Establishment of a Method To Rapidly Assay Bacterial Persister Metabolism

Mehmet A. Orman, Mark P. Brynildsen

Department of Chemical and Biological Engineering, Princeton University, Princeton, New Jersey, USA

Bacterial persisters exhibit an extraordinary tolerance to antibiotics that is dependent on their metabolic state. Although persister metabolism promises to be a rich source of antipersister strategies, there is relatively little known about the metabolism of these rare and transient phenotypic variants. To address this knowledge gap, we explored the use of several techniques, and we found that only one measured persister metabolism. This assay was based on the phenomenon of metabolite-enabled aminoglycoside killing of persisters, and we used it to characterize the metabolic heterogeneity of different persister populations. From these investigations, we determined that glycerol and glucose are the most ubiquitously used carbon sources by various types of *Escherichia coli* persisters, suggesting that these metabolites might prove beneficial to deliver in conjunction with aminoglycosides for the treatment of chronic and recurrent infections. In addition, we demonstrated that the persister metabolic assay developed here is amenable to high-throughput screening with the use of phenotype arrays.

Persisters are phenotypic variants that are tolerant to extraordinary concentrations of antibiotics. Persisters in biofilms have been hypothesized to underlie the proclivity of biofilm infections to relapse (1), and until recently there was no effective way to eliminate persisters. Two promising studies have demonstrated metabolism-dependent killing of persisters: one that potentiates aminoglycoside (AG) activity by stimulating proton motive force generation in persisters (2), and another that stimulates persister awakening (3). These methods suggest that knowledge of persister metabolic capabilities would expedite the discovery and development of antipersister therapies. Consequently, perturbations to numerous metabolic genes and regulators have been shown to alter persister levels (4–9, 30, 31), further supporting a central role for metabolism in maintenance of this phenotypic state. In addition, persisters have recently been shown to largely adopt a metabolically quiescent state that must be achieved and sustained by coordinated metabolic actions (10). Unfortunately, persister metabolism has not been sufficiently studied, and knowledge of the metabolic activities of these phenotypic variants remains limited. In part, this is due to difficulties associated with isolation of persisters from normally growing cultures, where current techniques provide only modest persister enrichment (11, 12).

In this study, we sought to measure the metabolic activities of natively generated *Escherichia coli* persisters, not those obtained synthetically by overexpression of a toxin (13) or treatment with persister-stimulating agents (14). First, we attempted to use the only isolation technique previously described to produce pure persister samples (15). In this method, normally growing bacteria are lysed with ampicillin (AMP), and the remaining cells, considered persisters, are sedimented by centrifugation. However, by using LIVE/DEAD staining, a fluorescent indicator of cell lysis, a metabolic assay, and persister measurements, we found that exponential-phase cultures of wild-type *E. coli* treated with AMP contained almost 100-fold more viable but nonculturable cells (VBNCs) than persisters and that VBNC metabolic activity obscured measurement of persister metabolism. In the absence of a high-fidelity isolation technique, it was necessary to base the measurements of persister metabolism on the fundamental definition of persistence, antibiotic tolerance exhibited by a reduced killing

rate in a biphasic kill curve, followed by growth resumption on standard media. This definition distinguishes persisters from normal and dead cells as well as VBNCs and, therefore, must be used to measure persister physiology in the absence of improved isolation techniques. Since persisters can only be killed by AGs if they metabolize carbon sources to generate proton motive force (PMF) (2), we adapted the AG potentiation assay to rapidly measure persister metabolism in a high-throughput manner with the use of phenotype microarrays. We used this method to measure the metabolism of various persisters (e.g., AMP and ofloxacin [OFX], during the exponential and stationary phases), and we found that glycerol and glucose were the most commonly metabolized substrates by *E. coli* persisters, establishing these carbon sources as prime candidates for combination therapy with AGs. The method described here can be used to explore persister metabolism and build back their active metabolic networks, while simultaneously probing for the best adjuvants for AG therapy of relapsing infections.

MATERIALS AND METHODS

Bacterial strains. All strains were derived from *E. coli* MG1655. The MG1655 *hipA7* mutant was a generous gift from the James J. Collins Lab (Boston University, Boston, MA). Generation of MO001, which contained a chromosomally integrated *lacI^q* promoter in place of the *lacI* promoter and a chromosomally integrated *T5p-mCherry* in place of *lacZ*Y_A, was described in a previous study (10). Genetic deletions (Δ *gldA*, Δ *glpK*, Δ *gldA* Δ *glpK*, and Δ *ptsI*) were transduced from the Keio Collection by using the standard P1 phage method (16). When necessary, the kanamycin resistance gene was removed using FLP recombinase (17). All mu-

Received 20 February 2013 Returned for modification 18 March 2013

Accepted 25 June 2013

Published ahead of print 1 July 2013

Address correspondence to Mark P. Brynildsen, mbrynild@princeton.edu.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.00372-13>.

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doi:10.1128/AAC.00372-13

tations were confirmed using PCR and/or DNA sequencing (Genewiz, South Plainfield, NJ).

Chemicals, media, and growth conditions. All chemicals, unless noted below, were purchased from Fisher Scientific or Sigma-Aldrich. A LIVE/DEAD bacterial viability kit was purchased from Life Technologies, Invitrogen (Grand Island, NY). Fluorescent particles for cell counting were purchased from Spherotech, Inc. (Lake Forest, IL). WST-1 was purchased from Dojindo Molecular Technologies (Rockville, MD), and isopropyl- β -D-thiogalactopyranoside (IPTG) was purchased from Gold Biotechnology (St. Louis, MO). Phenotype microarrays were purchased from Biolog, Inc. (Hayward, CA). LB medium (10 g/liter tryptone, 5 g/liter yeast extract, and 10 g/liter NaCl) and LB agar plates (LB with 15 g/liter agar) were used for planktonic growth and enumeration of CFU, respectively. When necessary, 0.22- μ m filter-sterilized pyruvate, α -ketoglutarate, or catalase solutions were aseptically spread on the surface of LB agar plates at concentrations of 0.1% pyruvate, 0.1% α -ketoglutarate, and 2,000 U catalase per plate (18). For persister assays, 200 μ g/ml AMP and 5 μ g/ml OFX were used. For the AG potentiation assay, 25 μ g/ml kanamycin (KAN) was used. For mutant selection, 50 μ g/ml KAN was utilized. To inhibit cytochrome activity, 1 mM KCN was used (2). To prepare the overnight cultures, cells from a 25% glycerol, -80°C stock were incubated in 2 ml LB medium at 37°C with shaking (250 rpm) for 24 h. The overnight cultures were diluted 1,000-fold in 50 ml of fresh LB medium in a 500-ml baffled flask and incubated for 90 min at 37°C and 250 rpm before antibiotic treatment. To enumerate CFU, cells were washed and diluted in phosphate-buffered saline (PBS), plated on LB agar, and incubated at 37°C for 16 h.

