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## Metabolite-Enabled Eradication of Bacterial Persisters by Aminoglycosides

Kyle R. Allison<sup>1</sup>, Mark P. Brynildsen<sup>1,4</sup>, and James J. Collins<sup>1,2,3,\*</sup>

<sup>1</sup>Howard Hughes Medical Institute, Department of Biomedical Engineering, Center for BioDynamics and Center for Advanced Biotechnology, Boston University, Boston, MA 02215, USA

<sup>2</sup>Boston University School of Medicine, 715 Albany Street, Boston, MA 02118, USA

<sup>3</sup>Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, MA 02118, USA

### Abstract

Bacterial persistence is a state in which a sub-population of dormant cells (persisters) tolerates antibiotic treatment<sup>1-4</sup>. Bacterial persisters have been implicated in biofilms and chronic and recurrent infections<sup>5-7</sup>. Despite this clinical relevance, there are currently no viable means for eradicating persisters. Here we show that specific metabolic stimuli enable aminoglycoside killing of both Gram-negative (*Escherichia coli*) and Gram-positive (*Staphylococcus aureus*) persisters. This potentiation is aminoglycoside-specific, does not rely on growth resumption, is effective in both aerobic and anaerobic conditions, and proceeds by generation of proton-motive force (PMF) which facilitates aminoglycoside uptake. Our results demonstrate that persisters, though dormant, are primed for metabolite uptake, central metabolism, and respiration. We show that aminoglycosides in combination with specific metabolites can be used to treat *E. coli* and *S. aureus* biofilms. Further, we demonstrate that this approach can improve treatment of chronic infection in a mouse urinary tract infection model. This work establishes a metabolic-based strategy for eradicating bacterial persisters and highlights the critical importance of metabolic environment to antibiotic treatment.

Researchers have shown that translation occurs at a reduced rate in persisters<sup>2,8</sup>, suggesting that persisters should be susceptible to the ribosome-targeting bactericidal aminoglycoside antibiotics<sup>9-13</sup>. However, despite continued translation, aminoglycosides have weak activity against dormant bacteria<sup>14, 15</sup>. Given the dormancy of persisters and the known energy requirement for aminoglycoside activity<sup>16</sup>, we reasoned that metabolic stimulation might potentiate aminoglycosides against bacterial persisters.

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\*Corresponding author: James Collins; [jcollins@bu.edu](mailto:jcollins@bu.edu); phone: (617) 353-0390..

<sup>4</sup>Present address: Department of Chemical and Biological Engineering, Princeton University, Princeton, NJ 08544, USA

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To test this, we screened metabolites for their ability to potentiate aminoglycosides against *Escherichia coli* persisters. We selected carbon sources to maximize coverage of glycolysis, the pentose-phosphate pathway (PPP) and the entner-douderooff pathway (EDP) (Fig. 1a, b). Persisters were isolated (Supplementary Information), re-suspended in minimal media supplemented with individual metabolites, and treated with aminoglycoside gentamicin for two hours.

We found that gentamicin was greatly potentiated by specific metabolic stimuli against persisters (Fig. 1a, b). Metabolites entering upper glycolysis (glucose, mannitol, and fructose) and pyruvate induced rapid gentamicin killing of persisters, reducing persister viability by three orders of magnitude. In contrast, metabolites that entered lower glycolysis (excepting pyruvate) caused little potentiation. Metabolites entering metabolism via the PPP or EDP (arabinose, ribose, and gluconate) also showed low potentiation. No killing was observed in the control, demonstrating that treated cells were persistent to gentamicin, in the absence of added metabolite. We verified that metabolite-enabled persister eradication was general to the aminoglycoside class by testing kanamycin and streptomycin (Supplementary Fig. 2).

We considered that potentiating metabolites might be reverting persisters to normally growing cells, which would render them susceptible to quinolone (DNA-damage) and  $\beta$ -lactam (cell-wall inhibition) antibiotics. To test this, we treated persisters in the presence and absence of mannitol with a member of each of the three major classes of bactericidal antibiotics: aminoglycosides, quinolones, and  $\beta$ -lactams. As seen in the metabolite screen, gentamicin rapidly eliminated metabolically-stimulated persisters (Fig. 1c). However, neither the  $\beta$ -lactam ampicillin nor quinolone ofloxacin showed appreciable killing of persisters in the presence or absence of mannitol. This result demonstrates that potentiation is aminoglycoside-specific and that cells were persistent to quinolones and  $\beta$ -lactams. It further suggests that metabolic stimuli under these conditions do not rapidly revert persisters to a growth state in which cell-wall and DNA synthesis are active. To further explore this, we tested growth of persisters on the metabolites used for aminoglycoside potentiation, and observed negligible growth of persisters eight hours after metabolite addition (Supplementary Figs 3 and 4). Taken together, these data suggest that the metabolic stimuli bolster a process specific to aminoglycosides, and do not revert persisters to normally growing cells.

