010; Russell et al., 2011). The *P. aeruginosa* H1-T6SS is under intricate regulation at both the post-transcriptional and post-translational level. Phosphorelay through the GacSA two-component system induces T6SS protein production via the regulatory small RNAs (sRNAs) RsmY and RsmZ, which relieve RsmA-mediated repression of translation of transcripts encoding T6SS proteins (Goodman et al., 2004; 2009; Lapouge et al., 2008; Moscoso et al., 2011; Ventre et al., 2006). Moreover, a threonine phosphorylation pathway regulates T6SS assembly and function via signal transduction through the membrane-associated TagQRST proteins, Fha1, and the kinase and phosphatase PpkA and PppA, respectively (Basler et al., 2013; Casabona et al., 2013; Hsu et al., 2009; Mougous et al., 2007).

P. aeruginosa undergoes dramatic evolution within the CF respiratory tract to transition to a chronic infection lifestyle (Folkesson et al., 2012; Winstanley et al., 2016), with mutations in evolved strains often occurring in *gacS*/*gacA* and T6SS structural genes (Bartell et al., 2019; Kordes et al., 2019; Marvig et al., 2015). Our characterization of T6S in the Bcc led us to hypothesize host adaptation by *P. aeruginosa* may open the door to subsequent Bcc

infections if the resident *P. aeruginosa* community loses T6SS activity. Here, we describe experiments conducted to test this hypothesis.

RESULTS

The *Bc*AU1054 T6SS mediates interbacterial competition

We selected *Burkholderia cenocepacia* strain AU1054 (*Bc*AU1054), which was isolated from the bloodstream of a CF patient, for our studies (Chen et al., 2001; Grigoriev et al., 2012). The *Bc*AU1054 genome encodes a predicted T6SS on chromosome 1 (BCEN_RS13060, *tssM*, through BCEN_RS13170, *tssL*) (Figure 1A). Presumably due to errors during the sequencing of this strain's genome (assembly GCA_000014085.1), multiple genes in this region were annotated as pseudogenes. We PCR amplified and sequenced these regions and found that each gene is actually intact (Table S1). Compared to the T6SS-encoding cluster of *B. cenocepacia* strain J2315 (*Bc*J2315), the *Bc*AU1054 T6SS gene cluster contains an additional region (BCEN_RS13075 (*tai1*) through BCEN_RS13110 (*vgrG1*)) that includes two predicted effector-immunity (E-I)-encoding gene pairs (*tae1-tai1* and *tle1-tli1*) (Figure S1). Immediately 3' to the *Bc*AU1054 core cluster (with the 5' to 3' direction corresponding to the sequence numbering in Figure 1A) is an additional region containing *vgrG2*, the predicted E-I-encoding pair *tne1-tni1*, and several genes predicted to be involved in phage or other mobile genetic elements.

To identify additional genes potentially encoding E-I pairs, we first analyzed genes located near the seven annotated *vgrG* genes (Figure 1A). VgrG proteins, along with PAAR-repeat proteins, form the puncturing tip of the T6SS needle and typically associate with effectors encoded by nearby genes (Pukatzki et al., 2007; Russell et al., 2014; Shneider et al., 2013). Based on previous nomenclature (Russell et al., 2014), we named predicted cell membrane-degrading effectors Tle for T6SS lipase effector, nucleic acid-degrading effectors Tne for T6SS nuclease effector, cell wall-degrading effectors Tae for T6SS amidase effector, and used Tpe for the T6SS pore-forming effector. We named cognate immunity proteins Tli, Tni, Tai, and Tpi. Three of the predicted E-I-encoding gene pairs (*tle1-tli1*, *tle3-tli3*, and *tpe1-tpi1*) are near ORFs encoding proteins with the domain of unknown function (DUF) 4123, which is a conserved chaperone domain for T6SS effectors (Liang et al., 2015) (Figure 1A). Protein secondary structure analysis using Phyre2 (Kelley et al., 2015) and HHpred (Zimmermann et al., 2018) predicted antibacterial enzymatic activities for all potential effectors (Table S2). A duplication of the 3' end of *tagL* flanking *tae1-tai1* (Figure 1A) suggests these predicted E-I-encoding genes inserted into the *Bc*AU1054 T6SS core cluster via a transposon, and Tae1 is predicted to have a glycosyl hydrolase domain. We identified the *tle4-tli4* gene pair by searching for DUFs shared among E-I-encoding genes, as DUF3304 is only present in the *Bc*AU1054 genome within *tli1*, *tli3*, and *tli4* (Table S2).

To determine if the *Bc*AU1054 T6SS mediates interbacterial competition, we generated an unmarked, in-frame deletion mutation in *hcp*, which encodes the inner tube protein of the T6S apparatus (the *Bc*AU1054 genome only has one *hcp* gene). Over 5 h coculture, *Bc*AU1054 outcompeted *Escherichia coli* DH5α by ~2 logs, whereas *Bc*AU1054 Δ*hcp* had no competitive advantage (Figure 1B). *Bc*AU1054 outcompeted another Bcc pathogen, *Burkholderia dolosa* strain AU0158 (*Bd*AU0158), by ~4 logs over 5 h, and *Bc*AU1054 Δ*hcp*

was outcompeted by *Bd*AU0158 by ~2 logs (Figure 1C). Ectopic expression of *hcp* from the genomic *att*Tn*7* site partially restored the ability of *Bc*AU1054 Δ*hcp* to outcompete *E. coli* DH5α and *Bd*AU0158 (Figures 1B and 1C). Growth rate differences did not determine competitive fitness as *Bc*AU1054 and *Bc*AU1054 Δ*hcp* had similar growth rates (Figure S2). The *Bc*AU1054 T6SS, therefore, is a potent weapon capable of killing competitor bacteria.

At least five *Bc*AU1054 T6SS effectors mediate interbacterial competition

To determine which predicted effectors are involved in T6SS-mediated interbacterial competition by *Bc*AU1054, we generated nine mutants, each containing an unmarked, in-frame deletion mutation in one of the predicted E-I-encoding gene pairs. We screened these mutants by engineering them to produce green fluorescent protein (GFP) and coculturing them, individually, with either wild-type (WT) or Δ*hcp Bc*AU1054 strains for ~20 h, and then measuring GFP fluorescence intensity and the OD_{600} of the cocultures. For four of the mutants (Δ*tle1*Δ*tli1*, Δ*tne1*Δ*tni1*, Δ*tne2*Δ*tni2*, and Δ*tpe1*Δ*tpi1*), the GFP/OD_{600} values for cocultures with WT *Bc*AU1054 were about half of what they were for cocultures with *Bc*AU1054 Δ*hcp*, indicating these mutants were outcompeted in a T6SS-dependent manner, presumably because they lack functional immunity proteins (Figure S3). We then cocultured each of these four mutants with WT and Δ*hcp Bc*AU1054 strains and measured competition quantitatively. In each case, the E-I deletion mutant was outcompeted by its parental strain in a T6SS-dependent manner (Figure 2A). Ectopic expression of the cognate immunity gene in each mutant rescued it from T6SS-mediated killing by the parental strain (Figure 2A), providing evidence that Tle1-Tli1, Tne1-Tni1, Tne2-Tni2, and Tpe1-Tpi1 are true antibacterial E-I pairs associated with the *Bc*AU1054 T6SS.

To determine if there are additional E-I-encoding gene pairs in *Bc*AU1054, we generated a mutant lacking all nine predicted E-I-encoding gene pairs (*Bc*AU1054 Δ9E-I) and assessed its ability to outcompete target bacteria. This nonuple mutant outcompeted *Bd*AU0158 by ~3.5 logs (slightly less than WT *Bc*AU1054) (Figure 2B), indicating at least one more effector delivered by the *Bc*AU1054 T6SS exists.

Susceptibility of *P. aeruginosa* CF isolates to the *Bc*AU1054 T6SS correlates with patient age

We next sought to determine whether the *Bc*AU1054 T6SS targets the prevalent CF pathogen *P. aeruginosa*. In cocultures with *P. aeruginosa* reference strain PAO1, PAO1 outcompeted both WT and Δ*hcp* strains of *Bc*AU1054, though showed a slightly (~0.5-log) greater ability to outcompete T6SS-active than T6SS-inactive *Bc*AU1054 (Figure 3A). These results are consistent with two theories on the regulation of H1-T6SS activity by PAO1: T6SS-dueling (Basler et al., 2013), in which the PAO1 H1-T6SS only deploys following antagonism by a neighboring cell, and the *P. aeruginosa* response to antagonism (PARA) (LeRoux et al., 2015), in which PAO1 activates aggressive behaviors, like T6SS activity, following detection of kin cell lysates. The ability of PAO1 to outcompete *Bc*AU1054 was dependent on the H1-T6SS, as a transposon insertion in *vipA1*, which encodes a protein necessary for H1-T6SS activity (Basler et al., 2013), caused PAO1 to be outcompeted by *Bc*AU1054 (Figure 3A).

