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# Mutation Rates and Fitness Genes in *Staphylococcus aureus* Treated with the Medicinal Plant *Synadenium glaucescens*

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## Abstract

Extracts, fractions and the pure compound epifriedelanol of the medicinal plant *Synadenium glaucescens* have antibacterial properties. Herbal products are generally considered less prone to resistance development than conventional antimicrobials, as they contain multiple compounds, which makes bacteria less likely to develop resistance. However, data supporting this notion are lacking. This study evaluated the development of resistance in *Staphylococcus aureus* subjected to extract, fractions and epifriedelanol of *S. glaucescens*. It also identified *S. aureus* fitness genes contributing to intrinsic resistance to extract of *S. glaucescens*. Fluctuation and gradient concentration assays were used to determine mutation rates and growth adaptation, respectively, which were lower following exposure to growth in crude extract than the pure compound epifriedelanol. By subjecting 1920 single gene mutants from the Nebraska Transposon Mutant Library to growth in the presence of extract of *S. glaucescens*, 12 genes were identified as important for natural resistance in *S. aureus* JE2; however, only mutation in the *hemB* gene decreased the minimum inhibitory concentration by greater than 4-fold (64-fold). In conclusion, purifying active antimicrobial compounds from *S. glaucescens* and using them as antibacterial substances as an alternative to crude extract increased the risk of resistance development. Further, the gene *hemB* appears to have a significant role in the natural resistance to the extracts obtained from *S. glaucescens* in this study.

**Keywords:** intrinsic resistance; mutation rate; fluctuation assay; gradient concentration; *Staphylococcus aureus*; *Synadenium glaucescens*; antimicrobials; antimicrobial resistance



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## 1. Introduction

Antibiotics are among the most important discoveries of the 20th century, having saved millions of lives from infectious diseases. Unfortunately, microbes have developed antimicrobial resistance (AMR) to many drugs accelerated by high selection pressure from the increasing use and misuse of antibiotics over the years [1]. Antimicrobial resistance (AMR) is ranked among the top ten global public health threats [2]. By 2050, AMR is anticipated to become a leading cause of death if appropriate measures are not taken [3]. The rise in the prevalence of AMR has led to an increased number of antibiotic treatment failures, including failures to treat infections caused by the medically important methicillin-resistant *Staphylococcus aureus* (MRSA) [4,5].

*S. aureus* is an opportunistic human and animal pathogen that causes a variety of diseases from mild skin infections to life-threatening systemic infections [6]. It is the most common cause of hospital-acquired infections [7]. A particularly fearsome variant is methicillin-resistant *S. aureus* (MRSA), associated with a 64% higher mortality rate than drug-sensitive *S. aureus* infections [8]. Resistance to many antibiotics is widespread in *S. aureus* [9].

To overcome the problem of AMR, there has been increasing interest in the search for novel antimicrobial compounds from medicinal plants [10,11]. Commonly, extracts from such plants contain complex mixtures of antimicrobial compounds [12]. There is a general belief that herbal products are more efficient and less prone to resistance development when used in their crude form, because such multiple compounds make it more difficult for bacteria to develop resistance, since they would need to mutate at multiple attack points simultaneously [11,13,14]. However, data to support this notion have not been produced.

The plant *Synadenium glaucescens* has antibacterial activity against Gram-positive bacteria, such as *S. aureus*, *Enterococcus faecalis* and *Streptococci* [15]. Recently, a study demonstrated that MIC values and results from time kill assays against *S. aureus* from the compound epifriedelanol (β-friedelanol), which was isolated from the root bark of this plant, were similar to MIC values and results from time kill assay of the full extracts of this plant. This suggests that this compound is likely a main active substance [15]. To standardize the production of antimicrobials, it may be beneficial to identify and produce purified compounds from the complex mixtures of plant extracts. Whether this will increase the risk of bacteria becoming resistant to plant medicine, and which genetic mechanisms would then be associated with resistance, remain unknown. This study ascertained resistance development in *S. aureus* towards crude extracts of *S. glaucescens*, as well as fractions, sub-fractions and the compound epifriedelanol to test the hypothesis that the frequency of resistance mutations would be affected by purity of the active substance. A further aim was to identify fitness genes in *S. aureus,* which influence the innate resistance of *S. aureus* towards *S. glaucescens*.

