Evolution towards Virulence in a *Burkholderia* Two-Component System

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ABSTRACT Bacteria in the *Burkholderia cepacia* complex (BCC) are significant pathogens for people with cystic fibrosis (CF) and are often extensively antibiotic resistant. Here, we assess the impacts of clinically observed mutations in *fixL*, which encodes the sensor histidine kinase FixL. FixL along with FixJ compose a two-component system that regulates multiple phenotypes. Mutations in *fixL* across two species, *B. dolosa* and *B. multivorans*, have shown evidence of positive selection during chronic lung infection in CF. Herein, we find that BCC carrying the conserved, ancestral *fixL* sequence have lower survival in macrophages and in murine pneumonia models than mutants carrying evolved *fixL* sequences associated with clinical decline in CF patients. In vitro phosphotransfer experiments found that one evolved FixL protein, W439S, has a reduced ability to autophosphorylate and phosphorylate FixJ, while *LacZ* reporter experiments demonstrate that *B. dolosa* carrying evolved *fixL* alleles has reduced *fix* pathway activity. Interestingly, *B. dolosa* carrying evolved *fixL* alleles was less fit in a soil assay than those strains carrying the ancestral allele, demonstrating that increased survival of these variants in macrophages and the murine lung comes at a potential expense in their environmental reservoir. Thus, modulation of the two-component system encoded by *fixL*J by point mutations is one mechanism that allows BCC to adapt to the host infection environment.

IMPORTANCE Infections caused by members of the *Burkholderia cepacia* complex (BCC) are a serious concern for patients with cystic fibrosis (CF) as these bacteria are often resistant to many antibiotics. During long-term infection of CF patients with BCC, mutations in genes encoding the FixLJ system often become prevalent, suggesting that these changes may benefit the bacteria during infection. The system encoded by *fixLJ* is involved in sensing oxygen and regulating many genes in response and is required for full virulence of the bacteria in a murine pneumonia model. Evolved *fixL* mutations seen later in infection improve bacterial persistence within macrophages and enhance infection within mice. However, these adaptations are short sighted because they reduce bacterial fitness within their natural habitat, soil.

KEYWORDS *Burkholderia*, evolution, two-component regulatory systems, virulence regulation

The *Burkholderia cepacia* complex (BCC) is a group of more than 20 species of closely related Gram-negative bacilli that can be dangerous respiratory pathogens for people with cystic fibrosis (CF) (1, 2). *B. cenocepacia* and *B. multivorans* are the most common species of BCC seen in infections among CF patients in the United States, although there is significant variability based on geographic region and institution...
(3–7), and *B. multivorans* has emerged as the predominant BCC species infecting CF patients in some regions (4–7). BCC members have caused several outbreaks within the CF community (2), including one outbreak of a highly antibiotic-resistant strain of *B. dolosa* among almost 40 CF patients in Boston (8) and another of *B. cenocepacia* in Toronto (9). BCC can also cause serious infections in individuals with chronic granulomatous disease (CGD) (10). Outbreaks of hospital-acquired BCC infections in non-CF and non-CGD patients have also been increasingly described and are often associated with contaminated medications (11–16), including a recent outbreak associated with contamination of the stool softener docusate with *B. contaminans* (15, 16).

Analysis of genomic diversity arising during *B. dolosa* and *B. multivorans* chronic infections in CF identified the two-component system (TCS) encoded by *fixLJ* as a pathway that is under positive selection, evident from genetic parallelism among many independent infections (17–19). TCSs are one mechanism that bacteria use to sense and respond to their environment (20). Our previous work determined that the TCS encoded by *fixLJ* senses oxygen depletion, is important for virulence in a murine model of pneumonia, and regulates ∼11% of the genome (21). Additionally, we found that the *fixLJ* system is involved in biofilm formation and motility as *B. dolosa* lacking *fixLJ* made more biofilm and had reduced motility (21). The TCS encoded by *fixLJ* was also critical for survival within THP-1-derived human macrophages. These experiments were conducted using a *fixLJ* deletion mutant to determine the effects of deletion of both genes. However, mutations observed in the clinic generate phenotypes that are more nuanced than these large deletions (22). Understanding how these clinically observed mutations alter pathogen phenotypes can inform treatment and prevention regimens. In the current study, we found that mutants carrying clinically observed *fixL* mutations (encoding single amino acid changes) were more virulent, had altered gene expression, and were less able to survive within soil than otherwise isogenic mutants carrying ancestral *fixL* variants. These results highlight the importance of the TCS pathway encoded by *fixLJ* in BCC pathogenesis and provide insight into the evolution of the BCC during chronic infection.

**RESULTS**

Mutations within the predicted sensory domain of BCC *fixL* are associated with a decline in lung function. In a series of published works involving more than 100 *B. dolosa* longitudinal isolates taken over 16 years from 14 different CF patients and 22 *B. multivorans* isolates taken from a single CF patient over 20 years, we identified mutations within *fixLJ* during chronic lung infection (17–19). The predicted domains of FixL along with the amino acid changes encoded by the clinically observed mutations are depicted in Fig. 1a. Most of the amino acid changes are within the predicted PAS and PAC domains, which are conserved sensory domains (23). Based on our previous work (21) and the predicted heme-binding pocket, it appears that the BCC *fixLJ* encodes an oxygen-sensing mechanism. Of note, the FixL proteins of *B. dolosa* (AK34_969) and *B. multivorans* (BMD20_10585) share 98% amino acid identity.

By retrospectively reviewing available clinical records from CF patients included in our previous *B. dolosa* whole-genome sequencing studies (17, 18), we compared the lung function of patients who were infected with *B. dolosa* isolates containing *fixL* mutations to determine if there was a correlation between *fixL* mutations and clinical outcomes. Of the 14 patients from our previous studies (17, 18), only 3 patients had sufficient lung function data that corresponded to the times when *B. dolosa* isolates were collected and sequenced. A steeper decline in lung function, measured by percent predicted forced expiratory volume in 1 s (ppFEV1), was seen in patient L after *B. dolosa* isolates with amino acid changes in the predicted sensory domain of FixL (red arrows) were detected. This slope differs in comparison to earlier time points, before such mutations were detected (Fig. 1c, purple and green arrows, red versus blue line; *P* = 0.05 comparing slopes using linear regression). The single green arrow among the purple arrows demonstrates that multiple lineages coexisted at the same time in the lung (17). Patient J (Fig. 1d) also had an increased rate of decline in lung function
after *B. dolosa* isolates with amino acid changes in the predicted sensory domain were detected. Another patient, patient M (Fig. 1b), who lacked isolates containing mutations in *fixL*, had a more modest decline in lung function over a similar period. These findings suggest a correlation between evolved *fixL* variants and decreased lung function.