PAO1 was originally isolated from a wound infection and has undergone decades of laboratory passage and diversification (Chandler et al., 2019; Holloway, 1955; Holloway and Morgan, 1986; Klockgether et al., 2010). To investigate T6SS-mediated interactions between *Bc*AU1054 and *P. aeruginosa* strains relevant to CF infection, we used collections of *P. aeruginosa* strains isolated from CF patients (Burns et al., 2001; Rosenfeld et al., 2001). *Bc*AU1054 did not outcompete any of the *P. aeruginosa* strains isolated from infants or young children (≤three years old) and was often slightly outcompeted by these strains (Figure 3B). By contrast, *Bc*AU1054 had the striking ability to outcompete nearly half of the *P. aeruginosa* strains isolated from teenagers and adults (11–31 years old) in a T6SS-dependent manner, oftentimes efficiently enough to prevent recovery of any *P. aeruginosa* from the cocultures (Figure 3D). We also determined if the nonuple E-I deletion mutant of *Bc*AU1054 could outcompete the susceptible *P. aeruginosa* strains. Although *Bc*AU1054 Δ9E-I was strongly outcompeted by PAO1, it retained a competitive advantage against C078C, C120C, C123D, and CEC118 (Figure S4). C120C was less susceptible to killing by *Bc*AU1054 Δ9E-I than were C078C, C123D, and CEC118 (Figure S4), suggesting C120C is less sensitive to the unidentified effector(s) associated with the *Bc*AU1054 T6SS, and *Bc*AU1054 T6SS effectors exhibit target strain-specific variability in toxicity. The ability of *Bc*AU1054 to kill *P. aeruginosa* from older CF patients correlates with the clinical presentation of Bcc infections, which arise only in teenagers and adults (Cystic Fibrosis Foundation, 2019).

Host-adapted *P. aeruginosa* isolates that are sensitive to the *Bc*AU1054 T6SS harbor T6SS-abrogating mutations

We sequenced the genomes of the *P. aeruginosa* clinical isolates used for the experiments described above. Of the four T6SS-susceptible isolates (C078C, C120C, C123D, and CEC118), three contain mutations in *gacS* or *gacA*, which encode a two-component system required for T6SS protein production (Table 1) (Goodman et al., 2004; Marden et al., 2013; Moscoso et al., 2011). C078C contains a missense mutation in *gacS* (*gacS*$_{G1715A}$), resulting in the variant protein GacS$_{G572D}$. C123D contains a genomic deletion spanning the *gacS* gene and the nearby *pirRSA* genes, which encode a siderophore iron-acquisition system (Ghysels et al., 2005). C120C contains a premature stop codon in *gacA* (*gacA*$_{C349T}$). The C078C genome also contains a premature stop codon in *pppA* (*pppA*$_{G111A}$). PppA is a post-translational regulator of H1-T6SS activity (Mougous et al., 2007), and is required for efficient T6SS-mediated competition (Basler et al., 2013). CEC118 has a small deletion in *fha1* (*fha1*$_{Δ404–424}$) resulting in the loss of seven amino acid residues from Fha1, another post-translational regulator of H1-T6SS activity (Mougous et al., 2007).

To investigate if the ability of *Bc*AU1054 to outcompete *P. aeruginosa* strains isolated from teenage/adult CF patients correlates with a loss of H1-T6SS activity in the *P. aeruginosa* strains, we assessed production of Hcp1, the major subunit protein of the H1-T6SS inner tube, during growth on agar. Every *P. aeruginosa* isolate that was outcompeted by the *Bc*AU1054 T6SS showed either negligible or diminished Hcp1 production compared to PAO1 (Figures 3D and 3E). CEC121, CEC122, and CEC116, which were not outcompeted by the *Bc*AU1054 T6SS, produced Hcp1 at levels similar to PAO1 (Figure 3E). Of the isolates producing diminished levels of Hcp1, two (C078C and CEC118) were strongly

outcompeted by *Bc*AU1054 and contain *pppA* or *fha1* mutations that likely abrogate T6SS activity independent of Hcp1 production. By contrast, mutations in genes encoding known post-translational T6SS regulators were not detected within CEC119 and CEC120, possibly explaining why these isolates were not strongly outcompeted by *Bc*AU1054. Every *P. aeruginosa* strain isolated from an infant or young child except CEC32 produced Hcp1 at or near levels similar to PAO1 (Figure 3C), which correlates with their resistance to being outcompeted by the *Bc*AU1054 T6SS (Figure 3B).

Restoration of H1-T6SS protein production can rescue host-adapted *P. aeruginosa* from T6SS-mediated elimination by *Bc*AU1054

Phosphorelay through the GacSA two-component system activates production of the sRNAs RsmY and RsmZ, which are required for T6SS protein production by *P. aeruginosa* (Goodman et al., 2009; 2004; Lapouge et al., 2008; Moscoso et al., 2011; Ventre et al., 2006). To determine if lack of *gacS*/*gacA* function is responsible for susceptibility of the *P. aeruginosa* strains with mutations in these genes, we introduced a plasmid (pJN-*rsmZ*) into C120C, C123D, and C078C to express *rsmZ* (induced by arabinose) independent of the GacSA phosphorelay (Intile et al., 2014; Janssen et al., 2018). The vector backbone (pJN105) served as a negative control. In competitions against *Bc*AU1054 on agar containing 0.1% arabinose, C120C pJN-*rsmZ* was rescued from T6SS-mediated elimination by *Bc*AU1054, while C120C pJN105 was not (Figure 4A). Consistent with this result, C120C pJN-*rsmZ*, but not C120C pJN105, produced Hcp1 when grown under inducing conditions (Figure 4D). pJN-*rsmZ* did not promote Hcp1 production in C123D or C078C (Figure 4D), and these strains were still strongly outcompeted by the *Bc*AU1054 T6SS (Figures 4B and 4C). C123D and C078C may harbor additional, unidentified mutations that render them T6SS-deficient, or the ectopic *rsmZ* strategy may simply not work in these strains. Because C078C also contains a premature stop codon in *pppA*, we delivered the WT *pppA* gene under control of a constitutive promoter to the *att*Tn*7* site, and also introduced pJN-*rsmZ*, into this strain, but expression of these genes failed to rescue C078C from T6SS-mediated elimination by *Bc*AU1054 (Figures 4E and 4F). Lastly, we delivered the WT *fha1* gene to the *att*Tn7 site of CEC118, as this isolate has a truncated *fha1*, but constitutive expression of full-length *fha1* did not rescue CEC118 from being outcompeted by *Bc*AU1054 (Figure 4G). It is not surprising that constitutive expression of full-length *pppA* and full-length *fha1* did not rescue C078C and CEC118, respectively, as both isolates were defective for Hcp1 production (Figure 3E). It is also possible the natively-produced truncated PppA and Fha1 variants act as dominant negatives in these strains.

Additional Bcc pathogens kill host-adapted *P. aeruginosa* in a T6SS-dependent manner

To investigate whether T6SS-mediated killing of host-adapted *P. aeruginosa* is a common feature of Bcc strains, we used *Burkholderia multivorans* strain CGD2M (*Bm*CGD2M) and *Bd*AU0158, which encode predicted T6SS-1 systems. *Bm*CGD2M and *Bd*AU0158 encode one and two additional predicted T6SSs, respectively. We generated plasmid disruption mutations in the *tssC1* genes of these strains' T6SS-1 clusters (*Bm*CGD2M *tssC1*::pAP82 and *Bd*AU0158 *tssC1*::pAP83), and competed these mutants and the parental strains against *P. aeruginosa* strains PAO1 and C078C. Both *Bm*CGD2M and *Bd*AU0158 strongly outcompeted C078C, but not PAO1, and they did so in a T6SS-dependent manner (Figure 5).

These data suggest T6S may provide many Bcc pathogens a competitive advantage against host-adapted *P. aeruginosa*.

B. cenocepacia isolates from CF patients with concurrent *P. aeruginosa* infections outcompete their paired *P. aeruginosa* isolates under T6SS-permissive conditions

Our data, together with data from other groups (Bartell et al., 2019; Kordes et al., 2019; Marvig et al., 2015), suggest T6SS-abrogating mutations can accumulate as *P. aeruginosa* evolves within the CF respiratory tract, and patients harboring T6SS-null *P. aeruginosa* may be susceptible to Bcc superinfections. To explore this hypothesis further, we acquired eight *B. cenocepacia-P. aeruginosa* co-infection pairs, each isolated from a separate concurrently-infected CF patient. During coculture on agar, seven of eight *B. cenocepacia* isolates outcompeted their paired *P. aeruginosa* isolate by as little as ~1 log (*Bc*AU7523 vs. *Pa*AU7618) or as great as ~4 logs (*Bc*AU29704 vs. *Pa*AU29744) (Figure 6A). Since genetic manipulation of recent human isolates is often not possible, we took advantage of the fact that growth in shaking liquid cultures is non-permissive for T6SS-mediated competition (Hood et al., 2010; Majerczyk et al., 2016; Russell et al., 2011; Speare et al., 2020), likely because cells are not in contact long enough to allow T6SS effector delivery to target cells. The competitive advantages of *B. cenocepacia* isolates over their paired *P. aeruginosa* isolates dropped dramatically during shaking liquid growth compared to growth on agar (Figure 6B). In two cases (*Pa*AU7618 and *Pa*AU19694), liquid growth provided *P. aeruginosa* a competitive advantage over its paired *B. cenocepacia* isolate (Figure 6B).

