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Genomic and Transcriptomic Insights into How Bacteria Withstand High Concentrations of Benzalkonium Chloride Biocides

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ABSTRACT Benzalkonium chlorides (BAC) are commonly used biocides in broad-spectrum disinfectant solutions. How microorganisms cope with BAC exposure remains poorly understood, despite its importance for disinfection and disinfectant-induced antibiotic resistance. To provide insights into these issues, we exposed two isolates of an opportunistic pathogen, *Pseudomonas aeruginosa*, to increasing concentrations of BAC. One isolate was preadapted to BAC, as it originated from a bioreactor fed with subinhibitory concentrations of BAC for 3 years, while the other originated from a bioreactor that received no BAC. Replicated populations of both isolates were able to survive high concentrations of BAC, up to 1,200 and 1,600 mg/liter for the non- and preadapted strains, respectively, exceeding typical application doses. Transcriptome sequencing (RNA-seq) analysis revealed upregulation of efflux pump genes and decreased expression of porins related to BAC transport as well as reduced growth rate. Increased expression of spermidine (a polycation) synthase genes and mutations in the *pmrB* (polymyxin resistance) gene, which cause a reduction in membrane negative charge, suggested that a major adaptation to exposure to the cationic surfactant BAC was to actively stabilize cell surface charge. Collectively, these results revealed that *P. aeruginosa* adapts to BAC exposure by a combination of mechanisms and provided genetic markers to monitor BAC-resistant organisms that may have applications in the practice of disinfection.

IMPORTANCE BAC are widely used as biocides in disinfectant solutions, food-processing lines, domestic households, and health care facilities. Due to their wide use and mode of action, there has been rising concern that BAC may promote antibiotic resistance. Consistent with this idea, at least 40 outbreaks have been attributed to infection by disinfectant- and antibiotic-resistant pathogens such as *P. aeruginosa*. However, the underlying molecular mechanisms that bacteria use to deal with BAC exposure remain poorly elucidated. Elucidating these mechanisms may be important for monitoring and limiting the spread of disinfectant-resistant pathogens. Using an integrated approach that combined genomics and transcriptomics with physiological characterization of BAC-adapted isolates, this study provided a comprehensive understanding of the BAC resistance mechanisms in *P. aeruginosa*. Our findings also revealed potential genetic markers to detect and monitor the abundance of BAC-resistant pathogens across clinical or environmental settings. This work contributes new knowledge about high concentrations of benzalkonium chlorides disinfectants-resistance mechanisms at the whole-cell genomic and transcriptomic level.

KEYWORDS *Pseudomonas aeruginosa*, adaptation, disinfectants, genomics, transcriptomics

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Benzalkonium chlorides (BAC) are among the most commonly used members of the quaternary ammonium compounds (QAC) as biocides. BAC act as cationic surfactants due to their amphiphilic property, i.e., a positively charged nitrogen atom in the center of a molecule with a C_8 to C_{18} *n*-alkyl chain. BAC have broad-spectrum biocidal activity against microbial, algal, fungal, and viral organisms based on the following modes of action (1): (i) adsorption and penetration of the BAC into the cell wall, (ii) interaction with the cytoplasmic membrane (lipid or protein) followed by membrane disorganization, (iii) leakage of intracellular low-molecular-weight material, (iv) degradation of proteins and nucleic acids, and (v) cell wall lysis by activity of autolytic enzymes (2, 3). BAC remain stable under various conditions in acidic, alkaline, and oxidative environments (4). Accordingly, BAC are widely employed in disinfectant solutions/formulations for food-processing lines, such as poultry facilities and dairy/agricultural settings, health care facilities, and domestic households, and are popular ingredients for over-the-counter products, such as cosmetics, hand sanitizers, and pharmaceuticals (5). Their extensive use and stability, especially under sediment-sorbed conditions (but not under nonsorbed, aerobic conditions where BAC can be quickly biodegraded; see below), have caused BAC concentrations to often be higher than those of other conventional organic pollutants (e.g., polyaromatic hydrocarbons) or other QAC representatives in nontarget environments (6, 7). BAC have been detected across diverse environments, such as river (at concentrations of ~6 μg/kg of body weight) and estuarine sediments (1.5 μg/kg), surface water (1.9 μg/liter), wastewater influent (170 μg/liter), and hospital wastewater, where they can reach concentrations of up to ~6.03 mg/liter (6–9).

Although BAC disinfectant solutions applied in hospital settings have a pH that is neutral to alkaline, are noncorrosive, nonstaining, and considered safe to apply on all washable surfaces (10, 11), at least 40 outbreaks have been attributed to infection by disinfectant-resistant pathogens contaminating antiseptic products, such as *Pseudomonas aeruginosa* (12–22). It has been hypothesized that the contaminating pathogens are associated with the water used in pharmaceutical processing facilities or the storage of BAC with cotton or gauze (22, 23). Thus, a better understanding of how microorganisms become resistant to BAC may help to better control such outbreak incidents. However, the underlying mechanisms of BAC resistance and their relative importance remain poorly elucidated (23) (see below for details). Furthermore, there is rising concern that widely used disinfectants such as BAC can promote antibiotic resistance (24–29). For instance, our previous study revealed that exposure to BAC can promote resistance to various antibiotics, including polymyxin B, and identified the molecular mechanisms for the resistance linkage between BAC and antibiotics (M. Kim, M. R. Weigand, S. Oh, J. K. Hatt, R. Krishnan, U. Tezel, S. G. Pavlostathis, and K. T. Konstantinidis, unpublished data). Antibiotic-resistant infections cause millions of casualties annually, emphasizing the need to better understand BAC resistance mechanisms that can also contribute to antibiotic resistance (30).

Several studies have reported BAC resistance in phylogenetically diverse microorganisms, most often related to the activation of efflux pump genes from four families, i.e., the major facilitator superfamily (MFS) (31, 32), the multidrug and toxic compound extrusion family (MATE) (33, 34), the resistance nodulation cell division family (RND) (35), and the small multidrug resistance family (SMR) (36, 37). In addition to efflux pump upregulation, dealkylation of BAC to benzyl dimethylamine (BDMA) and a long alkyl chain represents another important process for the development of BAC resistance (biodegradation), because BDMA is about 500 times less toxic than BAC (38–40). Furthermore, several studies have suggested that BAC-resistant bacteria show cell envelope modifications such as reduced negative charge of the cell surface, increased cell surface hydrophobicity, reduced cell surface roughness, and increased saturated fatty acid components on the cell surface, probably resulting in the decrease of cell membrane permeability and fluidity (41–43). Because the cell wall in Gram-positive bacteria and the outer membrane in Gram-negative bacteria act as barriers blocking the

