Title: Two resistance nodulation division-family efflux pumps in *Chromobacterium* species and their role in antibiotic resistance and tolerance

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ABSTRACT (250 words)

Very little is known of the antibiotic resistance mechanisms of members of the *Chromobacterium* genus. In previous studies of *Chromobacterium subtsugae* (formerly *C. violaceum*) strain CV017, we identified a resistance nodulation division (RND)-family efflux pump (CdeAB-OprM). Here, we show the *cdeAB-oprM* genes are widely distributed in members of the *Chromobacterium* genus. We use antimicrobial susceptibility testing with a CV017 *cdeAB-oprM* mutant to show the products of these genes confers resistance to a variety of antibiotics including ciprofloxacin, a clinically important antibiotic. We also identified a related RND-family pump, *cseAB-oprN*, in the genome of CV017 and other *C. subtsugae* species, that is not present in other members of the *Chromobacterium* genus. We demonstrate that CdeAB-OprM and CseAB-OprN are both transcriptionally induced in CV017 cells treated with sub-lethal antibiotic concentrations and they are important for induction of tolerance to different antibiotics. While CdeAB-OprM has a broad antibiotic specificity, the CseAB-OprN system is highly specific for a ribosome-targeting antibiotic produced by the saprophytic bacterium *Burkholderia thailandensis,* bactobolin. Finally, we use a previously developed *B. thailandensis-C. subtsugae* CV017 co-culture model to demonstrate that adding sub-lethal bactobolin at the beginning of co-culture growth increases the ability of CV017 to compete with *B. thailandensis* in a manner that is dependent on the CseAB-OprN system. Our results provide new information on the antibiotic resistance mechanisms of *Chromobacterium* species and highlight the importance of efflux pumps during competition with other bacterial species.

IMPORTANCE (150 words)

This study describes two closely related efflux pumps in members of the *Chromobacterium* genus, which includes opportunistic but often-fatal pathogens and species with highly versatile metabolic capabilities. Efflux pumps remove antibiotics from the cell and are important for antibiotic resistance. One of these pumps is broadly distributed in the *Chromobacterium* genus and increases resistance to clinically relevant antibiotics. The other efflux pump is present only in *Chromobacterium subtsugae* and is highly specific for bactobolin, an antibiotic produced by the soil saprophyte *Burkholderia thailandensis*. We demonstrate these pumps can be activated to increase resistance by their antibiotic substrates, and that this activation is important for *C. subtsugae* to survive in a laboratory competition experiment with *B. thailandensis.* These results have implications for managing antibiotic-resistant *Chromobacterium* infections, bioengineering of *Chromobacterium* species, and for understanding the evolution of efflux pumps.

INTRODUCTION

Members of the *Chromobacterium* genus are often found in water and soil environments of tropical and subtropical regions (1). The best-studied member, *Chromobacterium violaceum,* can cause occasional but often fatal infections in patients (2). *C. violaceum* and other members of this genus are also potentially useful in industry and ecological applications. *C. violaceum* has been shown to synthesize bioplastics (3, 4), hydrolyze plastic films (5), and solubilize gold (6, 7). *C. violaceum* and some other members of this genus can also kill insects and might be potentially useful in pest management (8, 9). The most well-known trait shared by many members of this genus is the ability to produce a purple pigment, violacein, which has a variety of biotechnologically interesting applications including as an antimicrobial (10) and anti-cancer therapeutic (11, 12). Production of violacein and other antibiotics is controlled by *N*-acylhomoserine lactone quorum sensing (13, 14), a type of cell-cell communication distributed widely in the Proteobacteria (15). Because production of violacein can be easily observed in cell cultures, a strain of *C. subtsugae* strain CV017 (formerly *C. violaceum* CV017) was developed for use as a biosensor of *N*-acyl homoserine lactone signals from the environment and other bacteria (13).

CV017 was also used in a dual-species laboratory model with another soil saprophyte, *Burkholderia thailandensis,* to understand quorum sensing involvement in antimicrobials production and interspecies competition (16). *B. thailandensis* and *C. subtsugae* can be isolated from similar soil and water environments of tropical regions, however, these were chosen for the co-culture model because they each grow similarly in the conditions of the experiment. Also, each species produces antibiotics that can kill the other species (16). In *B. thailandensis,* the antibiotic is bactobolin, which targets the 50S ribosomal subunit to block translation (17). We showed that an efflux pump in CV017 is important for bactobolin resistance, which we called CdeAB-OprM (18).

CdeAB-OprM is a predicted resistance nodulation division (RND) efflux pump. Drug efflux pumps are protein complexes which reside in the membrane and transport antimicrobials and toxins from the cytoplasm and periplasm to the extra-cellular space (19). The RND families are situated within the inner membrane and function in complex with two other proteins, an outer membrane channel and a periplasmic adaptor protein to form a tripartite efflux pump spanning both the inner and the outer membrane (19). Drug exporters belonging to the RND family play a key role in resistance to clinically relevant antibiotics in Proteobacteria (19). Resistance to antibiotics is generally due to mutation of an efflux pump regulator that causes an increase in transcription of the efflux pump genes and subsequently increases the antibiotic efflux activity of the pump, causing the cell to become more antibiotic resistant.

In this study, we explore the involvement of CdeAB-OprM in antibiotic resistance and how it is regulated. We show that CdeAB-OprM is involved in resistance to a variety of antibiotic classes and that this system is regulated by CdeR, a repressor of the *tetR* family. We also identified a new RND-family efflux pump CseAB-OprN, with high similarly to the CdeAB-OprM system. The *cseAB-oprN* genes are only present in *C. subtsugae*. We show that although CseAB-OprN does not appear to be important for antibiotic resistance, it does increase antibiotic tolerance in cells exposed to a sub-lethal antibiotic dose, and that this pump has a high degree of specificity and sensitivity to the *B. thailandensis* antibiotic bactobolin. The tolerance response relies on a LysR-family regulator encoded adjacent to the *cseAB-oprN* genes, CseR. Finally, we show that adding a sub-lethal dose of bactobolin to our *B. thailandensis*-*C. subtsugae* co-culture model increases the competitiveness of *C. subtsugae* in a manner dependent on CseAB-OprN and CseR. Together, our results describe two RND-efflux pumps found in members of the *Chromobacterium* genus and demonstrate how the evolution of antibiotic response might be important during competition with other species in the soil.