Given the energy dependence of aminoglycoside uptake<sup>16</sup>, we investigated if the metabolic stimuli screened were increasing aminoglycoside uptake. We measured uptake by fluorescently labeling gentamicin with Texas Red and analyzing by FACS. Cells were pre-incubated with metabolites for 30 minutes, prior to five-minute treatment with Gentamicin-Texas Red (Gent-TR) to determine uptake (Fig. 1d and Supplementary Fig. 10). Metabolites that induced substantial aminoglycoside killing were observed to induce high levels of aminoglycoside uptake, implying that increased uptake induced by these metabolites was responsible for aminoglycoside killing. Further, metabolites that caused low potentiation did not significantly increase aminoglycoside uptake.

The requirement of proton-motive force (PMF) for aminoglycoside uptake in exponentially growing bacteria has been studied extensively<sup>16</sup>. Though the complete mechanism of aminoglycoside uptake is unclear, it is known that a threshold PMF is required. We reasoned that, though metabolic stimuli are not rapidly stimulating growth of persisters, they may be promoting PMF, thereby facilitating uptake of and killing by aminoglycosides. To test this hypothesis, we pre-incubated persisters with the proton ionophore carbonyl cyanide *m*-chlorophenyl hydrazone (CCCP), which inhibits PMF, before treating them with metabolites in conjunction with gentamicin. Treatment with CCCP was found to abolish aminoglycoside potentiation by all of the carbon sources, demonstrating that PMF, induced by metabolites, is required for persister elimination (Fig. 2a and Supplementary Fig. 12). We next verified that the requirement for PMF was due to aminoglycoside uptake. We pre-incubated samples with CCCP and performed Gent-TR uptake experiments, and found that inhibiting PMF suppressed metabolite-induced uptake of aminoglycoside (Fig. 2b and Supplementary Fig. 13). Further, using the DiOC<sub>2</sub>(3) membrane stain, we verified that metabolites that induce aminoglycoside uptake and killing were also the ones that elevate PMF (Supplementary Figs 14 and 15). These results demonstrate that specific metabolites induce PMF in persisters, thereby facilitating aminoglycoside uptake and killing.

From these results, we hypothesized that aerobic respiration is primed in persisters and facilitates metabolic potentiation of aminoglycosides. We tested this using genetic knockout strains inactivated for each of the *E. coli* cytochrome quinol oxidases (bo, *cyoA*; bd-I, *cydB*; bd-II, *appB*), as well as potassium cyanide (KCN) to inhibit all cytochromes simultaneously. Wild-type persisters, with and without KCN, and enzymatically-inactivated persisters, were treated for two hours with gentamicin plus metabolites (Fig. 2c and Supplemental Fig. 16). Treatment with KCN abolished killing, consistent with work in rapidly growing bacteria<sup>17</sup>, demonstrating the necessity of aerobic respiration for aminoglycoside elimination of persisters under these conditions. The *cydB* strain, which lacks activity of the microaerobic cytochrome bd-I<sup>18, 19</sup>, suppressed killing by over two orders of magnitude, possibly as a result of its use in the oxygen-depleted and alkaline stationary phase cultures. Neither *cyoA* nor *appB* showed a significant effect. Though we found aerobic respiration was required for eradication in aerated conditions, we also found that metabolite-enabled eradication occurs anaerobically in conditions that support PMF (Supplementary Figs 17 and 18).

As aerobic respiration in *E. coli* is driven by NADH oxidation, we investigated the role of NADH utilization in this phenotype. Persister cells inactivated for NADH dehydrogenase I (*nuoI*), NADH dehydrogenase II (*ndh*), and both NADH dehydrogenases (*ndh nuoI*), were treated for two hours with gentamicin plus metabolites (Fig. 2d and Supplementary Fig. 19). We found that NADH dehydrogenase activity was important to this phenotype as gentamicin activity against the *ndh nuoI* strain was not potentiated by mannitol, fructose, or pyruvate, though there was slight potentiation by glucose (Supplementary Fig. 19a). Given that NADH drives electron transport, this requirement for NADH is not surprising though we found it is not essential for killing under all conditions (Supplementary Figs 18 and 20). Though both *ndh* and *nuoI* suppressed killing, the *nuoI* strain had a greater effect, possibly reflecting its direct contribution to PMF. Using a series of genetic

knockouts, we further determined that the enzyme pyruvate dehydrogenase was necessary for the observed phenotype, due to its NADH generation, whereas the PPP, EDP, and TCA cycle were not found to be necessary (Supplementary Figs 21-24).

