β-Lactam Antibiotics Enhance the Pathogenicity of Methicillin-Resistant *Staphylococcus aureus* via SarA-Controlled Lipoprotein-Like Cluster Expression

Weilong Shang, a Yifan Rao, b Ying Zheng, a Yi Yang, a Qiwen Hu, a Zhen Hu, a Jizhen Yuan, a Huagang Peng, a Kun Xiong, a Li Tan, a Shu Li, a Junmin Zhu, a Ming Li, a Xiaomi Hu, a Xuhu Mao, b,c Xiancai Rao a

a Department of Microbiology, College of Basic Medical Sciences, Army Medical University (Third Military Medical University), Key Laboratory of Microbial Engineering under the Educational Committee in Chongqing, Chongqing, China
b Institute of Modern Biopharmaceuticals, School of Life Sciences, Southwest University, Chongqing, China
c Department of Clinical Microbiology and Immunology, College of Medical Laboratory Science, Army Medical University (Third Military Medical University), Chongqing, China

**ABSTRACT** Methicillin-resistant *Staphylococcus aureus* (MRSA) resists nearly all β-lactam antibiotics that have a bactericidal activity. However, whether the empirically used β-lactams enhance MRSA pathogenicity in vivo remains unclear. In this study, we showed that a cluster of lipoprotein-like genes (*lpl*, *sa2275* to *sa2273*) was upregulated in MRSA in response to subinhibitory concentrations of β-lactam induction. The increasing expression of *lpl* by β-lactams was directly controlled by the global regulator SarA. The β-lactam-induced Lpls stimulated the production of interleukin-6 and tumor necrosis factor alpha in RAW 264.7 macrophages. The *lpl* deletion mutants (N315Δ*lpl* and USA300Δ*lpl*) decreased the proinflammatory cytokine levels in vitro and in vivo. Purified lipidated SA2275-his proteins could trigger a Toll-like-receptor-2 (TLR2)-dependent immune response in primary mouse bone marrow-derived macrophages and C57BL/6 mice. The bacterial loads of N315Δ*lpl* in the mouse kidney were lower than those of the wild-type N315. The β-lactam-treated MRSA exacerbated cutaneous infections in both BALB/c and C57BL/6 mice, presenting increased lesion size; destroyed skin structure; and easily promoted abscess formation compared with those of the untreated MRSA. However, the size of abscesses caused by the β-lactam-treated N315 was negligibly different from those caused by the untreated N315Δ*lpl* in C57BL/6 TLR2−/− mice. Our findings suggest that β-lactams must be used carefully because they might aggravate the outcome of MRSA infection compared to inaction in treatment.

**IMPORTANCE** β-Lactam antibiotics are widely applied to treat infectious diseases. However, certain poor disease outcomes caused by β-lactams remain poorly understood. In this study, we have identified a cluster of lipoprotein-like genes (*lpl*, *sa2275*–*sa2273*) that is upregulated in the major clinically prevalent MRSA clones in response to subinhibitory concentrations of β-lactam induction. The major highlight of this work is that β-lactams stimulate the expression of SarA, which directly binds to the *lpl* cluster promoter region and upregulates *lpl* expression in MRSA. Deletion of *lpl* significantly decreases proinflammatory cytokine levels in vitro and in vivo. The β-lactam-induced Lpls enhance host inflammatory responses by triggering the Toll-like-receptor-2-mediated expressions of interleukin-6 and tumor necrosis factor alpha. The β-lactam-induced Lpls are important virulence factors that enhance MRSA pathogenicity. These data elucidate that subinhibitory concentrations of β-lactams can exacerbate the outcomes of MRSA infection through induction of *lpl* controlled by the global regulator SarA.
KEYWORDS \(\beta\)-lactam antibiotics, methicillin-resistant *Staphylococcus aureus*, SarA, TLR2, lipoprotein-like genes, pathogenicity

