

Supplementary Results

Background

Bacterial persisters are a dormant sub-population of cells tolerant to antibiotic treatment¹⁻⁴ and are considered an important source of chronic and recurrent infection⁵⁻⁷. Persistence is distinguished from antibiotic resistance by the fact that persisters are not genetically different from antibiotic susceptible cells. Instead, persistence results from phenotypic population heterogeneity within an isogenic population. Viewed as part of an epigenetic survival strategy⁸, persisters forfeit rapid growth in order to gain tolerance to diverse stresses including antibiotics⁹. It has been suggested that, in some cases, phenotypic tolerance may be as relevant as antibiotic resistance to treatment failure^{5,6}. In these instances, current antibiotic therapy would not be sufficient for full recovery. Accordingly, viable methods for eliminating bacterial persisters need to be developed.

Genetic studies have uncovered a number of cellular phenomena involved in bacterial persistence, including toxin-antitoxin modules¹⁰⁻¹² and the stringent response¹³. In addition, glycerol¹⁴, glyoxylate¹⁵, and phosphate metabolism¹ have been found to affect persistence, suggesting that the cellular metabolic state plays a crucial role in the persister phenotype. Genetic studies have also suggested a role for the DNA damage response in the formation of persisters¹⁶⁻¹⁸, indicating that persistence may be, in part, inducible. This inducible aspect of persisters adds to recent phenomenological work suggesting that persistence is the result of a random switching event¹⁹. While these advances have expanded our understanding of persister formation, there is currently no method available for eliminating bacterial persisters.

Persister eradication is general to aminoglycosides

To determine whether the observed potentiation was specific to gentamicin or general to aminoglycosides, we tested the effects of adding the upper-glycolytic metabolites glucose, mannitol, and fructose to streptomycin and kanamycin. As seen with gentamicin, streptomycin and kanamycin activity was greatly enabled by these metabolites, and the antibiotics showed no appreciable killing in the absence of carbon source, demonstrating that metabolic potentiation was a general aminoglycoside phenomenon (Supplementary Fig. 2).

Metabolic stimuli do not induce rapid growth resumption

Due to the fact that metabolically stimulated persisters, though immediately susceptible to aminoglycosides, were not susceptible to the fluoroquinolone ofloxacin or the β -lactam ampicillin, we did not expect persisters to rapidly resume growth (Fig. 1c). Fluoroquinolones^{20, 21} and β -lactams target DNA topoisomerases and peptidoglycan synthesizing enzymes²², respectively, and, because these processes are necessary for growth, we reasoned that it was unlikely that persisters were reverting to normal growth. We tested this hypothesis in 96-well plates for all the carbon sources studied in the aminoglycoside potentiation screen and found, over an 8-hour period, metabolic stimuli were causing negligible cellular growth (Supplementary Figs 3 and 4) as measured by colony forming units (CFUs). This does not suggest that persisters remain entirely dormant in the presence of the metabolic stimuli used in this investigation. Clearly, they are catabolizing the metabolites. However, they do not have the required cell-wall biogenesis or DNA replication necessary for cell division. The absence of these processes suggests persisters are not reverting to a normal physiology. We verified that

all carbon sources used in this study could be used as primary carbon sources for exponentially growing *E. coli*.

Persisters can be resuscitated by carbon sources

Given that metabolic stimulation induced negligible growth of persisters in the previous assay, we sought to demonstrate that carbon sources at higher concentrations per persister could stimulate persister growth. To do this, we diluted persister cultures 1:100 into fresh minimal media with carbon source and determined growth during 20-hour incubation. We found that the carbon sources which caused high potentiation were the ones that caused the quickest revival of persisters, suggesting that these carbon sources are most efficiently metabolized by persisters (Supplementary Fig. 5). Further we found that all carbon sources were able to induce significant growth by 20 hours, with the exception of glycolate. These results demonstrate that persisters can be revived when the concentration of carbon source per persister is 100 times greater than is required for metabolite-enabled persister eradication and are consistent with previous studies²³.

Concentration dependence of metabolite-enabled persister eradication

We next sought to determine the dependence of the phenotype on the concentration of carbon source used. To test this, we treated persisters with gentamicin and a range of each of the following carbon sources: glucose, mannitol, fructose, and pyruvate. We found that, even with a nearly ten-fold reduction in carbon concentration (1.25 mM for glucose, mannitol, and fructose; 2.5 mM for pyruvate), aminoglycoside killing of persisters could still be potentiated by approximately two orders of magnitude

(Supplementary Fig. 6). These results demonstrate that metabolite-enabled eradication is effective even at significantly lower concentrations of metabolites.

Metabolite-enabled eradication of persisters is specific to aminoglycoside ribosomal inhibitors

To determine whether this phenomenon was due to the bactericidal property of aminoglycosides or perhaps their inhibition of the ribosome in changing metabolic environments, we tested the ability of three additional ribosomal inhibitors, all of which were bacteriostatic, to eliminate persisters in the presence of mannitol. Antibiotics from three additional classes of ribosomal inhibitors (tetracycline, spectinomycin, and chloramphenicol) were tested in conjunction with aminoglycoside-potentiating metabolite mannitol for their effect on persister survival. None of these ribosomal inhibitors induced any killing of persisters (Supplementary Fig. 7), demonstrating that killing was a result of metabolic potentiation of aminoglycoside activity rather than the result of an inability to translationally respond to changing metabolic conditions.

Metabolism of metabolite is required for persister eradication

Since rapid growth resumption of bacterial persisters was not induced by potentiating metabolites, we sought to determine whether potentiation required the entry of metabolites into central metabolism. We verified that metabolism of the carbon source was required for potentiation by testing a $\Delta mtlD$ mutant. *mtlD* encodes mannitol-1-phosphate-5-dehydrogenase, which is responsible for mannitol entry into glycolysis as fructose-6-phosphate (F-6-P), generating NADH in the process. The $\Delta mtlD$ strain

completely abolished gentamicin killing in the presence of mannitol, demonstrating that metabolic flux to glycolysis was necessary for potentiation (Supplementary Fig. 8).

Potentiating metabolites induce the uptake of aminoglycosides in stationary phase cells

Despite multiple efforts to isolate persisters^{24, 25}, an effective high-yield method for persisters formed in stationary phase has yet to be developed. Our assay for persister survival, though effective for measuring contributors to persister viability, is not amenable to direct observational studies such as microscopy and flow cytometry. The ofloxacin challenge kills susceptible stationary phase cells but does not lyse them or remove them from the culture. Further, β -lactams, which can lyse exponentially growing cells, are not able to lyse stationary phase cells. Hence, observational methods cannot distinguish between dead susceptible cells and persister cells, thereby confounding results. For this reason, we did not attempt to measure uptake in persisters directly, instead we focused on the parent culture from which the persisters are derived as a proxy. Stationary phase cells exhibit the same metabolic potentiation of aminoglycosides phenotype observed in persister cells (Supplementary Fig. 9). Without metabolic stimuli, the stationary phase culture is nearly completely tolerant to aminoglycosides. Upon the addition of specific carbon sources, rapid aminoglycoside potentiation is induced. We found that the same carbon sources which enabled killing in persisters also enabled killing of stationary phase cells.

We attempted to determine whether the metabolic potentiation observed in persisters could be explained by uptake of aminoglycosides by dormant (stationary phase) cells. We measured the uptake of Texas-Red labeled gentamicin (Gent-TR)

induced by metabolic stimuli using flow cytometry. We found that carbon sources which cause high potentiation of aminoglycoside killing in persisters also induce uptake of Gent-TR in stationary phase cells (Supplementary Fig. 10). Further, carbon sources which did not potentiate aminoglycosides against persisters induced negligible amounts of aminoglycoside uptake in stationary phase cells. Ribose, which has an intermediate level of aminoglycoside potentiation against persisters, has low aminoglycoside uptake in stationary phase cultures. The reason for this difference is unclear and requires further investigation.

The uptake of Gent-TR induced by glycolate is not included in the uptake figures as glycolate was found to induce the uptake of the unconjugated Texas Red fluorophore. This finding suggested that the Gent-TR signal measured in glycolate-induced uptake experiments was due to the effect of glycolate on Texas Red rather than glycolate on gentamicin uptake (Supplementary Fig. 11). No other carbon source tested was observed to increase the uptake of unlabeled Texas Red (Supplementary Fig. 11).

Metabolite-enabled uptake of aminoglycosides requires PMF

Uptake of aminoglycosides in exponentially growing bacteria is known to require proton-motive force (PMF)²⁶. Given that we have shown that PMF is also required for potentiation of aminoglycosides against persisters (Fig. 2b and Supplementary Fig. 12), we sought to test if PMF was required for metabolic-induced uptake in dormant bacteria. We pretreated cells with carbon sources shown to cause potentiation and the proton ionophore carbonyl cyanide m-chlorophenyl hydrazone (CCCP), and measured the subsequent uptake of Gent-TR by stationary phase cells using flow cytometry. We found that CCCP suppressed metabolic-induced uptake of Gent-TR, demonstrating that PMF is

required for aminoglycoside potentiation (Supplementary Fig. 13). This finding in stationary phase cells is consistent with what is known about aminoglycoside activity in exponentially growing cultures^{26, 27}.

Potentiating metabolites induce PMF

We directly measured the ability of glucose to induce changes in PMF using the membrane dye DiOC₂(3) in the presence and absence of the proton ionophore CCCP (Supplementary Fig. 14). We found that addition of glucose significantly increased the membrane potential of stationary phase *E. coli*. Further, this increase in membrane potential was blocked by CCCP, verifying that DiOC₂(3) was measuring PMF. Of note, addition of CCCP alone did not decrease the membrane potential suggesting that PMF in stationary phase cultures is already quite low. Taken together, these results show that the low PMF of dormant *E. coli* can be metabolically induced by glucose.

We further sought to investigate PMF induction by additional carbon sources, some of which potentiated aminoglycosides and some of which did not (Supplementary Fig. 15). We found that metabolites that potentiated aminoglycosides also induced significant increases in PMF, and that metabolites which did not induce substantial aminoglycoside killing had a significantly lesser effect on PMF. These results demonstrate that aminoglycoside-potentiating metabolites are inducing PMF.

Metabolite-enabled persister eradication can occur under anaerobic conditions if PMF is supported

Though we found aerobic respiration to be important to PMF-dependent eradication under our test conditions (Fig. 2c and Supplementary Fig. 16), PMF can also

be sustained under anaerobic conditions. Hence, PMF-dependent elimination of dormant bacteria should be possible under anaerobic conditions. We tested this by growing *E. coli* to stationary phase in an anaerobic chamber, then treating cultures with gentamicin and added carbon source (Supplementary Fig. 17). We found that, when added to cultures, upper-glycolytic carbon sources improved aminoglycoside killing by one to two orders of magnitude. Further, we found that addition of the terminal electron acceptor nitrate, added to boost PMF through anaerobic respiration, improved aminoglycoside killing by an additional order of magnitude in each case. These results, which agree with previous work showing the effect of nitrate on anaerobic aminoglycoside activity²⁸, demonstrate that metabolite-enabled elimination of persisters can occur in anaerobic environments and be improved by facilitating PMF generation.

PMF is required for metabolite-enabled persister eradication under anaerobic conditions

Given that metabolite-enabled aminoglycosides potentiation occurred under anaerobic conditions and could be further improved by facilitating PMF (Supplementary Fig. 17), we next sought to verify that PMF was required for this phenotype. In these experiments, we found that CCCP blocked all metabolite-enabled aminoglycoside activity, including in cultures that were supplemented with nitrate (Supplementary Fig. 18a), thereby demonstrating the requirement of PMF.

We also sought to determine the importance of NADH utilization for this phenotype under anaerobic conditions. Using the $\Delta ndh\Delta nuoI$ strain, we found that NADH utilization was not an absolute requirement for potentiation (Supplementary Fig. 18b). Glucose was capable of potentiating gentamicin in this strain under these

conditions. The fact that potentiation was less than in the wild-type strain under the same conditions suggests that, though not required, NADH utilization is important to aminoglycoside potentiation under anaerobic conditions.

NADH utilization is important to metabolite-enabled persister eradication, but not an absolute requirement

To investigate the utilization of NADH by the electron transport chain in this phenotype, we created a strain that lacked both NADH dehydrogenases ($\Delta ndh\Delta nuoI$). Using the $\Delta ndh\Delta nuoI$ strain, we found that NADH utilization was essential for potentiation induced by mannitol, fructose, and pyruvate (Fig. 2d, and Supplementary Fig. 19 b,c). Slight potentiation, however, was observed with glucose (Supplementary Fig. 19a), suggesting that, though NADH utilization is important to this phenotype, it is not an absolute requirement. Next, using CCCP, we sought to verify that PMF was required for glucose-enabled eradication, in this strain (Supplementary Fig. 20). We found that the small amount of aminoglycoside potentiation induced by glucose in the $\Delta ndh\Delta nuoI$ strain was abolished in the presence of CCCP, thereby demonstrating that PMF was an absolute requirement for this phenotype.

