The Mevalonate Auxotrophic Mutant of *Staphylococcus aureus* Can Adapt to Mevalonate Depletion

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In this study, we attempted to adopt the auxotrophic mevalonate synthase mutant (ΔmvaS mutant) of *Staphylococcus aureus* to study whether a nongrowing but viable cell population is tolerant to bactericidal antibiotics. The mevalonate-depleted nongrowing ΔmvaS mutant was found tolerant to antibiotics. Surprisingly, after prolonged cultivation, we obtained stable ΔmvaS variants that were able to grow without mevalonate, which suggested unknown mechanisms for compensating undecaprenyl pyrophosphate production without mevalonate in *S. aureus.*

**Bacterial persisters refer to a subpopulation of bacterial cells that survive prolonged exposure to multiple bactericidal antibiotics (1).** Persistence is regarded as a transient physiological state, as the persisters are genetically susceptible to antibiotics (1). Despite the first report describing the persister phenomenon over 60 years ago (2), the underlying mechanisms are still not fully understood. It is generally believed that a nongrowing, dormant state protects the bacteria from the killing of antibiotics (3–5). However, recent data suggest that some active defense mechanisms are involved (6–8), indicating that the mechanisms of persistence are rather redundant and diverse.

Tackling persisters is not easy, because persisters represent only a small cell population whose transient physiological changes are masked by the majority of nonpersisters. Single-cell strategy has been applied to solve this problem (9). In the current study, we aimed at establishing an alternative method to study persisters in *Staphylococcus aureus* on the scale of whole-cell populations. The idea is to put bacteria in an “artificial coma” from which they could be easily revived. The auxotrophic mevalonate synthase (mvaS) mutant of *S. aureus* turned out to meet the requirements.

Unlike many Gram-negative pathogens, in the Gram-positive low-GC pathogens, such as *S. aureus, Streptococcus pneumoniae,* and *Enterococcus faecalis,* the only route to isoprenoid production is the mevalonate pathway (10–12) (see Fig. S1 in the supplemental material). Notably, mevalonate is the precursor for undecaprenyl pyrophosphate, which is essential for cell wall biosynthesis. Deletion of mvaS that encodes the hydroxymethylglutaryl-coenzyme A (HMG-CoA) synthase leads to auxotrophy for mevalonate, as first proven in *E. coli* (11). We assumed that the ΔmvaS mutant could be a suitable candidate, because the auxotrophic ΔmvaS mutant can be handled in complex-rich medium with the usual carbon source and amino acids.

Here, we constructed an *S. aureus* mvaS deletion mutant to study nongrowing but viable cell populations toward antibiotic tolerance. To our surprise, however, the ΔmvaS mutant was able to adapt to mevalonate limitation and regrow after prolonged cultivation.

The mvaS gene was replaced in *S. aureus* strain SA113 by a kanamycin resistance cassette, which was subsequently excised by the Cre-lox system, resulting in a markerless deletion mutant (13) (see Fig. S2A in the supplemental material). As SA113 is defective in several global regulators, we also created the ΔmvaS markerless deletion in strain HG001, in which the global regulators *agr* and *sigB* are functional (14). The obtained marker-free mutants were complemented with plasmid pRB-mvaS, carrying the *mvaS* gene under the control of its native promoter (see Fig. S2B). Since both mutants showed essentially the same phenotype, we show here the results of the HG001ΔmvaS mutant as a representative.

The growth dependence on mevalonate of the ΔmvaS mutant was examined. Overnight cultures in tryptic soy broth (TSB) supplemented with 960 μM mevalonate were washed three times with fresh TSB, inoculated in fresh TSB to an optical density at 578 nm (OD578) of 0.1, and precultivated for 4 h. The precultivation in mevalonate-free medium was necessary for consuming the residual mevalonate. The precultivated bacterial cultures were further diluted in fresh TSB to an OD578 of 0.1 and cultivated aerobically. As expected, the growth of the HG001 ΔmvaS mutant was dependent on the presence of mevalonate. As shown in Fig. 1, in the presence of 960 μM mevalonate, the growth of the mutant was comparable to that of wild-type (wt) HG001 or the complemented mutant. In the absence of mevalonate, the OD of the ΔmvaS mutant increased slightly from 0.1 to 0.3 and stayed arrested over 24 h.