## 2. Materials and Methods

### 2.1. Bacterial Strains and Growth Media

*S. aureus* ATCC25923, livestock associated methicillin-resistant *S. aureus* CC398 (LA-MRSA) isolated from pigs (S0385) [16], and the community-associated methicillin-resistant *S. aureus* JE2 (CA-MRSA) isolate of USA300 [17] were used in the study. In addition, the published Nebraska Transposon Mutant Library (NTML) containing 1920 unique transposon mutants of *S. aureus* USA300 with single gene inactivation of non-essential genes was used [7]. A list of the genes investigated using mutants from this library appears in Supplementary File S1. Bacteria were propagated on Mueller Hinton agar (MHA), Trypton Soy Agar (TSA), Tryptic soy broth (TSB) or Mueller Hinton Broth (MHB) (all from Sigma Aldrich, St. Louis, MO, USA) and incubated at 37 °C for 18–24 h.

### 2.2. Plant Collection, Processing and Preparation of Extracts, Fractions, and Compounds

Fresh root bark of *S. glaucescens* was collected from Njombe district in the Southern Highlands of Tanzania as previously described [15]. The voucher specimen number HOS/FM 3672 for *S. glaucescens* was deposited at the herbarium of the Botany Department, College of Natural and Applied Sciences, University of Dar es Salaam, Tanzania. Plant names have been verified at http://www.theplantlist.org accessed on 4 August 2019. Crude extract from milled root bark and subfractions of this were obtained and analyzed exactly as published in a previous study [18]. Briefly, crude extracts were obtained by soaking in methanol solvent (Sigma Aldrich, Saint Louis, MO, USA) for 48 h then filtered and concentrated in a rotary evaporator (Büchi Labortechnik, Flawil, Switzerland) at 40 °C. The dried extract was stored at −20 °C before running bioassay. Further, the extract was fractionated sequentially with hexane, ethyl acetate and methanol solvents by vacuum liquid chromatography through a Buchner funnel. The resulting fractions were subjected to column chromatography to obtain sub-fractions: 20% ethyl acetate:methanol from ethyl acetate fraction and 20% methanol:ethyl-acetate from methanol fraction (chemicals all from Sigma Aldrich, Saint Louis, MO, USA), which was then purified to yield the compound epifriedelanol. A detailed description of techniques used for obtaining fractions and subfractions as well as techniques used to analyze sub-fractions and identify the structure of epifriedelanol has been published elsewhere [18]. A figure with the structure of epifriedelanol has been published in [15].

### 2.3. Minimum Inhibitory Concentration

The minimum inhibitory concentration (MIC) was determined by two-fold microdilution tests using 96-well microtiter plates. The protocol was conducted as described [19] with some modifications. Fifty microliters of MHB was placed into each well. To each well of the first row, 50 µL of the extract/fraction/epifriedelanol solution was added. Serial dilution was performed for each active ingredient to make a range of concentration starting with 24 to 0.047 µg/mL. Solvent control, dimethyl sulfoxide (DMSO from Sigma Aldrich, Saint Louis, MO, USA), was added in a ratio of 1:5 to the first well. Fifty microliters of the test organism was made in a concentration equivalent to 0.5 McFarland ($1.5 \times 10^8$ CFU/mL) and then adjusted to create an inoculum of $5 \times 10^6$ CFU/mL and added to each well. Two additional rows were used: sterility control containing blank culture medium, and growth control with bacteria and broth only. The microtiter plates were incubated at 37 °C for 18 h, and the last well observed with no growth of bacteria was considered MIC. The analyses were repeated three times.

### 2.4. Fluctuation Assay

Estimation of the de novo mutation rate was carried out by fluctuation assay, according to the published protocols [20,21], with some modifications. Bacteria were grown in MHB at 37 °C overnight with shaking. The primary culture was adjusted to optical density (OD600 nm) 0.3, which, by plate count assay, was determined to correspond to $10^9$ CFU/mL. A total of 100 µL of this culture was added to a tube containing 10 mL of MHB and thoroughly mixed. Afterwards, 22 µL of this solution was added to 44 mL of broth to reach a final bacterial density of 1000–5000 cells/mL in the primary culture. One milliliter of the primary culture was added to each of the 40 parallel culture tubes and incubated for 24 h with shaking at 125 rpm and 37 °C. These parallel cultures grew for many generations in the absence of selection pressure to allow them to mutate spontaneously. After incubation, 100 µL of the bacterial suspension from each tube was diluted. Then, 100 µL of the diluted culture was plated on an MHA agar plate without active ingredients to estimate the population size at the end of the growth period (Nt, determined by CFU/mL).

