Title: Efflux pumps in *Chromobacterium* species and their involvement in antibiotic tolerance and survival in a co-culture competition model

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Running title: Antibiotic tolerance in *Chromobacterium*

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ABSTRACT (250 words)

Members of the *Chromobacterium* genus includes opportunistic but often-fatal pathogens and soil saprophytes with highly versatile metabolic capabilities. In previous studies of *Chromobacterium subtsugae* (formerly *C. violaceum*) strain CV017, we identified a resistance nodulation division (RND)-family efflux pump (CdeAB-OprM) that confers resistance to several antibiotics including the bactobolin antibiotic produced by the soil saprophyte *Burkholderia thailandensis*. Here, we show the *cdeAB-oprM* genes increase *C. subtsugae* survival in a laboratory competition model with *B. thailandensis.* We also demonstrate that adding sublethal bactobolin concentrations to the co-culture increases *C. subtsugae* survival, but this effect is not through CdeAB-OprM. Instead, the increased survival requires a second, previously unreported pump we call CseAB-OprN. We show the CseAB-OprN genes are transcriptionally induced in cells exposed to sublethal bactobolin concentrations and that this causes an increase in bactobolin tolerance. Induction of this pump is through a bactobolin-responsive regulator, CseR. We also demonstrate that CseAB-OprN is highly specific and sensitive to bactobolin, while CdeAB-OprM appears to have a broader range of specificities. We examine the distribution of CseAB-OprN and CdeAB-OprM in members of the *Chromobacterium* genus and find that CseAB-OprN is limited to the non-pathogenic *C. subtsugae* strains, whereas CdeAB-OprM is more widely distributed among members of the *Chromobacterium* genus including the occasional pathogen *C. violaceum.* Our results provide new information on the antibiotic resistance mechanisms of *Chromobacterium* species and use co-culture competition experiments to highlight the importance of efflux pumps in saprophytic bacteria.

IMPORTANCE (150 words)

Antibiotic efflux pumps are best known for their role in increasing antibiotic resistance of a variety of pathogens. However, the role of these pumps in saprophytic species is much less well defined. This study describes the identification and characterization of two predicted efflux pump gene clusters in members of the *Chromobacterium* genus, which is primarily comprised of non-pathogenic saprophytes but also has several members known to occasionally cause highly fatal infections in humans. One of the predicted efflux pump gene clusters is present in every member of the *Chromobacterium* genus and shown to increase resistance to a broad range of antibiotics, including those that are clinically relevant. The other gene cluster confers resistance to a limited range of antibiotics and is found only in *Chromobacterium subtsugae,* a subset of this genus that is entirely nonpathogenic. We demonstrate these pumps can be activated by their antibiotic substrates to increase antibiotic tolerance. We also use a dual-species laboratory model to demonstrate that efflux pump activation by antibiotics is important for *C. subtsugae* to survive during competition with another antibiotic-producing bacteria. These results have implications for managing antibiotic-resistant *Chromobacterium* infections and for understanding the evolution of efflux pumps outside of the host.

73 **INTRODUCTION**
74
75 Members of the *Chromobacterium* genus are often found in water and soil environments
76 of tropical and subtropical regions (1), and cause occasional but often fatal infections in patients
77 (2). Many members of this genus are also potentially useful in industry and ecological applications
78 such as bioplastics synthesis (3, 4), hydrolyzing plastic films (5), solubilizing gold (6, 7), and pest
79 management (8, 9). The most well-known trait shared by many members of this genus is the
80 ability to produce a purple pigment, violacein, which has a variety of biotechnologically interesting
81 applications including as an antimicrobial (10) and anti-cancer therapeutic (11, 12). Production of
82 violacein and other antibiotics is controlled by *N*-acylhomoserine lactone (AHL) quorum sensing
83 (13, 14), a type of cell-cell communication distributed widely in the Proteobacteria (15). Because
84 production of violacein can be easily observed in cell cultures, a strain of *C. subtsugae* strain
85 CV017 (formerly *C. violaceum* CV017) is commonly used as a biosensor of AHL signals from the
86 environment and other bacteria (13). This strain has also been used to study mechanisms of
87 interspecies competition using a laboratory model developed with another soil saprophyte,
88 *Burkholderia thailandensis* (16, 17).
89 We are interested in understanding mechanisms that are important for competition with
90 other strains and species in polymicrobial communities, and studying these mechanisms using
91 relatively simple laboratory models. Our *B. thailandensis*-*C. subtsugae* model was previously
92 used to show how quorum sensing-controlled antibiotics can change the dynamics of competition
93 between species (16, 17). Here, we use this model to explore mechanisms of antibiotic defense.
94 We initially chose *B. thailandensis* and *C. subtsugae* for our model because they each grow
95 similarly in the conditions of the experiment, however, these species can also be isolated from
96 similar tropical soil and water environments. Also, in our laboratory conditions, both species
97 produce antibiotics that can kill the other species, and we wished to explore the effects of these
98 antibiotics in our earlier experiments (16). In the case of *C. subtsugae*, the involved antibiotic(s)
99 remain unknown, and have a relatively small but significant role in the outcome of competition. In

100 the case of *B. thailandensis* the antibiotic is bactobolin, which has a relatively large effect in the
101 co-culture model (16). Bactobolin is a broad-spectrum antibiotic that targets the 50S ribosomal
102 subunit to block translation (17).

103 Our previous studies identified a *C. subtsugae* predicted antibiotic efflux pump gene
104 cluster that contributes to bactobolin resistance (18). We named the predicted efflux pump
105 CdeAB-OprM. CdeAB-OprM belongs to the nodulation division (RND) efflux pump family. RND-
106 family efflux pumps are situated within the inner membrane and involve three proteins: an inner
107 membrane pump, an outer membrane channel, and a periplasmic adaptor protein. Together, the
108 proteins form a tripartite efflux pump spanning both the inner and the outer membrane (19). Drug
109 exporters belonging to the RND family play a key role in resistance to clinically relevant antibiotics
110 in Proteobacteria (19). Resistance to antibiotics is generally due to mutation of a transcription
111 regulator that increases production of the efflux pump and causes the cell to become more
112 antibiotic resistant. Although the involvement of efflux pumps in antibiotic-resistant infections has
113 been well established, much less is known about the factors that contribute to the evolution of
114 efflux pumps outside of a host infection. The presence of efflux pumps in many saprophytic
115 bacteria suggest these pumps are also important in other contexts, for example for managing the
116 toxicity of endogenous antibiotics (20). Efflux pumps might also be important for defending against
117 antibiotics produced by other species during competition.