To determine the specific phenotypes of evolved *fixL* alleles, we generated otherwise isogenic mutant strains in reference isolate *B. dolosa* AU1058. The *fixL* deletion strain (21) was complemented with the ancestral *fixL* sequence or the evolved sequence found in strain AU0158 (encoding a *FixL* W439S amino acid change). It is worth pointing out that the reference strain AU0158 is itself an evolved strain as it was isolated 3 years after initial *B. dolosa* infection. Strains containing the *fixL* alleles that were associated with lower lung function in patient L (Fig. 1c) were also generated, encoding *FixL* G345S or R347H. These mutants were also complemented with the conserved ancestral sequence of *fixJ* along with ~600 bp upstream, allowing for expression from its native promoter, using a mini-Tn7-based vector, allowing for long-term stability without the need for selection by the insertion into the chromosome (24). We also compared the effects that *fixL* sequence variants have on virulence in *B. multivorans* by generating otherwise isogenic mutants within *B. multivorans* VC7102 (BM2) by replacing a fragment of the *fixL* gene in its native location with a fragment with the desired evolved mutation (25).

*B. multivorans* VC7102 was isolated from a patient with CF with a chronic BCC infection and later had isolates with *fixL* mutations (19). We found that the *B. dolosa* construct carrying the ancestral *fixL* genotype had a slight decrease in growth when grown in vitro at 37°C with ambient oxygen (Fig. S1 in the supplemental material) compared to *B. dolosa* constructs carrying evolved *fixL* genotypes.
This decrease was only noticeable during log-phase growth, and by 24 h, there was only a slight, albeit statistically significant, growth defect. Consistent with our previous findings (21), *B. dolosa* lacking *fixL* had a greater defect in growth than *B. dolosa* carrying any *fixL* genotype. *B. multivorans* carrying ancestral *fixL* genotypes also had a slight growth defect during the log phase compared to *B. multivorans* carrying evolved *fixL* genotypes that recovered by 24 h of growth (Fig. S1).

**BCC strains carrying evolved *fixL* alleles are better able to survive within human macrophages and a murine pneumonia model but are less able to survive in soil.** We compared the virulence of the BCC *fixL* mutants by measuring their ability to invade/ survive within macrophages and within the lungs and spleen in a murine pneumonia model. To measure bacterial invasion/survival within macrophages, we used THP-1 human monocyte cells treated with phorbol 12-myristate 13-acetate (PMA) to differentiate them into macrophage-like cells. *B. dolosa* mutants carrying the evolved *fixL* allele (encoding *FixL* W439S) or the evolved *fixL* alleles associated with a period of clinical decline (encoding *FixL* G345S or R347H) were detected at significantly increased levels (2- to 3-fold higher) compared to mutants carrying the ancestral *fixL* sequence or lacking *fixL* (empty vector) (Fig. 2a). One of the two *B. multivorans* mutants carrying the evolved *fixL* allele (encoding *FixL* T353M) had increased bacterial loads within THP-1-derived macrophages compared to the ancestral strain (VC7102) (Fig. 2b). This increase in bacterial load within the THP-1-derived macrophages could be a function of either increased uptake of the bacteria or increased survival within the macrophage. Our previous work found that *B. dolosa* lacking *fixL* was equally able to be taken up by macrophages while it was less able to survive within macrophages than *B. dolosa* containing *fixL* (21). To determine the mechanism of the increased bacterial load, we conducted a series of time course experiments where we varied the length of the infection before the addition of kanamycin (which kills the extracellular bacteria) to determine the uptake/invasion of the bacteria by the macrophages. We also varied the length of the kanamycin exposure to determine the ability of the bacteria to survive once intracellular. We found that the *B. dolosa* mutant carrying the evolved *fixL* allele (encoding *FixL* W439S) had significantly increased survival (increased bacterial load) within macrophages at 4 and 8 h after a 2-h infection compared to the strain with the *B. dolosa* ancestral *fixL* genotype or the strain lacking *fixL* (Fig. 2c). We also compared the uptake of the *B. dolosa* *fixL* mutants by THP-1-derived macrophages (Fig. 2d) and found that all the *fixL* mutants had equal uptake at initial time points. *B. dolosa* carrying the evolved *fixL* allele (encoding *FixL* W439S) had an increased number of intracellular bacteria at the 2-h time point, which was likely due to its increased ability to survive within the macrophages. These data demonstrate that *B. dolosa* strains carrying evolved *fixL* alleles are better able to survive within macrophages than strains carrying the ancestral *fixL* allele, making these strains carrying evolved *fixL* alleles more virulent.

Our previous work found a correlation between the ability to survive within THP-1-derived macrophages and the ability to persist within murine lungs and to disseminate and persist in the spleen after intranasal inoculation (21). In order to measure the ability of the *fixL* mutants to persist in vivo, C57BL/6 mice were intranasally infected with these BCC mutants using similar methods. Mice infected with *B. dolosa* carrying the evolved *fixL* allele (encoding *FixL* W439S) had 4- to 5-fold higher levels of bacteria within the lungs and spleen than mice infected with *B. dolosa* carrying the ancestral *fixL* allele or lacking *fixL* (empty vector) (Fig. 2e), indicating that these strains carrying evolved *fixL* alleles are more virulent. Mice that were infected with *B. multivorans* VC7102 carrying the evolved *fixL* allele (encoding *FixL* T353M) had significantly increased bacterial loads in the lungs and spleen compared to mice infected with bacteria carrying the ancestral *fixL* allele, while the other evolved *fixL* mutation (encoding *FixL* R400G) did not (Fig. 2f). The *FixL* T353M-expressing *B. multivorans* mutant also was better able to survive within the THP-1-derived macrophages than the ancestral strain, while the *FixL* R400G-expressing *B. multivorans* mutant was not (Fig. 2b). It is likely that the mutation encoding the R400G mutation in *FixL* confers a benefit that was not investigated in this study.
**FIG 2** *Burkholderia cepacia* complex strains with evolved *fixL* alleles are more virulent in human macrophages and a murine pneumonia model but are less able to survive in soil. PMA-treated THP-1 human macrophages were infected with \( \sim 2 \times 10^6 \) CFU/well (MOI of \( \sim 10:1 \)) of (a) *B. dolosa* strain (Continued on next page)
To determine if the fixL mutations that confer an increased level of virulence within the host came at the expense of survival in the soil, a natural ecological niche of the BCC, we measured the ability of the B. dolosa fixL mutants to survive within soil for 10 days. We found that all three of the strains carrying evolved fixL alleles (encoding FixL W439S) had an ~80 to 85% reduction in the ability to survive within soil compared to the strain carrying the ancestral fixL allele (Fig. 2g). Bacteria lacking fixLJ had reduced survival in the soil, demonstrating the importance of the fixL gene to survival both within the host and in the environment.