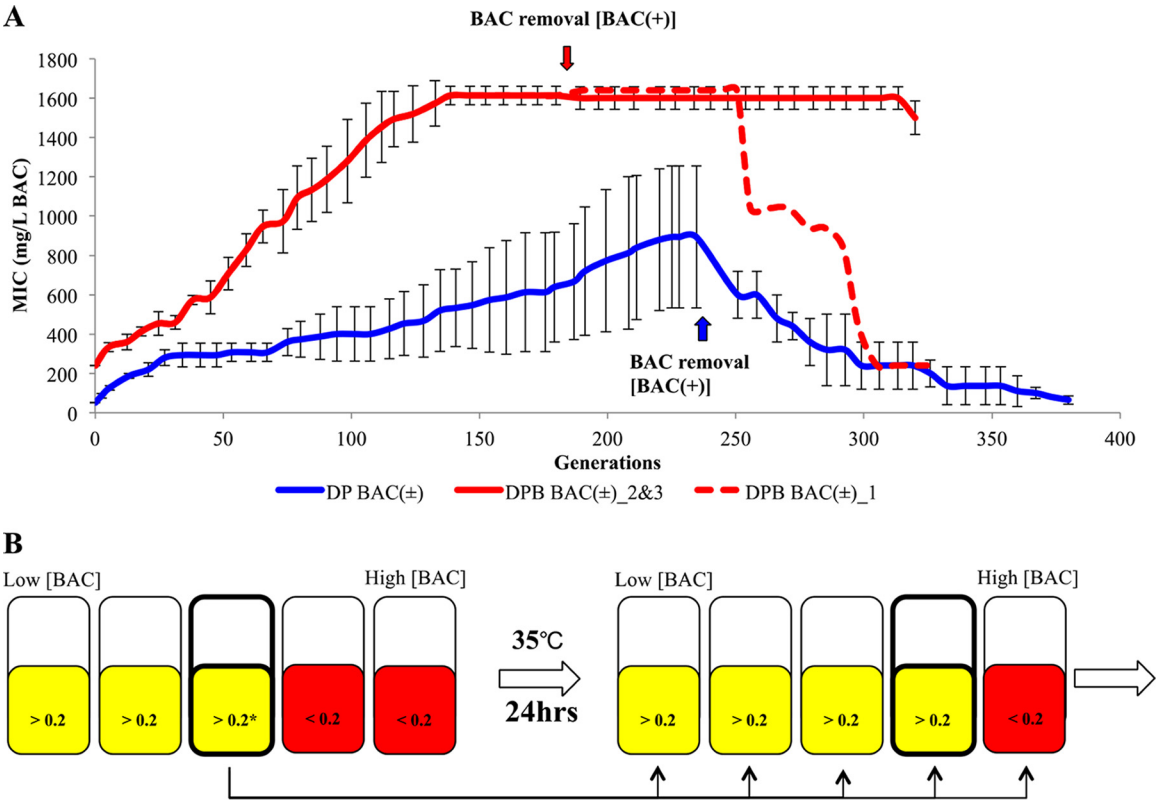

FIG 1 Schematic graphs of the adaptive evolution experiment. (A) Changes in MIC of BAC. Each adaptive evolution experiment is divided into two phases: BAC exposure, where BAC concentration was increased after each round until reaching the maximum BAC concentration in which growth was observed, and a BAC-free phase, where BAC was removed from the growth media. Red and blue lines represent *P. aeruginosa* DPB and *P. aeruginosa* DP populations, respectively. (B) A schematic representation of the experimental design. A 1% aliquot from the culture that showed a greater than 0.2 relative growth ratio compared to that of the control (measured by optical density; yellow test tubes) for the highest concentration of BAC tested was used as the inoculum (boldface black marks the inoculum tube for the example shown) for the next round with higher BAC concentrations. Red tubes represent no growth.

entrance and access of BAC to the inner cell membrane, such cell surface modifications are expected to be beneficial to BAC resistance.

Although the above-mentioned studies attempted to link specific phenotypic properties, such as the active removal of BAC by efflux pumps and reduction of the uptake, adsorption, and permeability of BAC via several types of cell surface modifications to BAC resistance, there is a lack of a comprehensive understanding of BAC resistance mechanisms at the whole-cell level, e.g., whether or not a cell employs all these mechanisms and their relative importance in coping with high BAC concentrations. Further, it remains unclear what specific mutations are selected by BAC exposure. Such mutations could also serve as biomarkers for detecting BAC-resistant microorganisms as well.

To provide new insights into these questions, we exposed two strains (individual colonies) of *P. aeruginosa*, strains DP and DPB, to increasing concentrations of BAC during an adaptive evolution experiment (Fig. 1). DP and DPB strains originated from the same ancestor from a river sediment inoculum, but DP was subsequently propagated for 3 years in a fed-batch aerobic bioreactor with a dextrin peptone mix (DP) as a sole carbon source and never saw BAC during this incubation period, while DPB originated from a bioreactor fed with DP plus 50 mg/liter BAC. Neither of the strains was able to degrade BAC in pure culture based on high-performance liquid chromatography (HPLC) results; hence, biodegradation was excluded as a potential mechanism to cope with BAC exposure in the present study. We have previously sequenced the genomes of the two strains in order to evaluate whether or not the genetic adaptations, which have occurred under BAC-supplemented versus BAC-free bioreactor conditions,

have conferred increased antibiotic resistance. Briefly, we found that the two genomes differed by five point mutations, one small (9-bp) insertion, and two genomic islands, one of which appeared to be an integrative and conjugative element (ICE) likely acquired during growth in the BAC-supplemented bioreactor. At least two of these genomic changes were selected by BAC exposure and conferred increased resistance to a couple of antibiotics, which we will report in detail elsewhere (Kim et al., unpublished data). Here, we focus on cellular adaptation to BAC exposure by investigating mutations occurring in the genome of the two strains with different histories of BAC exposure within short periods of time (e.g., 100 to 200 generations) and under increasing BAC concentrations, as well as gene expression differences between BAC and non-BAC growth conditions.

RESULTS AND DISCUSSION

Resistance levels of *P. aeruginosa* exposed to increasing BAC concentrations. The initial BAC MIC for *P. aeruginosa* DP and *P. aeruginosa* DPB was 50 mg/liter BAC and 240 mg/liter BAC, respectively (Fig. 1A), consistent with their history of BAC exposure and accumulated genomic modifications, i.e., presence of an ICE encoding a BAC efflux pump (i.e., *sugE*-A) in *P. aeruginosa* DPB and lack of the ICE in *P. aeruginosa* DP (see also gene expression results below). For the adaptive evolution experiment (Fig. 1), six test tubes containing a range of BAC concentrations (e.g., a range from 200 mg/liter BAC to 400 mg/liter BAC) in Luria broth (LB) medium were inoculated with a 1% aliquot of a *P. aeruginosa* DP or DPB overnight culture and allowed to grow for 24 h in triplicate. Subsequently, a 1% aliquot of the adapted population from the tube among the six tubes that showed growth at the highest MIC was transferred to six new tubes with increasing BAC concentrations daily. The process was continued for multiple rounds until no growth was observed in any of the six tubes [BAC(+) populations]. All *P. aeruginosa* DP BAC(+) populations showed increased MIC for BAC after growth under increasing BAC concentrations for approximately 240 generations [DP BAC(+)_1, 920 mg/liter BAC; DP BAC(+)_2, 1,240 mg/liter BAC; DP BAC(+)_3, 520 mg/liter BAC]. *P. aeruginosa* DPB BAC(+) populations showed even higher MIC for BAC after growth with BAC for about 180 generations [DPB BAC(+)_1, 1,640 mg/liter BAC; DP BAC(+)_2, 1,560 mg/liter BAC; DP BAC(+)_3, 1,640 mg/liter BAC]. No biodegradation of BAC was observed by any of the populations, indicating that the BAC resistance mechanisms were physiological (e.g., differential gene expression) and/or genetic (DNA mutations).