LIVE/DEAD staining assay. SYTO 9 and propidium iodide (PI) staining were performed according to the manufacturer's instructions. Exponential-phase and AMP-treated cell cultures were washed and diluted in sterile 0.85% NaCl buffer to reach a final density of 1×10^6 cells/ml. Then, cells were stained with SYTO 9 and PI simultaneously, at concentrations of 5 μ M and 30 μ M, respectively, and incubated at room temperature for approximately 15 min before flow cytometry analysis. As controls, approximately 1×10^6 exponential-phase cells were either incubated in 1 ml of 70% ethanol solution for 1 h or sonicated at 10% amplitude with a sonic dismembrator (Fisher Scientific, Pittsburgh, PA) in 1 ml of 0.85% NaCl buffer on ice for 30 min prior to staining.

Fluorescent indicator (mCherry) of cell lysis assay. MO001 cells were cultured as described above, except that 1 mM IPTG was included in the overnight and exponential growth cultures. After AMP treatment, 1-ml samples were collected at the indicated time points, washed, diluted in PBS, and analyzed by flow cytometry to count the unlysed cells. For the control, exponential-phase cells were sonicated in PBS as described above prior to the flow cytometry assay.

Flow cytometry analysis. All samples were analyzed with an LSRII flow cytometer (BD Biosciences, San Jose, CA). Microorganisms were identified using forward and side scatter parameters (FSC and SSC). SYTO 9/PI-stained cells and mCherry-expressing cells were assayed with lasers emitting at 488 nm and 560 nm, respectively, and fluorescence intensities were collected by using a green fluorescence (525/50 nm) bandpass filter for SYTO 9 and a red fluorescence (610/20 nm) bandpass filter for both PI and mCherry. Data were acquired and analyzed using FACSDiVa software (BD Biosciences, San Jose, CA).

Cell sorting. All cell sorting experiments were performed using a FACSVantage SE cell sorter with DiVa (BD Biosciences, San Jose, CA) at 16 lb/in² with a 70- μ m nozzle. Microorganisms were determined using forward and side scatter parameters. mCherry-positive cells were identified by measuring red fluorescence (561-nm excitation with a 615/30-bandpass filter). Cells were sorted using sterile $1 \times$ PBS as sheath fluid in the sorter. mCherry-positive cells after AMP treatment (approximately 10,000 cells) were sorted in microcentrifuge tubes, plated on LB agar, and incubated at 37°C for 16 h to count CFU. These data were used to calculate the persister/VBNC ratio. To determine the VBNC levels prior to antibiotic treatment, single cells from exponential-phase MG1655 cultures were

delivered to individual wells of 96-well plates filled with 200 μ l LB agar. The plates were incubated for 16 h at 37°C . Wells that failed to grow were inoculated with a dead cell or VBNC, whereas wells with measureable growth were inoculated with a normal cell or a persister.

Persister and VBNC enumeration. MIC ranges for MG1655 cells were found to be 1.5 to 3 μ g AMP/ml, 0.075 to 0.15 μ g OFX/ml, and 3 to 6 μ g KAN/ml by using the method based on serial 2-fold dilutions of antibiotics in LB medium (10). Exponential-phase cultures (50 ml) were treated with either AMP (200 μ g/ml) or OFX (5 μ g/ml), while stationary-phase cultures (2 ml) were only treated with OFX (5 μ g/ml), and all cultures were incubated at 37°C and 250 rpm. To enumerate the persister levels, samples were collected at the indicated time points, washed, and serially diluted in PBS, and then 10- μ l samples were plated on LB agar. VBNC levels at the indicated time points during the AMP treatment were enumerated by flow cytometry with fluorescent counting particles with either SYTO 9/PI-stained wild-type cells or mCherry-expressing cells as described above. To determine whether VBNCs from these experiments could be resuscitated (18), antibiotic-treated samples were plated on LB, LB plus pyruvate (0.1%), LB plus α -ketoglutarate (0.1%), or LB plus catalase (2,000 U/plate).

Tetrazolium assay. For the tetrazolium assay, the detection reagent was prepared according to the methods described by Tsukutani and co-workers (19). WST-1 and 2-methyl-1,4-naphthoquinone were dissolved in distilled water and ethanol at concentrations of 11.1 mM and 8 mM, respectively, and then they were mixed at a ratio of 9:1, yielding 10 mM tetrazolium salt and 0.8 mM electron mediator. The reagent was filter sterilized with a membrane filter (0.2 μ m).

Cells from 50-ml exponential-phase cultures treated with AMP (5 or 20 h) were pelleted at 4,000 rpm for 15 min using a centrifuge (5810R; Eppendorf AG, Hamburg, Germany) and resuspended in 1 ml of sterile $1.25 \times$ M9 salt solution, including AMP (125 μ g/ml). Then, 80 μ l of microbial resuspension, 10 μ l of detection reagent, and 10 μ l of carbon source solution (600 mM carbon) were added to each well of 96-well microtiter plates. This yielded approximately 10^7 live cells (VBNCs plus persisters), 1 mM WST-1, 0.08 mM electron mediator, and 60 mM carbon per well. As a control, 10 μ l sterile water was added to this mixture instead of a carbon source. We note that all carbon sources were dissolved in distilled water and then filter sterilized. Ninety-six-well plates were covered with sterile, gas-permeable sealing membranes (Breath-Easy; Sigma-Aldrich). Cell cultures were incubated at 37°C and 250 rpm, and the A_{438} was measured at the indicated time points by using a Synergy H1 hybrid multimode microplate reader (BioTek, Winooski, VT). The same procedures were applied to stationary- and exponential-phase cultures before AMP treatment to measure their metabolic activities. The cell density in all samples was $\sim 10^7$ live cells/well.

To understand if the debris from lysed cells affected formazan production, 2 ml of exponential-phase cells before the AMP treatment was washed with $1.25 \times$ M9 salt solution as described above and sonicated at 10% amplitude for 30 min on ice. This method did not result in protein denaturation, which was confirmed by measuring the fluorescence intensities of samples before and after sonication using a microplate reader (560-nm excitation, 610-nm emission). Lysis of all cells was confirmed by flow cytometry. A colorimetric microbial assay was performed for both sonicated and unsonicated exponential-phase cells that had been chilled on ice as described above.

Aminoglycoside potentiation assay. For the AG potentiation assay, cells from 50-ml exponential-phase cultures treated with AMP (200 μ g/ml) or OFX (5 μ g/ml) were pelleted and suspended in 1 ml of sterile $1.25 \times$ M9 salt solution as described above without antibiotic. Then, 80 μ l of cell suspension, 10 μ l of KAN solution, and 10 μ l of carbon source solution were mixed in each well of the plates, resulting in $\sim 10^5$ AMP or $\sim 10^6$ OFX persisters, 25 μ g/ml KAN, and 60 mM carbon per well. The plates were covered with sterile, gas-permeable sealing membranes and incubated at 37°C and 250 rpm, and 5- μ l samples were removed from each well at the indicated time points and diluted 60-fold in PBS into another 96-well

plate, to reduce the antibiotic concentrations to sub-MIC levels. Then, 10- μ l aliquots of these samples were further diluted and plated onto LB agar. Where indicated, KCN, AMP, or OFX was added to a 1.25 $\times$ M9 suspension buffer (controls). The final concentrations of these chemicals in the cultures were, respectively, 1 mM, 100 μ g/ml, and 5 μ g/ml. The same procedures were applied to stationary-phase OFX persisters. Overnight cultures in 2 ml LB were treated with OFX (5 μ g/ml) to obtain stationary-phase persisters.