Source of NADH in persister eradication

Having demonstrated the importance of NADH utilization, we next sought to determine the metabolic pathways used by carbon sources for NADH generation in persisters. We proceeded by creating knockouts of the most direct metabolic pathways by which the carbon sources glucose, mannitol, fructose, and pyruvate could be catabolized to produce NADH; the enzymatic steps that were inactivated are displayed in

Supplementary Table 1. We then tested the ability of glucose, mannitol, fructose and pyruvate to potentiate aminoglycoside activity in persisters of each of these strains (Supplementary Figs 21 and 22). Given that PMF generation was required, we only knocked out enzymes required for the production of reducing equivalents branching at different locations along central metabolism.

The removal of glucose 6-phosphate-1-dehydrogenase (Δzwf) did not have a significant effect on the aminoglycoside killing induced by the four carbon sources used (Supplementary Figs 21 and 22a), suggesting that the PPP and EDP, and the NADPH generated via these processes, do not play a significant role in aminoglycoside potentiation. The removal of 6-phosphofructokinase ($\Delta pfkA\Delta pfkB$), on the other hand, abolished potentiation by the upper-glycolytic carbon sources, but had no effect on pyruvate potentiation (Supplementary Figs 21 and 22b). This result demonstrates that carbon from upper glycolytic carbon sources must pass to lower glycolysis or to pyruvate and possibly the TCA cycle. That pyruvate potentiation is not affected in the $\Delta pfkA\Delta pfkB$ strain demonstrates that this genetic perturbation does not itself increase the tolerance of persisters. The removal of pyruvate oxidase ($\Delta poxB$), which passes electrons directly to the quinone pool without NADH, did not have a significant effect on the aminoglycoside killing induced by the four carbon sources used (Supplementary Figs 21 and 22c), indicating that pyruvate oxidase is not required for supplying reducing potential. The removal of citrate synthase activity ($\Delta gltA\Delta prpC$) slightly suppressed potentiation of aminoglycosides (Supplementary Figs 21 and 22d), suggesting a minor role for the TCA cycle in this phenomenon. Though fructose had reduced potentiation ability in the $\Delta gltA\Delta prpC$ strain at two hours, by four hours it had potentiated gentamicin

to nearly the same extent as the other carbon sources (Supplementary Fig. 22d). These results were unexpected given the importance of NADH for aminoglycoside potentiation and the large NADH-generating capacity of the TCA cycle, and thereby suggests that NADH is being produced from another source.

Alternatively, the removal of pyruvate dehydrogenase ($\Delta aceE$), which eliminates pyruvate dehydrogenase activity²⁹, abolished potentiation by all carbon sources used, suggesting that it, or a downstream process, is critical to aminoglycoside potentiation against persisters (Supplementary Figs 21 and 22e). Pyruvate dehydrogenase produces NADH and acetyl-CoA. The $\Delta gltA\Delta prpC$ strain demonstrated that entry of acetyl-CoA into the TCA cycle is not required when pyruvate dehydrogenase is present. Therefore, if acetyl-CoA is important for potentiation, it is through a TCA-cycle-independent process.

Pyruvate dehydrogenase is required due to NADH generation

We next sought to determine if acetyl-CoA plays a role in this phenotype. To distinguish between a requirement for NADH and a requirement for acetyl-CoA, we repeated the previous experiment but supplemented acetate which is converted by acetyl-CoA synthetase (*acs*) to acetyl-CoA. Given that acetate on its own was not seen to potentiate aminoglycoside killing in the $\Delta aceE$ or wild-type strains, a rescue of aminoglycoside potentiation in the $\Delta aceE$ strain would suggest either that an acetyl-CoA-dependent biosynthetic process was crucial for potentiation or that the TCA cycle was crucial for NADH generation in the absence of pyruvate dehydrogenase. We found that adding acetate rescued a significant amount of the aminoglycoside potentiation induced by all four carbon sources in the $\Delta aceE$ strain (Supplementary Figs 23a and 24a),

demonstrating that the requirement for pyruvate dehydrogenase could be complemented by acetate addition for these carbon sources.

To determine whether acetate supplementation of the $\Delta aceE$ strain was important for the synthesis of biosynthetic acetyl-CoA or, alternatively, for a compensating generation of NADH by the TCA cycle, we tested a triple knockout ($\Delta gltA\Delta prpC\Delta aceE$) which inactivates both pyruvate dehydrogenase and citrate synthase activity. In this strain, the supplemented acetate can only be utilized in biosynthetic processes that do not branch from the TCA cycle. We found that the added $\Delta gltA\Delta prpC$ modification eliminated the rescued potentiation by acetate, demonstrating that the acetate supplementation of the $\Delta aceE$ strain rescued potentiation by generating NADH in the TCA cycle (Supplementary Figs 23b and 24b). This result also suggests that acetyl-CoA generation is not responsible for potentiation. Hence the importance of pyruvate dehydrogenase in potentiation is due to its NADH.

Metabolite-enabled elimination of *E. coli* biofilms and dependence on pH

Biofilms are a major source of hospital-acquired infections, due to their growth on medical devices and surfaces, and are also found in chronic infections unrelated to medical devices³⁰. The highly compact structure of biofilms thwarts immune attack and limits the diffusion, and hence activity, of β -lactams³¹ and reactive oxygen species^{30, 32}. Quinolones and aminoglycosides, however, have been shown to readily diffuse in both Gram-negative³³ and Gram-positive³¹ biofilms. The tolerance of biofilms to antibiotics that are not diffusion-limited has been attributed to metabolic population heterogeneity and bacterial persistence^{7, 30, 32, 34}. It is worth noting that at high concentrations and long-

time scales aminoglycosides are moderately effective against some biofilms^{35, 36}. We reasoned that metabolic stimulation might facilitate the elimination of the biofilm persister population by aminoglycosides. To test this hypothesis, we grew *E. coli* biofilms, as previously described³⁷, and treated them for four hours with ofloxacin, mannitol, gentamicin, and mannitol plus gentamicin (Fig. 3b). As quinolones have high efficacy against Gram-negative biofilms compared to other antibiotics^{33, 38}, ofloxacin was used as a benchmark for high biofilm killing. Ofloxacin reduced the viable cells in biofilms by almost two orders of magnitude, suggesting that greater than 1% of the biofilms were persisters. Mannitol and gentamicin in combination reduced the viable cells in biofilms by greater than four orders of magnitude, demonstrating a reduction of biofilm persisters by 2.5 orders of magnitude. Mannitol and gentamicin alone had no detectable effect on biofilm viability. We also tested the ability of fructose to potentiate aminoglycoside elimination of biofilm persisters and observed similar results (Fig. 3c).

Given the known dependence of aminoglycoside activity on pH, we determined the pH dependence for metabolite-enabled biofilm killing²⁶. We found that metabolite-enabled elimination of biofilms by aminoglycosides was a pH dependent phenomenon, being effective at pH values from 6 to 8, but not under acidic conditions at pH 5 (Supplementary Fig. 25).

High tolerance of *E. coli* biofilms to aminoglycosides

Given that we used aminoglycoside concentrations low enough to be used in the body, we were not surprised to observe negligible killing of biofilms after 4 hours. To test whether killing could be observed at longer timescales, we performed biofilm assays in which treatment was extended to 8 hours (Supplementary Fig. 26). We found that

gentamicin alone has an effect on biofilm viability at 8 hours, approximately a log-decrease in viability, but the effect can be potentiated substantially by mannitol.

Fructose transport is up-regulated in stationary phase *S. aureus*

Given that glucose, mannitol, and fructose enter glycolysis very near one another, we reasoned that differences in their ability to potentiate aminoglycosides in *S. aureus* persisters may have been due to enzymes responsible for their catabolism. To explore this possibility, we examined stationary phase and exponential phase *S. aureus* microarray data³⁹ and found that the expression of glucose-specific PTS transport (*ptsG*) was down more than 3 fold, manitol-specific PTS transport (*mtlF*) was unchanged, and putative fructose PTS transport (SACOL2663) was increased more than 20 fold in stationary phase *S. aureus* cultures when compared to exponential phase cultures (Supplementary Table 3). This provides evidence that the differences in potentiation by glucose, mannitol, and fructose could be attributed to the cellular ability of dormant *S. aureus* to catabolize these carbon sources, and that *S. aureus* persisters may be primed for rapid fructose uptake. It remains unclear why pyruvate did not potentiate aminoglycoside killing in *S. aureus* persisters. Reduced potentiation could be a result of reduced pyruvate transport, which could not be determined by expression analysis, as there is no annotated pyruvate transporter in *S. aureus*. This demonstrates the importance of a carbon source's ability to be actively metabolized for potentiation of aminoglycosides. It further suggests the importance of understanding the metabolism of and environment encountered by pathogens in developing treatments. It is also possible that the NADH contribution of pyruvate dehydrogenase to the potentiation mechanism in *S. aureus* is not as critical as was found in *E. coli*.

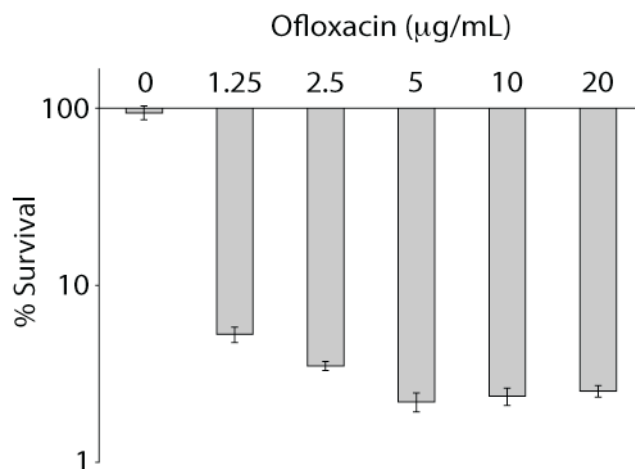
Supplementary References

1. Lewis, K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* **5**, 48-56 (2007).
2. Gefen, O. & Balaban, N.Q. The importance of being persistent: heterogeneity of bacterial populations under antibiotic stress. *FEMS Microbiol Rev* **33**, 704-717 (2009).
3. Bigger, J.W. Treatment of staphylococcal infections with penicillin by intermittent sterilization. *Lancet* **ii**, 497-500 (1944).
4. Jayaraman, R. Bacterial persistence: some new insights into an old phenomenon. *J Biosci* **33**, 795-805 (2008).
5. Smith, P.A. & Romesberg, F.E. Combating bacteria and drug resistance by inhibiting mechanisms of persistence and adaptation. *Nat Chem Biol* **3**, 549-556 (2007).
6. Levin, B.R. & Rozen, D.E. Non-inherited antibiotic resistance. *Nat Rev Microbiol* **4**, 556-562 (2006).
7. Lewis, K. Persister cells and the riddle of biofilm survival. *Biochemistry (Mosc)* **70**, 267-274 (2005).
8. Kussell, E., Kishony, R., Balaban, N.Q. & Leibler, S. Bacterial persistence: a model of survival in changing environments. *Genetics* **169**, 1807-1814 (2005).
9. Harrison, J.J. et al. Persister cells mediate tolerance to metal oxyanions in *Escherichia coli*. *Microbiology* **151**, 3181-3195 (2005).
10. Keren, I., Kaldalu, N., Spoering, A., Wang, Y. & Lewis, K. Persister cells and tolerance to antimicrobials. *FEMS Microbiol Lett* **230**, 13-18 (2004).
11. Moyed, H.S. & Bertrand, K.P. *hipA*, a newly recognized gene of *Escherichia coli* K-12 that affects frequency of persistence after inhibition of murein synthesis. *J Bacteriol* **155**, 768-775 (1983).
12. Rotem, E. et al. Regulation of phenotypic variability by a threshold-based mechanism underlies bacterial persistence. *Proc Natl Acad Sci U S A* **107**, 12541-12546 (2010).
13. Korch, S.B., Henderson, T.A. & Hill, T.M. Characterization of the *hipA7* allele of *Escherichia coli* and evidence that high persistence is governed by (p)ppGpp synthesis. *Mol Microbiol* **50**, 1199-1213 (2003).
14. Spoering, A.L., Vulic, M. & Lewis, K. GlpD and PlsB participate in persister cell formation in *Escherichia coli*. *J Bacteriol* **188**, 5136-5144 (2006).
15. McKinney, J.D. et al. Persistence of *Mycobacterium tuberculosis* in macrophages and mice requires the glyoxylate shunt enzyme isocitrate lyase. *Nature* **406**, 735-738 (2000).
16. Dorr, T., Vulic, M. & Lewis, K. Ciprofloxacin causes persister formation by inducing the TisB toxin in *Escherichia coli*. *PLoS Biol* **8**, e1000317.
17. Hansen, S., Lewis, K. & Vulic, M. Role of global regulators and nucleotide metabolism in antibiotic tolerance in *Escherichia coli*. *Antimicrob Agents Chemother* **52**, 2718-2726 (2008).
18. Dorr, T., Lewis, K. & Vulic, M. SOS response induces persistence to fluoroquinolones in *Escherichia coli*. *PLoS Genet* **5**, e1000760 (2009).