P. aeruginosa DP and DPB BAC(+) populations were allowed to evolve in LB only (no BAC added) after they reached the maximum BAC concentration that they were able to withstand [here referred to as BAC(−) populations; Fig. 1] in order to test how quickly the BAC resistance phenotype could be lost (if at all). Interestingly, while all three *P. aeruginosa* DP BAC(−) populations reverted back to similar levels of the initial MIC (50 mg/liter BAC) within 150 generations after transfer to BAC-free medium, two of the *P. aeruginosa* DPB BAC(−) populations maintained high BAC resistance, with MIC for BAC similar to those of the DPB BAC(+) populations. The third *P. aeruginosa* DPB BAC(−) lost BAC tolerance, with MIC for BAC reverting to the initial MIC (240 mg/liter BAC) within about 140 generations of transferring to BAC-free medium, similar to the results observed for DP BAC(−) populations. These observed phenotypic differences called for further genomic and transcriptomic investigations to elucidate the molecular mechanisms for BAC resistance employed by the adapted populations.

Fixed mutations in only a single gene, *pmrB*, in all BAC-adapted populations. Whole-genome sequencing identified 29 single-nucleotide polymorphisms (SNPs) and 17 DIPs (deletions, insertions, and other polymorphisms) in *P. aeruginosa* DP BAC(+) and BAC(−) populations and 22 SNPs and 16 DIPs in *P. aeruginosa* DPB BAC(+) and BAC(−) populations relative to the control population and the ancestor, respectively (see Table S1A, B, C, and D in the supplemental material). Several additional mutations identified in all *P. aeruginosa* DP and DPB populations showed lower frequencies (range, 50 to 80%) and were excluded from further analysis in order to focus on fixed mutations, which are more likely to underlie adaption. Based on the number of fixed

mutations, the estimated spontaneous mutation rate was 6.49×10^{-9} mutations per base per generation for *P. aeruginosa* DP BAC(+) populations, which was higher (3.85-fold; *P* value of 0.016 by Student's *t* test) than that of the *P. aeruginosa* DP BAC(−) populations (1.68×10^{-9} mutations per base per generation). On the other hand, the estimated spontaneous mutation rate for *P. aeruginosa* DPB BAC(+) populations was 7.34-fold higher than that of the *P. aeruginosa* DPB BAC(−) populations (7.72×10^{-9} versus 1.05×10^{-9}), which was significant (*P* value of <0.01 by Student's *t* test). Therefore, higher mutation rates were observed during BAC adaptation than during growth on BAC-free medium for both *P. aeruginosa* isolates, and the change in mutation rate was higher for DPB populations, which withstood higher BAC concentrations.

Notably, all DP BAC(+)/BAC(−) and DPB BAC(+)/BAC(−) samples had fixed mutations only in a single gene in common, *pmrB*, albeit at different locations within the gene. Mutations in *pmrB* are known to confer polymyxin antibiotic resistance, and the level of polymyxin antibiotic resistance depends on the type of mutation in *pmrB* (e.g., location and combinations of mutations) (44). Apparently, these mutations were selected by BAC exposure and represent a common mechanism to cope with BAC toxicity, at least for *P. aeruginosa* (note that polymyxin is also a membrane-disrupting agent, similar to BAC) (45, 46). In particular, mutations in *pmrB* are known to lead to constitutive activation of the *pmrA* regulon, and expression of *pmrA* leads to the expression of *arnBCADTEF*, which is responsible for the addition of 4-amino-L-arabinose (L-Ara4N) to the phosphate groups of lipid A, resulting in reduction of the net negative charge on the outer membrane (44, 47, 48). The reduction in net negative charge could increase bacterial BAC tolerance by reducing adsorption of the positively charged BAC molecules to the cell wall.

The loss of BAC resistance in DP BAC(−) populations was attributable to the acquisition of different mutations in other parts of *pmrAB* or other regulatory genes selected by BAC(−) growth conditions (Table S1C and E). In particular, *P. aeruginosa* DP BAC(−)_2 and DP BAC(−)_3 subpopulations had additional mutations at different locations in *pmrB* and *pmrA* than *P. aeruginosa* DP BAC(+) populations (e.g., SNPs in *pmrB* in >50% of the population), and *P. aeruginosa* DP BAC(−)_1 had a 15-bp insertion mutation in the quorum-sensing regulator gene *lasR*, which controls more than 300 genes, including various efflux pump genes (e.g., MFS and RND family) (49, 50) (Table S1C). These results may explain the loss of BAC resistance in all DP BAC(−) populations, although direct experimental verification of these results will be the subject of future research.

Other than *pmrB*, no consistent mutations were identified in a functional gene that was shared by all of the triplicate populations of either DP or DPB strains. The latter findings indicated that additional adaptations to BAC exposure have been at the level of cell physiology and/or gene regulation. Therefore, we conducted transcriptomic analysis for *P. aeruginosa* DPB BAC(+) and BAC(−) populations both under BAC-free and subinhibitory BAC concentrations in order to identify molecular mechanisms for BAC adaptation.

Gene expression under BAC-exposed conditions. To further investigate the molecular mechanisms for BAC resistance in *P. aeruginosa* DPB, we first conducted pairwise comparisons of gene expression profiles determined by transcriptome sequencing (RNA-seq) of the ancestral population and its evolved control population (under BAC-free medium, LB) of *P. aeruginosa* DPB when grown with BAC-free versus BAC-exposed media (i.e., subinhibitory concentrations of BAC were used). These pairwise comparisons identified 1,071 differentially expressed genes in the ancestor and 1,474 differentially expressed genes in the control out of the total of 6,038 predicted genes present in the genome (Table S2). Notably, expression of efflux pump genes (i.e., *sugE*-A, *sugE*-B, ATP-binding cassette [ABC] family, and an RND family member) in the ICE increased in both the ancestral and control populations under the BAC-exposed versus BAC-free conditions (Fig. 2). The expression level of *sugE*-A in particular was

