Supplementary material for "A Gateway-compatible allelic exchange system for generation of in-frame and unmarked gene deletions in *Burkholderia cenocepacia*" by Fazli et al.

Killing of *Burkholderia cenocepacia* by chromosomally encoded single copy or plasmid encoded multicopy *pheS* gene

We used three *B. cenocepacia* strains to test the effectiveness of the engineered *pheS* gene in killing *B. cenocepacia* cells in the presence of 0.1% cPhe when expressed as a single copy or a multicopy gene. The *Bcam1349* merodiploid strain served as an example for a bacterium with a single copy *pheS* gene. This strain harbours a single copy *pheS* gene on the gene replacement vector pENTRPEX18Tp-SceI-*pheS-Bcam1349*, which is integrated into the chromosome. Trimethoprim (Tp) was added to the growth medium to maintain the merodiploid state. As an example of multicopy *pheS* gene, we transformed the wild type *B. cenocepacia* strain with the plasmid pBBR1MCS-Km-*pheS* (1). We also transformed the wild type *B. cenocepacia* strain with the plasmid pBBR1MCS2, which served as the vector control strain. Kanamycin (Km) was added to the growth medium for plasmid maintenance. The strains were grown in LB medium with appropriate antibiotics overnight at 37°C. One ml of the overnight grown cultures was harvested, washed twice in 1ml 0.9% NaCl and serially diluted in 0.9% NaCl. Approximately $2x10^5$ CFU were plated on AB-agar medium with appropriate antibiotics and with or without 0.1% cPhe. The plates were incubated at 37°C for 48 hours. The results indicate that the engineered *pheS* gene was not efficient in killing *B. cenocepacia* cells in the presence of cPhe when expressed as a single copy on the chromosome, but it effectively killed almost all *B. cenocepacia* cells when expressed from the multicopy plasmid pBBR1MCS-Km-*pheS* (Fig. S1), demonstrating that the mutant *pheS* gene provides effective counter selection in *B. cenocepacia* when it is present in multiple copies in the cells.

