Phenotypic Switching of Staphylococcus aureus Mu50 Into a Large Colony Variant Enhances Heritable Resistance Against β-Lactam Antibiotics

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Phenotypic heterogeneity within a bacterial population may confer new functionality and allow microorganisms to adapt to fluctuating environments. Previous work has suggested that Staphylococcus aureus could form small colony variants to avoid elimination by therapeutic antibiotics and host immunity systems. Here we show that a reversible non-pigment large colony morphology (Mu50ΔlcpA-LC) was observed in S. aureus Mu50 after knocking out lcpA, coding for the LytR-CpsA-Psr family A protein. Mu50ΔlcpA-LC increased resistance to β-lactam antibiotics, in addition, the enlarged cell size, enhanced spreading ability on solid medium, and reduced biofilm formation, suggesting better abilities for bacterial expansion. Moreover, the expression of spa encoding protein A was significantly increased in Mu50ΔlcpA-LC. This study shows that besides the small colony variants, S. aureus could fight against antibiotics and host immunity through phenotype switching into a large colony variant.

Keywords: Staphylococcus aureus, MRSA, phenotype switching, large colony, β-Lactams, lcpA, spa

INTRODUCTION

Staphylococcus aureus is an important human opportunistic pathogen that commonly colonizes the skin, mucosal surfaces, and soft tissues. When host barriers are disrupted, S. aureus can cause diverse clinical syndromes, ranging from skin abscess to more serious infections such as pneumonia, bacteremia, endocarditis, and osteomyelitis (Turner et al., 2019). S. aureus has been notoriously able to acquire resistance to a variety of antibiotics. The targets for β-lactam antibiotics are known as penicillin-binding proteins (PBPs). β-Lactam antibiotics inhibit the last step in peptidoglycan synthesis by acylating the penicillin-binding proteins involved in cross-linking peptides to form peptidoglycan, which is a vital constituent of the bacterial cell wall. However, the increasing use of β-lactams has resulted in the worldwide spread of the methicillin-resistant S. aureus (MRSA; Lee et al., 2018). The resistance of MRSA is usually conferred by acquisition of a low-affinity penicillin-binding protein 2A (Hartman and Tomasz, 1984).

Other proteins such as LytR-CpsA-Psr (LCP) family members than PBPs also play important roles in bacterial cell wall synthesis. S. aureus possesses three lcp genes, including lcpA.
(also named msrR), lcpB, and lcpC. These three LCP proteins of \textit{S. aureus}, in particular LcpA, could catalyze the linkage of wall teichoic acid (WTA) to synthetic uncrisscrosslinked peptidoglycan (PGN) oligomers \textit{in vitro} (Schaef er et al., 2017; Schaef er et al., 2018). LcpA can glycosylate the wall attachment protein GspA in the gram-positive bacterial \textit{Actinomyces oris} (Siegel et al., 2019). However, this latter function has not been demonstrated in \textit{S. aureus}. Similarly, LcpC in \textit{S. aureus} catalyzed attachment of the capsular polysaccharide to PGN (Rausch et al., 2019). In addition, lcpA contributed to oxacillin resistance in two MRSA strains COLn and USA300 (Hub scher et al., 2009; Schaefer et al., 2017) and a methicillin-susceptible \textit{S. aureus} ( MSSA) MSSA1112 (Hub scher et al., 2009). The deficiency in lcpA reduced the \(\beta\)-lactam resistance in two MRSA strains BA01611 and Mu50 and an MSSA strain Newman (Li et al., 2020). Interestingly, BA01611\(\Delta\)lcpC had decreased teicoplanin resistance but Mu50\(\Delta\)lcpA had increased teicoplanin resistance (Li et al., 2020). Teicoplanin is a semisynthetic glycopeptide antibiotic with a spectrum of activity similar to vancomycin. Both teicoplanin and vancomycin inhibit cell biosynthesis. Mu50 is a vancomycin-intermediate \textit{S. aureus} strain (VISA), which possesses active cell wall biosynthesis, leading to a thicker cell wall than the vancomycin-susceptible \textit{S. aureus} (VSSA) strains (Cui et al., 2000; Katayama et al., 2016). Indeed, BA01611, a VSSA strain, has a thinner envelope than that of Mu50 (Li et al., 2020). It seems that the deficiency of lcpC had different effects on Mu50 and BA01611. Thus, we hypothesized that lcpA might also have different effects on resistances in Mu50 and BA01611. To confirm the hypothesis, we constructed the Mu50\(\Delta\)lcpA and BA01611\(\Delta\)lcpA strains. We found that the lcpA deletion had different effects on resistances between these two strains. In addition, we observed co-existence of larger colonies and normal colonies in Mu50\(\Delta\)lcpA strain populations, in comparison with the wild type (WT) of Mu50. This type of phenotype variation has never been reported in \textit{S. aureus} before.

