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Impacts of Global Transcriptional Regulators on Persister Metabolism

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Running Title: Global Transcriptional Regulators and Persister Metabolism

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ABSTRACT

Bacterial persisters are phenotypic variants with an extraordinary capacity to tolerate antibiotics, and they are hypothesized to be a main cause of chronic and relapsing infections. Recent evidence has suggested that the metabolism of persisters can be targeted to develop therapeutic counter-measures; however, knowledge of persister metabolism remains limited due to difficulties associated with isolating these rare and transient phenotypic variants. By using a technique to measure persister catabolic activity, which is based on the ability of metabolites to enable aminoglycoside (AG) killing of persisters, we investigated the role of seven global transcriptional regulators (ArcA, Cra, CRP, DksA, FNR, Lrp, and RpoS) on persister metabolism. We found that removal of CRP resulted in a loss of AG potentiation in persisters for all metabolites tested. These results highlight a central role for cAMP/CRP in persister metabolism, as its perturbation can significantly diminish the metabolic capabilities of persisters, and effectively eliminate the ability of AGs to eradicate these troublesome bacteria.

Key Words: Cyclic AMP, CRP, aminoglycoside, persister cells

INTRODUCTION

Bacterial persisters are phenotypic variants that are highly tolerant to antibiotics (1). It is believed that they are a main culprit of the proclivity of biofilm infections to relapse, which imposes a substantial burden on the healthcare system (2, 3). When a bacterial population is treated with bactericidal antibiotics, biphasic killing is observed, where the death of normal cells is characterized by an initial, rapid killing rate, and the presence of persisters is illuminated by a second regime with a much slower rate of cell death (4, 5). When these survivors are re-cultured, the resulting population exhibits antibiotic sensitivities identical to that of the original culture, demonstrating that persisters are not antibiotic-resistant mutants, but phenotypic variants (1, 5, 6).

While persisters largely arise from dormant subpopulations (2, 4, 7, 8), recent studies have demonstrated that they remain metabolically active, with the capacity to catabolize specific carbon sources and generate proton motive force through respiration (9, 10). This metabolic activity, specifically proton motive force generation, enables aminoglycoside (AG) transport into cells that results in killing of persisters, and several enzymes needed for this process have been identified (9, 10). Knowledge of the enzymes and metabolic pathways present in persisters, as well as how they can be altered, could prove useful for the development of anti-persister therapies. A fundamental question in this regard is: what are the cellular components responsible for defining the metabolic network in persisters? Due to the strong dependence of metabolism on transcriptional regulation (11, 12), the goal of this study was to determine the importance of several global transcriptional regulators to persister metabolism. To do this, we measured catabolic activity in persisters from Δ*arcA*, Δ*cra*, Δ*crp*, Δ*dksA*, Δ*fnr*, Δ*lrp* and Δ*rpoS* mutants,

and discovered that CRP along with its metabolite co-factor cyclic-AMP, which is synthesized by CyaA, are critical regulators of persister metabolism.

MATERIALS AND METHODS

Bacterial strains

All strains were derived from *E. coli* MG1655. Standard P1 phage transduction was employed to transfer the genetic deletions (Δ*arcA*, Δ*cra*, Δ*crp*, Δ*dksA*, Δ*fnr*, Δ*lrp* and Δ*rpoS*) from the Keio Collection to *E. coli* MG1655 (13). The kanamycin resistance gene (KAN^R) was removed from these strains using FLP recombinase when required (14). To complement Δ*crp* and Δ*cyaA*, the native promoters and genes were amplified from *E. coli* MG1655 genomic DNA using primers 5'-CTAGTAGCTCGAGTTTTGCTACTCCACTGCGTC-3' and 5'-GCATCATCCTGCAGGTTAACGAGTGCCGTAAACGA-3' for *crp* and primers 5'-CTAGTAGCTCGAGAGTGTGCCTGCCAGAGTGCA-3' and 5'-GCATCATCCTGCAGGTCACGAAAAATATTGCTGTA-3' for *cyaA*. The amplified genes were digested with XhoI and SbfI (New England Biolabs, Ipswich, MA) and cloned into pUA66 (15). All gene deletion and cloning constructions were confirmed by colony PCR and/or DNA sequencing (Genewiz, South Plainfield, NJ).

