Mevalonate auxotrophic mutant of *Staphylococcus aureus* can adapt to mevalonate depletion

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Abstract

In this study we attempted to adopt the auxotrophic mevalonate synthase mutant (ΔmvaS) of *Staphylococcus aureus* to study whether a non-growing but viable cell population is tolerant to bactericidal antibiotics. The mevalonate-depleted non-growing ΔmvaS 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*.

Introduction

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 persisters phenomenon over 60 years ago (2), the underlying mechanisms are still not fully understood. It is generally believed that a non-growing, 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 non-persisters. 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 also be easily revived. The auxotrophic mevalonate synthase (mvaS) mutant of *S. aureus* turned out to meet the requirements. Unlike many
Gram-negative bacteria, 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) (Fig. S1). Notably, mevalonate is the precursor for undecaprenyl pyrophosphate that is essential for cell wall biosynthesis. Deletion of *mvaS* that encodes the hydroxymethylglutaryl-CoA (HMG-CoA) synthase leads to auxotrophy for mevalonate, as first proven in *Streptococcus pneumonia* (12). We assumed that Δ*mvaS* could be a suitable candidate also because that the auxotrophic Δ*mvaS* can be handled in complex rich medium with usual carbon source and amino acids.

Here we constructed an *S. aureus* Δ*mvaS* deletion mutant to study non-growing but viable cell populations towards antibiotics tolerance. To our surprise, however, Δ*mvaS* was able to adapt to mevalonate limitation and re-grow after prolonged cultivation.

**Results and Discussion**

The *mvaS* gene was replaced in *S. aureus* strain SA113 by a kanamycin resistance cassette, which was subsequently excised by Cre-lox system resulting in a marker-less deletion mutant (13) (Fig. S2A). As SA113 is defective in several global regulators, we also created the Δ*mvaS* marker-less 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 (Fig. S2B). Since both mutants showed essentially the same phenotype, we show hereafter the results of HG001 Δ*mvaS* as a representative.

The growth dependence on mevalonate of Δ*mvaS* was examined. Overnight cultures in TSB (tryptic soy broth) supplemented with 960 μM mevalonate were washed three
times with fresh TSB, inoculated in fresh TSB to OD$_{578}$ of 0.1 and pre-cultivated for 4 h. The pre-cultivation in mevalonate-free medium was necessary for consuming up the residual mevalonate. The pre-cultivated bacterial cultures were further diluted in fresh TSB to OD$_{578}$ 0.1 and cultivated aerobically. As expected, the growth of HG001ΔmvaS 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 HG001 wild type (wt) or the complemented mutant. In the absence of mevalonate, the OD of ΔmvaS increased slightly from 0.1 to 0.3 and stayed arrested over 24 h.

Next, we tested the hypothesis of whether non-growing cell populations are tolerant to bactericidal antibiotics with the mevalonate-starved ΔmvaS. Bacteria were cultivated likewise as the growth assay. After 4 hrs' pre-cultivation, HG001 wt, ΔmvaS 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 ≈3x10$^7$ and the ΔmvaS was ≈1x10$^7$ (Fig. 2, dotted lines). Subsequently, several bactericidal antibiotics of 1xMIC and 10xMIC concentrations were added into the bacterial cultures, which were further incubated for 6 h.

Fig. 2 shows the effect of bactericidal antibiotics on ΔmvaS. In the absence of the antibiotics, the HG001 wt and the complemented mutant grew from 3x10$^7$ to about 3x10$^9$ CFU/ml while the ΔmvaS did not grow and the CFU/ml stayed constant at 1x10$^7$ during the incubation time. In the presence of 10xMIC (20 µg/ml) gentamicin, the CFU/ml of the wt and the complemented mutant decreased drastically (from 3x10$^7$ to 10$^2$ CFU/ml). In contrast, the CFU/ml of ΔmvaS was completely unaffected and stayed constant at 1x10$^7$ (Fig. 2A). The non-growing but viable ΔmvaS appeared tolerant to gentamicin. Indeed, similar results were found with rifampicin, ciprofloxacin and daptomycin (Fig. 2B-D). Rifampicin and daptomycin had a slight killing activity with the
mutant (Fig. 2B, 2D). But the effect was still mild compared to that of wt where the CFU/ml decreased more than 3 log units while in the mutant it was only 1 log unit drop.