Most microorganisms such as \textit{S. aureus} live in groups of varying degrees of complexity. Various subpopulations within the group are responsible for the division of labor, which provide bacteria populations more benefits to survive different host environments and therapeutic antimicrobial stresses (Ackermann, 2015). Colony morphology variety is one of the most common phenotypic diversity. The subpopulation with distinct colony morphology usually has different phenotypes for antibiotic resistance, motility and virulence. For example, Clostridiodides difficile could switch their colony morphology between the rough colony and smooth colony. The rough colony variants could swim rapidly and induced more serious infections \textit{in vivo}, while the smooth strain tended to colonize \textit{in vivo} but had attenuated virulence (Garrett et al., 2019). \textit{Streptococcus pneumoniae} underwent spontaneous phase variation between opaque and transparent colony. The transparent variant had more advantages on adhesion to epithelial cells and nasopharyngeal colonization than the opaque counterparts (Li et al., 2016). In \textit{Mycobacterium tuberculosis}, the reversible genetic frameshift mutations could lead to reversible drug resistances and a phenotype switching between large colony and small colony (Safi et al., 2019). In \textit{S. aureus}, the phenotypic switching between normal colony and small colony within an isogenic population commonly occurs. For example, Cui et al. (2012) found the small colony variants (SCVs) Mu50\(\Delta\)lcpA, which had the same general features of the Mu50 genome, could revert back to normal-size colonies, caused by almost half of the chromosome inversion between homologous regions of SaPlm4 and SaPlm1. Guerillot et al. (2019) found that the \textit{S. aureus} NRS384 produced reversible SCVs caused by chromosome reversion between two homologous regions of hsdM-hsdS genes. Switching into the SCV is a direct response of \textit{S. aureus} to environmental stresses, such as antibiotic stresses and host immune responses. However, whether \textit{S. aureus} uses other types of phenotypic diversity to adapt to environmental changes has not been reported.

Here, we report an unexpected observation of phenotypic variation into large colonies in Mu50\(\Delta\)lcpA but not in BA01611\(\Delta\)lcpA. This phenotypic variation has not been reported before in other lcpA null mutant strains of previous studies (Hub scher et al., 2009; Schaefer et al., 2017). Thus, the objective of this study was to investigate the biological consequence of this variant.

**MATERIALS AND METHODS**

**Bacterial Strains, Plasmids, and Growth Conditions**

Bacterial strains and plasmids used in this study are listed in Supplementary Table S1. We used two strains: the representative clinical MRSA-VISA strain Mu50 and the MRSA-VSSA strain BA01611 that isolated from bovine mastitis. \textit{S. aureus} strains were grown in tryptic soy broth (TSB) medium, unless stated otherwise. \textit{E. coli} DH5\(\alpha\) cells were cultivated in lysogeny broth (LB) medium with appropriate carbenicillin (Sigma-Aldrich Co., St. Louis, MO, United States).

**Construction of lcpA Deletion and Complementary Strains**

Deletion and complementation of the lcpA gene were performed by the allelic replacement method according to the published method (Wu et al., 2018). Briefly, to establish the lcpA null mutants of Mu50 and BA01611, the DNA fragments flanking the lcpA were amplified, then ligated into the pKZ2 vector \textit{via} Gibson Assembly, producing p\(\Delta\)lcpA. The primer sets were P1/P2 and P3/P4 for plasmid p\(\Delta\)lcpA (Supplementary Table S2). The plasmid p\(\Delta\)lcpA was electroporated into \textit{S. aureus}. The integration and loss of p\(\Delta\)lcpA and the selection of transconjugants were performed as previously described (Wu et al., 2018). The deletion of lcpA was verified by PCR amplification (Primers P5 and P6, see Supplementary Table S2) and sequencing.