RESULTS

Distribution of* cdeAB-oprM *and* cseAB-oprN *in* Chromobacterium*.

We constructed a phylogenetic tree using 32 *Chromobacterium* genomes and an outgroup (*Aquitaliea magnus*) to analyze efflux pump genes in the *Chromobacterium* genus (Fig. 1A, Fig. S1 and Table S1). We initially used 16S rRNA sequences to construct the tree, however, we could not confidently predict some of the groupings, particularly the relationship of CV017 with other species. Thus, we constructed the tree using a set of 140 orthologous proteins (see Materials and Methods) to increase confidence of relationship predictions. Using this method, CV017 grouped with the *C. subtsugae* strains, consistent with the new classification of this strain as *C. subtsugae*. We were also able to make predictions of species associations for some of the unnamed species, for example, strain F49 grouped with the *C. subtsugae* clade.

To determine the distribution of the *cdeAB-oprM* genes in the *Chromobacterium* genus, we searched for the *C. subtsugae* CV017 *cdeB* gene in the 32 *Chromobacterium* genomes. We identified *cdeB* homologs in all of the strains, with nucleotide sequence identities ranging from 85-100% to CV017 *cdeB*. In CV017, *cdeB* is flanked by a putative periplasmic efflux pump-associated protein (*cdeA*) and outer membrane component (*oprM*) (Fig. 1B), typical of many RND-family efflux pump gene clusters. There was a similar gene arrangement in each of the other *Chromobacterium* species. We also identified second *cdeB* homolog in CV017, which we named *cseB*. *cseB* has 80% nucleotide identity to *cdeB* (74% protein sequence identity). *cseB* is similarly flanked by a *cdeA* homolog (*cseA*) and an *oprM* homolog (*oprN*). However, there is no apparent regulator encoded upstream of *cseA.* Instead, we noted a predicted LysR-family regulator encoded downstream of *oprN,* which we call *cseR.* We identified *cseB* homologs only in CV017, F49 and other members of the *C. subtsugae* species. In these other species, the *cseB* genes were >99% identical to one another, and the organization of genes surrounding *cseB* was conserved with that of the *cseAB-oprN* genes in CV017. We did not identify an apparent *cseB* homolog in one species of *C. subtsugae* (MWU2920) or any of the other *Chromobacterium*

strains. We predicted that the *cseAB-oprN* gene cluster might have arisen from the *cdeAB-oprM* genes via a duplication event that occurred in an ancestor of *C. subtsugae,* however, generation of a second tree using the sequences of *cseB* and *cdeB* only did not support this hypothesis (Fig. S2). Instead, the alignments show *cseB* groups with *cdeB* from *Chromobacterium* species C61, suggesting that it might have arisen from a horizontal gene transfer event from an ancestor of this strain.

Antibiotic susceptibility of* cdeAB-oprM *and* cseAB-oprN *mutants.

We previously demonstrated that deleting the *cdeAB-oprM* genes from CV017 increases susceptibility to the ribosome-targeting antibiotics bactobolin and another ribosome-targeting antibiotic, tetracycline, and the DNA intercalating agent ethidium bromide (18). These data support the idea that CdeAB-OprM system is an efflux pump that increases resistance to a range of toxic substrates. We sought to characterize the involvement of the CdeAB-OprM pump in resistance to a wider range of antibiotics and similarly characterize the CseAB-OprN system. Thus, we deleted *cseB* in CV017 and tested susceptibility of the Δ*cseB* and Δ*cdeAB-oprM* mutants to bactobolin and tetracycline, and other ribosome-targeting antibiotics chloramphenicol, erythromycin (a macrolide), and gentamicin and kanamycin (both aminoglycosides). We also tested imipenem (a cell wall-targeting β-lactam antibiotic), and the DNA gyrase inhibitor ciprofloxacin. The results (Table 1) show that deleting the *cdeAB-oprM* genes increased susceptibility to erythromycin, chloramphenicol and ciprofloxacin, but not to either of the aminoglycoside antibiotics. Surprisingly, deleting *cseB* had no effect on susceptibility to any of the antibiotics tested. There was also no effect of deleting *cseB* in the Δ*cdeAB-oprM* mutant. These results show that CdeAB-OprM increases resistance to a wider range of antibiotics than reported previously, and that CseAB-OprN does not appear to influence resistance of any of these antibiotics in the conditions of our experiments.

CdeR is a CdeAB-OprM repressor.

cdeR is encoded adjacent to the *cdeAB-oprM* gene cluster and predicted to encode a TetR-family transcriptional regulator, which typically function as transcriptional repressors by binding to the promoters of their target genes (the hallmark of this family, TetR, binds to the promoter of the *tetA* gene encoding the tetracycline pump) (20). Previously, we reported a CdeR mutant (CdeR H127Y) that increased transcription of *cdeA* and *cdeB*, and also increased bactobolin resistance (18). These results are consistent with the idea that CdeR is a repressor, and the CdeR H127Y mutation abolishes repressor activity. As further validation that CdeR is a repressor, we deleted *cdeR* in CV017, and tested the consequence of the deletion on *cdeAB* transcription and antibiotic susceptibility. Similar to the CdeR H127Y mutation, deleting *cdeR* increased transcription of *cdeA* and *cdeB* (Table 1 and Fig. S3). The Δ*cdeR* mutant was also more resistant than the CV017 parent to each of the apparent CdeAB-OprM substrates (tetracycline, erythromycin, chloramphenicol and ciprofloxacin, Table 1). The Δ*cdeR,* Δ*cdeAB-oprM* double mutant was not resistant to these antibiotics, showing MICs similar to that of the Δ*cdeAB-oprM* single mutant. Together, these results support that CdeR functions as a specific repressor of *cdeAB-oprM* transcription.

CseAB-OprN and CdeAB-OprM are important for antibiotic tolerance.