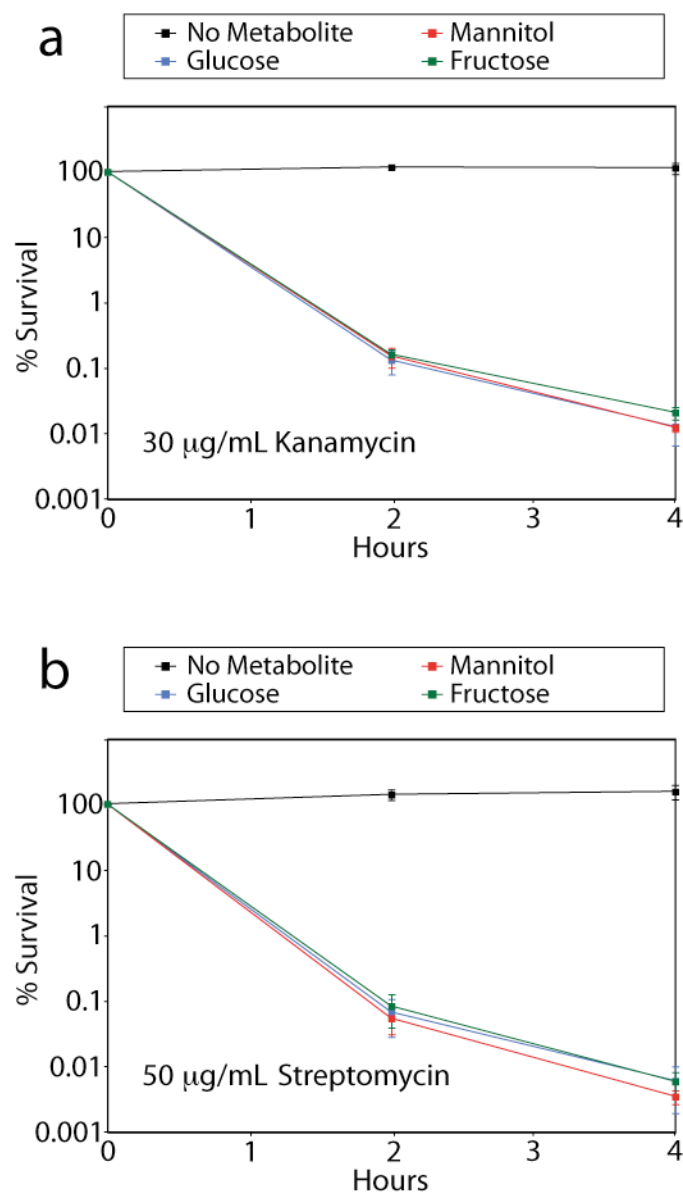
19. Balaban, N.Q., Merrin, J., Chait, R., Kowalik, L. & Leibler, S. Bacterial persistence as a phenotypic switch. *Science* **305**, 1622-1625 (2004).
20. Drlica, K. & Zhao, X. DNA gyrase, topoisomerase IV, and the 4-quinolones. *Microbiol Mol Biol Rev* **61**, 377-392 (1997).
21. Drlica, K. Mechanism of fluoroquinolone action. *Curr Opin Microbiol* **2**, 504-508 (1999).
22. Tomasz, A. The mechanism of the irreversible antimicrobial effects of penicillins: how the beta-lactam antibiotics kill and lyse bacteria. *Annu Rev Microbiol* **33**, 113-137 (1979).
23. Joers, A., Kaldalu, N. & Tenson, T. The frequency of persisters in *Escherichia coli* reflects the kinetics of awakening from dormancy. *J Bacteriol* **192**, 3379-3384 (2010).
24. Keren, I., Shah, D., Spoering, A., Kaldalu, N. & Lewis, K. Specialized persister cells and the mechanism of multidrug tolerance in *Escherichia coli*. *J Bacteriol* **186**, 8172-8180 (2004).
25. Shah, D. et al. Persisters: a distinct physiological state of *E. coli*. *BMC Microbiol* **6**, 53 (2006).
26. Taber, H.W., Mueller, J.P., Miller, P.F. & Arrow, A.S. Bacterial uptake of aminoglycoside antibiotics. *Microbiol Rev* **51**, 439-457 (1987).
27. Davis, B.D. Mechanism of bactericidal action of aminoglycosides. *Microbiol Rev* **51**, 341-350 (1987).
28. Campbell, B.D. & Kadner, R.J. Relation of aerobiosis and ionic strength to the uptake of dihydrostreptomycin in *Escherichia coli*. *Biochim Biophys Acta* **593**, 1-10 (1980).
29. Guest, J.R., Roberts, R.E. & Stephens, P.E. Hybrid plasmids containing the pyruvate dehydrogenase complex genes and gene-DNA relationships in the 2 to 3 minute region of the *Escherichia coli* chromosome. *J Gen Microbiol* **129**, 671-680 (1983).
30. Costerton, J.W., Stewart, P.S. & Greenberg, E.P. Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318-1322 (1999).
31. Singh, R., Ray, P., Das, A. & Sharma, M. Penetration of antibiotics through *Staphylococcus aureus* and *Staphylococcus epidermidis* biofilms. *J Antimicrob Chemother* **65**, 1955-1958 (2010).
32. Mah, T.F. & O'Toole, G.A. Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* **9**, 34-39 (2001).
33. Walters, M.C., 3rd, Roe, F., Bugnicourt, A., Franklin, M.J. & Stewart, P.S. Contributions of antibiotic penetration, oxygen limitation, and low metabolic activity to tolerance of *Pseudomonas aeruginosa* biofilms to ciprofloxacin and tobramycin. *Antimicrob Agents Chemother* **47**, 317-323 (2003).
34. Lewis, K. Persister Cells. *Annu Rev Microbiol* **64**, 357-372 (2010).
35. Olson, M.E., Ceri, H., Morck, D.W., Buret, A.G. & Read, R.R. Biofilm bacteria: formation and comparative susceptibility to antibiotics. *Can J Vet Res* **66**, 86-92 (2002).
36. Rediske, A.M. et al. Pulsed ultrasound enhances the killing of *Escherichia coli* biofilms by aminoglycoside antibiotics in vivo. *Antimicrob Agents Chemother* **44**, 771-772 (2000).

37. Lu, T.K. & Collins, J.J. Dispersing biofilms with engineered enzymatic bacteriophage. *Proc Natl Acad Sci U S A* **104**, 11197-11202 (2007).
38. Spoering, A.L. & Lewis, K. Biofilms and planktonic cells of *Pseudomonas aeruginosa* have similar resistance to killing by antimicrobials. *J Bacteriol* **183**, 6746-6751 (2001).
39. Majerczyk, C.D. et al. Direct targets of CodY in *Staphylococcus aureus*. *J Bacteriol* **192**, 2861-2877 (2010).

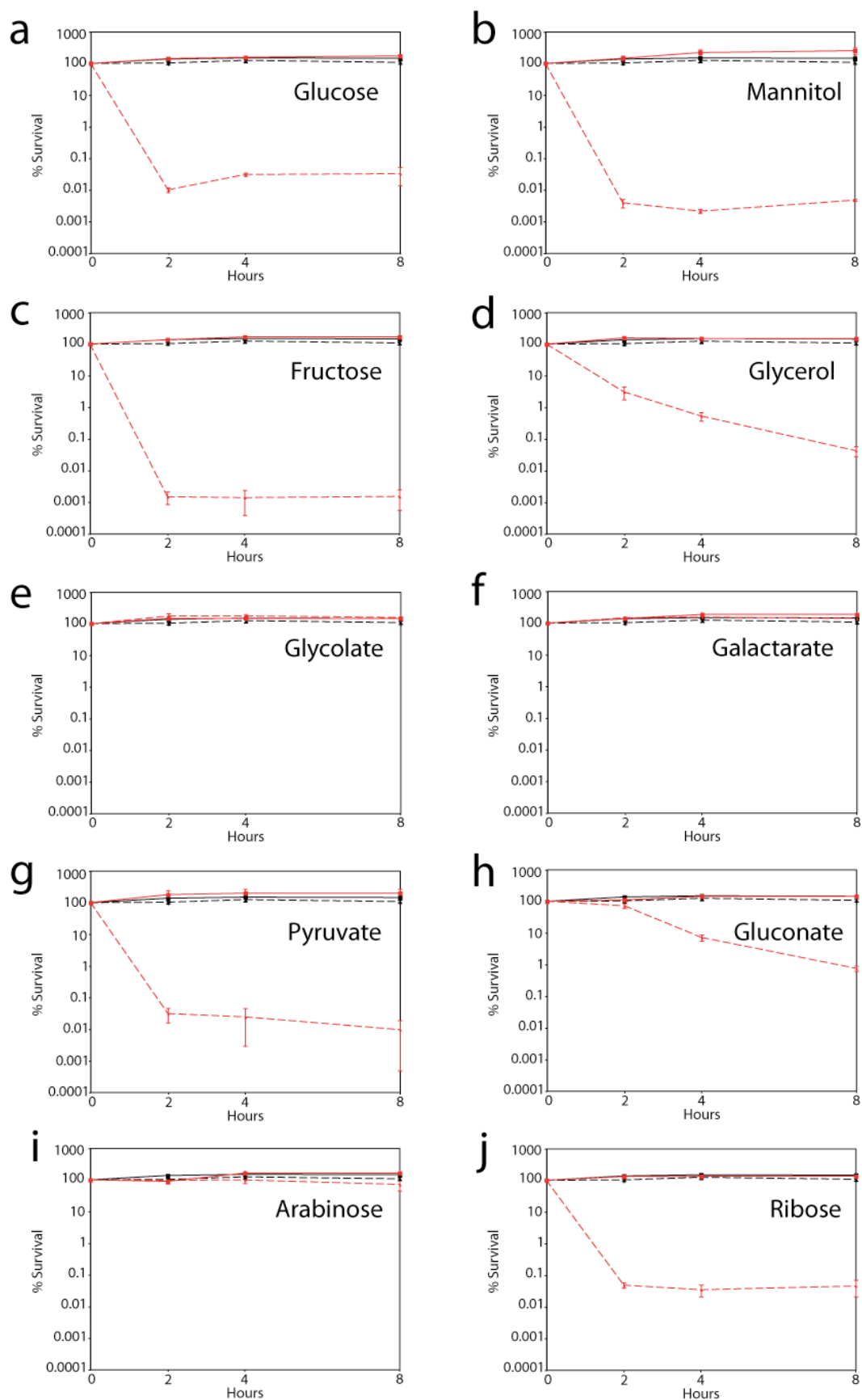
Supplementary Figures



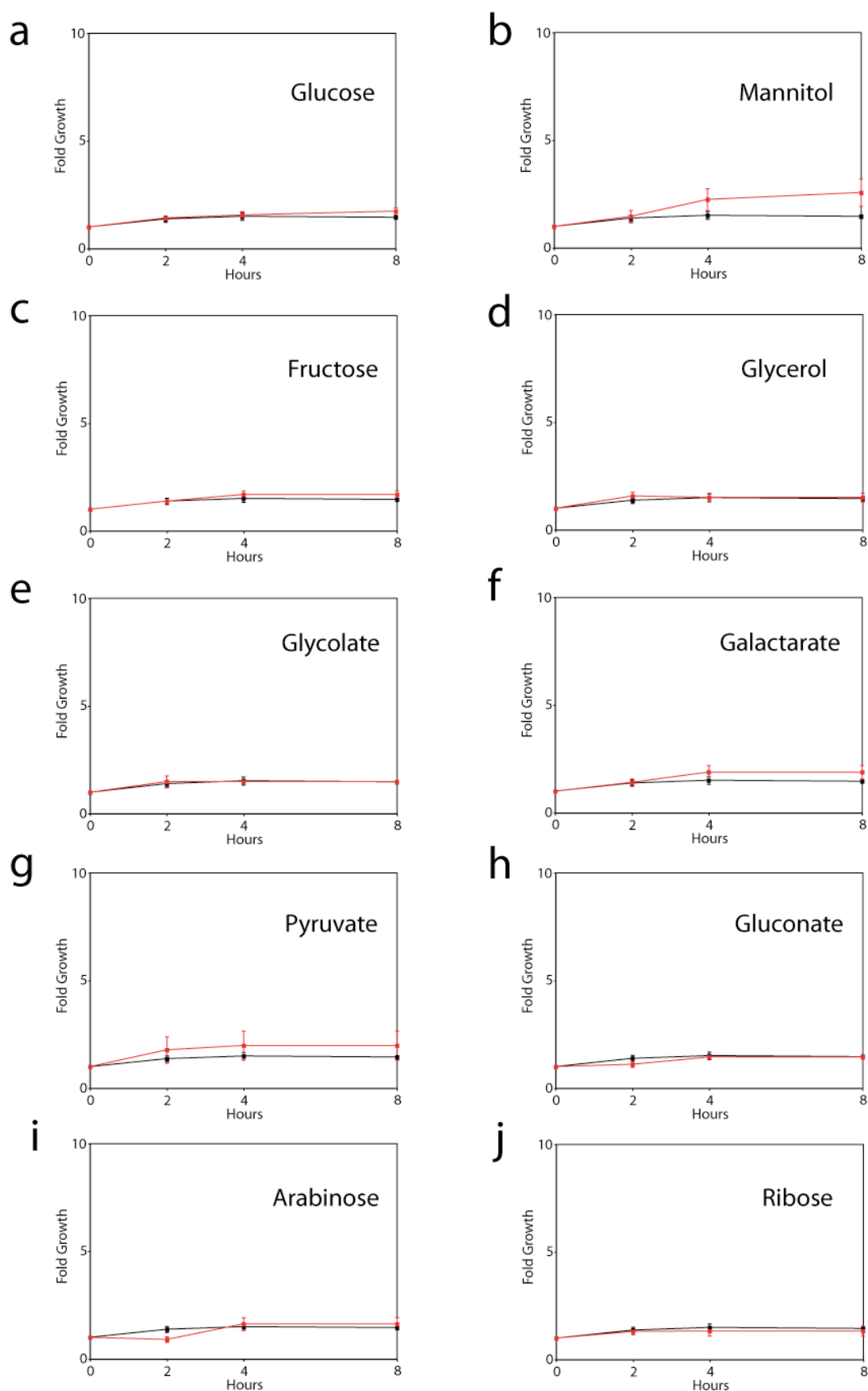
Supplementary Figure 1. Increasing concentrations of ofloxacin do not decrease survival. Percent survival of stationary phase wild-type *E. coli* after 4-hour treatment with indicated concentrations of ofloxacin.