To identify potential genetic explanations for the competitive disadvantages of the *P. aeruginosa* co-infection isolates, we PCR-amplified the genes involved in H1-T6SS production/activity that are mutated in the *P. aeruginosa* strains isolated from adults for which we have whole-genome sequence information (Table 1) and sequenced these PCR products. Three isolates contain mutations in *gacS* or *gacA* (*Pa*AU7618 contains a $gacA_{G175A}$ mutation resulting in $GacA_{G59S}$, *Pa*AU19694 contains a $gacA_{C162A}$ mutation resulting in $GacA_{D54E}$, and *Pa*AU23781 contains a premature stop codon in *gacS* ($gacS_{G1568A}$)) (Table 1). *Pa*AU4391 contains a small deletion in *fha1* ($fha1_{\Delta405–425}$) that is nearly identical to the mutation in CEC118 ($fha1_{\Delta404–424}$) (Table 1). Western blotting showed negligible or diminished Hcp1 production by five *P. aeruginosa* co-infection isolates (*Pa*AU7618, *Pa*AU10617, *Pa*AU19694, *Pa*AU22775, and *Pa*AU23781) compared to PAO1 (Figure 6C). *Pa*AU4391, *Pa*AU5159, and *Pa*AU29744 produced Hcp1 at levels similar to PAO1 (Figure 6C), though *Pa*AU4391 has an $fha1_{\Delta405–425}$ mutation that may abrogate T6SS activity without affecting protein production. *Pa*AU5159 and *Pa*AU29744 may harbor mutations in other genes important for post-translational regulation of T6SS activity. Together, our results suggest Bcc pathogens may only be able to invade a *P. aeruginosa*-colonized CF respiratory tract if the *P. aeruginosa* population, or at least a subpopulation, has lost T6SS activity.

DISCUSSION

The underlying reasons for the propensity of Bcc pathogens to infect only older CF patients, and to cause superinfections in those colonized with *P. aeruginosa* (Folescu et al., 2015;

McCloskey et al., 2001; Whiteford et al., 1995) are unknown. During our investigation of T6S in the Bcc, we found that none of the *P. aeruginosa* strains isolated from infant or child CF patients, but almost half of the strains isolated from teenage and adult CF patients, were susceptible to T6SS-mediated killing by *Bc*AU1054. Additional Bcc pathogens (*Bm*CGD2M and *Bd*AU0158) also efficiently outcompeted susceptible *P. aeruginosa* strains via T6SS activity, and seven of eight *B. cenocepacia* strains from patients with concurrent *P. aeruginosa* infections outcompeted their paired *P. aeruginosa* strains under conditions promoting T6SS-mediated interactions. These data suggest that one reason Bcc pathogens are restricted to infecting older CF patients is because only in these patients are resident *P. aeruginosa* susceptible to T6SS-mediated competition by Bcc bacteria.

We found that differential susceptibility of *P. aeruginosa* strains to T6SS-mediated competition by Bcc pathogens depends on T6SS functionality in *P. aeruginosa*. Disruption of *vipA1* to inactivate the H1-T6SS converted PAO1 from being resistant to T6SS-mediated competition by *Bc*AU1054 to being outcompeted by four logs. We found that all of the susceptible *P. aeruginosa* strains isolated from teenagers or adults harbor mutations predicted to abrogate production and/or function of their T6SSs, all failed to produce substantial amounts of Hcp1, and for one strain, elimination by *Bc*AU1054 was prevented by activating production of its T6SS proteins. Consistent with these observations, *B. cenocepacia* isolates strongly outcompeted their co-isolated *P. aeruginosa* strains when the bacteria were cocultured on a solid surface (conducive to contact-dependent interactions) and not when cocultured in liquid medium. The *P. aeruginosa* co-infection isolates were also typically deficient in Hcp1 production. These data indicate that, at least for the *P. aeruginosa* strains studied here, the main factor in determining susceptibility to T6SS-mediated competition by Bcc bacteria is whether *P. aeruginosa* produces a functional T6SS.

The T6S-abrogating mutations we identified in *P. aeruginosa* CF isolates in this study fell into two classes: those in genes encoding post-translational regulators of T6SS activity (*pppA* and *fha1*), and those in genes encoding the GacSA two-component regulatory system. Fha1 is required for the initial assembly of the T6S apparatus, whereas PppA is required for disassembly of apparatuses and recycling of T6SS proteins into new apparatuses. Mutations in *pppA* or *fha1* are expected to prevent efficient T6SS activity without affecting production of individual T6SS components (Basler et al., 2013; Mougous et al., 2007). Consistent with this expectation, Hcp1 was detectable in *Pa*AU4391, which contains a small deletion in *fha1*, but this co-infection isolate was outcompeted by its paired *B. cenocepacia* isolate. By contrast, mutations in *gacA* or *gacS* are expected to prevent production of the entire T6S apparatus. GacS is one of four hybrid sensor kinases that controls phosphorylation, and hence activation, of the GacA response regulator. LadS functions with GacS to activate GacA, while RetS blocks GacS activity, thereby inhibiting GacA activation (Chambonnier et al., 2016; Goodman et al., 2009). The PA1611-encoded sensor kinase promotes GacA activation by relieving RetS inhibition of GacS (Kong et al., 2013). When active, GacA induces production of two sRNAs, RsmY and RsmZ, which bind to, and prevent activity of, RsmA, a pleiotropic global regulator that impedes translation of many target genes (Brencic and Lory, 2009; Brencic et al., 2009). When not inhibited by RsmY or RsmZ, RsmA activity results in production of factors associated with acute infection (e.g., flagella, type III secretion, type IV pili) and lack of production of factors and phenotypes associated with

chronic infection (e.g., exopolysaccharide production, biofilm, T6S). The RetS/PA1611/LadS/GacSA signaling pathway is therefore considered to function as a switch between acute and chronic infection modes (Balasubramanian et al., 2013; Goodman et al., 2009; 2004).

While there is evidence that the genes encoding the RetS/PA1611/LadS/GacSA signaling pathway are intact when *P. aeruginosa* establishes infection initially in the CF lung, mutations arise in *retS* within some strains over time (*e.g.*, 11/36 clone types in the 2015 Marvig et al. study), and, at least for those studied, all *retS*-mutated strains acquire subsequent mutations in *gacS*/*gacA* or *rsmA* (Bartell et al., 2019; Marvig et al., 2015). Our data are consistent with these reports, as three out of nine teenage/adult *P. aeruginosa* isolates used in our study were *gacS*/*gacA* mutants and also contained nonsynonymous *retS* mutations, though it is unknown whether these mutations affect RetS function. Three out of eight *P. aeruginosa* co-infection isolates contained *gacS*/*gacA* mutations and did not produce Hcp1; their *retS* statuses are unknown. Thus, there appears to be a selection for lack of GacSA activity following mutation of *retS* within the CF respiratory tract, and we envisage this selection could be either T6S-independent or T6S-dependent; a Gac-regulated target other than T6S may drive this selection, with loss of T6S being simply a consequence of Gac inactivation, or T6S itself could be what is selected against. We and others (Kordes et al., 2019; Marvig et al., 2015) have detected mutations in genes encoding proteins specific for T6SS assembly and function in *P. aeruginosa* strains isolated from older CF patients, supporting the hypothesis that T6S may be disadvantageous to *P. aeruginosa* during chronic infection in the CF lung.

Why might *P. aeruginosa* lose T6SS activity in later stages of host colonization? Given the polymicrobial nature of the CF respiratory tract, it is reasonable to hypothesize that maintaining a potent antibacterial weapon like the T6SS would be beneficial. However, *P. aeruginosa* T6SS proteins are immunogenic (Mougous et al., 2006), and avoiding the host immune response could be equally, or more, beneficial. Additionally, production of T6SSs is energetically costly, and while T6SS-mediated competition may be worth the cost during early stages of infection, these structures may be dispensable once *P. aeruginosa* has established its niche. As indicated by the proportion of reads in metagenomic samples, *P. aeruginosa* can constitute over 90% of all bacterial cells within the airways of certain CF patients (Carmody et al., 2015; 2013; J. Zhao et al., 2012). Under these conditions, T6S-mediated interspecies competition should not be required. Loss of T6S by bacteria colonizing humans has been shown with gut resident *Bacteroides* spp., as T6SS-proficient *Bacteroides* are more prevalent in the unstable infant gut microbiota than they are in adult gut microbiota where individual *Bacteroides* spp. or strains predominate (Verster et al., 2017). One might expect that similar selective pressures would act on Bcc during CF infection. However, the *B. cenocepacia* T6SS is required for murine infection (Hunt et al., 2004), and at least some Bcc strains produce a T6SS effector (TecA) that promotes survival within macrophages (Aubert et al., 2016; 2015; Rosales-Reyes et al., 2012), suggesting there is a strong selective advantage for Bcc pathogens to remain T6SS-active while infecting the CF lung.

Although *P. aeruginosa* and Bcc bacteria ultimately colonize different sites in the CF airways (Schwab et al., 2014), Bcc pathogens must traverse the lumen, where *P. aeruginosa* can exist in large populations, before invading host cells. Therefore, transient Bcc-*P. aeruginosa* interactions likely occur, and our data support the hypothesis that the outcome of these interactions depends on the T6S proficiency of the resident *P. aeruginosa*. *P. aeruginosa* populations within individual CF patients exhibit genotypic and phenotypic diversity across different regions of the respiratory tract (Jorth et al., 2015), and thus Bcc bacteria may only need to interact with a subpopulation of *P. aeruginosa* that has lost T6SS activity in order to initiate an infection and invade host cells. Experiments using animal models and human microbiome analyses have shown that T6SS-mediated competition occurs within mammalian intestines (M. C. Anderson et al., 2017; Sana et al., 2016; Verster et al., 2017; Wexler et al., 2016; W. Zhao et al., 2018), though whether such interactions occur in the CF respiratory tract is unknown. These questions would be better addressed with animal models of CF disease. Unfortunately, a dearth of robust, efficient animal models for chronic bacterial infections has inhibited progress in the understanding of these infections (Fisher et al., 2011; Kukavica-Ibrulj and Levesque, 2008; Semaniakou et al., 2018).