**BCC strains carrying evolved fixL alleles are more motile, make less biofilm, and have altered gene expression.** Our previous work showed that B. dolosa lacking fixLJ produced increased levels of biofilm and had decreased motility compared to B. dolosa containing fixLJ (21). We found that both B. dolosa and B. multivorans carrying evolved fixL alleles produced significantly less biofilm than otherwise isogenic strains carrying the ancestral fixL sequence (Fig. 3a and b), although overall biofilm production in this assay was low. To further evaluate the potential contribution of biofilm in the in vitro invasion assay described above, we performed Wright-Giemsa staining of THP-derived macrophages infected with B. dolosa. We observed that B. dolosa carrying either the ancestral fixL sequence or an evolved fixL sequence (encoding FixL W439S) was not seen in biofilm-like clusters but was instead observed to exist as single or small clusters made up of no more than 4 cells, with ~80% of the aggregates consisting of 1 to 2 bacterial cells (Fig. S2). B. dolosa and B. multivorans mutants with evolved fixL alleles also had increased motility compared to mutants carrying the ancestral fixL allele (Fig. 3c and d). Interestingly, B. dolosa carrying the ancestral fixL allele was completely nonmotile, where the diameter plotted in Fig. 3c (12 mm) was the diameter of the 10-μl drop placed on the agar surface. B. dolosa carrying an empty vector, which lacks fixLJ, still had the ability to swim, albeit at lower levels than B. dolosa carrying an evolved fixL allele (Fig. 3c).

To identify the differentially expressed genes that were responsible for the various observed phenotypes, we measured global transcript levels using RNA sequencing (RNA-seq) of bacteria grown in vitro to log phase (Table S1). Previously we found that ~11% of the genome was differentially expressed in a B. dolosa fixLJ deletion mutant compared to the parental strain (21). Here, the B. dolosa FixL W439S-expressing mutant had 205 genes that were significantly downregulated and 302 genes that were significantly upregulated (q value of <0.05, at least 2-fold difference; Fig. 3e) compared to B. dolosa carrying the ancestral fixL allele. We sought to identify pathways that were differentially regulated by analyzing enriched Gene Ontology (GO) terms of the genes that were significantly differentially expressed. Surprisingly, there were no GO terms that were enriched among genes that were significantly upregulated in B. dolosa carrying the ancestral fixL allele compared to B. dolosa carrying the evolved fixL allele. However, there were four GO terms that were enriched among genes that were differentially upregulated in B. dolosa carrying the evolved fixL allele compared to B. dolosa
Burkholderia cepacia complex strains with evolved fixL alleles were more motile, made less biofilm, and had altered gene expression. Biofilm formation of (a) B. dolosa strain AU0158 or (b) B. multivorans strain VC7102 mutants carrying fixL alleles on PVC plates as measured by crystal violet staining at 48 h. Means from three separate experiments with 5 to 6 replicates per experiment are plotted with error bars representing one standard deviation; *, P < 0.05 by ANOVA with Tukey’s multiple-comparison test compared to ΔfixL/FixL/J or VC7102. Motility of (c) B. dolosa strain AU0158 or (d) B. multivorans strain VC7102 mutants carrying fixL alleles on low-density (0.3%) LB agar and swimming distance were measured after incubation for 48 h. Means from three separate experiments with 3 to 4 replicates per experiment are plotted with error bars representing one standard deviation; *, P < 0.05 by ANOVA with Tukey’s multiple-comparison test compared to construct carrying ancestral fixL allele; #, P < 0.05 by ANOVA with Tukey’s multiple-comparison test. (e) Volcano plot depicting the differential regulation of genes. Green dots signify genes with expression 2-fold lower in the B. dolosa strain AU0158 mutant carrying the evolved fixL allele (encoding FixL W439S) than in a mutant carrying the ancestral fixL allele, with a q-value of <0.05. Red dots signify genes with expression 2-fold higher in the mutant carrying the evolved fixL allele (encoding FixL W439S) than in a mutant carrying the ancestral fixL allele, with a q-value of <0.05. (f) GO terms that were enriched with an adjusted P value of <0.05 among genes that were statistically upregulated (q-value of <0.05, at least 2-fold) in B. dolosa carrying the evolved fixL allele (encoding FixL W439S) relative to B. dolosa carrying the ancestral fixL allele.
carrying the ancestral fixL allele (encoding FixL W439S; Fig. 3f). Three of these GO terms are associated with motility or chemotaxis and the fourth is associated with signal transduction. A subset of the significantly upregulated genes associated with motility and flagellar assembly in the B. dolosa FixL W439S-expressing mutant are listed in Table 1. Additionally, we confirmed the differential expression levels of fliC and motA transcripts in B. dolosa carrying the ancestral fixL allele, evolved fixL allele (encoding FixL W439S), and empty vectors using reverse transcription-quantitative PCR (qRT-PCR) (Fig. S3).