Supplementary Figure 2. Metabolite-enabled persister eradication is general to aminoglycosides. Percent survival of wild-type *E. coli* persisters treated with no metabolite (black), glucose (blue), mannitol (red), and fructose (green) plus: **a**, 30 µg/mL kanamycin; or **b**, 50 µg/mL streptomycin.

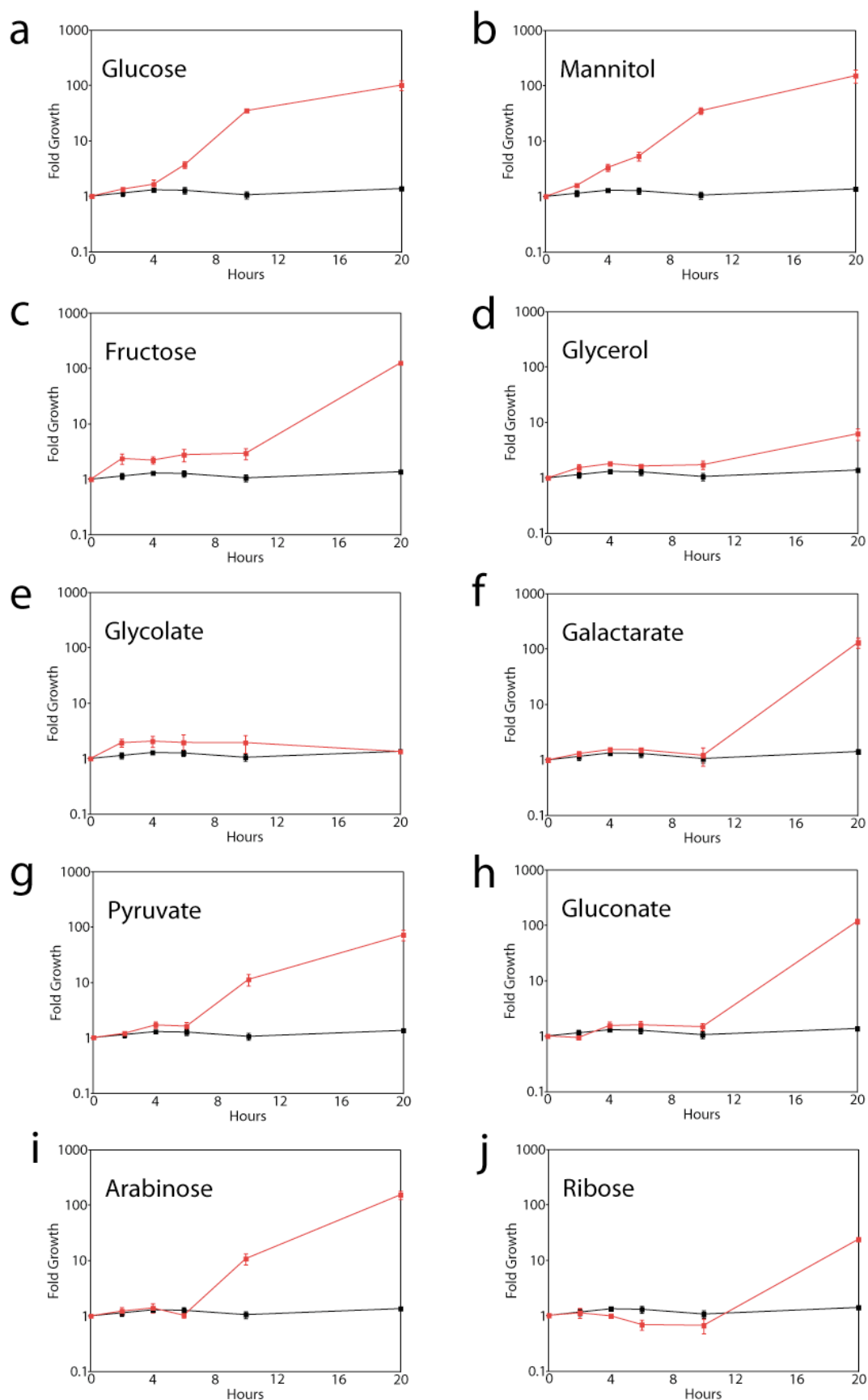


Supplementary Figure 3. Metabolite potentiation of gentamicin activity and ability to induce growth in *E. coli* persisters. Persister cells were incubated for four hours in M9 minimal media plus carbon sources entering metabolism at specific points in glycolysis, the pentose-phosphate pathway, and the entner-douderooff pathway. Solid red lines indicate persisters treated with carbon sources; dotted red lines indicate persisters treated with 10 $\mu\text{g/mL}$ gentamicin and carbon sources. In all plots, solid black lines indicate no treatment and dotted black lines indicate treatment with 10 $\mu\text{g/mL}$ gentamicin. Plots **a-j** show survival data for persisters in M9 plus the following carbon sources: **a**, 10 mM glucose; **b**, 10 mM mannitol; **c**, 10 mM fructose; **d**, 20 mM glycerol; **e**, 30 mM glycolate; **f**, 10 mM galactarate; **g**, 20 mM pyruvate; **h**, 10 mM gluconate; **i**, 12 mM arabinose; and **j**, 12 mM ribose.

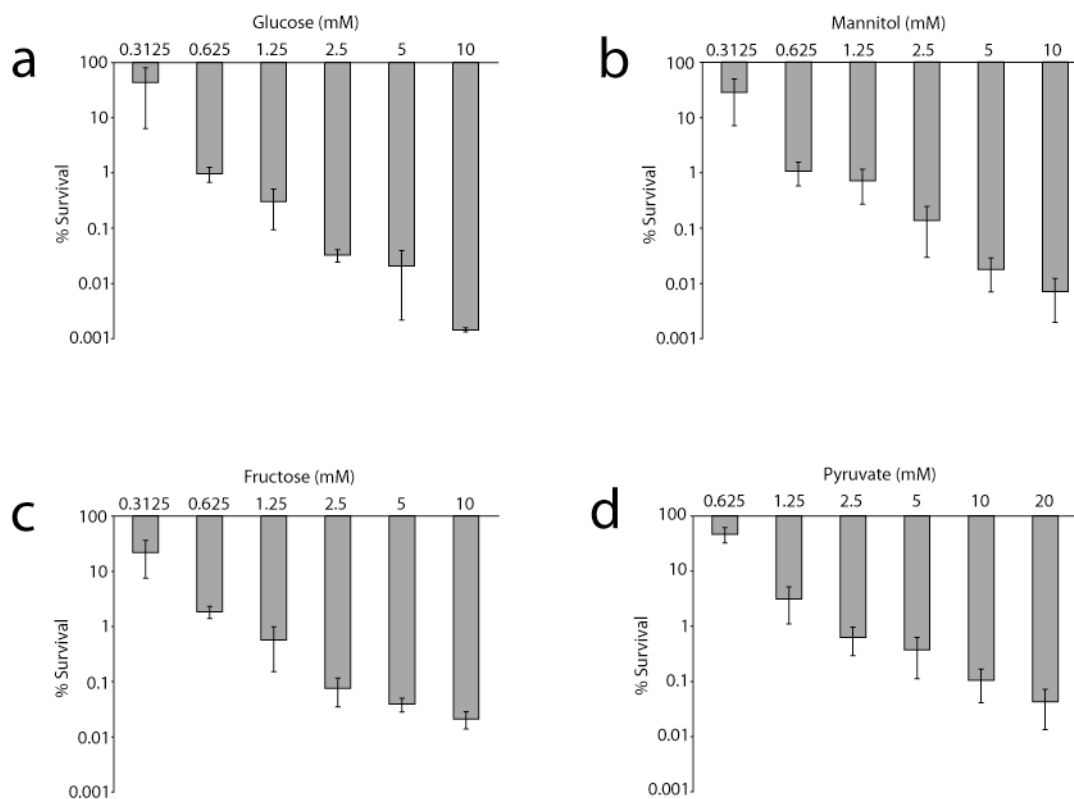


Supplementary Figure 4. Metabolite ability to induce growth in *E. coli* persisters.

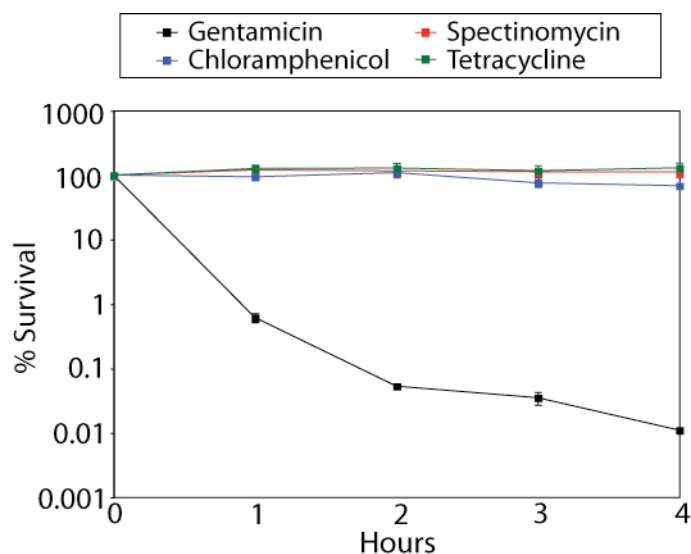
Growth data from Supplementary Figure 3 were plotted on a linear scale to show fold growth induced by metabolites. In all plots, black lines indicate no treatment and red lines indicate treatment with carbon source. Plots **a-j** fold growth data for persisters in M9 plus the following carbon sources: **a**, 10 mM glucose; **b**, 10 mM mannitol; **c**, 10 mM fructose; **d**, 20 mM glycerol; **e**, 30 mM glycolate; **f**, 10 mM galactarate; **g**, 20 mM pyruvate; **h**, 10 mM gluconate; **i**, 12 mM arabinose; and **j**, 12 mM ribose.



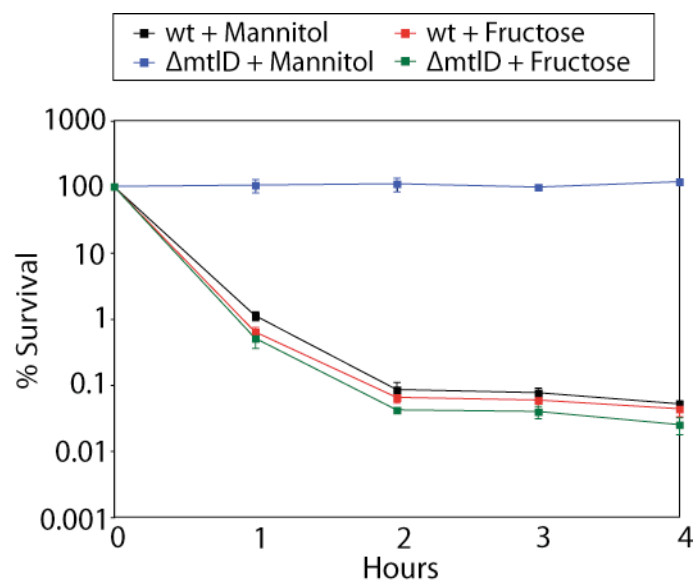
Supplementary Figure 5. Metabolite-induced resuscitation of persisters after 100-fold dilution. Persister cells were incubated for four hours in M9 minimal media plus carbon sources entering metabolism at specific points in glycolysis, the pentose-phosphate pathway, and the entner-douderooff pathway. Solid red lines indicate growth of persisters on carbon sources. In all plots, solid black lines indicate cell counts of persisters in M9 without carbon source. Plots **a-j** show growth of persisters in M9 plus the following carbon sources: **a**, 10 mM glucose; **b**, 10 mM mannitol; **c**, 10 mM fructose; **d**, 20 mM glycerol; **e**, 30 mM glycolate; **f**, 10 mM galactarate; **g**, 20 mM pyruvate; **h**, 10 mM gluconate; **i**, 12 mM arabinose; and **j**, 12 mM ribose.