*Methicillin* (MET)-resistant *Staphylococcus aureus* (MRSA) is a leading pathogen with notable pathogenic effects. MRSA causes a wide range of diseases, including acute skin and soft tissue infections, chronic and persistent endocarditis, osteomyelitis, and pneumonia (1, 2). MRSA infections cause higher morbidity and mortality than infections by MET-susceptible *S. aureus* (MSSA) (3, 4). However, the underlying mechanisms of these effects remain unclear. Studies have suggested that inappropriate treatments or unidentified virulence factors contribute to poor outcomes of MRSA infections (5, 6). Owing to failure to initially recognize MRSA infection, between 30% and 80% of individuals infected with MRSA have been reported to be inappropriately treated with \(\beta\)-lactam antibiotics (7, 8). Low levels of antibiotics can induce extracellular DNA release, biofilm formation, and virulence factor production (9, 10). Accumulated data have revealed that subinhibitory concentrations of \(\beta\)-lactam antibiotics can promote *S. aureus* pathogenicity by increasing the expression of alpha-toxin, Panton-Valentine leukocidin (PVL), enterotoxins, or staphylococcal protein A (SpA) *in vitro* (9–13). The contributions of certain altered virulence factors to MRSA pathogenicity *in vivo* and the molecular mechanisms underlying \(\beta\)-lactam-modulated MRSA pathogenicity remain largely unknown.

Over the past few decades, the global virulence regulon staphylococcal accessory (sar) and accessory gene regulators (agr) have been recognized to play central roles in *S. aureus* pathogenesis (14, 15). SarA is a pleiotropic global regulator that modulates the expression of approximately 120 genes in *S. aureus* via agr-dependent or -independent pathways (16). As a classic transcription factor, SarA can activate expressions of certain genes, for example, *agr* and *hla*, and repress expressions of others, such as *cna* and *sspA* (17). Treatment of *S. aureus* strains with subinhibitory concentrations of \(\beta\)-lactams showed increased SarA expression (12). However, whether \(\beta\)-lactam-induced SarA modulates other virulence factors to contribute to MRSA pathogenicity remains an important issue that must be addressed.

Lipoproteins (Lpps) are an abundant family of proteins anchored to the bacterial membrane and account for at least 2% of bacterial proteomes (18, 19). *S. aureus* encodes 55 to 70 putative Lpps, and approximately 50% of these Lpps are annotated as chaperones or transporters of amino acids, peptides, iron, and zinc (18). Several Lpps include major Toll-like receptor 2 (TLR2) ligands that play important roles in *S. aureus* infection and host inflammatory response (20). Several staphylococcal Lpps can trigger host cell invasion, increase bacterial pathogenicity, and contribute to the epidemic of CC8 and CC5 strains (21, 22). Other authors proposed that more than 30% of Lpps in *S. aureus* are hypothetically conserved proteins with unknown functions (19). Most virulent MRSA strains, such as USA300, carry a conserved genomic island termed \(\nu S a\alpha\), which is a nonphage and non-staphylococcal cassette chromosome genomic island that contains numerous homologous tandem-arranged *lpp* genes, which are referred to as “tandem Lpps” or “lipoprotein-like” (*lpl*) (18, 23, 24). This *lpl* cluster possibly represents the paralogous genes that have diverged after a duplication event in *S. aureus* (18). MRSA USA300, belonging to the clonal complex CC8, carries 15 (22%) hypothetical Lpls. Among these Lpls, nine are specific to the \(\nu S a\alpha\) island (18). In contrast, N315, belonging to the clonal complex CC5, carries 12 (21%) hypothetical Lpls. Among these Lpls, nine Lpl proteins are specific to the \(\nu S a\alpha\) island (locus 0) and three Lpls are encoded by the genome (locus III) (see Table S1 in the supplemental material) (24). However, the exact roles of Lpls remain unclear.

In this study, we demonstrated that an *lpl* cluster outside the \(\nu S a\alpha\) island was upregulated in response to subinhibitory concentrations of \(\beta\)-lactam induction. We observed that the increasing expression of *lpl* after \(\beta\)-lactam treatment was directly controlled by the global regulator SarA. We also showed that the \(\beta\)-lactam-induced Lpls are important virulence factors that enhance MRSA pathogenicity by triggering the...
TLR2-dependent expressions of interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α).

