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Persister formation in *Staphylococcus aureus* is associated with ATP depletion

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Persisters are dormant phenotypic variants of bacterial cells that are tolerant to killing by antibiotics[1]. Persisters are associated with chronic infections and antibiotic treatment failure[1–3]. In *Escherichia coli*, toxin–antitoxin modules have been linked to persister formation[4–6]. The mechanism of persister formation in Gram-positive bacteria is unknown. *Staphylococcus aureus* is a major human pathogen, responsible for a variety of chronic and relapsing infections such as osteomyelitis, endocarditis and infections of implanted devices. Deleting toxin–antitoxin modules in *S. aureus* did not affect the level of persisters. Here, we show that *S. aureus* persisters are produced due to a stochastic entrance into the stationary phase accompanied by a drop in intracellular adenosine triphosphate. Cells expressing stationary-state markers are present throughout the growth phase, and increase in frequency with cell density. Cell sorting revealed that the expression of stationary markers is associated with a 100–1,000-fold increase in the likelihood of survival to antibiotic challenge. The adenosine triphosphate level of the cell is predictive of bactericidal antibiotic efficacy and explains bacterial tolerance to antibiotics.

Antibiotic resistance is a major human health problem[7]. However, most pathogens that cause hard-to-treat chronic infections are not drug-resistant[1,3,8]. There is mounting evidence that drug-tolerant persister cells contribute to this phenomenon[2,9–12]. Persister cells are phenotypic variants that survive lethal doses of antibiotics and are genetically identical to their drug-susceptible kin. The mechanism of persister formation has been extensively studied in the closely related Gram-negative organisms *Escherichia coli* and *Salmonella* Typhimurium[1,13,14].

In *E. coli*, isolated persisters express toxin–antitoxin (TA) modules[15], most of which code for mRNA endonucleases called interferases[16]. Although deletion of individual interferases has no phenotype, a knockout of ten TAs produced a decrease in persisters in both a growing culture and in the stationary phase[4]. A small fraction of persisters form in *E. coli* when cells stochastically express the HipA toxin[12]. HipA is a protein kinase[17] that phosphorylates glutamyl aminoacyl-tRNA synthetase, inhibiting protein synthesis[18,19]. Selection for increased drug tolerance *in vitro* led to the identification of a *hipA7* mutant allele that produces up to 1,000-fold more persisters than the wild type[6]. We recently identified *hipA7* strains among patients with chronic urinary tract infections[12]. Similarly, *hip* mutants are common among isolates of *Pseudomonas aeruginosa* from patients with cystic fibrosis[11] and from patients with chronic *Candida albicans* infections[20]. In *S.* Typhimurium, TA modules are responsible for a sharp increase in persisters when the pathogen infects macrophages[9]. These findings provide a link between persisters and clinical manifestation of disease. Little is known about the mechanism of persister formation in Gram-positive species.

We first sought to examine the role of TAs in persister formation in *S. aureus*. There are three known type II TAs in *S. aureus*: *mazEF*, *relBE* homologues *axe1/txe1* and *axe2/txe2*. These are the only three TAs predicted in *S. aureus* 8325, the parental strain of HG001 and HG003. An additional phage-associated TA has been identified in *S. aureus* Newman using the TAfinder tool[21] but overexpression of the potential toxin did not inhibit growth (Supplementary Fig. 1). We therefore continued with analysis of the three active type II TAs. The toxins from all three modules are RNA endonucleases[22]. We constructed a triple knockout in the TAs (Δ3TA) and examined the strain's ability to form persisters. Ciprofloxacin causes a characteristic biphasic killing of wild-type *S. aureus* with a subpopulation of surviving persisters (Supplementary Fig. 2 and Fig. 1a). Unexpectedly, knockout of all TAs had no effect on the level of persisters in exponentially growing or stationary-phase cells (Fig. 1a and Supplementary Fig. 3). A similar result was obtained with oxacillin, vancomycin and rifampicin (Fig. 1b). This is in stark contrast to *E. coli*, where a knockout of ten toxin endonucleases produces a decrease in persisters[4]. It remains possible that these TAs or as yet unannotated TAs play a role in persister formation under a specific environmental condition, but we see no evidence of a role for the TAs we examined, in persister formation under regular growth conditions.

The stringent response has also been linked to persister formation in *E. coli*[23]. In *S. aureus*, Rsh (a homologue of RelA/SpoT) contains both a synthase and hydrolase domain for (p)ppGpp (ref. 24). In response to starvation, the level of (p)ppGpp rises, leading to downregulation of genes involved in proliferation, protein synthesis and replication, and increased expression of genes involved in survival and stress responses. Most of these genes are regulated by the repressor CodY (ref. 24). We tested persister levels in a *codY* mutant and in an *rsh* mutant (rsh_{syn}) that has a mutated synthase domain and does not produce an increase in (p)ppGpp in response to amino-acid starvation[24]. There was no effect of either a *codY* or an rsh_{syn} mutation on the level of persisters in the exponential and stationary phases of growth (Fig. 1c,d). *S. aureus* possesses two minor ppGpp synthases, RelP and RelQ, that have been associated with the response to cell wall stress. We examined persister formation in a strain deleted in rsh_{syn}, *relP* and *relQ*. Again, this strain produced persisters tolerant to ciprofloxacin or gentamicin at levels similar to those of the wild type (Supplementary Fig. 2). The mutant did not exhibit a growth defect and entered the stationary phase in a similar manner to the wild type, suggesting that the stringent response is not a significant regulator of the stationary phase under these conditions (Supplementary Fig. 3).

