Magnesium Links Starvation-Mediated Antibiotic Persistence to ATP

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ABSTRACT Bacterial persisters emerge and increase in numbers over time as a bacterial culture grows from log phase to stationary phase. However, the underlying basis of the inevitable tendency is unclear. In this study, we investigated the role of nutrients in starvation-mediated persister formation of Staphylococcus aureus. By screening of nutrient components, we found that starvation-induced persister formation of log-phase cultures could be reversed by addition of magnesium (Mg$$^{2+}$$) but not amino acids, nucleotides, or other salts. Further, deprivation of extracellular Mg$$^{2+}$$ reduced cytoplasmic ATP, inducing persistence without affecting cytoplasmic Mg$$^{2+}$$ or membrane potential. Finally, we showed that Mg$$^{2+}$$ reduced expression of stationary cell marker genes, capSA and arcA. These findings indicate a connection between Mg$$^{2+}$$ levels and ATP, which represents metabolic status and mediates antibiotic persistence during growth.

IMPORTANCE Various genes have been identified to be involved in bacterial persister formation regardless of the presence or absence of persister genes. Despite recent discoveries of the roles of ATP and membrane potential in persister formation, the key element that triggers change of ATP or membrane potential remains elusive. Our work demonstrates that Mg$$^{2+}$$ instead of other ions or nutrient components is the key element for persistence by inducing a decrease of cytoplasmic ATP, which subsequently induces persister formation. In addition, we observed tight regulation of genes for Mg$$^{2+}$$ transport in different growth phases in S. aureus. These findings indicate that despite being a key nutrient, Mg$$^{2+}$$ also served as a key signal in persister formation during growth.

KEYWORDS ATP, Staphylococcus aureus, antibiotic persistence, magnesium
poly(dC)/RmlB transduces signals of starvation to mediated persistence in *Pseudomonas aeruginosa*. Two expected consequences of starvation, decreases in ATP levels and membrane potential, proved to be two important aspects in persister formation (3, 10, 11). However, there are still missing links such as signal sensing and regulatory mechanisms between starvation and ATP/membrane potential-mediated persister formation.

To study the effects of starvation on *Staphylococcus aureus* persister cell formation, we examined persister levels of log-phase cultures under nutrient deprivation by resuspending the bacterial cells in saline or supernatants of stationary-phase cultures. As shown in Fig. 1a to d, treatment of log-phase cultures with stationary-phase supernatants significantly induced persister formation compared to that with log-phase supernatants, and treatment with saline caused even stronger induction of persisters. To investigate the key nutrient that mediates persister formation, we used a chemically defined medium (Hussain-Hastings-White modified medium [HHWm]) that supports *S. aureus* growth and persister formation (see Fig. S1 in the supplemental material) (12).
The components of HHWM were grouped into six groups including amino acids, sugar, major salts, trace salts, nucleotides, and trace compounds (concentrations of each component are listed in Table S1). By testing the persister formation of log-phase S. aureus treated with saline or saline supplemented with different nutrient groups diluted to the same concentration as in HHWM, we found that only the major salt group reduced persistence (Fig. 1e). A further dissection with each salt component showed that it was MgSO4 that dampened induction of persisters (Fig. 1f). Since an extra control Na2SO4 showed no effect in reversing persister formation, it was confirmed that Mg2+ but not SO42− was the functioning factor. Indeed, addition of Mg2+ up to 5 mM showed the strongest inhibition of persister formation (Fig. 1g). We further showed that the effects of Mg2+ are applicable in different S. aureus strains, including methicillin-resistant strains USA300_FPR3757, USA500 (13), and a reference model strain, HG003 (Fig. S2) (14). Treatment with EDTA, a cation-chelating agent, significantly enhanced persister formation, which could be inhibited by Mg2+. Although Ca2+ was unable to hinder persister induction by nutrition depletion, it could free Mg2+ and offset the effects of EDTA on persister formation (Fig. 1h), probably by its higher affinity for EDTA than Mg2+ (15).

We then tested whether disruption of magnesium transport or efflux could affect persister formation. MgtE is a vital Mg2+ transporter in S. aureus Newman (16), and YhdP is the efflux pump for Mg2+ (17). We constructed a mutant strain of yhdP by homologous recombination but could not obtain a knockout mutant of mgtE. Instead, we used an antisense RNA (asRNA) plasmid that targets mgtE to address the role of mgtE in persister formation. Silencing of the essential mgtE inhibited growth, while the yhdP mutant showed no growth defect (Fig. S3). Silencing mgtE caused significant increase of persisters, while mutation of YhdP did not affect persister formation of either log- or stationary-phase cultures. As a control, asRNA against yycF, which is also an essential gene, did not increase persister formation (Fig. 1i).

