



# 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, *cap5A* 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

Persisters, defined as a subpopulation of bacterial cells that survive lethal doses of antibiotics by entry into dormancy, play a key role in persistent bacterial infections (1, 2). Multiple genes from different physiological processes, including DNA repair, protein synthesis, toxin-antitoxin, and energy production, have been shown to affect persister formation (3–7). However, most persister genes are neither essential nor conserved for persistence among different bacteria (7). Meanwhile, the phenomenon that the number of persisters of a given culture increases as the culture ages and enters stationary phase is ubiquitous among all bacterial species (8). Therefore, the formation of bacterial persisters is a complicated process, with multiple redundant mechanisms that lead to profound changes in physiological status. Recent studies showed that starvation is a major trigger of antibiotic persistence. Starvation of *Escherichia coli* cells in biofilms produces higher tolerance to ofloxacin (9). Chen et al. (33) showed that

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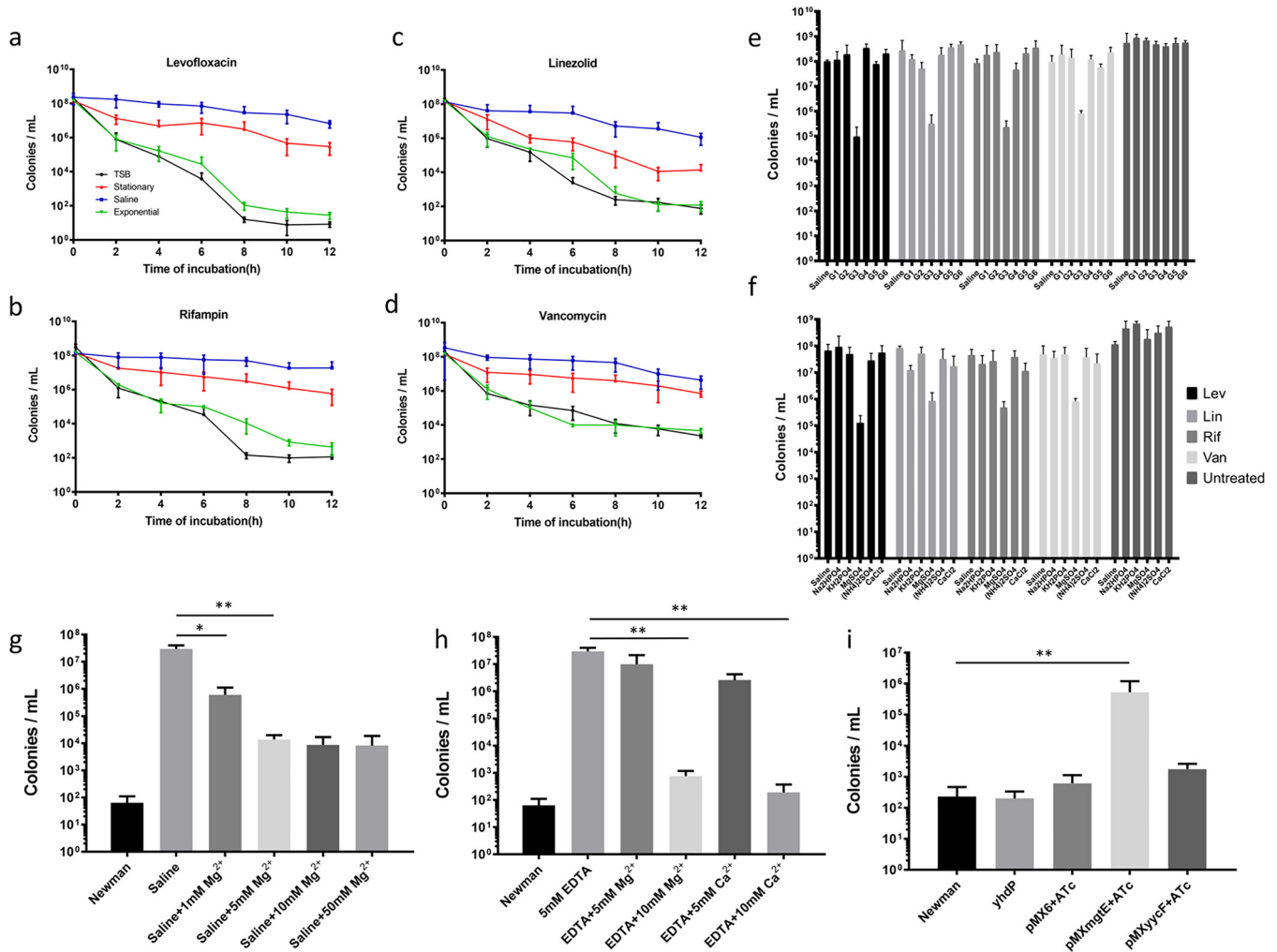
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**FIG 1** Magnesium dampens starvation-induced persistence. (a to d) Persister levels of *S. aureus* Newman log-phase cultures treated with saline or supernatant of stationary-phase cultures. Bacterial samples were treated with different antibiotics as described in the text for 8 h, and CFU counting was performed every 2 h. (e) Persister levels of Newman log-phase cultures treated with saline and different groups of HHWm. (f) Persister levels of Newman log-phase cultures treated with saline and different components from the major salt group of HHWm. (g) Levels of persisters against levofloxacin of Newman exponential cultures treated with saline and different concentration of  $Mg^{2+}$ . (h) Levels of persisters against levofloxacin from Newman exponential cultures treated with EDTA and  $Mg^{2+}$  or  $Ca^{2+}$ . (i) Levels of persisters against levofloxacin from exponential cultures of the *yhdP* mutant strain or Newman with induced *asRNA* against *mgE*. Results are expressed as CFU count with comparison to untreated cultures. Data are the average results from at least two independent experiments, each with three biological replicates. Significant differences are indicated with asterisks: \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (two-way ANOVA). Standard deviations are represented with error bars.