The *cseR* gene adjacent to *cseAB-oprN* is also predicted to encode a transcriptional regulator, which belongs to the broadly distributed LysR-type regulator family (21). Most LysR-family regulators function as activators and require binding of a ligand, such as an antibiotic, to effectively activate transcription of their target genes (21). Consistent with the idea that CseR is not a repressor, deleting *cseR* had no effect on resistance to any of the antibiotics tested (Table 1). To test the idea that CseR might be an antibiotic-responsive activator, we developed an

experiment to measure changes in antibiotic susceptibility (MIC) after exposure to sublethal antibiotic concentrations, which we call induction of antibiotic tolerance. We incubated CV017 cells with a sub-lethal concentration of antibiotic (1/2 the MIC determined in Table 1), then assessed MIC of the treated cells and identically grown untreated cells. We began by testing bactobolin, because efflux pumps are commonly activated by their own antibiotic substrates (19), and the high relatedness of CseB and CdeB suggest these systems might have overlapping antibiotic specificity. We found that after 6 h of incubation with sub-MIC bactobolin, the MIC of bactobolin-treated cells increased by 3.2 (± 0.2)-fold compared with identically-grown untreated cells (Fig. 2A), demonstrating a clear induction of bactobolin tolerance. We performed similar experiments with our Δ*cdeAB-oprM* and Δ*cseB* mutants. Our results showed sublethal bactobolin increased the MIC of the Δ*cdeAB-oprM* mutant similar to wild type. However, the MIC did not increase for the Δ*cseB* mutant. These results support that induction of bactobolin tolerance requires CseAB-OprN but not CdeAB-OprM.

To test whether CseAB-OprN is also involved in inducing resistance to other antibiotics, we tested erythromycin, ciprofloxacin, and tetracycline for induction of tolerance. We observed no significant resistance increase after exposure to a sub-lethal erythromycin and ciprofloxacin showed evidence of a small but not significant change (Fig. S4). However, exposure to sub-MIC concentrations of tetracycline increased tetracycline resistance 3.9 (± 0.9)-fold, similar to that of bactobolin (Fig. 2B). Surprisingly, no induction of resistance was observed in the Δ*cdeAB-oprM* mutant, whereas a significant 2.7 (± 1.3)-fold increase was observed in the Δ*cseB* mutant. These results suggest CdeAB-OprM is primarily responsible for induction of tetracycline tolerance.

CseR is an activator of the *cseAB-oprN* genes.

Because CseR is genetically linked to CseAB-OprN, and this system is involved in induction of bactobolin tolerance, we predicted CseR is important for bactobolin tolerance. Consistent with

this prediction, Δ*cseR* cells did not induce bactobolin tolerance as well as wild type, increasing the MIC by only 1.8 (± 0.7)-fold ($p < 0.0005$ compared with wild type induction). Surprisingly, Δ*cseR* cells were similarly impaired for induction of tetracycline tolerance. We also measured *cdeA* and *cseA* transcripts in wild type or the Δ*cseR* mutant treated with bactobolin or tetracycline, or with no antibiotic (Fig. 3). Our results show that *cseA* transcription was 2.4-fold higher in bactobolin-treated cells compared with untreated cells, and the increase was dependent on *cseR.* There was no change in *cseA* transcription in tetracycline-treated cells. These results are consistent with the idea that CseR activates transcription of the *cseAB-oprN* genes, and that the response is specific to bactobolin. *cdeA* transcription was also antibiotic inducible. We observed a 3-fold increase in *cdeA* transcription in cells treated with either bactobolin or tetracycline. However, CseR was not required for the increase in *cdeA* transcription with either antibiotic. Our results suggest CseR has a direct role in regulating *cseAB-oprN* transcription in response to bactobolin antibiotic, and might also participate in regulating CdeAB-OprM in response to tetracycline.

Specificity and response to bactobolin

We found it interesting that *cdeA* was transcriptionally activated by bactobolin similar to *cseA* (Fig. 3), but the CdeAB-OprM system was not important for bactobolin tolerance (Fig. 2). These results suggest bactobolin induces CdeAB-OprM system but the induction or export activity might be relatively weak. To understand the CdeAB-OprM interaction with bactobolin, we performed a cross-tolerance experiment. We incubated cells with sub-MIC bactobolin and subjected those cells to an MIC experiment with tetracycline. Our result showed that incubation with sub-MIC bactobolin increased resistance to tetracycline in a manner that was dependent on CdeAB-OprM (Fig. 4A). These results support the conclusion that the CdeAB-OprM pump can be induced by sub-MIC bactobolin. We also incubated cells with sub-MIC concentrations of

tetracycline and subjected those cells to an MIC with bactobolin. Our results showed that tetracycline induction increased resistance to bactobolin in a manner that was also dependent on the CdeAB-OprM system (Fig. 4B). Thus, bactobolin can serve as a substrate of CdeAB-OprM, consistent with results of the MIC experiments (Table 1). However, the induction requires tetracycline, suggesting that tetracycline induces CdeAB-OprM more strongly than bactobolin. The results are also consistent with the conclusion from the quantitative PCR data that tetracycline specifically induces the CdeAB-OprM pump, whereas bactobolin can induce both pumps. Together, our results support that CseAB-OprN has a high level of sensitivity and specificity for bactobolin, whereas CdeAB-OprM recognizes bactobolin to a lesser degree, but can also respond to other antibiotics.

CseAB-OprN is important for induction of bactobolin resistance in a laboratory co-culture model

We used our *B. thailandensis-C. subtsugae* co-culture model to test the role of CseAB-OprN and tolerance induction during competition. In this model, *B. thailandensis* typically outcompetes *C. subtsugae* (CV017) primarily due to production of bactobolin (18). To test the role of CseAB-OprN in the competition model, we combined exponentially growing cultures of *B. thailandensis* and *C. subtsugae* and enumerated each species after 24 h by colony counting on selective media. The results of competition with the wild-type strain was similar to previously published results: *B. thailandensis* outcompeted *C. subtsugae* and dominated the culture at the end of the experiment (Fig. 5, 'wild type,' open circles). However, when sub-MIC bactobolin was supplied in the culture, *C. subtsugae* gained an advantage over *B. thailandensis* (Fig. 5, filled circles). These results show that sub-MIC bactobolin exposure can increase *C. subtsugae* competitiveness in our co-culture model. The increase in competitiveness not observed in a Δ*cseB* mutant, and thus was due to CseAB-OprN (Fig. 5, Δ*cseB,* open circles). We observed a similar competition defect in a Δ*cseR* mutant. These results are consistent with the role of CseAB-

OprN in the induction of bactobolin resistance, and highlight the potential role of this response in competition with other antibiotic-producing species.