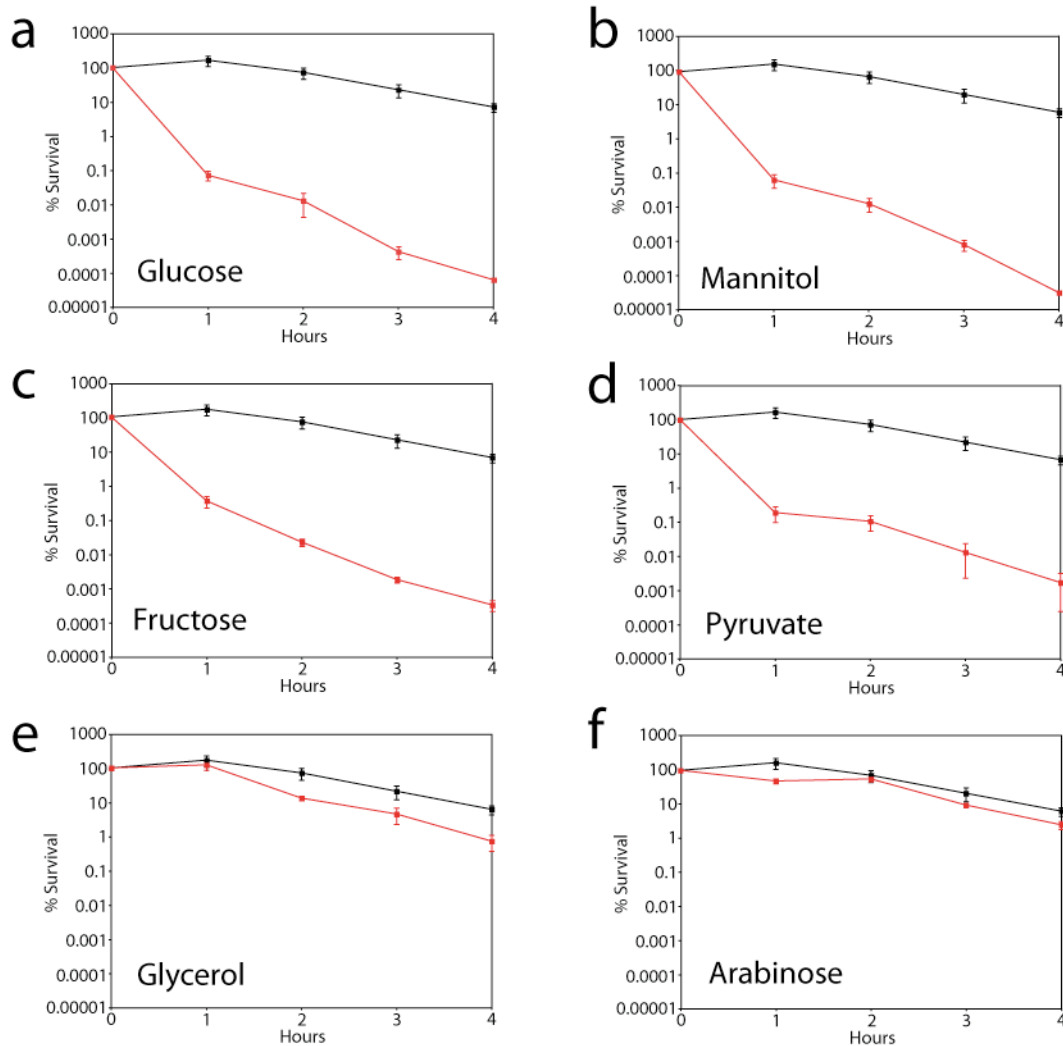
Supplementary Figure 6. Dependence of metabolite-enabled persister eradication on metabolite concentration. Persister cells were treated with 10 $\mu\text{g/mL}$ gentamicin for four hours in M9 minimal media plus indicated concentrations of metabolites: **a**, glucose; **b**, mannitol; **c** fructose; and **d**, pyruvate.



Supplementary Figure 7. Static ribosomal inhibitors do not cause metabolite-enabled killing of persistent *E. coli*. Percent survival of wild-type *E. coli* persists after treatment with 10 mM mannitol and the following ribosomal inhibitors: 10 $\mu\text{g/mL}$ gentamicin (black squares), 100 $\mu\text{g/mL}$ spectinomycin (red squares), 50 $\mu\text{g/mL}$ chloramphenicol (blue squares), and 40 $\mu\text{g/mL}$ gentamicin (green squares).



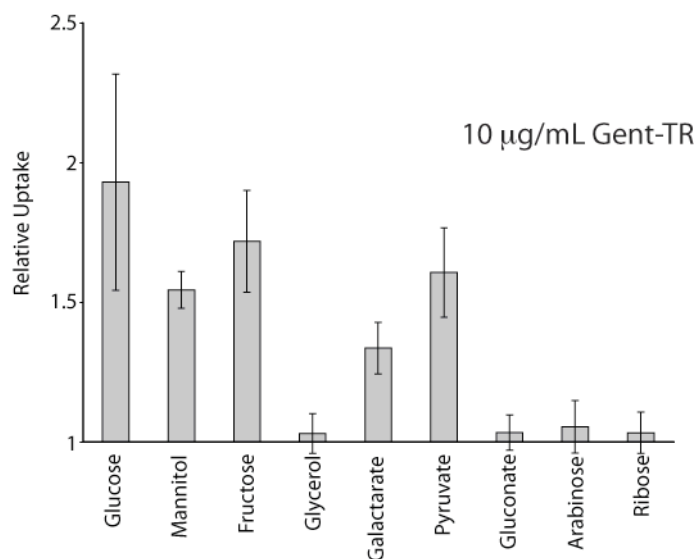
Supplementary Figure 8. Metabolism of carbon source is required for metabolite-enabled potentiation of aminoglycosides. Survival of wild-type and $\Delta mtlD$ *E. coli* (EMG2) persisters treated with 10 $\mu\text{g/mL}$ gentamicin plus 10 mM mannitol, or 10 mM fructose.



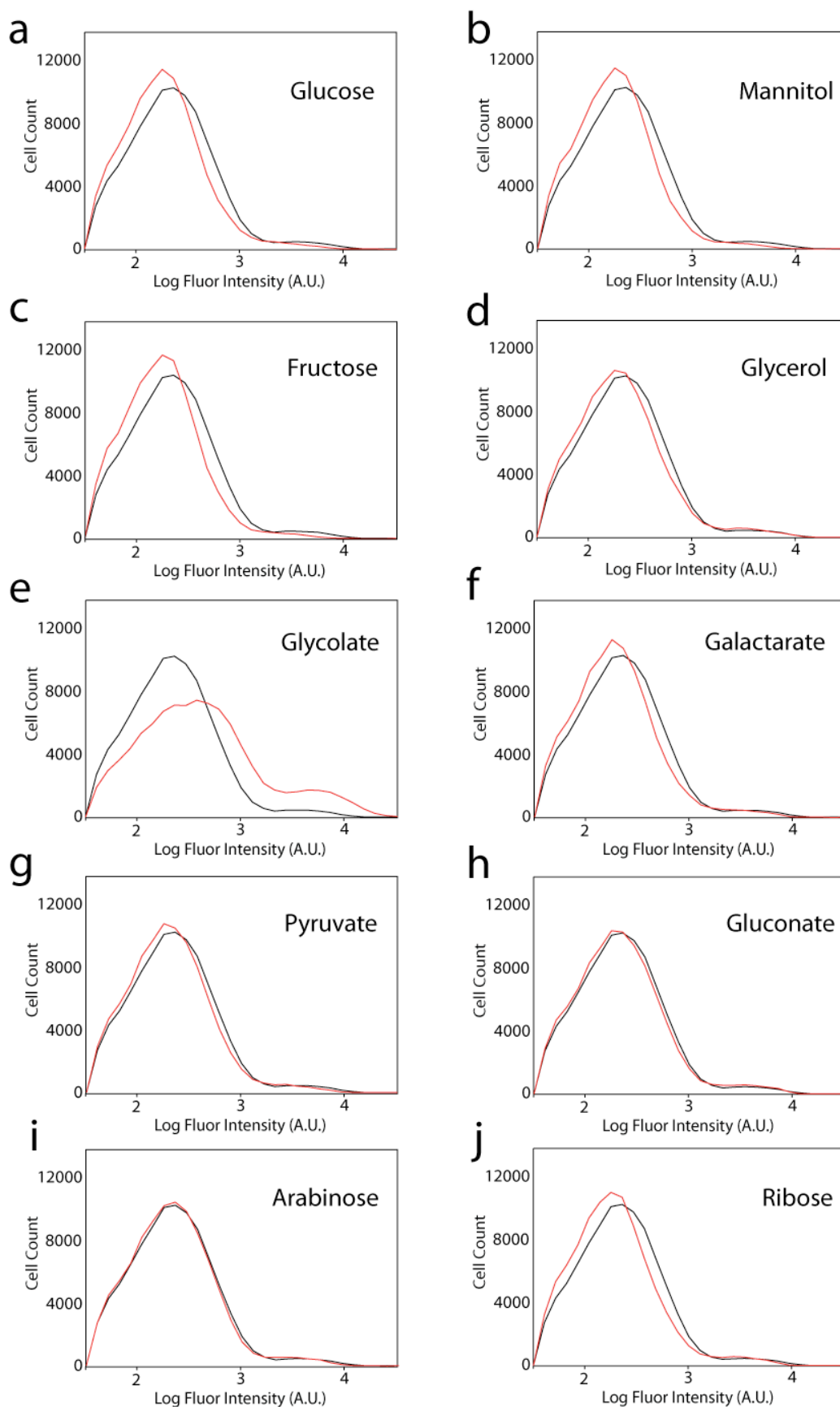
Supplementary Figure 9. Metabolite-enabled eradication of stationary phase *E. coli*.

Survival of wild-type (EMG2) in stationary phase treated with 10 $\mu\text{g/mL}$ gentamicin with and without carbon source. In all plots, black lines indicate cultures treated solely with gentamicin and red lines indicate cultures treated with gentamicin plus carbon source.

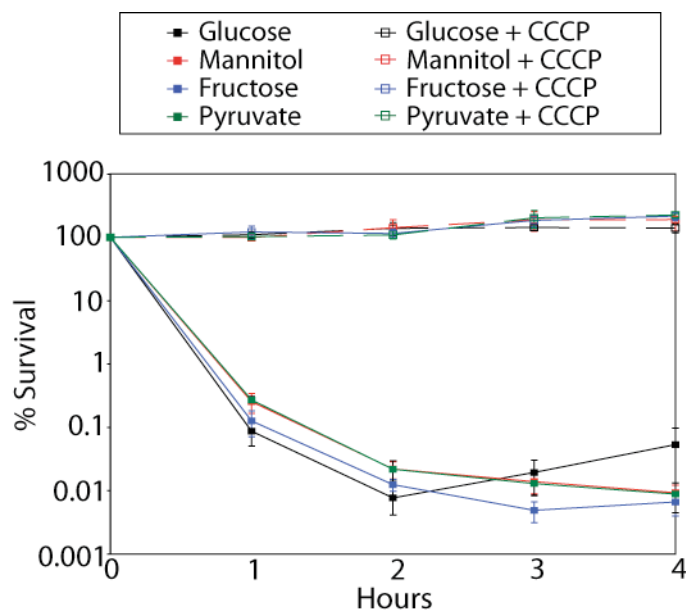
Plots **a-f** show percent survival of persisters in M9 plus the following carbon sources: **a**, 10 mM glucose; **b**, 10 mM mannitol; **c**, 10 mM fructose; **d**, 20 mM pyruvate; **e**, 20 mM glycerol; and **f**, 12 mM arabinose.



Supplementary Figure 10. Gentamicin uptake induced by carbon sources in stationary phase *E. coli*. Median uptake of 10 µg/mL gentamicin-Texas Red (Gent-TR) induced by 30 minutes of incubation on carbon sources. Median values were normalized by uptake in the no-carbon control. The normalized median values presented are the average of three independent experiments. Error bars signify the SEM of median values.