These results demonstrate that persisters are primed for specific biochemical processes, including central metabolism, that allow PMF induction. This resumption of central metabolism and respiration in persisters, however, is not sufficient in the time-scales examined to support other processes necessary for cellular growth, such as cell-wall biogenesis and DNA replication. Thus, persisters treated with specific metabolites appear to be in an energized but non-dividing state that facilitates their elimination by aminoglycosides. On the basis of these findings, we propose the following mechanism for metabolite-enabled eradication of persisters by aminoglycosides (Fig. 3a). Certain metabolites—glucose, mannitol, fructose, and pyruvate—are transported to the cytoplasm, some by their specific PTS enzymes, and enter glycolysis, where their catabolism generates NADH. NADH is oxidized by enzymes in the electron transport chain, which in turn contribute to PMF. The elevated PMF facilitates the uptake of aminoglycosides which bind to the ribosome causing mistranslation-induced cell death.

We next investigated if this mechanism was applicable to clinically relevant cases, such as bacterial biofilms. We reasoned that metabolic stimulation might facilitate aminoglycoside elimination of biofilm persisters. To test this hypothesis, we grew *E. coli* biofilms, and treated them for four hours with ofloxacin, mannitol, gentamicin, and mannitol plus gentamicin (Fig. 3b). Ofloxacin (which is efficient against Gram-negative biofilms<sup>15, 20</sup>) reduced biofilm viability by almost two orders of magnitude, suggesting that greater than 1% of the biofilms were persisters. Mannitol and gentamicin in combination reduced biofilm viability by over 4 orders of magnitude, demonstrating a reduction of biofilm persisters by 2.5 orders of magnitude. We also tested the ability of fructose to induce biofilm elimination and observed similar results (Fig. 3c).

To determine the clinical relevance of metabolic potentiation of aminoglycosides *in vivo*, we tested the ability of mannitol in combination with gentamicin to treat chronic, biofilm-associated infection in a mouse model. Mice had catheters colonized with uropathogenic *E. coli* biofilms implanted in their urinary tracts (Fig. 3d). Two days after surgery, mice received no treatment or intravenous treatment with gentamicin or gentamicin and mannitol for three days, after which the catheters were removed and biofilm viability was determined. Gentamicin alone had no effect, whereas gentamicin in combination with mannitol reduced the viability of the catheter biofilms by nearly 1.5 orders of magnitude (Fig. 3d). We also found that treatment with gentamicin and mannitol inhibited the spread of bacterial infection to the kidneys, as compared to treatment with gentamicin alone and the no treatment control (Supplementary Fig. 27). These *in vivo* results demonstrate the feasibility of our approach for clinical use.

Having demonstrated that certain metabolites can enable aminoglycoside activity in Gram-negative (*E. coli*) bacterial persisters and biofilms, we sought to determine whether a similar phenomenon existed in Gram-positive bacteria. Persisters of the Gram-positive pathogen *Staphylococcus aureus* were treated with gentamicin in conjunction with metabolites. After

an initial hour of no killing, gentamicin with fructose rapidly eliminated persistent *S. aureus* (Fig. 4a). Curiously, mannitol, glucose, and pyruvate, which showed strong potentiation against *E. coli* persisters, showed little potentiation in *S. aureus*. Using expression analysis of *S. aureus* microarrays, we present data suggesting this lack of potentiation results from differential expression of metabolite transporters (Supplementary Table 3). We next tested whether fructose-enabled killing of *S. aureus* was unique to aminoglycosides or general to other classes of bactericidal antibiotics. As with *E. coli*, we found that metabolite-enabled killing of *S. aureus* persisters was aminoglycoside-specific (Fig. 4b), suggesting that *S. aureus* persisters were not reverting to normally growing cells.