118 In this study, we use our co-culture model to explore the importance of efflux pumps during
119 interspecies competition. We demonstrate that CdeAB-OprM increases *C. subtsugae* survival
120 during competition with bactobolin-producing *B. thailandensis.* We also demonstrate that adding
121 a sublethal concentration of bactobolin to the co-culture experiments increases *C. subtsugae*
122 survival in the co-culture model, suggesting that *C. subtsugae* defense mechanisms are activated
123 by exposure to the low doses of bactobolin. However, increased survival is not dependent on
124 CdeAB-OprM. Instead, we find a newly identified pump, CseAB-OprN, and show this pump is
125 important for the increased survival in co-culture. We also demonstrate the involvement of CseAB-

126 OprN in a specific response to bactobolin and that this gene cluster is activated by sublethal
127 bactobolin concentrations through a LysR-family regulator encoded adjacent to the *cseAB-oprN*
128 genes, CseR. We show that the *cseAB-oprN* genes are limited to the *C. subtsugae* strains, which
129 are not known to be pathogenic, and that the genes encoding CdeAB-OprM are widely distributed
130 in the *Chromobacterium* genus. Together, our results describe two RND-efflux pumps found in
131 members of the *Chromobacterium* genus and demonstrate how the evolution of antibiotic
132 response might be important during competition with other species in the soil.
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134

135 **RESULTS**
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138 **Sublethal bactobolin increases *C. subtsugae* survival in co-culture.**

139 In many bacteria, exposure to sublethal antibiotic concentrations reversibly increases
140 antibiotic resistance, often through the activation of antibiotic efflux pumps (19), to cause induction
141 of antibiotic tolerance. We used our *B. thailandensis-C. subtsugae* co-culture model to test
142 whether addition of a sublethal antibiotic (bactobolin) alters the outcome of competition through
143 induction of antibiotic tolerance. We combined exponentially growing pure cultures of *B.*
144 *thailandensis* and *C. subtsugae* and added sublethal concentrations of bactobolin from *B.*
145 *thailandensis* filtered culture fluid at the beginning of co-culture growth. We used 1/2 the minimal
146 bactobolin concentration that inhibits growth (1/2 MIC, which was 0.5-0.6% culture fluid in the total
147 co-culture). With no added bactobolin, *B. thailandensis* outcompeted *C. subtsugae* in co-cultures
148 consistent with previous results (16, 18)(Fig. 1, 'wild type,' filled circles). However, when sub-MIC
149 bactobolin was supplied in the culture, *C. subtsugae* gained an advantage over *B. thailandensis*
150 (Fig. 1 'wild type,' open circles). In identical experiments with culture fluid from a *B. thailandensis*
151 bactobolin mutant, we observed no effect on co-culture outcomes (Fig. S1). These results show
152 that exposure to sub-MIC bactobolin increases *C. subtsugae* survival in co-culture competition
153 with *B. thailandensis*.
154

155 **Increased survival in response to sublethal bactobolin is through a newly identified efflux**
156 **pump, CseAB-OprN in *C. substugae***

157 Because our previous results showed CdeAB-OprM is important for bactobolin resistance,
158 we predicted the CdeAB-OprM efflux pump might increase competitiveness in response to
159 bactobolin (18). To test this hypothesis, we compared the competitive ability of a *C. subtsugae*
160 Δ*cdeAB-oprM* mutant with that of wild type in our co-culture experiments. In co-cultures with no
161 added bactobolin, we observed that the Δ*cdeAB-oprM* mutant was less competitive than wild type

162 (Fig. 1). This result is consistent with the role of the *cdeAB-oprM* genes in bactobolin resistance
163 (18). However, when sublethal bactobolin was added to the co-culture the competitive ability of
164 the Δ*cdeAB-oprM* mutant increased by 1000-fold (Fig. 1). The increase was similar to that of wild-
165 type *C. subtsugae*. These results show that although the CdeAB-OprM pump is important for
166 bactobolin resistance, this pump is not responsible for the change in the outcome of competition
167 following exposure to sublethal bactobolin.

168 We predicted a related pump with similar substrate specificity might be involved in the
169 response to sublethal bactobolin in co-cultures. We searched the genome of CV017 for a gene
170 related to *cdeB* and found one that is 80% identical to *cdeB* at the nucleotide level, which we call
171 *cseB.* The *cseB* gene is flanked by genes with similarity to the *cdeA* and *oprM* genes (*cseA* and
172 *oprN*) (Fig. 1B). Upstream of the *cdeAB-oprM* genes there is a gene encoding a putative TetR-
173 family repressor called *cdeR*, which we previously showed is involved in regulating the *cdeAB-*
174 *oprM* genes (18). However, there is no homologous gene to *cdeR* upstream of the *cseAB-oprN*
175 gene cluster. Instead, downstream of the *cseAB-oprN* genes there is a gene encoding a predicted
176 LysR family transcription activator, which we call *cseR* (21). To test the role of *cseAB-oprN* and
177 *cseR* in our co-culture model, we deleted the *cseB* and *cseR* genes in CV017 and performed co-
178 culture experiments with the deletion mutants. We observed that the competitive ability of both
179 Δ*cseB* and Δ*cseR* was unchanged after exposure to sublethal bactobolin (Fig. 1, Δ*cseB* and
180 Δ*cseR,* filled circles). Thus, both CseR and CseAB-OprN are important for *C. subtsugae* to
181 increase competitiveness in response to sublethal bactobolin.

182

183 ***Role of CseAB-OprN and CdeAB-OprM in antibiotic susceptibility and tolerance***

184 To further characterize the newly identified CseAB-OprN and the CdeAB-OprM efflux
185 pump, we determined the minimum inhibitory concentration (MIC) of bactobolin for *C. subtsugae*
186 wild type and strains defective for each of the pumps or their putative regulators (Table 1).

187 Consistent with previously reported results (18), the Δ*cdeAB-oprM* mutant was about 3-fold more
188 susceptible to bactobolin than wild type. Deleting *cdeR* increased bactobolin resistance by 6-fold,
189 and the increase was not observed in a *cdeR, cdeAB-oprM* double mutant. These results suggest
190 CdeR is represses bactobolin resistance through the CdeAB-OprM system. We also observed
191 *cdeA* and *cdeB* transcripts were increased in a *cdeR* mutant compared with wild type (Fig. S2).
192 Together, these results support the idea that CdeAB-OprM increases bactobolin resistance and
193 CdeR is a transcriptional repressor of the *cdeAB-oprM* gene cluster. In contrast, the bactobolin
194 MIC for the Δ*cseB* mutant was identical to that of wild type (Table 1). Thus, the CseAB-OprN
195 pump does not contribute to bactobolin resistance in standard MIC experiments.