To construct the complementary strains Mu50\(\Delta\)lcpA-LC::lcpA, Mu50\(\Delta\)lcpA-NC::lcpA and BA01611\(\Delta\)lcpA::lcpA, the lcpA was integrated back into the chromosomes of lcpA null mutants through the same procedure (Wu et al., 2018). The primer
sets were P7/P8 and P10/P11 for pΔlcpA-ΔlcpA^E146K as well as P8/P9 and P10/P11 for pΔlcpA-ΔlcpA (Supplementary Table S2). Moreover, a restriction site XhoI was inserted behind the lcpA transcriptional terminator for distinguishing the complements from the WT strains. The complementation of lcpA was confirmed by PCR (Primers P5 and P6, see Supplementary Table S2) and sequencing.

Assessment of the Phenotype Switching

About 0.5 × 10^3 CFU of Mu50ΔlcpA was spread on TSA plates and was grown at 37°C for 48 h. Independent colonies of the Mu50ΔlcpA-LC and Mu50ΔlcpA-NC were picked from the Mu50ΔlcpA plate according to colony size and inoculated in TSB for 12 h, and then the cultures were diluted and plated on TSA plates. These plates were incubated at 37°C for 48 h. This passage was repeated four times. The number of the large colonies and small colonies on the plates of every experiment were used to estimate the switching frequency. The possibility of the phenotypic switching being attributed to the impurity of inoculating bacteria was excluded by the clonal passage.

Measurement of Staphyloxanthin Production

Each strain was cultured in TSB at 37°C for 24 h. Cells were harvested from 1.7 ml cultures by centrifugation at 10,000 × g and washed twice with PBS. The cell pellets were suspended in 0.4 ml methanol and incubated at 55°C for 3 min. Cells were removed by centrifugation at 15,000 × g. The absorption spectra of the supernatant extracts were measured at OD_{480}. The experiment was repeated three times.

Measurement of Colony Diameter

About 0.5 × 10^3 CFU per Mu50 isogenic strain was spread on TSA plates and was incubated at 37°C for 48 h. To detect the contribution of growth to the colony expansion without passive swelling, these strains were spread on agar plates as described above and were initially incubated at 37°C for 24 h and then were incubated subsequently at 4°C for 24 h. All plates were imaged at 24 h, 36 h, and 48 h with a Bio-Rad ChemiDoc™ XRS™ system (CA, United States) in transmission model. The diameters of 50 random colonies per strain were measured at 24 h, 36 h, and 48 h.

Colony-Spreading Assay

The bacterial spreading ability was detected as the published method (Kaito and Sekimizu, 2007). Mu50 isogenic strains were incubated in TSB for 12 h. TSB supplemented with 0.24% agar was autoclaved and poured onto a plate (90 mm diameter). The plates were spotted with the bacterial cultures (2 μl) and incubated at 37°C for 48 h. The diameters of colonies were measured at 24 h, and the colonies were imaged at 24 h and 48 h using a Bio-Rad ChemiDoc™ XRS™ system (CA, United States) in transmission model. The experiment was repeated three times.

Biofilm Formation Assay

Biofilm formation was performed as the published study (Garcia-Betancur et al., 2017). Briefly, overnight bacterial cultures were diluted (1:200) in TSB plus 0.25% glucose before being added to a 96-well microtiter plate (100 μl per well). The plates were incubated at 37°C for 24 h without shaking and were then washed three times with PBS, dried at 65°C for 45 min, and stained with 0.1% crystal violet for 15 min. The stained crystal violet was solubilized for quantitative analysis using acetic acid at 33%. The absorption spectra of suspension were measured at OD_{595}. The experiment was repeated three times.

Antimicrobial Susceptibility Testing

The minimum inhibitory concentration (MIC) of three β-lactams (penicillin, cefazolin, and oxacillin; Tokyo Chemical Industry Co., Shanghai, China) against S. aureus were determined firstly by broth microdilution according to the published method (Wiegand et al., 2008). Briefly, three antimicrobial stock solutions were serially diluted twofold in 96-well microtiter plates with fresh Mueller–Hinton (MH) broth containing 2% NaCl. Then bacteria were added into each well, resulting in a final inoculum of 1×10^4 CFU/ml. Plates were incubated at 37°C for 24 h. The MIC was read as the lowest concentration that prevented visible growth. The experiment was repeated three times.