DISCUSSION

In this study, we demonstrate antibiotic specificity and regulation of two efflux pumps of *Chromobacterium subtsugae* CV017. One of these, CseAB-OprN, had not been previously reported and is limited to the *C. subtsugae* species. The other, CdeAB-OprM, is widely distributed members of the *Chromobacterium* genus. CdeAB-OprM has a broad range of specificity for antibiotics such as tetracycline, erythromycin, chloramphenicol and ciprofloxacin. As ciprofloxacin is one of the recommended treatments for infections with pathogenic *Chromobacterium* (2), the results suggest this pump might be important in cases of antibiotic failures. To date, there are very few publications on *Chromobacterium* mechanisms of antibiotic resistance and drug export (18, 22, 23). Results of this study will be useful for identifying resistance mechanisms in clinical isolates and for future *Chromobacterium* research.

Our results demonstrate that both CdeAB-OprM and CseAB-OprN are induced by their antibiotics substrates. Such a response is typical of other pumps such as the aminoglycoside-responsive MexXY pump of *Pseudomonas aeruginosa* (24) and the trimethoprim-responsive BpeEF-OprC pump of *Burkholderia pseudomallei* (25). Because many antibiotic resistance factors are costly to produce, waiting to induce their production until needed might allow a valuable metabolic cost savings (26). The ability to respond to antibiotic stress might be an important survival strategy in infected patients undergoing antibiotic therapy (27), and, as highlighted by our co-culture model, such responses might also be important during competition with other bacteria. Competitive interactions with other bacteria might also be an important factor shaping the evolution of antibiotic production. Many antibiotics are regulated by quorum sensing in response to changes in population density, for example *B. thailandensis* bactobolin is regulated by the BtaR2-I2 quorum sensing system (28). Quorum sensing regulation of antibiotics might be a strategy to avoid tolerance induction of competitors (29). By waiting to produce antibiotic until high density, the population is able to produce a sudden dose of antibiotic to kill the competitor. Quorum-sensing regulation delays the production of bactobolin until the population reaches

stationary phase (30). This growth stage occurs around 12 hr in our co-culture model (18). We were able to induce antibiotic tolerance of *C. subtsugae* by adding sub-MIC bactobolin to the co-culture at the start of the experiment. These results highlight how delaying antibiotic production until a sufficient killing dose can be produced might be useful to avert tolerance induction in competitors.

Our initial attempt to determine the phylogeny of the members of the *Chromobacterium* genus by 16S sequences provided a limited view of the relationships between the species. The alternative method of determining phylogeny with a set of 140 orthologous proteins provided a much higher level of confidence of relationship predictions. Using this method, we were able to validate the new classification of strain CV017 as a *C. subtsugae* strain. We were also able to group each of the previously unspecified strains with close relatives to assign likely species to these strains. The phylogenetic tree we generated suggests the *Chromobacterium* genus can be separated into three distinct groups. First is the *C. haemolyticum* group, a pathogenic species with a pronounced ability to haemolyze human erythrocytes (31), second is a group of non-pathogens including *C. subtsugae* and *C. sphagnii,* and third is a group including *C. violaceum, C. piscinae, C. amazonense, C. vaccinnii,* and *C. pseudoviolaceum.* Although the pathogenic ability of *C. violaceum* has been reported, the other members of this group were isolated from soil and water environments and their virulence potential remains unclear.

Our phylogenetic analysis suggests that the *cseAB-oprN* genes were acquired through horizontal transfer of the *cdeAB-oprM* cluster from another species, rather than through duplication of the *cdeAB-oprM* genes already in the ancestral strain (Fig. 1A and S1). The finding that horizontal transfer of resistance genes occurred in saprophytic species supports the idea that horizontal transfer can lead to the dissemination of antibiotic resistance genes in polymicrobial communities of the soil (32). The spread of resistance might have been helpful for surviving antibiotics produced by other bacteria in the soil. The acquisition of new resistance genes might also promote tolerance to self-produced antibiotics (33). *Chromobacterium* is known to produce

an arsenal of antimicrobial-active molecules (34), although many have not been well characterized. The hypothesis that CseAB-OprN or CdeAB-OprM provides resistance to a self-produced antibiotic can be tested experimentally, and such a line of experimental questioning might shed light on how antibiotic resistance mechanisms and their regulatory circuits evolve in multispecies communities.

MATERIALS AND METHODS

Bacterial culture conditions and strains. All strains were grown in Luria-Bertani (LB) broth, LB containing morpholine-propanesulfonic acid (LB-MOPS; 50 mM; pH 7), or on LB with 1.5% (wt/vol) Bacto-Agar. All broth cultures were incubated with shaking at 30°C (*C. subtsugae* or *C. subtsugae-B. thailandensis* co-cultures) or 37°C (*B. thailandensis* or *E. coli*). As a source of bactobolin, we used filtered *B. thailandensis* culture fluid prepared as described previously (18). Filtered *B. thailandensis* culture fluid was stored at 4°C for up to 1 month prior to use and used directly for experiments. For strain constructions, we used gentamicin at 50 µg ml−1 (*C. subtsugae*) or 15 µg ml−1 (*E. coli*). For selection from co-cultures we used gentamicin at 100 µg ml−1 (*B. thailandensis*) and trimethoprim at 100 µg ml−1 (*C. subtsugae*).

Bacterial strains and plasmids used in this study are listed in Tables S1-S3. For co-cultures, we used the wild-type *B. thailandensis* strain E264 (35). *C. subtsugae* strain CV017 (referred to as wild type, previously known as *C. violaceum* CV017) is a derivative of strain ATCC31532 (36) with a transposon insertion in gene CV_RS05185 causing overexpression of violacein (37, 38). All *C. subtsugae* mutant strains were constructed from CV017 using allelic exchange and methods described previously (16). The constructs for generating deletions in *cseB, cdeR* and *cseR* were made by introducing

PCR-generated amplicons or synthetic gene fragments (IDT or Genscript) into pEX18Gm-derived delivery plasmid (39). The mutation was introduced to *C. subtsugae* by conjugation and transconjugants were selected on gentamicin. Transconjugants were transferred to no salt-LB + 15 % sucrose to select for plasmid excision and correct clones were identified by PCR-based screening. All strains and PCR-generated plasmids were verified by PCR amplification and sequencing.