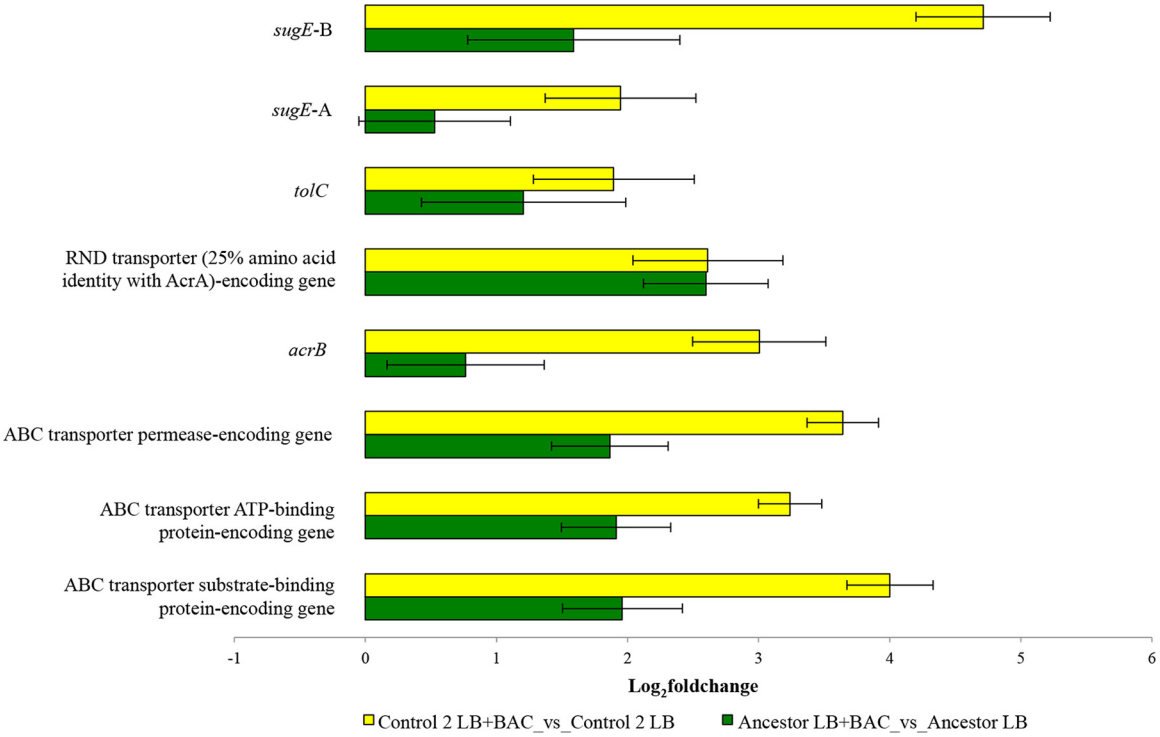

FIG 2 Overexpression of efflux pump genes present in the ICE when BAC is added to the growth media. Green bars represent comparisons between the *P. aeruginosa* DPB ancestor in LB growth medium and LB plus BAC (LB+BAC) medium (AL_versus_AB); yellow shows comparisons between *P. aeruginosa* DPB_Control_2 in LB and LB+BAC (C2L_versus_C2B). AL, *P. aeruginosa* DPB ancestor in LB growth medium; AB, *P. aeruginosa* DPB ancestor in LB+BAC medium; C2L, *P. aeruginosa* DPB_Control_2 in LB medium; C2B, *P. aeruginosa* DPB_Control_2 in LB+BAC medium.

slightly increased in the ancestor (1.4-fold higher) but not statistically significantly (adjusted *P* value [P_{adj}] of >0.01) and increased significantly in the control population (4-fold higher, with P_{adj} of <0.01) under BAC-exposed conditions. *sugE*-A has also been shown previously to confer increased BAC resistance in *Enterobacter cloacae* (51). Collectively, these results suggested that the ICE conferred BAC resistance based on the presence of *sugE*-A and transport proteins.

Furthermore, three protein sequences of the ICE carry an RND transporter that showed >95% amino acid sequence identity with the AcrAB-TolC efflux pump in *Salmonella enterica* subsp. *enterica* serovar Typhimurium, except for AcrA, which showed 25% amino acid sequence identity. BAC resistance mutants of *S.* Typhimurium showed increased expression of *arcB* and deletion mutants for *acrAB*, and *tolC* showed increased susceptibility to BAC (52). Similar to the *sugE*-A genes mentioned above, the ancestor showed slightly increased, but not significant, expression of *acrB* and *tolC* (1.7- and 2.3-fold higher, respectively, with P_{adj} value of >0.01), and the control population showed significantly increased expression of all these genes (Fig. 2). These results suggested that the efflux pump genes also are responsible, at least in part, for BAC resistance.

Further, the different degrees of overexpression of these genes under BAC-exposed conditions in the control and the ancestor population compared to those for the BAC-free conditions may be attributable to the exposure history of the populations. The control grew for ~330 generations in LB medium before the MIC-for-BAC test was performed, while the ancestor experienced growth for ~3 years in a BAC-amended bioreactor before the test. Thus, the ancestor was perhaps (more) preadapted to the presence of BAC, which likely accounted, at least in part, for lower induction of the efflux pump genes present in the ICE in BAC-exposed relative to BAC-free conditions. Consistent with this explanation, background ICE gene expression in the ancestor was

higher, by 2.8-fold, on average than that in the control under BAC-free conditions (*P* value of <0.01 by Student's *t* test), but no such significant difference was observed under BAC-exposed conditions. It is also important to note that the ICE was likely present in the highly complex microbial community of the starting river sediment inoculum, given that *P. aeruginosa* DPB, which encoded the ICE, and DP isolates represented descendants of the same ancestor in the original inoculum based on sequence analysis (e.g., it is less likely that the ICE was brought into the bioreactors through aerial transport in the meantime). However, the exact genomic context in which the ICE resided in the original inoculum remains unknown.

In order to further corroborate these findings and provide insights into the specific adaptations that enable evolved *P. aeruginosa* DPB strains to withstand high concentrations of BAC, we conducted pairwise comparisons of gene expression between the ancestral population and the *P. aeruginosa* DPB BAC(+) populations under both BAC-free (Table S3 and S4) and BAC-exposed conditions (Table S5 and S6). Differentially expressed genes in the evolved control population versus the ancestor were considered to be the result of stochastic processes or selection by the growth conditions (e.g., the bottle effect), and these genes were removed from the comparison of the ancestor versus *P. aeruginosa* DPB BAC(+), as was also performed in several previous studies (e.g., reference 53). In the pairwise comparison between the ancestor and *P. aeruginosa* DPB BAC(+) populations under BAC-exposed conditions, we were able to identify 303 differentially expressed genes that were shared among the three *P. aeruginosa* DPB BAC(+) populations (Table S5), while 381, 272, and 710 differentially expressed genes were specific to *P. aeruginosa* DPB BAC(+)_1, *P. aeruginosa* DPB BAC(+)_2, and *P. aeruginosa* DPB BAC(+)_3, respectively (Table S6). This number of genes was significantly larger than that from a similar comparison under BAC-free conditions (with 61 differentially expressed genes in common), indicating that important physiological adaptations to BAC exposure have taken place in the replicate *P. aeruginosa* DPB BAC(+) populations and underlay their (shared) high MIC levels for BAC. Consistent with the latter interpretations, the total number of differentially expressed genes ($n = 373$) found in the comparison between *P. aeruginosa* DPB BAC(+)_1 and *P. aeruginosa* DPB BAC(−)_1, which lost BAC resistance, under BAC-exposed conditions was higher than the number of such genes ($n = 16$) found in the comparison between *P. aeruginosa* DPB BAC(+)_2 and *P. aeruginosa* DPB BAC(−)_2, which maintained MIC for BAC, under BAC-exposed conditions (Table S7 and S8). Further, no differentially expressed genes were found between the replicate populations, e.g., three replicates of the BAC(+)_1 population, under the same conditions (i.e., BAC free or BAC amended), indicating that the differences identified by our RNA-seq analysis above reflected largely biologically relevant adaptations and not merely higher noise of RNA versus DNA data sets and protocols. Therefore, our subsequent analysis was focused on these 303 genes differentially expressed in *P. aeruginosa* DPB BAC(+) populations compared to the ancestor under BAC-exposed conditions.