Chemicals, media, and growth conditions

All chemicals were purchased from Fisher Scientific or Sigma-Aldrich. LB medium (10 g/L -Tryptone, 5 g/L Yeast Extract, and 10 g/L NaCl) and LB-agar (LB + 15 g/L agar) were prepared from the components and autoclaved at 121 °C for 30 min to achieve sterilization. LB medium and agar were used for planktonic growth and colony formation unit (CFU) measurements,

93 respectively. For mutant selection, 50 µg/mL kanamycin (KAN) and 25 µg/mL chloramphenicol
94 (CM) were used. For persister assays and AG potentiation assays, 5 µg/mL ofloxacin (OFL) and
95 10 µg/mL gentamicin (GENT) were used, respectively. To inhibit cytochrome activity during
96 AG assays, 5 mM potassium cyanide (KCN) was used. CM stock solution (25 mg/mL) was
97 dissolved in ethanol, whereas GENT (10 mg/mL), KCN (1 mM), KAN (50 mg/mL) and OFL (5
98 mg/mL) stock solutions were dissolved in deionized (DI) H_2O. OFL stock solution was titrated
99 with 1 M sodium hydroxide until the OFL fully dissolved. To prepare 1.25X M9 salt solution,
100 2.5 mL of 1 M $MgSO_4$ and 125 µL and 1M $CaCl_2$ were first mixed with 747.5mL DI H_2O, then
101 250 mL of 5X M9 salt solution (33.9g/L dibasic sodium phosphate, 15 g/L monobasic potassium
102 phosphate, 5 g/L ammonium chloride, and 2.5 g/L sodium chloride) that had been autoclaved at
103 121 °C for 30 min was added. Carbon sources (glucose, glycerol, fructose, mannitol, gluconate,
104 succinate, pyruvate, arabinose, fumarate, lactose and acetate) were dissolved in DI H_2O to
105 prepare stock solutions (600 mM carbon). The 1.25X M9 salt solution, KCN, CM, GENT, OFL
106 and carbon source stock solutions were filter-sterilized with 0.22µm filters. Overnight cultures
107 were inoculated from -80 ºC frozen stocks stored in 25% glycerol and grown in 2 mL of LB in a
108 test tube at 37 ºC with shaking at 250 rpm for 24 h.

109 **Persister assay**

110 Following overnight growth, cultures were diluted to optical density at 600nm (OD_{600}) of
111 0.2 in 1 mL of fresh LB in a test tube and treated with 5 µg/mL of OFL immediately. At desired
112 time points, 100 µL samples were collected from antibiotic-treated cultures, mixed with 900 µL
113 phosphate buffer saline (PBS) in microcentrifuge tubes, and the cells were pelleted by
114 centrifugation at 15,000 rpm for 3 minutes. To dilute the antibiotic concentration, 900 µL of
115 supernatant was removed and the cell pellets were resuspended with 900 µL of PBS. This

procedure was repeated until the antibiotic concentration was below the minimal inhibitory concentration (MIC). We have previously demonstrated that the MIC for MG1655 was 0.075-0.15 µg/mL OFL (7). After washing the cells, cell pellets were resuspended in the remaining 100 µL of PBS. Then, 10 µL of sample from each cell suspension was added into 90 µL PBS in a 96-well round bottom plate, and serially diluted. 10 µL of each dilution was spotted on LB agar plates, which were then incubated at 37 °C for 16 h before the CFUs were counted. For each spot, 10 to 100 colonies were counted.