Next, we tested the hypothesis of whether nongrowing cell populations are tolerant to bactericidal antibiotics with the mevalonate-starved ΔmvaS mutant. Bacteria were cultivated like in the growth assay. After 4 h of precultivation, wt HG001, the ΔmvaS mutant, and the complemented mutant were diluted into fresh TSB and further grown for 1 h. At this time point, the CFU/ml of the wt was ≈3×10⁷, and that of the ΔmvaS mutant was ≈1×10⁷ (Fig. 2, dotted lines). Subsequently, several bactericidal antibiotics of 1× MIC and 10× MIC were added into the bacterial cultures, which were further incubated for 6 h.

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Figure 2 shows the effect of bactericidal antibiotics on the ΔmvaS mutant. In the absence of the antibiotics, wt HG001 and the complemented mutant grew from $3 \times 10^7$ to about $3 \times 10^9$ CFU/ml, while the ΔmvaS mutant did not grow and the CFU/ml stayed constant at $1 \times 10^7$ CFU/ml during the incubation time. In the presence of 10× MIC (20 μg/ml) of gentamicin, the CFU/ml of the wt and the complemented mutant decreased drastically (from $3 \times 10^7$ to $10^2$ CFU/ml). In contrast, the CFU/ml of the ΔmvaS mutant was completely unaffected and stayed constant at $1 \times 10^7$ (Fig. 2A). The nongrowing but viable ΔmvaS mutant appeared tolerant to gentamicin. Indeed, similar results were found with rifampin, ciprofloxacin, and daptomycin (Fig. 2B to D). Rifampin and daptomycin had a slight killing activity with the mutant (Fig. 2B and D); however, the effect was still mild compared to that of the wt, where the CFU/ml decreased more than 3 log units, while in the mutant it was only a 1-log-unit drop. When mevalonate (960 μM) was supplemented in the medium, the mutant was revived and became as susceptible as the wt parent to antibiotics (Fig. 2A, illustrated only with gentamicin). We concluded that the mevalonate-depleted nongrowing ΔmvaS mutant was tolerant to multiple bactericidal antibiotics; resuscitating the same cell population with mevalonate made them susceptible to antibiotics again.

In the next step, we aimed at testing the prolonged persistence of the ΔmvaS mutant. The same cultivation procedures as those described above (with 120 μM mevalonate in the overnight culture) were performed to cultivate the bacteria for 9 days. The OD$_{578}$ and CFU/ml were monitored every 24 h. During the prolonged cultivation, the wt HG001 and complemented mutant grew 2 log units (from $10^7$ to $10^9$ CFU/ml) within the first 24 h, which then gradually declined and reached a steady state of $\approx 10^8$ CFU/ml on the fourth day (Fig. 3A). In contrast, the CFU/ml of the ΔmvaS mutant decreased from $10^7$ to $10^2$ within 24 h. Unexpectedly, afterward the CFU/ml of the ΔmvaS mutant steadily increased and reached a level of $\approx 10^8$ CFU/ml on the fourth day, at which point the level was the same as that of the wt (Fig. 3A). The OD$_{578}$ of the ΔmvaS mutant showed a similar tendency,
where it stayed arrested for 3 days and increased to a steady level of ~3 on the fifth day (Fig. 3B). It seemed that the ΔmvaS mutant was able to adapt to mevalonate limitation and start to regrow after prolonged cultivation. Interestingly, when we reinoculated the ΔmvaS mutant from any time point after it was adapted (from day 5 on) (data not shown). The reinoculating assay indicated that once the mutant was adapted, it no longer needed the long lag phase (the first 3 days) to resume growth, suggesting that the adaptation was stable.