Competition assay. To understand if VBNCs interfere with the AG potentiation assay (e.g., consume a carbon source and generate a product that potentiates AG activity in persisters), a competition assay was performed. Antibiotic-treated samples of wild-type and mutant (Δ *gldA* Δ *glpK* or Δ *ptsI*) cells from either exponential- or stationary-phase cultures were pelleted and suspended in 1 ml of sterile 1.25 $\times$ M9 salt solution as described above. Wild-type and mutant cells (Δ *gldA* Δ *glpK* or Δ *ptsI*) were mixed so as to obtain an approximate 50/50 proportion of persisters. Then, 80 μ l of cell suspension, 10 μ l of KAN solution, and 10 μ l of carbon source solution were mixed in each well of the plates, resulting in $\sim 10^5$ persisters, 25 μ g/ml KAN, and 60 mM carbon per well. For controls, wild-type-only and mutant-only cell suspensions were treated with KAN. All mixed cultures had the same amount of wild-type or mutant persisters as wild-type-only or mutant-only cultures. Where indicated, KCN and AMP were added to 1.25 $\times$ M9 suspension buffer. Where indicated, cell densities were decreased (10-fold) to minimize the effects of surrounding cells on persisters, and KAN and carbon source concentrations were kept constant. The plates were then covered with sterile, gas-permeable sealing membranes and were incubated at 37°C and 250 rpm. After 2 h of incubation, cell cultures (100 μ l) were transferred to sterile microcentrifuge tubes and washed with PBS to remove the antibiotics, and 10- μ l aliquots of these samples were further diluted and plated onto LB agar. The remaining 90 μ l of the cultures was also plated in case the CFU were not measured from the 10- μ l spot. To confirm that mixed-cell cultures were enriched with mutants after 2 h of incubation with KAN and the nonutilized carbon source (glycerol for Δ *gldA* Δ *glpK* and glucose for Δ *ptsI*), at least 24 colonies from mixed cultures were grown in 200 μ l M9-glycerol or M9-glucose medium (60 mM carbon), in 96-well plates, for 24 h at 37°C and 250 rpm. This allowed quantification of the proportion of surviving cells that were mutants, since Δ *gldA* Δ *glpK* and Δ *ptsI* cannot grow in M9-glycerol or M9-glucose medium, respectively, whereas wild-type cells can grow in both media.

Phenotype microarray assay. Phenotype microarrays (PM) were used to measure AG potentiation and cell growth in a high-throughput manner. Stationary-phase cells in 2 ml LB treated with OFX for 5 h were washed and diluted 10-fold in 1 $\times$ M9 salt solution, including 25 μ g/ml KAN, and 100- μ l aliquots of samples from this cell suspension were added to the wells of a PM. Plates were covered with sterile, gas-permeable sealing membranes and incubated at 250 rpm and 37°C. At the indicated time points, 5- μ l samples were removed for CFU enumeration as described above. When indicated, KCN or AMP was added to the 1 $\times$ M9 suspension buffer (controls). To determine the cell growth rates, stationary-phase cells without antibiotic treatment were washed and diluted 10-fold in 1 $\times$ M9 salt solution, and 100- μ l aliquots of the cell suspension were added to wells of a PM. Cells were incubated at 250 rpm and 37°C for 6 h, and at the indicated time points, 10- μ l aliquots of cultures were mixed with 290 μ l of 1 $\times$ M9 salt solution to measure the optical density at 600 nm.

Statistical analysis. All experiments were independently repeated at least 3 times. Pairwise comparison was performed using a two-tailed *t* test, and a *P* value threshold of 0.05 was used to identify statistically significant differences. Each data point is represented by the mean value $\pm$ the standard error.

RESULTS

Ampicillin-lysed cultures include more VBNCs than persisters. Persisters cannot be isolated from untreated cultures, due to iso-

lation difficulties that originate from their transient nature, low abundance, and a lack of high-fidelity biomarkers. Fluorescence-activated cell sorting (FACS) methods used for persister isolation based on an rRNA reporter (an indicator of protein synthesis) (11) and a fluorescent indicator of cell division (10, 12) provide samples that are enriched in persisters but remain highly contaminated with other cell types, such as normal cells and VBNCs. Another technique that has been described for persister isolation involves treatment of cultures with β -lactams and sedimentation of nonlysed cells (15, 20). As evidence that the sedimented cells are persisters, Keren and colleagues performed LIVE/DEAD (SYTO/PI) staining to show that all remaining cells stained as live cells (15). However, the claim that these sedimented cells are all persisters has come under scrutiny due to additional studies that have demonstrated that cell lysis by β -lactams leaves many more cells with intact membranes than there are persisters in the population (7, 12, 21). Here, we sought to determine if β -lactam lysis would be useful for isolation of wild-type *E. coli* persisters for metabolic measurements. After 24 h of incubation in LB, the overnight cells were inoculated in fresh medium and cultured for 90 min (doubling time, ~ 25 min). Exponential-phase cells were then treated with AMP, live cells were counted by flow cytometry, and CFU were measured by plating on LB agar. The LIVE/DEAD probes, SYTO 9 and PI, which are, respectively, green and red fluorescent nucleic acid stains that differ in their ability to penetrate bacterial cells (22), were used to enumerate live cells, as Keren and colleagues had done (15). While SYTO 9 can stain all cells, PI only stains cells with damaged membranes (qualified as dead). To determine the gates of live and dead cell subpopulations, exponential-phase cells were treated with ethanol (standard control) or sonicated until all cells were killed and then stained with SYTO 9 and PI dyes (Fig. 1A). We reasoned that sonication was an important control due to the fact that AMP lyses cells. As depicted in Fig. 1A, dead cells were differentially stained by these two dyes. When we compared these data with a stained AMP-treated culture (Fig. 1B and C), sonicated samples more closely resembled AMP-treated cells than ethanol-treated cells. When we monitored the live cell and persister levels for 5 h during AMP treatment, we found that the level of live cells was almost 2 orders of magnitude higher than the level of persisters (Fig. 1G), consistent with previous studies (7, 12, 21). Cells that cannot form a colony on standard media but stain as live are known as VBNCs (23). Under specific circumstances, VBNCs have been observed to return to culturability by plating on media with antioxidants, such as pyruvate, α -ketoglutarate, or catalase (18). To determine if such resuscitation was possible under our experimental conditions, antibiotic-treated samples were plated on LB alone, LB with pyruvate, LB with α -ketoglutarate, and LB with catalase, and CFU were measured. As depicted in Fig. S1 in the supplemental material, the addition of these components could not resuscitate VBNCs, suggesting that the distinction between VBNC and persister was robust to the medium variation in this study.

