LETTER

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Metabolite-enabled eradication of bacterial persisters by aminoglycosides

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

Translation occurs at a reduced rate in bacterial persisters[2,8], indicating that they should be susceptible to aminoglycosides, which are ribosome-targeting bactericidal antibiotics[9–13]. However, aminoglycosides have weak activity against dormant bacteria, despite continued translation in these cells[14,15]. Given the dormancy of persisters and the known energy requirement for aminoglycoside activity[16], we reasoned that metabolic stimulation might potentiate aminoglycosides against bacterial persisters.

To test this, we screened metabolites for their ability to potentiate aminoglycosides against *E. coli* persisters. We selected carbon sources to maximize coverage of glycolysis, the pentose-phosphate pathway (PPP) and the Entner-Doudoroff pathway (EDP) (Fig. 1a, b). Persisters were isolated (see Methods), re-suspended in minimal media supplemented with individual metabolites and then treated for 2 h with the aminoglycoside gentamicin.

We found that gentamicin was significantly potentiated against persisters by specific metabolic stimuli (Fig. 1a, b). Metabolites that enter upper glycolysis (glucose, mannitol and fructose), as well as pyruvate, induced rapid killing of persisters by gentamicin, reducing persister viability by three orders of magnitude. In contrast, metabolites that enter lower glycolysis (except for 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 the treated cells were persistent to gentamicin in the absence of an added metabolite. We verified that the metabolite-enabled eradication of persisters was general to the aminoglycoside class of antibiotics by testing kanamycin and streptomycin (Supplementary Fig. 2).

We considered that the potentiating metabolites might cause persisters to revert to normally growing cells, which would render them susceptible to quinolone (DNA-damaging) and β-lactam (cell-wall-inhibiting) 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 β-lactams. As seen in the metabolite screen, gentamicin rapidly eliminated metabolically stimulated persisters (Fig. 1c). However, neither the β-lactam ampicillin nor the quinolone ofloxacin showed appreciable killing of persisters in either the presence or the absence of mannitol. This shows that potentiation is aminoglycoside-specific and that cells were persistent to quinolones and β-lactams. It further indicates that metabolic stimuli under these conditions do not rapidly revert persisters to a growth state in which cell-wall synthesis and DNA synthesis are active. To explore this further, we tested the growth of persisters on the metabolites used for aminoglycoside potentiation and observed negligible growth of persisters 8 h after metabolite addition (Supplementary Figs 3 and 4). Taken together, these data indicate that the metabolic stimuli bolster a process that is specific to aminoglycosides and do not cause persisters to revert to normally growing cells.

Given that aminoglycoside uptake is energy-dependent[16], we investigated whether the metabolic stimuli screened here could increase aminoglycoside uptake. We measured uptake by fluorescently labelling gentamicin with Texas red and analysing cells by fluorescence-activated cell sorting (FACS). Cells were pre-incubated with metabolites for 30 min before a 5-min treatment with gentamicin-Texas red (Fig. 1d and Supplementary Fig. 10). Metabolites that induced substantial killing by aminoglycosides also induced high levels of aminoglycoside uptake, implying that the increased uptake was responsible for the killing. Furthermore, 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[16]. Although the complete mechanism of aminoglycoside uptake is unclear, it is known that a threshold PMF is required. We reasoned that, although metabolic stimuli do not rapidly stimulate the growth of persisters, they may promote PMF, thereby facilitating the uptake of aminoglycosides and subsequent bacterial killing. 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 gentamicin-Texas red uptake experiments, showing that inhibiting PMF suppressed the metabolite-induced uptake of aminoglycoside (Fig. 2b and Supplementary Fig. 13). Furthermore, using the membrane stain 3,3′-diethyloxacarbocyanine iodide ($DiOC_2(3)$), we verified that metabolites that induced aminoglycoside uptake and bacterial killing were also the ones that induced an elevated