196 We also tested other antibiotics such as tetracycline, chloramphenicol, erythromycin (a
197 macrolide), gentamicin and kanamycin (both aminoglycosides), imipenem (a cell wall-targeting β-
198 lactam antibiotic), and ciprofloxacin (DNA gyrase inhibitor). In the case of tetracycline,
199 erythromycin, chloramphenicol and ciprofloxacin, the *cdeAB-oprM* mutant was more susceptible
200 than wild type. However, as with bactobolin, the Δ*cseB* mutant was not more susceptible than
201 wild type to any of the tested antibiotics. We also tested a *cseB, cdeAB-oprM* double mutant for
202 sensitivity to each of the antibiotics, and the double mutant showed the same susceptibility
203 phenotypes as the *cdeAB-oprM* single mutant. The results show that CseAB-OprN does not play
204 a significant role in increasing resistance in standard susceptibility experiments.

205 Although CseAB-OprN was not important in standard antibiotic susceptibility testing, the
206 role of this pump in bactobolin-treated co-cultures (Fig. 1) supports the idea that this pump could
207 be important for inducing a bactobolin tolerance response. To test this hypothesis, we developed
208 an experiment to assess antibiotic tolerance by measuring the change in antibiotic susceptibility
209 (MIC) following exposure to sublethal antibiotic concentrations. We grew CV017 cells for 6 h with
210 1/2 MIC of bactobolin (determined in Table 1), then compared the MIC of bactobolin-exposed
211 cells with that of identically grown cells with no prior bactobolin exposure. We found that the MIC

212 of bactobolin-exposed cells was 3.2 (± 0.2)-fold higher than identically treated cells not exposed
213 to any antibiotic (Fig. 2A). Thus, we were able to observe induction of bactobolin tolerance in *C.*
214 *subtsugae* in our experiment. We performed similar experiments with the *C. subtsugae* Δ*cdeAB-*
215 *oprM,* Δ*cseB* or Δ*cseR* mutants. Our results showed exposure to sublethal bactobolin increased
216 the MIC of the Δ*cdeAB-oprM* mutant 3.4 (± 1.2)-fold, similar to the induction observed with wild
217 type. However, there was no significant MIC change observed with Δ*cseB* mutant cells following
218 sublethal bactobolin exposure. These results support that the CseAB-OprN pump, and not the
219 CdeAB-OprM pump, is important for bactobolin tolerance. Δ*cseR* mutant cells also induced
220 bactobolin tolerance, but the change in MIC of 1.8 (± 0.2)-fold for the Δ*cseR* mutant was
221 significantly less than that of wild type ($p < 0.0005$) supporting that CseR also plays a role in
222 bactobolin tolerance.

223 To test whether CseAB-OprN is involved in inducing resistance to antibiotics other than
224 bactobolin, we used a similar approach to assess tolerance responses to erythromycin,
225 ciprofloxacin, and tetracycline. We observed no significant change in MIC to erythromycin or
226 ciprofloxacin after sublethal exposure (Fig. S3). However, exposure to sublethal concentrations
227 of tetracycline increased tetracycline MIC by 3.9 (± 0.9)-fold, similar to that of bactobolin (Fig. 2B).
228 Surprisingly, a similar 2.7 (± 1.3)-fold increase in MIC was observed for the Δ*cseB* mutant. And
229 no increase was observed for the Δ*cdeAB-oprM* mutant. These results show *C. subtsugae*
230 induces tetracycline tolerance through the CdeAB-OprM system and not through CseAB-OprN.
231

232 **Specificity of CseAB-OprN and CdeAB-OprM**

233 We found it interesting that the CdeAB-OprM system was important for bactobolin
234 resistance, but not tolerance (Fig. 2). These results suggest bactobolin might be weakly
235 recognized as an inducer by the CdeAB-OprM system or serve as a poor substrate for export. To
236 understand the interaction of each pump with bactobolin, we exposed cells to sublethal

237 concentrations of bactobolin and then determined the MIC of tetracycline. We predicted that if
238 bactobolin can induce the CdeAB-OprN pump but serves as a poor substrate for export, we would
239 observe an increase in tetracycline MIC in response to bactobolin induction in a manner
240 dependent on CdeAB-OprM. Consistent with this prediction, our result showed that incubation
241 with sublethal bactobolin increased the tetracycline MIC and the response was dependent on
242 CdeAB-OprM (Fig. 3A). These results support the conclusion that the CdeAB-OprM pump can be
243 induced by sublethal bactobolin. We also performed the reverse experiment; we incubated cells
244 with sublethal tetracycline and subjected those cells to a bactobolin MIC. In this experiment,
245 tetracycline increased the bactobolin MIC in a manner that was also dependent on the CdeAB-
246 OprM system (Fig. 3B). Thus, tetracycline and bactobolin can both serve to induce tolerance
247 through CdeAB-OprM, although the effects of bactobolin were only observed in combination with
248 tetracycline suggesting bactobolin is a relatively poor substrate of this pump. In contrast,
249 tetracycline was unable to induce tolerance or be recognized by the CseAB-OprN pump,
250 suggesting this antibiotic is only recognized by the CdeAB-OprM pump. Together, our results
251 support the conclusion that CseAB-OprN has a high level of sensitivity and specificity for
252 bactobolin, whereas CdeAB-OprM recognizes a broader range of antibiotics.
253
254 **CseR is a bactobolin-responsive *cseAB-oprN* gene activator.**
255 Because CseR is important in co-culture and antibiotic tolerance experiments and also
256 belongs to the LysR transcriptional regulator family, we hypothesized that CseR might be an
257 activator of the CseAB-OprN system. To test this hypothesis, we measured *cseA* transcripts in
258 wild type or the Δ*cseR* mutant cells exposed to bactobolin, tetraycline or no antibiotic (Fig. 4). Our
259 results showed a small but significant 2.4-fold increase in *cseA* transcripts in bactobolin-treated
260 cells compared with untreated cells or tetracycline-treated cells. The increase in bactobolin-
261 treated cells was dependent on *cseR.* Thus, CseR activates transcription of the *cseAB-oprN*
262 genes in response to bactobolin. We also measured induction of *cdeA* transcription in these cells

263 to assess the specificity of CseR gene activation. We observed a 3-fold increase in *cdeA*
264 transcription in cells treated with either bactobolin or tetracycline that was independent of CseR.
265 Thus, CseR specifically activates *cseAB-oprN* transcription in response to bactobolin antibiotic.
266 We also observed that *cdeA* transcription was induced by bactobolin or tetracycline in a CseR-
267 independent manner, which was consistent with the results of our antibiotic tolerance experiments
268 (Figs. 2 and 3).