When mevalonate (960 µM) was supplemented in the medium, the mutant was revived and became as susceptible as the parent wt to antibiotics (Fig. 2A, illustrated only with gentamycin). We concluded that the mevalonate depleted non-growing ΔmvaS 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 ΔmvaS. Same cultivation procedures 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 HG001 wt 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 decreased from $10^7$ to $10^2$ within 24 h. Unexpectedly, afterwards the CFU/ml of the ΔmvaS steadily increased and reached a final level of $\approx 10^8$ CFU/ml on the fourth day where it merged with the wt (Fig. 3A). The OD_{578} of ΔmvaS showed a similar tendency where it stayed arrest for 3 days and increased to a steady level of $\approx 3$ on the fifth day (Fig. 3B). It seemed that the ΔmvaS was able to adapt to mevalonate limitation and start to re-grow after prolonged cultivation. Interestingly, when we re-inoculated the ΔmvaS culture on the seventh day into fresh TSB, it started to grow immediately and reached an OD_{578} of $\approx 3$ within 2 days (Fig. 3B). In fact the same happened if we re-inoculated the ΔmvaS from any time point after it was adapted (from Day 5 on) (data not shown). The re-inoculating 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 inoculum (1:10^2, 1:10^3 and 1:10^4) or omitting the 4 hrs’ pre-cultivation. Under all conditions tested, the ΔmvaS was able to re-grow to an OD<sub>578</sub> of 2-3 after certain days and the re-growth was stable (data not shown). It was also confirmed by PCR that all the adapted ΔmvaS still carried the mvaS marker-less mutation (data not shown).

Further, samples from the prolonged cultivation were subjected for microscopy analysis. After 1 or 2 days’ cultivation in the absence of mevalonate, ΔmvaS appeared very sick under microscopy. Cells were swollen, translucent and heterogeneous in shape and size (Fig. 4Ac-f, phase contrast images). ΔmvaS also showed a strong binding to Vancomycin BODIPY® FL-conjugate (Van-FL) that preferentially binds to the D-Ala-D-Ala residues of uncross-linked peptidoglycan (Fig. 4Ac). Abnormal septum rings were frequently observed, which were rare in the wt (Fig. 4Ad, e). Some dividing cells were short of septum (Fig. 4Ac, 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, ΔmvaS steadily adapted to almost normal morphology. In the samples from the fourth and fifth day, the cell size became homogenous with only a few enlarged cells (Fig. 4Ag, h). The cell wall stained by Van-FL appeared similar to that of the wt with normal septum formation. When the adapted ΔmvaS was grown on TSB agar plate (TSA) without mevalonate, it formed homogenous small white colonies after several days’ incubation, whereas the non-adapted mutant was unable to form visible colonies (Fig. 4B). In the presence of mevalonate, both adapted and non-adapted ΔmvaS formed normal yellow colonies like the wt (Fig. 4B). These changes implied that the adapted ΔmvaS was able to adopt some rescue systems to optimize the growth and compensate the shortage of
undecaprenyl pyrophosphate in the absence of mevalonate.

It has been shown previously that an mvaA mutant (mvaA 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 HG001ΔmvaS was avirulent in a ten-days 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 low mevalonate environment. It would be interesting to test the adapted ΔmvaS in the future infection studies.

Taken together, we found that the S. aureus ΔmvaS was auxotrophic for mevalonate for only 24 h under tested conditions; afterwards it started to adapt and resume growth without mevalonate. The growth arrest within the first 24 h rendered the mutant tolerant towards multiple bactericidal antibiotics. The finding that ΔmvaS 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 potential antibacterial target.

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References


Figure legends

**Fig. 1.** Growth of HG001 wt, ΔmvaS, and the complemented mutant ΔmvaS (pRBmvaS) in TSB. White circle, HG001 wt; white triangle, ΔmvaS cultivated without mevalonate; black triangle, ΔmvaS supplemented with 960 µM mevalonate; white square, complemented mutant. Error bars represent the standard deviation of three independent experiments.

**Fig. 2.** The non-growing ΔmvaS is tolerant towards multiple bactericidal antibiotics. The CFU/ml of HG001 wt, mevalonate-depleted ΔmvaS and the complemented mutant after 6 hrs' incubation with various bactericidal antibiotics are shown: (A) gentamicin, (B) rifampicin, (C) ciprofloxacin and (D) daptomycin. The dotted lines indicate the starting CFU/ml (≈10⁷) before antibiotics were added. The resuscitation experiment where the ΔmvaS was cultivated with 960 µM mevalonate is shown in A with gentamycin. White bar, without antibiotics; light gray bar, addition of 1xMIC antibiotics; dark gray bar, addition of 10xMIC antibiotics. Error bars represent the standard deviation of three independent experiments.

**Fig. 3.** Prolonged cultivation leads to adaption of ΔmvaS. (A) CFU/ml of HG001 wt, ΔmvaS and the complemented mutant grown over 9 days. Black circle, wt; white triangle, ΔmvaS; cross, complemented mutant. (B) The growth of re-inoculating ΔmvaS from the seventh day's culture into new fresh TSB without mevalonate. Arrow indicates re-inoculation; white triangle, ΔmvaS; white square, re-inoculated ΔmvaS. Error bars
Fig. 4. (A) Morphological changes of ΔmvaS during prolonged cultivation. (a-b) HG001 wt grown in TSB for 3 h (a) and 5 days (b); (c-h) ΔmvaS 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 of Fig. 3. (B) Comparative colony morphology of HG001, the non-adapted and the adapted ΔmvaS on TSA plates with and without mevalonate. In the absence of mevalonate, the non-adapted ΔmvaS was unable to form to visible colonies (not shown).