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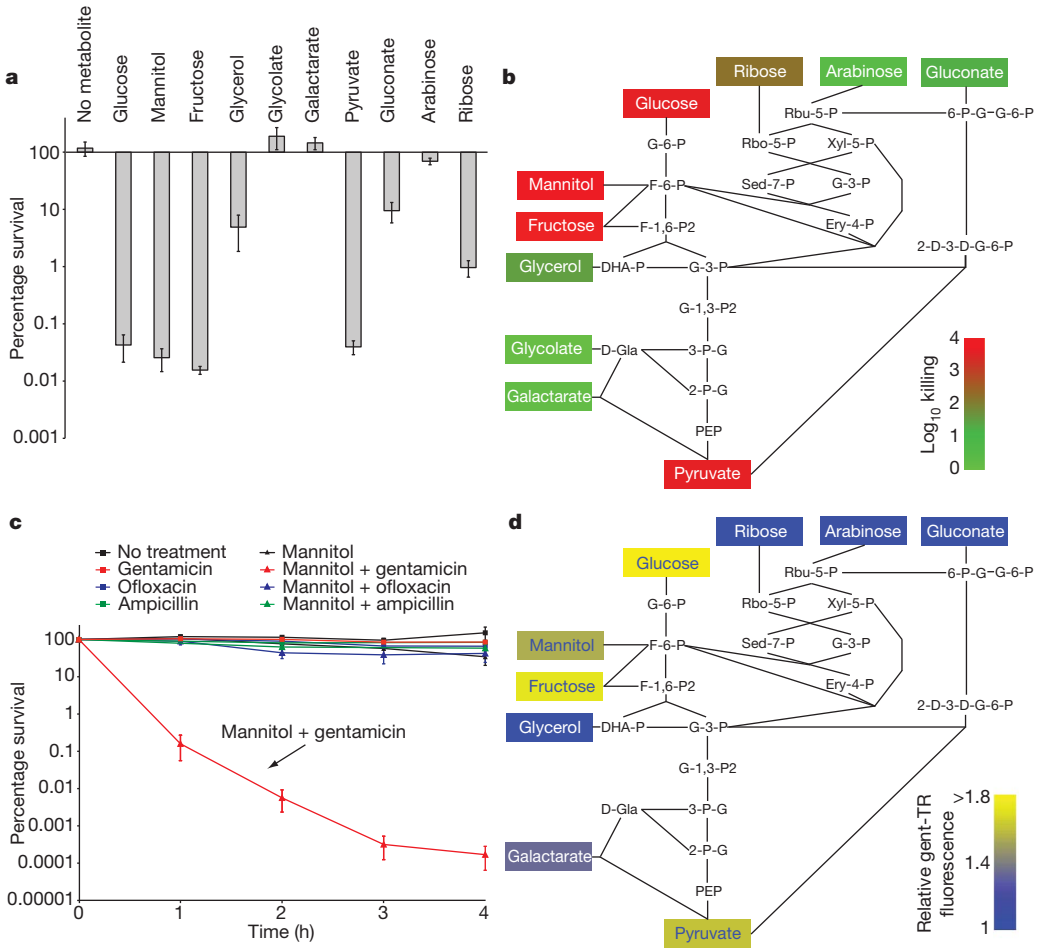

Figure 1 | Specific metabolites enable killing of *E. coli* persisters by aminoglycosides. **a**, Percentage survival of persisters after 2-h treatment with gentamicin and metabolites. **b**, Efficiency of metabolite-enabled elimination of persisters, superimposed on a metabolic network. Metabolites are colour-coded according the aminoglycoside killing which they induce ($\log_{10}$ killing). The following metabolic intermediates are depicted: glucose-6-phosphate (G-6-P), fructose-6-phosphate (F-6-P), fructose-1,6-bisphosphate (F-1,6-P2), dihydroxyacetone phosphate (DHAP), glyceraldehyde-3-phosphate (G-3-P), 1,3-bisphospho-glycerate (G-1,3-P2), 3-phospho-glycerate (3-P-G), 2-phospho-glycerate (2-P-G), phosphoenolpyruvate (PEP), D-glycerate (D-Gla), ribulose-5-phosphate (Rbu-5-P), ribose-5-phosphate (Rbo-5-P), xylulose-5-phosphate (Xyl-5-P), glyceraldehyde-3-phosphate (G-3-P), erythrose-4-phosphate (Ery-4-P), sedoheptulose-7-phosphate (Sed-7-P), 6-phospho-gluconate (6-P-G) and 2-dehydro-3-deoxy-gluconate-6-phosphate (2-D-3-D-G-6-P). **c**, Percentage survival of persisters after treatment with mannitol and different classes of antibiotics. **d**, Metabolite-enabled uptake of gentamicin-Texas red (gent-TR) by stationary-phase cells, superimposed on a metabolic network (see also Supplementary Fig. 10). Metabolites are colour-coded according to the relative uptake that they induce. Means ± s.e.m. are presented in **a** and **c** ($n \geq 3$).