Given that aminoglycoside activity in growing *S. aureus* is dependent on PMF<sup>21,22</sup>, we tested whether persister elimination mediated by fructose required PMF. We found that the potentiation of aminoglycoside by fructose in *S. aureus*, as in *E. coli*, requires PMF generation (Fig. 4c), suggesting that the PMF-requiring mechanism of aminoglycoside persister elimination exists in both Gram-negative and Gram-positive bacteria. We also investigated if gentamicin with fructose could be used to treat *S. aureus* biofilms. We found that the viability of *S. aureus* biofilms was reduced by nearly 1.5 orders of magnitude when treated for four hours with fructose and gentamicin (Fig. 4d).

Here we established a metabolic-based approach for eradicating persisters, one effective against both Gram-negative and Gram-positive bacteria. The metabolite-mediated potentiation proceeds by PMF generation, which we found is necessary for aminoglycoside uptake and killing in persisters. This work adds to a growing understanding of the role played by metabolism in killing by bactericidal antibiotics<sup>13,23,24</sup> and broadens our understanding of persister physiology. Moreover, our findings imply the benefit of delivering PMF-stimulating metabolites as adjuvants to aminoglycosides in the treatment of chronic bacterial infections.

## Methods Summary

In all experiments, bacterial cells were cultured in 25mL Luria-Bertani broth (LB) for 16 hours at 37°C, 300RPM, and 80% humidity in 250mL flasks. Unless otherwise noted, the following concentrations were used: 10 µg/mL gentamicin, 100 µg/mL ampicillin, 5 µg/mL ofloxacin, 20 µM CCCP, 1 mM KCN. The concentration of all carbon sources added to potentiate aminoglycosides was normalized to deliver 60 mM carbon (e.g., 10 mM glucose, 20 mM pyruvate, etc.). *E. coli* (K12 EMG2) and *S. aureus* (ATCC 25923) were the two parent strains used in this study. Knockouts (Supplementary Table 1 and 2) were constructed by P1-phage transduction from the Keio knockout collection. In *E. coli*, non-persister stationary phase cells were killed by treatment with 5 µg/mL ofloxacin for 4 hours<sup>25, 26</sup>. Samples were then washed with phosphate buffered saline (PBS) and suspended in M9 salts with carbon source and antibiotic to determine metabolite-enabled killing of persisters. At specified time points, 10 µL aliquots of samples were removed, serially diluted, and spot-plated onto LB agar plates to determine colony forming units/mL (CFU/mL) and survival. Gent-TR was made as previously described<sup>27</sup>. Aminoglycoside uptake was measured by incubating stationary phase samples with 10 µg/mL Gent-TR for 5 minutes at 37°C, 300RPM, and 80% humidity. 100 µL of each sample was then washed and resuspended in

PBS and analyzed on a BD FACS Aria II flow cytometer. Biofilm survival assays were performed as previously described<sup>28</sup>. Raw microarray data for *S. aureus* were downloaded from the Gene Expression Omnibus (GEO) series GSE20973<sup>29</sup> and processed with RMA express using background adjustment, quantile normalization, and median polish summarization to compute RMA expression values<sup>30</sup>. Mouse experiments were performed with female Charles River Balb/C mice in collaboration with ViviSource Laboratories and conformed to the ViviSource IACUC policies and Procedural Guidelines.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

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## Appendix

### Methods

#### Antibiotics and Chemicals

The following concentrations of antibiotics were used in this study: 10 µg/mL gentamicin, 30 µg/mL kanamycin, 50 µg/mL streptomycin, 5 µg/mL ofloxacin, 100 µg/mL ampicillin, 40 µg/mL tetracycline, 50 µg/mL chloramphenicol, and 100 µg/mL spectinomycin. 20 µM of carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used in experiments to suppress proton-motive force. 1 mM potassium cyanide (KCN) was used to inhibit aerobic respiration. Stock solutions were made by suspending CCCP in DMSO at 500 µM stored at 4°C. All antibiotics and chemicals were purchased from Sigma and Fisher.

#### Media and Growth Conditions

All stationary phase samples were prepared in the following way: cells from frozen stock were grown at 37°C, 300RPM, and 80% humidity in Luria-Bertani (LB) broth to an optical density (OD<sub>600</sub>) of 0.3. Cells were then diluted 1:1000 in 25mL LB and grown for 16 hours at 37°C, 300RPM, and 80% humidity in 250mL flasks. This experimental set-up ensured that, regardless of the conditions used in assays, the initial population of persisters or stationary phase cells was prepared in a uniform manner consistent with previous work<sup>25, 26</sup>.