269 Many LysR-family activators require a ligand for activation of target genes. To test if
270 increasing CseR production is sufficient to activate the CseAB-OprN system or if bactobolin is
271 required for this activation, we introduced the *cseR* gene into plasmid pBBR-MCS5 downstream
272 of the constitutive T3 promoter and introduced this plasmid to the *C. subtsugae* Δ*cseR* strain. As
273 a control, we also made a similar pBBR-MCS5 *cdeR* plasmid and introduced this plasmid to the
274 *C. subtsugae* Δ*cdeR* strain. While the pBBR-*cdeR* plasmid markedly decreased the Δ*cdeR* mutant
275 bactobolin MIC, there were no effects of expressing *cseR* in the Δ*cseR* mutant (Table S1).
276 However, *cseR*-expressing cells exposed to sublethal bactobolin significantly increased
277 bactobolin tolerance compared with the plasmid-only control or cells not exposed to bactobolin
278 (Fig. S3). Thus, constitutive CseR production does not appear to be sufficient to induce tolerance,
279 and bactobolin likely serves as a ligand activator of the CseR regulator.

280

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

282 We were interested in evaluating the distribution of the *cdeAB-oprM* and *cdeAB-oprM* genes
283 in the *Chromobacterium* genus. We constructed a phylogenetic tree using 32 *Chromobacterium*
284 genomes and an outgroup (*Aquitaliea magnus*) (Fig. 5, Fig. S5 and Table S2). We constructed
285 the tree using a set of 140 orthologous proteins (see Materials and Methods) to increase
286 confidence of relationship predictions. Using this method, CV017 grouped with the *C. subtsugae*
287 strains, consistent with the new classification of this strain. We were also able to make confident

288 predictions of species associations for some of the unnamed species, for example, strain F49
289 grouped with the *C. subtsugae* clade. It is notable that the species known to cause infections in
290 humans, *C. violaceum* and *C. haemolyticum,* grouped separately from *C. subtsugae*. Thus *C.*
291 *subtsugae* may be representative of a primarily non-pathogenic clade of *Chromobacterium.*

292 We identified *cdeB* homologs in all of the strains ranging from 85-100% nucleotide sequence
293 identity to CV017 *cdeB*. Each of these strains had a similar arrangement of *cdeA, oprM* and *cdeR*
294 genes surrounding *cdeB* (Fig. 5B). We used a best-reciprocal BLAST search to discern *cdeB* and
295 *cseB* homologs, and identified *cdeB* homologs only in CV017, F49 and other members of the *C.*
296 *subtsugae* species with the exception of MWU2920. In these other species, the *cseB* genes were
297 >99% identical to one another, and the organization of genes surrounding *cseB* was conserved
298 with that of the *cseAB-oprN* genes in CV017. We predicted that the *cseAB-oprN* gene cluster
299 might have arisen from the *cdeAB-oprM* genes via a duplication event that occurred in an ancestor
300 of *C. subtsugae*; however, generation of a second tree using the *cseB* and *cdeB* gene homologs
301 did not support this hypothesis (Fig. S6). Instead, the alignments show *cseB* groups with *cdeB*
302 from *Chromobacterium* species C61, suggesting that *cseB* might have arisen from a horizontal
303 gene transfer event from an ancestor of this strain.
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310 **DISCUSSION**
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312 In this study, we demonstrate antibiotic specificity and regulation of two efflux pumps of
313 *Chromobacterium subtsugae* CV017. One of these, CseAB-OprN, had not been previously
314 reported and is limited to the *C. subtsugae* species. The other, CdeAB-OprM, is widely distributed
315 in members of the *Chromobacterium* genus. CdeAB-OprM has a broad range of specificity for
316 antibiotics such as tetracycline, erythromycin, chloramphenicol and ciprofloxacin. As ciprofloxacin
317 is one of the recommended treatments for infections with pathogenic *Chromobacterium* (2), the
318 results suggest CdeAB-OprM might be important in cases of antibiotic failures during
319 *Chromobacterium* infections. To date, there are very few publications on *Chromobacterium*
320 mechanisms of antibiotic resistance and drug export (18, 22, 23). Results of this study will be
321 useful for identifying resistance mechanisms in clinical isolates and for future *Chromobacterium*
322 research. As *Chromobacterium* members are primarily saprophytic non-pathogens, our results
323 are also important for understanding the ecology and evolution of efflux pumps.
324 Our results demonstrate that both CdeAB-OprM and CseAB-OprN are induced by their
325 antibiotic substrates. Such a response is typical of related pumps such as the aminoglycoside-
326 responsive MexXY pump of *Pseudomonas aeruginosa* (24) and the trimethoprim-responsive
327 BpeEF-OprC pump of *Burkholderia pseudomallei* (25). Because many antibiotic resistance
328 factors are costly to produce, waiting to induce their production until needed might allow a valuable
329 metabolic cost savings (26). The ability to respond to antibiotic stress might be an important
330 strategy for bacteria to survive antibiotics during treatment of infected patients (27). Such
331 responses might also be important during competition with other bacteria (28). Our co-culture
332 model highlights the importance of tolerance induction during competition and how such
333 regulation might evolve in soil bacteria. The induction of tolerance has been proposed to be a
334 type of 'danger sensing,' which is important for sensing and responding to threats posed by other
335 bacteria (28). Sublethal antibiotics serves as a warning that antibiotic-producing competitors are
336 nearby and might soon deliver higher killing doses.

337 Competitive interactions with other bacteria might also be an important factor shaping the
338 evolution of antibiotic production. Many antibiotics are regulated by quorum sensing, which
339 activates production in response to changes in population density; for example in *B. thailandensis,*
340 bactobolin is regulated by the BtaR2-I2 quorum-sensing system (29). Placing antibiotics under
341 the control of quorum-sensing systems might be a strategy to avert induction of antibiotic
342 tolerance by other species (30). Quorum-sensing control of antibiotics might serve to delay
343 antibiotic production until the population can produce a sudden killing dose of antibiotic.
344 Bactobolin production is activated by quorum sensing when the population reaches stationary
345 phase (31) and sufficient cells have accumulated to produce killing dose. Adding bactobolin at
346 the start of the experiment exposes *C. substugae* to low doses prior to the accumulation of the
347 killing dose. The early exposure allowed *C. subtsugae* to mount a defense response and survive
348 the higher killing doses. Thus regulatory mechanisms that delay antibiotic production might
349 increase the killing effect of the antibiotics, allowing an effective and efficient 'sneak attack' on the
350 competitor (30).