We also identified several differentially expressed genes with homologs in other bacteria that have been shown to play a role in cyclic diguanylate monophosphate (c-di-GMP) metabolism that are important for motility and biofilm regulation (Table S2). Notably, a homolog of the predicted phosphodiesterase cpdA that could hydrolyze c-di-GMP, AK34_1958, was significantly downregulated in B. dolosa carrying the ancestral fixL allele compared to in B. dolosa carrying the evolved fixL allele (~8.5-fold; Table S2). B. pseudomallei (26) and B. cenocepacia (27, 28) mutants lacking cpdA had reduced motility, consistent with the B. dolosa strain carrying the ancestral fixL allele, which had lower cpdA transcript levels and lower motility than B. dolosa carrying the evolved fixL allele. Similar to B. pseudomallei and B. cenocepacia cpdA, B. dolosa AU0158 cpdA has predicted GGDEF and EAL domains, but only the EAL domain responsible for phosphodiesterase activity is predicted to be enzymatically active based on amino acid sequence at the catalytic site. B. pseudomallei (26) and B. cenocepacia (27) mutants lacking cpdA had increased levels c-di-GMP. The expression changes of these genes (Table S2) suggest that decreased intracellular c-di-GMP levels may be found in the B. dolosa mutants carrying evolved fixL alleles, and this may explain the increased motility and decreased biofilm seen in these mutants (26, 28, 29). But, surprisingly, there was a significant increase in intracellular c-di-GMP levels found in the B. dolosa mutant carrying the evolved fixL allele (Fig. S4).

Evolved fixL alleles downregulate fix pathway activity. Most two-component systems function by regulating gene expression when activated by a specific signal. The first component, the sensor kinase, senses the signal and autophosphorylates. The phosphate is then transferred to the second component, the response regulator, which can then regulate gene transcription (20). We conducted in vitro phosphorylation assays with recombinant truncated FixL and full-length FixJ proteins to measure the ability of the different FixL variants to phosphorylate themselves and then subsequently FixJ. The recombinant FixL was truncated to exclude the predicted transmembrane domains to facilitate protein purification, so the recombinant protein began at amino acid 329. The recombinant FixL proteins still retain the predicted PAS/PAC

### Table 1

| Gene name (genome designation) | Description | Fold change in B. dolosa carrying evolved fixL allele (encoding FixL W439S) |
|-------------------------------|-------------|--------------------------------------------------------------------------------|
| AK34_2913                     | Flagellin protein FliC                             | 205.8                                                                           |
| AK34_2901                     | Flagellar motor rotation protein MotA             | 46.8                                                                            |
| AK34_2900                     | Flagellar motor rotation protein MotB             | 19.9                                                                            |
| AK34_2902                     | Flagellar transcriptional activator FlhC         | 24.9                                                                            |
| AK34_2903                     | Flagellar transcriptional regulator FlhD          | 10.9                                                                            |
| AK34_2914                     | Flagellar cap protein FliD                        | 22.7                                                                            |
| AK34_2885                     | Flagellar biosynthesis protein FliA               | 46.5                                                                            |
| AK34_2886                     | Flagellar biosynthesis protein FliB               | 28.1                                                                            |
| AK34_83                       | Flagellar basal-body P-ring formation protein FlgA| 18.2                                                                            |
| AK34_86                       | Flagellar basal-body rod modification protein FldD| 49.8                                                                            |
| AK34_87                       | Flagellar hook protein FliE                       | 32.0                                                                            |
| AK34_88                       | Flagellar basal-body rod protein FldF             | 50.5                                                                            |
| AK34_2914                     | Flagellar cap protein FliD                        | 22.7                                                                            |
| AK34_3043                     | Flagellar motor switch protein FimM              | 17.2                                                                            |
| AK34_3044                     | Flagellar motor switch protein FimN              | 9.0                                                                             |
sensory domain (with oxygen-binding heme moiety) and histidine kinase domain. The use of similar truncated recombinant proteins in *in vitro* phosphotransfer experiments has been reported for other two-component systems (30–32). We hypothesized that FixL variants that had different phenotypes would have differing levels of autophosphorylation and/or phosphotransfer. Figure 4a shows that the ancestral FixL had higher levels of autophosphorylation than the evolved FixL W439S. When the levels of autophosphorylation were quantified, the evolved FixL W439S had approximately 50% of the level of autophosphorylation of the ancestral sequence (Fig. 4b). Interestingly, the other two evolved FixL proteins, G345S and R347H, had increased levels of autophosphorylation compared to the ancestral protein (Fig. 4a and b) despite making less biofilm and being more motile like the other evolved protein FixL W439S. Equal amounts of each of the purified protein were used in each reaction, and each protein preparation was >90% pure (Fig. S5). When the autophosphorylation levels were quantified relative to the 1-min time point, only the FixL W439S autophosphorylation levels decreased, while the other three variants increased or stayed the same (Fig. S6).

We also measured the ability of the recombinant FixL proteins to phosphorylate the response regulator FixJ. As the evolved FixL W439S protein had lower levels of autophosphorylated FixL, that variant had lower levels of phosphotransfer to FixJ than the ancestral protein (Fig. 4c and d). The evolved FixL W439S protein had a different phosphotransfer profile compared to the ancestral protein and the other two evolved proteins when the level of phosphorylated FixJ was quantified relative to the level of autophosphorylated FixL. The FixL W439S protein had a more rapid phosphotransfer to FixJ and potentially a greater level of dephosphorylation of FixJ (Fig. 4d). The other two evolved FixL proteins R347H and G345S had similar levels of phosphotransfer to ancestral FixL (Fig. 4d) despite having an increased ability to autophosphorylate compared to ancestral FixL (Fig. 4b). To further explore and quantify the effects of *fixL* mutations on the *fix* pathway in *B. dolosa*, we also measured *fix* activity using a LacZ reporter conjugated into *B. dolosa* carrying the various *fixL* alleles (21). We found that *B. dolosa* carrying the ancestral and the evolved *fixL* alleles (encoding FixL W439S, G345S, or R347H) had an increase in *fix* pathway activity when the construct was grown in low oxygen (<5%), demonstrating that all alleles are activated in low oxygen (Fig. 4e).

Consistent with the *in vitro* phosphorylation experiments, *B. dolosa* carrying the ancestral *fixL* sequence had higher *fix* pathway activity than *B. dolosa* carrying the evolved *fixL* allele (encoding FixL W439S) when grown in either ambient or low oxygen. *B. dolosa* carrying the other two evolved *fixL* alleles (encoding FixL G345S or R347H) had lower *fix* pathway activity than *B. dolosa* carrying the ancestral allele in both ambient and low oxygen, demonstrating decreased *fix* pathway activity in all the *B. dolosa* mutants carrying evolved *fixL* alleles.