PMF (Supplementary Figs 14 and 15). These results demonstrate that specific metabolites induce PMF in persisters, thereby facilitating aminoglycoside uptake and bacterial killing.

From these results, we hypothesized that aerobic respiration is primed in persisters and that it facilitates the metabolic potentiation of aminoglycosides. We tested this using genetic knockout strains inactivated for each of the *E. coli* cytochrome quinol oxidases (bo, Δ*cyoA*; bd-I, Δ*cydB*; bd-II, Δ*appB*). We also used potassium cyanide to inhibit all cytochromes simultaneously. Wild-type persisters, with and without potassium cyanide, and enzymatically inactivated persisters were treated for 2 h with gentamicin plus metabolites (Fig. 2c and Supplementary Fig. 16). Treatment with potassium cyanide abolished bacterial killing, consistent with studies of rapidly growing bacteria[17], demonstrating the necessity of aerobic respiration for elimination of persisters by aminoglycosides under these conditions. The Δ*cydB* mutation, which abolishes activity of the microaerobic cytochrome bd-I[18,19], suppressed killing by more than two orders of magnitude, possibly owing to the use of cytochrome bd-I in oxygen-depleted and alkaline stationary-phase cultures. Neither Δ*cyoA* nor Δ*appB* showed a significant effect on bacterial killing. Although we found that aerobic respiration was required for eradication of bacteria in aerated conditions, we also found that metabolite-enabled eradication occurs anaerobically in conditions that support PMF (Supplementary Figs 17 and 18).

Because 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 (Δ*nuoI*), NADH dehydrogenase II (Δ*ndh*) and both NADH dehydrogenases (Δ*ndh*Δ*nuoI*) were treated for 2 h with gentamicin plus metabolites (Fig. 2d and Supplementary Fig. 19). We found that NADH dehydrogenase activity was important for this phenotype because gentamicin activity against the Δ*ndh*Δ*nuoI* strain was not potentiated by mannitol, fructose or pyruvate, although there was slight potentiation by glucose (Supplementary Fig. 19a). Given that NADH drives electron transport, this requirement for NADH is not surprising, although we found that it is not essential for killing under all conditions (Supplementary Figs 18 and 20). Both the *ndh* and *nuoI* deletions suppressed killing but the Δ*nuoI* strain was the more resistant of the two, possibly reflecting the direct contribution of NADH dehydrogenase I to PMF. Using a series of genetic knockouts, we further determined that the enzyme pyruvate dehydrogenase was necessary for the observed phenotype, owing to its generation of NADH, whereas the PPP, EDP and TCA cycle were not necessary (Supplementary Figs 21–24).