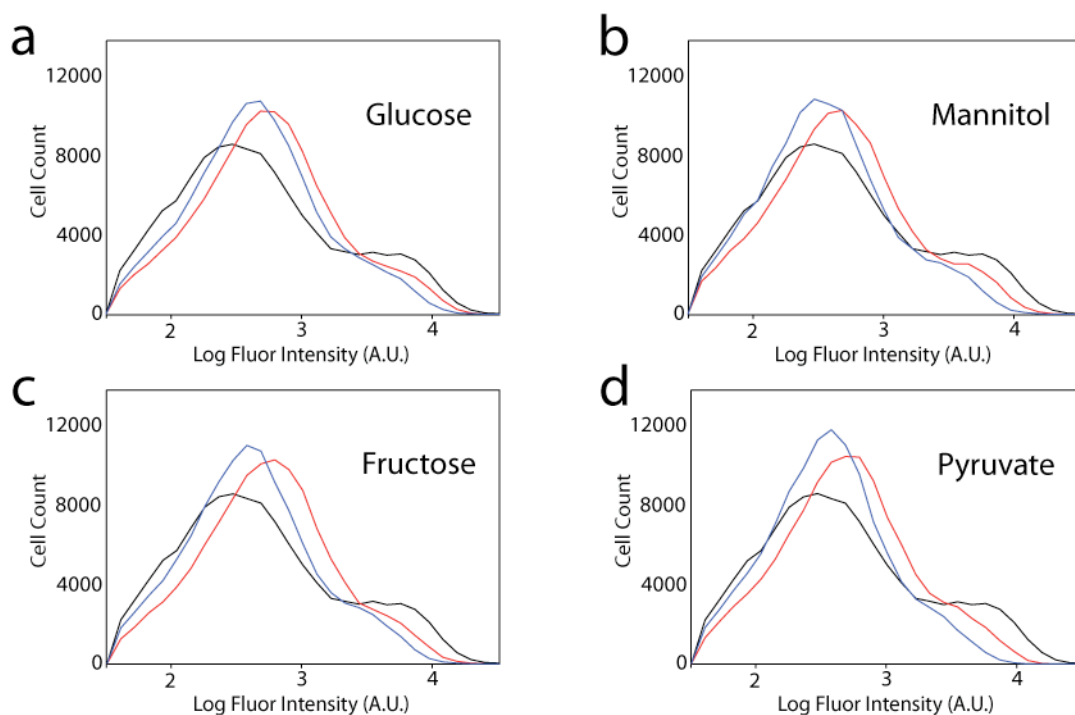


Supplementary Figure 11. Glycolate affects the uptake of Texas Red probe in stationary phase *E. coli*. Representative histograms of uptake of Texas Red by stationary phase *E. coli* treated with the following carbon sources: **a**, 10 mM glucose; **b**, 10 mM mannitol; **c**, 10 mM fructose; **d**, 20 mM glycerol; **e**, 30 mM glycolate; **f**, 10 mM galactarate; **g**, 20 mM pyruvate; **h**, 10 mM gluconate; **i**, 12 mM arabinose; and **j**, 12 mM ribose. Red lines indicate samples treated with carbons source and black lines indicate samples that were not treated with carbon source.

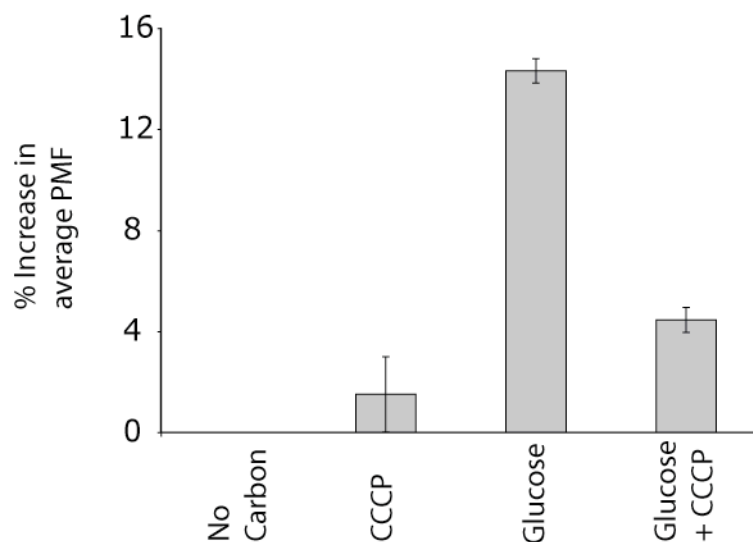


Supplementary Figure 12. CCCP abolishes metabolite-enabled potentiation in *E.*

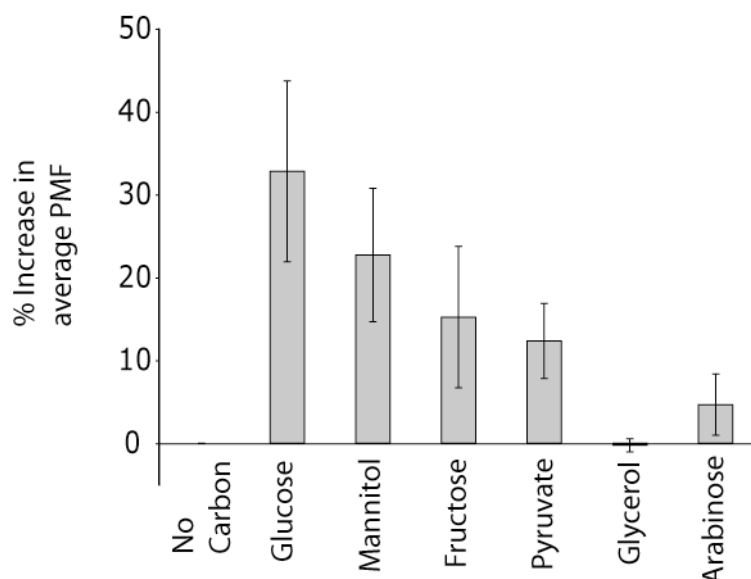
***coli* persisters.** Percent survival of wild-type *E. coli* persisters after treatment with 10 $\mu\text{g/mL}$ gentamicin plus uptake-potentiating carbon sources (solid lines and solid squares) or uptake-potentiating carbon sources with 20 μM CCCP (dashed lines and open squares).



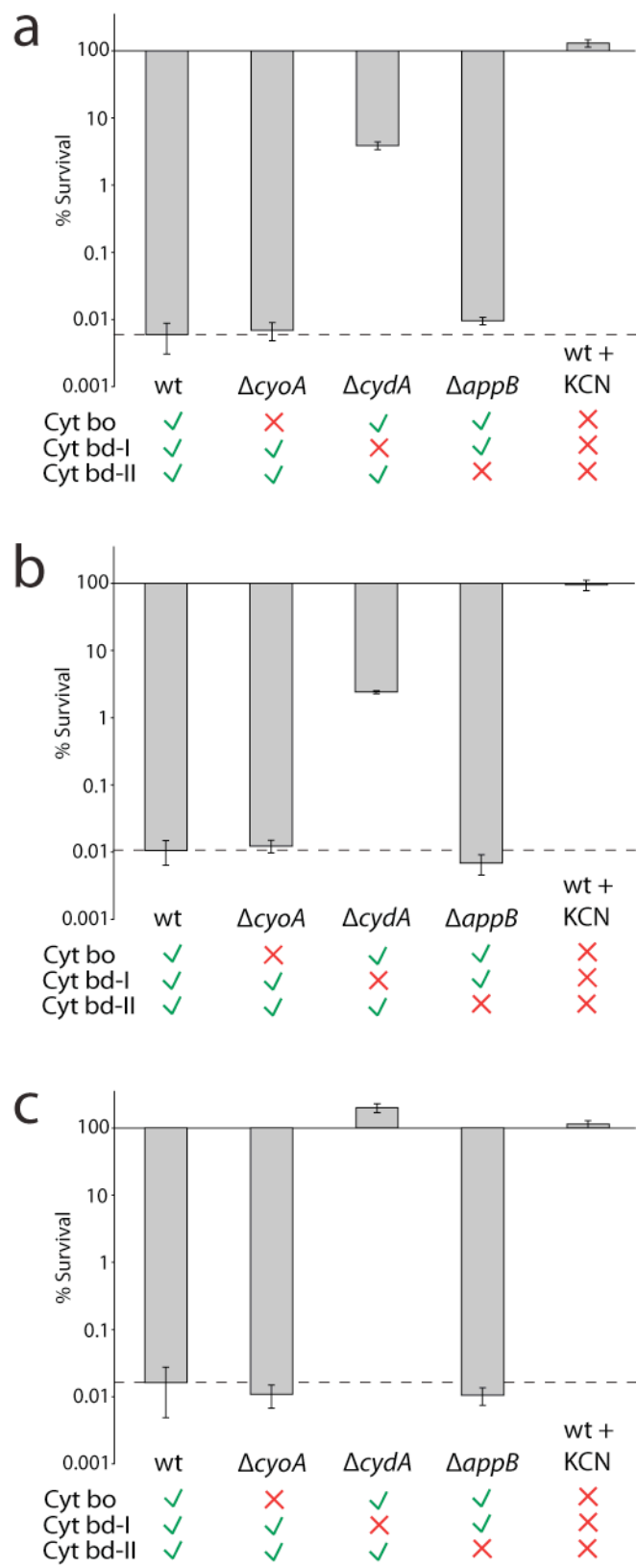
Supplementary Figure 13. Metabolite-enabled aminoglycoside uptake requires PMF. Representative measurements of uptake of 10 μ g/mL fluorescently labeled gentamicin (Gent-TR) by stationary phase cells after 30 minutes incubation with no carbon source (black lines), carbon source (red lines), or carbon source and 20 μ M CCCP (blue lines). The following carbon sources were used: **a**, 10 mM glucose, **b**, 10 mM mannitol, **c**, 10 mM fructose, and **d**, 20 mM pyruvate.



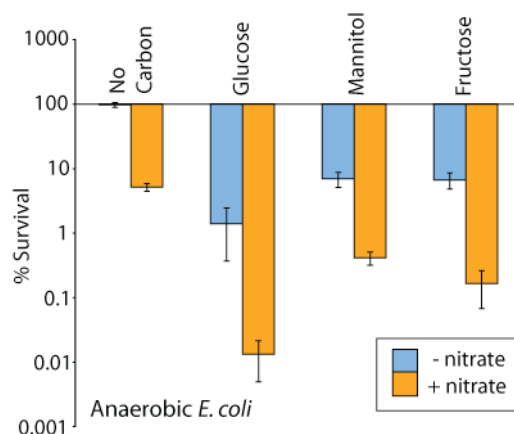
Supplementary Figure 14. Glucose induces PMF in dormant *E. coli*. Mean percent increase in membrane potential, measured as red/green from DiOC₂(3) (see Methods), induced by no treatment, 20 μ M CCCP, 10 mM glucose, and 10 mM glucose plus 20 μ M CCCP.



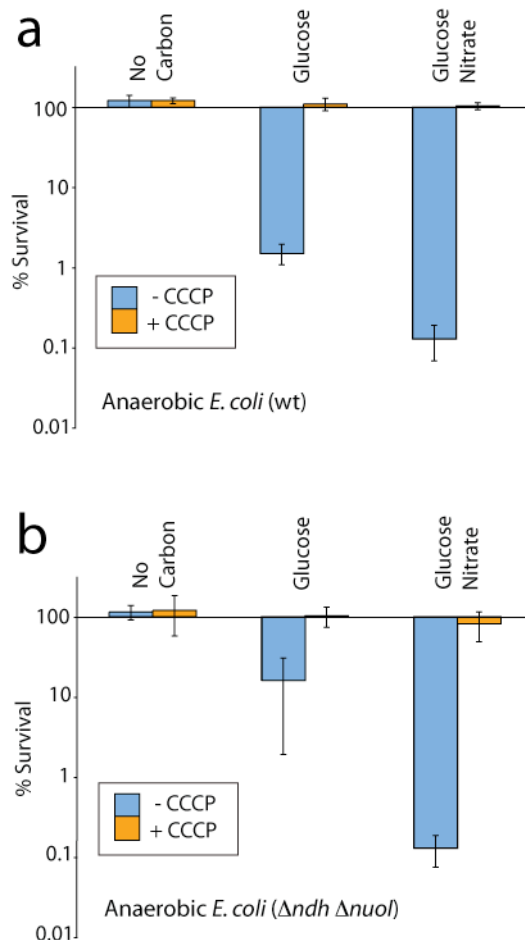
Supplementary Figure 15. Induction of PMF in dormant *E. coli* by different metabolites. Mean percent increase in membrane potential, measured as red/green from DiOC₂(3) (see Methods), induced by no carbon, 10 mM glucose, 10 mM mannitol, 10 mM fructose, 20 mM pyruvate, 20 mM glycerol, and 12 mM arabinose.