Restricted growth as an adaptation to high BAC concentrations. With a subinhibitory concentration of BAC in the growth medium, expression of phosphorus utilization-related genes significantly decreased in the evolved DPB BAC(+) populations compared to that of the ancestor (Fig. 3A and Table S5). It is unlikely that these expression patterns were attributable to the lower phosphorus concentration or different phosphorus species in the LB medium, because the control population, which was propagated on LB, did not show similar gene expression patterns (note also that expression differences in the control versus ancestor were removed from further analysis in order to avoid such effects of LB growth medium, as noted above). Significantly decreased expression (-2.24-$\log_2$ fold change, on average; P_{adj} value of <0.01) of the phosphate-specific transport (*pst*) operon (*pstSCAB* and *phoU*) and the *phoB* gene, encoding a transcription activator of the two-component regulatory system *phoBR*, was observed in all *P. aeruginosa* DPB BAC(+) populations. *P. aeruginosa* possesses a *pst* system (high-affinity phosphate transport) which is activated by phos-

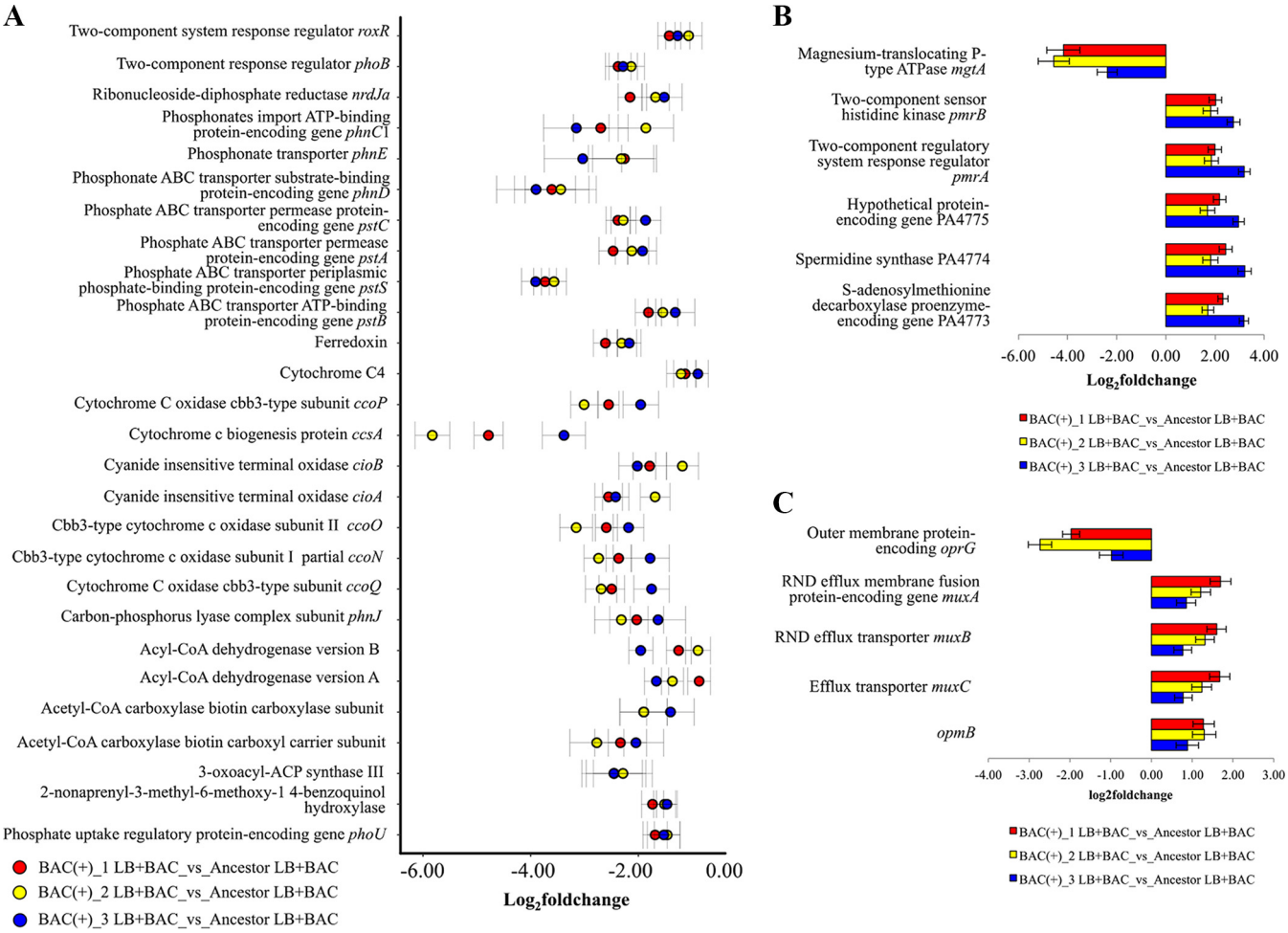

FIG 3 Gene expression changes in BAC-adapted *P. aeruginosa* DPB populations. Average $\log_2$ fold change values of three replicate for each population and ancestor were used. (A) Decreased expression of genes related to growth in *P. aeruginosa* DPB BAC(+) populations versus their ancestor in LB+BAC media [AB_versus_BAC(+)]. (B) Overexpression of spermidine synthesis genes and *pmrAB* and decreased expression of Mg^{2+} transport ATPase. (C) Overexpression of *muxABC-opmB* RND efflux pump and decreased expression of *oprG* porin for the same comparison. AB, *P. aeruginosa* DPB ancestor in LB+BAC medium; BAC(+)_1B, *P. aeruginosa* DPB BAC(+)_1 population in LB+BAC medium; BAC(+)_2B, *P. aeruginosa* DPB BAC(+)_1 population in LB+BAC medium; BAC(+)_3B, *P. aeruginosa* DPB BAC(+)_3 population in LB+BAC medium.

phate limitation (54), leading to phosphate taxis, and deletion of the *pstCAB* and *phoU* genes results in restraint of phosphate taxis even under conditions of phosphate excess (55). Therefore, decreased expression of the *pst* operon suggested lower uptake of phosphate. Furthermore, significantly decreased expression of the phosphonate utilization pathway (-2.69-$\log_2$ fold change, on average; P_{adj} value of <0.01) was observed in all *P. aeruginosa* DPB BAC(+) populations, especially the ATP-binding protein of the phosphonate ABC transport system *phnC*, ABC transporter substrate-binding protein *phnD*, phosphonate transporter *phnE*, and carbon-phosphorus lyase complex subunit *phnJ*. Phosphonates are P compounds (valence of +3) with a C-P bond instead of the more common C-O-P bond (valence of +5 for P) found in phosphate esters, so the strength of the C-P bond makes phosphonates resistant to chemical and enzymatic hydrolysis (56). Some microorganisms are known to be able to utilize phosphonates as a P source, and phosphonate utilization pathways have been reported with detailed genetic and biochemical evidence (57, 58). Therefore, the lower expression of these genes was also consistent with decreased phosphorus utilization as an effect of exposure to high BAC concentrations.