The following concentrations of carbon sources were used in this study: 10 mM glucose, 10 mM mannitol, 10 mM fructose, 20 mM glycerol, 30 mM glycolate, 10 mM galactarate, 20 mM pyruvate, 10 mM gluconate, 12 mM arabinose, and 12 mM ribose. 10 mM acetate was used as a supplement and 30 mM acetate was used as potentiating metabolite as a control. Carbon sources were purchased from Sigma or Fisher. In *E. coli* persister assays, samples were resuspended in M9 minimal media salts plus carbon source. In all experiments using CCCP, samples were pretreated for 5 minutes with the proton ionophore before antibiotic addition.

## Strains

*Escherichia coli* (K12 EMG2) and *Staphylococcus aureus* (ATCC 25923) were the two parent strains used in this study. All *E. coli* knockouts used in this study (see Supplementary Table 2) were transduced into EMG2 strain from strains in the KEIO knock-out collection using the P1 phage method. All strains were cured prior to assays using pCP20 and standard lab procedures.

## Persister Assays

For *E. coli* persister assays, samples were grown to stationary phase as described above. Cultures were then treated for 4 hours with 5 µg/mL ofloxacin at the above stated growth conditions. Previous work has demonstrated that treatment under these conditions for 3 hours eliminates all susceptible non-persister cells<sup>25</sup>. We verified that remaining cells were persisters by increasing the amount of added ofloxacin to 20 µg/mL and noted no further decrease in viability (see Supplementary Fig. 1). Persistence was further verified by demonstrating that none of the bactericidal antibiotics used in this study caused killing of cells when added without carbon source (see Fig. 1c). Samples were then washed with 10mL 1X filtered PBS and re-suspended in M9 minimal media. Carbon sources and antibiotics were added, and samples were incubated at 37°C, 300RPM, and 80% humidity. Resuspension of samples in defined minimal media allowed us to precisely and specifically test the effect of different carbon sources on persister viability, without the possible confounding factors that a more complex media would present. For *S. aureus* persister experiments, cells were grown at 37°C and 300RPM in LB broth to an OD600 of 0.3. Cells were then diluted 1:1000 in 25mL LB and grown for 16 hours at 37°C and 300RPM in 250mL flasks. Cultures were treated with carbon source and antibiotic. Previous work has shown that, approximately all, stationary phase *S. aureus* cells are persistent<sup>25</sup>.

At specified time points, 10 µL aliquots of samples were removed, serially diluted, and spot-plated onto LB agar plates to determine colony forming units/mL (CFU/mL). Only dilutions that yielded between 10-100 colonies were counted. Survival was determined by dividing a replicate's CFU/mL at each time point by the initial CFU/mL for the replicate.

For persister resuscitation experiments (Supplementary Fig. 5), cells that had been treated with ofloxacin, washed and re-suspended in M9 were then diluted 1:100 in M9 plus carbon source and incubated at 37°C, 300RPM, and 80% humidity.

## Gentamicin Texas-Red Uptake

Gentamicin Texas-Red (Gent-TR) was made as previously described<sup>27</sup>. At 4°C, 1 mg of Texas Red (Invitrogen) was dissolved in 50 µL of high-quality anhydrous N,N-dimethylformamide. The dissolved Texas Red was slowly added to solution to 2.3 mL of a 10 mg/mL gentamicin in a 100 mM K<sub>2</sub>CO<sub>3</sub> solution at 4°C.

Uptake induced by carbon sources in stationary phase cells was determined by adding concentrated carbon sources (see **Media and Growth Conditions** for concentrations) to stationary phase cultures and incubating them for 30 minutes at 37°C, 300RPM, and 80% humidity. Concentrated Gent-TR was then added to samples to a final concentration of 10

$\mu\text{g/mL}$  and samples were incubated for 5 minutes. 100  $\mu\text{L}$  of each sample was then washed with 1 mL of phosphate buffered saline (PBS) and resuspended in 1 mL of PBS. 200  $\mu\text{L}$  of the resuspended sample was then added to 800  $\mu\text{L}$  of PBS in flow tubes. Samples were analyzed on a BD FACS Aria II flow cytometer with the following settings: mCherry voltage, 650; FSC threshold, 1,000; recorded events, 100,000; and gated-out mCherry events, <30.