351 Our phylogenetic analysis suggests that the *cseAB-oprN* genes were acquired through
352 horizontal transfer of the *cdeAB-oprM* cluster from another species, rather than through
353 duplication of the *cdeAB-oprM* genes in the ancestral strain (Fig. 5A and S6). The finding that
354 horizontal transfer of resistance genes occurred in saprophytic species supports the idea that
355 horizontal transfer can lead to the dissemination of antibiotic resistance genes in polymicrobial
356 communities of the soil (32). Our co-culture experiments also support the idea that the spread of
357 resistance might have been helpful for surviving antibiotics produced by other bacteria in the soil.
358 The acquisition of new resistance genes might be important for surviving antibiotics produced by
359 other species, as demonstrated by our co-culture model, or it might also promote tolerance to
360 self-produced antibiotics (33). *Chromobacterium* is known to produce an arsenal of antimicrobial-
361 active molecules (34), although many have not been well characterized. The hypothesis that

362 CseAB-OprN or CdeAB-OprM provides resistance to a self-produced antibiotic can be tested
363 experimentally.
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366 **MATERIALS AND METHODS**
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368 **Bacterial culture conditions and strains.** All strains were grown in Luria-Bertani (LB)
369 broth, LB containing morpholine-propanesulfonic acid (LB-MOPS; 50 mM; pH 7), or on
370 LB with 1.5% (wt/vol) Bacto-Agar. All broth cultures were incubated with shaking at 30°C
371 (*C. subtsugae* or *C. subtsugae-B. thailandensis* co-cultures) or 37°C (*B. thailandensis* or
372 *E. coli*). As a source of bactobolin, we used filtered *B. thailandensis* culture fluid prepared
373 as described previously (18). Filtered *B. thailandensis* culture fluid was stored at 4°C for
374 up to 1 month prior to use and used directly for experiments. For strain constructions, we
375 used gentamicin at 50 µg ml−1 (*C. subtsugae*) or 15 µg ml−1 (*E. coli*). For selection from
376 co-cultures we used gentamicin at 100 µg ml−1 (*B. thailandensis*) and trimethoprim at
377 100 µg ml−1 (*C. subtsugae*).
378 Bacterial strains and plasmids used in this study are listed in Tables S3-S4. For
379 co-cultures, we used the wild-type *B. thailandensis* strain E264 (35). *C. subtsugae* strain
380 CV017 (referred to as wild type, previously known as *C. violaceum* CV017) is a derivative
381 of strain ATCC31532 (36) with a transposon insertion in gene CV_RS05185 causing
382 overexpression of violacein (37, 38). All *C. subtsugae* mutant strains were constructed
383 from CV017 using allelic exchange and methods described previously (16). The
384 constructs for generating deletions in *cseB, cdeR* and *cseR* were made by introducing
385 PCR-generated amplicons or synthetic gene fragments (IDT or Genscript) into
386 pEX18Gm-derived delivery plasmid (39). The mutation was introduced to *C. subtsugae*

387 by conjugation and transconjugants were selected on gentamicin. Transconjugants were
388 transferred to no salt-LB + 15 % sucrose (wt/vol) to select for plasmid excision and correct
389 clones were identified by PCR-based screening. *cseR* and *cdeR* expression plasmids
390 were made using pBBR-MCS5 (40) with PCR-generated *cseR* or *cdeR* fragments introduced
391 downstream of the T3 promoter. All strains and PCR-generated plasmids were verified by
392 PCR amplification and sequencing.
393
394 **Co-culture experiments**. Co-culture experiments were conducted in 10 ml LB-MOPS
395 medium in 125 ml baffled flasks. The inoculum was from logarithmic-phase pure cultures
396 of *C. subtsugae* and *B. thailandensis*. The initial OD_{600} in the co-culture was 0.05 for *B.*
397 *thailandensis* (2-4x10^7 cells per ml) and 0.005 for *C. subtsugae* (2-4x10^6 cells per ml).
398 When indicated, cultures also contained *B. thailandensis* culture fluid at a concentration
399 corresponding with ½ of the MIC for the *C. subtsugae* strain used in the experiment. After
400 inoculating, co-cultures were incubated at 30ºC with shaking at 230 r.p.m for 24 h. Colony
401 forming units of each species were determined by using differential antibiotic selection on
402 LB agar plates. *B. thailandensis* was selected with gentamicin and *C. subtsugae* was
403 selected with trimethoprim.
404
405 **Sequencing the *cse* gene locus.** *cseB* (CV_15400) was initially identified in the
406 assembled CV017 genome (GCA_001510755.1) on scaffold 169 (LKIW01000078). 274
407 bases downstream of *cseB* is the 3' end of scaffold 169. As this sequence did not overlap
408 with any of the other 211 scaffolds of CV017, we used a BLAST search to identify similar
409 sequence in a similar strain (*C. subtsugae* PRAA4-1, GCA_001676875.1) This search

410 identified sequence containing a *cseB*-like gene in scaffold 30 of PRAA4-1, corresponding
411 with CV017 scaffold 192 (LKIW01000104). We used alignments of CV017 scaffold 169
412 and 192 with the PRAA4-1 sequence to design oligonucleotides to amplify the region
413 between scaffolds 169 and 192. We Sanger sequenced the amplified PCR product to
414 verify the correct orientation of the scaffolds and identify the previously unknown 39
415 nucleotides linking forward-facing scaffold 169 with reverse-facing scaffold 192. This was
416 GCCGCCGCCGACAGCCAGCGCCAGCGCGTCAGCGCGACG. CV017 scaffold 169
417 was updated through NCBI (LKIW01000078.2) and scaffold 192 was made secondary to
418 this new scaffold so searches for either will give the new version of scaffold 169.
419
420 **Antibiotic susceptibility and tolerance experiments.** Antibiotic susceptibility (Table 1)
421 was determined by minimum inhibitory concentration (MIC) according to the 2003
422 guidelines of the Clinical and Laboratory Standards Institute (NCCLS), using a modified
423 microtiter method. Antibiotics were added to LB-MOPS in a 100 ul well of a 96-well plate,
424 and 10 successive 2-fold dilutions were made. For each antibiotic, 3 different starting
425 concentrations of antibiotic were used to increase sensitivity of the experiment. *C.*
426 *subtsugae* inocula were prepared by diluting logarithmic-phase cells from LB-MOPS
427 cultures to an optical density at 600 nm (OD_{600}) of 0.005 ($6x10^6$ CFU) in each well. Plates
428 were sealed with Breathe-Easy strips (Fisher Scientific, fishersci.com) and grown for 24
429 h with shaking at 30°C. The MIC was defined as the lowest concentration of antibiotic
430 (µg/ml) in which bacterial growth in the well was not measurable by determining the
431 optical density at 600 nm (OD_{600}) on a 96-well plate reader.