To rule out artifacts and further confirm this finding, we tried different experimental conditions, such as using different concentrations (960 or 120 μM) of mevalonate in the overnight culture, washing the overnight culture more thoroughly, reducing the starting inoculums (1:10², 1:10³, and 1:10⁴), or omitting the precultivation. Under all conditions tested, the ΔmvaS mutant was able to regrow to an OD₅₇₈ of 2 to 3 after certain days, and the regrowth was stable (data not shown). It was also confirmed by PCR that all the adapted ΔmvaS mutants still carried the mvaS markerless mutation (data not shown). Further, samples from the prolonged cultivation were subjected for microscopy analysis. After 1 or 2 days of cultivation in the absence of mevalonate, the ΔmvaS mutant appeared very sick under microscopy. Cells were swollen, translucent, and heterogeneous in shape and size (Fig. 4Ac to f, phase-contrast images). Abnormal septum rings were frequently observed, which were rare in the wt (Fig. 4Ad and e). Some dividing cells were short of septum (Fig. 4Ac and d). These severe defects in septum formation and cell division were probably due to the cell wall biosynthesis defect of the mvaS mutant lacking undecaprenyl pyrophosphate. However, after prolonged cultivation, the ΔmvaS mutant steadily adapted to almost normal morphology. In the samples from the fourth and fifth days, the cell size became homogenous, with only a few enlarged cells (Fig. 4Ag and h). The cell wall stained by Van-FL appeared similar to that of the wt with normal septum formation. When the adapted ΔmvaS mutant was grown on a TSB agar plate (tryptic soy agar [TSA]) without mevalonate, it formed homogenous, small, white colonies after several days of incubation, whereas the nonadapted mutant was unable to form visible colonies (not shown).

FIG 3 Prolonged cultivation leads to adaption of the ΔmvaS mutant. (A) CFU/ml of wt HG001, the ΔmvaS mutant, and the complemented mutant grown over 9 days. Black circle, wt; white triangle, ΔmvaS mutant; ex, complemented mutant. (B) The growth after reinoculating the ΔmvaS mutant from the seventh day’s culture into new fresh TSB without mevalonate. Arrow, reinoculation; white triangles, ΔmvaS mutant; white squares, reinoculated ΔmvaS mutant. Error bars represent the standard deviations from three independent experiments.

FIG 4 (A) Morphological changes of the ΔmvaS mutant during prolonged cultivation. (a and b) Wild-type HG001 grown in TSB for 3 h (a) and 5 days (b); (c to h) the ΔmvaS mutant grown in TSB without mevalonate for 1 day (c, d), 2 days (e, f), 4 days (g), and 5 days (h). Microscopy images: gray, phase-contrast images; green, fluorescent Van-FL staining. Scale bar, 2 μm. The number of cultivation days corresponds to that for Fig. 3. (B) Comparative colony morphology of HG001 and the nonadapted and the adapted ΔmvaS mutants on TSA plates with and without mevalonate. In the absence of mevalonate, the nonadapted ΔmvaS mutant was unable to form visible colonies (not shown).
encodes 3-hydroxy-3-methylglutaryl coenzyme A reductase) was avirulent in a murine hematogenous pyelonephritis infection model (15). Consistent with this, we found that our Hg001 ΔmvaS mutant was avirulent in a 10-day murine bacteremia model (data not shown). Apparently, S. aureus does not possess a high-affinity mevalonate transporter, and the administered mutant was not yet adapted to a low-mevalonate environment. It would be interesting to test the adapted ΔmvaS mutant in future infection studies.

Taken together, we found that the S. aureus ΔmvaS mutant was auxotrophic for mevalonate for only 24 h under tested conditions; afterward, it started to adapt and resume growth without mevalonate. The growth arrest within the first 24 h rendered the mutant tolerant toward multiple bactericidal antibiotics. The finding that the ΔmvaS mutant can adapt to mevalonate depletion after prolonged cultivation is fairly interesting. Once adapted, the mutant is stable and consistently grows without mevalonate. It demonstrates once again how flexible and adaptable bacteria can be to survive stressful environments; it also raises concerns on proposing the mevalonate pathway as a potential antibacterial target.

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