### Membrane Potential Measurements

We used the *BacLight*<sup>TM</sup> Bacterial Membrane Potential Kit (B34950, Invitrogen) to assess changes in proton-motive force induced by metabolites. Membrane potential induced by carbon sources in stationary phase cells was determined by adding concentrated carbon sources (see **Media and Growth Conditions** for concentrations) and 10  $\mu\text{L}$  DiOC<sub>2</sub>(3) (membrane stain) to stationary phase cultures, followed by incubation for 30 minutes at 37°C, 300RPM, and 80% humidity. 10  $\mu\text{L}$  of culture was added to 1 mL of PBS in flow tubes immediately before analyzing. Samples were analyzed on a BD FACS Aria II flow cytometer with settings optimized according to the *BacLight*<sup>TM</sup> kit manual. Settings used were: FITC voltage, 250; mCherry voltage, 650; FSC threshold, 1,000; recorded events, 100,000; and gated-out FSC and SSC outliers before data acquisition. The red/green (mCherry/FITC) values for each cell were determined and normalized, then compared to samples without metabolite to determine relative PMF change.

### Anaerobic Experiments

*E. coli* were grown to an optical density (OD<sub>600</sub>) of 0.3, and then diluted 1:1000 in 25mL anaerobic LB with 10 mM NaNO<sub>3</sub> and grown for 16 hours at 37°C, 200RPM, 1.5-2.0% hydrogen, <50 ppm oxygen in 250mL flasks. Cultures were then treated with carbon source and metabolite in the presence and absence of an additional 10 mM NaNO<sub>3</sub>. The additional NaNO<sub>3</sub> was added to determine if increasing the concentration of the terminal electron acceptor could increase aminoglycoside potentiation.

### Biofilm Assay

Overnight cultures grown in LB were diluted 1:200 into pre-warmed LB which was then added to MBEC plates (Innovotech) at 150  $\mu\text{L}$  per well. Plates were incubated at 35°C, 150 RPM for 24 hours, then pegs were washed in a microtiter plate with 200  $\mu\text{L}$  1X PBS per well. Pegs were then added to a microtiter plate containing 200  $\mu\text{L}$  M9 minimal salts (for *E. coli*) or sterofiltered, stationary phase media (for *S. aureus*) plus carbon source and antibiotic. Plates were incubated at 35°C, 150 RPM for 4 hours, then pegs were washed twice in microtiter plates with 200  $\mu\text{L}$  1X PBS per well. To dislodge biofilm cells, pegs were placed in a microtiter plate with 145  $\mu\text{L}$  1X PBS per well and sonicated in water bath for 30 minutes at 40 kHz. Serial dilutions and spot plating were performed to determine viable CFU/peg. For determination of the dependence of *E. coli* biofilm elimination on pH, we carried out the above procedure in M9 salts buffered to appropriate pH with citric acid as opposed to KH<sub>2</sub>PO<sub>4</sub>, which is typically used for M9.



## Mouse Chronic Urinary Tract Infection Assay

Female Charles River Balb/C mice (weighing 22-26 g) received surgical implantation in the urinary tract of 6 mm PE50 catheter tubing that had been incubated in cultures of uropathogenic *E. coli* for 24 hours to form biofilms. 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. Seven to eight mice were included in each group. Twenty-four hours after the last treatment, catheter tubing was extracted to determine biofilm viability and kidneys were removed to determine bacterial load. Mouse materials were provided by ViviSource Laboratories, a facility approved by the U.S. Department of Agriculture and by the Office of Laboratory Animal Welfare, where all *in vivo* experimental work was performed. The study conformed with the ViviSource IACUC policies and Procedural Guidelines.

## *S. aureus* Microarray Analysis

Raw microarray data (.CEL files) for two exponential (GSM524189, GSM524193) and two stationary phase (GSM524362, GSM524363) *S. aureus* cultures were downloaded from the Gene Expression Omnibus (GEO) series GSE20973<sup>29</sup>. The data were processed with RMA express using background adjustment, quantile normalization, and median polish summarization to compute RMA expression values<sup>30</sup>. Mean expression values were calculated for both exponential and stationary phase data, and the relative fold changes (stationary/exponential) are reported in Supplementary Table 3.