While our data are consistent with T6SS-mediated competition between Bcc pathogens and *P. aeruginosa* affecting susceptibility of older CF patients to the Bcc, we hypothesize additional factors may prevent Bcc infections in young patients. *Staphylococcus aureus* is the most prevalent pathogen of young CF patients (Cystic Fibrosis Foundation, 2019), and *S. aureus* colonization could preclude Bcc infection. Additionally, changes in the immune response, physiology, and/or nutritional environment of the CF respiratory tract over time could cause these tissues to be more hospitable to Bcc pathogens later in the lives of CF patients compared to those in infants and children. CF patients are often on antibiotic regimens to treat opportunistic infections, and regular use of antibiotics may promote Bcc pathogen colonization of older patients. Other unknown factors could also be at play.

In our studies, the T6SS-1 provided strong competitive advantages to three Bcc pathogens (*Bc*AU1054, *Bm*CGD2M, and *Bd*AU0158) against host-adapted *P. aeruginosa*. Gene clusters encoding T6SS-1 are prevalent throughout the Bcc (Spiewak et al., 2019), suggesting T6SS-mediated killing of host-adapted *P. aeruginosa* may be a common asset of Bcc pathogens. The role of additional T6SSs in Bcc pathogens remains unknown, but it appears that interbacterial antagonism is mostly mediated by the T6SS-1, at least under the conditions used in this study. Our investigation of the *Bc*AU1054 T6SS revealed four *bona fide* antibacterial E-I pairs; however, our bioinformatic prediction of E-I pair-encoding genes missed at least one gene pair, as *Bc*AU1054 Δ9E-I maintained a strong competitive advantage against *Bd*AU0158. The unidentified effector(s) is/are not encoded by gene(s) near *vgrG* genes, nor are there shared domains between the effectors we identified and the unidentified effector(s), suggesting the unidentified effector(s) may be members of an uncharacterized class of T6SS toxins. Our screening for antibacterial effectors and follow-up competition experiments were specific to intrastrain antagonism (*Bc*AU1054 vs. *Bc*AU1054) under one condition (LSLB agar at 37°C). The predicted E-I pairs that our screen suggested were not important for intrastrain competition may be important for interstrain/interspecies competition or competition under different conditions (e.g., temperature, salt, pH); similar conditional efficiency has been demonstrated for *P. aeruginosa* T6SS effectors (LaCourse et

al., 2018). Supporting this hypothesis, *Bc*AU1054 Δ9E-I outcompeted T6SS-null *P. aeruginosa* teenage/adult isolates to varying degrees, suggesting the additional, unidentified effector(s) have prey cell-specific activity.

There is growing appreciation for the genotypic and phenotypic diversity of *P. aeruginosa* within the CF respiratory tract (Folkesson et al., 2012; Jorth et al., 2015; Winstanley et al., 2016). Although reference strains are powerful tools for studying bacterial pathogens, they do not always perfectly represent the strains currently infecting humans. Our investigations illuminate differences between PAO1 and recently collected *P. aeruginosa* CF isolates specific to T6SS-mediated competition against Bcc pathogens, as well as demonstrate varying abilities of *P. aeruginosa* CF isolates to compete against *Bc*AU1054. Our data support a model in which resident *P. aeruginosa* populations must evolve to lose T6SS activity in order for Bcc pathogens to colonize the CF respiratory tract. If true, not only is the Bcc T6SS an important colonization factor, but assessing the T6S potential of resident *P. aeruginosa* could predict susceptibility of CF patients to deadly Bcc superinfections.

STAR METHODS TEXT

RESOURCE AVAILABILITY

Lead Contact—Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Peggy A. Cotter (peggy_cotter@med.unc.edu).

Materials Availability—Bacterial strains and plasmids generated in this study are available upon request from the Lead Contact.

Data and Code Availability—Sequencing reads generated as part of this study are available at the NCBI Sequencing Read Archive: PRJNA607994 and PRJNA609958.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Bacterial strains and growth conditions—All bacterial strains in this study were cultured at 37°C in low salt lysogeny broth (LSLB: 10 g/L tryptone, 5 g/L yeast extract, 5 g/L sodium chloride) or on LSLB agar (1.5% agar). Antibiotics to select for *Burkholderia* strains were used at the following concentrations, when applicable: 30 μg/mL gentamicin, 250 μg/mL kanamycin, 50 μg/mL trimethoprim, 40 μg/mL tetracycline. Antibiotics to select for *P. aeruginosa* strains were used at the following concentrations, when applicable: 20 or 35 μg/mL chloramphenicol, 20 μg/mL nalidixic acid, 50μg/mL trimethoprim, 75 μg/mL gentamicin, 40 μg/mL tetracycline. 20 μg/mL nalidixic acid was used to select for *E. coli* DH5α, when applicable. LSLB agar was supplemented with 200 μg/mL 2,6-diaminopimelic acid to support the growth of *E. coli* strain RHO3.

METHOD DETAILS

Genetic manipulations—*E. coli* strain RHO3 was used to conjugate plasmids into *Burkholderia* spp. and *P. aeruginosa*. The pEXKm5 allelic exchange vector (López et al., 2009) was used to generate unmarked, in-frame deletion mutations in *Bc*AU1054. Briefly,

~500 nucleotides 5' to and including the first three codons of the gene to be deleted were fused to ~500 nucleotides 3' to and including the last three codons of the gene by overlap extension PCR and cloned into pEXKm5. Following selection of *Bc*AU1054 merodiploids with the plasmids integrated into the chromosome, cells were grown for 4 h in YT broth (10 g/L yeast extract, 10 g/L tryptone) at 37°C with aeration, subcultured 1:1000 in fresh YT broth, and grown overnight at 37°C with aeration. After overnight growth, cells that lost the cointegrated plasmid following the second homologous recombination step were selected on YT agar (1.5% agar) containing 25% sucrose and 100 μg/mL 5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (X-Gluc). Deletion mutants were screened for by PCR and verified by sequencing regions spanning the deletions.

The pUC18T-mini-Tn7T suite of plasmids (Choi et al., 2005) was used to deliver antibiotic resistance gene cassettes to the *att*Tn7 sites of *Bc*AU1054 and *P. aeruginosa*. The trimethoprim resistance-conferring plasmid pUC18T-mini-Tn7T-Tp was generated in this study by restriction digesting out *dhfRII* from pUC18T-mini-Tn7-Tp-P_{S12}-mCherry (LeRoux et al., 2012) using MscI and NcoI and ligating into digested pUC18T-mini-Tn7T-Km (Choi et al., 2005) lacking *nptII* (the kanamycin resistance-conferring gene). pUC18-miniTn7-*kan*-*gfp* (Norris et al., 2010) was used to generate GFP-producing *Bc*AU1054 E-I deletion mutants. Complemented *Bc*AU1054 mutant strains (with either *hcp* or T6SS immunity-encoding genes) were generated by PCR amplifying the genes of interest and cloning the sequences into pUCS12Km, with genes expressed off the constitutive ribosomal S12 subunit gene promoter of *Burkholderia thailandensis* strain E264 (M. S. Anderson et al., 2012). *Bc*AU1054 strains constitutively expressing *lacZ* were generated using pECG10 (M. S. Anderson et al., 2012). *P. aeruginosa* isolates C078C and CEC118 were complemented with *pppA* and *fha1* genes from PAO1, respectively, by cloning these sequences into pUCS12Km, digesting out the genes and upstream constitutive promoter P_{S12}, and cloning these fragments into pUC18T-mini-Tn7T-Tet (M. S. Anderson et al., 2012). For all pUC18T-mini-Tn7T-based cassette delivery to the *att*Tn7 sites of *Bc*AU1054 and *P. aeruginosa*, the transposase-encoding pTNS3 helper plasmid was used in triparental conjugation. *Bm*CGD2M *tssC1*::pAP82 and *Bd*AU0158 *tssC1*::pAP83 were generated by cloning ~500 internal nucleotides of the *tssC1* genes into pUC18T-mini-Tn7T-Km, conjugating the plasmids into *Bm*CGD2M and *Bd*AU1058, and selecting for plasmid cointegrants on kanamycin.

Interbacterial competition experiments—All competition experiments were conducted for 5 h on LSLB agar at 37°C, with an ~1:1 starting cell ratio of inhibitor and target strains, unless stated otherwise. Cells were collected from overnight liquid cultures, centrifuged for 2 min at 15,000 rpm, washed in 1X phosphate buffered saline (PBS), diluted to an OD_{600} of 1.0, and equal volumes of inhibitor and target cells were mixed. For *Bc*AU1054 vs. *E. coli* DH5α competitions, *Bc*AU1054 1.0 OD_{600} cell suspensions were diluted 1:3 in 1X PBS before mixing with DH5α 1.0 OD_{600} cell suspensions to attain an ~1:1 starting cell ratio. 20 μL spots of mixtures were plated on LSLB agar in 24-well plates, allowed to dry, and incubated at 37°C for 5 h. Starting mixtures were also serially diluted and plated on antibiotic-containing selective media to enumerate inhibitor and target strains at the initial time point. Following 5 h, competition spots were resuspended in 1 mL 1X PBS

within wells, serially diluted, and plated on antibiotic-containing selective media to separately grow inhibitor and target strains. Colony counts at the initial and 5 h time points allowed for competitive index (C.I.) calculations as follows: C.I. = (inhibitor$_{f5}$/target$_{f5}$)/(inhibitor$_{f0}$/target$_{f0}$). A positive $\log_{10}$ C.I. indicates the inhibitor strain outcompeted the target strain, a negative $\log_{10}$ C.I. indicates the target strain outcompeted the inhibitor strain, and a $\log_{10}$ C.I. of ~0 indicates neither strain had a competitive advantage. For cocultures from which no target bacteria were recovered, the target colony count was set at the limit of detection (one colony forming unit at a 10^{-2} dilution) and indicated by grey-filled diamonds; thus, the calculated C.I.'s for these competitions are likely underestimations.