RESULTS

**β-Lactam antibiotics stimulated lpl expression in MRSA.** β-Lactam antibiotics block the cell wall synthesis of bacteria to exert antimicrobial effects. In contrast, subinhibitory concentrations of β-lactam antibiotics are reported to induce the production of *S. aureus* toxins (12). A globally prevalent sequence type 5 (ST5) MRSA strain N315 (25) was tested for its antibiotic response to identify new factors contributing to MRSA pathogenicity. The MICs of oxacillin (OXA), MET, cefoxitin (FOX), imipenem (IMI), meropenem (MER), chloramphenicol (CHL), vancomycin (VAN), kanamycin (KAN), and erythromycin (ERY) against N315 were determined (see Table S2 in the supplemental material). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed that a protein band of approximately 30 kDa was upregulated in subinhibitory concentrations of OXA-, MET-, FOX-, IMI-, or MER-treated N315 compared with the untreated control (Fig. 1A). In contrast, CHL, VAN, KAN, and ERY showed no induction effects on this protein band. Further observations indicated that subinhibitory concentrations of OXA exerted a broad-spectrum induction effect on other major clinically prevalent MRSA clones (see Fig. S1A in the supplemental material).

The protein band was excised from the SDS-PAGE gel and analyzed through liquid chromatography tandem mass spectrometry (LC-MS/MS) to characterize the β-lactam-induced proteins in MRSA strains. The detected peptides matched with 68 proteins in the N315 proteome (see Table S3 in the supplemental material). Most known metabolic enzymes were excluded, and three putative Lpls, SA2273 (30.8 kDa), SA2274 (30.1 kDa), and SA2275 (30.4 kDa), which are encoded by a consecutive gene cluster, were selected on the basis of theoretical molecular weights for analysis (Fig. 1B; see Table S1). SA2273, SA2274, and SA2275 were annotated as Lpls in the N315 genome (locus III) (GenBank accession no. BA000018.3) (24). A typical Lpp precursor contains a signal peptide at the N-terminal end, and a characteristic conserved three-amino-acid lipobox is detected in front of the invariable cysteine ([LVI] (ASTG) (GA) C)](19, 20). Both SA2275 and SA2273 possess signal peptides and “lipobox” sequences, whereas SA2274 comprises a transmembrane helix domain at the N-terminal end (see Fig. S1B in the supplemental material). These proteins were annotated as Lpls that belong to a domain of unknown function 576 (DUF576) protein family on the Pfam database (26) and account for more than 62.6% of amino acid identity (see Fig. S1C in the supplemental material). To verify whether sa2275, sa2274, and sa2273 are cotranscribed, reverse transcription-PCR (RT-PCR) of sa2273 was performed with RNA extracted from wild-type N315 by specific primers. Results on the template of genomic DNA or RNA revealed that sa2275, sa2274, and sa2273 were cotranscribed from the sa2275 promoter (Fig. 1B). We further examined the influence of β-lactams on lpl expression. Quantitative RT-PCR (RT-qPCR) showed that the mRNA levels of sa2275, sa2274, and sa2273 were upregulated from the sa2275 promoter (Fig. 1B). We further examined the influence of β-lactams on lpl expression. Quantitative RT-PCR (RT-qPCR) showed that the mRNA levels of sa2275, sa2274, and sa2273 were upregulated from the sa2275 promoter (Fig. 1B). We further examined the influence of β-lactams on lpl expression. Quantitative RT-PCR (RT-qPCR) showed that the mRNA levels of sa2275, sa2274, and sa2273 were upregulated in N315 after treatment with subinhibitory concentrations of OXA (Fig. 1C). The identities of β-lactam-induced proteins were verified through Western blot analysis (Fig. 1D). Two protein bands were detected by mouse anti-SA2275 antibody in Western blot experiments, and this result was probably due to the high identities of Lpls at the amino acid level. Western blot analysis also demonstrated that the protein levels of Lpls in N315 total cell lysates (Fig. 1E) and culture supernatant (see Fig. S1D in the supplemental material) increased in a dose-dependent manner after OXA treatment. Lpl expression was also upregulated in N315 after MET treatment (see Fig. S1E in the supplemental material). Furthermore, the Lpl expression by N315 increased in a time-dependent manner after OXA treatment (Fig. 1F). These results verified that MRSA Lpls could be released from bacteria, and their production was influenced by subinhibitory concentrations of β-lactam antibiotics.