Phylogeny of *Chromobacterium* species and efflux pump genes. *Chromobacterium* species used for phylogenetic analyses are listed in Table S3. Annotated protein sequences from assembled genomes of *Chromobacterium* and *Aquitaliea magnusonii* (outgroup) were retrieved from the National Center for Biotechnology Information (NCBI, June 2018, Table S3). Comparisons using 16S rRNA provided low confidence predictions of phylogeny, thus we performed an analysis using a set of 140 single-copy orthologs, which were identified using several steps. Orthologous proteins were initially identified by using blastp to carry out reciprocal best blast hits of each protein from each strain against a protein database made of all the proteins in our strain set ((40), options: -max_target_seqs 1), to find orthologs with best blast hit between all possible pairs of species, and no more than 10% variation in protein length. We aligned this group of 171 proteins individually using Muscle version 3.8.31 ((41), options –diags), then reordered sequences using the stable.py script provided by the Muscle developer. Finally, we removed any orthologous protein groups with less than 75% average pairwise identity, less than 30% of sites identical or 100% of sites identical. This last step ensured that all proteins would have intermediate levels of divergence and left the final set of 140

orthologs used for phylogenetic tree construction. Finally, we concatenated the protein sequences in each alignment to create one single alignment consisting of 25,351 amino acids. After inspection of this alignment, we found that several pairs of isolates had very low divergence (less than 10 amino acid differences across the entire alignment). We decided to remove one of each of these pairs to reduce redundancy (removed isolates were *C. violaceum* strains LK6, LK30, H5525 and 16-419B). C. *subtsugae* F49 and *C. subtsugae* CV017 are identical across the alignment of orthologous proteins but both were retained because CV017 is the focus of the paper.

We generated phylogenetic trees using neighbor joining, maximum likelihood and Bayesian methods. A simple neighbor joining (NJ) tree implemented in Geneious version 10.1.3 (http://www.geneious.com) with a Jukes-Cantor substitution model and 100 bootstrap replicates and *A. magnusonii* as an outgroup. We constructed a maximum likelihood tree using RaxML version 8.2.11 (42) with a Gamma BLOSSUM62 protein model, 100 bootstrap replicates with a parsimony random seed of 1 and *A. manusonii* as an outgroup. We used Mr. Bayes version 3.2.6 (43) to construct a tree using Bayesian methods with a Poisson rate matrix, gamma rate variation with 4 categories, and *A. manusonii* as an outgroup, an initial chain length of 1,100,000 with four heated chains at a temperature of 0.2, the subsampling frequency was 2000 generations after an initial burn-in length of 100,000 generations. However, after 332,000 generations, the standard deviation of split frequencies was less than 0.01 and so the tree search was terminated. For all three methods, consensus trees were built based on 50% majority rule, and the three trees were compared using the RaxML maximum likelihood tree as a backbone. We elected to use a concatenated sequence to build our tree because our concern is

about the overall evolutionary history of the genus and not how specific genes trees might differ from species tree.

To reconstruct the evolutionary history of the two efflux pumps, we compared DNA sequence of the pump genes to improve our ability to resolve evolutionary distances between closely related isolates. We retrieved the DNA sequences by performing a tblastn (44) of the amino acid sequence for that protein against all *Chromobacterium* genome assemblies and then located the correct sequence based on isolate name. Note that one sequence was split among two scaffolds in *C. subtsugae* MWU2387 and was therefore not annotated so we pieced together the appropriate sequence. To find orthologs of the second pump (*cseB*) we performed a tblastn search of the amino acid sequence of CdeB from CV017 against all assembled *Chromobacterium* species. Reciprocal best blast searches confirmed the presence of this second pump in only a subset of the *C. subtsugae* isolates (including CV017). We also searched for similar sequences outside of *Chromobacterium* to find evidence for horizontal transfer of the pump. Finally, we constructed phylogenetic trees of the DNA sequence for the two pumps similar to what was described above but with nucleotides instead of amino acids.

Sequencing the *cse* gene locus. *cseB* (CV_15400) was initially identified on scaffold 169 of the assembled CV017 genome (GCA_001510755.1). 274 bases downstream of *cseB* is the 3' end of scaffold 169. As this sequence did not overlap with any of the other 211 scaffolds of CV017, we used a BLAST search to identify similar sequence in a similar strain (*C. subtsugae* PRAA4-1, GCA_001676875.1) This search identified sequence containing a *cseB*-like gene in scaffold 30 of PRAA4-1, corresponding with scaffold 192

in CV017. We used alignments of CV017 scaffold 169 and 192 with the PRAA4-1 sequence to design oligonucleotides to amplify the region between scaffolds 169 and 192. We Sanger sequenced the amplified PCR product to verify the correct orientation of the scaffolds and identify the previously unknown 39 nucleotides linking forward-facing scaffold 169 with reverse-facing scaffold 192. This was GCCGCCGCCGACAGCCAGCGCCAGCGCGTCAGCGCGACG.

Antibiotic susceptibility and tolerance experiments. Antibiotic susceptibility was determined by minimum inhibitory concentration (MIC) according to the 2003 guidelines of the Clinical and Laboratory Standards Institute (NCCLS), using a modified microtiter method. Antibiotics were added to LB-MOPS in a 100 ul well of a 96-well plate, and diluted 2-fold down the row of 10 wells. For each antibiotic, 3 different starting concentrations of antibiotic were used to increase sensitivity of the experiment. *C. subtsugae* inocula were prepared by diluting logarithmic-phase cells from LB-MOPS cultures to an optical density at 600 nm (OD_{600}) of 0.005 ($6x10^6$ CFU) in each well. Plates were sealed with Breathe-Easy strips (Fisher Scientific, fishersci.com) and grown for 24 h with shaking at 30°C. The MIC was defined as the lowest concentration of antibiotic (µg/ml) in which bacterial growth in the well was not measurable by determining the optical density at 600 nm (OD_{600}) on a 96-well plate reader.

To assess changes in MIC induced by exposure to sub-lethal antibiotic (tolerance), logarithmic-phase *C. subtsugae* cells were diluted to an OD_{600} of 0.1 in 10 ml LB-MOPS in a 125-ml culture flask. The culture medium contained antibiotic at ½ of the MIC determined by the method described above (this varied for each strain), or no antibiotic

(untreated), and incubated for 6 hours with shaking at 30°C. These cultures were then directly used to determine MIC using methods described above.