Liquid competitions between *B. cenocepacia-P. aeruginosa* co-infection isolates were set up following the above protocol, except 20 μL of cell mixtures were inoculated into 1 mL LSLB and grown for 5 h at 37°C shaking at 220 rpm. For competitions between *Bc*AU1054 and *P. aeruginosa* clinical isolates, *Bc*AU1054 WT and Δ*hcp* strains constitutively expressing *lacZ* were used and inocula/competitions were plated onto antibiotic-containing LSLB agar with 40 μg/mL 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal) to help differentiate between *P. aeruginosa* and *Bc*AU1054 colonies. Competitions between *Bc*AU1054 and pJN-*rsmZ*/pJN105-harboring *P. aeruginosa* teenage/adult isolates were conducted on LSLB agar containing 0.1% L-arabinose.

***Bc*AU1054 T6SS E/I screen—**Cocultures were set up following the same protocol as in the interbacterial competition experiments. For monocultures, cell suspensions (at an OD_{600} of 1.0) were mixed 1:1 with 1X PBS before plating. For cocultures and monocultures, 20 μL spots were plated on LSLB agar within 24-well plates, spots were allowed to dry, and plates were incubated at 37°C for ~20 h. Following incubation, the cultures were resuspended in 1 mL 1X PBS within wells, 100 μL were added to 96-well plates, and OD_{600} values and GFP fluorescence intensities (485 nm excitation, 530 nm emission) were measured on a PerkinElmer Wallac VICTOR$^3{}_{TM}$ plate reader.

Hcp1 immunoblotting—*P. aeruginosa* strains were swabbed onto LSLB agar and grown overnight at 37°C. For pJN-*rsmZ*/pJN105-harboring *P. aeruginosa* teenage/adult isolates, strains were swabbed onto LSLB agar containing 75 μg/mL gentamicin and 0.1% L-arabinose and grown overnight at 37°C. Following overnight incubation, cells were scraped off plate, resuspended in 1 mL cold 1X PBS, centrifuged for 2 min at 15,000 rpm, washed in 1 mL cold 1X PBS, and diluted to an OD_{600} of 5.0. Cells were then centrifuged for 2 min at 15,000 rpm and resuspended in 200 μL 2X SDS-PAGE sample loading buffer (6X SDS-PAGE sample loading buffer: 375 mM Tris-HCl, 9% sodium dodecyl sulfate (SDS), 50% glycerol, 0.03% bromophenol blue, 1.3 M β-mercaptoethanol), boiled at 99°C for 15 min, and samples were sheared 10 times through a 26G needle. Samples were resolved on 12% SDS-PAGE gels (5 μL loaded), transferred to nitrocellulose membranes, and membranes were blocked with 5% (w/v) non-fat dry milk in 1X PBS for 1 h with rotation at room temperature (RT). Membranes were then washed three times in 1X PBS and incubated with anti-Hcp1 polyclonal peptide antibody (diluted 1:1000 in 5% (w/v) non-fat dry milk in 1X PBS+0.1% Tween®20 (PBS-T)) for 1 h with rotation at RT. Membranes were then washed three times in 1X PBS-T, incubated with IRDye® 800CW goat anti-rabbit IgG secondary

antibody (diluted 1:25,000 in 5% (w/v) non-fat dry milk in 1X PBS-T) for 30 min with rotation at RT, washed three times in 1X PBS, and imaged on a LI-COR Odyssey® fluorescence imager.

Sequencing—Genomic DNA was purified from *P. aeruginosa* isolates C078C, C120C, and C123D using the Promega Wizard Genomic DNA Purification Kit. Paired-end TruSeq (Illumina) libraries were generated and sequenced on the Illumina MiSeq 2×150 platform at the High-Throughput Sequencing Facility at the University of North Carolina at Chapel Hill. Demultiplexed FASTQ files were mapped to the PAO1 reference genome (assembly GCA_000006765.1) using the Geneious Prime standard assembler. Sequencing reads can be accessed in BioProject PRJNA609958.

To sequence *P. aeruginosa* CEC isolate genomes, genomic DNA was isolated using a GenElute Bacterial Genomic DNA Kit (Sigma Aldrich, NA2110; St. Louis, MO) following kit instructions with the following exception: all DNA was eluted in 400uL of ultra-pure DEPC-treated water (ThermoFisher Scientific, Waltham, MA). Concentration of DNA preps was determined using a NanoDrop 1000 (ThermoFisher Scientific, Waltham, MA). All preps were stored at −20C. The 150bp sequencing reads from the Illumina platform were assembled using spades v.3.7.1 with careful mismatch correction and the assemblies were filtered to contain only contigs ≥500bp with ≥5X k-mer coverage. The assemblies were further examined for characteristics that would suggest the genome was of high quality (<400 contigs) and potentially *P. aeruginosa*. All reads and assemblies are deposited at NCBI under BioProject PRJNA607994.

Specific *P. aeruginosa* co-infection isolate genes were sequenced by PCR-amplifying genes of interest and submitting the PCR products for Sanger sequencing.

Bioinformatic analysis of *Bc*AU1054 T6SS-encoding genes and effector proteins—The *Bc*AU1054 and *Bc*J2315 (genome assembly GCA_000009485.1) T6SS-encoding core clusters were aligned in Geneious Prime using the Mauve plugin (Darling et al., 2004). Phyre2 (Kelley et al., 2015) and HHpred (Zimmermann et al., 2018) were used to predict the secondary structures and catalytic activities of potential *Bc*AU1054 T6SS effector proteins.

QUANTIFICATION AND STATISTICAL ANALYSES

All statistical significance was calculated by Mann-Whitney tests using GraphPad Prism v. 8. Specific details of statistical analysis, including total number of samples and replicates, can be found in the figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- *Burkholderia cepacia* complex (Bcc) pathogens produce functional antibacterial T6SSs
- Bcc pathogens use T6SSs to outcompete host-adapted *P. aeruginosa* CF isolates
- Host-adapted *P. aeruginosa* CF isolates harbor T6SS-abrogating mutations
- T6SS-mediated competition dynamics may restrict Bcc infections to older CF patients

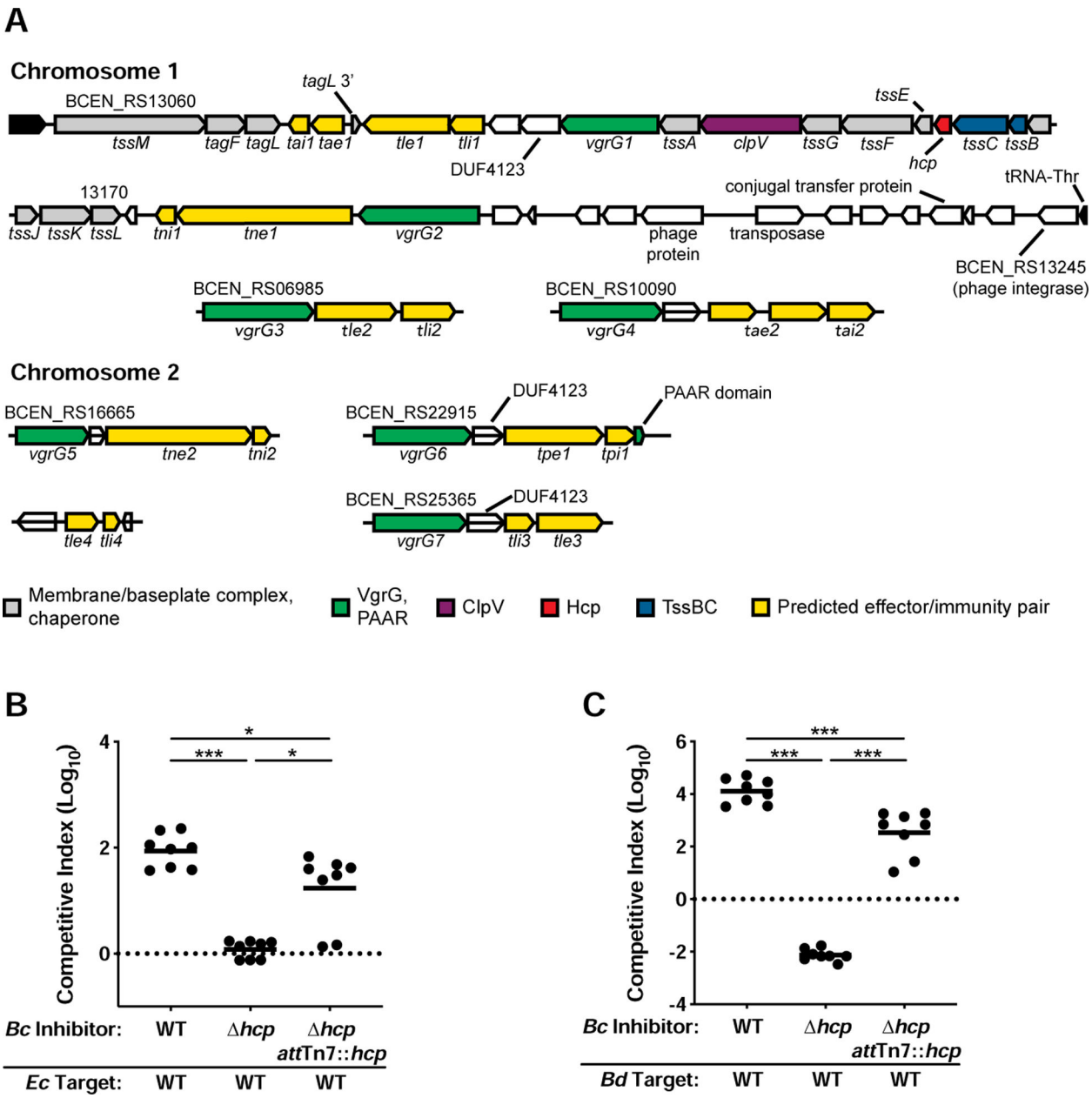

Figure 1. The *Bc*AU1054 T6SS mediates interbacterial competition.