The susceptibilities of three β-lactams and four other antibiotics were further detected using the agar dilution technique following the published method (Wiegand et al., 2008). The bacterial suspensions were serially diluted tenfold in MH broth, giving final bacterial densities ranging from 10^5 CFU/ml to 10^6 CFU/ml. During the preliminary study, 1 μl of diluted bacterial suspensions was spotted onto the MH agar plates that had incorporated different concentrations of the antibiotics and 2% NaCl. Plates were incubated at 37°C for 24 h before checking the visible colonies. The bacteria showed different degrees of resistance to antibiotics. Then, the antibiotic concentration with the most obvious resistance difference was chosen to repeat this test three times. Images were captured using a Bio-Rad ChemiDoc™ XRS™ system (CA, United States).

The growth curves of S. aureus in TSB with β-lactam antibiotics were performed as the published method (Dilworth et al., 2014) with some modifications. In the preliminary study, overnight bacterial cultures were diluted to a starting density of 10^5 CFU/ml in TSB. Then several different β-lactam concentrations below the minimum inhibitory concentrations (MICs) were added into cultures. Then the antibiotic concentrations at which the mutant strains displayed the most obvious resistant differences were chosen to repeat the experiment. Cultures were shaken at 37°C for 8 h and the OD_{600} was determined at 1 h intervals. Moreover, the 48-h growth curves in TSB without β-lactams were determined through the same procedure, and the CFUs were determined on antibiotic-free TSA plates. The experiment was repeated three times.

Transmission Electron Microscopy

Mu50 isogenic strains were grown to stationary phase and were collected by centrifugation. The pellet was incubated in
a fixative solution [2.5% glutaraldehyde in 0.05 M sodium phosphate buffer (pH 7.4)] overnight at 4°C. Transmission electron microscopy samples were prepared at the Department of Electron Microscope of XI’AN JiaoTong University Health Science Center. Images were captured on the Hitachi H-7650 instrument. The fraction of the cells with the complete septum was counted according to the septum growth (400 cells per strain were counted). The cell area of 300 cells per strain was measure using the ImageJ plugin.

RNA Extraction and qRT-PCR Analysis
Bacteria at the exponential phase and stationary phase were harvested and mechanically lysed using glass beads. RNA was isolated from the supernatant using E.Z.N.A.*HP Total RNA Kit (Omega, Norcross, United States) and treated with DNasel (Transgen, Beijing, China). RNA was reverse-transcribed to cDNA using Transcript One-step gDNA Removal and cDNA Synthesis SuperMix (Transgen, Beijing, China). The qRT-PCR was performed using TransStart Tip Green qPCR SuperMix (Transgen, Beijing, China) on a CFX96 Real-Time PCR System (Bio-Rad). The qRT-PCR primers were shown in Supplementary Table S2.

Statistical Analysis
Statistical analyses were conducted using SPSS 23.0 (SPSS Inc., Chicago, IL). One-way ANOVA followed by LSD or Dunnett’s T3 multiple comparisons test was performed. Significant differences were defined by values of p (two tailed)<0.05 (*), <0.01 (**), and<0.001(***)

RESULTS
Reversible Large Colonies With Altered Phenotypes Are Detected in Mu50ΔlcpA Strain
To investigate the effect of lcpA on antibiotic resistance in S. aureus, the lcpA gene was removed from chromosomes of Mu50 and BA01611 strains, producing the Mu50ΔlcpA and BA01611ΔlcpA strains, respectively. During the experiment, we unexpectedly observed that the Mu50ΔlcpA exhibited two distinct colony morphologies on agar plates: a large flat colony (Mu50ΔlcpA-LC) and a normal colony (Mu50ΔlcpA-NC) that was similar to Mu50 WT (Figure 1A, panel 1). Mu50ΔlcpA-NC had decreased pigment production compared with Mu50 WT, while the pigment of Mu50ΔlcpA-LC was significantly less than that of Mu50ΔlcpA-NC (Figure 1B). To test the stability of their colony morphology, a large colony and a normal colony were picked from the Mu50ΔlcpA agar plate and incubated in tryptic soy broth (TSB) for 12h. Then, each culture was diluted and spread on TSA agar (TSA) plates. Results showed that a few large colonies always appeared in Mu50ΔlcpA-NC populations, while a few normal colonies always appeared in Mu50ΔlcpA-LC populations (Figure 1A, panel 2 and 3). From four repeated experiments, the colony count showed that both the large and normal switching colonies appeared in bacteria populations at a frequency of about 0.1%. This heterogeneity in colony morphology was not observed in Mu50 WT and all BA01611 isogenic strains. These results suggest that the deficiency of lcpA might induce an unstable phenotype in Mu50.