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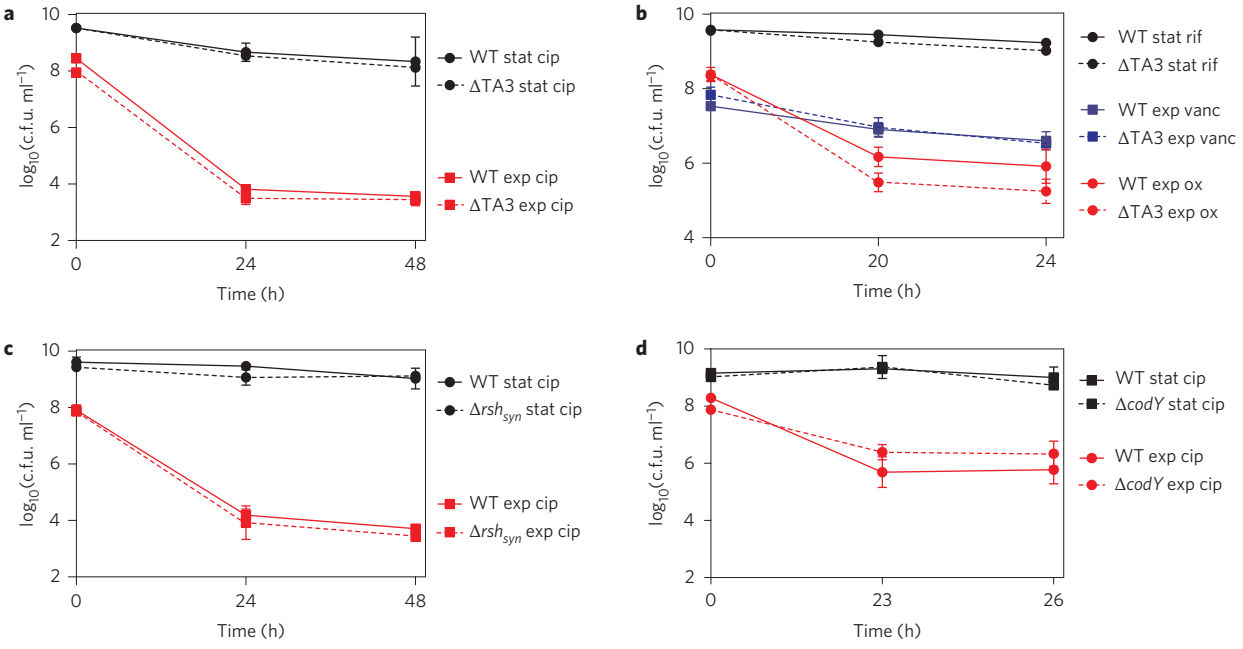

Figure 1 | Toxin–antitoxin modules and stringent response do not control persister formation in *S. aureus*. **a**–**d**, The contribution of TA modules *mazEF*, *axe1-txe1* and *axe2-txe2* in strain Newman (**a**,**b**), the stringent response element *rsh* in strain HG001 (**c**) and the stringent response regulator *codY* in strain SH1000 (**d**) to persister formation in *S. aureus*. Strains were grown for 4 h to the mid-exponential phase (exp) or overnight to the stationary (stat) phase in Mueller–Hinton broth (MHB) and challenged with either ciprofloxacin (cip), vancomycin (vanc), oxacillin (ox) or rifampicin (rif) (10 × MIC). Aliquots were removed at the indicated time points, washed and plated to enumerate survivors. All experiments were performed in biological triplicates. Error bars represent standard deviations.

It is known that *S. aureus* exhibits complete tolerance to many antibiotics in the stationary state, which is another important distinction between this pathogen and *E. coli*[2,25]. It appears that *S. aureus* cells in a stationary state exhibit antibiotic tolerance similar to persisters. We reasoned that persisters in the exponential phase may be cells that have entered the stationary phase early. To examine this we used two reporters of the stationary phase. The promoter of the capsular polysaccharide operon, P*cap5A*, has been shown to be activated in the stationary phase[26,27]. An increase in relative fluorescence of a strain carrying P*cap5A*-GFP over time in a growing culture confirmed the suitability of this promoter as a marker of the stationary phase (Fig. 2a,b). The promoter of the arginine deiminase pathway, P*arcA*, was used as a second marker, because proteomic analysis showed that the ArcA protein accumulates specifically in the stationary phase, increasing in abundance 10.5-fold relative to the exponential phase. Analysis of P*arcA* fused to *gfp* confirmed that this promoter is activated specifically in the stationary phase (Supplementary Fig. 4). Analysis with real-time, quantitative, reverse transcription PCR (qRT-PCR) showed that transcript levels of *cap5A* and *arcA* increase 3.88- and 25.38-fold, respectively, in the stationary phase. These promoters were inserted upstream of gfp_{uvr} in plasmid pALC1434 to yield P*cap5A*::*gfp* and P*arcA*::*gfp*.