**DISCUSSION**

In previous work, we demonstrated that the BCC two-component system encoded by *fixL* shows evidence of positive selective pressure during chronic lung infection in CF patients (17–19) and is critical for full BCC virulence (21). In this study, we evaluated the function of evolved *fixL* sequence variations in both *B. dolosa* and *B. multivorans* by generating otherwise isogenic mutants carrying the evolved *fixL* alleles. These mutants carrying evolved *fixL* alleles were more virulent (as having increased bacterial levels within tissue or macrophages), more motile, and produced less biofilm (Fig. 2 and 3). The *fixL* mutations that occurred during chronic infection downregulated *fix* pathway signaling, demonstrating that high *fix* pathway activity is associated with lowered virulence (Fig. 4).

We found that BCC carrying ancestral *fixL* sequences had reduced motility and reduced virulence in both murine and macrophage models compared to variants carrying evolved sequences (Fig. 2 and 3), suggesting that reduced motility was contributing to the reduced virulence, as has been suggested by other studies (33–35). Our previous work demonstrated that *B. dolosa* lacking flagella were equally virulent as parental flagellated strains,
suggesting that motility plays a minimal role in the infection models evaluated (21). We analyzed the transcriptomes of *B. dolosa* mutants carrying ancestral or evolved fixL alleles to understand the mechanisms of the altered phenotypes seen between the two mutants. Among the genes that were identified to be differentially expressed between *B. dolosa*
carrying different fixL sequences were genes that have homologs in other bacterial species that are involved in c-di-GMP metabolism (Table S2 in the supplemental material) (26, 28, 29). Surprisingly, B. dolosa carrying the ancestral fixL sequence that produced more biofilm had lower levels of c-di-GMP than B. dolosa carrying the evolved fixL allele (Fig. S3). The increased biofilm seen in B. dolosa carrying the ancestral fixL allele and in B. dolosa lacking fixLJ was independent of increased c-di-GMP levels, indicating that there are additional pathways involved in biofilm formation. Furthermore, high levels of c-di-GMP have been shown to stimulate the production of extracellular polysaccharides that leads to increased biofilm formation (28, 36). Interestingly, the genes that are responsible for c-di-GMP-induced production of polysaccharides (Burkholderia cenocepacia exopolysaccharide [Bep], poly-N-acetylg glucosamine [PNAG], and cepacian) (37–39) were not differentially expressed by RNA-seq analysis (Table S1), suggesting that c-di-GMP-independent mechanisms of biofilm production are involved.

One potential c-di-GMP-independent mechanism of biofilm production involves the wsp system that can promote biofilm formation in B. cenocepacia without direct activation of a diguanylate cyclase (40, 41). Multiple components of the wsp system were 2- to 4-fold upregulated in B. dolosa carrying the ancestral fixL allele (Table S2), suggesting a potential role of the wsp system in fix pathway-mediated biofilm formation. The contribution of biofilm formation to BCC virulence remains unclear, as mutants carrying ancestral fixL sequences produced more biofilm and were less pathogenic in our murine model of pneumonia than isogenic constructs with evolved fixL alleles (Fig. 2 and 3). Similarly, in our previous study, B. dolosa fixLJ deletion mutants made more biofilm and were less pathogenic than the parental strain that contained fixLJ (21). These findings suggesting that BCC biofilms may not be beneficial for infection are supported by a study of CF lung explants from patients infected with Pseudomonas aeruginosa and/or BCC that were stained using species-specific antibodies (42). BCC bacteria were rarely found in biofilm-like structures, while P. aeruginosa were often found in such structures, suggesting that BCC may not form large biofilms during infection but may form small aggregates. The conversion of BCC from a mucoid, biofilm-producing state to a nonmucoid phenotype is often observed during chronic infection and is correlated with worse clinical outcomes (notably different from P. aeruginosa, where mucoid strains are associated with clinical decline) (43). Interestingly, these more virulent isolates are found early during infection for P. aeruginosa but are found later during infection for CF patients infected with BCC (43, 44).

Since increased biofilm formation is not associated with increased BCC virulence (Fig. 2 and 3), we hypothesized that biofilm formation may allow for B. dolosa to better survive within the soil. But, the ability to make biofilms does not, on its own, confer the ability to survive within soil, as B. dolosa lacking fixLJ was unable to persist in the soil at high levels (Fig. 2) despite making higher levels of biofilm (Fig. 3). The mutations in fixLJ that confer increased virulence are likely dead-end mutations, as they make the bacteria less able to survive in the natural BCC reservoir. It is possible that these mutations make the bacteria more transmissible between human hosts; future work will investigate this.

To better understand the mechanism of altered phenotypes seen in the mutants carrying different fixL alleles, we examined genes that had the largest magnitude of differential expression observed by RNA-seq. One such gene was an AraC family transcription regulator (AK34_4608) that was significantly downregulated (~95-fold) in B. dolosa carrying the evolved fixL allele (Table S2). Expression of this gene is potentially detrimental to bacterial dissemination since a homolog of this gene was found to be downregulated in B. cenocepacia blood isolates taken from CF patients with cepacia syndrome compared to in lung isolates taken at same time (45). Additionally, a homolog of this same gene was found to be downregulated in B. pseudomallei isolates from CF patients taken at late versus early time points (46). Other genes that were differentially expressed encode a putative CidA/CidB-like holin/antiholin system (AK34_3040, AK34_3041) that was significantly downregulated (~64- and 42-fold, respectively) in
**TABLE 2** Strains used in this study