Additionally, 10 µL of the diluted culture from each tube was inoculated on agar plates supplemented with 750 µg/mL of the extract, fractions, sub-fractions, and epifriedelanol. This concentration was based on pre-experiments establishing the concentration needed in agar medium to prevent the growth of the *S. aureus* strains assayed. The plates were allowed to dry and incubated at 37 °C for 24–48 h. The number of colonies observed on the plates were counted for analysis. The number of mutational events, m, was estimated using R-package flan version 0.9 as described in [20]. Once m and Nt were obtained, estimation of the mutation rate (µ) was obtained using the following formula:

$$(\mathrm{i}): \ \mu = \frac{\mathrm{m}}{\mathrm{Nt}} \text{ where Nt } = (\text{Number of bacteria cells in the culture}) \quad (1)$$

Subsequently, the upper and lower bounds of m were divided by Nt in a similar manner to create confidence intervals for the mutation rate.

### 2.5. Gradient Concentration Assay

A gradient agar plate technique in which *S. aureus* was grown in the presence of increasing concentrations of extract, fractions, sub-fractions and epifriedelanol was used as described [22]. Briefly, preparation of the gradient concentration agar plates was carried out by pouring melted MHA into a tilted 13 cm round petri-dish to cover the entire bottom surface. The plate was allowed to solidify in a slanted position. Then, an extra layer of agar with solutions of the extracts/fractions/compound at 350 or 750 µg/mL in 20 mL agar was added to the agar plate in a horizontal position and allowed to solidify. The plates were left at room temperature for 24 h to allow equilibrium diffusion of the extract. Individual bacterial colonies were seeded into 0.7 mL of MHB and incubated at 37 °C for 2 h. The culture was then adjusted to a 0.5 McFarland standard ($1.5 \times 10^8$ CFU/mL). Following this, the culture was diluted to a concentration of $1 \times 10^6$ CFU/mL by adding 10 µL of the inoculum to 1 mL of broth. Finally, 1 µL of the diluted culture was streaked onto a plate supplemented with the extract. Plates were incubated at 37 °C for 24 h. Colonies were swabbed from the growth at the highest concentration, suspended in 0.9% sodium chloride, adjusted to $1 \times 10^5$ CFU/mL and sub-cultivated on the gradient concentration plate until full growth appeared on the streaked line. Control plates with no extracts were included.

### 2.6. Screening for Mutants with Increased Susceptibility to the Extract

The NTML mutant strains were tested to determine their level of susceptibility against extract, as previously described [23], but with some minor alterations. The strains from the frozen stock at −80 °C of NTML were immediately transferred using a Deutz 96 cryoreplicator from the 96-well plate onto TSA plates with an extract concentration of 375 µg/mL to allow the growth of mutants. After 24 h of incubation at 37 °C, the plates were visually examined to determine whether specific mutants had not grown. Where the mutants had not grown was considered to have increased in susceptibility.

### 2.7. Statistical Analysis

Dunnett's multiple comparison test in GraphPad Prism version 9 was used for comparison of the mutation rate between groups of extract, fractions, sub-fractions and epifriedelanol. A two-tailed *p*-value of <0.05 was considered statistically significant.

## 3. Results

### 3.1. Measuring Mutation Rate with Fluctuation Assay

The crude extract, fractions, sub-fractions and epifriedelanol demonstrated a minimum inhibitory concentration of 3–6 µg/mL against *S. aureus* strains ATCC25923, LA-MRSA and

CA-MRSA. However, as fluctuation assays are dependent on scoring of growth of bacteria on solid media, knowledge of the concentrations of extract, fractions and epifriedelanol needed to inhibit growth on MHA was required. Growth was observed at 375 µg/mL, while no growth occurred at 750 µg/mL. Thus, fluctuation assay was carried out with 750 µg/mL of extracts, fractions, sub-fractions and epifriedelanol.