Adaptation to high concentrations of BAC resulted in additional significant changes, mostly a decrease in expression of genes related to various metabolic pathways, such

TABLE 1 Growth characteristic parameters in *P. aeruginosa* DPB ancestor and BAC(+) populations at subinhibitory concentrations of BAC[a]

Parameter	Value for:			
	Ancestor with BAC	BAC(+)_1 with BAC	BAC(+)_2 with BAC	BAC(+)_3 with BAC
Maximal growth rate (OD_{600}/h)	0.15 ± 0.01	0.11 ± 0.004	0.06 ± 0.01	0.13 ± 0.04
Lag time (h)	4.36 ± 0.44	6.69 ± 0.24	2.67 ± 0.37	6.4 ± 0.33

[a]See procedures in Materials and Methods for details on how cells were grown and how growth rate was measured.

as fatty acid biosynthesis, the nitrogen cycle, and energy metabolism in all *P. aeruginosa* DPB BAC(+) populations compared to the ancestor (Fig. 3A). First, decreased expression of acetyl-coenzyme A (CoA) carboxylase biotin carboxyl carrier protein subunit, acetyl-CoA carboxylase biotin carboxylase subunit, and 3-oxoacyl-ACP synthase III, which are involved in the fatty acid biosynthetic process (−2.17-$\log_2$ fold change, on average; P_{adj} value of <0.01), and decreased expression of acyl-CoA dehydrogenase (−1.33-$\log_2$ fold change, on average; P_{adj} value of <0.01), which is the initial step of fatty acid beta-oxidation, were observed. Second, the expression levels of almost all genes related to energy metabolism (i.e., the electron transport system) were decreased. For instance, the expression of genes involved in an electron transfer chain, such as those encoding the cbb_3-type cytochrome *c* oxidase subunits (i.e., *ccoN*, *ccoO*, *ccoQ*, and *ccoP*), the cytochrome *c* biogenesis gene *ccsA*, the cytochrome c_4, 2-nonaprenyl-3-methyl-6-methoxy-1,4-benzoquinol hydroxylase, which is part of the ubiquinone (cofactor) biosynthetic pathway, and ferredoxin decreased by 2.43-$\log_2$ fold, on average (P_{adj} value of <0.01), relative to the ancestor. Furthermore, significantly decreased expression of *roxR* (encoding a response regulator of the RoxSR two-component regulatory system) and cyanide-insensitive terminal oxidases (i.e., *cioA* and *cioB*) was observed in all *P. aeruginosa* DPB BAC(+) populations (−1.71-$\log_2$ fold change, on average; P_{adj} value of <0.01). This result was consistent with previous findings that RoxR is a direct positive transcriptional regulator of a cyanide-insensitive oxidase that is part of the electron transport system in *P. aeruginosa* (59). Finally, we observed significantly reduced expression of ribonucleoside-diphosphate reductase (−1.78-$\log_2$ fold change, on average; P_{adj} value of <0.01), which is responsible for the production of the precursors necessary for DNA synthesis. Altogether, reduced expression of genes related to cell energy metabolism, various metabolic pathways, and decreased uptake and utilization of phosphorus suggested that evolved *P. aeruginosa* DPB BAC(+) populations adapted to high concentrations of BAC, altering their growth characteristics (e.g., lag time or growth rate).

Reduction of the growth rate is known to be beneficial for bacterial populations exposed to antibiotics such as beta-lactams, which are cell envelope-disrupting agents like BACs, and other stress-exposed conditions (60, 61). Therefore, low growth rate might be beneficial for the high concentration of BAC adaptation. Consistent with these interpretations, we noted a lower growth rate in all DPB BAC(+) populations growing in 1,000 mg/liter BAC relative to that of the ancestor grown in the presence of 100 mg/liter BAC (*P* value of <0.01 by Student's *t* test) (Table 1). Furthermore, the average total cell count of the ancestor during the stationary phase under BAC-exposed conditions (100 mg/liter BAC) was 3.87×10^7 CFU/ml, which was 2.5-fold higher than that (1.52×10^7 CFU/ml) of *P. aeruginosa* DPB BAC(+) populations under BAC-exposed conditions (1,000 mg/liter BAC) (*P* value of <0.05 by Student's *t* test).

Stress-related genes. We also examined additional genes that were suspected to be involved in BAC resistance based on the (known) mode of action of BAC. Exposure to sublethal concentrations of cetyltrimethylammonium bromide, another member of the QAC family, induces oxidative stress by generating reactive oxygen species (ROS; e.g., superoxide and hydrogen peroxide) in *Escherichia coli* cells (62). Accordingly,

bacteria subjected to BAC are known to induce genes for ROS-scavenging antioxidants such as catalase and alkyl hydroperoxide reductase (63, 64). The expression of the gene encoding universal stress protein A (UspA) is also elevated in response to various kinds of stress conditions, including oxidant exposure, and in the presence of polymyxin or other antibiotics (65). However, when *P. aeruginosa* DPB BAC(+) populations were exposed to subinhibitory concentrations of BAC, the expression of alkyl hydroperoxide reductase, catalase, and *uspA* decreased by an average $\log_2$ fold of 1.49 (P_{adj} value of <0.01) relative to the ancestor (Table S5), indicating that expression of these stress-related genes does not represent a major physiological adaptation to BAC exposure, at least in the *P. aeruginosa* strains. On the other hand, we observed elevated expression of the stress-induced gene *ygiW* (2.64-$\log_2$ fold increase, on average; P_{adj} value of <0.01) in all *P. aeruginosa* DPB BAC(+) populations (Table S5). The *E. coli ygiW* mutant is sensitive to hydrogen peroxide and produces elevated levels of biofilm with the expression of flagellar biosynthesis genes (66); *ygiW* is also known to be induced by hydrogen peroxide (67). Although the mechanisms by which *ygiW* protects cells from hydrogen peroxide remain unknown, the increased expression of *ygiW* in all *P. aeruginosa* DPB BAC(+) populations is consistent with the previous studies that found that QAC induce oxidative stress by generating ROS (62). Overall, these results indicate that protection from the oxidative stress induced by exposure to a subinhibitory concentration of BAC is not the primary BAC resistance mechanism that allows *P. aeruginosa* DPB BAC(+) populations to withstand higher concentrations of BAC than their non-adapted ancestor.