To corroborate the LIVE/DEAD staining, we measured the level of intact cells by using a fluorescent protein (12). In this method, the fluorescent protein is expressed and then cells are treated with AMP. Normal cells lyse and their fluorescent signals are lost, whereas unlysed cells retain their high fluorescence. Here, we used an *mCherry* gene under the control of a strong, synthetic, IPTG-inducible promoter (T5) that was knocked into the chromosome of an *E. coli* strain carrying a chromosomally integrated

FIG 1 Enumeration of persister and VBNC levels in exponential-phase cultures. (A) Wild-type live and dead cells (sonicated or ethanol treated) stained with SYTO9/PI were analyzed by flow cytometry to determine the live and dead cell regions. (B and C) Exponential-phase cells were treated with AMP for 5 h, and samples were taken before and after the treatment for staining with SYTO9/PI. (D) Similarly, mCherry-expressing live and dead (sonicated) cells (MO001) were analyzed by flow cytometry to determine the live and dead cell cell counts. (E and F) Samples taken before and after the AMP treatment were assayed with flow cytometry. K indicates the cell count values (y axis) should be multiplied by 1,000. (G) VBNC and persister levels were monitored in both wild-type and MO001 cell types for 5 h during the AMP treatment. Significant differences were not observed when persister levels ($P > 0.30$) or VBNC levels ($P > 0.90$) of wild-type and MO001 strains were compared after 5 h of AMP treatment. (H) VBNC and persister levels of *hipA7* mutant were monitored for 5 h during the AMP treatment. A significant difference between wild-type and *hipA7* strains was not observed when VBNC levels ($P > 0.10$) were compared after 5 h of AMP treatment; however, persister levels were significantly higher in the *hipA7* strain than wild-type ($P < 0.001$). CFU were enumerated from antibiotic-treated samples by plating on LB agar, whereas live cells were enumerated by counting the unlysed cells using flow cytometry as a function of time. Error bars indicate the standard errors of the means.

LacI^q promoter mutation (MO001) (10). IPTG was applied to induce *mCherry* expression during both the overnight and exponential growth period to maintain the high fluorescent signals of unlysed cells. As a control, exponential-phase MO001 cells expressing mCherry were sonicated to lyse the population and determine the region of lysed cells on the flow diagram (Fig. 1D), which was consistent with that of AMP-treated cells (Fig. 1E and F). Using this technique, the level of live cells in exponential MO001 cultures was monitored for 5 h during AMP treatment and found to be statistically indistinguishable from the level of live cells in wild-type cultures, as enumerated with SYTO/PI (Fig. 1G). Further, persister levels of wild-type and MO001 cells were statistically indistinguishable (Fig. 1G), and when cells from the live

subpopulation of an antibiotic-treated MO001 cell culture were sorted, only $0.59\% \pm 0.21\%$ of those cells regrew, a result that was consistent with the 2 orders of magnitude difference observed between the levels of live cells and persisters when using LIVE/DEAD staining (see Fig. S2 in the supplemental material). These results demonstrated that AMP-treated wild-type cultures contain significantly more VBNCs than persisters. We also performed analogous experiments with a *hipA7* mutant and found that the level of VBNCs was comparable to that of the wild type, whereas the level of persisters was over an order of magnitude higher (Fig. 1H). This result agrees with a previous study that demonstrated that the AMP lysis technique was insufficient to isolate persisters from *hipA7* cultures (7).

To determine whether VBNCs may be problematic for persister isolation from untreated cultures, we measured the proportions of normal cells, dead cells, VBNCs, and persisters in wild-type cultures prior to antibiotic treatment. Using single-cell-mode FACS, single cells were delivered to individual wells of 96-well plates filled with LB agar. Wells that produced a colony arise from a normal cell or persister, whereas wells without a colony received either a dead cell or VBNC. The frequency of dead cells was quantified using LIVE/DEAD staining, and the frequency of persisters was quantified in antibiotic tolerance assays. With this combination of assays, each subpopulation could be quantified, and the results are presented in Table S1 of the supplemental material. Notably, we observed that VBNCs were far more abundant than persisters in untreated wild-type samples. When analogous experiments were performed on the *hipA7* mutant, the proportions of normal cells, dead cells, and VBNCs were comparable to that of the wild type, whereas the proportion of persisters was over an order of magnitude higher (Fig. 1H; see also Table S1 in the supplemental material). These data demonstrated that VBNCs are more abundant than persisters in untreated cultures and confirmed that, similar to the β -lactam technique (15, 20), methods for persister isolation from untreated samples are also complicated by the presence of VBNCs (11).

VBNCs exhibit significantly reduced metabolic activity. Although VBNCs preserve their membrane integrity, it remained unclear whether VBNCs exhibited significant metabolic activity. If VBNCs were metabolically inactive, AMP-treated samples might still be useful for measurement of persister metabolic activities. To determine whether AMP-treated samples exhibited metabolic activity, we adapted an assay that uses a water-soluble tetrazolium salt (WST-1) in conjunction with an electron mediator (2-methyl-1,4-naphthoquinone) to facilitate dye reduction (see Materials and Methods). WST-1 is reduced extracellularly to its soluble formazan by electron transport across the membrane of metabolically active cells (19, 24). The color change during formazan production can be detected by absorbance measurements, which correlate with the cellular dehydrogenase and reductase activities (24). This method enabled us to test numerous carbon sources simultaneously.

To measure metabolic activity from AMP-treated samples, cells were washed in M9 minimal medium without a carbon source to remove LB, and approximately 1×10^7 nonlysed cells (VBNCs plus persisters) were incubated in 100 μ l/well M9 medium containing various carbon sources (60 mM carbon) in a 96-well plate. AMP at 100 μ g/ml was also added to prevent growth resumption. A carbon source-free control was used to measure the background reduction of the water-soluble tetrazolium salt by the different cell suspensions. When absorbance measurements were taken at the indicated time points (Fig. 2A), the nonlysed cells could metabolize several of the carbon sources tested, including glycerol, glucose, mannitol, fructose, and succinate (see Fig. S3 in the supplemental material for all carbon sources tested). To verify that debris from lysed cells was not responsible for the measured WST-1 reduction, we measured the metabolic activities of samples that were lysed by a sonication procedure that did not denature proteins (see Fig. S4 in the supplemental material), and we found that cell debris was an insignificant source of formazan production (see Fig. S4). However, it was unclear whether VBNCs or persisters were responsible for the observed metabolic activity. Although the VBNC levels were ~ 2 orders of magnitude higher

than persister levels, the VBNCs could have been far less metabolically active. Therefore, we sought to measure metabolic activity from samples where the abundance of one cell type was approximately constant and the abundance of the other cell type was altered by orders of magnitude. Interestingly, when exponential-phase cells were treated with 200 μ g/ml AMP for 20 h, VBNC levels did not change, whereas persister levels decreased by ~ 100 -fold (Fig. 2C and D). This phenomenon allowed determination of whether the metabolic signal obtained from AMP-treated samples was from VBNCs or persisters. As depicted in Fig. 2A, B, and E, 20-h AMP-treated samples displayed a metabolic activity that was strikingly similar to that obtained from 5-h AMP-treated samples (see Fig. S3 in the supplemental material for all carbon sources tested). This experiment suggested that the metabolic activity of AMP-lysed cultures is dominated by VBNCs that mask the contribution by persisters. To ensure that the WST-1 absorbance measurements showed metabolic activity, VBNCs from mutants that cannot utilize glycerol and glucose, $\Delta glpK \Delta glcA$ and $\Delta ptsI$, respectively (see Fig. S5 in the supplemental material), were assayed for their ability to reduce WST-1. As depicted in Fig. 2F and G, VBNCs from the $\Delta glpK \Delta glcA$ mutants were unable to reduce WST-1 in the presence of glycerol, whereas VBNCs from $\Delta ptsI$ mutants were unable to reduce WST-1 in the presence of glucose.