To further analyze the colony morphology of Mu50ΔlcpA-LC, the Mu50ΔlcpA was spread on TSA plates and incubated firstly at 37°C for 24h, then subsequently incubated at 37°C or 4°C for 24h. As shown in Figures 1C,D, bacterial colonies could keep on expanding within 48h at 37°C, and the colony diameter of Mu50ΔlcpA-LC was significantly larger than Mu50 WT and Mu50ΔlcpA-NC strains on average. When incubated for 48h at 37°C, the colony diameter of Mu50ΔlcpA-LC was 66.4% larger than Mu50 WT, while Mu50ΔlcpA-NC was 0.04% smaller than Mu50 WT. However, the Mu50ΔlcpA-LC colonies were not expanded when bacterial growth was inhibited by low temperature (4°C) from 24th to 48th hour (Figure 1D). These results suggest that the Mu50ΔlcpA-LC had a growth advantage on solid medium at 37°C. However, the lcpA complementary strain Mu50ΔlcpA-LC::lcpA still maintained the large colony morphology (Figure 1D), and the lcpA complementation did not restore the decreased pigment production of Mu50ΔlcpA-LC but partially worked in Mu50ΔlcpA-NC (Figure 1B). These results suggest that other unknown factors than the lcpA also contribute to the large colony morphology and decreased pigmentation of Mu50ΔlcpA-LC.

In addition to the differences in colony morphology and pigmentation, Mu50ΔlcpA-LC also showed a stronger spreading ability on soft agar plates in the spreading ability assay and decreased biofilm formation in the crystal violet staining assay when compared with Mu50ΔlcpA-NC and Mu50 WT strains (Figures 1E,F). There were no differences in spreading ability and biofilm production between Mu50ΔlcpA-NC and Mu50 WT (Figures 1E,F). Unexpectedly, the lcpA complementation resulted in a significantly lower biofilm production in Mu50ΔlcpA-LC and Mu50ΔlcpA-NC strains (Figure 1F). These results suggest that the stronger spreading ability and reduced biofilm formation helped Mu50ΔlcpA-LC to expand on agar plates.

Large and Normal Colony Isolates of Mu50ΔlcpA Exhibit Distinct Susceptibility to β-Lactam Antibiotics
To test the resistance of Mu50ΔlcpA-LC against β-lactam antibiotics, the MICs of all strains against three β-lactam, oxacillin, cefazolin, and penicillin were first determined by broth microdilution. As shown in Table 1, the BA01611ΔlcpA had reduced MICs for all three β-lactam antibiotics and the complementation with lcpA gene in BA01611ΔlcpA restored the decreased MICs. However, the Mu50 WT, Mu50ΔlcpA-LC, Mu50ΔlcpA-NC, and Mu50ΔlcpA-NC::lcpA had same MICs for these β-lactams. Unexpectedly, Mu50ΔlcpA-LC::lcpA displayed significantly decreased MICs of β-lactams (Table 1). This result revealed that the lcpA exerted strain-specific effects on β-lactam resistance in S. aureus. lcpA contributed to β-lactam resistance in BA01611, but it was not necessary for Mu50ΔlcpA-NC.
FIGURE 1 | Continued
However, lcpA significantly decreased the β-lactam resistance in Mu50ΔlcpA-LC. The resistance of lcpA null mutants against antibiotics was further investigated by agar dilution, first using a multiple concentration gradient and then using a fixed concentration, at which the difference of resistance against the antibiotics was most obvious. As shown in Figure 2A, compared with the WT strains and complementary strains, the Mu50ΔlcpA-LC had the highest resistances against β-lactams, while the resistances of Mu50ΔlcpA-LC::lcpA decreased sharply. In contrast, the BA01611ΔlcpA was more susceptible to β-lactams than its WT and complementary strains (Figure 2A). Besides, susceptibilities to glycopeptide antibiotics (vancomycin and teicoplanin), tetracycline, and clindamycin were also measured. The BA01611ΔlcpA was more susceptible to vancomycin but not to teicoplanin than its WT and complementary strains. Mu50ΔlcpA-LC was more sensitive to teicoplanin and vancomycin than its WT and complementary strains, while Mu50ΔlcpA-NC was more resistant to vancomycin and more sensitive to teicoplanin (Supplementary Figure S1). β-Lactams and glycopeptides act on the cell wall, while the drug target site of tetracycline and clindamycin is the intracellular ribosome. Both BA01611ΔlcpA and Mu50ΔlcpA-NC showed increased susceptibility to tetracycline and clindamycin, while Mu50ΔlcpA-LC had same resistances to these two antibiotics as the wild type (Supplementary Figure S1). These results showed again that the effect of deleting lcpA on antibiotic resistance was strain-specific. For BA01611, deleting lcpA reduced resistance to most cell wall-targeting antibiotics and ribosome-targeting antibiotics. For Mu50ΔlcpA-NC, deleting lcpA had no significant effect on resistance to most cell wall-targeting antibiotics but decreased its ribosome-targeting antibiotic resistance. For Mu50ΔlcpA-LC, deleting lcpA increased the resistance to all cell-wall targeted antibiotics but had no effect on resistance to ribosome-targeting antibiotics. These results suggested that Mu50ΔlcpA could protect its bacterial population against not only cell-wall-targeted antibiotics but also ribosome-targeting antibiotics by switching into Mu50ΔlcpA-LC.