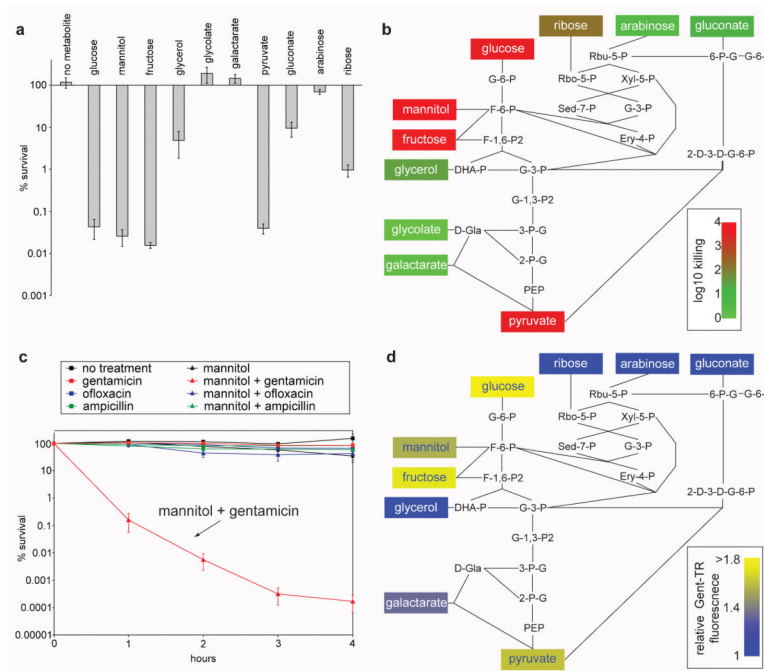
## Software

MATLAB (Mathworks, Natick, MA) was used for processing flow cytometric data, analyzing microarray data, and generating scaled heat maps using the `imagesc()` function. Microsoft Excel was used to plot survival assays. All figures were formatted with Adobe Illustrator.

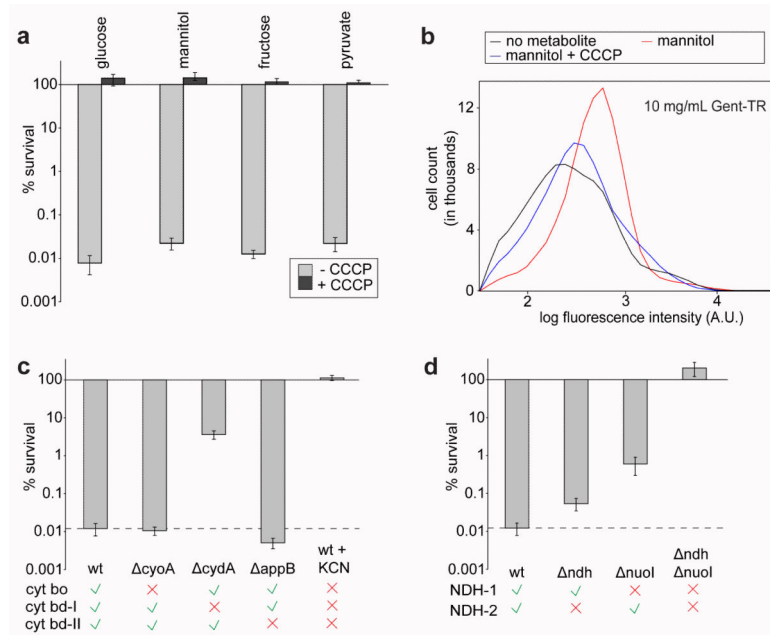
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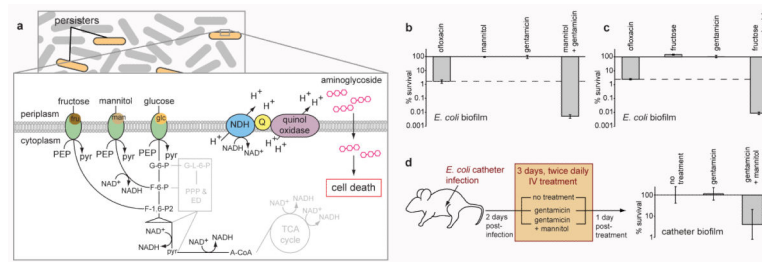


**Figure 1. Specific metabolites enable aminoglycoside killing of *E. coli* persisters**  
**a**, Survival of persisters after 2-hour treatment with gentamicin and respective metabolite. **b**, Metabolite-induced persister elimination superimposed on metabolic network. **c**, Survival of persisters after the following treatments: no treatment (black squares), mannitol (black triangles), gentamicin (red squares), gentamicin and mannitol (red triangles), ofloxacin (blue squares), ofloxacin and mannitol (blue triangles), ampicillin (green squares), or ampicillin and mannitol (green triangles). **d**, Metabolite-induced Gent-TR uptake by stationary phase cells superimposed on metabolic network (see also Supplementary Fig. 10). Mean  $\pm$  s.e.m. are presented (n = 3).