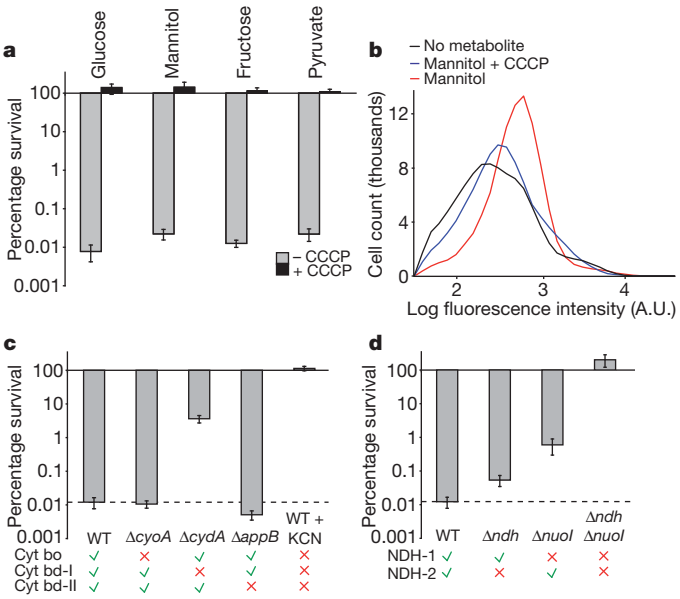

Figure 2 | Metabolite-enabled aminoglycoside uptake and bacterial killing requires PMF produced by the oxidative electron transport chain. **a**, Percentage survival of persisters after treatment with gentamicin plus uptake-potentiating metabolites with CCCP (dark grey bars) and without CCCP (light grey bars). **b**, Representative measurement of gentamicin-Texas red uptake by stationary-phase cells after incubation with no metabolite, mannitol or mannitol and CCCP (see also Supplementary Fig. 13). A.U., arbitrary units. **c**, Percentage survival of persisters in wild-type (WT) and cytochrome-inactivated strains after treatment with gentamicin plus mannitol (see also Supplementary Fig. 16). The presence (green ticks) or absence (red crosses) of functional complexes is indicated below test conditions. **d**, Percentage survival of persisters in wild-type and NADH-dehydrogenase-inactivated strains after treatment with gentamicin plus mannitol (see also Supplementary Fig. 19). The presence or absence of functional complexes is indicated as in **c**. Means ± s.e.m. are presented throughout ($n \geq 3$).

These results show that persisters are primed for specific biochemical processes that allow PMF induction, including central metabolism. However, in the timescales examined here, this resumption of central metabolism and respiration was not sufficient to support other processes necessary for cellular growth, such as cell-wall biogenesis and DNA replication. Thus, persisters treated with specific metabolites seem 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 phosphotransferase system enzymes; they enter glycolysis, where their catabolism generates NADH, and this NADH is then oxidized by enzymes in the electron transport chain, which contribute to PMF. The elevated PMF facilitates the uptake of aminoglycosides, which bind to the ribosome and cause cell death via mistranslation.

We next investigated whether this mechanism was applicable to clinically relevant cases, such as bacterial biofilms. We reasoned that metabolic stimulation might facilitate the elimination of biofilm persisters by aminoglycosides. To test this hypothesis, we grew *E. coli* biofilms and treated them for 4 h with ofloxacin, mannitol, gentamicin and mannitol plus gentamicin (Fig. 3b). Ofloxacin, which is efficient against Gram-negative biofilms[15,20], reduced biofilm viability by almost two orders of magnitude, indicating that more than 1% of the bacteria in the biofilms were persisters. Mannitol and gentamicin, in combination, reduced biofilm viability by more than four orders of magnitude, representing a reduction in biofilm persisters of 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 gentamicin in combination with mannitol to treat chronic, biofilm-associated infection in a mouse model. Catheters colonized with uropathogenic *E. coli* biofilms were implanted in the urinary tracts of mice (Fig. 3d). Two days after surgery, mice received either no treatment or intravenous treatment with gentamicin or gentamicin and mannitol for 3 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,