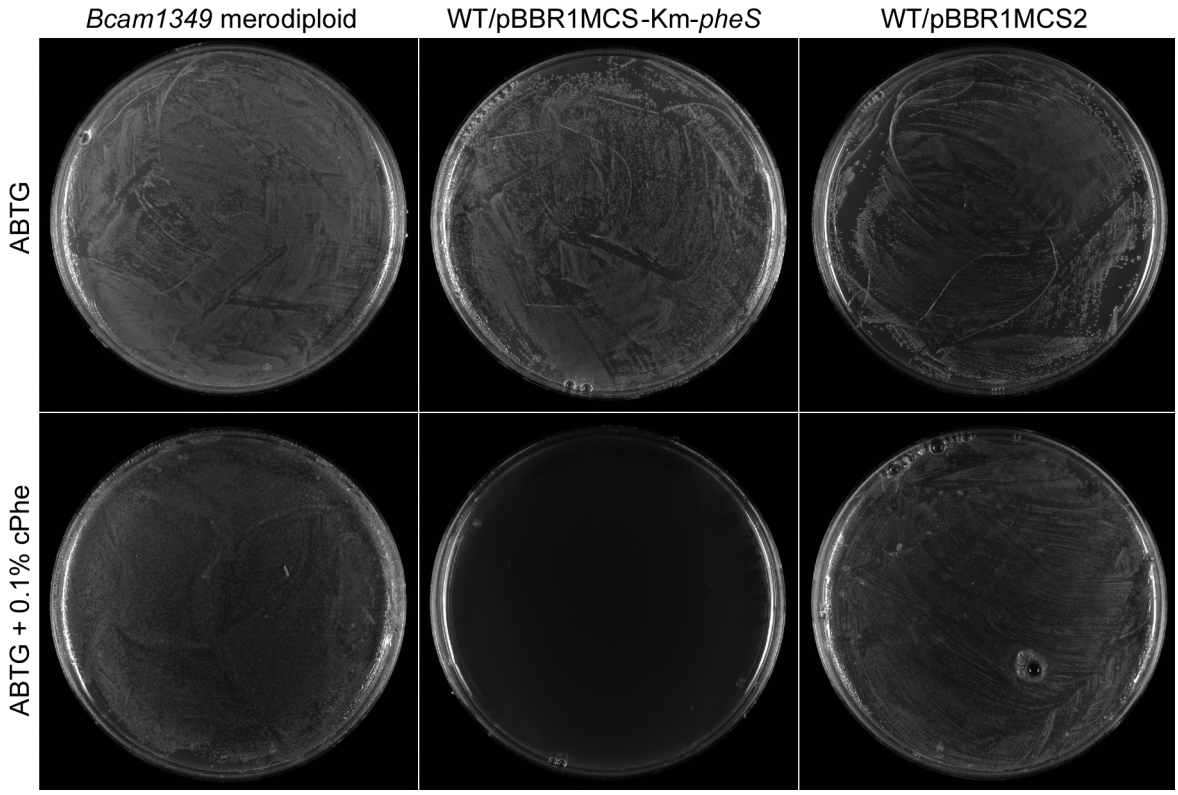

Fig. S1. Killing of *B. cenocepacia* strains by the engineered *pheS* gene in the presence of 0.1% cPhe when expressed in single or multiple copies. *Bcam1349* merodiploid is the single copy *pheS* gene containing strain, WT/pBBR1MCS-Km-pheS is the multicopy *pheS* gene containing strain, and WT/pBBR1MCS2 is the vector control strain. Both Tp and Km were used at 100 μg/ml. An identical amount of cells was plated on AB-agar medium with or without 0.1% cPhe. The images of the plates were acquired after 48 hours of incubation at 37°C.

Construction of the *Burkholderia thailandensis phzF* (BTH_I0859) deletion mutant

Using the allelic exchange system described here, we have successfully deleted the *phzF* (BTH_I0949) gene encoding a putative phenazine biosynthesis protein in *B. thailandensis*. *phzF* is the first gene of a predicted operon containing five genes.

We constructed the gene replacement vector pENTRPEX18Tp-SceI-*pheS*-*phzF* as follows. The ~0.5-kb upstream and downstream fragments of *phzF* gene were amplified using the primer pairs Phz_UpF/Phz_UpR and Phz_DnF/Phz_DnR, respectively (The primer sequences are available upon request). Both fragments were fused together using the primers GW-*attB*1 and GW-*attB*2 in splicing-by-overlap-extension (SOE) PCR to generate the *phzF* deletion allele. The final PCR product was then purified and verified by restriction analysis. BP clonase reaction for recombinational transfer of the mutant allele into the allelic exchange vector pDONRPEX18Tp-pheS was performed at 25 °C overnight as described in the Gateway cloning manual (Invitrogen), using only half of the recommended amount of BP Clonase II enzyme mix (Invitrogen). The BP clonase reaction product was transferred into chemically competent *E. coli* DH5α cells. The transformants growing on LB-agar plates containing 50 µg Tp mL^{-1} were streaked on LB-agar plates containing 50 µg Tp mL^{-1} for purification, plasmid isolation, restriction analysis and partial sequencing.

The gene replacement vector pENTRPEX18Tp-SceI-*pheS*-*phzF* was introduced into *B. thailandensis* via tri-parental mating as described previously (2). The co-integrants were selected for Tp resistance on LB-agar plates containing 100 µg Tp mL^{-1} and 100 µg Amp mL^{-1}. Eight Tp resistant colonies were streaked on the same selective plates, and the growing colonies were screened for integration of the plasmid by colony PCR using the primers Phz_UpF and Phz_DnR. A single positive merodiploid clone was transformed with pDAI-SceI-*pheS* by tri-parental mating to stimulate the second homologous recombination event and resolve the merodiploid state. The transconjugants were screened for Tet resistance on LB-agar plates containing 120 µg Tet mL^{-1} and 100 µg Amp mL^{-1}. Batches of 10 Tet resistant colonies were screened for the loss of the wild type allele and the presence of the desired gene deletion by colony PCR using the primers Phz_UpF and Phz_DnR. Two positive clones were purified by streaking and growing on the same selective plates. Thereafter a single colony for each clone was picked and grown in 1 ml AB-glucose medium containing 0.1% (w/v) cPhe at 37 °C overnight in order to stimulate the loss of pDAI-SceI-*pheS* via the counter-selectable marker *pheS* on the plasmid. Ten-fold serial dilutions of the overnight grown cultures were plated on LB-agar plates without any antibiotic, and 20 of the growing colonies for each clone were patched on LB-agar plates with or without tetracycline to screen for Tet sensitivity, which indicated

the loss of the plasmid pDAI-SceI-*pheS*. A single positive colony for each clone was selected and stored at -80 °C.

References

1. Barrett AR, Kang Y, Inamasu KS, Son MS, Vukovich JM, Hoang TT. 2008. Genetic tools for allelic replacement in Burkholderia species. Appl. Environ. Microbiol. 74:4498-508.
2. Fazli M, O'Connell A, Nilsson M, Niehaus K, Dow JM, Givskov M, Ryan RP, Tolker-Nielsen T. 2011. The CRP/FNR family protein Bcam1349 is a c-di-GMP effector that regulates biofilm formation in the respiratory pathogen Burkholderia cenocepacia. Mol. Microbiol. 82:327-41.