Spermidine. Extracellular DNA can function as a nutrient source, a biofilm component, and a cation chelator, which can result in growth inhibition, thereby having implications for antibiotic resistance (68). Because DNA can sequester divalent metal cations, exposure to high levels of extracellular DNA induces the Mg^{2+}-responsive PhoPQ and PmrAB two-component systems, which control many genes required for virulence and antimicrobial peptide resistance (68). Exposure to sublethal concentrations of extracellular DNA and membrane-targeting antibiotics induces expression of genes *PA4773* to *PA4775* of the *P. aeruginosa* type strain, which are putatively annotated as spermidine and other polyamine synthesis genes. Polyamines are produced under Mg^{2+}-limiting conditions and can act as an organic polycation to bind lipopolysaccharide (LPS). Thus, these polyamines can stabilize and protect the outer membrane against antibiotic (i.e., polymyxin B) and oxidative (i.e., hydrogen peroxide) damage (69). Interestingly, both expression of the spermidine synthase gene operon, which is almost identical to genes *PA4773* to *PA4775*, and expression of *pmrAB* operon genes, which are located downstream of the spermidine synthase operon (i.e., *PA4776* and *PA4777*), were significantly increased by a $\log_2$ fold change of 2.39 and 2.27, on average (P_{adj} value of <0.01), respectively, in all *P. aeruginosa* DPB BAC(+) samples compared to the ancestor (Fig. 3B and Table S5). Furthermore, significantly decreased expression of Mg^{2+} transport ATPase (*mgtA*) was also observed (-3.71-$\log_2$ fold change, on average; P_{adj} value of <0.01) (Fig. 3B and Table S5). Therefore, these results indicate that a chloride ion in BAC acts as a cation (e.g., Mg^{2+}) chelator, destabilizing the cellular membrane, and *P. aeruginosa* DPB BAC(+) populations evolved to express more spermidine synthase genes when exposed to BAC conditions in order to protect cells from membrane-damaging BAC and/or oxidative stress possibly induced by BAC exposure.

Efflux pumps and porins. In addition to the ICE efflux pump genes mentioned above, we also examined the chromosomal ones. MuxABC-OpmB is an RND-type multidrug efflux pump that has been reported to confer resistance to novobiocin, aztreonam, macrolides, and tetracycline (70), and inactivation of the MuxABC-OpmB transporter leads to ampicillin and carbenicillin susceptibility (71). Moreover, the *muxABC-opmB* genes expressed by a metabolically active subpopulation in *P. aeruginosa* biofilm when exposed to colistin (polymyxin E) was necessary for colistin resistance but did not affect resistance to the chelator EDTA, the detergent SDS, or

chlorhexidine (72). A modest increased expression of the *muxABC-opmB* genes (+1.21-$\log_2$ fold change, on average; P_{adj} value of <0.01) (Fig. 3C and Table S5) indicated that BAC also is a substrate for this efflux pump. However, the latter finding awaits experimental verification, because none of the previous studies of the *muxABC-opmB* genes was focused on BAC. OprG is one of the major outer membrane proteins (e.g., OprD, OprE, OprF, and OprH) in *P. aeruginosa* (73). The function of OprG has not been clearly elucidated, and several studies reported that decreased expression of *oprG* was related to increased resistance to antibiotics such as tetracycline and kanamycin (74). However, other studies reported that expression of *oprG* under iron-rich anaerobic conditions was increased, but OprG was not involved in iron or antibiotic uptake (75), and interestingly, OprG might mediate the diffusion of small hydrophobic molecules across the outer membrane based on structural analysis (76). Because the first step of the mode of action for BAC is adsorption and penetration of the BAC into the cell wall and BAC has a long hydrophobic alkyl chain, which can be a substrate for OprG, decreased expression of the *oprG* gene by a 1.89-$\log_2$ fold change, on average (P_{adj} value of <0.01), in all *P. aeruginosa* DPB BAC(+) populations compared to the ancestor at subinhibitory concentrations of BAC might be beneficial for preventing BAC from entering the cells (Fig. 3C and Table S5). However, further experimental tests are necessary to test these hypotheses in order to clarify the mechanisms of BAC resistance.

Conclusions and outlook. It should be noted that BAC are often combined with other chemicals (e.g., excipients) in typical disinfectant formulations, which could increase BAC potency (77); no excipients were used in our study. Nonetheless, experimental adaptation to increasing concentrations of BAC rendered *P. aeruginosa* resistant to high BAC concentrations, ranging from 480 mg/liter BAC to 1,600 mg/liter BAC, which are comparable to, or even higher than, those used in practice for disinfection, e.g., typically between 400 and 500 mg/liter (78). Given the high stability of BAC in the environment and several outbreaks linked to contaminated QAC-based antiseptic solutions, these results underscored the need for further investigation of the mechanisms of BAC resistance.

Our genomic and transcriptomic analysis on BAC-evolved *P. aeruginosa* suggested that BAC resistance occurred at both the level of cell physiology (e.g., gene regulation) and genomic adaptations (e.g., fixed mutations). After 3 years of exposure to a subinhibitory concentration of BAC within the DPB bioreactor, *P. aeruginosa* acquired an ICE encoding several efflux pump proteins that were likely responsible for BAC resistance based on bioinformatics gene function annotation. In the present study, we showed that these genes were also overexpressed under BAC-exposed conditions compared to BAC-free conditions (Fig. 2), further corroborating the bioinformatics predictions that the genes are related to BAC resistance. Moreover, the physiological adaptations to high BAC concentrations appeared to include a combination of reduction of phosphorus utilization/uptake and energy production (e.g., electron transfer chains), slower growth in general, and altered expression of efflux pump genes and porins. These extensive and multifaceted differences at the transcriptome level contrasted with those at the DNA level, which revealed only a single gene in common that had undergone mutations during BAC exposure (*pmrB*). Specifically, after experimental adaptation to high concentrations of BAC, fixed mutations in *pmrB* allowed *P. aeruginosa* to be resistant to BAC via expression of genes related to stabilization of cell surface charge (e.g., 4-amino-L-arabinose and spermidine synthesis). Therefore, several independent mechanisms were employed by evolved *P. aeruginosa* populations to tolerate ~7-fold higher BAC concentrations relative to that of its ancestral population.

Our results revealed that reduction of growth and stabilization of cell surface charge represented two of the major BAC resistance mechanisms. This finding suggested that highly sensitive, culture-independent detection methods, such as quantitative PCR, can be used to monitor the microbial contamination of disinfectant manufacturing facilities or hospital disinfectant stocks in order to prevent outbreaks associated with contaminated stocks or storage (22, 23). In particular, mutations in *pmrB* can be used as

potential biomarkers to detect BAC-resistant organisms, especially *P. aeruginosa*. Furthermore, because our results suggested that stabilizing cell surface charge by the production of cationic polyamines represents another major BAC resistance mechanism, adjusting (e.g., lowering) the pH of the water in the antiseptic processing lines could be helpful in removing any remaining BAC resistance pathogens. However, adjusting the pH might not always be easy or even possible. Finally, BAC-evolved *P. aeruginosa* showed changes in the expression of several virulence factors (e.g., type III secretion systems and genes related to motility) under both BAC-free and BAC-exposed conditions (Tables S3, S4, S5, and S6). These results implied that exposure (and resistance) to BAC of pathogens also alters their virulence, e.g., decreased expression of type III secretion systems and motility genes in BAC-adapted populations or increased expression of pyoverdine biosynthesis genes (Tables S3, S4, S5, and S6), which warrants further investigation.