Aminoglycoside potentiation assay

After 5 h of OFL treatment, cells were pelleted and resuspended in 1 mL of 1.25X M9 salt solution. The cells were washed again in 1 mL of 1.25X M9 to remove the antibiotic and residual LB, and the cell concentrations were adjusted such that the final concentration was ~10^5 persisters/mL. To enumerate CFUs in the cell suspension, 10 µL of sample was serially-diluted and plated on LB agar. Then, 80 µL of cell suspension, 10 µL of GENT solution (100 µg/mL) and 10 µL of carbon source solution (600 mM) were mixed in each well of 96-well flat-bottom plates, resulting in ~10^4 persisters, 10 µg/mL GENT, and 60 mM of carbon per well. For the no carbon source control, 10 µL of DI H_2O was added instead of a carbon source. To inhibit cellular respiration, 50 mM KCN was added to the GENT stock solution, and 10 µL of this mixture was mixed with 80 µL of cell suspension and 10 µL of carbon source solution, thus introducing 5 mM KCN in each well. Sample plates were sealed with sterile, gas-permeable Breathe-Easy membranes and incubated at 37 ºC and 250 rpm for 2 h. After incubation, 100 µL cell cultures from each well were transferred to microcentrifuge tubes with 900 µL of PBS. Cells were pelleted at 15,000 rpm for 3 min, and 900 µL of supernatant was removed. This washing step was repeated twice to dilute the GENT concentration below its MIC (16). After washing the

cells, cell pellets were resuspended in 100 µL of the remaining supernatant, and 10 µL of samples were serially diluted and plated on LB agar. The remaining 90 µL of samples were also plated on LB agar to improve the limit of detection. The plates were incubated at 37°C for 16 h, and CFUs were counted.

Gentamicin sensitivity assay

E. coli MG1655Δ*crp* and Δ*cyaA* were inoculated from -80 ºC frozen stocks stored in 25% glycerol into 2 mL of LB. Cells were grown for 37 ºC with shaking at 250 rpm for 4 h before being diluted in 2 mL of M9 with 10 mM glucose (M9-glucose) and grown for 16 h. Cells were diluted into 25 mL of M9-glucose to an OD_{600} of 0.01 and cultured for 6 h until they reached exponential phase. 100 µL of each culture was removed, serially diluted, and plated to enumerate CFUs prior to GENT treatment. The remaining cells were then treated with 10 µg/mL of GENT for 2 h. 1 mL of sample was removed from each culture, and the samples were pelleted, washed, serially-diluted, and plated as described above. The plates were incubated for 16 h before CFUs were counted.

Statistical Analysis

Three biological replicates were performed for each experimental condition, and a two-tailed t-test was performed for pairwise comparisons. p-values ≤ 0.05 were considered significant and each data point has been represented by mean value ± standard error.

RESULTS

Screen of transcriptional regulator mutants to identify those that are critical for metabolite-enabled AG potentiation in persisters.

Due to their low abundance, transient nature, and similarities to the more highly abundant viable but non-culturable cell (VBNC) phenotype, highly pure persister samples have yet to be isolated (10). In the absence of such methods, we developed an approach to measure metabolic activity in persisters that utilizes the phenomenon of metabolite-enabled aminoglycoside (AG) potentiation (9). In brief, AG uptake is dependent on proton motive force, and therefore, this drug class has an impaired ability to kill de-energized cells (9). The vast majority of persisters are metabolically dormant (7), and within such a state AGs are ineffective (9). In previous work, we discovered that specific metabolites produce AG killing of persisters, and that such potentiation was dependent on catabolism of the substrate to generate proton motive force through respiration (9, 10). This study demonstrated that persisters were metabolically active, and we have since shown that the assay can be used to measure persister metabolism from antibiotic-treated cultures. Notably, the approach circumvents the need to segregate persisters from other cell-types, because within antibiotic-treated populations that have reached the second regime of biphasic killing, the only cells that remain culturable are persisters. Therefore, survival data from samples treated with an AG with or without a metabolite can be used to infer persister catabolism (10). Using this assay, we analyzed the impacts of Δ*arcA*, Δ*cra*, Δ*crp*, Δ*dksA*, Δ*fnr*, Δ*lrp* and Δ*rpoS* on the ability of persisters to consume carbon sources and generate proton motive force aerobically. We focused on these transcriptional regulators due to their systems-level roles in regulating metabolism, and our overall goal to identify the molecular mediators responsible for establishing the persister metabolic network. Collectively, these seven global regulators govern diverse aspects of *E. coli* metabolism: CRP and Cra participate in controlling the expression of many enzymes including those within central metabolism; Lrp is involved in regulating amino acid metabolism; ArcA and FNR coordinate control of aerobic and anaerobic respiration; and