Droplet digital PCR. RNA was harvested from *C. violaceum* as described in the figure legends. RNA was prepared using the RNeasy® Mini Kit (Qiagen) following the manufacturer instruction with a modification in the DNA digestion step as described previously (18). Droplet digital PCR was performed on a Bio-Rad's QX200 Droplet Digital PCR (ddPCR) System using Eva Green Supermix. Each reaction mixture contained 1 ng μl^{-1} of cDNA template, 0.25 µM of each primer, 10 µl Eva Green Supermix, and 8 µl H_2O in a 20-µl volume. After generating 40 µl of oil droplets, 40 rounds of PCR were conducted using the following cycling conditions: 94°C for 20 sec, 60°C for 20 sec, and 72°C for 20 sec. Absolute transcript levels were determined using the Bio-Rad QuantaSoft Software. In all cases a no template control was run with no detectable transcripts.

Co-culture experiments. Co-culture experiments were conducted in 10 ml LB-MOPS medium in 125 ml baffled flasks. The inoculum was from logarithmic-phase pure cultures of *C. subtsugae* and *B. thailandensis*. The initial OD_{600} in the co-culture was 0.05 for *B. thailandensis* (2-4x10^7 cells per ml) and 0.005 for *C. subtsugae* (2-4x10^6 cells per ml). When indicated, cultures also contained *B. thailandensis* culture fluid at a concentration corresponding with ½ of the MIC for the *C. subtsugae* strain used in the experiment. After inoculating, co-cultures were incubated at 30ºC with shaking at 230 r.p.m for 24 h. Colony forming units of each species were determined by using differential antibiotic selection on

LB agar plates. *B. thailandensis* was selected with gentamicin and *C. subtsugae* was selected with trimethoprim.

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Table 1. Antimicrobial susceptibility of *C. violaceum* (or C. *subtsugae*) strains

	Minimum inhibitory concentration (MIC)[a]							
	Bact[b]	Tet	Gent	Kan	Ery	Chlo	Imip	Cipro
Cv strain	(%)				μg.ml^{-1}			ng ml^{-1}
Wild type	1.3±0.2	1.1±0.1	23±3	40	14±2	45.3±14	1.1±0.1	10±4
Δ*cdeAB*[c]	0.4±0.2	0.5± 0.1	23±3	38±4	7±1	12.5	1	2±1
Δ*cdeR*	8	5±1	36±5	41±4	75±10	106±31	1.1±0.1	110±12
Δ*cseB*	1.5±0.4	1.1±0.3	25±5	45±7	12±1	53±6	1	9±1
Δ*cdeAB* Δ*cseB*	0.3±0.1	0.4±0.2	26.7±3	37.5±4	6±2	54±7	1.1±0.1	2±1
Δ*cdeRAB*[d]	0.5±0.1	0.4	45±5	47.5±3.5	45±5.8	45.3±14	1	350±70
Δ*cseR*	1.4±0.4	1.0±0.3	25±5	35±7	16±1	54±7	1	8±1

[a]The minimum inhibitory concentration (MIC) of *B. thailandensis* culture fluid bactobolin, tetracycline (Tet), gentamicin (Gent), kanamycin (Kan), erythromycin (Ery), ciprofloxacin (Cipro), chloramphenicol (Chlo), and imipenem (Imip). Results are the average of three independent experiments and the range is indicated when it was not zero.

[b]Results are from a single preparation of *B. thailandensis* fluid. Results with other preparations were similar. There were no observed growth defects in identical experiments with 3% culture fluid from a *B. thailandensis* bactobolin-deficient mutant.

[c]Δ*cdeAB* indicates the strain Δ*cdeAB-oprM*

[d]Δ*cdeRAB*, indicates the strain Δ*cdeAB-oprM*, Δ*cdeR*

FIGURE LEGENDS

Fig. 1. A) Phylogenetic tree of *Chromobacterium* species. Tree construction is described in materials and methods. Circles at each node represent bootstrap values based on neighbor joining (blue circles), maximum likelihood (red circles) and Bayesian (yellow circles) methods. Filled circles, >95% support; unfilled circles, >50% support; missing circles, <50% support. *, all three methods showed >95% support. Triangles represent several highly similar strains; *Chromobacterium sphagnii* (37-2 and 14-B11), *Chromobacterium violaceum* (L_1B5_1, LK17, LK15, GHS51, 16-454, CV1192, CV1197, GN5 and 12472), and *C. vaccinii* (MWU205 and 21-1). A full representation of the species in the phylogenetic tree is shown in Fig. S1. Red box indicates species encoding the *cseAB-oprN* genes. Red arrow, node where species with the *cseAB-oprN* gene cluster separate from other species. **B) Illustration of *cdeRAB-oprM* and *cseAB-oprN-cseR* gene clusters.** Shading indicates amino acid identity. Dark grey, 80-89% identical. Light grey, 60-69% identical. The regulators (*cdeR* and *cseR*) share no identity.

Fig. 2. Antibiotic tolerance of *C. subtsugae* CV017 strains. Antibiotic minimum inhibitory concentration (MIC) was determined of cells treated for 4 h with antibiotic at a sub-MIC (1/2 MIC from Table 1) (white bars) or no antibiotic (black bars). MIC and sub-MIC were with A) bactobolin or B) tetracycline. Bactobolin MIC is given as the final concentration of filtered culture fluid (% FCF). Final MIC is shown as the average and standard error of 4-6 biological replicates for each strain. Statistical significance by *t*-test; *, $P < 0.03$.

Fig. 3. Antibiotic induction of *cdeA* and *cseA* transcription. Exponentially growing cultures were diluted to an optical density of 600 nm (OD_{600}) of 0.1 and incubated for 4 h with no antibiotic (black bars), or a sub-minimum inhibitory concentration (sub-MIC) of bactobolin (grey bars) or tetracycline (white bars) prior to harvesting RNA. Sub-MIC concentrations are 1/2 the MIC as indicated in Table 1. Quantitative digital PCR was used to measure *cseA* (panel A) or *cdeA* (panel

B) transcripts, and results were normalized to the *rpoD* housekeeping gene and shown as *rpoD*-adjusted transcripts. The values represent the mean of three independent experiments and the error bars represent the standard error of the mean. Statistical analysis by t- test compared with untreated wild type: *, $p \leq 0.02$

Fig. 4. Specificity of tolerance responses. Antibiotic minimum inhibitory concentration (MIC) was determined of cells treated for 6 h with antibiotic at a sub-MIC (1/2 MIC from Table 1) (white bars) or no antibiotic (black bars). MIC and sub-MIC were with A) bactobolin and tetraycline or B) tetracycline and bactobolin, respectively. Bactobolin MIC is given as the final concentration of filtered culture fluid (% FCF). Sub-MIC concentrations are 1/2 the MIC as indicated in Table 1. Final MIC is shown as the average and standard error of 3-4 biological replicates for each strain. *, statistical significance by *t*-test; *, $P < 0.03$.