It is important to note that our experimental design (e.g., semicontinuously fed, mixed-batch culture) represents a good model for engineered biological wastewater treatment systems used in poultry, meat, and pharmaceutical processing facilities. For instance, BAC often accumulate in such facilities due to the continuous input and adsorption to biomass (79); thus, our experimental design based on increasing BAC concentrations (Fig. 1B) simulates this scenario well. Further, our results showed that the triplicate BAC-adapted *P. aeruginosa* DPB populations were able to maintain their high MIC even after BAC was removed from the feed for at least one hundred generations. Hence, our findings are, in general, realistic with respect to practice.

MATERIALS AND METHODS

Adaptive evolution experiments. The BAC used in the experiments described here consisted of a 60:40 mixture of benzyldimethyldodecylammonium chloride and benzyldimethyltetradecylammonium chloride (C_{12}BDMA-Cl and C_{14}BDMA-Cl, respectively; Sigma-Aldrich). Six test tubes containing a range of BAC concentrations (e.g., a range from 200 mg/liter BAC to 400 mg/liter BAC) in 10 ml Luria broth (LB) medium were inoculated with a 1% aliquot of *P. aeruginosa* DP or DPB and allowed to grow for 24 h in triplicate at 35°C, using an orbital shaker at 225 rpm. The 1% aliquot originated from an overnight culture grown from a single colony of each isolate. Subsequently, a 1% aliquot of the adapted population from the tube among the six tubes that showed growth at the highest MIC was transferred to six new tubes with increasing BAC concentrations daily. The process was continued for multiple rounds until no growth was observed in any of the six tubes [BAC(+) populations]. Growth was defined as an optical density (OD) ratio of >0.2 relative to the control culture grown in LB medium (no BAC). Inoculum from three BAC(+) populations (replicates) was then transferred to fresh LB medium with no BAC added [BAC(−) populations] daily for about one hundred generations (Fig. 1A and B). Control experiments were conducted in parallel with the same original inoculum (i.e., ancestor strains) but no BAC added to the medium. *P. aeruginosa* DP and DPB control populations (i.e., Control_1 and Control_2) were sampled when BAC(+) and BAC(−) populations were sampled, respectively. The number of generations of the cultures was measured every 24 h by counting CFU. MIC was determined as the concentration at which ≥80% inhibition in cell growth was observed by OD values at 600 nm (OD_{600}) compared to that of the control (no BAC in the medium). The BAC antimicrobial susceptibility test was performed with the microdilution procedure in LB medium (80). All populations were tested for their ability to biodegrade BAC using HPLC as described previously (39).

DNA extraction, RNA extraction, and sequencing. DNA of all *P. aeruginosa* populations from the adaptive evolution experiment was extracted using the QIAamp DNA blood minikit (Qiagen, Germany). DNA sequencing libraries were prepared using the Nextera XT DNA library preparation kit (Illumina, San Diego, CA) and were sequenced using an in-house Illumina MiSeq instrument. RNA of *P. aeruginosa* DPB populations from the adaptive evolution experiment was extracted as previously described (81). Briefly, cells from each *P. aeruginosa* population grown in LB or LB+BAC medium were harvested during mid-log phase based on OD_{600} measurements. rRNA was depleted from total RNA using the Ribo-Zero rRNA removal kit (Illumina, San Diego, CA), and cDNA libraries were constructed using the ScriptSeq v2 RNA-Seq library preparation kit (Illumina, San Diego, CA) according to the manufacturer's instructions. All constructed cDNA libraries were sequenced using either an Illumina MiSeq instrument or an Illumina HiSEQ 2500 instrument at the Georgia Institute of Technology.

Bioinformatics sequence analysis. Raw Illumina reads were trimmed, assembled, and functionally annotated as described previously (82). To identify single-nucleotide polymorphisms (SNPs) and genomic modifications such as deletions, insertions, and other polymorphisms (DIPs) between *P. aeruginosa* DP and DPB control, BAC(+), and BAC(−) populations versus their *P. aeruginosa* DP and DPB ancestors, breseq was used (consensus mode and Phred quality score [*Q*] cutoff of 15) (83, 84). Mutations identified in the genomes of the evolved control populations (no BAC in the LB growth medium) versus its ancestor were considered to be the result of stochastic processes or selection by the growth conditions (e.g., bottle effect) and were removed from further analysis. The estimated spontaneous mutation rates were

calculated with the equation $\mu = m/(GN)$, where μ is the mutation rate per base per generation, m is the total number of observed mutations (sum of the number of SNPs and DIPs that were observed at higher than 80% frequency in the output of breseq), G is the size of the genome that was assembled, and N is the number of generations (85).

All transcriptomic reads were filtered using SortMeRNA v2.0 (86) to remove remaining rRNA sequences. Non-rRNA reads were mapped to *P. aeruginosa* DPB assembled contigs using Bowtie 2 (87), and read count tables against predicted genes were generated by featureCounts v1.4.6-p3 (88). The output read count tables were used as input for DESeq2 (89) to obtain the lists of differentially expressed genes in pairwise comparisons after adjusting for the false-discovery rate by using the Benjamini-Hochberg method, which provided the adjusted *P* value (P_{adj}; cutoff of a P_{adj} value of <0.01 and number of read counts of >1). Differentially expressed genes from the comparison between control populations and ancestors that were also observed in the comparison of BAC(+) or BAC(−) populations against the ancestor were discarded from further analysis, similar to the mutations described above.

Growth of *P. aeruginosa* DPB ancestor and BAC(+) populations at subinhibitory concentrations of BAC. To assess growth characteristics of populations, i.e., λ (lag time) and μ (maximal growth rate), the following experiment was performed. The growth medium contained subinhibitory concentrations of BAC, which were 100 mg/liter BAC and 1,000 mg/liter BAC for the ancestor and BAC(+) populations, respectively. All tested inocula were first grown in LB growth medium and sampled at the end of the exponential phase in order to be subsequently inoculated (diluted) in the above-mentioned BAC-supplemented media to a final concentration of 5×10^5 CFU/ml. Cell growth was performed in four replicates on 24-well plates and was measured at 600 nm using a spectrophotometer. Lag time and maximal growth rate were estimated by fitting the data with the spline fit in the grofit R package (90).

Accession number(s). The isolate genome sequences were deposited at DDBJ/ENA/NCBI under the accession numbers MTLL00000000 (*P. aeruginosa* DP) and MTLM00000000 (*P. aeruginosa* DPB), and all genomic and transcriptomic sequences of *P. aeruginosa* DPB BAC(+) and BAC(−) populations can be found under BioProject code PRJNA184698.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00197-18.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.6 MB.

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We have no conflicts of interest to declare.

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