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.

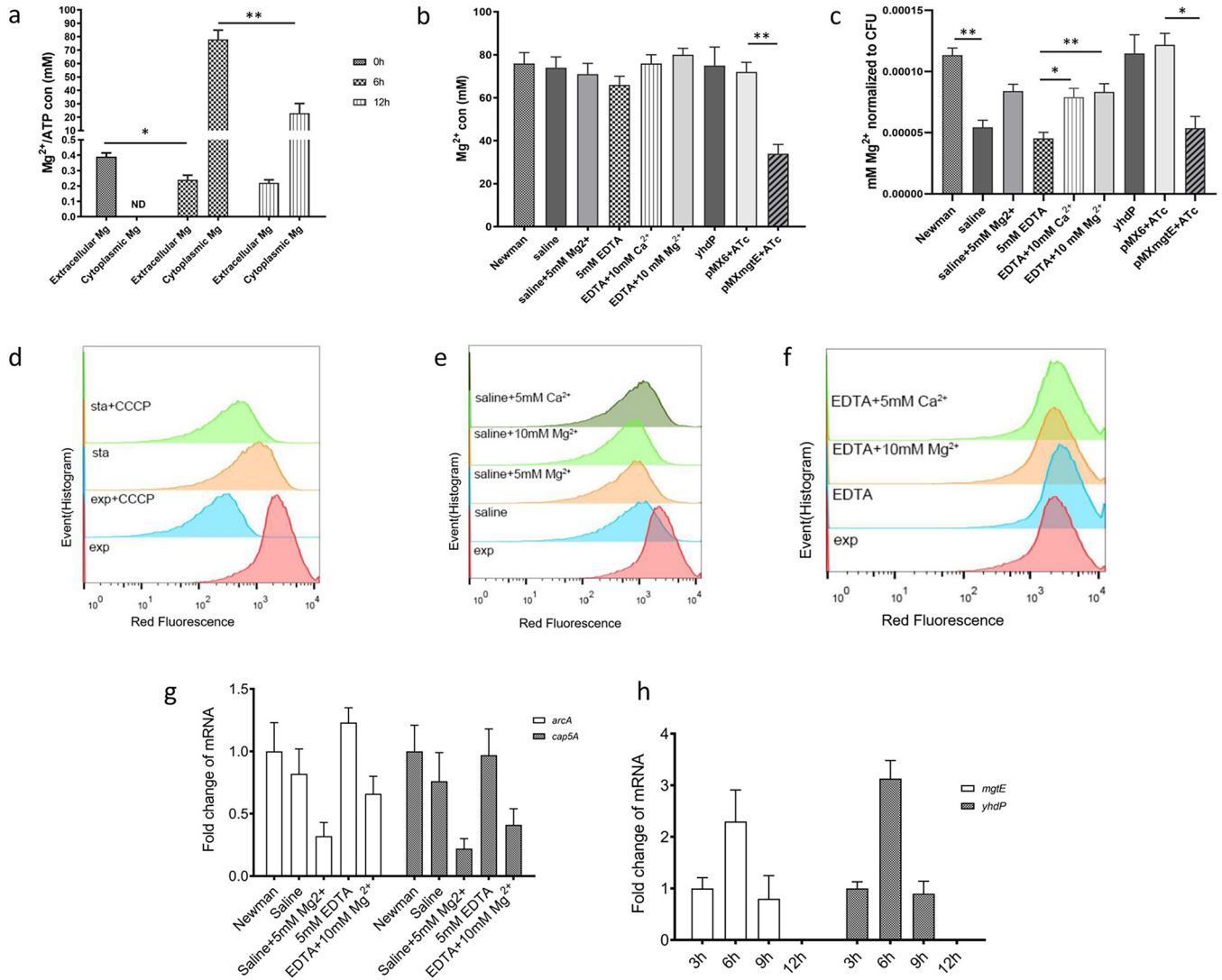
To study the effects of starvation on *Staphylococcus aureus* persister cell formation, we examined persister levels of log-phase cultures under nutrient deprivation by resuspending the bacterial cells in saline or supernatants of stationary-phase cultures. As shown in Fig. 1a to d, treatment of log-phase 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  $\text{MgSO}_4$  that dampened induction of persisters (Fig. 1f). Since an extra control  $\text{Na}_2\text{SO}_4$  showed no effect in reversing persister formation, it was confirmed that  $\text{Mg}^{2+}$  but not  $\text{SO}_4^{2-}$  was the functioning factor. Indeed, addition of  $\text{Mg}^{2+}$  up to 5 mM showed the strongest inhibition of persister formation (Fig. 1g). We further showed that the effects of  $\text{Mg}^{2+}$  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  $\text{Mg}^{2+}$ . Although  $\text{Ca}^{2+}$  was unable to hinder persister induction by nutrition depletion, it could free  $\text{Mg}^{2+}$  and offset the effects of EDTA on persister formation (Fig. 1h), probably by its higher affinity for EDTA than  $\text{Mg}^{2+}$  (15).