Since the WST-1 assay as applied to AMP-treated samples indicates VBNC metabolism, we compared the metabolism of VBNCs with that of stationary- and exponential-phase cells from the same cultures. Overnight cells, exponential-phase cells (before AMP treatment), and VBNCs (after AMP treatment) were similarly incubated in M9 minimal medium as described above. We defined the metabolic activity rates as the slopes of the A_{438} during the first 2 h of incubation with WST-1. As shown in Fig. 3, VBNC metabolism was significantly reduced (approximately 5-fold and 2-fold compared to exponential- and stationary-phase cells, respectively) but surprisingly similar to that of exponential- and stationary-phase cells. Glucose, glycerol, mannitol, and fructose were highly utilized by all three cell types.

Aminoglycoside potentiation can identify persister metabolic activities. Since VBNCs are orders of magnitude more abundant than persisters and these nonlysed cells have metabolic activity, the metabolic activity of persisters could not be assayed by the WST-1 method. The inability to isolate persisters to homogeneity, either from normally growing or antibiotic-treated cultures, precludes direct measurement of persister metabolism. The distinguishing characteristic of persisters that is not shared with other cell types (e.g., normal, dead, or VBNCs) is an enhanced ability to tolerate antibiotic treatment (second regimen of a biphasic kill curve) followed by resumption of growth on standard media. Recently, we discovered that persisters can metabolize specific carbon sources and become susceptible to AG (2). AG susceptibility was conferred by increased AG uptake, facilitated by catabolism of carbon sources to generate a proton motive force. Notably, persister killing could be eliminated through perturbations to the respiratory chain, including treatment with KCN, which blocks cytochrome oxidoreductase activity. Since AG potentiation is measured by a reduction in CFU, it provided an opportunity to measure persister metabolism in antibiotic-treated samples, where persisters are the only cell type capable of colony formation on standard media.

To adapt the AG potentiation phenomenon for the purpose of rapidly measuring persister metabolism, we sought to (i) identify

FIG 2 Metabolic activity measurements of ampicillin-treated cultures. (A) Live cells (VBNCs and persisters) after 5 h of AMP treatment were incubated in M9 minimal medium with various carbon sources, WST-1, and an electron mediator in 96-well plates, and absorbance (A_{438}) was measured at the indicated time points. (B) Similarly, absorbance was measured from 20-h AMP-treated cultures. (C to E) Persister and VBNC levels, as well as absorbance measurements from 5- and 20-h AMP-treated samples, were compared. Persister levels were significantly different ($P < 0.001$), whereas VBNC levels and absorbance measurements for both cultures were similar ($P > 0.5$). VBNCs were enumerated by counting mCherry-positive cells in the antibiotic-treated samples using flow cytometry. (F and G) $\Delta glpK \Delta glpA$ and $\Delta ptsI$ cells after 5 h of AMP treatment were incubated in M9 minimal medium with glycerol, glucose, or no carbon (control), and absorbance was measured at the indicated time points. Error bars indicate the standard errors of the means.

conditions where many metabolites could be assayed in a single experiment without the need for wash steps and (ii) confirm that the assay results were attributable to persister metabolism. To accomplish the first task, the AG assay was scaled to a 96-well plate filled with 100- μ l culture volumes and 25 μ g/ml of KAN (see Materials and Methods). After exponential-phase cells in LB were treated with AMP for 5 h, cells were washed and incubated with KAN in M9 minimal medium with different carbon sources (60 mM carbon). To confirm that persisters remained nonreplicative and retained their antibiotic tolerance, AMP was used as a control (Fig. 4A). To confirm that the same AG potentiation phenomenon was being measured, KCN was used to block respiration and pro-

ton motive force generation. In addition, a control that provided no carbon source was used to measure inherent AG activity, from which enhancements from metabolite exposure could be quantified (Fig. 4A). Results from this assay (Fig. 4B) demonstrated that glycerol and glucose efficiently potentiated AG activity in AMP persisters from exponential-phase cultures (see Fig. S6A in the supplemental material for the potentiation of other carbon sources). To demonstrate that this assay measured metabolic activity, we confirmed that AG potentiation by glycerol was eliminated in the $\Delta glpA \Delta glpK$ strain and AG potentiation by glucose was eliminated in the $\Delta ptsI$ strain (Fig. 4C).

To accomplish the second task, we sought to confirm that the

FIG 3 Comparison of metabolic activities of exponential- and stationary-phase cells and VBNCs. Stationary- and exponential-phase cells as well as VBNCs from 5-h AMP-treated exponential-phase cultures were incubated in M9 minimal medium with various carbon sources, and absorbance was measured during the first 2 h of incubation with WST-1 and an electron mediator. Metabolic activity rates were defined as the slopes of absorbance curves during the first 2 h (n is the number of total live cells determined by flow cytometry). In each group, metabolic activity rates with different carbon sources were compared to baseline values (no-carbon control) by using the t test. Error bars indicate the standard errors of the means.

dead cells and VBNCs in antibiotic-treated samples were not influencing assay results. We reasoned that since VBNCs are the dominant metabolically active subpopulation in antibiotic-treated samples, it was possible that they might consume a carbon source and generate a product that would potentiate AG activity in persisters, thus interfering with interpretation of assay results. To determine if such a phenomenon was present, we performed competition assays where antibiotic-treated samples of the wild type and mutants ($\Delta gldA \Delta glpK$ or $\Delta ptsI$) were mixed so as to obtain an approximate 50/50 proportion of persisters (see Materials and Methods). The mixed samples were then assayed for AG potentiation with the differentiating carbon source. If persisters were not influenced by surrounding cells, treatment of wild-type and $\Delta gldA \Delta glpK$ samples with glycerol and KAN or wild-type and $\Delta ptsI$ samples with glucose and KAN would yield a 2-fold reduction in the persister level, and all remaining persisters would be $\Delta gldA \Delta glpK$ or $\Delta ptsI$. In wild-type plus $\Delta gldA \Delta glpK$ samples, upon glycerol and KAN treatment all remaining CFU were $\Delta gldA \Delta glpK$, but a significant portion of $\Delta gldA \Delta glpK$ persisters were eliminated (10-fold reduction) (Fig. 5A). With the controls, we found that AMP failed to eliminate persisters, and KCN prevented AG-dependent killing. These data demonstrated that wild-type persisters were far more susceptible to AG potentiation with glycerol than $\Delta gldA \Delta glpK$ cells in mixed samples, and we identified an interesting phenomenon that was not apparent in the sole $\Delta gldA \Delta glpK$ cultures. Elimination of $\Delta gldA \Delta glpK$ persisters by glycerol and KAN in mixed samples may be attributable to any subpopulation within the wild-type sample (dead, VBNC, or persister cells), since assays performed without those cells failed to reduce CFU (Fig. 4C). We reasoned that reducing the total cell density of the assay would minimize the effects of surrounding cells and enhance the direct effect of the carbon source on persisters. As demonstrated in Fig. 5B, a 10-fold reduction in cell density yielded conditions under which all wild-type persisters in a mixed sample were eliminated by glycerol and KAN, whereas all $\Delta gldA \Delta glpK$ persisters survived. These data demonstrated assay conditions that exclusively reported persister metabolic activity, since persisters must consume the carbon source and drive respiration in order to be eliminated,

and all surviving persisters were those unable to metabolize the carbon source. When these experiments were performed with wild-type plus $\Delta ptsI$ samples (Fig. 5C and D), a significant elimination of $\Delta ptsI$ persisters by glucose and KAN was not observed under the assay conditions with a high or low cell density, verifying that persisters consume glucose to potentiate AG activity.