| Strain          | Notes                        | Source               |
|-----------------|------------------------------|----------------------|
| **E. coli**     |                              |                      |
| NEB S-alpha competent E. coli | DH5α derivative cloning strain | New England BioLabs |
| RHO3            | Mobilizer strain, Km⁶; SM10(λpir) Δasd::FRT ΔaphA::FRT | 25                   |
| BL21(DE3)       | Protein expression strain    | Invitrogen           |
| **BCC**         |                              |                      |
| B. dolosa AU0158| Clinical isolate             | John LiPuma          |
| B. multivorans VC7102 |                          | 19                   |
| ΔfixL + W4395 fixLJ | B. dolosa ΔfixL + pfxLJ carrying W4395 FixL and FixJ, integrated at attTn7 site downstream of AK34_4894 | 21                   |
| ΔfixL + empty vector | B. dolosa ΔfixL + empty pUC1BT-mini-Tn7-Tp integrated at attTn7 site downstream of AK34_4894 | 21                   |
| ΔfixL + fixLJ   | B. dolosa ΔfixL + pfxLJ carrying W439 FixL and FixJ, integrated at attTn7 site downstream of AK34_4894 | This study           |
| ΔfixL + R437H fixLJ | B. dolosa ΔfixL + pfxLJ carrying R437H FixL and FixJ, integrated at attTn7 site downstream of AK34_4894 | This study           |
| ΔfixL + G345S fixLJ | B. dolosa ΔfixL + pfxLJ carrying G345S FixL and FixJ, integrated at attTn7 site downstream of AK34_4894 | This study           |
| VC7102 (FixL T353M) | VC7102 with T353M mutation in native fixL gene using pBM_FixL_T353M | This study           |
| VC7102 (FixL R400G) | VC7102 with R400G mutation in native fixL gene using pBM_FixL_R400G | This study           |
| ΔfixL + W4395 fixLJ/pfx- reporter | B. dolosa ΔfixL complemented with W4395 fixLJ carrying pfxK reporter | 21                   |
| ΔfixL + empty vector/pfxK-reporter | B. dolosa ΔfixL complemented with empty vector carrying pfxK reporter | 21                   |
| ΔfixL + fixLJ/pfxK-reporter | B. dolosa ΔfixL complemented with fixLJ carrying pfxK reporter | This study           |
| ΔfixL + R437H fixLJ/pfxK-reporter | B. dolosa ΔfixL complemented with R437H fixLJ carrying pfxK reporter | This study           |
| ΔfixL + G345S fixLJ/pfxK-reporter | B. dolosa ΔfixL complemented with G345S fixLJ carrying pfxK reporter | This study           |

*Km, kanamycin.

B. dolosa carrying the evolved fixL allele (Table S2). Homologs of this system were found to be upregulated in P. aeruginosa when grown in vitro with Staphylococcus aureus (47), suggesting that upregulation of this system may result in more autolysis and increased biofilm formation (48). Further investigation is needed to determine the role of these genes in BCC virulence and their role in fix pathway-mediated phenotypes.

In this study, we have characterized the effects of mutations within the oxygen-sensing two-component system encoded by fixLJ that arise during chronic infection in people with CF. Some of these mutations were associated with a period of clinical decline. Host-evolved BCC carrying fixL mutants were more virulent, more motile, and produced less biofilm. In contrast, P. aeruginosa becomes less motile, produces more biofilm, shows increased antibiotic resistance, and has increased auxotrophy in chronic infections, ultimately leading to the evolution of reduced virulence of late isolates in animal models of infection (49, 50). Less is known about phenotypic changes that occur during chronic BCC infection, and most prior work has focused on B. cenocepacia (51). The findings from this study demonstrate a novel way that the BCC adapts to the host by making the bacteria more pathogenic at the cost of being less able to survive within the environment.

**MATERIALS AND METHODS**

**Clinical data.** Records of B. dolosa-infected patients were reviewed under Boston Children’s Hospital institutional review board protocol number M10-08-0370.

**Bacterial strains, plasmids, cell lines, and growth conditions.** All strains used and generated in this study are listed in Table 2. B. dolosa strain AU0158 was obtained from John LiPuma (University of Michigan) and is an early isolate from the index patient from the B. dolosa outbreak (about 3 years into the outbreak). BCC and E. coli were grown on LB plates or in LB medium and were supplemented with the following additives: ampicillin (100 μg/ml), kanamycin (50 μg/ml for E. coli, 1 mg/ml for BCC), trimethoprim (100 μg/ml for E. coli, 1 mg/ml for BCC), gentamicin (15 or 50 μg/ml), chloramphenicol (20 μg/ml), or dianaminopicolinic acid (200 μg/ml). Plasmids that were used in this study are listed in Table 3. The human monocytic cell line THP-1 was obtained from ATCC and grown at 37°C with 5% CO₂. THP-1 cells were cultured in RPMI 1640 medium containing 2 mM l-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4,500 mg/liter glucose, and 1,500 mg/liter sodium bicarbonate supplemented with 10% heat-inactivated fetal calf serum (FCS) (Invitrogen) and 0.05 mM 2-mercaptoethanol. Low-oxygen environments were generated using the CampyGen gas generating system (Thermo Fisher), and the low-oxygen concentration (<5%) is based on the manufacturer’s specifications.
**TABLE 3** Plasmids used in this study

| Plasmid Name | Notes | Source |
|--------------|-------|--------|
| pEXKm5      | Km', sacB, gusA | 25 |
| pTN53       | Amp', helper plasmid for mini-Tn7 integration into attTn7 site | 53 |
| pRK2013     | Km' conjugation helper | 52 |
| pSCrhaB2    | Tp', ori<sub>kan</sub>hphR, rhaS | 69 |
| pUC18T-mini-Tn7-Tp | Amp', Tp' on mini-Tn7-Tp; mobilizable | 52 |
| pfxL/W439S  | pUC18T-mini-Tn7-Tp carrying W439S FixL and FixJ with 670 bp upstream flanking | 21 |
| pfxL-unc    | pUC18T-mini-Tn7-Tp carrying FixL and FixJ with 670 bp upstream flanking | This study |
| pfxL/R347H  | pUC18T-mini-Tn7-Tp carrying R347H FixL and FixJ with 670 bp upstream flanking | This study |
| pfxL/G455S  | pUC18T-mini-Tn7-Tp carrying G455S FixL and FixJ with 670 bp upstream flanking | This study |
| pBM_FixL_T353M | pEXKm5 containing 1.5-kbp fragment encoding T353M FixL mutation | This study |
| pBM_FixL_R400G | pEXKm5 containing 1.5-kbp fragment encoding R400G FixL mutation | This study |
| pfxK-reporter | pSCrhaB2 carrying B. dolosa fixK-lacZ fusion with Km' in place of Tp' | 21 |
| pENTR       | Gateway cloning entry vector | Invitrogen |
| pTRX-HIS-DEST | Expression vector for response regulator Amp' | 30 |
| pHIS-MBP-DEST | Expression vector for histidine kinase Amp' | 30 |
| pENTR-FixJ  | B. dolosa AU0158 fixJ cloned into pENTR | This study |
| pENTR-FixL  | B. dolosa AU0158 fixl amino acids 329 to 851 cloned into pENTR | This study |
| pFixL_Expression | B. dolosa fixl cloned into pTRX-HIS-DEST for purification | This study |
| pFixL_W439_expression | B. dolosa fixL containing W439 sequence cloned into pHIS-MBP-DEST for purification | This study |
| pFixL_W439S_expression | B. dolosa fixL containing W439S sequence cloned into pHIS-MBP-DEST for purification | This study |
| pFixL_R347H_expression | B. dolosa fixL containing R347H sequence cloned into pHIS-MBP-DEST for purification | This study |
| pFixL_G455S_expression | B. dolosa fixL containing G455S sequence cloned into pHIS-MBP-DEST for purification | This study |