432 To assess changes in MIC induced by exposure to sublethal antibiotic (tolerance),
433 logarithmic-phase *C. subtsugae* cells were diluted to an OD_{600} of 0.1 in 10 ml LB-MOPS
434 in a 125-ml culture flask. The culture medium contained antibiotic at ½ of the MIC
435 determined by the method described above (this varied for each strain), or no antibiotic
436 (untreated), and incubated for 6 hours with shaking at 30°C. These cultures were then
437 directly used to determine MIC using methods described above.
438

439 **Droplet digital PCR**. RNA was harvested from *C. subtsugae* as described in the figure
440 legends. RNA was prepared using the RNeasy® Mini Kit (Qiagen) following the
441 manufacturer instruction with a modification in the DNA digestion step as described
442 previously (18). Droplet digital PCR was performed on a Bio-Rad's QX200 Droplet Digital
443 PCR (ddPCR) System using Eva Green Supermix. Each reaction mixture contained 1 ng
444 $\mu l^{-}1$ of cDNA template, 0.25 µM of each primer, 10 µl Eva Green Supermix, and 8 µl H_2O
445 in a 20-µl volume. After generating 40 µl of oil droplets, 40 rounds of PCR were conducted
446 using the following cycling conditions: 94°C for 20 sec, 60°C for 20 sec, and 72°C for 20
447 sec. Absolute transcript levels were determined using the Bio-Rad QuantaSoft Software.
448 In all cases a no-template control was run with no detectable transcripts.
449

450 **Phylogeny of *Chromobacterium* species and efflux pump genes.** *Chromobacterium*
451 species used for phylogenetic analyses are listed in Table S4. Annotated protein
452 sequences from assembled genomes of *Chromobacterium* and *Aquitaliea magnusonii*
453 (outgroup) were retrieved from the National Center for Biotechnology Information (NCBI,
454 June 2018, Table S3). Comparisons using 16S rRNA provided low confidence predictions

455 of phylogeny, thus we performed an analysis using a set of 140 single-copy orthologs,
456 which were identified using several steps. Orthologous proteins were initially identified by
457 using blastp to carry out reciprocal best BLAST hits of each protein from each strain
458 against a protein database made of all the proteins in our strain set ((41), options: -
459 max_target_seqs 1), to find orthologs with best BLAST hit between all possible pairs of
460 species, and no more than 10% variation in protein length. We aligned this group of 171
461 proteins individually using Muscle version 3.8.31 ((42), options –diags), then reordered
462 sequences using the stable.py script provided by the Muscle developer. Finally, we
463 removed any orthologous protein groups with less than 75% average pairwise identity,
464 less than 30% of sites identical or 100% of sites identical. This last step ensured that all
465 proteins would have intermediate levels of divergence and left the final set of 140
466 orthologs used for phylogenetic tree construction. Finally, we concatenated the protein
467 sequences in each alignment to create one single alignment consisting of 25,351 amino
468 acids. After inspection of this alignment, we found that several pairs of isolates had very
469 low divergence (less than 10 amino acid differences across the entire alignment). We
470 decided to remove one of each of these pairs to reduce redundancy (removed isolates
471 were *C. violaceum* strains LK6, LK30, H5525 and 16-419B). C. *subtsugae* F49 and *C.*
472 *subtsugae* CV017 are identical across the alignment of orthologous proteins but both
473 were retained because CV017 is the focus of the paper.

474 We generated phylogenetic trees using neighbor joining, maximum likelihood and
475 Bayesian methods. A simple neighbor joining (NJ) tree implemented in Geneious version
476 10.1.3 (http://www.geneious.com) with a Jukes-Cantor substitution model and 100
477 bootstrap replicates and *A. magnusonii* as an outgroup. We constructed a maximum

478 likelihood tree using RaxML version 8.2.11 (43) with a Gamma BLOSSUM62 protein
479 model, 100 bootstrap replicates with a parsimony random seed of 1 and *A. manusonii* as
480 an outgroup. We used Mr. Bayes version 3.2.6 (44) to construct a tree using Bayesian
481 methods with a Poisson rate matrix, gamma rate variation with 4 categories, and *A.*
482 *manusonii* as an outgroup, an initial chain length of 1,100,000 with four heated chains at
483 a temperature of 0.2, the subsampling frequency was 2000 generations after an initial
484 burn-in length of 100,000 generations. However, after 332,000 generations, the standard
485 deviation of split frequencies was less than 0.01 and so the tree search was terminated.
486 For all three methods, consensus trees were built based on 50% majority rule, and the
487 three trees were compared using the RaxML maximum likelihood tree as a backbone.
488 We elected to use a concatenated sequence to build our tree because our concern is
489 about the overall evolutionary history of the genus and not how specific genes trees might
490 differ from species tree.

491 To reconstruct the evolutionary history of the two efflux pumps, we compared DNA
492 sequence of the pump genes to improve our ability to resolve evolutionary distances
493 between closely related isolates. We retrieved the DNA sequences by performing a
494 tblastn (45) of the amino acid sequence for that protein against all *Chromobacterium*
495 genome assemblies and then located the correct sequence based on isolate name. Note
496 that one sequence was split among two scaffolds in *C. subtsugae* MWU2387 and was
497 therefore not annotated so we pieced together the appropriate sequence. To find
498 orthologs of the second pump (*cseB*) we performed a tblastn search of the amino acid
499 sequence of CdeB from CV017 against all assembled *Chromobacterium* species.
500 Reciprocal best BLAST searches confirmed the presence of this second pump in only a

501 subset of the *C. subtsugae* isolates (including CV017). We also searched for similar
502 sequences outside of *Chromobacterium* to find evidence for horizontal transfer of the
503 pump. Finally, we constructed phylogenetic trees of the DNA sequence for the two pumps
504 similar to what was described above but with nucleotides instead of amino acids.
505

506
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508
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515 input on sequence analysis.

516
517 **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*cdeRAB*[d]	0.5±0.1	0.4	45±5	47.5±3.5	45±5.8	45.3±14	1	350±70
Δ*cseB*	1.5±0.4	1.1±0.3	25±5	45±7	12±1	53±6	1	9±1
Δ*cseR*	1.4±0.4	1.0±0.3	25±5	35±7	16±1	54±7	1	8±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

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

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

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

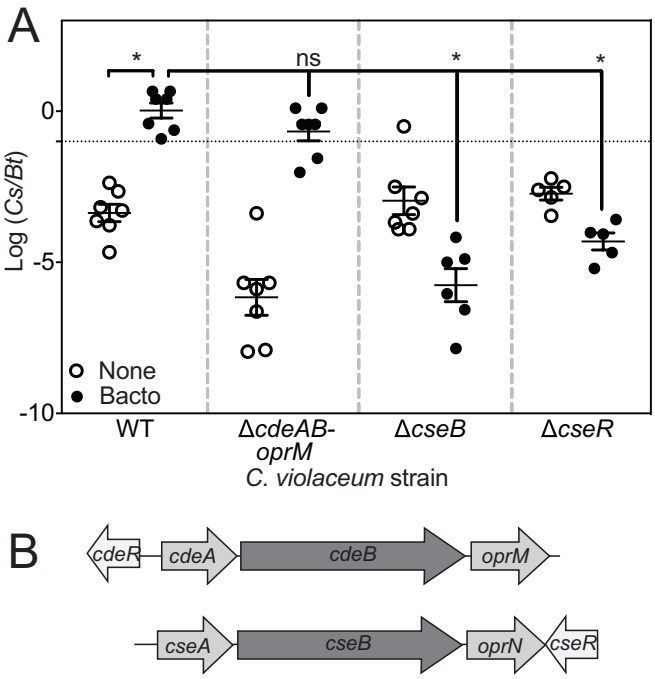
537 [d]Δ*cdeRAB* indicates the strain Δ*cdeAB-oprM*, Δ*cdeR*