Since the low-density assay was not amenable to high-throughput screening, due to the need for wash steps to remove KAN, we used the high-density assay as a prescreen, to identify carbon sources with the potential to be metabolized by persisters, followed by the low-density assay for a conclusive demonstration of persister metabolism. We noted that carbon sources that did not reduce CFU in the high-density assay also failed to reduce CFU in the low-density assay, as confirmed with malate and gluconate (Fig. 5E).

Glycerol and glucose are the most desirable carbon sources in different persisters. Due to recent literature suggesting heterogeneity in persister populations (6, 8, 25), we sought to measure the metabolic activity of OFX persisters obtained from exponential-phase cultures. Exponential-phase cells were treated with OFX for 5 h (Fig. 6A), and then the high-density AG potentiation assay was performed as described above. We confirmed that OFX persisters remained nonreplicative, AG potentiation was eliminated by blocking respiration with KCN (Fig. 6B), and that the AG potentiation reflected metabolic activity (Fig. 6C and D). We observed that glycerol and glucose most strongly potentiated AG activity in both AMP and OFX persisters obtained from exponential-phase cultures (Fig. 4B and 6C). Furthermore, AG potentiation largely eliminated OFX persisters when mannitol, pyruvate, succinate, or fructose was present (Fig. 6C; see also Fig. S6B in the supplemental material). For glucose and glycerol, we confirmed with the low-density assay that persisters metabolized these substrates (see Fig. S7 in the supplemental material).

To understand how persister metabolism varies as a function of growth stage, we applied the AG potentiation assay to stationary-phase persisters obtained after 5 h of OFX treatment of overnight cells. Among the tested carbon sources, malate, fumarate, succinate, lactose, arabinose and gluconate did not potentiate the

FIG 4 AG potentiation assay for ampicillin persisters. (A) After exponential-phase cells were treated with AMP for 5 h, cells were incubated with KAN (25 μ g/ml) in M9 minimal medium with different carbon sources using 96-well plates. For controls, AMP (100 μ g/ml) and KCN (1 mM) were used. AMP is required to confirm that persisters are nonreplicative in M9-minimal medium, whereas KCN blocks respiration and eliminates AG potentiation. (B) The survival fraction was monitored based on CFU. (C) Similarly, $\Delta glpK \Delta gldA$ (deficient in glycerol utilization) and $\Delta ptsI$ (deficient in glucose utilization) persisters were incubated with KAN in M9 minimal medium, and the survival fraction was monitored. Error bars indicate the standard errors of the means.

AG activity (see Fig. S8 in the supplemental material). However, glycerol, pyruvate, mannitol, or glucose, or to a lesser extent fructose, efficiently potentiated AG activity (Fig. 7). This was in contrast to the results from exponential-phase persisters, where only glycerol and glucose were the major carbon sources to potentiate AG activity in persisters. For glucose and glycerol, we confirmed with the low-density assay that stationary-phase OFX persisters metabolized these substrates (see Fig. S9 in the supplemental material). These potentiation substrates differed slightly from those identified previously (2), and this difference was likely caused by the assay conditions, which included 100- μ l samples treated in a 96-well plate. For example, increased AG activity in glycerol samples identified here might have been due to more efficient oxygenation, since the glycerol degradation pathway requires glycerol-3-phosphate dehydrogenase, an aerobic respiratory enzyme that catalyzes the oxidation of glycerol-3-phosphate to dihydroxyacetone phosphate.

The AG metabolic assay can be successfully applied to phenotype microarrays. We demonstrated that the AG potentiation method provides information regarding the metabolic capabilities of persisters, and we wanted to translate the high-density portion of the assay to a high-throughput screening format. Using a PM with stationary-phase persisters to OFX (5-h treatment), we tested 95 different carbon sources in a high-throughput manner to iden-

tify substrates that persisters catabolized for respiratory activity (Fig. 8). The controls included a cell suspension without any metabolite but including KAN (Fig. 8, box with bold outline) and analogous plates treated with KAN plus KCN or AMP only. All controls produced negligible killing of persisters (data not shown). We identified that glycerol, glucose, glycerol phosphate, glucose-6-phosphate, and glucose-1-phosphate all effectively potentiated AG activity in persisters. The carbon sources mannose, fructose, sorbitol, pyruvate, methyl pyruvate, lactate, and acetate potentiated AG activity in comparison to the no-carbon control, but did so less effectively than glucose and glycerol. Further, this assay showed that thymidine, uridine, and inosine could potentiate AG activity in persisters, suggesting that persisters may have an active nucleotide salvage pathway (Fig. 8).

After demonstrating that phenotype arrays could be used to perform the high-density assay, we sought to determine whether growth of normal cells on a substrate was predictive of AG potentiation in persisters. Therefore, we measured the ability of normal cells to grow in the PM plate and compared those results to AG potentiation results from persisters. As depicted in Table S2 of the supplemental material, there does appear to be a slight dependency between the ability of a metabolite to support normal cell growth and to potentiate AG against persisters. However, although normal cells can utilize maltotriose as efficiently as glucose,

FIG 5 Competition assay. (A) After 5 h of treatment of exponential-phase cells with AMP, wild-type only, $\Delta gldA \Delta gfpK$ only, and mixed cell cultures (approximate 50/50 proportion of persisters) were treated for 2 h as indicated. (B) The same experiments were repeated following a 10-fold reduction in cell densities. (C and D) Similar experiments were performed for wild-type and $\Delta ptsI$ at both high (C) and low (D) cell densities. When KCN was used with KAN or a carbon source was absent, AG potentiation was eliminated. AMP confirmed that persisters are nonreplicative in M9-minimal medium. (E) AG potentiation of various carbon sources was tested in low-cell-density assays. Note that, in low-cell-density assays, no detectable CFU were counted after 2 h of treatment with KAN and glycerol (B and E). Mixed cultures had the same number of wild-type persisters as wild-type-only cultures and the same number of mutant persisters as mutant-only cultures. Error bars indicate the standard errors of the means.

glycerol, and glucose-6-phosphate for growth, this substrate cannot effectively potentiate AG activity in persisters. Further, specific substrates, such as methyl pyruvate and acetic acid, were poor growth substrates but led to significant potentiation of AG in persisters. This interesting phenomenon, which has not been observed previously, might be clinically desirable, since until now the AG adjuvants identified have all been able to support rapid bacterial growth.