**β-Lactam-induced Lpl expression in MRSA was directly controlled by SarA.** As inducers, β-lactams may trigger global regulatory networks to modulate virulence in *S. aureus*; the global virulence regulons sar and agr play critical roles in virulence factor...
Global regulators can recognize specific motifs in the promoter regions of a certain gene, thereby controlling gene expression (14). We analyzed the binding motif of SarA (27, 28) in the promoter regions of lpl and discovered a typically predicted SarA box (Fig. 2A). Electrophoretic mobility shift assay (EMSA) results showed that recombinant SarA-his proteins bound to the lpl cluster promoter region that carried the putative SarA binding box (Fig. 2B). No shifting band was observed when the AT-rich SarA box was mutated to become GC rich (Fig. 2C). To investigate whether β-lactam-stimulated sarA can regulate the expression of lpl. Western blot analysis indicated that both SarA and Lpls increased in a dose-dependent manner in response to β-lactam antibiotic treatment (Fig. 2D). Deletion of sarA (N315ΔsarA) reduced Lpl levels in N315 (Fig. 2E). The sarA-overexpressing strain (N315ΔsarA/pLI-sara) produced more Lpls than the wild-type N315 strain, whereas the empty pLI50-transformed strain production (12, 14). Global regulators can recognize specific motifs in the promoter regions of a certain gene, thereby controlling gene expression (14). We analyzed the binding motif of SarA (27, 28) in the promoter regions of lpl and discovered a typically predicted SarA box (Fig. 2A). Electrophoretic mobility shift assay (EMSA) results showed that recombinant SarA-his proteins bound to the lpl cluster promoter region that carried the putative SarA binding box (Fig. 2B). No shifting band was observed when the AT-rich SarA box was mutated to become GC rich (Fig. 2C). To investigate whether β-lactam-stimulated sarA can regulate the expression of lpl. Western blot analysis indicated that both SarA and Lpls increased in a dose-dependent manner in response to β-lactam antibiotic treatment (Fig. 2D). Deletion of sarA (N315ΔsarA) reduced Lpl levels in N315 (Fig. 2E). The sarA-overexpressing strain (N315ΔsarA/pLI-sara) produced more Lpls than the wild-type N315 strain, whereas the empty pLI50-transformed strain production (12, 14). Global regulators can recognize specific motifs in the promoter regions of a certain gene, thereby controlling gene expression (14). We analyzed the binding motif of SarA (27, 28) in the promoter regions of lpl and discovered a typically predicted SarA box (Fig. 2A). Electrophoretic mobility shift assay (EMSA) results showed that recombinant SarA-his proteins bound to the lpl cluster promoter region that carried the putative SarA binding box (Fig. 2B). No shifting band was observed when the AT-rich SarA box was mutated to become GC rich (Fig. 2C). To investigate whether β-lactam-stimulated sarA can regulate the expression of lpl. Western blot analysis indicated that both SarA and Lpls increased in a dose-dependent manner in response to β-lactam antibiotic treatment (Fig. 2D). Deletion of sarA (N315ΔsarA) reduced Lpl levels in N315 (Fig. 2E). The sarA-overexpressing strain (N315ΔsarA/pLI-sara) produced more Lpls than the wild-type N315 strain, whereas the empty pLI50-transformed strain production (12, 14). Global regulators can recognize specific motifs in the promoter regions of a certain gene, thereby controlling gene expression (14). We analyzed the binding motif of SarA (27, 28) in the promoter regions of lpl and discovered a typically predicted SarA box (Fig. 2A). Electrophoretic mobility shift assay (EMSA) results showed that recombinant SarA-his proteins bound to the lpl cluster promoter region that carried the putative SarA binding box (Fig. 2B). No shifting band was observed when the AT-rich SarA box was mutated to become GC rich (Fig. 2C). To investigate whether β-lactam-stimulated sarA can regulate the expression of lpl. Western blot analysis indicated that both SarA and Lpls increased in a dose-dependent manner in response to β-lactam antibiotic treatment (Fig. 2D). Deletion of sarA (N315ΔsarA) reduced Lpl levels in N315 (Fig. 2E). The sarA-overexpressing strain (N315ΔsarA/pLI-sara) produced more Lpls than the wild-type N315 strain, whereas the empty pLI50-transformed strain
SarA bound to the lpl cluster promoter region to control β-lactam-induced Lpl expression in MRSA. (A) Predicted SarA box in the promoter regions of the lpl cluster. (B) EMSA detected the interaction between wild-type lpl promoter region (lpl-P) and SarA-his proteins. (C) EMSA by lpl promoter mutant (lpl-PM). (D) Western blot analysis of SarA and Lpls in N315 treated with different concentrations of OXA. (E) Deletion of sarA decreased Lpl expression in N315. (F) Western blot analysis of SarA and Lpls in N315 and N315ΔsarA postcultured in BHI with or without OXA treatment. (G) Lpl levels in N315ΔagrA increased after treatment with different concentrations of OXA. (H) Lpl levels in USA300ΔagrA increased after treatment with different concentrations of OXA. Western blot assays and EMSAs were repeated three times, and representative gels are shown. Molecular weights of the protein marker (M) are indicated on the left. LC, loading control.