538
539

540 **FIGURE LEGENDS**
541
542 **Fig. 1. A) *B. thailandensis-C. subtsugae* competition.** Co-cultures were of wild-type *B.*
543 *thailandensis* (*Bt*) and *C. subtsugae* (*Cs*) CV017 or CV017 mutant strains. The black dashed line
544 indicates the starting 1:10 ratio of *Cs* to *Bt*. The ratio of *Cs* to *Bt* (*Cs/Bt*) after 24 h was determined
545 by selective plating and colony counts. Open circles, cultures with no added antibiotic. Filled
546 circles, co-cultures grown with sublethal bactobolin provided by adding *B. thailandensis* filtered
547 culture fluid (0.5-0.6%) at the start of the co-culture experiment. The solid lines represent means
548 for each group. The vertical bars show the standard error of the mean for each group. *****,
549 statistically significant by student's *t*-test compared with bactobolin-treated wild type ($p < 0.001$).
550 **B) Illustration of *cdeRAB-oprM* and *cseAB-oprN-cseR* gene clusters.** Shading indicates
551 nucleotide sequence identity. Dark grey, 80-89% identical. Light grey, 60-69% identical. The
552 regulators (*cdeR* and *cseR*) share no identity.
553
554 **Fig. 2. Antibiotic tolerance of *C. subtsugae* CV017 strains.** Antibiotic tolerance is the change
555 in minimum inhibitory concentration (MIC) of cells exposed to sublethal antibiotic (white bars)
556 compared with that of identically treated cells with no antibiotic exposure (black bars). Sublethal
557 antibiotic concentrations are 1/2 the MIC as indicated in Table 1 and used to treat cells for 6 h
558 just prior to MIC determinations. Cells were treated for 6 h with A) Bactobolin or B) tetracyline
559 and the same antibiotic was used for MICs. Bactobolin MIC is given as the final concentration of
560 filtered culture fluid (% FCF). B) Tetracycline was used for sublethal exposure and MIC
561 determinations. Final MIC is shown as the average and standard error of 4-6 biological replicates
562 for each strain. Statistical significance by *t*-test; *, $P < 0.03$.
563
564
565 **Fig. 3. Specificity of tolerance responses.** Antibiotic tolerance experiments were performed as
566 described for Fig. 2. In this case, sublethal exposure and MIC determinations were with different
567 antibiotics: A) bactobolin and tetraycline or B) tetraycline and bactobolin, respectively. Final MIC

568 is shown as the average and standard error of 3-4 biological replicates for each strain. *, statistical
569 significance by *t*-test; *, $P < 0.03$.
570
571 **Fig. 4. Antibiotic induction of the efflux pump genes** *cseA* (A) or *cdeA* (B). Transcripts were
572 measured from exponentially growing cells treated for 4 h with no antibiotic (black bars) or a
573 sublethal concentration of bactobolin (grey bars) or tetracycline (white bars). Sublethal antibiotic
574 concentrations are 1/2 the MIC as indicated in Table 1. Results are shown as *rpoD*-adjusted
575 transcripts. The values represent the mean of three independent experiments and the vertical
576 bars represent the standard error of the mean. Statistical analysis by t- test compared with
577 untreated wild type: *, $p \leq 0.02$
578
579
580 **Fig. 5. Phylogenetic tree of *Chromobacterium* species.** Tree construction is described in
581 materials and methods. Circles at each node represent bootstrap values based on neighbor
582 joining (blue circles), maximum likelihood (red circles) and Bayesian (yellow circles) methods.
583 Filled circles, >95% support; unfilled circles, >50% support; missing circles, <50% support. *, all
584 three methods showed >95% support. Triangles represent several highly similar strains;
585 *Chromobacterium sphagnii* (37-2 and 14-B11), *Chromobacterium violaceum* (L_1B5_1, LK17,
586 LK15, GHS51, 16-454, CV1192, CV1197, GN5 and 12472), and *C. vaccinii* (MWU205 and 21-1).
587 A full representation of the species in the phylogenetic tree is shown in Fig. S1. Red box indicates
588 species encoding the *cseAB-oprN* genes. Red arrow, node where species with the *cseAB-oprN*
589 gene cluster separate from other species.
590
591
592
593

594 **Fig. 1.**
595



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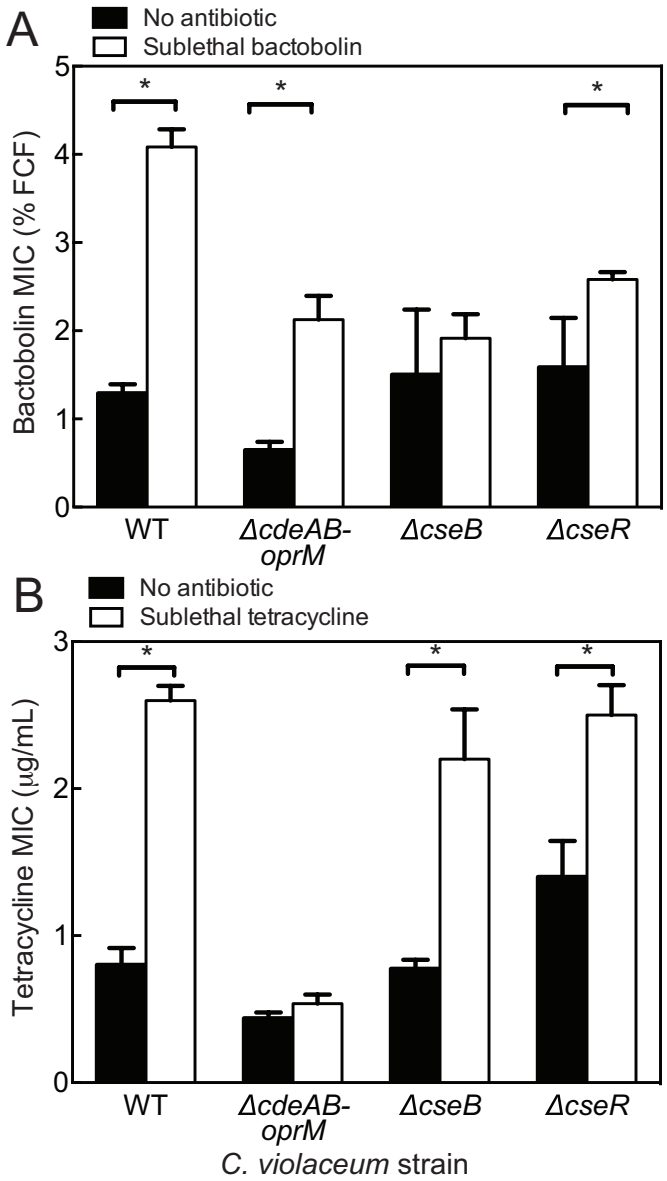
600 **Fig. 2.**
601