Mg2+ is the most abundant multivalent cation in all living cells. Bacteria maintain a high concentration of cytoplasmic Mg2+, which results in a concentration gradient up to hundreds of times across the cell membrane (18). Magnesium participates in all biological pathways that produce or consume ATP (19). During entry into stationary phase, persister formation has been correlated with a decrease of cytoplasmic ATP (3, 10). However, the direct link between growth status and change of ATP levels is missing. We speculated that a change in extracellular or cytoplasmic Mg2+ concentration could affect persister formation and that ATP participated in this effect. First, we measured the concentrations of extracellular and cytoplasmic Mg2+ in log phase or stationary phase. The result is depicted in Fig. 2a, demonstrating that the Mg2+ in culture medium was significantly consumed from inoculation to log phase but remained at a stable concentration until stationary phase. The cytoplasmic Mg2+ of stationary-phase cells decreased by ~70%, compared with that of log-phase cells, showing a similar trend as the drop in ATP from log phase to stationary phase in S. aureus (3). It is worth noting that from inoculation to log phase, the bacteria consumed one-third of the Mg2+ in the medium, but the concentration of Mg2+ in the medium was not significantly decreased during growth from log phase to stationary phase, while the cytoplasmic Mg2+ of bacteria was reduced by two-thirds. With an ~5-fold increase of bacterial population from late exponential phase to stationary phase, it appears that the daughter cells went through loss of cytoplasmic magnesium during reproduction.

To study whether the drop in extracellular or cytoplasmic Mg2+ could cause a decrease of cytoplasmic ATP, the effects of treatment with saline or EDTA on cytoplasmic Mg or ATP were determined. While we found little influence on cytoplasmic Mg2+ by treatment with saline or EDTA, the ATP levels were significantly reduced. Like persistence, the effects of saline on cytoplasmic ATP could be dampened by addition of Mg2+, and the effects of EDTA could be dampened by addition of Mg2+ or Ca2+ (Fig. 2b and c). The concentration of cytoplasmic Mg2+ decreased under induction of
mgtE asRNA but was not affected by depletion of yhdP (Fig. 2b). Similar effects were observed on the concentration of cytoplasmic ATP (Fig. 2c).

It is intriguing that depletion of Mg\(^{2+}\) from medium caused a rapid drop in cytoplasmic ATP without affecting the concentration of cytoplasmic Mg\(^{2+}\), indicating that magnesium serves as a signal that bacteria utilize to cope with the approaching magnesium starvation by reducing ATP as a counterstrategy. A similar strategy has been demonstrated by a series of studies in *Salmonella enterica*, which copes with low magnesium stress by reducing cytoplasmic ATP to allow translation by ribosomes (20).

The PhoP/PhoQ two-component system (TCS) is the sensor for extracellular Mg\(^{2+}\), and the virulence protein MgtC mediates reduction of ATP production (21). However, in *S. aureus* the sensor of either extracellular or cytoplasmic Mg\(^{2+}\) remains unknown, and there is no close homolog of MgtC or M-box, the magnesium-sensing riboswitch RNA (22). PhoPQ is the typical extracellular Mg\(^{2+}\) sensor in Gram-negative bacteria but not in *S. aureus*. We investigated the roles of several TCSs (PhoPR, ArlRS, SrrAB, and GraRS)
by detecting the persister level of their mutants. None of them seemed to be involved in starvation-triggered antibiotic persistence (Fig. S4).

Membrane potential has been found to be responsible for persister induction by inactivation of tricarboxylic acid (TCA) genes in *S. aureus* (11). However, a recent study showed that extracellular magnesium suppresses membrane potential-mediated hyperpolarization and protects *Bacillus subtilis* from ribosome-targeting antibiotics (23). Here, in *S. aureus* we showed that treatment with saline significantly reduced the membrane potential of the log-phase cultures, and while addition of Mg\(^{2+}\) reduced persister cell formation, it caused a slight decrease in membrane potential of saline-treated cultures (Fig. 2d to f). Therefore, the sensitization of persisters by magnesium was not due to recovery of membrane potential.

Two reporter genes, *arcA* and *cap5A*, have been found to be activated in stationary phase and used as indicators for persister formation (24). The expression of *arcA* and *cap5A* was slightly induced by treatment with saline or EDTA but significantly repressed by addition of Mg\(^{2+}\) (Fig. 2g). We also detected the expression of *mgtE* or *yhdP*, each of which was upregulated from inoculation to log phase, while during entry to stationary phase the two genes were silenced to undetectable levels (Fig. 2h). This indicates that *S. aureus* shuts down magnesium transport and export after entry into stationary phase, although the nutrition in the supernatant of a stationary-phase culture is able to support a new round of bacterial growth to stationary phase (unpublished data). This might provide an explanation for our observation that addition of extra Mg\(^{2+}\) to stationary-phase cultures could not reduce persisters of stationary-phase cultures (Fig. S5). Further studies on sensors for extracellular and cytoplasmic Mg\(^{2+}\) will provide clues for unraveling the detailed mechanism of magnesium-mediated persister formation in *S. aureus*.