Flow cytometry was then used to track cells expressing high levels of the stationary-phase markers (termed bright) at hourly intervals from the early exponential phase to the stationary phase (Fig. 2c,d). We found that a subpopulation of cells express stationary markers in the early exponential phase, and their frequency increases with the rise in the density of the population (Fig. 2e). This suggests that the stationary phase does not initiate in a uniform manner, but is a heterogeneous process.

We next sought to determine whether the subpopulation of stationary-phase cells in a growing culture were in fact persisters. For this, we used fluorescence-activated cell sorting (FACS). *S. aureus* HG003 P*cap5A*::*gfp* or HG003 P*arcA*::*gfp* were grown to the mid-exponential or stationary phase and analysed by FACS (Fig. 3a,b). To examine whether the bright cells were persisters, the exponential-phase culture was exposed to a lethal dose of ciprofloxacin (10 × MIC, minimal inhibitory concentration) for 24 h. The culture was then re-analysed by FACS, and cells were gated into 'bright', 'middle' and 'dim' populations based on the expression of P*cap5A*::*gfp* or P*arcA*::*gfp* (Fig. 3a,b). Cells were then sorted onto Mueller–Hinton agar (MHA) in 96 spots to enumerate survivors from each population (32 spots for each population: bright, middle, dim). The lethal dose of ciprofloxacin causes ~3 logs of killing in the total culture, so cells were sorted onto MHA plates at 1, 10, 100, 1,000 and 5,000 per spot to achieve viable counts for each population (representative plate, Fig. 3c,d). The bright population had 100–1,000-fold more survivors than the middle and dim populations with both markers. We chose to compare only the middle and bright fractions for quantification as the dim fraction had <100% sorting efficiency (Fig. 3e,f).

To determine whether the expression of capsular polysaccharide contributes to ciprofloxacin tolerance, we transformed plasmid P*cap5A*::*gfp* into a *cap5A* mutant strain and repeated the cell sorting experiment. Disrupting the *cap5A* gene did not alter the expression profiles of P*cap5A*::*gfp* (Supplementary Fig. 5). Similarly, the bright cells in a *cap5A* mutant also exhibited a 100-fold enrichment for cells tolerant to ciprofloxacin in the exponential phase compared to the middle fraction, showing that entry into the stationary phase—rather than levels of the CapA protein—affect persister formation (Fig. 3e). We also examined persister formation in an *arcA* mutant and found it to be similar to the wild-type strain (Supplementary Fig. 6). As a control for stationary-phase reporters, we repeated the experiment using a promoter that is also expressed in the exponential phase (P*spa*::*gfp*). In this case, the bright population had no enrichment of persisters compared to the middle of the population (Supplementary Fig. 5). This shows that expression of a stationary marker, rather than expression of GFP per se, determines whether a cell is a persister.

We wanted to further examine any potential role for the stringent response and tested the expression of the persister markers in the