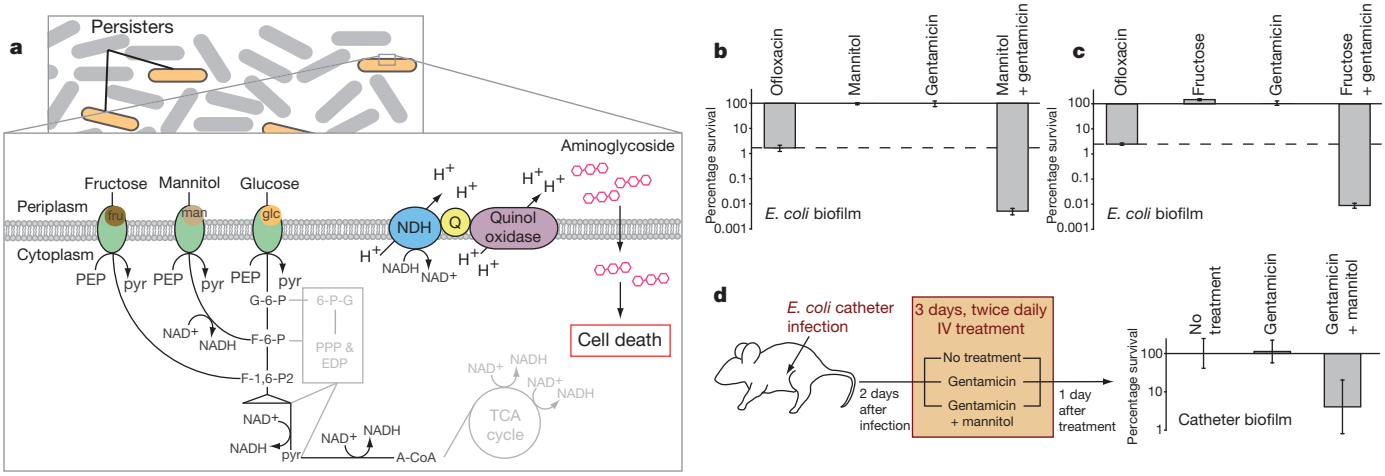

Figure 3 | Mechanism for metabolite-enabled eradication of persisters (a) **and clinically relevant experiments** (b–d)**.** **a**, Metabolite-enabled eradication of persisters proceeds through catabolism of carbon sources, thereby generating NADH, the production of which does not require the PPP, EDP or TCA cycle. The electron transport chain oxidizes NADH and contributes to PMF, which facilitates aminoglycoside uptake and the killing of persisters. The quinones (Q), NADH dehydrogenases (NDH) and central metabolism, including pyruvate (pyr) and acetyl-CoA (A-CoA), are depicted. **b**, Percentage survival of *E. coli* biofilms after treatment with ofloxacin, mannitol, gentamicin or mannitol plus gentamicin. Because quinolones have high efficacy against Gram-negative biofilms, as compared to other antibiotics[15,20], ofloxacin was used as a benchmark for high biofilm killing. **c**, Percentage survival of *E. coli* biofilms after treatment with ofloxacin, fructose, gentamicin or fructose plus gentamicin. **d**, Schematic of *in vivo* experiments in mice (left panel). The right panel shows survival of *E. coli* biofilms on urinary-tract-inserted catheters after treatment with gentamicin (1 mg kg^{-1}) or mannitol (1.5 g kg^{-1}) plus gentamicin (1 mg kg^{-1}). Means ± s.e.m. are presented throughout ($n \geq 3$).