Mutation rates in *S. aureus* ATCC25923, LA-MRSA and CA-MRSA were then estimated using Luria and Delbrück fluctuation assay. The estimated mutation rate ranges from $4.50 \times 10^{-10}$ to $6.14 \times 10^{-9}$ per generation for *S. aureus* ATCC25923 (Figure 1A), $5.44 \times 10^{-10}$ to $3.28 \times 10^{-9}$ per generation for LA-MRSA CC398 (Figure 1B) and $2.65 \times 10^{-10}$ to $3.58 \times 10^{-9}$ per generation for CA-MRSA (Figure 1C). Generally, mutation rates were observed to increase from crude extract to pure compound against all the strains tested except for the ethyl acetate fraction in *S. aureus* ATCC25923 and sub-fraction of ethyl acetate in LA-MRSA CC398, where there was no significant difference with the extract. While mutation rates thus increased with purification, statistical analysis only showed significant differences to crude extracts, while no significant difference in the mutation rate of epifriedelanol was observed compared to the methanol fraction, methanol subfraction, ethyl acetate fraction, and ethyl acetate subfraction against *S. aureus* strains ATCC25923, LA-MRSA and CA-MRSA ($p > 0.05$), except for the ethyl acetate subfraction, which exhibited a significant difference against *S. aureus* ATCC25923 ($p < 0.05$). Colonies from plates with bacteria growing in the presence of extracts at 750 µg/mL were subjected to MIC determination. Generally, the MIC only increased moderately in the range of 2–4-fold. Numbers of colonies observed with the different strains and MIC of selected mutants can be seen in Supplementary File S2, Tables S1–S4.

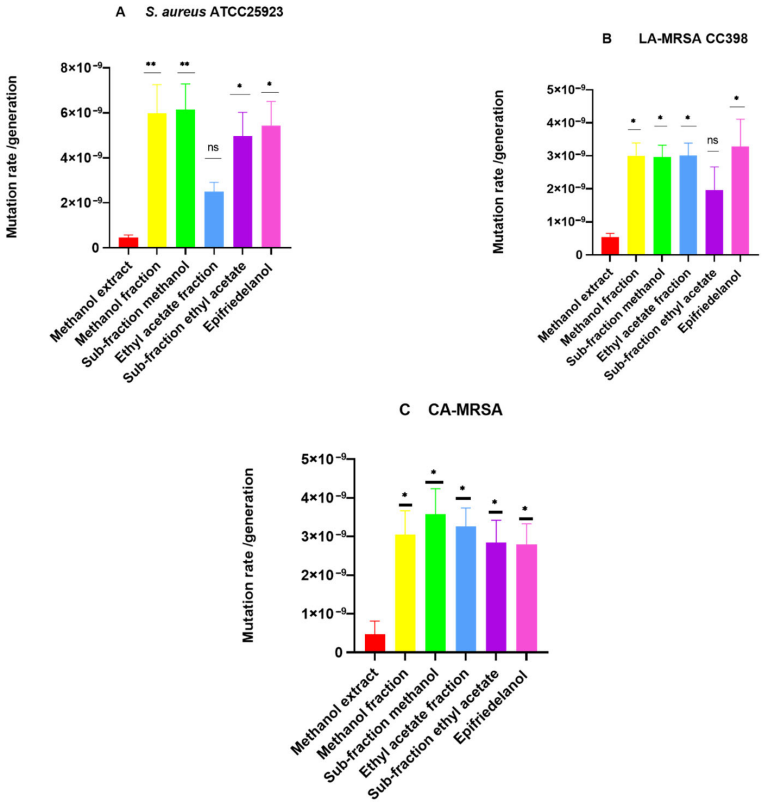


**Figure 1.** Estimation of mutation rates in *S. aureus* ATCC25923 (**A**), LA-MRSA (**B**) and CA-MRSA (**C**) towards crude methanol extract, fractions and sub-fractions of this extract and epifriedelanol obtained from the medicinal plant *Synadenium glaucescens*. Error bars represent 95% confidence intervals calculated as explained in the protocol. Statistical significance is shown as * $p < 0.05$, ** $p < 0.01$; ns: not significant with reference to the crude methanol extract.

### 3.2. Adaptation to Growth on Gradient Agar

To confirm that it would be easier for bacteria to adapt to purified epifriedelanol than to crude extract, *S. aureus* ATCC 25923 and LA-MRSA CC398 were subjected to growth in a gradient concentration assay. The strains passaged daily on MHA plates incorporated with 375 μg/mL of the crude methanol extract took 5 days to reach full-length growth. The adapted bacteria from the highest concentration on the gradient plate were re-streaked on a new gradient plate with 750 μg/mL of the crude extract. Full-length growth on this plate was never reached, even after daily passage for another 24 days. The strains tested with fractions, sub-fractions, and epifriedelanol at 375 μg/mL achieved full growth after 2 days; this growth was sustained for two additional passages before transferring to plates with 750 μg/mL, where full growth of the streak was not obtained, and the experiment ended after 5 days.