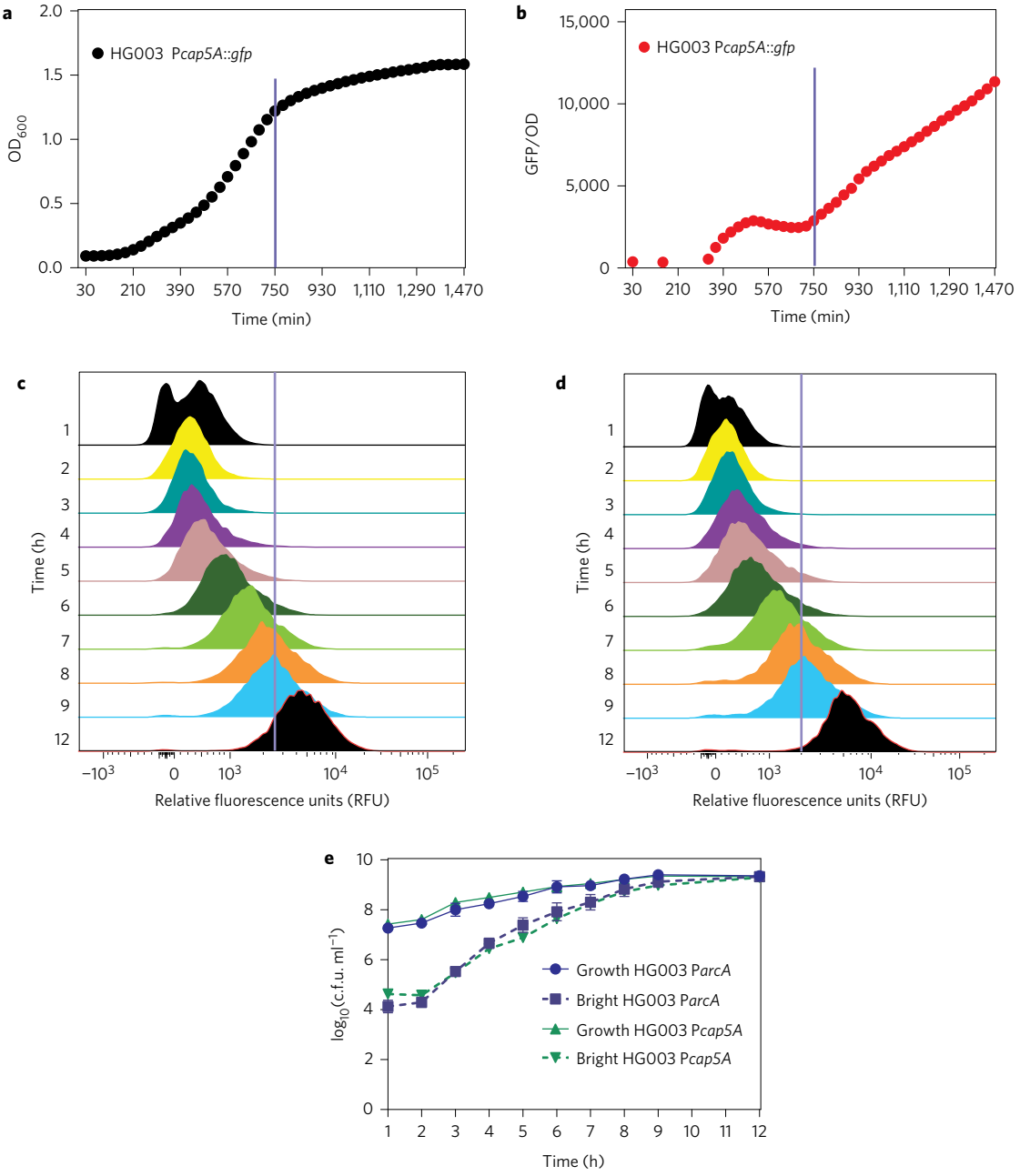

Figure 2 | Activation of stationary markers is heterogeneous. a,**b**, Growth (optical density at 600 nm, OD_{600}) (**a**) and GFP expression of HG003 P*cap5A*::*gfp* (**b**) versus time. Blue vertical lines represent entrance into the stationary phase. **c**,**d**, Distribution of the GFP signal in P*cap5A*::*gfp* (**c**) and P*arcA*::*gfp* (**d**) at hourly intervals. The cutoff for the bright fraction is indicated by a blue vertical line representing the fluorescence intensity of 90% of cells in a stationary-phase population. This cutoff represents the level of expression in a stationary-phase culture. **e**, A subpopulation of stationary-phase cells, defined as cells with stationary-phase levels of expression of P*arcA* and P*cap5A*, is always present and increases with population density. All experiments were performed in biological triplicates. Standard deviations are indicated. The results in **c** and **d** are representative of one replicate.

rsh_{syn} mutant background. Neither *cap5A* nor *arcA* promoter activity was significantly affected by mutation of rsh_{syn} (Supplementary Fig. 4).

We reasoned that a decrease in the energy level of the cell in the stationary phase could lead to antibiotic tolerance. Killing by bactericidal antibiotics results from corrupting active targets[1]. Aminoglycosides kill by causing mistranslation, which leads to the production of toxic peptides[28]. Fluoroquinolones inhibit the religation step of DNA gyrase and topoisomerase, causing double strand breaks[29]. β-Lactams lead to a futile cycle of peptidoglycan synthesis and autolysis[30]. A decrease in adenosine tri-phosphate (ATP) would decrease the activity of ATP-dependent antibiotic targets such as gyrase, topoisomerase and RNA polymerase, leading to antibiotic tolerance, and ATP has previously been suggested to impact survival to antibiotics[5,31,32,33].

We examined the ATP levels of an exponential- and stationary-phase population and indeed found that ATP levels decrease significantly in the stationary phase (Fig. 4a). We then found that emulating stationary-phase ATP levels in an exponential-phase population by decreasing it with arsenate resulted in a 325-fold induction in persister formation (Fig. 4b). ATP levels are lowered by arsenate as it forms a rapidly hydrolysable adenosine di-phosphate (ADP)-As, producing a futile cycle[34]. Interestingly, we found that stationary-phase-specific promoters were also activated in response to arsenate (Fig. 4c). Hence, these promoters are