(A) Core cluster (top two lines) and accessory genes encoding the *Bc*AU1054 T6SS and predicted effector-immunity (E-I) pairs (see Table S2). The legend indicates function of protein products. White ORFs encode DUF4123 T6SS adapter proteins, or proteins not known to be associated with the T6SS. (B and C) Competition experiments between inhibitor *Bc*AU1054 and target *E. coli* (B) and *B. dolosa* (C). WT, Δ*hcp*, and Δ*hcp* *att*Tn7::*hcp* *Bc*AU1054 inhibitor strains used in each. Circles represent individual cocultures from two biological replicates, each with four technical replicates. Solid horizontal lines represent mean $\log_{10}$ C.I. values. Dotted horizontal lines ($\log_{10}$ C.I. = 0) indicate no competitive advantage for either strain. **P*<0.05, ***P*<0.0005, Mann-Whitney test.

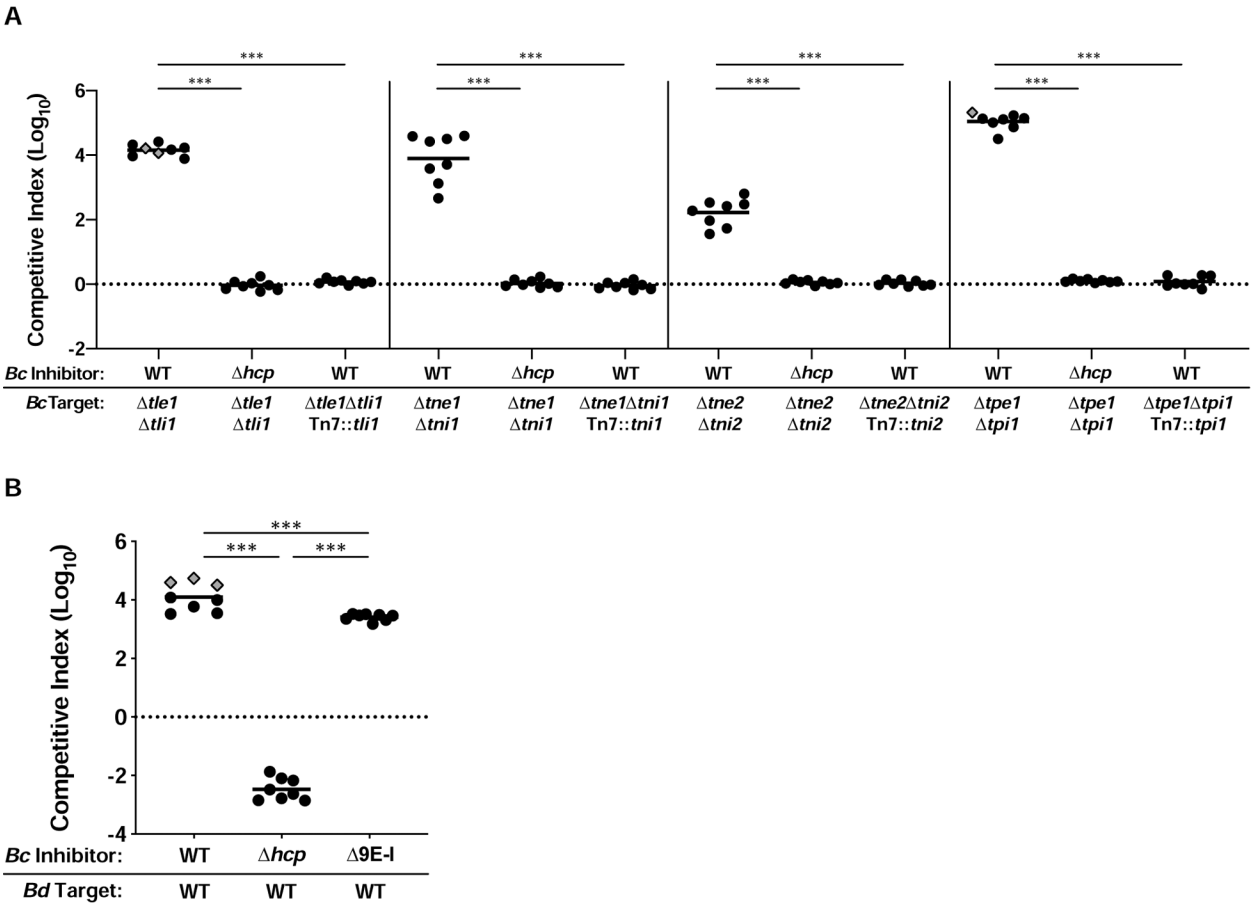

Figure 2. At least five *Bc*AU1054 T6SS effectors mediate interbacterial competition.

(A) Competition experiments between WT and Δ*hcp Bc*AU1054 inhibitor strains and *Bc*AU1054 mutants lacking E-I-encoding genes, including mutants complemented with cognate immunity genes. (B) Competition experiments between WT, Δ*hcp*, and Δ9E-I *Bc*AU1054 inhibitor and *Bd*AU0158 target strains. For (A and B), circles/diamonds represent individual cocultures from two biological replicates, each with four technical replicates. Grey-filled diamonds represent competitions from which no target cells were recovered. Solid horizontal lines represent mean $\log_{10}$ C.I. values. Dotted horizontal lines ($\log_{10}$ C.I. = 0) indicate no competitive advantage for either strain. ***P<0.0005, Mann-Whitney test.

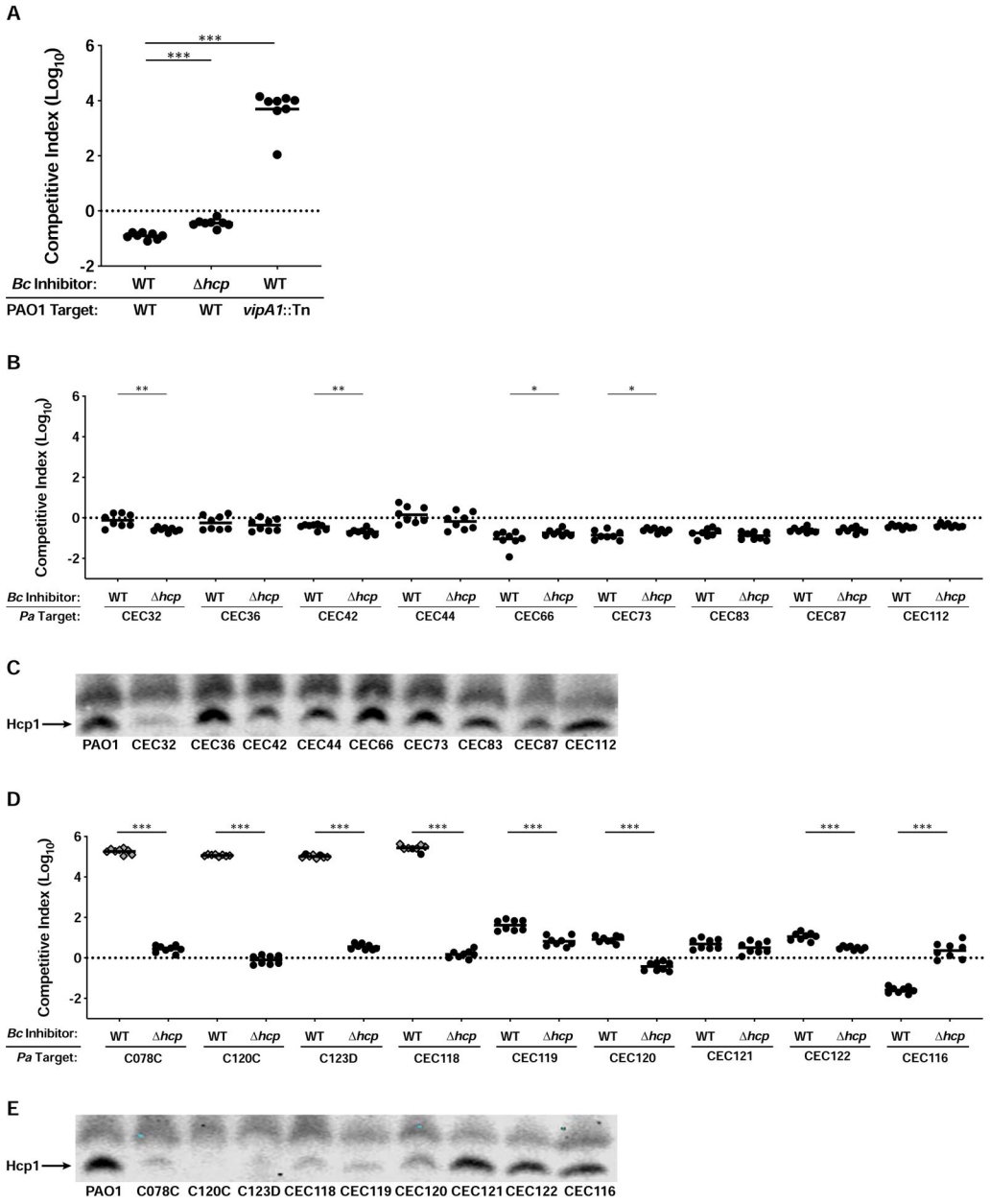

Figure 3. Susceptibility of *P. aeruginosa* CF isolates to the *Bc*AU1054 T6SS correlates with patient age.