RpoS and DksA are regulators of the general stress and stringent responses, respectively (17-24). Prior to measuring persister catabolism, we verified that five hours of ofloxacin treatment was sufficient to enumerate persisters within cultures of wild-type and the seven deletion strains (Fig. 1). Persisters were then subjected to the AG potentiation assay, where samples were washed, resuspended in M9 media, and exposed to a panel of 11 carbon sources (60 mM carbon) in the presence of 10 μg/mL gentamicin (GENT) (Fig. 2A). To quantify the level of GENT potentiation that was metabolite independent, a no carbon source control was included. We also included controls where samples were treated with 5 mM KCN in addition to carbon sources and GENT to confirm that persister killing was consistent with the mechanism of AG potentiation identified previously (10) (Fig. 2B-I).

Results from this screen demonstrated that, with the exception of Δ*crp*, glucose, fructose, and pyruvate strongly potentiated AG killing in the persisters examined. In most cases, the survival fraction of persisters treated with these three carbon sources decreased by 100-fold or more. Upon inhibiting the electron transport chain with KCN, AG potentiation was significantly reduced in all samples. From this screen, we found that the panel of carbon sources tested could not potentiate AG killing persisters derived from Δ*crp*, suggesting an essential role for CRP in establishing the metabolic network of persisters.

Crp is a key regulatory protein in persister metabolism.

Results from the screen demonstrated that the deletion of *crp*, encoding the catabolite activator protein, eliminated the ability of metabolites to stimulate AG killing of persisters. To confirm that the reduction in potentiation was due to loss of CRP, we cloned *crp* under the control of its native promoter into pUA66, a low copy vector, to produce pUA66-*crp*. When

206 persisters were enumerated in Δ*crp* with pUA66-*crp* and Δ*crp* with pUA66 (empty control
207 plasmid), the persister abundances were comparable to that of Δ*crp* (Fig. 3A). We then examined
208 the effect of *crp* complementation on carbon source metabolism and AG potentiation, and we
209 found that pUA66-*crp* restored metabolic stimulation of AG killing in Δ*crp*, whereas pUA66 did
210 not (Fig. 3B-E). Similar to Δ*crp*, a loss of CFUs was not detected in Δ*crp* with pUA66,
211 demonstrating that the vector did not interfere with carbon source consumption or AG
212 sensitivity.

213 Since CRP activates catabolic gene expression when it is bound to cyclic adenosine
214 monophosphate (cAMP), which is synthesized by adenylate cyclase encoded by *cyaA*, we
215 generated Δ*cyaA* and its complementation strains and assessed both persister levels and carbon
216 source consumption. Persister levels within Δ*cyaA*, Δ*cyaA* with pUA66, and Δ*cyaA* with
217 pUA66-*cyaA* were found to be comparable (Fig. 4A), whereas Δ*cyaA*, much like Δ*crp*, resulted
218 in a broad reduction in the array of carbon sources that persisters could consume to potentiate
219 AG activity (Fig. 4B-E). In addition, pUA66-*cyaA*, but not the control plasmid (pUA66),
220 restored carbon source consumption to persisters.

221 We note that *E. coli* lacking *crp* or *cyaA* may exhibit increased tolerance toward AG (25-
222 27). To ensure that the reduction in metabolite-enabled AG killing observed in Δ*crp* and Δ*cyaA*
223 was due to alterations in persister metabolism rather than an inability of AGs to kill these
224 mutants, we assessed whether GENT could kill Δ*crp* and Δ*cyaA* in M9 minimal media, which
225 was the media used for AG potentiation assays. As depicted in Fig. 5, GENT could kill > 99.5%
226 of Δ*crp* and Δ*cyaA* within 2 hours (>100-fold reduction in CFUs levels), which was the length of
227 the time used in AG potentiation assays. This demonstrated that Δ*crp* and Δ*cyaA* could be killed
228 with the AG, media, and time-scale used in the persister catabolism assays, confirming that the

lack of killing observed in Δ*crp* and Δ*cyaA* persisters was not due to AG tolerance, but rather a phenotypic inability to consume metabolites and potentiate AGs.