Fig. 5. *B. thailandensis-C. subtsugae* competition. Co-cultures were of wild-type *B. thailandensis* and *C. subtsugae* wild-type CV017 or mutant strains. The black dashed line indicates the starting 10:1 ratio of *B. thailandensis* to *C. subtsugae*. The log ratio of *C. subtsugae* to *B. thailandensis* (*Cs/Bt*) after 24 h was determined by selective plating and colony counts. Open circles, cultures with no added antibiotic. Filled circles, co-cultures were treated with a sub-lethal concentration of bactobolin from *B. thailandensis* filtered culture fluid (see Materials and Methods) added to culture medium at the start of the co-culture experiment. The solid lines represent means for each group. The vertical bars show the standard error of the mean for each group. *****, statistically significant by student's *t*-test compared with bactobolin-treated wild type ($p < 0.001$).

Fig. 1.

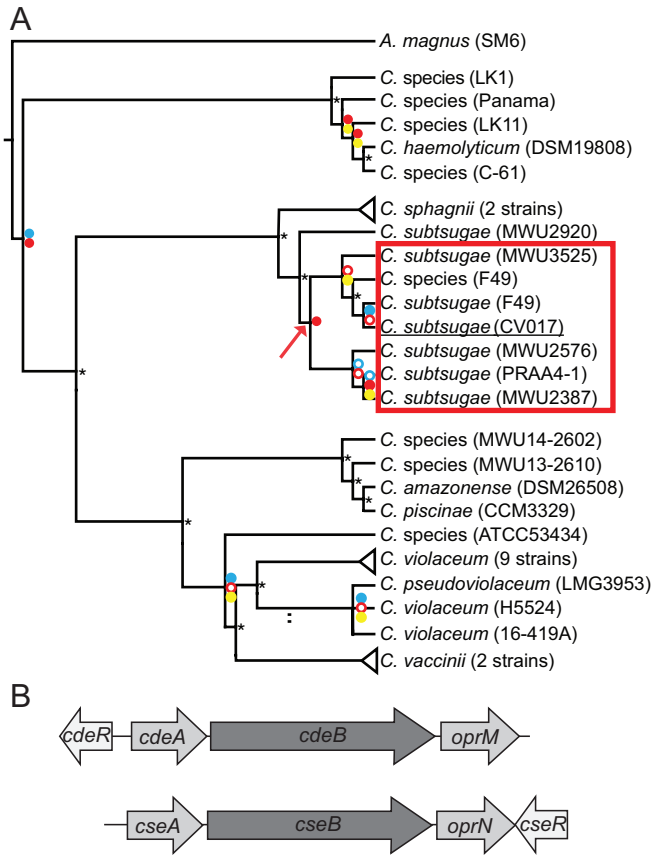

A
A. magnus (SM6)
C. species (LK1)
C. species (Panama)
C. species (LK11)
C. haemolyticum (DSM19808)
C. species (C-61)
C. sphagnii (2 strains)
C. subtsugae (MWU2920)
C. subtsugae (MWU3525)
C. species (F49)
C. subtsugae (F49)
C. subtsugae (CV017)
C. subtsugae (MWU2576)
C. subtsugae (PRAA4-1)
C. subtsugae (MWU2387)
C. species (MWU14-2602)
C. species (MWU13-2610)
C. amazonense (DSM26508)
C. piscinae (CCM3329)
C. species (ATCC53434)
C. violaceum (9 strains)
C. pseudoviolaceum (LMG3953)
C. violaceum (H5524)
C. violaceum (16-419A)
C. vaccinii (2 strains)
B
cdeR
cdeA
cdeB
oprM
cseA
cseB
oprN
cseR

Fig. 2.

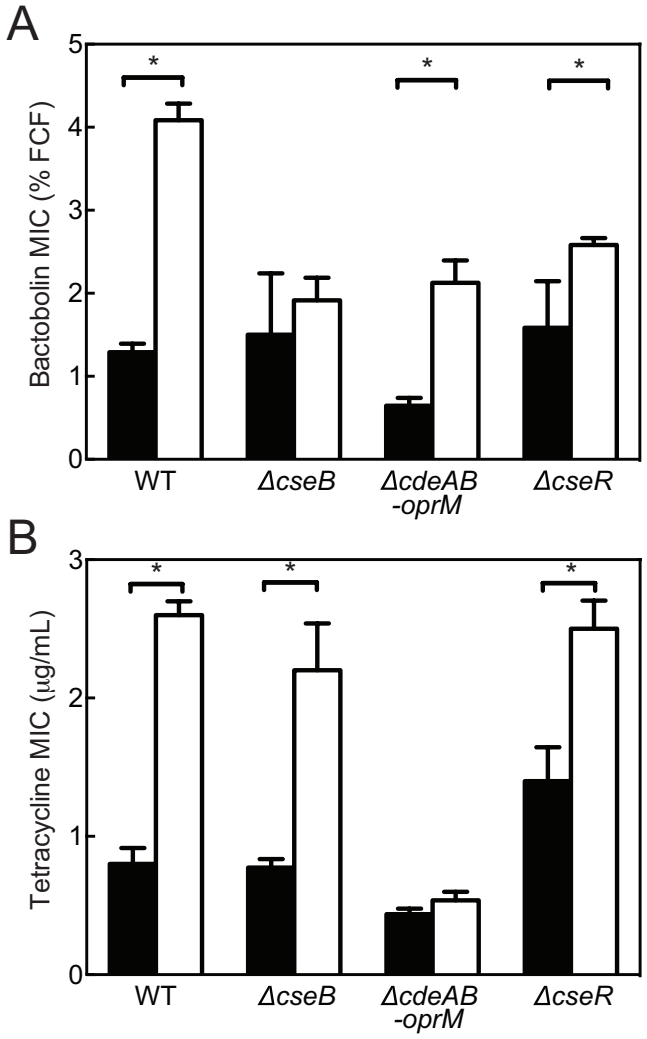

A
Bactobolin MIC (% FCF)
5
4
3
2
1
0
*
*
*
WT
ΔcseB
ΔcdeAB
-oprM
ΔcseR
B
Tetracycline MIC (μg/mL)
3
2
1
0
*
*
*
WT
ΔcseB
ΔcdeAB
-oprM
ΔcseR

Fig. 3.