(N315ΔsarA/pLJ50) caused no such effect. The Lpls presented no significant change in the sarA mutant after OXA treatment compared with untreated N315ΔsarA (Fig. 2F). However, OXA treatment significantly increased the Lpl levels in N315. These data indicate that S. aureus SarA can directly bind to the lpl cluster promoter region, thereby upregulating lpl expression in the presence of β-lactams. The AT-rich motif (ATTTAAT) in the promoter regions of lpl is essential for SarA binding and regulation.

We then examined whether AgrA can upregulate the expression of lpl. Western blot analysis showed that N315 and N315ΔagrA produced similar amounts of Lpls, and Lpl levels in N315ΔagrA increased after OXA treatment (Fig. 2G). Studies have reported that N315 contains a defective agr (29). We then tested the effect of agr on lpl expression in an agr-positive MRSA USA300 strain. USA300ΔagrA expressed similar amounts of Lpls as the wild-type strain, and Lpl levels in USA300ΔagrA also increased after OXA treatment (Fig. 2H). These data indicate that MRSA agr plays no role in the regulation of Lpl expression. The β-lactam-induced Lpl expression in MRSA may be controlled by SarA through an agr-independent pathway.

β-Lactam-induced Lpls triggered TLR2-dependent proinflammatory cytokine production by macrophages. The rSaα-specific Lpl proteins of MRSA USA300 enhanced the production of IL-6 and TNF-α in innate immune cells (21). In N315, the β-lactam-inducible lpl genes are not in the rSaα island and encode Lpls belonging to the DUF576 protein family (24, 26). To determine whether Lpls contribute to innate
immune stimulation, we constructed a markerless deletion mutant, N315Δ/lpl, and a complement strain, N315Δ/lpl/pLI-lpl, for macrophage infection (see Fig. S2A in the supplemental material). The production of IL-6 and TNF-α in cell culture supernatant were determined 6 h postinfection through enzyme-linked immunosorbent assay (ELISA). Phosphate-buffered saline (PBS) served as negative controls. Approximately 5 × 10^6 BMDMs or BMDM TLR2^−/− cells were stimulated with different amounts of purified lipidated SA2275-his proteins (50, 100, and 500 ng/ml). IL-6 (C) and TNF-α (D) levels in cell culture supernatant were determined after 6 h of treatment. The unlipidated SA2275-his (-sp) (500 ng/ml)-stimulated cells served as the negative control, whereas LPS-induced cells (200 ng/ml) served as the positive control. The experiments in duplicate were conducted at least three times. Error bars indicate SD. Statistical significances were calculated by Student’s t test; ns, no statistical significance. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