To corroborate the result obtained by agar dilution, the growth curves in TSB containing β-lactam antibiotics were investigated. All Mu50 isogenic strains had same growth rates in TSB without antibiotics (Figure 2B). As shown in Figure 2C, the Mu50ΔlcpA-LC strain had higher resistance than Mu50 WT evidenced by more growth advantages after 3h incubation, while the resistance to β-lactams of Mu50ΔlcpA-NC was similar to Mu50 WT. The mixed strain Mu50ΔlcpA (including Mu50ΔlcpA-LC and Mu50ΔlcpA-NC) also displayed higher antibiotic resistance than Mu50 WT and Mu50ΔlcpA-NC but lower than Mu50ΔlcpA-LC, suggesting the increased β-lactam resistance of Mu50ΔlcpA was ascribed to the Mu50ΔlcpA-LC cells. The growth of Mu50ΔlcpA-LC::lcpA was inhibited in antibiotics (Figure 2C). These results prove again that the Mu50ΔlcpA-LC had increased resistance against β-lactams. Collectively, deleting lcpA decreased β-lactam resistance in BA01611ΔlcpA but increased β-lactam resistance in Mu50ΔlcpA-LC, based on the agar dilution assay and the growth curve test.

**Most Mu50ΔlcpA-LC Cells Possess a Complete Septum and Have a Large Cell Size**

To investigate the cell envelope changes in Mu50ΔlcpA-LC, the cells of Mu50 WT, Mu50ΔlcpA-LC, and Mu50ΔlcpA-NC at the stationary phase were imaged by transmission electron microscopy (TEM). As shown in Figure 3A, no defect in cell walls in all strains was observed. However, a larger fraction of Mu50ΔlcpA-LC cells had a complete closed septum compared with Mu50 WT and Mu50ΔlcpA-NC. As quantified in Figure 3B, a complete septum was observed in 60.5 ± 2.9% of the Mu50ΔlcpA-LC cells compared to 16.3 ± 2.7% of the Mu50ΔlcpA-NC cells and 11.6 ± 3.1% of the Mu50 WT cells.

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**TABLE 1** β-lactam MICs (µg/ml) of wild-type and lcpA mutant strains.

| Strains            | Oxacillin | Cefazolin | Penicillin |
|--------------------|-----------|-----------|------------|
| BA01611            | 32        | 64        | 512        |
| BA01611ΔlcpA       | 16        | 16        | 256        |
| BA01611ΔlcpA::lcpA | 32        | 64        | 512        |
| Mu50              | 256       | 128       | 16         |
| Mu50ΔlcpA          | 256       | 128       | 16         |
| Mu50ΔlcpA-LC       | 256       | 256       | 16         |
| Mu50ΔlcpA-NC       | 256       | 128       | 16         |
| Mu50ΔlcpA-LC::lcpA | 8         | 32        | 4          |
| Mu50ΔlcpA-NC::lcpA | 256       | 128       | 16         |