DISCUSSION

Metabolism has been emerging as a key modulator of persistence (5, 28). Recent studies have demonstrated that metabolic stresses are important sources of persisters in both planktonic cultures and biofilms (29-34). In fact, persistence can be regarded as a metabolic program, where shut-down of metabolic processes participates in entry into this quasi-dormant state; metabolic activity during stasis maintains viability; and reactivation of metabolism is required for reawakening and growth after the conclusion of antibiotic treatment. The importance of metabolism is further highlighted by the discovery of anti-persister strategies which depend on metabolic stimulation in persisters (9, 35-38). These findings advocate the need for greater understanding of persister metabolism. Unfortunately, measurement of persister metabolic activities with standard methods is not currently possible, due to inabilities associated with isolating persisters (10, 39, 40). However, with the AG potentiation assay we are able to measure persister metabolism even in the presence of other cell types, such as VBNCs, because the method deduces metabolic activity from culturability data, and the defining characteristic of persistence is retention of culturability following prolonged antibiotic treatment (10). In addition, the AG potentiation assay not only identifies carbon sources that persisters consume to drive proton motive force generation, but it simultaneously identifies metabolite adjuvants to be used with AGs to kill persisters, which is important information that is therapeutically relevant (10).

With the AG potentiation assay, we directly assessed the contribution of a set of global transcriptional regulators to persister metabolism. We focused on these regulators, because we sought to identify the cellular components responsible for defining the metabolic network in

252 persisters, and transcriptional regulation has been found to play a pivotal role in metabolism (11,
253 12). Deletion of any of the seven global regulators in our initial screen did not significantly alter
254 the stationary phase persister levels, which is consistent with previous observations (41).
255 Interestingly, we found that Δ*crp* and Δ*cyaA* persisters consumed a narrower panel of carbon
256 sources compared to wild-type *E. coli*. CRP and CyaA are two key players in catabolite
257 repression/activation, regulating the hierarchy of carbon source usage across diverse lineages of
258 bacteria. In *E. coli*, their vast regulon consists of 188 genes, and they have been shown to play a
259 role in a plethora of cellular processes including persister formation (31, 42-44). Here, our
260 results have now identified CRP and CyaA as critical regulators of persister metabolism.

261 When cellular concentrations of preferred carbon sources, such as glucose are low, CyaA is
262 activated, resulting in synthesis and accumulation of cAMP. The binding of cAMP to CRP
263 enables it to activate genes for the catabolism of secondary carbon sources. The results presented
264 here suggest that catabolic regulation remains active in persisters, and the absence of either CRP
265 or CyaA has wide-spread effects on their metabolic networks. In particular, the ability of
266 persisters to catabolize many substrates is lost in Δ*crp* and Δ*cyaA*, thereby suggesting two
267 mutational routes *E. coli* could use to avoid AG-killing of persisters. Alternatively, synthetic
268 activation of CRP represents one possible route to improve killing of persisters with AGs.

269

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387

388 **FIGURE LEGENDS**

389 **Figure 1. Enumeration of persisters in wild-type and deletion mutants of transcriptional**

390 **regulators.**

391 Overnight cultures were diluted to OD_{600} of 0.2 in fresh LB and treated with 5 µg/mL OFL. CFU

392 levels were monitored for 5 h during treatment, and survival fractions were determined by

393 dividing CFU counts at each time point by that at t=0 h .

394 **Figure 2. Aminoglycoside potentiation assays in persisters and mutants.**

395 (A) Persisters were treated with GENT (10 µg/mL) and carbon sources (normalized to 60 mM

396 carbon) in M9 minimal media for 2 h. After AG treatment, persister survival fraction was

397 calculated from CFUs present in the original culture and after carbon source and GENT

398 treatment. Survival fraction of each strain and treatment condition was normalized to no carbon

399 source (DI H_2O) control of the same strain. * denotes statistical comparison performed between

400 each carbon source-treated sample and the no carbon source control of each strain ($p \leq 0.05$). (B-I)

401 Survival fractions of *E. coli* MG1655 and its global regulator deletion strains after being treated

402 with GENT and carbon sources (black bars) or GENT, carbon sources, and 5 mM KCN (gray

403 bars).