FIG 3 Antibiotic-induced MRSA Lpls stimulated proinflammatory cytokine production by macrophages. IL-6 (A) and TNF-α (B) levels elevated by RAW 264.7 macrophages stimulated with N315, N315Δ/lpl, N315Δ/lpl/pLI-lpl, or N315Δ/lpl/pLI50 at the MOI of 30. The levels of IL-6 and TNF-α in cell culture supernatant were determined 6 h postinfection through enzyme-linked immunosorbent assay (ELISA). Phosphate-buffered saline (PBS) served as negative controls. Approximately 5 × 10^6 BMDMs or BMDM TLR2^−/− cells were stimulated with different amounts of purified lipidated SA2275-his proteins (50, 100, and 500 ng/ml). IL-6 (C) and TNF-α (D) levels in cell culture supernatant were determined after 6 h of treatment. The unlipidated SA2275-his (-sp) (500 ng/ml)-stimulated cells served as the negative control, whereas LPS-induced cells (200 ng/ml) served as the positive control. The experiments in duplicate were conducted at least three times. Error bars indicate SD. Statistical significances were calculated by Student’s t test; ns, no statistical significance. *, P < 0.05; **, P < 0.01; ***, P < 0.001.

macrophages, mouse bone marrow-derived macrophages (BMDMs) of C57BL/6 mice were isolated and characterized and then stimulated with different amounts of purified lipidated SA2275-his proteins. SA2275-his proteins increased the production of IL-6 and TNF-α in BMDMs in a concentration-dependent manner, whereas unlipidated SA2275-his (-sp) proteins showed no immunomodulatory effect on the production of IL-6 and TNF-α in BMDMs (Fig. 3C and D). However, the lipidated SA2275-his proteins could not induce the production of IL-6 and TNF-α in BMDMs derived from TLR2-deficient C57BL/6 mice (TLR2−/− BMDMs) (Fig. 3C and D). In summary, these data suggest that the enhancement by purified recombinant SA2275-his proteins of proinflammatory cytokine production by macrophages is TLR2 dependent, and correctly lipidated Lpls are needed for the recognition of TLR2 receptors to trigger the immune response by macrophages (31).

MRSA Lpls enhanced proinflammatory cytokine production in mice. We determined whether the β-lactam responsible for Lpls contributed to cytokine stimulation in vivo. The levels of IL-6 and TNF-α in BALB/c mice 6 h after challenge with N315Δlpl were significantly lower than those with N315 administered (P < 0.01). The N315Δlpl/pLl-ipl strain stimulated higher levels of IL-6 and TNF-α in mice than the N315Δlpl/pLl150 strain did (Fig. 4A and B). Moreover, C57BL/6 and C57BL/6 TLR2−/− mice were administered different concentrations of purified lipidated SA2275-his proteins through tail vein injection. The levels of IL-6 and TNF-α in C57BL/6 mice increased after SA2275-his proteins were administered, whereas this phenomenon was absent in C57BL/6 TLR2−/− mice (Fig. 4C and D). Overall, these data suggest that the systemic inflammatory response in MRSA infection may be associated with Lpl expression and that MRSA Lpls stimulate a TLR2-dependent host immune response.

β-Lactam-induced Lpls promoted the pathogenicity of MRSA. To investigate whether β-lactam-induced Lpls enhance colonization by MRSA, we determined the bacterial burden in the organs of a mouse model. BALB/c mice were infected intravenously with pGFP plasmid-transformed N315Δlpl and N315 for 5 days (see Table S4 in the supplemental material), and bacterial colonization was tracked through an in vivo imaging system. The fluorescence intensity of green fluorescent protein (GFP) in the kidneys of mice injected with N315 was significantly higher than in those infected with N315Δlpl (Fig. 5A; see Fig. S3A in the supplemental material). Consistent with the radiant efficiency, the bacterial loads in the kidneys of N315-infected mice were also significantly higher than those of N315Δlpl-infected ones (Fig. 5B).