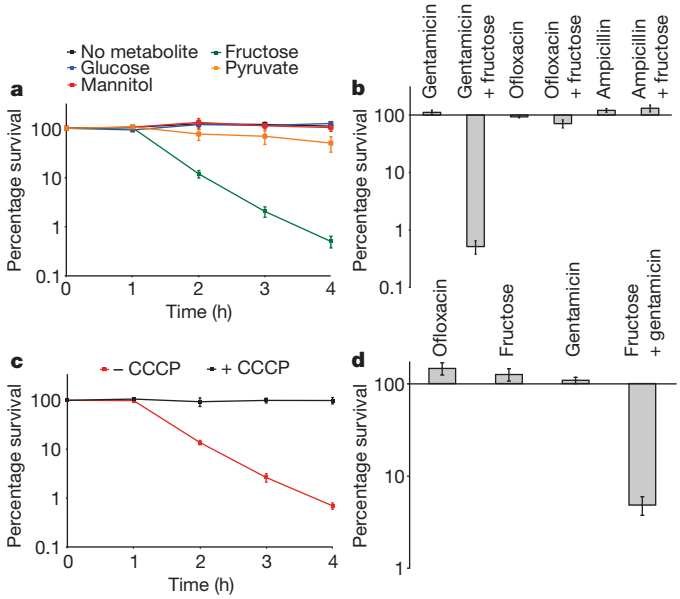

Figure 4 | Fructose induces PMF-dependent killing of *S. aureus* persisters by an aminoglycoside. **a**, Percentage survival of *S. aureus* persisters after treatment with gentamicin and different metabolites. **b**, Percentage survival of *S. aureus* persisters after 4-h treatment with fructose and different classes of antibiotics. **c**, Percentage survival of *S. aureus* persisters after 4-h treatment with gentamicin and fructose, with or without CCCP. **d**, Percentage survival of *S. aureus* biofilms after 4-h treatment with ofloxacin, fructose, gentamicin or fructose plus gentamicin. Means ± s.e.m. are presented throughout ($n \geq 3$).

as compared to treatment with gentamicin alone and to the untreated control (Supplementary Fig. 27). These *in vivo* results demonstrate the feasibility of our approach for clinical use.

Having shown 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 *S. aureus* were treated with gentamicin in conjunction with metabolites. After an initial hour in which no killing was seen, gentamicin with fructose rapidly eliminated persistent *S. aureus* (Fig. 4a). Interestingly, mannitol, glucose and pyruvate, which showed strong potentiation against *E. coli* persisters, showed little potentiation against *S. aureus*. Expression analysis using *S. aureus* microarrays indicated that this lack of potentiation results from differential expression of metabolite transporters in growing versus dormant *S. aureus* (Supplementary Table 3). We next tested whether the 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 specific to aminoglycosides (Fig. 4b), indicating that *S. aureus* persisters did not revert to normally growing cells when treated with metabolites.

Given that the activity of aminoglycosides in growing *S. aureus* is dependent on PMF[21,22], we tested whether the elimination of persisters mediated by fructose also required PMF. We found that the potentiation of aminoglycosides by fructose in *S. aureus*, as in *E. coli*, requires PMF generation (Fig. 4c), indicating that the PMF-requiring mechanism exists in both Gram-negative and Gram-positive bacteria. We also investigated whether 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 4 h with fructose and gentamicin (Fig. 4d).

Here we have established an approach for eradicating persisters which is based on metabolism and is effective against both Gram-negative and Gram-positive bacteria. The metabolite-mediated potentiation occurs via PMF generation, which we found was necessary for aminoglycoside uptake and killing of persisters. This work adds to a growing understanding of the role of metabolism in killing by bactericidal antibiotics[13,23,24] and broadens our understanding of persister physiology. Moreover, our findings imply that delivering PMF-stimulating metabolites as adjuvants to aminoglycosides would be beneficial in the treatment of chronic bacterial infections.

METHODS SUMMARY

In all experiments, bacterial cells were cultured in 25 ml Luria-Bertani broth (LB) for 16 h at 37 °C, 300 r.p.m. and 80% humidity in 250 ml flasks. Unless otherwise noted, the following concentrations were used: 10 μg ml^{-1} gentamicin, 100 μg ml^{-1} ampicillin, 5 μg ml^{-1} ofloxacin, 20 μM CCCP and 1 mM potassium cyanide. The concentration of all carbon sources added to potentiate aminoglycosides was normalized to deliver 60 mM carbon (for example, 10 mM glucose, 20 mM pyruvate, etc.). The parent strains used in this study were *E. coli* (K12 EMG2) and *S. aureus* (ATCC 25923). Knockouts (Supplementary Tables 1 and 2) were constructed by P1 phage transduction from the Keio knockout collection. In *E. coli*, non-persister cells in stationary phase were killed by treatment with 5 μg ml^{-1} ofloxacin for 4 h (refs 25, 26). Samples were then washed with phosphate buffered saline (PBS) and suspended in M9 salts with a carbon source and antibiotic to determine metabolite-enabled killing of persisters. At specified time points, 10-μl aliquots were removed, serially diluted and spot-plated onto LB agar plates to determine colony-forming units per ml (c.f.u. ml^{-1}) and survival. Gentamicin-Texas red was made as previously described[27]. Aminoglycoside uptake was measured by incubating stationary-phase samples with 10 μg ml^{-1} gentamicin-Texas red for 5 min at 37 °C, 300 r.p.m. and 80% humidity. 100 μl of each sample was then washed, re-suspended in PBS and analysed on a BD FACS Aria II flow cytometer. Biofilm survival assays were performed as previously described[28]. Raw microarray data for *S. aureus* were downloaded from the Gene Expression Omnibus series GSE20973 (ref. 29) and processed with RMA express using background adjustment, quantile normalization and median polish summarization to compute RMA expression values[30]. Mouse experiments were performed with female Charles River BALB/c mice in collaboration with ViviSource Laboratories and conformed to the ViviSource institutional animal care and use policies and procedural guidelines.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions All authors designed the study, analysed results and wrote the manuscript. Experiments were performed by K.R.A. and M.P.B.