404 **Figure 3. Complementation of the Δ*crp* mutant.**

405 (A) Persister levels of Δ*crp*, Δ*crp* with pUA66 (empty vector), and Δ*crp* with pUA66-*crp* were

406 monitored for 5 h during OFL treatment. (B) Persisters were treated with GENT (10 µg/mL) and

407 carbon sources (60 mM carbon) in M9 minimal media for 2 h. Persister survival fractions of each

408 data point was normalized to the no carbon source (DI H_2O) control. * denotes statistical

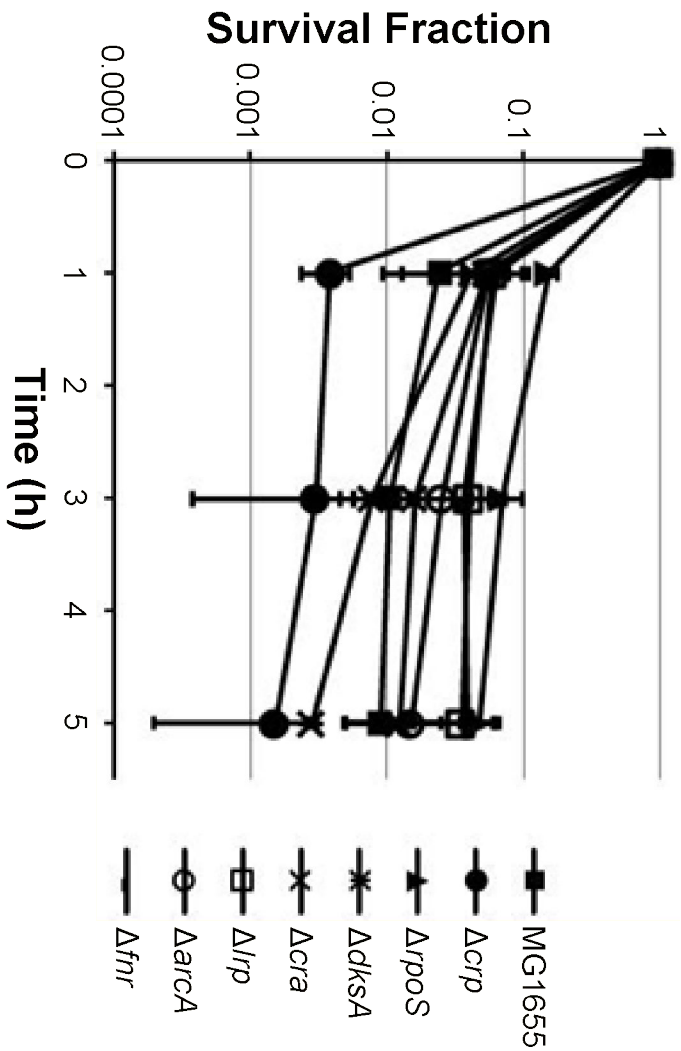
comparison performed between each carbon source-treated sample and the DI H_2O control of their respective strain ($p \leq 0.05$). Survival fractions of *E. coli* MG1655Δ*crp* (C), Δ*crp* with pUA66 (D), and Δ*crp* with pUA66-*crp* (E) after being treated with GENT and carbon sources (black bars) or GENT, carbon sources, and 5 mM KCN (gray bars).