(A) Competition experiments between WT and Δ*hcp Bc*AU1054 inhibitor strains and WT and *vipA1*::Tn PAO1 target strains. (B) Competition experiments between WT and Δ*hcp Bc*AU1054 inhibitor strains and *P. aeruginosa* infant/child CF isolate targets. (C) Immunoblot for Hcp1 production by PAO1 and *P. aeruginosa* infant/child CF isolates. (D) Competition experiments between WT and Δ*hcp Bc*AU1054 inhibitor strains and *P. aeruginosa* teenage/adult CF isolate targets. (E) Immunoblot for Hcp1 production by PAO1 and *P. aeruginosa* teenage/adult CF isolates. For (A, B, and D), circles/diamonds represent individual cocultures from two biological replicates, each with four technical replicates. Grey-filled diamonds represent competitions from which no target cells were recovered.

Solid horizontal lines represent mean $\log_{10}$ C.I. values. Dotted horizontal lines ($\log_{10}$ C.I. = 0) indicate no competitive advantage for either strain. *P<0.05, **P<0.005, ***P<0.0005, Mann-Whitney test. For (C and E), non-specific band above Hcp1 serves as loading control. Blots are representative of at least two experiments per strain.

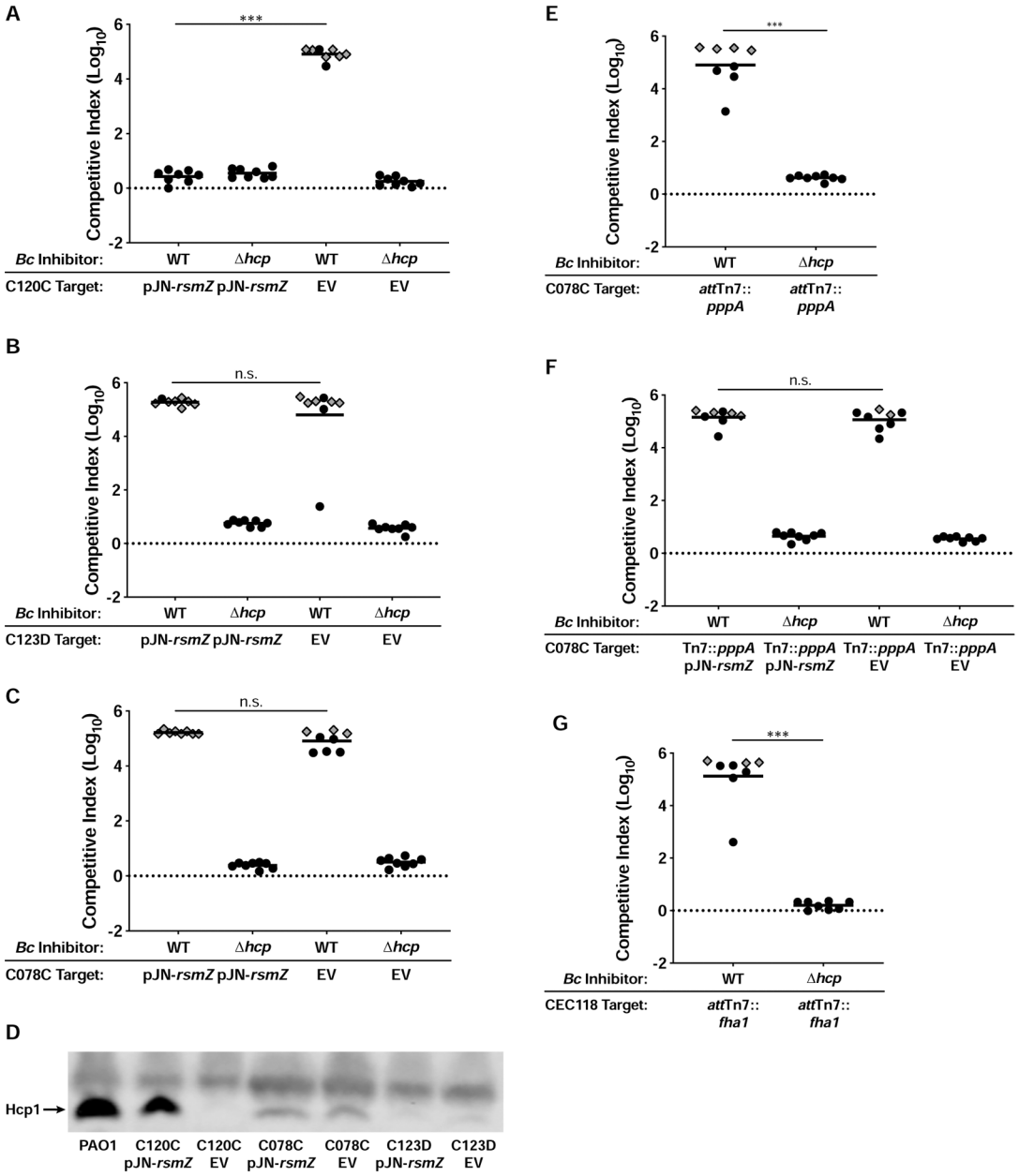

Figure 4. Restoration of H1-T6SS protein production can rescue host-adapted *P. aeruginosa* from T6SS-mediated elimination by *Bc*AU1054.

(A, B, and C) Competition experiments between WT and Δ*hcp Bc*AU1054 inhibitors and *P. aeruginosa* teenage/adult CF isolates C120C (A), C123D (B), and C078C (C) harboring the pJN-*rsmZ* and pJN105 (EV) plasmids. Competitions conducted on agar containing 0.1% arabinose. (D) Immunoblot for Hcp1 production by C120C, C078C, and C123D harboring pJN-*rsmZ* and pJN105 (EV) during growth on agar containing 0.1% arabinose, and PAO1 for comparison. Non-specific band above Hcp1 serves as loading control. The blot is representative of at least two experiments per strain. (E) Competition experiments between WT and Δ*hcp Bc*AU1054 inhibitor and C078C *att*Tn7::*pppA* target strains. (F) Competition experiments between WT and Δ*hcp Bc*AU1054 inhibitor and C078C *att*Tn7::*pppA* target

strains harboring pJN-*rsmZ* and pJN105 (EV). Competitions conducted on agar containing 0.1% arabinose. (G) Competition experiments between WT and Δ*hcp Bc*AU1054 inhibitor and CEC118 *att*Tn7::*fha1* target strains. For (A, B, C, E, F, and G), circles/diamonds represent individual cocultures from two biological replicates, each with four technical replicates. Grey-filled diamonds represent competitions from which no target cells were recovered. Solid horizontal lines represent mean $\log_{10}$ C.I. values. Dotted horizontal lines ($\log_{10}$ C.I. = 0) indicate no competitive advantage for either strain. n.s.=not significant, *** P<0.0005, Mann-Whitney test.

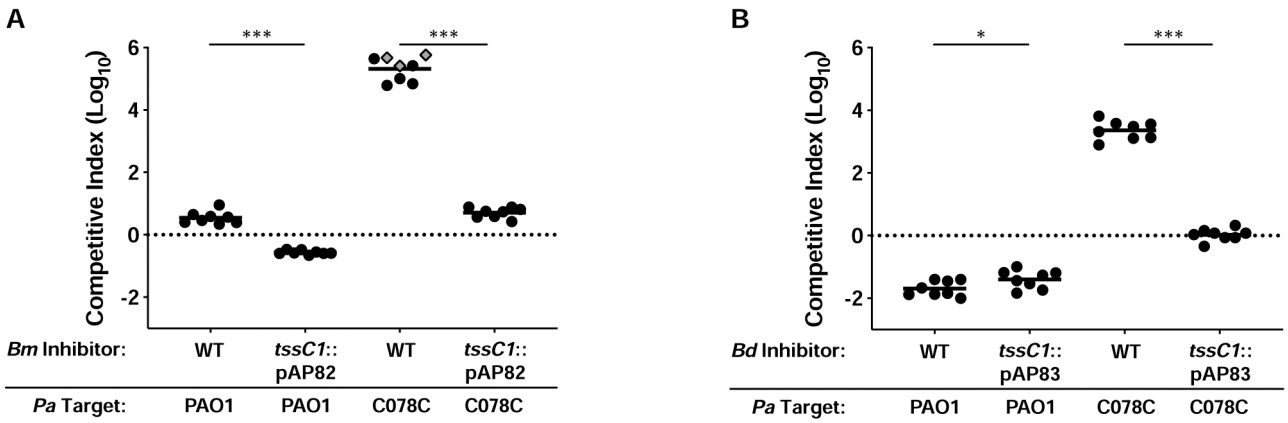

Figure 5. Additional Bcc pathogens kill host-adapted *P. aeruginosa* in a T6SS-dependent manner.

(A) Competition experiments between WT and *tssC1*::pAP82 *Bm*CGD2M inhibitor strains and PAO1 and C078C target strains. (B) Competition experiments between WT and *tssC1*::pAP83 *Bd*AU0158 inhibitor strains and PAO1 and C078C target strains. For (A and B), circles/diamonds represent individual cocultures from two biological replicates, each with four technical replicates. Grey-filled diamonds represent competitions from which no target cells were recovered. Solid horizontal lines represent mean $\log_{10}$ C.I. values. Dotted horizontal lines ($\log_{10}$ C.I. = 0) indicate no competitive advantage for either strain. *P<0.05, ***P<0.0005, Mann-Whitney test.