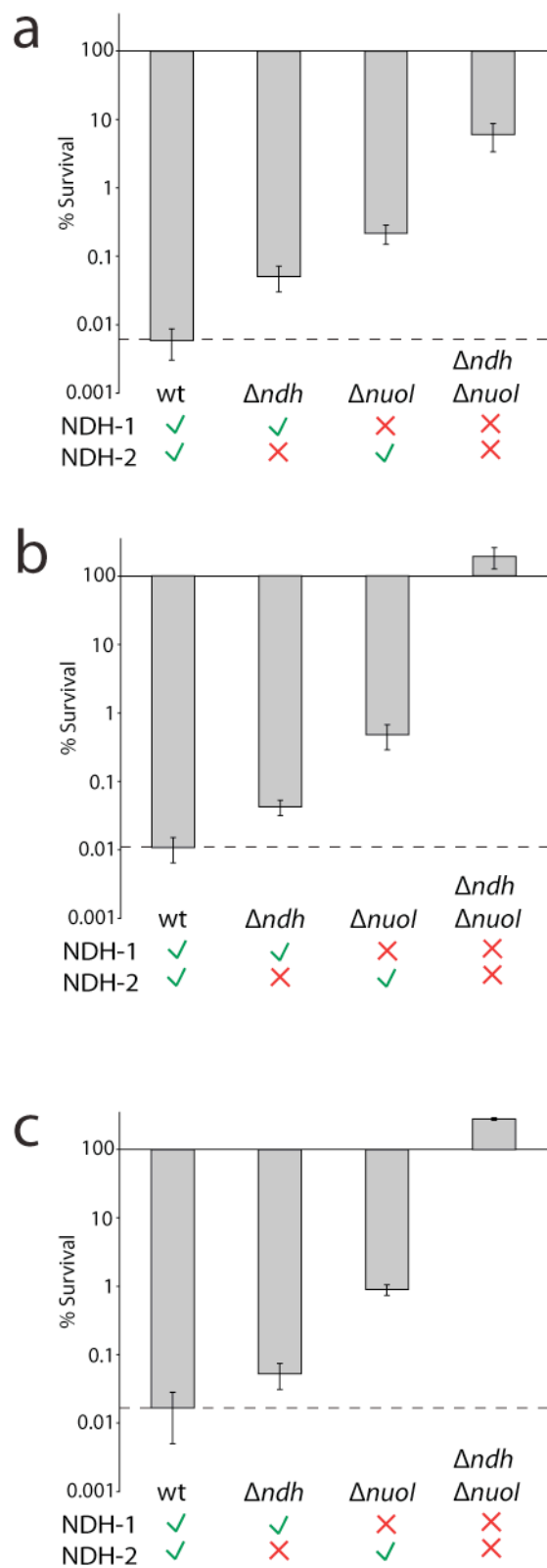
Supplementary Figure 16. Aerobic respiration is necessary for the observed metabolite-enabled eradication of bacterial persisters. Percent survival of persisters in strains with genetic perturbations to the three *E. coli* cytochromes after treatment with 10 µg/mL gentamicin plus: **a**, 10 mM glucose, **b**, 10 mM fructose, and **c**, 20 mM pyruvate. The presence (green checks) and absence (red X's) of functional cytochrome complexes is indicated below the tested conditions.



Supplementary Figure 17. Anaerobic metabolite-enabled eradication of dormant bacteria. Percent survival of stationary phase wild-type *E. coli* (EMG2) after 4-hour treatment with 10 mM indicated carbon source plus 10 µg/mL gentamicin (blue bars) or 10 mM nitrate and 10 µg/mL gentamicin (orange bars). Growth and treatment of cultures were conducted in an anaerobic chamber.

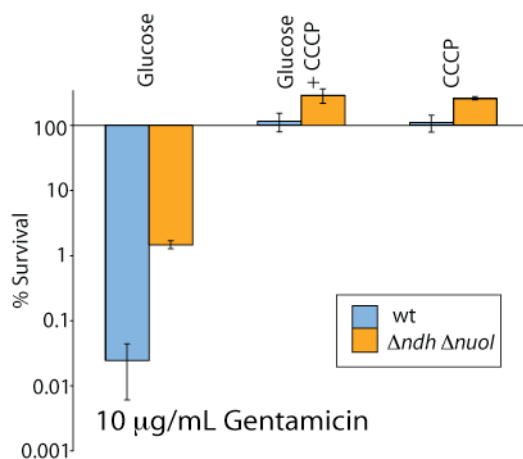


Supplementary Figure 18. Anaerobic metabolite-enabled eradication of dormant bacteria requires PMF. Percent survival of stationary phase wild-type (**a**) and $\Delta ndh \Delta nuoI$ (**b**) *E. coli* (EMG2) after 4-hour treatment with 10 $\mu\text{g/mL}$ gentamicin, with (orange bars) and without (blue bars) 20 μM CCCP, and with 10 mM glucose and 10 mM nitrate. Growth and treatment of cultures were conducted in an anaerobic chamber.

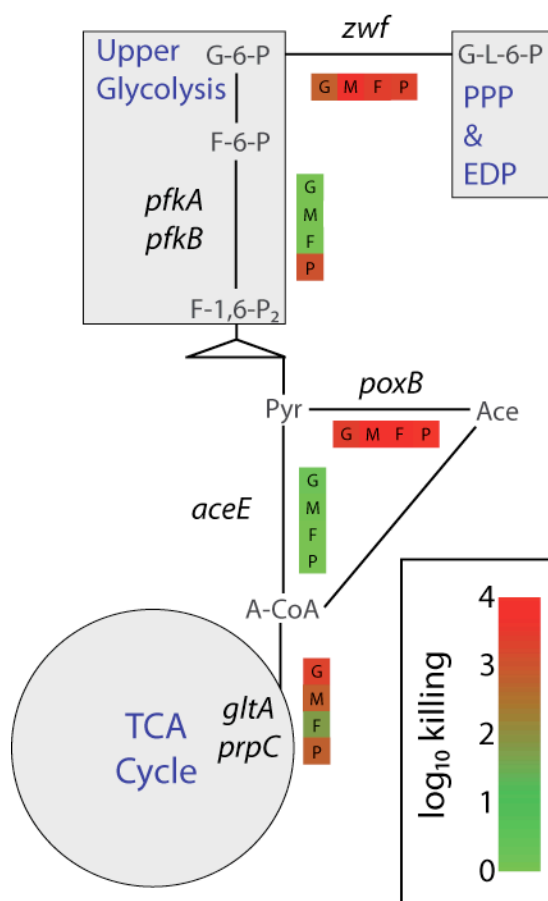


Supplementary Figure 19. NADH dehydrogenase activity is important for metabolite-enabled eradication of bacterial persisters by most carbon sources.

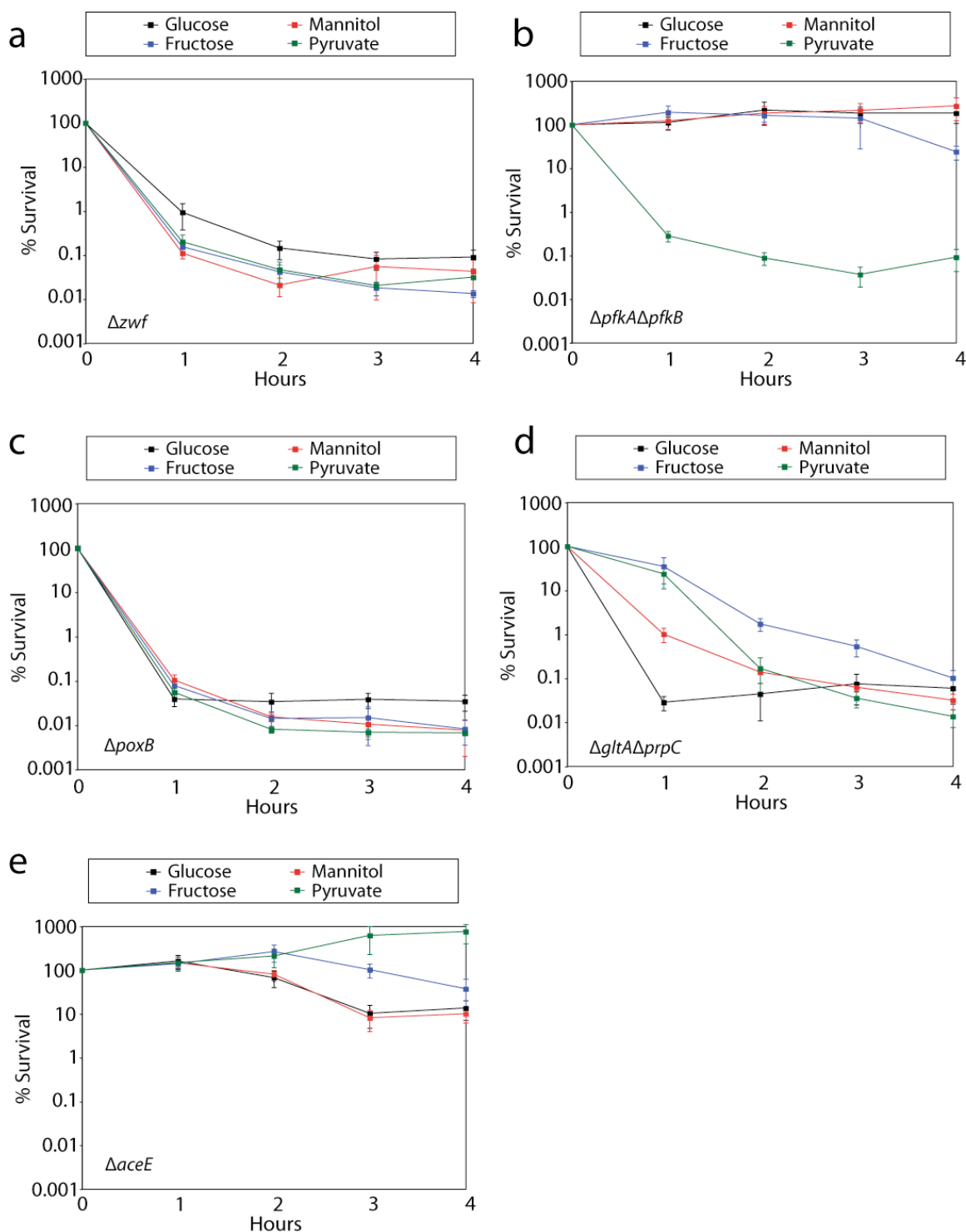
Percent survival of persisters in strains with genetic perturbations to the two *E. coli* NADH dehydrogenase complexes after treatment with 10 µg/mL gentamicin plus: **a**, 10 mM glucose, **b**, 10 mM fructose, and **c**, 20 mM pyruvate. The presence (green checks) and absence (red X's) of functional NADH dehydrogenase complexes is indicated below the tested strains.



Supplementary Figure 20. PMF is required for metabolite-enabled eradication of bacterial persisters in the $\Delta ndh \Delta nuoI$ strain. Percent survival of persisters in the wild-type (blue) and $\Delta ndh \Delta nuoI$ (orange) *E. coli* strains after treatment with 10 μ g/mL gentamicin plus 10 mM glucose, 20 μ M CCCP, or both 10 mM glucose and 20 μ M CCCP.

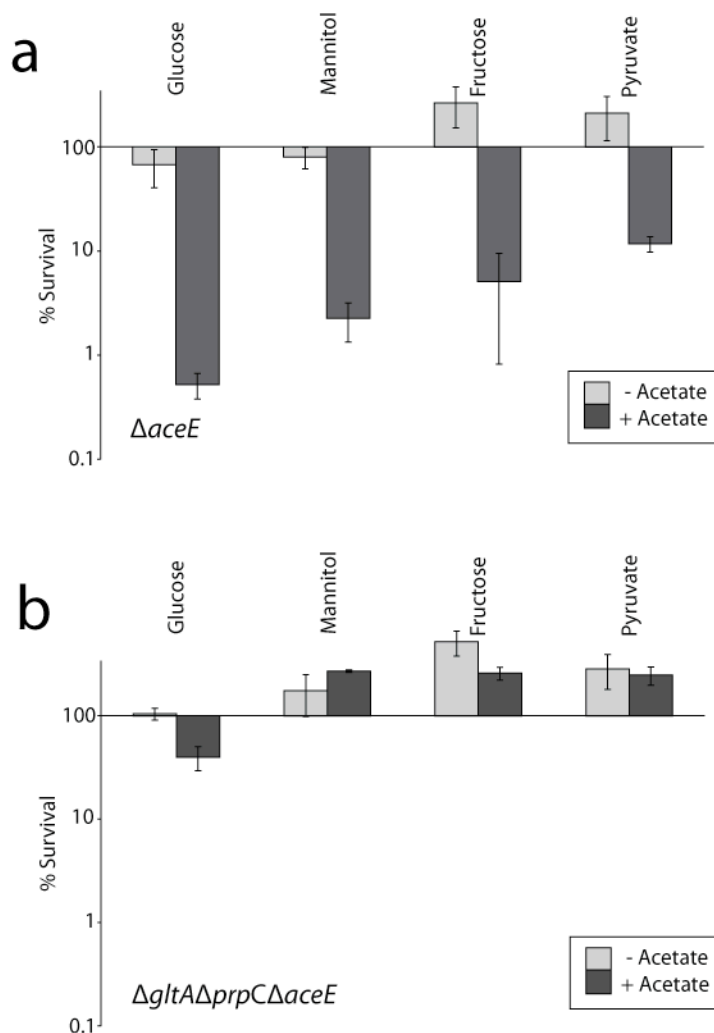


Supplementary Figure 21. Source of NADH production in aminoglycoside killing of *E. coli* persisters. Heat maps of gentamicin potentiation by carbon sources in genetic knockouts overlaid on central metabolism. The color of the box around each letter (“G” for glucose, “M” for mannitol, “F” for fructose, and “P” for pyruvate) indicates the aminoglycoside potentiation induced by carbon sources in a knockout strain. The knocked-out enzymatic steps are labeled opposite the heat maps to which they correspond (see also Supplementary Fig. 22).