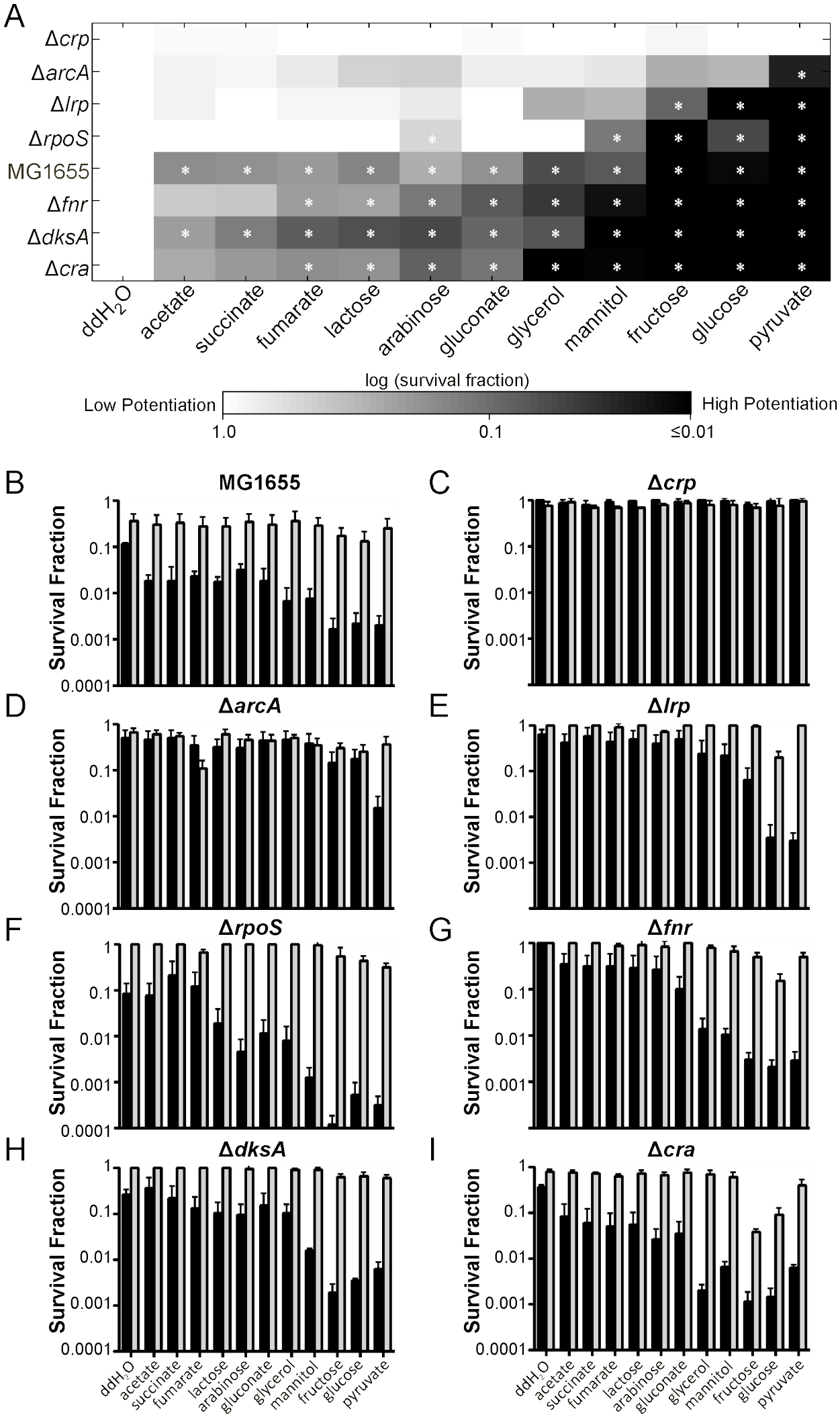
Figure 4. Complementation of the Δ*cyaA* mutant.