*Amp, ampicillin; Km, kanamycin; Tp, trimethoprim.*

**Genetic manipulations and strain construction.** To generate *B. dolosa* mutants carrying fixL mutations, we introduced the desired mutations using the QS site-directed mutagenesis kit (New England Biolabs) into pfxL (21). pfxL contains the AU0158 fixL sequence along with 670 bp upstream within the pUC18-mini-Tn7-Tp back bone, allowing for stable chromosomal integration at an attTn7 site (24, 52), and was renamed pfxL/W439S for this study. Point mutations were verified by Sanger sequencing. The *B. dolosa* fixL complementation vectors and the corresponding empty vector controls were conjugated into the AU0158 fixL deletion mutant with pRK2013 and pTN53 using published procedures (21). Conjugants were selected for by plating on LB agar containing trimethoprim (1 mg/ml) and gentamicin (50 μg/ml). Insertions into the attTn7 site downstream of AK34, 4894 were confirmed by PCR. To generate *B. multivorans* VC7102 mutants carrying fixL mutations, we introduced the desired fixL mutations in the native fixL gene using the suicide plasmid pEXKm5 (25). Briefly, approximately 1 kb upstream and downstream of the desired mutation were PCR amplified, as was pEXKm5, and then the two fragments were joined using the NEBuilder HiFi DNA assembly master mix (New England BioLabs) per the manufacturer’s protocol. The plasmids were Sanger sequenced and transformed into RHO3 E. coli and then conjugated into *B. multivorans* VC7102 (24, 53). Conjugants were selected for on LB with kanamycin at 1 mg/ml. To resolve merodiploidy, conjugants were counterselected against by plating on LB with 15% (wt/vol) sucrose and incubated for 2 days at 30°C. Clones were screened for the introduction of the desired mutation by PCR and Sanger sequencing.

Expression vectors for FixL and FixL His<sub>6</sub>-tagged proteins were generated using the Gateway high-throughput recombinational cloning system (Invitrogen). The entire fixL gene or fixL amino acids 329 to 851 (lacking transmembrane domains) were amplified from *B. dolosa* AU0158 and cloned in pENTR Gateway LR clonase reactions were used to move fixL or fixl into pHIS-MBP-DEST or pTRX-HIS-DEST, respectively, for expression (30). fixL mutations were introduced using a QS site-directed mutagenesis kit (New England Biolabs), and sequences were confirmed by Sanger sequencing. To generate fixK reporter strains, a fixK-lacZ fusion reporter was conjugated into *B. dolosa* constructs as previously described (21).

**Bacterial invasion assays.** The ability of *B. dolosa* to invade and persist within macrophages was determined using published protocols (21). Human THP-1 monocyes were differentiated into macrophages by seeding 1 ml into 24-well plates at 8 x 10<sup>5</sup> cells/ml with 200 nM phorbol 12-myristate 13-acetate (PMA), THP-1-derived macrophages were infected with log-phase grown BCC washed in RPMI three times at ~2 x 10<sup>5</sup> CFU/well (multiplicity of infection [MOI] of ~10:1). Plates were spun at 500 g for 5 min to synchronize infection and were then incubated for 15 min to 2 h at 37°C with 5% CO<sub>2</sub>. To determine the total number of bacteria, wells were treated with 100 μl of 10% Triton X-100 lysis buffer (final concentration, 1% Triton X-100), serially diluted, and plated to enumerate the number of bacteria. To determine the number of intracellular bacteria, infected wells were washed two times with phosphate-buffered saline (PBS) and then incubated with RPMI + 10% heat-inactivated FCS with kanamycin (1 mg/ml) for 1 to 24 h. Monolayers were washed three times with PBS and lysed with 1% Triton X-100, serially diluted, and plated to enumerate the number of bacteria. In some experiments, THP-1 cells were differentiated into macrophage-like cells using PMA described above on 8-well chamber slides (Lab-Tek) and were infected with 2 x 10<sup>5</sup> CFU for 2 h, and cells were washed with PBS 4 times and stained and fixed with a Hema 3 stain set (Fisher Scientific) per the manufacturer’s protocol.
Murine model of pneumonia. All animal protocols and procedures were approved by the Boston Children's Hospital Institutional Animal Care and Use Committee (assurance number A3303-01). The specific protocol number is 18-01-3617R. All animal protocols are compliant with the NIH Office of Laboratory Animal Welfare, the Guide for the Care and Use of Laboratory Animals, the US Animal Welfare Act, and the PHS Policy on Humane Care and Use of Laboratory Animals. Female C57BL/6 mice 6 to 8 weeks of age were obtained from Taconic Biosciences. Mice were maintained at the animal facilities at Boston Children's Hospital. Mice were anesthetized with ketamine (100 mg/kg) and xylazine (13.3 mg/kg) given intraperitoneally. While the mice were held in dorsal recumbency, 10 μl of inoculum was instilled in each nostril (20 μl total). The inoculum consisted of log-phase B. dolosa washed in PBS and diluted to a concentration of ~2 × 10^6 CFU/ml (4 × 10^6 CFU/mouse). Mice were euthanized 7 days after infection by CO₂ overdose, and lungs and spleens were aseptically removed. Lungs and spleens were weighed and placed into 1 ml of 1% protease peptone in water, homogenized, and then serially diluted and plated on oxidation/fermentation-polymyxin-bacitracin-lactose (OPFBL) plates.

Soil survival assays. Top soil (coast of Maine) was autoclaved twice after removing stones and other large debris. One gram of soil was then placed into a 14-ml polystyrene tube. B. dolosa was grown overnight in M9 medium supplemented with 20 μM succinate, 2 μM MgSO₄, and 0.1 μM CaCl₂ at 37°C with shaking at 200 rpm. B. dolosa inocula were then washed in M9 and diluted to 1 to 3 × 10⁶ CFU/ml in M9. Two-hundred microliters of each bacterial suspension was added to the tubes containing 1 g of soil. Soil tubes were incubated for 10 days at 30°C without agitation. After that, 1 ml of 1% protease peptone in water with 1% Triton X-100 was added to each tube, and the tubes were vigorously vortexed. Soil was allowed to settle for 15 min, after which bacteria were enumerated by serial plating on Trypticase soy agar (TSA) plates.