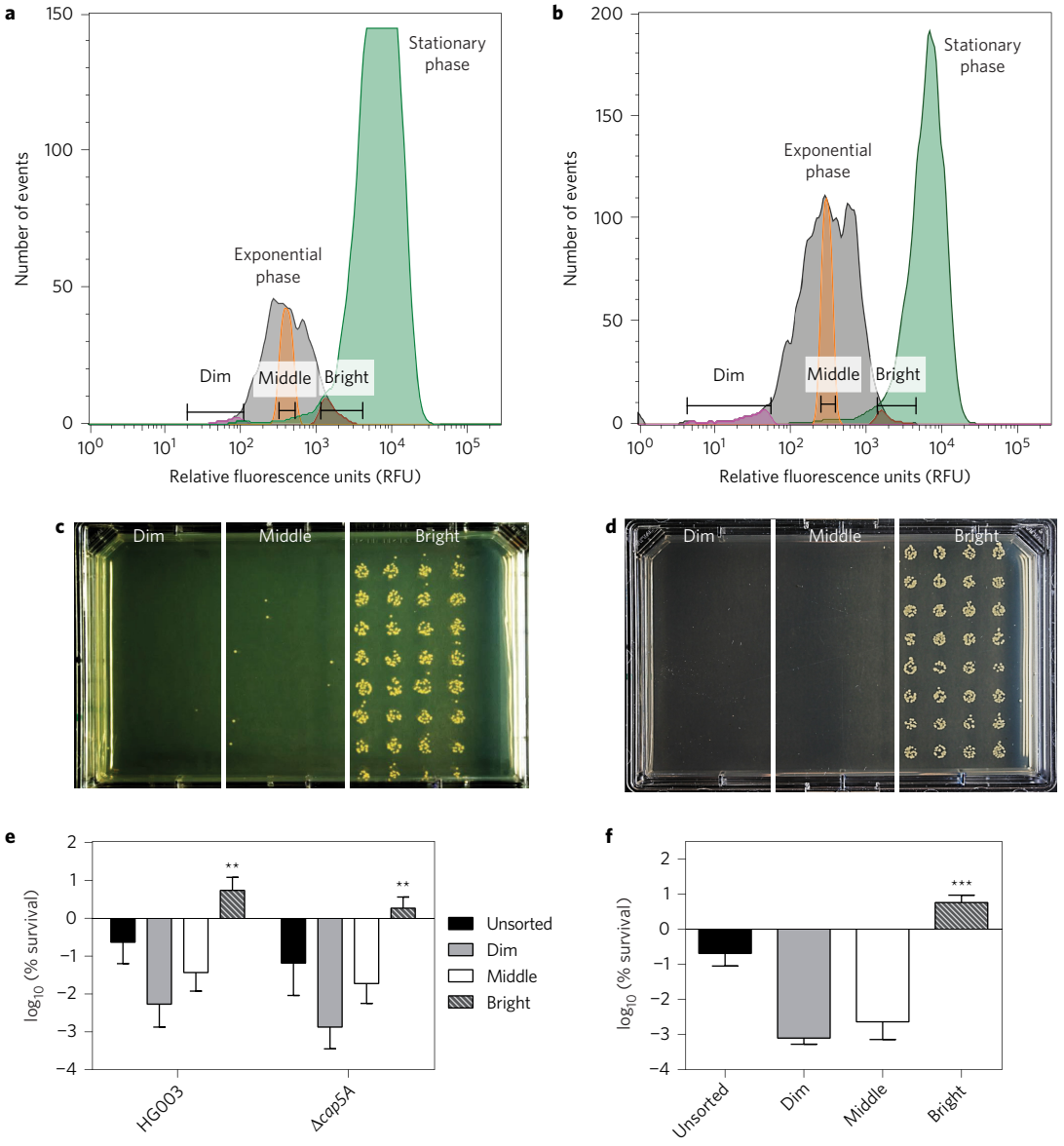

Figure 3 | Persister sorting using stationary markers P*cap5A* and P*arcA*. a,b, Expression of P*cap5A*::*gfp* (**a**) or P*arcA*::*gfp* (**b**) in the exponential phase following ciprofloxacin challenge (grey peak) and in the stationary phase (green peak), measured by FACS. Exponential-phase cells were gated into three populations depending on the expression of GFP: dim (pink peak), middle (orange peak) or bright (red peak, cells expressing stationary-phase levels of reporter in the exponential phase). **c**,**d**, Cells were sorted based on dim, middle or bright GFP expression onto MHA plates at 1,000 events/spot for P*cap5A*::*gfp* (**c**) and P*arcA*::*gfp* (**d**). Representative plates are shown. **e**,**f**, Survivors from each population of HG003 or Δ*cap5A* harbouring P*cap5A*::*gfp* (**e**) and P*arcA*::*gfp* (**f**) were counted following incubation overnight at 37 °C. Asterisks indicate statistical significance between middle and bright populations, determined using Student's *t* test (**$P < 0.005$ or ***$P < 0.0005$). All experiments were performed in biological triplicates. Standard deviations are indicated. The results in **a**–**d** are representative of one replicate.

activated in the stationary phase as ATP levels in the cells drop. The P*cap5A* and P*arcA* promoters then enable single-cell detection of ATP, linking a decrease in the energy level to antibiotic tolerance in individual persisters.

It was thus clear that cells with reduced ATP levels are antibiotic-tolerant and express markers of this phenotypic state. What remained unclear was whether a transcriptional response was necessary for persister formation. To examine this, we again induced persister formation with arsenate, but we also included a 15 min pre-incubation with rifampicin at 1 × MIC, which was sufficient to inhibit the induction of stationary markers (Fig. 4c) but did not cause cell death (Supplementary Fig. 7). Inhibition of transcription did not impede persister induction (Fig. 4d) (Supplementary Fig. 7). This shows that, although a specific transcriptional response that includes expression of P*cap5A* and P*arcA* is induced in response to low ATP, this response is not required for antibiotic tolerance. Rather, tolerance of both stationary populations and persisters can be explained by a drop in ATP, which will result in a decrease in the activity of drug targets. To further test whether ATP levels determine persister formation, we examined killing in a medium where ATP concentration is expected to increase. Supplementing tryptic soy broth (TSB) medium with glucose increased ATP significantly and resulted in a 100-fold reduction in persisters (Supplementary Fig. 8).