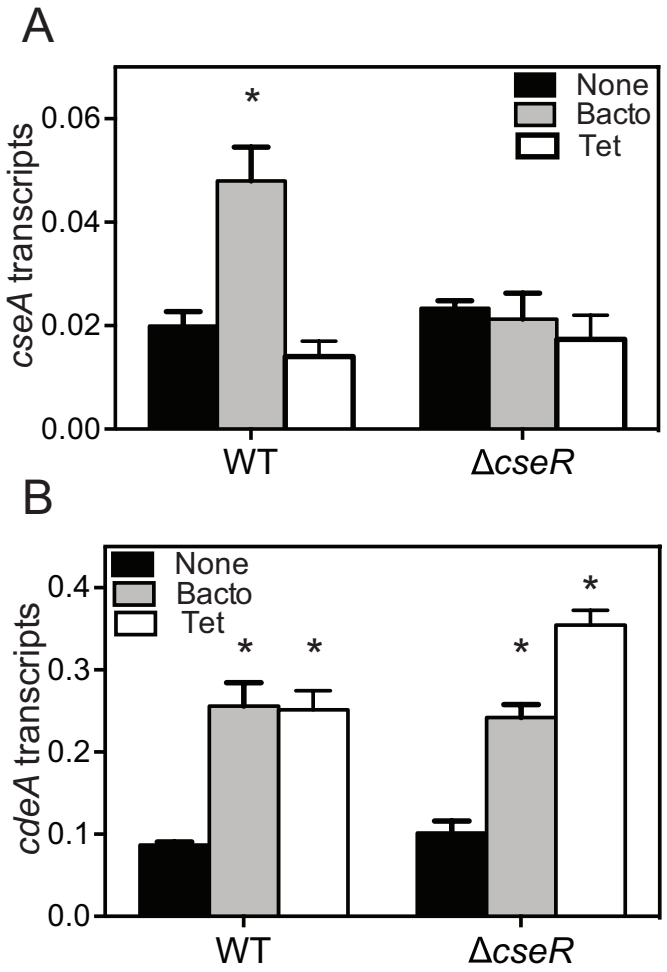

A
None
Bacto
Tet
*
0.06
0.04
0.02
0.00
cseA transcripts
WT
ΔcseR
B
None
Bacto
Tet
*
*
*
*
0.4
0.3
0.2
0.1
0.0
cdeA transcripts
WT
ΔcseR

Fig. 4

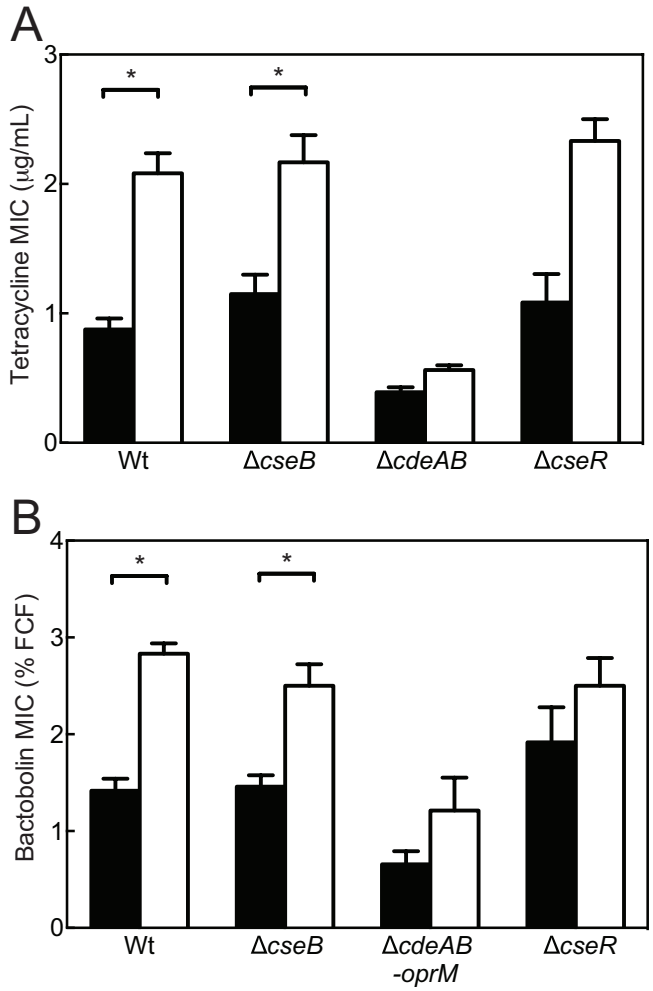
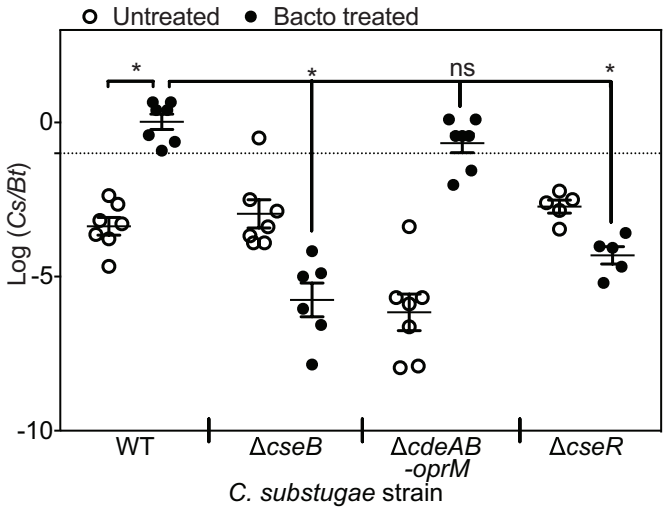

A
Tetracycline MIC (μg/mL)
0
1
2
3
*
*
Wt
ΔcseB
ΔcdeAB
ΔcseR
B
Bactobolin MIC (% FCF)
0
1
2
3
4
*
*
Wt
ΔcseB
ΔcdeAB
-oprM
ΔcseR

Fig. 5.


Untreated
Bacto treated
*
*
ns
*
0
-5
-10
Log (Cs/Bt)
WT
ΔcseB
ΔcdeAB
-oprM
ΔcseR
C. substugae strain

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