DISCUSSION

Recent studies have found that persisters can be eliminated by targeting their metabolism (2, 3). Persisters are arguably the most clinically important cell type in an antibiotic-treated culture, since they are the only cell type capable of resuming growth once antibiotics have killed all normal cells and been removed from the system. Increased knowledge and understanding of persister metabolism will facilitate the development of antipersister therapies

that increase antibiotic activity in persisters, disrupt persister homeostasis, and/or promote exit from the phenotype. However, measurement of persister metabolism is difficult and must be performed under experimental conditions where alternative cell types (e.g., normal cells and VBNCs) do not interfere with measurements. Unfortunately, current isolation techniques do not provide the purity necessary to perform metabolic measurements directly. The method of Shah and colleagues produced persister samples where ~70% of the population was not antibiotic tolerant (11). Further, the abundance of persisters in their normal cell sample (~2%) was approximately 100-fold higher than the level that is commonly observed from exponentially growing wild-type *E. coli* in rich media (10, 26). This suggests that the antibiotic tolerance measured by Shah and colleagues did not reflect the persistence phenotype. The β -lactam isolation method of Keren and colleagues (15), which we have shown here, produces orders of magnitude more VBNCs than persisters, and the VBNCs retain

FIG 6 AG potentiation assay for ofloxacin persisters. (A to C) OFX persisters (5-h treatment) (A) were similarly incubated with KAN in M9 minimal medium with different carbon sources, and the survival fraction was monitored by CFU (B and C). For controls, AMP only, OFX only, or both KCN and KAN were used (B). (D) Survival fractions for $\Delta gldA \Delta gldK$ and $\Delta ptsI$ persisters were monitored during the AG potentiation assay. Error bars indicate the standard errors of the means.

sufficient metabolic activity to preclude direct measurement of persister metabolism from the samples. Microscopy-based techniques applied to characterize the persister physiology (27, 28) are also difficult to use for isolation due to the similarities between persisters and VBNCs. Both cell types stain as live cells, retain

metabolic activity, and often appear as nongrowing. The main distinction between persisters and VBNCs is the ability of persisters to resume normal growth after antibiotic treatment. However, upon growth resumption, the cell is no longer a persister, and thus experimental conditions where VBNCs are significantly less abundant than persisters are a prerequisite for isolation by microscopy. Unfortunately, we also demonstrated here that under normal culturing conditions, VBNCs are in much greater abundance than persisters in untreated samples.

In the absence of high-fidelity persister isolation techniques, any phenotypic measurement of persister physiology must be based on their ability to tolerate antibiotics and resume growth on standard media. Inspired by the phenomenon of metabolite-enabled AG potentiation, we developed a high-throughput method to measure persister metabolism that is not influenced by the presence of other cell types, including VBNCs. An important aspect to note is that the standard method by which phenotype microarrays measure metabolic activity involves reduction of a water-soluble tetrazolium salt (29), and we demonstrated here that such an assay measures VBNC metabolism and not the metabolism of persisters in antibiotic-treated samples. In contrast, AG potentiation measures the metabolism of persisters, because it is based on a loss of culturability. This loss in culturability of persisters may arise from cell death or a transition to the VBNC state, which are both possible outcomes. Unfortunately, distinguishing between these out-

FIG 7 Metabolic properties of stationary-phase persisters. After stationary-phase cells were treated with OFX for 5 h, cells were incubated with KAN in M9 minimal medium with different carbon sources, and the survival fraction was monitored based on CFU. Error bars indicate the standard errors of the means.

Negative control 0.870±0.049 0.777±0.074	L-Arabinose 0.761±0.062 0.159±0.081	N-Acetyl-D-Glucosamine 0.142±0.072 0.014±0.011	D-Saccharic Acid 0.680±0.193 0.446±0.122	Succinic Acid 0.408±0.136 0.157±0.051	D-Galactose 0.538±0.151 0.375±0.098	L-Aspartic Acid 0.749±0.102 0.393±0.164	L-Proline 0.740±0.119 0.506±0.199	D-Alanine 0.568±0.101 0.450±0.225	D-Trehalose 0.556±0.122 0.326±0.109	D-Mannose 0.161±0.067 0.015±0.008	Dulcitol 0.662±0.134 0.525±0.098
D-Serine 0.394±0.124 0.106±0.051	D-Sorbitol 0.125±0.059 0.021±0.005	Glycerol 0.004±0.002 0.000±0.000	L-Fucose 0.569±0.186 0.308±0.094	D-Gluconic Acid 0.377±0.124 0.147±0.042	D-Gluconic Acid 0.326±0.088 0.097±0.030	D,L-α-Glycerol P. 0.016±0.008 0.000±0.000	D-Xylose 0.679±0.196 0.488±0.209	L-Lactic Acid 0.378±0.122 0.072±0.012	Formic Acid 0.504±0.071 0.430±0.063	D-Mannitol 0.081±0.032 0.006±0.002	L-Glutamic Acid 0.416±0.043 0.552±0.176
D-Glucose-6-Phosphate 0.045±0.018 0.003±0.001	D-Galactonic Acid-γ-Lactone 0.428±0.099 0.393±0.164	D,L-Malic Acid 0.463±0.132 0.378±0.173	D-Ribose 0.480±0.024 0.428±0.150	Tween 20 0.312±0.119 0.231±0.088	L-Rhamnose 0.597±0.131 0.604±0.106	Fructose 0.201±0.091 0.029±0.015	Acetic Acid 0.293±0.210 0.023±0.009	α-D-Glucose 0.073±0.050 0.008±0.003	Maltose 0.437±0.020 0.251±0.039	D-Melibiose 0.668±0.101 0.475±0.089	Thymidine 0.226±0.054 0.072±0.010
L-Asparagine 0.572±0.117 0.349±0.186	D-Aspartic acid 0.580±0.103 0.507±0.107	D-Glucosaminic Acid 0.694±0.170 0.445±0.077	1,2-Propanediol 0.411±0.128 0.481±0.202	Tween 40 0.452±0.171 0.328±0.119	α-Keto-Glutaric Acid 0.366±0.111 0.455±0.126	α-Keto-Butyric Acid 0.716±0.050 0.527±0.166	α-Methyl-D-Galactoside 0.491±0.124 0.518±0.103	α-D-Lactose 0.456±0.089 0.329±0.112	Lactulose 0.374±0.077 0.476±0.151	Sucrose 0.588±0.136 0.542±0.181	Uridine 0.235±0.145 0.056±0.021
L-Glutamine 0.510±0.220 0.310±0.184	m-Tartaric Acid 0.909±0.077 0.566±0.088	D-Glucose-1-Phosphate 0.090±0.062 0.008±0.003	D-Fructose-6-Phosphate 0.311±0.178 0.041±0.016	Tween 80 0.377±0.145 0.294±0.102	α-Hydroxy Glutaric A.-γ-Lactone 0.616±0.186 0.695±0.159	α-Hydroxy Butyric Acid 0.461±0.174 0.430±0.193	β-Methyl-D-Glucoside 0.271±0.150 0.152±0.038	Adonitol 0.444±0.209 0.381±0.080	Maltotriose 0.282±0.192 0.105±0.025	2-Deoxy Adenosine 0.507±0.223 0.112±0.024	Adenosine 0.395±0.173 0.117±0.037
Glycyl-L-Aspartic Acid 0.542±0.208 0.126±0.034	Citric Acid 0.690±0.142 0.505±0.134	m-Inositol 0.520±0.144 0.416±0.083	D-Threonine 0.485±0.238 0.376±0.112	Fumaric Acid 0.615±0.173 0.461±0.220	Bromo Succinic Acid 0.310±0.075 0.440±0.083	Propionic Acid 0.423±0.122 0.447±0.152	Mucic Acid 0.622±0.031 0.534±0.117	Glycolic Acid 0.541±0.111 0.491±0.026	Glyoxylic Acid 0.649±0.195 0.705±0.133	D-Cellobiose 0.511±0.217 0.319±0.065	Inosine 0.228±0.147 0.028±0.013
Glycyl-L-Glutamic Acid 0.564±0.172 0.242±0.107	Tricarballic Acid 0.577±0.125 0.500±0.112	L-Serine 0.476±0.153 0.284±0.132	L-Threonine 0.692±0.212 0.436±0.194	L-Alanine 0.551±0.189 0.244±0.058	L-Alanyl-Glycine 0.486±0.164 0.320±0.061	Acetoacetic acid 0.533±0.162 0.339±0.127	N-Acetyl-β-D-Mannosamine 0.171±0.082 0.068±0.026	Mono Methyl Succinate 0.459±0.138 0.236±0.079	Methyl Pyruvate 0.326±0.131 0.023±0.005	D-Malic Acid 0.895±0.029 0.657±0.137	L-Malic Acid 0.722±0.162 0.317±0.104
Glycyl-L-Proline 0.447±0.157 0.480±0.173	p-Hydroxy Phenyl Acetic Acid 0.541±0.159 0.464±0.113	m-Hydroxy Phenyl Acetic Acid 0.572±0.124 0.479±0.166	Tyramine 0.719±0.092 0.579±0.162	D-Psicose 0.498±0.179 0.255±0.015	L-Lyxose 0.636±0.149 0.426±0.059	Glucuronamide 0.460±0.186 0.226±0.115	Pyruvic acid 0.385±0.148 0.022±0.008	L-Galactonic Acid-γ-Lactone 0.689±0.139 0.544±0.180	D-Galacturonic Acid 0.543±0.253 0.115±0.034	Phenylethyl amine 0.749±0.102 0.548±0.067	2-Aminoethanol 0.611±0.105 0.451±0.038