Promoters of *arcA* and *cap5A* are induced when ATP drops in the stationary phase or in the presence of arsenate. Cells expressing these markers are highly enriched for persisters. Low ATP can lead to tolerance of a stationary culture, and explains antibiotic tolerance of a persister subpopulation. This work links the phenomena of

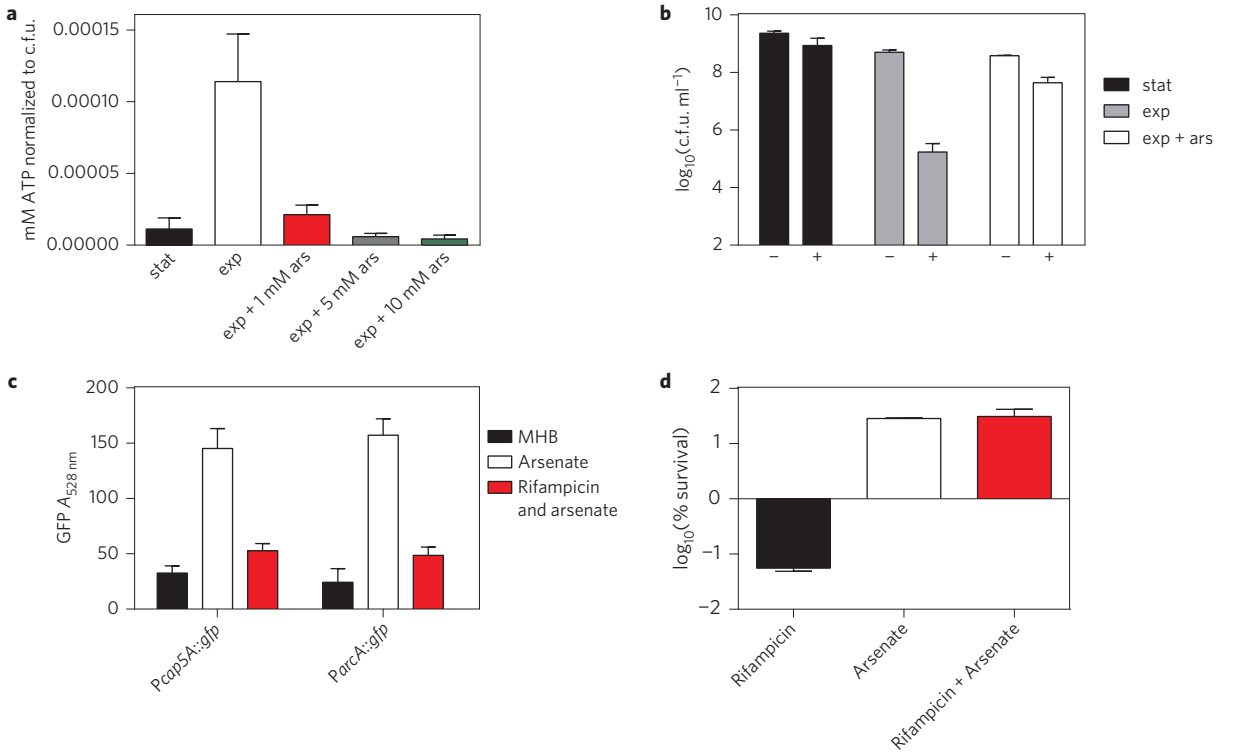

Figure 4 | Reduction in ATP induces persister formation and expression of stationary-phase markers. a, Titering arsenate to produce stationary-phase levels of ATP. Arsenate was added to an exponential-phase population of *S. aureus* for 15 min before measuring ATP. **b**, A decrease in ATP results in a 325-fold induction of persisters in the exponential phase. On the *x* axis '−' indicates cell count before addition of ciprofloxacin and '+' represents cell count after 24 h incubation in 10 × MIC of ciprofloxacin (0.4 μg ml^{-1}). **c**, P*cap5A*::*gfp* and P*arcA*::*gfp* are induced by depletion of ATP. **d**, Inhibition of transcriptional response by adding 0.1 μg ml^{-1} rifampicin 15 min prior to ciprofloxacin does not protect from killing. ATP depletion (30 min of 1 mM arsenate) protects against ciprofloxacin killing. Addition of rifampicin 15 min before ATP depletion does not inhibit this persister induction. Results are represented as log % survival after 24 h ciprofloxacin treatment. All experiments were performed in biological triplicates. Standard deviations are shown.

population-wide tolerance and persister cell tolerance. A growing population contains cells that enter into the stationary state early and these become antibiotic-tolerant persisters. Persisters form as cells lose ATP. Entrance into the stationary state is stochastic, with the frequency of persisters increasing with cell density.