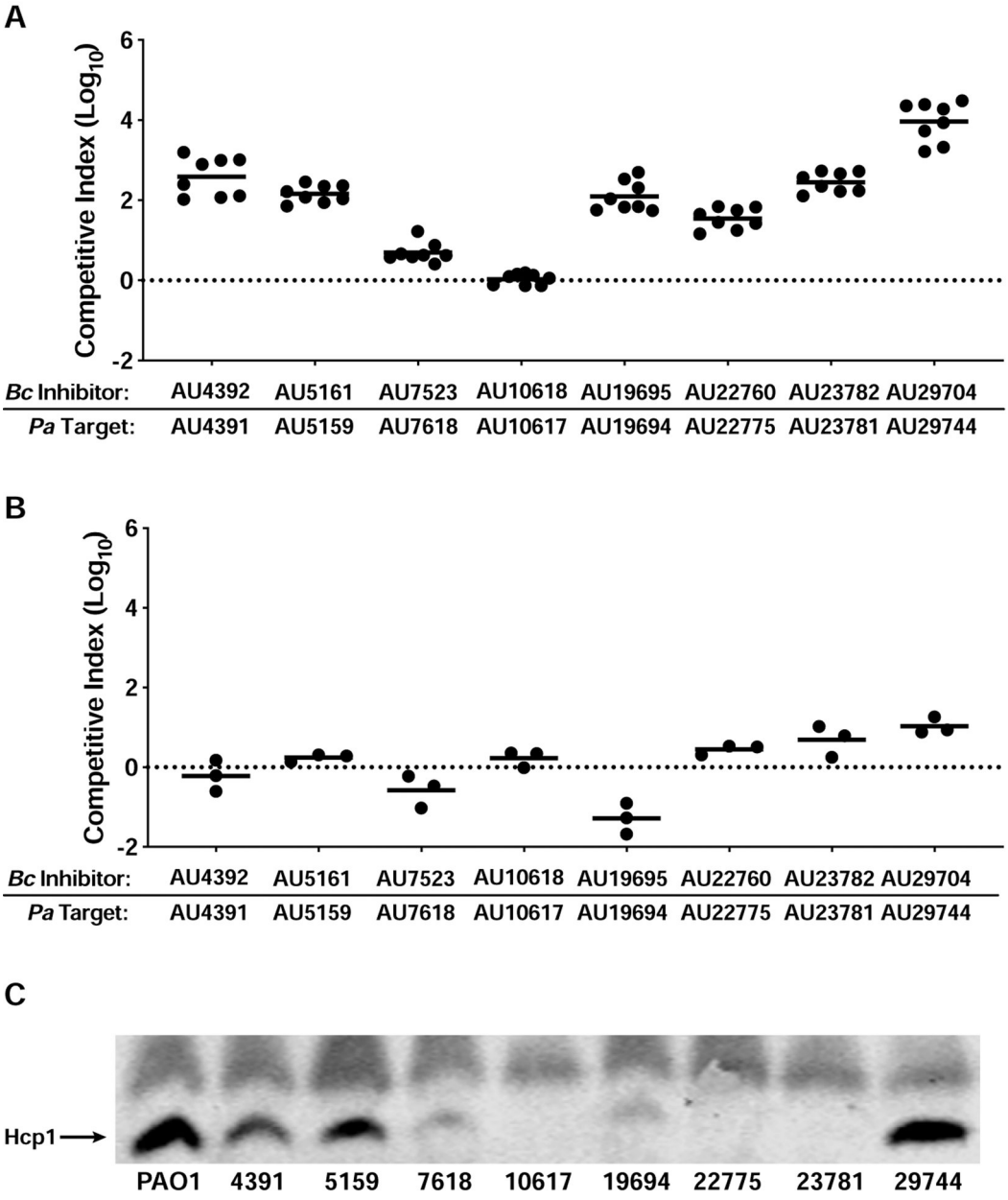

Figure 6. *B. cenocepacia* isolates from CF patients with concurrent *P. aeruginosa* infections outcompete their paired *P. aeruginosa* isolates under T6SS-permissive conditions.

(A and B) Competition experiments between *B. cenocepacia-P. aeruginosa* co-infection isolate pairs on agar (A) and in shaking liquid culture (B). In (A), circles represent individual cocultures from two biological replicates, each with four technical replicates. In (B), circles represent individual cocultures from three biological replicates. Solid horizontal lines represent mean $\log_{10}$ C.I. values. Dotted horizontal lines ($\log_{10}$ C.I. = 0) indicate no competitive advantage for either strain. (C) Immunoblot for Hcp1 production by *P. aeruginosa* co-infection isolates, and PAO1 for comparison. Non-specific band above Hcp1 serves as loading control. The blot is representative of at least two experiments per strain.

Table 1.

Competition sensitivity, Hcp1 production, and putative T6SS-abrogating mutations of *P. aeruginosa* CF isolates used in this study.

***P. aeruginosa* CF Isolate**	**Patient age (in years) at isolation**	**Susceptibility to *Bc*AU1054 T6SS, or to *B. cenocepacia* paired isolate**	**Hcp-1 production**	**Putative T6SS-abrogating mutation(s)**	**RetS substitutions in *gacS/gacA* mutants**
Infant/Child Isolates					
CEC32	1	−	+	N/A	N/A
CEC36	<1	−	+++	N/A	N/A
CEC42	2	−	+++	N/A	N/A
CEC44	2	−	+++	N/A	N/A
CEC66	1	−	+++	N/A	N/A
CEC73	3	−	+++	N/A	N/A
CEC83	1	−	+++	N/A	N/A
CEC87	<1	−	+++	N/A	N/A
CEC112	3	−	+++	N/A	N/A
Teenage/Adult Isolates					
C078C	31	+++	+	*gacS*$_{G1715A}$ (GacS$_{G572D}$), *pppA*$_{G111A}$ (PppA$_{TRUNC}$)	RetS$_{A46v}$, RetS$_{R144H}$
C120C	12	+++	−	*gacA*$_{C349T}$ (GacA$_{TRUNC}$)	RetS$_{A46v}$, RetS$_{L856Q}$
C123D	19	+++		−	RetS$_{A46V}$
CEC118	17	+++	+	*fha1*$_{Δ404–424}$ (Fha1$_{Δ134–140}$)	N/A
CEC119	25	+	+	N/D	N/A
CEC120	11	+	+	N/D	N/A
CEC121	19	−	+++	N/A	N/A
CEC122	18	+/−	+++	N/A	N/A
CEC116	11	−	+++	N/A	N/A
Co-Infection Isolates 1					
*Pa*AU4391	39	+++	+++	*fha1*$_{Δ405–425}$ (Fha1$_{Δ135–141}$)	N/A
*Pa*AU5159	26	++	+++	N/D	N/A
*Pa*AU7618	33	+	+	*gacA*$_{G175A}$ (GacA$_{G59S}$)	N/D
*Pa*AU10617	17	−	−	N/D	N/A
*Pa*AU19694	36	+++	+	*gacA*$_{C162A}$ (GacA$_{D54E}$)	N/D
*Pa*AU22775	38	+	−	N/D	N/A
*Pa*AU23781	39	+	−	*gacS*$_{G1568A}$ (GacS$_{TRUNC}$)	N/D
*Pa*AU29744	33	+++	+++	N/D	N/A

For susceptibility to competition against *B. cenocepacia* and Hcp1 production status, see Figures 3 and 6.

N/A, not applicable.

N/D, not determined.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-PAO1 Hcp1 polyclonal peptide antibody	Gift from Dr. John Mekalanos	N/A
IRDye® 800CW Goat anti-rabbit IgG Secondary Antibody	LI-COR Biosciences	926–32211; RRID: AB_621843
Bacterial and Virus Strains		
All bacterial strains used in this study listed in Table S3		
Chemicals, Peptides, and Recombinant Proteins		
Gentamicin	Gold Biotechnology	G-400–25
Kanamycin	Gold Biotechnology	K-120–25
Nalidixic acid	Sigma-Aldrich	N4382
Trimethoprim	Gold Biotechnology	T-350–5
Tetracycline	Fisher Scientific	BP912–100
Chloramphenicol	Fisher Scientific	BP904–100
5-bromo-4-chloro-3-indoxyl-β-D-glucuronide (X-Gluc)	Gold Biotechnology	G1281C1
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)	Gold Biotechnology	X4281C
L-arabinose	Gold Biotechnology	A-300–100
D-sucrose	Fisher Scientific	BP220–1
2,6-diaminopimelic acid	Alfa Aesar	AAB22391–06
Critical Commercial Assays		
Wizard Genomic DNA Purification Kit	Promega	A1120
GenElute™ Bacterial Genomic DNA Kit	Sigma-Aldrich	NA2110
Deposited Data		
P. aeruginosa isolate whole genome sequences	This study	NCBI Sequence Read Archive: PRJNA607994 and PRJNA609958
Recombinant DNA		
All plasmids used in this study listed in Table S4		
Software and Algorithms		
Geneious Prime	Geneious, Software, Newark, New Jersey, USA	http://www.geneious.com; RRID: SCR_010519
Phyre2	(Kelley et al., 2015)	http://www.sbg.bio.ic.ac.uk/~phyre2; RRID: SCR_010270
HHPred	(Zimmermann et al., 2018)	https://toolkit.tuebingen.mpg.de/tools/hhpred; RRID: SCR_010276
Mauve	(Darling et al., 2004)	http://gel.ahabs.wisc.edu/mauve/; RRID: SCR_012852
GraphPad Prism version 8.0 for Mac	GraphPad, Software, La Jolla, California, USA	http://www.graphpad.com; RRID: SCR_022798