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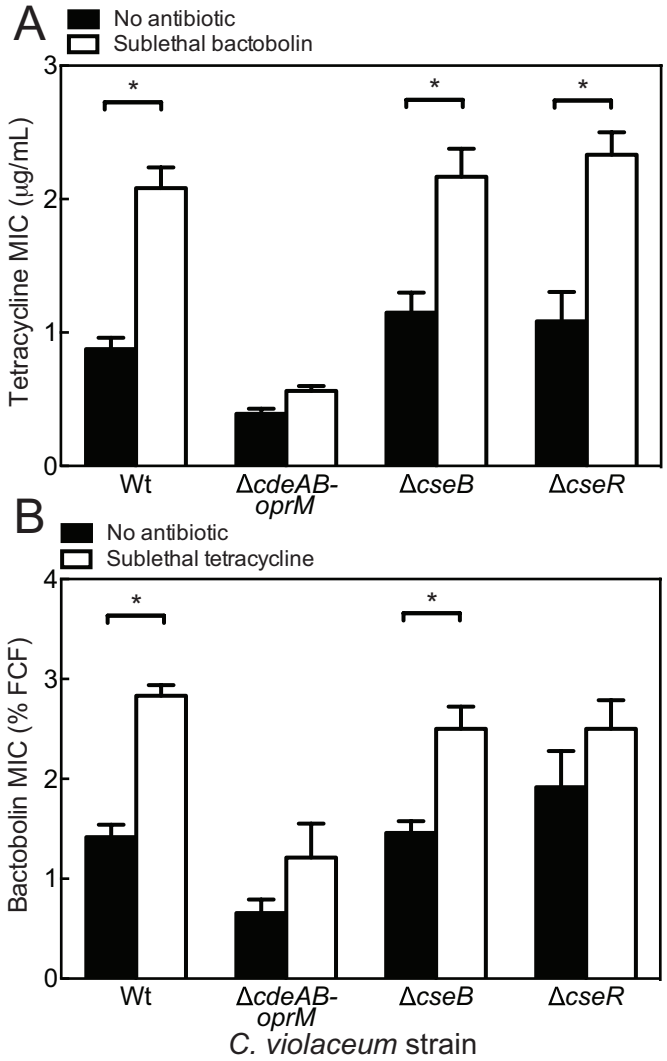
607 **Fig. 3.**



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614 **Fig. 4**
615

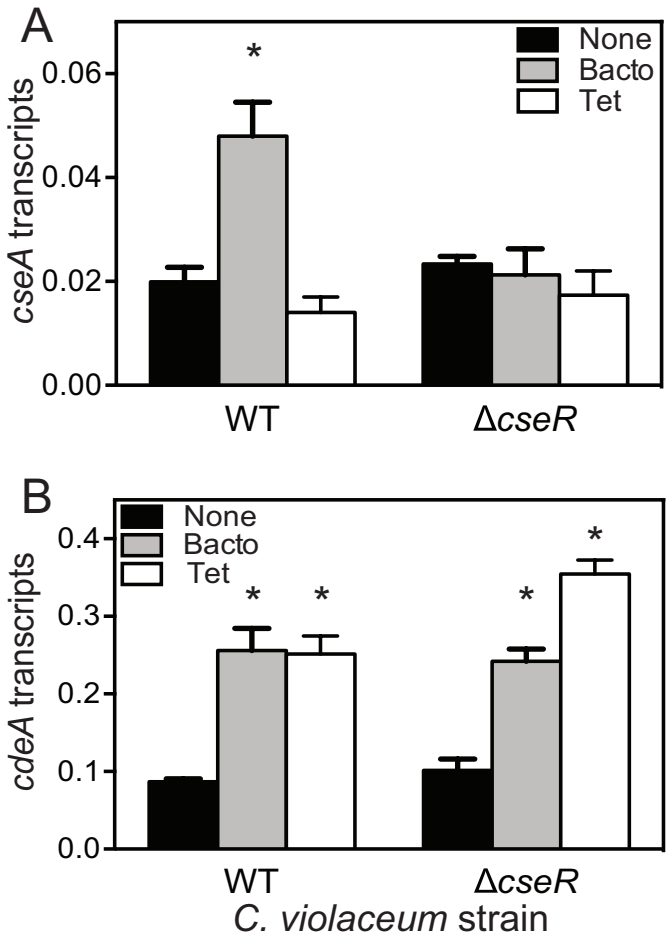

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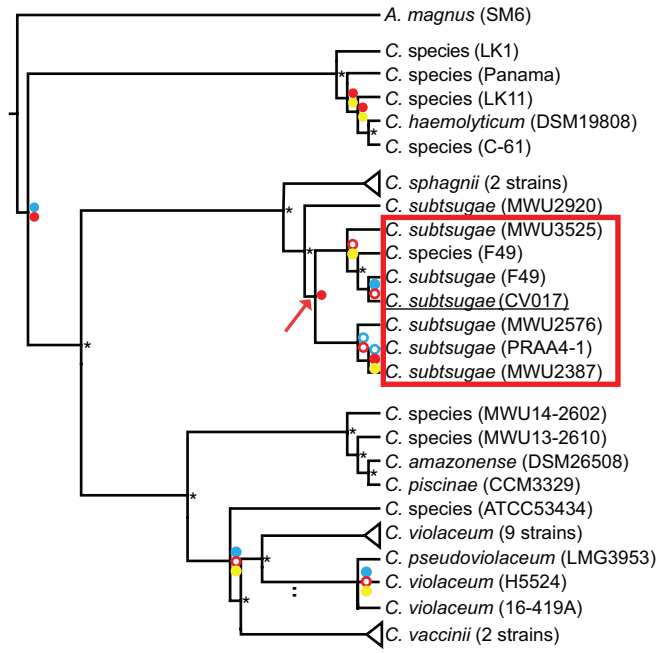
Fig. 5.


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)

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