Our measurements of ATP in single persister cells by FACS have been performed with two different reporters, P*arcA*-GFP and P*cap5A*-GFP. Both are ATP sensors, but the detection requires transcription and translation of GFP. To establish direct causality, it would be interesting to perform single-cell detection of ATP in persisters more directly, such as with a fluorescence resonance energy transfer (FRET)-based sensor[35], once it is adapted to *S. aureus*.

Interestingly, tolerance to clinically relevant daptomycin was also observed in the stationary phase[36]. Also, a recent study shows that altered levels of inorganic phosphate and polyphosphate in daptomycin-tolerant cells could be related to a depletion of ATP (ref. 37).

A recent study shows that population heterogeneity and capsular polysaccharide expressing subpopulations also occur *in vivo* in persistent carriers of *S. aureus*[27]. The role of ATP levels in the recalcitrance of *S. aureus* infection should be examined, and ATP levels of cells during infection may be an important determinant of the success or failure of antibiotic therapy.

Understanding how persisters form will improve our ability to control chronic infections. We recently identified a compound capable of killing persisters, acyldepsipeptide 4 (ADEP4). ADEP4 targets ClpP and converts it into a non-specific protease, which forces both growing and dormant cells to self-digest[2]. Importantly, ADEP4 dissociates the protease from its ATP-dependent chaperones, and the dysregulated proteolysis does not require ATP. In combination with rifampicin (to decrease resistance development), ADEP4 eradicated a biofilm both *in vitro* and in a mouse model of a chronic *S. aureus* infection. This shows that persisters can be killed by a compound that does not require an ATP-dependent target. In this regard, it is interesting to note that stationary cells of *S. aureus* exhibit considerable tolerance to daptomycin, a membrane-acting antibiotic[36,37]. Why dormant cells would be tolerant to this compound is an interesting problem that remains to be solved.

This study suggests that a new mechanism of persister formation, loss of energy leading to drug tolerance, operates in *S. aureus*. It is possible that this is a general mechanism of tolerance that also governs persister formation in other bacteria.

Methods

Bacterial strains and growth conditions. *S. aureus* were cultured in Mueller–Hinton broth (MHB) or tryptic soy broth (TSB), with or without added glucose. TSB and TSB without glucose were buffered to pH 7.0 using 100 mM MOPS. Bacteria were routinely grown at 37 °C and 225 r.p.m. Media were supplemented with chloramphenicol (10 μg ml^{-1}) to maintain plasmids where necessary. MSSA strains Newman, SH1000 and HG001 were used to analyse the role of TA modules and stringent response as mutations of interest had previously been constructed and characterized in these backgrounds[22,24,38]. The model strain HG003 was used for all subsequent experiments. For *E. coli* experiments, growth of the overexpression strain was compared to an empty vector control in a plate reader over 16 h at 37 °C in LB medium supplemented with 0.2% arabinose.

Strain construction. For construction of reporter plasmids, primers P*cap5A*_1 (gcgcgaattctctatctgataataatcatc), P*cap5A*_2 (ggcctctagactaatgtactttccattatt), P*spa*_1 (gcaggaattctttccgaaattaaacctcagc), P*spa*_2 (gcagtctagaattaataccccctgtatgta), P*arcA*_1 (gcgcgaattcaaaatgtatatttgaccca) and P*arcA*_2 (ggcctctagatctatttcctccttttatct) flanked by restriction sites *EcoRI* and *XbaI* were used to amplify predicted promoter sequences of *cap5A*, *spa* and *arcA*, respectively. The promoter regions were cloned upstream of gfp_{uvr} into the *EcoRI* and *XbaI* sites of plasmid pALC1434 (ref. 39).

A Newman strain was created containing deletions for all three known type II TA systems (Newman ΔTA3). Using Newman Δ*mazEF* (ALC4072) as a starting strain, the *axe1*/*txe1* and *axe2*/*txe2* operons were deleted by sequential allelic exchange using the pMAD plasmids pALC6480 and pALC6481 (ref. 40), respectively. Deletion of these genes was verified by PCR analysis and chromosomal DNA sequencing. For hypothetical toxin overexpression, the primers P*tox*_1 gcgcgaattcatggaagaaactttaa and P*tox*_2 gcgcggtaccttatgcaatttaaaaa were used to amplify the toxin and the fragment was digested with *EcoRI* and *KpnI* and cloned into the pBAD33 vector upstream of an arabinose-inducible promoter, digested with the same restriction enzymes.

Persister assays. Strains were grown to the mid-exponential or stationary phase (~16 h) in MHB in 14 ml round-bottomed snap-cap culture tubes. Cells were plated for colony-forming unit (c.f.u.) counts and challenged with the antibiotics ciprofloxacin, rifampicin, vancomycin, gentamicin or oxacillin (4.0, 0.4, 10, 5 and 1.5 μg ml^{-1}, respectively). At each timepoint, an aliquot of cells was removed, washed with 1% NaCl and plated to enumerate survivors. All experiments were performed in biological triplicates. Averages and standard deviations are representative of three biological replicates. Rifampicin-resistant mutants arise spontaneously at a frequency of ~2.3×10^{-8}. Rifampicin killing in the exponential phase selected for the proliferation of rifampicin-resistant mutants, which had repopulated the exponential-phase cultures by 24 h (Supplementary Fig. 9). For this reason, levels of persisters tolerant to rifampicin were examined in the stationary phase only.