LC, large colony; NC, normal colony.
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FIGURE 2 | β-Lactam susceptibility assay for BA01611 and Mu50 isogenic strains. (A) β-Lactam resistance was measured by agar dilution for BA01611 and Mu50 isogenic strains. Bacteria cultures were diluted from 10^8 to 10^4 CFU/ml, then 1 μl of bacterial suspensions was spotted on the MHA + 2% NaCl medium with different antibiotics. Images of MHA plates represent one out of three experiments showing similar results. (B) Growth curves of Mu50 isogenic strains in TSB without antibiotics showing by CFU/ml. (C) Growth curves of BA01611 and Mu50 isogenic strains in TSB with β-lactams showing by OD_{600}. Error bars correspond to SEM from three independent experiments. One-way ANOVA with LSD or Dunnett’s T3 test for multiple comparisons, *p < 0.05, **p < 0.01, ***p < 0.001.
There were also many cells (10.8 ± 1.7%) with the incomplete septum in Mu50ΔlcA-LC. In contrast, a large proportion of Mu50 WT cells (85.6 ± 3.7%) and Mu50ΔlcA-NC cells (78.3 ± 4.2%) were non-dividing, while only 28.7 ± 4.0% of Mu50ΔlcA-LC cells had no septum. Furthermore, the individual cell sizes of Mu50ΔlcA-LC and Mu50ΔlcA-NC cells were 21.7 and 11.7% larger than that of the Mu50 WT (Figure 3C). In addition, Mu50ΔlcA-LC cell area was significantly larger than Mu50ΔlcA-NC (Figure 3C). These results suggest that most Mu50ΔlcA-LC cells with the closed septum may increase the cell size (Figure 3C) by halting in inward progression of cell splitting, which may contribute to the expansion of the large colonies.

**Mu50ΔlcA-LC Has a Significant High Level of spa Expression**

Lower pigmentation, decreased biofilm formation, higher spreading ability and increased resistance to a Bsa bacteriocin that targets cell wall have been reported for a variant of a clinical MRSA strain, in comparison with its parent strain (Koch et al., 2014). The authors attribute the changes to a
mutation in the rsbW gene, which upregulated the agr system, leading to changes in expression levels of agr downstream-regulated genes, such as a decrease in spa gene and an increase in psm gene (Koch et al., 2014). But Mu50 is an agr-defected strain although it possesses an agr system (Sakoulas et al., 2002). To investigate whether the phenotype switching of Mu50ΔlcpA-LC was also induced by activating the agr system, we performed the qRT-PCR assay. The result showed that the agr system was not activated in Mu50ΔlcpA-LC (data not shown). On the contrary, the expression of spa was significantly increased in Mu50ΔlcpA-LC, in comparison with Mu50 WT, increased about five hundred times at the exponential phase and about 5.8 times at the stationary phase (Figure 4), respectively. These results suggest that the phenotype switching of Mu50ΔlcpA-LC was not induced by upregulating agr system.

**DISCUSSION**

The phenotypic diversity can spontaneously emerge in an isogenic population, allowing bacteria to survive dynamic environments (Ackermann, 2015). Here, we first identified a reversible large colony variant Mu50ΔlcpA-LC from an isogenic population of *S. aureus* Mu50ΔlcpA. Mu50ΔlcpA-LC is distinct broadly in phenotypes, with decreased susceptibility to β-lactam antibiotics, high spreading ability, reduced biofilm formation, low pigmentation, and a significant increase in spa expression level. Our results suggest that the *S. aureus* can evolve in response to antibiotic stress and host immunity system by phenotypic switching.

Mu50ΔlcpA-LC forms large colonies due to its growth advantage on solid medium (Figure 1D). It has been known that the formation of SCVs has been directly associated with its slow growth (Proctor et al., 2006). In Mu50ΔlcpA-LC, switching to the large colony phenotype may promote these bacterial cells to move to more suitable places for nutrition and colonization *in vivo*, due to their higher spreading ability and decreased biofilm formation (Figures 1E,F). The high spreading ability promoted motility of bacterial cells on surfaces (Koch et al., 2014). The less polymeric matrix of biofilm reduced restrictions on the expansion of colony on 1% agar plate in *Vibrio cholerae* (Yan et al., 2017). In addition, the TEM assay showed that the individual cell size of Mu50ΔlcpA-LC was 21.7% larger than that of Mu50 WT, while the colony diameter of Mu50ΔlcpA-LC was 66.4% larger than that of Mu50 WT. Importantly, Mu50ΔlcpA-LC did not differ from WT in terms of the growth rate (Figure 2B). It has been reported that a phase variant with an unchanged growth rate could expand rapidly and form large colonies due to its elongated cells and cell chains in rod bacteria *Clostridioides difficile* (Garrett et al., 2019). On the other hand, Kriegeskorte et al. (2014) reported that the enlarged cells with incomplete or multiple division planes of ΔthvA mutant of *S. aureus* could form small colonies, possibly due to the lower growth rate and severe growth defects of the mutant. Therefore, the large cell size of Mu50ΔlcpA-LC, combined with its high spreading ability and low biofilm formation, could contribute to the colony expansion.