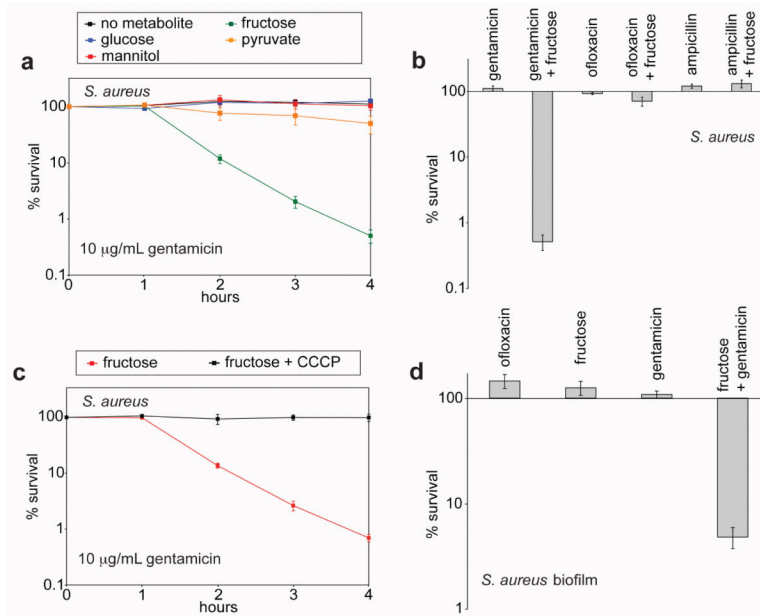
**Figure 2. Metabolite-enabled aminoglycoside uptake and killing requires PMF produced by oxidative electron transport chain**

**a**, Survival of persisters after treatment with gentamicin plus uptake-potentiating metabolites with (dark grey bars) and without CCCP (light grey bars). **b**, Representative uptake measurement of Gent-TR by stationary phase cells after incubation with no sugar (black lines), mannitol (red lines), or mannitol and CCCP (blue lines) (see also Supplementary Fig. 13). **c**, Survival of persisters in cytochrome-inactivated strains after treatment with gentamicin plus mannitol (see also Supplementary Fig. 16). **d**, Percent survival of persisters in NADH-dehydrogenase-inactivated strains after treatment with gentamicin plus mannitol (see also Supplementary Fig. 19). Presence (green checks) and absence (red X's) of functional complexes is indicated below test conditions. Mean  $\pm$  s.e.m. are presented (n = 3).



**Figure 3. Mechanism for metabolite-enabled eradication of persisters and clinically relevant experiments**

**a**, Metabolite-enabled persister eradication proceeds through catabolism of carbon sources thereby generating NADH, the production of which does not require the PPP, EDP, or TCA cycle. The electron transport chain oxidizes NADH and contributes to PMF, which facilitates aminoglycoside uptake and killing of persisters. **b**, Survival of *E. coli* biofilms after treatment with ofloxacin, mannitol, gentamicin, or mannitol plus gentamicin. As quinolones have high efficacy against Gram-negative biofilms compared to other antibiotics<sup>15, 20</sup>, ofloxacin was used as a benchmark for high biofilm killing. **c**, Survival of *E. coli* biofilms after treatment with ofloxacin, fructose, gentamicin, or fructose plus gentamicin. **d**, Schematic of *in vivo* experiments in mice (left). Survival of *E. coli* biofilms on urinary-tract-inserted catheters after treatment with gentamicin (1 mg/Kg) or mannitol (1.5 g/Kg) and gentamicin (1 mg/Kg) (right). Mean  $\pm$  s.e.m. are presented (n = 3).



**Figure 4. Fructose induces PMF-dependent aminoglycoside killing of *S. aureus* persisters**  
**a**, Survival of *S. aureus* persisters after treatment with gentamicin plus no metabolite (black squares), glucose (blue squares), mannitol (red squares), fructose (green squares), or pyruvate (orange squares). **b**, Survival of *S. aureus* persisters after 4-hour treatment with gentamicin, gentamicin and fructose, ofloxacin, ofloxacin and fructose, ampicillin, or ampicillin and fructose. **c**, Survival of *S. aureus* persisters after 4-hour treatment with gentamicin and fructose with (dark grey bars) or without CCCP (light grey bars). **d**, Survival of *S. aureus* biofilms after 4-hour treatment with ofloxacin, fructose, gentamicin, or fructose plus gentamicin. Mean  $\pm$  s.e.m. are presented (n = 3).