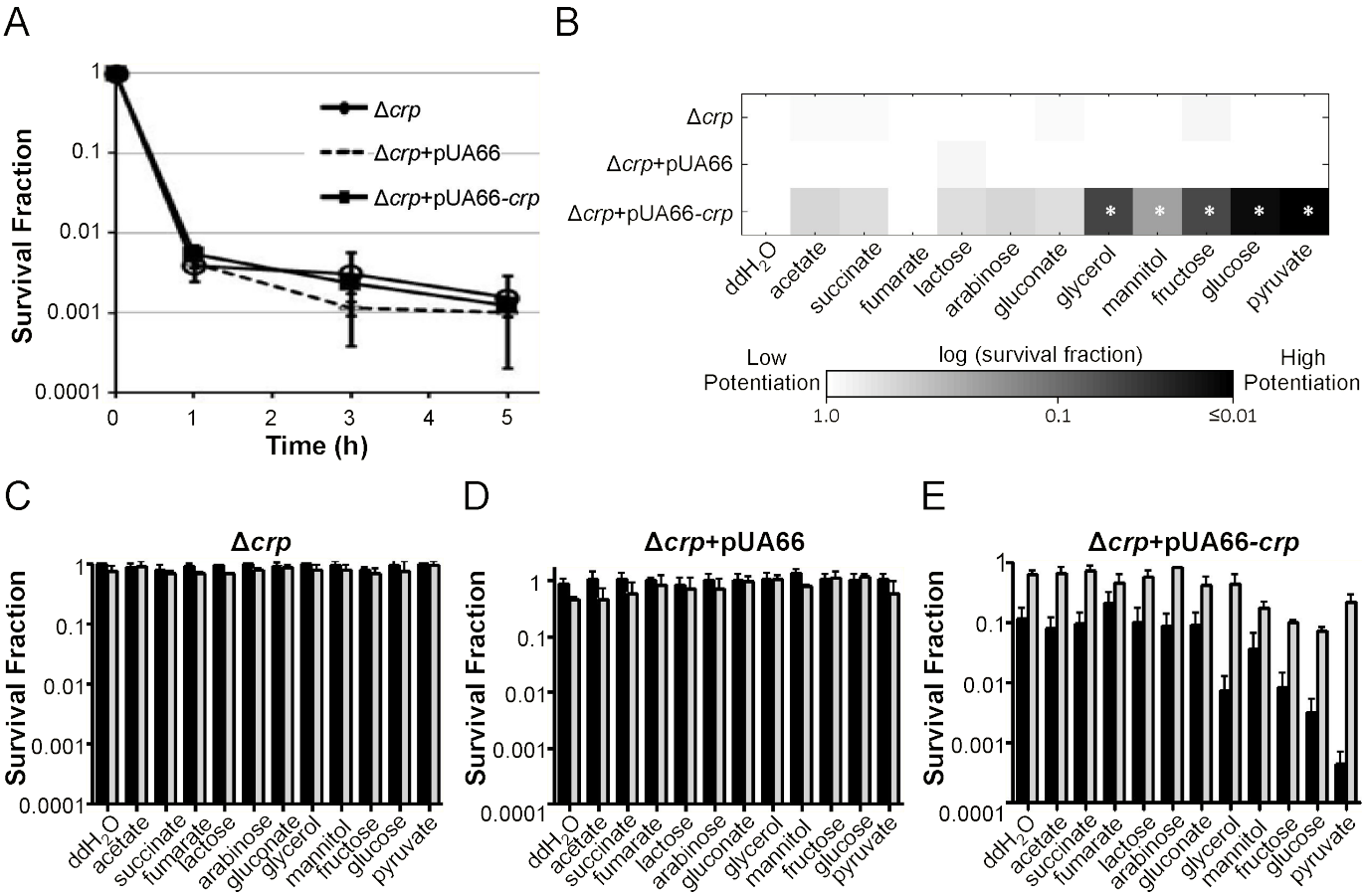
(A) Persister levels of Δ*cyaA*, Δ*cyaA* with pUA66 (empty vector), and Δ*cyaA* with pUA66-*cyaA* were monitored for 5 h during OFL treatment. (B) Persisters were treated with GENT (10 µg/mL) and carbon sources (normalized to 60 mM carbon) in M9 minimal media for 2 h. Persister survival fraction of each data point was normalized to no carbon source (DI H_2O) control. * denotes statistical comparison performed between each carbon source-treated sample and the DI H_2O control of their respective strain ($p \leq 0.05$). Survival fractions of *E. coli* MG1655Δ*cyaA* (C), Δ*cyaA* with pUA66 (D), and Δ*cyaA* with pUA66-*cyaA* (E) after being treated with GENT and carbon sources (black bars) or GENT, carbon sources, and 5 mM KCN (gray bars).

Figure 5. Sensitivity of *E. coli* MG1655Δ*crp* and MG1655Δ*cyaA* toward GENT.

The two strains were inoculated in M9-glucose to an OD_{600} of 0.01 and propagated for 6 h to exponential phase before being treated with 10 µg/mL of GENT for 2 h. Survival fraction was determined from CFU counts at t=0 h and t=2 h of GENT treatment.







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