**Bacterial strains and genetic manipulations.** *S. aureus* Newman and USA300_ FPR3757 were used, unless otherwise stated, for this study. Mutant strains of USA300 were selected from the sequence-defined transposon mutant library of *S. aureus* (25). The *yhdP* mutant of the Newman strain was obtained with plasmid pMX10-yhdP, which was constructed by inserting two DNA fragments beside *yhdP* into the plasmid pMX10 (26), according to the standard homologous recombination procedure (27). The antisense RNA plasmid against *mgtE* or *yycF* was constructed by inserting a DNA fragment that covered the ribosome binding site and the following 100 bp of the coding sequence of the target gene into pMX6 (28). Plasmid constructions were performed with *E. coli* strain DC10B (29). More details of strains and plasmids are listed in Table S2, and sequences of primers used in this study are listed in Table S3.

**Mg\(^{2+}\) quantitation.** For cytoplasmic Mg\(^{2+}\), the bacterial cells were collected and resuspended in PBS containing 100 μg of lysostaphin (Sigma, Germany). The cells were thoroughly lysed at 37°C for 0.5 to 1 h. The Mg\(^{2+}\) concentration was detected with the QuantiChrom magnesium assay kit (Bioassay Systems, Hayward, CA), according to the recommended protocol. The final concentration was normalized to CFU and average volume of *S. aureus* in log phase (0.33 μm\(^3\)) or stationary phase (0.23 μm\(^3\)) (30).

**Persistor assays.** The cultures grown to exponential phase (OD\(_{600}\) = 0.5) were centrifuged and resuspended with saline, 5 mM EDTA, or 5 or 10 mM MgSO\(_4\). The cultures were incubated at 37°C for 30 min and treated with 10× to 100× MIC of levofloxacin (50 μg/ml), linezolid (25 μg/ml), rifampin (1 μg/ml), or vancomycin (50 μg/ml) for 8 to 12 h. Antibiotics were removed by washing, and serial dilutions of each sample were performed with 10-μl aliquots spotted on tryptic soy agar (TSA) plates for CFU counting. Results were obtained from three biological duplicates, and the data were assessed with the t test.

**ATP assays.** The cytoplasmic ATP concentration was measured with the BacTiterGlo microbial cell viability assay kit (Promega, Madison, WI). The luminescence of each sample was detected with the FB12 luminometer (Berthold, Pforzheim, Germany) in three independent experiments. The data were normalized with the CFU count of each sample.
Detection of membrane potential. The membrane potential was measured with the BacLight bacterial membrane potential kit (Molecular Probes, Eugene, OR), where the cultures were treated with saline, EDTA, or MgSO4. The samples were mixed with 10 μl fluorescent membrane potential indicator dye diethyloxacarbocyanine iodide (DiOC2) and incubated at 37°C for another 30 min. The fluorescent signals were recorded by an LSRFortessa flow cytometry analyzer (BD, CA) counting 50,000 cells. Membrane potential was indicated by the ratio between channel F3 (red fluorescence) and F1 (green fluorescence) using FlowJo (BD, CA).

qRT-PCR. RNA samples were extracted according to the method previously reported (31). Briefly, the pellets were collected by centrifugation and resuspended in 100 μl diethylpyrocarbonate (DEPC)-H2O and 100 μl phenol-chloroform (1:1). The samples were incubated at 70°C for 30 min and then centrifuged. RNA from the supernatants was purified with the RNasy minikit (Qiagen, Hilden, Germany) according to the protocol provided. Reverse transcription was performed with a cDNA synthesis kit (Bio-Rad, Hercules, CA). The qRT-PCR experiments were carried out with the SYBR green PCR kit (TaKaRa, Japan) on the LightCycler 480 System (Roche, Branchburg, NJ). The primer sequences are listed in Table S3. The data were obtained from three independent experiments, and the threshold cycle \(2^{-\Delta \Delta CT}\) method (32) was used for analysis of relative gene expression.

Statistical analyses. The significance of experimental differences in persister assay, Mg2+ measurement, and intracellular ATP assay was evaluated with the two-tailed unpaired t test (two groups).

SUPPLEMENTAL MATERIAL
Supplemental material is available online only.
FIG S1, TIF file, 0.4 MB.
FIG S2, TIF file, 0.2 MB.
FIG S3, TIF file, 0.3 MB.
FIG S4, TIF file, 0.2 MB.
FIG S5, TIF file, 0.2 MB.
TABLE S1, DOCX file, 0.02 MB.
TABLE S2, DOCX file, 0.02 MB.
TABLE S3, DOCX file, 0.02 MB.

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