Arsenate and rifampicin persister assays. Strains were grown to the mid-exponential phase in MHB media. Where indicated, rifampicin (0.01 μg ml^{-1}) was added for 15 min and/or arsenate (1 mM) for 30 min before ciprofloxacin challenge for 24 h (10 × MIC).

Flow cytometry and FACS analysis using *gfp* reporters. Fluorescent protein level was analysed with a BD Aria II flow cytometer (BD Biosciences) with a 70 μm nozzle. Cell population was detected using using forward scatter (FSC) and side scatter (SSC) parameters and fluorescence was analysed with an emitting laser of 488 nm and bandpass filter of 525/15 nm, using a FACS ARIA II (Becton Dickinson). Strains harbouring plasmids P*cap5A*::*gfp*, P*arcA*::*gfp* or P*spa*::*gfp* were grown to the mid-exponential and stationary phase in MHB containing 10 μg ml^{-1} chloramphenicol. For growth curve construction, the population was gated so that over 90% of the stationary-phase population was designated bright. These gates were applied to all timepoints. At each timepoint, c.f.u.s were measured and the number of stationary-phase cells was calculated by multiplying the percentage of cells in the bright fraction by the total cell number. An overnight culture was subcultured 1:100 into fresh MHB and grown for 3 h. A volume of 300 μl of this was added to 3 ml of MHB to begin the growth curve. This subculturing step removed any carry-over of stationary-phase cells from the stationary-phase culture. For FACS analysis of persisters, strains were exposed to ciprofloxacin for 24 h. Before the challenge, an aliquot of the culture was diluted and plated for c.f.u. counts. Challenged cells were washed and plated to enumerate survivors. Cells pre- and post-antibiotic challenge were analysed by FACS. A gate was drawn based on stationary-phase expression of P*cap5A*::*gfp* or P*arcA*::*gfp*. Exponential-phase cells expressing stationary-phase levels of P*cap5A*::*gfp* or P*arcA*::*gfp* were termed bright. Two gates were drawn within the exponential-phase P*cap5A*::*gfp* expression peak and termed middle and dim, respectively. To calculate the percentage survival of each population following antibiotic challenge, we first calculated the sorting efficiency from each population before antibiotic challenge. Events (cells) from each population were sorted in a 96-well format with 32 spots for each population; dim, middle and bright. One event per spot (for 32 spots) and colonies were counted following incubation. For the middle and bright fractions we achieved 100% sorting efficiency (32 colonies), but the sorting efficiency for the dim fraction was lower, ~90% or 29 colonies. This indicated that not all events in the 'dim' fraction were cells. For this reason we chose to focus on the differences between the bright population and the middle or bulk of the population. Following antibiotic challenge, cells (events) from each population were sorted onto MHA plates in a 96-well format at 1, 10, 100, 1,000 and 5,000 per spot (32 spots/population) to enumerate survivors. A similar method was applied for all reporters. Ciprofloxacin treatment did not affect the expression of any reporters used in this study. A control experiment was performed where samples were sonicated for 5 min in a sonicating water bath before cell sorting. Sonication had no impact on the sorting results, confirming that cell aggregation was not influencing the FACS experiments. Cells were analysed and sorted using FACS-Diva software. Figures were generated using FlowJo software. Experiments were performed in triplicate. Error bars represent the standard deviations of the means and statistical significance was determined by the Student's *t* test.

Proteomic analysis. Biological duplicates were grown in MHB and collected in the mid-exponential and stationary phases of growth. Samples were labelled and fractionated and mass spectrometry was performed as previously described[2].

Real-time qRT–PCR. RNA was isolated from the exponential-phase population after 4 h of growth and from the stationary phase after 16 h of growth using a QIAGEN RNA purification kit. Samples were treated with Turbo DNase, and RNA integrity was confirmed on a bioanalyser. Reverse transcriptase was used to convert to cDNA according to the manufacturer's instructions. Serial tenfold dilutions of genomic DNA were used to construct standard curves for each set of primers. qRT–PCR was performed using SYBR green enzyme. Fold change was calculated based on the cycle number required to achieve a pre-designated quantity of signal normalized to a 16S rRNA control.

ATP assays. ATP levels of the stationary and mid-exponential cultures with the addition of various concentrations of arsenate were measured using a Promega BacTiter Glo kit according to the manufacturer's instructions.

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Author contributions

B.P.C. and S.E.R. designed the study, performed experiments, analysed results and wrote the paper. A.B.G., A.S.N. and E.A.Z. performed experiments. N.P.G. created the triple TA mutant strain. G.C. and J.N.A. designed the study and analysed results. A.L.C. designed the study. K.L. designed the study, analysed results and wrote the paper.

Additional information

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Competing interests

The authors declare no competing financial interests.