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METHODS

Antibiotics and chemicals. The following concentrations of antibiotics were used in this study: 10 μg ml^{-1} gentamicin, 30 μg ml^{-1} kanamycin, 50 μg ml^{-1} streptomycin, 5 μg ml^{-1} ofloxacin, 100 μg ml^{-1} ampicillin, 40 μg ml^{-1} tetracycline, 50 μg ml^{-1} chloramphenicol and 100 μg ml^{-1} spectinomycin. 20 μM carbonyl cyanide m-chlorophenyl hydrazone (CCCP) was used in experiments to suppress proton-motive force. 1 mM potassium cyanide was used to inhibit aerobic respiration. A stock solution of CCCP was made in dimethylsulphoxide at 500 μM and 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, 300 r.p.m. and 80% humidity in Luria-Bertani (LB) broth to an optical density at 600 nm (OD_{600}) of 0.3. Cells were then diluted 1:1,000 in 25 ml LB and grown for 16 h at 37 °C, 300 r.p.m. and 80% humidity in 250 ml 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[25,26].

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 a potentiating metabolite in control experiments. Carbon sources were purchased from Sigma or Fisher. In *E. coli* persister assays, samples were re-suspended in M9 minimal media salts plus a carbon source. In all experiments using CCCP, samples were pre-treated for 5 min with the proton ionophore before antibiotic addition.

Strains. The parent strains used in this study were *E. coli* (K12 EMG2) and *S. aureus* (ATCC 25923). All *E. coli* knockouts (see Supplementary Table 2) were transduced into the EMG2 strain from strains in the KEIO knockout collection using the P1 phage method. All strains were cured using pCP20 and standard laboratory procedures before their use in assays.

Persister assays. For *E. coli* persister assays, samples were grown to stationary phase as described above. Cultures were then treated for 4 h with 5 μg ml^{-1} ofloxacin in the above-stated growth conditions. Previous work has demonstrated that treatment for 3 h under these conditions eliminates all susceptible non-persister cells[25]. We verified that the remaining cells were persisters by increasing the amount of ofloxacin added to the culture to 20 μg ml^{-1} 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 killed cells when added without a carbon source (see Fig. 1c). Samples were then washed with 10 ml of 1× filtered PBS and re-suspended in M9 minimal media. Carbon sources and antibiotics were added and samples were incubated at 37 °C, 300 r.p.m. and 80% humidity. Re-suspension of samples in defined minimal media allowed us to test the effects of different carbon sources on persister viability precisely and specifically, without the possible confounding factors that a more complex medium would present. For *S. aureus* persister experiments, cells were grown at 37 °C and 300 r.p.m. in LB broth to an OD_{600} of 0.3. Cells were then diluted 1:1,000 in 25 ml LB and grown for 16 h at 37 °C and 300 r.p.m. in 250 ml flasks before treatment with a carbon source and antibiotic. Previous work has shown that almost all stationary-phase *S. aureus* cells are persistent[25].

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 per ml (c.f.u. ml^{-1}). Only dilutions that yielded 10–100 colonies were counted. Survival was determined by dividing the c.f.u. ml^{-1} of a sample at each time point by the initial c.f.u. ml^{-1} for that sample.