We then tested whether disruption of magnesium transport or efflux could affect persister formation. *MgtE* is a vital  $\text{Mg}^{2+}$  transporter in *S. aureus* Newman (16), and *YhdP* is the efflux pump for  $\text{Mg}^{2+}$  (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).

$\text{Mg}^{2+}$  is the most abundant multivalent cation in all living cells. Bacteria maintain a high concentration of cytoplasmic  $\text{Mg}^{2+}$ , 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  $\text{Mg}^{2+}$  concentration could affect persister formation and that ATP participated in this effect. First, we measured the concentrations of extracellular and cytoplasmic  $\text{Mg}^{2+}$  in log phase or stationary phase. The result is depicted in Fig. 2a, demonstrating that the  $\text{Mg}^{2+}$  in culture medium was significantly consumed from inoculation to log phase but remained at a stable concentration until stationary phase. The cytoplasmic  $\text{Mg}^{2+}$  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  $\text{Mg}^{2+}$  in the medium, but the concentration of  $\text{Mg}^{2+}$  in the medium was not significantly decreased during growth from log phase to stationary phase, while the cytoplasmic  $\text{Mg}^{2+}$  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  $\text{Mg}^{2+}$  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  $\text{Mg}^{2+}$  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  $\text{Mg}^{2+}$ , and the effects of EDTA could be dampened by addition of  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  (Fig. 2b and c). The concentration of cytoplasmic  $\text{Mg}^{2+}$  decreased under induction of



**FIG 2** ATP but not membrane potential mediates Mg<sup>2+</sup>-associated persistence. (a) Quantitation of cytoplasmic and extracellular concentrations of Mg<sup>2+</sup> as well as cytoplasmic ATP in growth. (b) Detection of cytoplasmic Mg<sup>2+</sup> and ATP levels of Newman strain treated with saline or EDTA. (c) Antisense RNA of *mgtE* decreases cytoplasmic Mg<sup>2+</sup> and ATP. (d to f) Impacts of growth phase, saline, or EDTA on membrane potential of the Newman strain. The red fluorescence that indicates membrane potential was analyzed by fluorescence-activated cell sorting. sta, stationary phase; exp, exponential phase; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone. (g) Mg represses expression of *arcA* and *cap5A* in Newman exponential cultures treated with saline or EDTA, detected by qRT-PCR. (h) Expression levels of *mgtE* and *yhdP* in different growth phases. Data represent the results from three independent experiments with standard deviations represented with error bars. Significant differences are indicated with asterisks: \*, *P* < 0.05; \*\*, *P* < 0.01 (two-way ANOVA); ND, no data.

*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<sup>2+</sup> from medium caused a rapid drop in cytoplasmic ATP without affecting the concentration of cytoplasmic Mg<sup>2+</sup>, 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<sup>2+</sup>, and the virulence protein MgtC mediates reduction of ATP production (21). However, in *S. aureus* the sensor of either extracellular or cytoplasmic Mg<sup>2+</sup> 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<sup>2+</sup> 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 anti-sense 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  $\mu$ g 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  $\mu$ m<sup>3</sup>) or stationary phase (0.23  $\mu$ m<sup>3</sup>) (30).

**Persister 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 $\times$  to 100 $\times$  MIC of levofloxacin (50  $\mu$ g/ml), linezolid (25  $\mu$ g/ml), rifampin (1  $\mu$ g/ml), or vancomycin (50  $\mu$ g/ml) for 8 to 12 h. Antibiotics were removed by washing, and serial dilutions of each sample were performed with 10- $\mu$ l 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 BacTiter-Glo 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  $MgSO_4$ . The samples were mixed with 10  $\mu$ l fluorescent membrane potential indicator dye diethyloxycarbocyanine iodide ( $DiOC_2$ ) 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  $\mu$ l diethylpyrocarbonate (DEPC)- $H_2O$  and 100  $\mu$ l 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 RNeasy 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,  $Mg^{2+}$  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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