### 3.3. Screening for Mutants with Hyper-Susceptibility to Crude Extract of S. glaucescens

Colonies of 1920 single gene inactivation mutants in the NTML library were screened at 375 μg/mL using an agar plate assay to identify mutants that were not able to grow at this concentration. Twelve mutants were unable to grow on the plate, and these were subjected to MIC determination. Eleven of the mutants displayed a 2-fold decrease in MIC only, while the mutant with inactivation of the gene *hemB* displayed a 64-fold increase in susceptibility when compared to the MIC of the wild type of CA-MRSA (Table 1).

**Table 1.** MIC (μg/mL) of methanol extract of *S. glaucescens* against the wild type and selected mutants of *S. aureus* JE2 (CA- MRSA).

| S/No | Strain | Inactivated Gene | MIC (μg/mL) |
|---|---|---|---|
| 1 | Wild type of *S. aureus* JE2 SAUSA300 | - | 6 |
| 2 | SAUSA300_1615 | *hemB* | 0.094 |
| 3 | SAUSA300_1473 | *nusB* | 3 |
| 4 | SAUSA300_2406 | *cap5A* | 3 |
| 5 | SAUSA300_0228 | *Fade* | 3 |
| 6 | SAUSA300_1995 | *scrR* | 3 |
| 7 | SAUSA300_1343 | *Nth* | 3 |
| 8 | SAUSA300_0605 | *sarA* | 3 |
| 9 | SAUSA300_2550 | *nrdG* | 3 |
| 10 | SAUSA300_1222 | *Nuc* | 3 |
| 11 | SAUSA300_0889 | *oppD* | 3 |
| 12 | SAUSA300_1139 | *sucD* | 3 |
| 13 | SAUSA300_1359 | unknown | 3 |

## 4. Discussion

The central questions posed in the current study were whether refinement of plant medicines of *S. glaucescens* with antimicrobial activity by purification from crude extract to fractions, sub-fractions and purified compounds would increase the risk of *S. aureus* developing resistance to the antimicrobial substance, and which genes in *S. aureus* are important for intrinsic resistance of this bacterium against crude extracts from this plant.

The Luria and Delbrück fluctuation test has been used widely as a reference method to calculate and compare de novo mutation rates raised by microbes [20]. The analyses showed that mutation rates were significantly lower ($p < 0.05$) for strains selected on plates with crude extract than on plates with fractions, sub-fractions and epifriedelanol, except for two strains against a subfraction with ethyl acetate extraction. The reason why this subfraction was different from other subfractions and why the difference was only seen in

two of our three *S. aureus* strains tested are not known but may mean that this subfraction is more like the crude extract than the other subfractions. Further characterization is needed to determine this. Previous studies have shown that the likelihood of selecting resistant colonies was lower when bacteria were plated on a synergistic combination of antimicrobial substances than on individual substances [24,25]. In line with this, the low rate of mutations towards crude extract from *S. glaucescens* may be because the crude extract contains a mixture of antimicrobial substances. This explanation is challenged by the fact that the purified compound epifriedelanol has the same antimicrobial activity as the full extracts of this plant [15]. This activity has been determined by two-fold MIC assays, which are not very precise. A possible explanation is that mutation to growth in the presence of epifriedelanol happens in genes that determine the natural resistance towards other substances present in the crude extract, thus making it more difficult to mutate towards resistance to the crude extract. In terms of mechanistic understanding, this study is thus incomplete, and further studies are indicated to understand this apparent contradiction. The mutation rates determined by fluctuation assay in the current study were similar to natural rate of mutations in most microbial species, which ranges from $10^{-9}$ to $10^{-10}$ [24]. Strains with higher mutation rates have been shown to have an increased likelihood of acquisition of drug resistances, which may led to rapid selection of resistance and increased risk of multi drug resistance [26]; however, we saw no indications of high mutator strains in this study.