FIG 8 Persister survival fractions determined in the phenotype microarray. Stationary-phase cells treated with OFX for 5 h were incubated with KAN in M9 minimal medium with different carbon sources in a PM, and the survival fraction was monitored based on CFU. Note that in each cell, the first number indicates the first-hour survival fraction, and the second number indicates the second-hour survival fraction. Black, second-hour survival fraction < 0.01; dark grey, 0.01 < second-hour survival fraction < 0.05; light grey, 0.05 < second-hour survival fraction < 0.1. The no-carbon control is highlighted with bold outline. Error bars indicate the standard errors of the means.

comes is not technically possible, given the extremely low abundance of persisters compared to VBNCs and dead cells in antibiotic-treated samples (see Fig. S10 in the supplemental material). However, regardless of whether a persister dies or becomes a VBNC by AG potentiation, the method described here still measures persister metabolism, since either transition is facilitated by metabolic events within persisters; this was demonstrated both genetically with knockouts and chemically with KCN.

With the high-cell-density AG potentiation assay, we rapidly tested various carbon sources, as a prescreen, to identify those with the potential to be metabolized by persisters. We then performed a low-cell-density AG potentiation assay to unambiguously measure persister metabolism, and we found that glycerol and glucose were the most commonly catabolized carbon sources. We note that the need for both the high-density and low-density AG potentiation assays originated from the scalability of the high-density assay to high-throughput screening, and the results of the competition assay (wild-type and mutant mixed samples), where low-cell-density conditions demonstrated that all surviving persisters were those that could not metabolize the carbon source and that all persisters that could not metabolize the carbon source survived in the assay. Though we did not identify the underlying cause as to why *ΔgldA ΔglpK* persisters were killed in the presence of glycerol, KAN, and antibiotic-treated wild-type cells under high

cell density conditions, we postulate that wild-type VBNCs, which are the dominant metabolically active subpopulation within antibiotic-treated samples (Fig. 2), were most likely metabolizing glycerol and excreting a substrate that *ΔgldA ΔglpK* could consume to drive respiration and AG uptake. The absence of this phenomenon for *ΔptsI* cells in mixed culture may originate from VBNCs not processing glucose to a product that potentiates AG activity in *ΔptsI* persisters.

The results demonstrating that diverse persister populations (exponential- and stationary-phase persisters; AMP and OFX persisters) consume glycerol and glucose suggest that there might be an active core metabolic network in persisters that is independent of the antibiotic tolerance mechanism and growth stage, suggesting the feasibility of a universal persister elimination strategy. In addition, we identified novel carbon sources that potentiate AG activity in persisters, including mannose, sorbitol, acetic acid, lactic acid, methyl pyruvate, inosine, thymidine, and uridine. To confirm that persisters metabolize these carbon sources, low-density assays will need to be performed. Of these carbon sources, methyl pyruvate and acetate are particularly interesting, since they do not support robust growth of *E. coli* but are efficient at potentiating the activity of AGs in persisters. Such metabolic adjuvants are potentially attractive, as they would not facilitate outgrowth of

potential survivors after the majority of persisters had been eliminated.

The technique presented here is the first method to rapidly assay persister metabolism, and we demonstrated that it is amenable to high-throughput screening through the use of phenotype microarrays. This assay, which includes both a high- and low-cell-density portion, provides a platform to more effectively explore the metabolic potential of persisters and provides data in the form of metabolic input (consumed substrate) and output (cytochrome activity). However, the network between input and output in persisters is not delineated with this assay. To explore such connections in persisters, genetic knockouts can be used (2), and we performed such an analysis here to determine that GlpK and not GldA was responsible for glycerol catabolism in persisters (see Fig. S11 in the supplemental material). To comprehensively identify the active metabolic network of persisters, the metabolic data can be analyzed with computational approaches to identify candidate networks capable of generating the metabolic output from the input. These candidate networks can then be analyzed to identify genetic perturbations for validation. In this manner, an iterative experimental and computational approach can be employed to reconstruct the metabolic networks of persisters and uncover novel strategies to eliminate persisters as a source of chronic and recurrent infection.

ACKNOWLEDGMENTS

We thank Christina J. DeCoste for technical support with flow cytometry and James J. Collins and Kyle R. Allison for providing the MG1655 *hipA7* strain. We also acknowledge Brittany Williams and Nicolas Ugaz for their help with the WST-1 assay. We thank the National BioResource Project (NIG, Japan) for their support of the distribution of the Keio collection.

Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under award number R21AI105342, the Department of the Army under award number W81XWH-12-2-0138, and with startup funds from Princeton University.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the funding agencies.

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