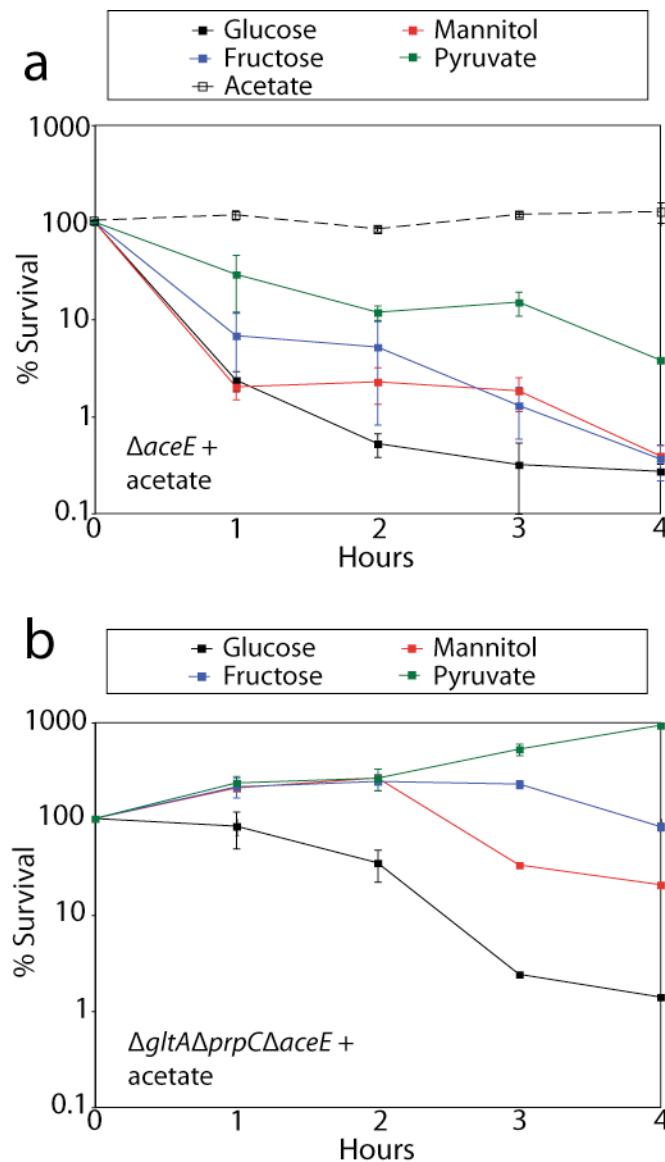


Supplementary Figure 22. Potentiation of aminoglycoside killing of *E. coli* persisters by carbon sources in metabolic mutants. Survival of **a**, Δzwf , **b**, $\Delta pfkA\Delta pfkB$, **c**,

Δ *poxB*, **d**, Δ *gltA* Δ *prpC*, and **e**, Δ *aceE* *E. coli* (EMG2) persisters treated with 10 μ g/mL gentamicin plus 10 mM glucose (black squares), 10 mM mannitol, (red squares), 10 mM fructose (blue squares), or 20 mM pyruvate (green squares).

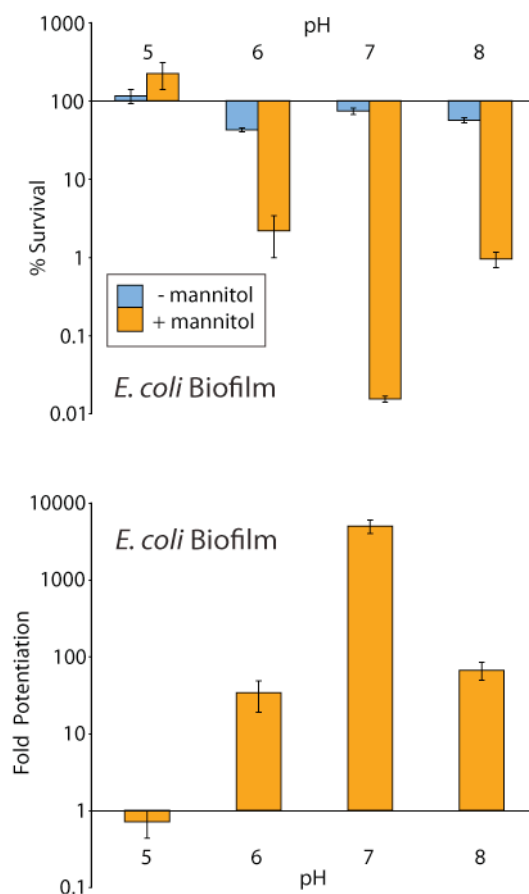


Supplementary Figure 23. NADH production by pyruvate dehydrogenase induces aminoglycoside killing of *E. coli* persisters. **a**, Percent survival of $\Delta aceE$ *E. coli* persisters after 2-hour treatment with 10 μ g/mL gentamicin and potentiating carbon source without acetate (light grey bars) or with 10 mM acetate (dark grey bars). **b**, Percent survival of $\Delta gltA \Delta prpC \Delta aceE$ *E. coli* after 2-hour treatment with 10 μ g/mL gentamicin and potentiating carbon source without acetate (light grey bars) or with 10 mM acetate (dark grey bars) (see also Supplementary Fig. 24).

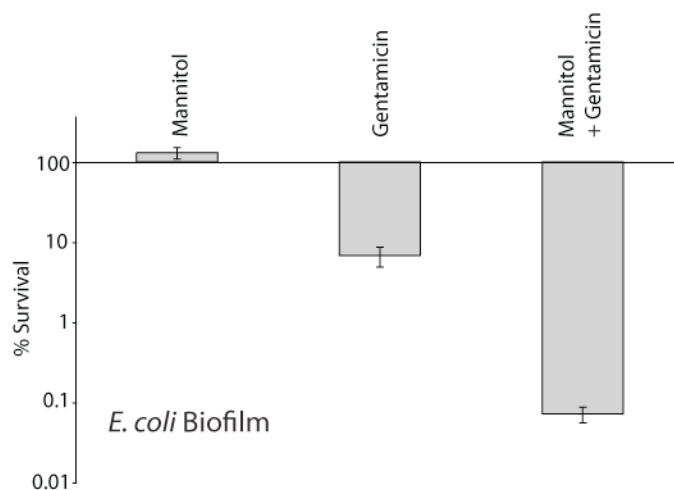


Supplementary Figure 24. Pyruvate dehydrogenase activity, due to its NADH generation, is important to the observed metabolite-enabled potentiation in persisters. **a**, Survival of $\Delta aceE$ persisters after treatment with 10 μ g/mL gentamicin, 10 mM acetate, and one of the following carbon sources: 10 mM glucose (black squares), 10 mM mannitol, (red squares), 10 mM fructose (blue squares), or 20 mM pyruvate (green squares). Survival of $\Delta aceE$ persisters after treatment with 10 μ g/mL gentamicin and 30

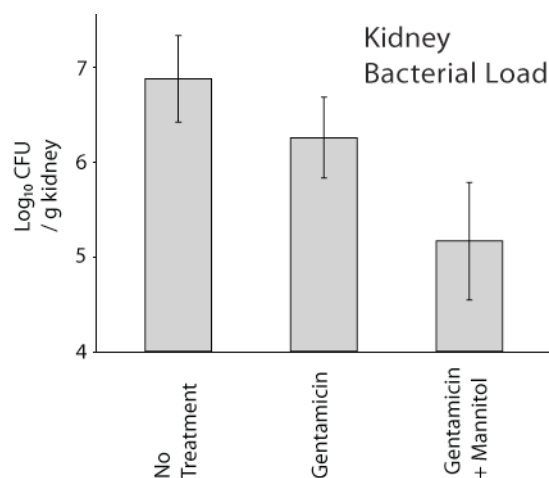
mM acetate (black dashed lines and open squares). **b**, Survival of $\Delta gltA \Delta prpC \Delta aceE$ persists after treatment with 10 $\mu\text{g/mL}$ gentamicin, 10 mM acetate, and one of the following carbon sources: 10 mM glucose (black squares), 10 mM mannitol, (red squares), 10 mM fructose (blue squares), or 20 mM pyruvate (green squares).



Supplementary Figure 25. Dependence of biofilm eradication on pH. **a**, Percent survival of wild-type *E. coli* (EMG2) biofilms after 4-hour treatment with 10 μ g/mL gentamicin (blue bars) or 10 mM mannitol plus 10 μ g/mL gentamicin (orange bars) in M9 buffered to the indicated pH. **b**, Fold gentamicin potentiation induced by 10 mM mannitol against biofilms at indicated pH (as determined from data presented in **b**).



Supplementary Figure 26. Biofilm eradication at longer timescales. Percent survival of wild-type *E. coli* (EMG2) biofilms after 8-hour treatment with 10 mM mannitol, 10 $\mu\text{g/mL}$ gentamicin or 10 mM mannitol plus 10 $\mu\text{g/mL}$ gentamicin in M9 minimal media.



Supplementary Figure 27. Mannitol plus gentamicin stops spread of infection to mouse kidneys. Mice had catheters colonized with uropathogenic *E. coli* biofilms surgically implanted in their urinary tracts. Forty-eight hours after surgery, mice received no treatment or twice-daily, intravenous treatment with gentamicin (1 mg/Kg) or mannitol (1.5 g/Kg) and gentamicin (1 mg/Kg) for three days. Twenty-four hours after the last treatment, kidneys were removed and bacterial load was determined and plotted as log₁₀ CFU/g kidney.

Supplementary Table 1: Enzymes inactivated to determine source of NADH generation.

Enzyme	Gene(s)	Reducing Equivalents
glucose 6-phosphate-1 dehydrogenase	<i>zwf</i>	NADPH*
6-phosphofructokinase	<i>pfkA pfkB</i>	NADH
citrate/methylcitrate synthase	<i>gltA prpC</i>	NADH
pyruvate dehydrogenase	<i>aceE</i>	NADH

*NADPH can be converted to NADH by pyridine nucleotide transhydrogenase (*sthA*)

Supplementary Table 2. Check primers for genetic knock-out strains.

Gene	Forward Primer	Reverse Primer
<i>mtlD</i>	CTCCTCACGGAGAGGGTTT	TCAAGCACACGGTTTTTCAA
<i>zwf</i>	CGTAATCGCACGGGTGGATAA	TTATGACTGAAACGCCTGTAACCG
<i>pfkA</i>	CCAGGGAGGGTAAACGGTCTATG	GGTTTCAGGGTAAAGGAATCTGCC
<i>pfkB</i>	ATTTCTTCACTTTCCGCTGATTCG	ATTTCCCTCATCATCCGTCATAGTG
<i>poxB</i>	TGGTCGGGTAAACGGTATCAC	ACCGTTAGTGCCTCCTTTCTCT
<i>glfA</i>	TAAAGCCAGGTTGATGTGCGAA	AAGTATTGGGTGCTGATAATTTGAGC
<i>prpC</i>	GACCCTACAAATGATAACAATGACGAGG	GACGATATCAACGATTTTACGATCAAA
<i>aceE</i>	GTGAGCGTTCTCTGCGTCGTCT	TCTCTTTCACGGTGCCAGCAA
<i>cyoA</i>	TTCCCGTAAAATGCCCCACAC	TAATGCCAGCGATCGTAACC
<i>cydB</i>	GCCCAAGCAGCCTGAAAA	CGGTGATTACCCCAAACGAA
<i>appB</i>	TGCAGAAATATGCCCCGTCTG	AGGGTGGAGAGCGAACACAT
<i>ndh</i>	GCGAAGAACATTTTCATTGCTGTA	GATCGCGCTGTTCTCTCAAG
<i>nuoI</i>	TGTCCTTCGGCTGGAAAATC	CTCGCAAGGTCGCAAGTATG
<i>kanR</i>		GGTCCGCCACACCCAGCC

Supplementary Table 3. Stationary phase fold regulation of metabolite transporters.

Gene Name	UniProt Accession #	Exponential Replicate 1	Exponential Replicate 2	Stationary Replicate 1	Stationary Replicate 2	Fold change (stationary / exponential)	Description
<i>crr</i>	Q5HFZ9, P60856, P60857	100.18	107.50	120.26	131.12	1.21	Glucose-specific phosphotransferase enzyme IIA component
<i>ptsG</i>	Q5HJI3, Q7A807, Q99X32	233.59	218.12	72.59	60.48	0.29	PTS system glucose-specific EIICBA component
<i>mtlA</i>	Q5HE48, Q7A4B3, Q99SA3	5.59	4.61	3.21	3.28	0.64	PTS system mannitol-specific EIICB component
<i>mtlF</i>	Q5HE46, P0A0D7, P0A0D8	23.64	16.75	14.62	23.53	0.94	Mannitol-specific phosphotransferase enzyme IIA component
<i>SACOL2663</i>	Q5HCQ6, Q7A374, Q99QZ7	8.93	8.18	206.91	150.47	20.88	PTS system, fructose-specific IIBC components