In confirmation of the results from the fluctuation study, gradient concentration assay demonstrated that it took a longer time for the strains to adapt to growth with crude extract, compared to when they were sub-cultured on gradient plates with fractions, subfractions, and epifriedelanol. Interestingly, even though growth adaptation was observed, the propagated colonies only showed a 2–4-fold increase in MIC (Supplementary Table S4), which falls below the commonly accepted 4-fold increase threshold for reliably identifying resistant strains [27]. We can only speculate why it is difficult to generate mutations that cause significantly increased MIC values above 4-fold.

To try to understand intrinsic resistance towards the antimicrobial substance(s) in *S. glaucescens*, we searched for genes, where gene knock-out would increase susceptibility to the crude extract. One gene, the *hemB* gene, stood out as particularly important, as the *hemB* mutant showed a 64-fold decrease in MIC compared to the wild type-strain JE2 of the NTML library screened. *hemB* encodes for a heme biosynthesis enzyme crucial for oxygen transport and energy production in *S. aureus* [28]. In accordance with previous observations, the *hemB* mutants formed small colonies attributed to a lack of hemin biosynthesis, which, in turn, interferes with the functioning of the respiratory chain and subsequently impacts ATP synthesis [28,29]. Previous research has shown that the gene significantly influences the susceptibility of *S. aureus* towards tomatidine and *N*,*N*′-dicyclohexylcarbodiimide (DCCD) with MICs of 0.12 μg/mL and 2–8 μg/mL for the mutant in contrast to an MIC of >128 μg/mL in the wild type [30–32]. It was reported that tomatidine and *N*,*N*′- dicyclohexylcarbodiimide (DCCD) target ATP synthase in SCV with a defective electron transport chain (ETC) [32]. This may indicate that epifriedelanol also targets ATP synthesis and/or that natural resistance towards the active substance in the crude extract is energy-dependent. Further studies are indicated to investigate this, and such testing should include both crude extract, fractions and purified compound(s).

*S. aureus* has a propensity to generate transient variations, such as switching from the normal to SCV phenotype and then back to the normal phenotype. This flipping is an essential component of the pathophysiology of *S. aureus* infections when causing chronic infections [33]. SCVs of *S. aureus* are naturally resistant to gentamicin, but combining gentamicin with tomatidine or *N*,*N*′-dicyclohexylcarbodiimide (DCCD) restores their sensi-

tivity to the antibiotic [30–32]. In the current study, we have not combined the plant extract with other antimicrobials, although previous studies from other scholars have shown and there may be an avenue for combination treatment between plant medicine and traditional antibiotics [34]. It would be interesting to combine epifriedelanol with gentamicin to see if there is synergy between the two compounds. Other genes identified in our screen of susceptibility genes are associated with the formation of SCVs (*aroD* and *menD*) [35,36]. However, the changes in MIC caused by mutation in these genes were small, and they were deemed less interesting.

## 5. Conclusions

In conclusion, *S. aureus* mutations to active substances from *S. glaucescens* developed in a higher frequency and more rapidly to subfractions and purified substances than to the crude extract. Furthermore, *S. aureus* mutation in the *hemB* gene significantly increased susceptibility towards crude extract of *S. glaucescens*, indicating that this gene plays a role in resistance. Studies report on the rate of mutation against antibacterial ingredients derived from plants, such as the present study, are generally scarce. We recommend that future studies should include this aspect, when characterizing herbal antimicrobials, particularly if the extracts are purified to enhance their activity or standardization of active substances.

**Supplementary Materials:** The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/app15158753/s1: Supplementary File S1: List of mutants in the NTML library tested; Supplementary File S2: Supplementary Tables S1–S3: Number of colonies observed on plates with 750 μg/mL in the fluctuation assays with the three strains tested. Supplementary Table S4: Minimum inhibitory concentration of Staphylococcus aureus strains from fluctuation assay.

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## Abbreviations

The following abbreviations are used in this manuscript:

| | |
|---|---|
| AMR | Antimicrobial resistance |
| LA-MRSA | Livestock-associated methicillin-resistant *S. aureus* |
| CA-MRSA | Community-associated methicillin-resistant *S. aureus* |
| NTML | Nebraska Transposon Mutant Library |
| MHA | Mueller Hinton agar |
| TSA | Trypton Soy Agar |
| TSB | Tryptic soy broth |
| MHB | Mueller Hinton Broth |
| ETC | Electron Transport Chain |
| DCCD | *N*,*N*′-dicyclohexylcarbodiimide |
| ATP | Adenosine Triphosphate |
| SCV | Small Colony Variant |
| MIC | Minimum Inhibitory Concentration |

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