Mu50ΔlcpA-Negative had same MICs against all three β-lactam antibiotics, while BA01611ΔlcpA reduced MICs against all three β-lactam antibiotics (Table 1). Decreased resistance of BA01611ΔlcpA could be due to the decreased level of PGN cross-linking that was controlled by WTA (Atianno et al., 2010). However, Mu50 could resist lcpA deletion through its active PGN synthesis and thicker cell wall, as observed by Cui et al. (2000). The TEM assay also proved that lacking lcpA did not lead to a defective cell wall in Mu50ΔlcpA-Neg (Figure 3A). Broth microdilution showed that Mu50ΔlcpA-LC had same β-lactam MICs as WT and Mu50ΔlcpA-Neg strains (Table 1). The precision of broth microdilution was considered to be plus or minus 1 twofold concentration. To increase the precision, we used agar dilution and the growth curve method, with more concentration gradients. The increased antibiotic resistance of Mu50ΔlcpA-LC against β-lactams was demonstrated by agar dilution and the growth curve test.

**FIGURE 4** | qRT-PCR analysis for expression of spa in Mu50 isogenic strains grown at the exponential phase and the stationary phase. Genes were normalized with the expression level of gyrB. Error bars correspond to SEM, five independent experiments. One-way ANOVA with LSD test for multiple comparisons, “p<0.05, ““p<0.01, “***p<0.001"
Several phenotypic changes we observed in Mu50ΔlcpA-LC are similar to what has been reported for an un-pigmented variant “W” developed from a clinical MRSA strain (Koch et al., 2014). Both Mu50ΔlcpA-LC and the “W” variant had a high spreading ability, decreased biofilm formation and low pigmentation. However, mechanisms for such phenotypic changes could be different. The “W” variant produced the lantibiotic bacteriocin of *S. aureus* (Bsa) which targets bacterial cell wall precursors to remove niche competitors in the intra-clonal competitive environment (Koch et al., 2014). However, Mu50 does not contain the *bsa* gene cluster and had a growth advantage in cell wall-targeting antibiotics (Figure 2). In addition, the “W” variant with RsbW mutation caused hyperactivation of the *agr* system (Koch et al., 2014). However, Mu50 is an *agr*-deficient strain that carries several silent mutations in *agrA* (Sakoulas et al., 2002). Indeed, in this study, all the Mu50 isogenic strains express the *RNAIII* at extremely low levels (data not shown). Furthermore, the *spa* gene expression, which was inhibited by *agr* in the “W” variant, was unexpectedly increased significantly in Mu50ΔlcpA-LC than other isogenic strains at the exponential and stationary phases (Figure 4). Despite unknown mechanisms for phenotypic changes in Mu50ΔlcpA-LC, the activation of *spa* expression may allow Mu50ΔlcpA-LC to escape the host immunity, since *spa* encodes the surface protein A that binds the Fc region of immunoglobulin G (IgG) and facilitates the bacteria evasion from the host immune response (Atkins et al., 2008).

The changed phenotypes of Mu50ΔlcpA-LC, including the possession of septum at the stationary phase, β-lactam antibiotic resistance, spreading ability, the staphyloxanthin production (a component of cell functional membrane microdomain) and the upregulated *spa* encoding the surface protein A are all associated with the cell wall structure (Kaito and Sekimizu, 2007; García-Fernández et al., 2017). Thus, it is plausible that the distinct phenotypes of Mu50ΔlcpA-LC are associated with alteration of its cell envelope. Besides, phenotypic diversity could also occur independently of genetic variation by methods such as epigenetic modification of chromatin and stochastic gene expression (Ackermann, 2015). Therefore, further investigation is needed to find the mechanisms of the phenotypic diversity of Mu50ΔlcpA.

These findings from this study will aid in our understanding of the ability of *S. aureus* to adapt to fluctuating environments through the development of strain-level variation. The variant in this study may allow *S. aureus* community to evade environmental stresses such as antibiotics and immune system.
to colonize rapidly in hosts, causing persistent infections (Figure 5).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

XZ and YS conceived and designed the experiments and wrote the manuscript. YS collected the data and performed the analysis. ML and MN participated in data collection. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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