Biofilm formation. The ability to form biofilms on polyvinyl chloride (PVC) plates was determined using published methods (54). Briefly, overnight cultures were diluted in Trypticase soy broth (TSB) with 1% glucose and pipetted into wells of a 96-well PVC plate. Plates were incubated for 48 h at 37°C, and unattached bacteria were washed with water. Biofilms were stained with 0.5% crystal violet, excess stain was washed away, and stain was solubilized with 33% acetic acid. The solution was transferred to a flat-bottom plate, and then biofilm amount was quantified by measuring optical density at 540 nm (OD₅₄₀).

Motility assay. The ability of B. dolosa to swim was measured in low-density LB agar using a modification of published methods (55). Briefly, 10 μl of overnight B. dolosa culture was plated in the center of low-density (0.3% agar) LB plates. Plates were incubated agar side down for 48 h at 37°C when swimming diameter was measured. The swimming diameter was visible throughout the thickness of the agar.

RNA-seq. RNA was isolated from log-phase B. dolosa (two biological replicates per construct) using the Ribopure bacterial RNA purification kit (Ambion) per the manufacturer’s protocol, and contaminant DNA was removed using DNase. RNA was processed, and libraries were generated as previously published (21). Samples were sequenced using single-end 50 bp reads using the Illumina HiSeq platform. Data analysis was done using Galaxy (https://usegalaxy.org) (56). Reads (9 to 12 million reads per replicate) were trimmed using the Trimmomatic tool (57) and mapped to the B. dolosa AU0158 genome (GenBank assembly accession number GCA_000959505.1) (58) using BowTie2 with very sensitive local preset settings (59). Differentially expressed genes were identified using CuffDiff using the Benjamin-Hochberg procedure to determine the q value (P value corrected for multiple comparisons) (60). Reads were deposited to BioProject PRJNA579568. GO terms that were enriched among genes that were differentially regulated were identified using GoSeq using a Wallenius approximation and a Benjamini-Hochberg test to determine a corrected P value (61). GO terms that were considered to be enriched had a corrected P value of <0.05.

qRT-PCR. cDNA was synthesized from 2 μg of RNA using the ProtoScript II first strand cDNA synthesis kit (New England BioLabs) per the manufacturer’s protocol. cDNA was cleaned using a QIAquick PCR purification kit (Qiagen). Oligonucleotides to amplify gyrB, rpoD, and flIC were previously published (21), and motA was amplified using 5′-GTGAAGATCGGGCTCTTGT-3′ and 5′-GGACGCTATATGGAGCTGTG-3′. Genes were amplified using oligos FastStart essential DNA green master mix (Roche) per the manufacturer’s protocol. Expression was determined relative to B. dolosa AU0158 carrying the evolved fixL allele (encoding FixL W439S) normalized by gyrB (AK34_3072) or rpoD (AK34_4533) expression using the threshold cycle (ΔΔCₚ) method (62). Both gyrB and rpoD had similar expression by RNA-seq between AU0158 and the fixL deletion mutant, and these genes have been used to normalize expression in B. cepacia in other studies (63, 64).

Protein expression and purification. FixL and FixJ expression vectors were transformed into E. coli BL21(DE3) cells, and protein was expressed and purified using nickel affinity columns following published protocols (30).

In vitro phosphorylation. Autophosphorylation and phosphotransfer assays were done as previously described (65). Briefly, FixL variants were used at a final concentration of 2.5 μM mixed with a final concentration of 5 mM MgCl₂, 0.5 mM ATP, and 2.5 μCi of [γ-³²P]ATP (stock of 6,000 Ci/mmol, 10 μCi/ml; PerkinElmer). Autophosphorylation reactions were performed at 30°C with ambient oxygen and were stopped at the indicated time points by the addition of 4 × sample buffer (200 mM Tris-HCl at pH 6.8, 400 mM dithiothreitol (DTT), 8% SDS, 0.4% bromphenol blue, 40% glycerol). For phosphotransfer assays, FixL variants were autophosphorylated using the above parameters at 30°C for 15 min and then incubated with reaction mixtures containing the response regulator FixJ and MgCl₂ at final concentrations of 5 μM and 5 mM, respectively. Phosphotransfer reactions were run at 30°C with ambient oxygen. Reactions were stopped at the indicated time points with the addition of 4 × sample buffer. Samples were then run on an “any kD” Bio-Rad mini-protein TGX gels for 50 min at 150 V. Gels were exposed to phosphor screens for 4 to 5 h so that phosphorylated protein bands could be observed. Screens were
imaged using the Typhoon-FLA9500 imager with a "phosphor" setting and a resolution of 50 μm. Band intensity of phosphorylated proteins was quantified using ImageJ.

**Reporter assay.** BCC carrying a fxk-lacZ reporter plasmid was grown overnight in LB with kanamycin (1 mg/ml). Cultures were subcultured in LB in ambient oxygen or LB that had been degassed in the CampyGen gas generating system (Thermo Fisher). Cultures were grown in ambient oxygen with shaking (200 rpm) at 37°C or within the CampyGen gas generating system at 37°C for 4 to 6 h. The level of fxk pathway-driven LacZ activity was measured by determining Miller units following published procedures (66).

**c-di-GMP quantification.** B. dolosa constructs were grown to stationary phase when 50-ml aliquots were spun down, and c-di-GMP was extracted using ice-cold extraction buffer (methanol:acetonitrile:distilled water, 40:40:20 + 0.1 N formic acid). c-di-GMP levels were measured using mass spectroscopy as previously described (67).

**Data availability.** RNA-seq reads have been deposited to BioProject under accession number PRJNA579568 (https://www.ncbi.nlm.nih.gov/bioproject/579568).

**SUPPLEMENTAL MATERIAL**

Supplemental material is available online only.

**FIG S1**, TIF file, 0.2 MB.  
**FIG S2**, PDF file, 0.4 MB.  
**FIG S3**, TIF file, 0.3 MB.  
**FIG S4**, TIF file, 0.1 MB.  
**FIG S5**, PDF file, 0.2 MB.  
**FIG S6**, TIF file, 0.1 MB.  
**TABLE S1**, XLSX file, 0.5 MB.  
**TABLE S2**, DOCX file, 0.01 MB.

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The authors have declared that no conflict of interest exists.

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