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 a carbon source and incubated at 37 °C, 300 r.p.m. and 80% humidity.

Gentamicin-Texas red uptake. Gentamicin-Texas red was made as previously described[27]. 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 added slowly to 2.3 ml of 10 mg ml^{-1} gentamicin solution in 100 mM K_2CO_3 at 4 °C.

Uptake of gentamicin-Texas red induced by carbon sources in stationary-phase cells was determined by adding concentrated carbon sources (see section on media and growth conditions for concentrations) to stationary-phase cultures and incubating them for 30 min at 37 °C, 300 r.p.m. and 80% humidity. Concentrated gentamicin-Texas red was then added to a final concentration of 10 μg ml^{-1} and samples were incubated for 5 min. 100 μl of each sample was then washed with 1 ml of PBS and re-suspended in 1 ml of PBS. 200 μl of the re-suspended sample was then added to 800 μl of PBS in flow tubes. Samples were analysed on a BD FACS Aria II flow cytometer with the following settings: mCherry voltage, 650 V; FSC threshold, 1,000; recorded events, 100,000; gated-out mCherry events, <30.

Membrane potential measurements. We used the BacLight 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 section on media and growth conditions for concentrations) and 10 μl $DiOC_2(3)$ (membrane stain) to stationary-phase cultures, followed by incubation for 30 min at 37 °C, 300 r.p.m. and 80% humidity. 10 μl of culture was added to 1 ml of PBS in flow tubes immediately before analysing. Samples were analysed on a BD FACS Aria II flow cytometer with settings optimized according to the BacLight kit manual. Settings used were: FITC voltage, 250 V; mCherry voltage, 650 V; FSC threshold, 1,000; recorded events, 100,000. FSC and SSC outliers were gated out by visual inspection before data acquisition. The red/green (mCherry/FITC) values for each cell were determined and normalized, then compared to samples without metabolite to determine the relative change in PMF.

Anaerobic experiments. *Escherichia coli* were grown to an OD_{600} of 0.3, then diluted 1:1,000 in 25 ml anaerobic LB with 10 mM $NaNO_3$ and grown for 16 h at 37 °C, 200 r.p.m., 1.5–2.0% hydrogen and <50 p.p.m. oxygen in 250 ml flasks. Cultures were then treated with a carbon source and metabolite in the presence or absence of an additional 10 mM $NaNO_3$. The additional $NaNO_3$ was added to determine whether 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 μl per well. Plates were incubated at 35 °C, 150 r.p.m. for 24 h, then pegs were washed in a microtitre plate with 200 μl of 1× PBS per well. Pegs were then added to a microtitre plate containing 200 μl M9 minimal salts (for *E. coli*) or sterile-filtered, stationary-phase media (for *S. aureus*), plus a carbon source and antibiotic. Plates were incubated at 35 °C, 150 r.p.m. for 4 h, then pegs were washed twice in microtiter plates with 200 μl of 1× PBS per well. To dislodge biofilm cells, pegs were placed in a microtiter plate with 145 μl of 1× PBS per well and sonicated in a water bath for 30 min at 40 kHz. Serial dilutions and spot-plating were performed to determine viable c.f.u. per peg. For determination of the dependence of *E. coli* biofilm elimination on pH, we carried out the above procedure in M9 salts buffered to an appropriate pH with citric acid, as opposed to KH_2PO_4, 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 h to form biofilms. 48 h after surgery, mice received either no treatment or twice-daily, intravenous treatment with gentamicin (1 mg kg^{-1}) or mannitol (1.5 g kg^{-1}) plus gentamicin (1 mg kg^{-1}) for 3 days. Seven or eight mice were included in each group. 24 h 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 US Department of Agriculture and by the Office of Laboratory Animal Welfare, where all *in vivo* experimental work was performed. The study conformed with ViviSource institutional animal care and use policies and procedural guidelines.

***Staphylococcus 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 (ref. 29). The data were processed with RMA express using background adjustment, quantile normalization and median polish summarization to compute RMA expression values[30]. 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) was used for processing flow cytometric data, analysing 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.