We then investigated whether Lpls enhance the pathogenicity of MRSA. A mouse subcutaneous infection model was established to evaluate the contribution of OXA-induced Lpls to skin and soft tissue infections. BALB/c mice were injected subcutaneously in both flanks with N315 and N315Δlpl. Then, the mice were intraperitoneally injected with 1 µg of OXA per gram of weight twice a day for 14 days. The course of infection was monitored every day. The PBS-treated N315- and N315Δlpl-infected mice served as controls. The mouse skin abscesses caused by OXA-treated N315 were significantly larger than those caused by OXA-treated N315Δlpl, PBS-treated N315, and PBS-treated N315Δlpl between 2 and 11 days postinfection (Fig. 5C), and this observation was further shown in the photographs of skin abscesses of BALB/c mice 7 days postinfection (Fig. 5D; see Fig. S3B in the supplemental material). Histological examinations indicated that the corium layer of OXA-treated N315-challenged BALB/c mice exhibited extensive inflammation with leukocyte infiltration, more flake-like abscess formation, and destroyed skin structure compared with the OXA-treated N315Δlpl-, PBS-treated N315-, and N315Δlpl-infected mice (see Fig. S3C in the supplemental material). In contrast, the skin of OXA-treated N315Δlpl-challenged mice displayed similar leukocyte infiltration and sporadic abscess formation relative to those of PBS-treated N315Δlpl-infected mice. These pathological phenomena might be caused by β-lactam-induced MRSA Lpls, which stimulated higher levels of IL-6 and TNF-α in mice (see Fig. S3D and E in the supplemental material), thereby promoting an exuberant, systemic inflammatory response.
To further determine whether MRSA Lpl-promoted pathogenicity of MRSA is associated with TLR2, we subcutaneously challenged C57BL/6 and C57BL/6 TLR2−/− mice in both flanks with N315 and N315Δlpl. Results showed that skin abscesses in wild-type C57BL/6 mice caused by OXA-treated N315 were larger than those caused by OXA-treated N315Δlpl, PBS-treated N315, and N315Δlpl between 4 and 10 days postinfection (see Fig. S4A in the supplemental material). This observation was further shown in the photographs of skin abscesses of C57BL/6 mice at 7 days postinfection (see Fig. S4B in the supplemental material). N315 and N315Δlpl caused larger skin lesions in C57BL/6 TLR2−/− mice than those in C57BL/6 mice (Fig. 5E and F; see Fig. S4B and C in the supplemental material), consistent with the previous results (32). However, the skin abscesses in C57BL/6 TLR2−/− mice caused by OXA-treated N315 were negligibly different from those caused by OXA-treated N315Δlpl, PBS-treated N315, and N315Δlpl (Fig. 5E and F; see Fig. S4B and C). Overall, these data confirmed that β-lactam-induced Lpls aggravated host TLR2-dependent MRSA infections.
**DISCUSSION**

MRSA is distinct from MSSA in terms of the acquisition of a genetic element called staphylococcal cassette chromosome mec, in which mecA encodes an alternative penicillin-binding protein 2a (PBP2a) with a low affinity for β-lactams (24). Thus, MRSA strains are resistant to nearly all β-lactam antibiotics (3). As antibiotics, β-lactams bind to PBPs and inhibit the transpeptidation and transglycosylation of the cell wall, resulting in a weakened cell wall and inducing cell lysis and death (33). This type of antibiotic, particularly cephalosporins and β-lactam-β-lactamase inhibitor combinations, has been empirically used for clinical treatments of infectious diseases (34). Subinhibitory
concentrations of antistaphylococcal agents may occur due to either antibiotic-resistant microorganisms or pharmacokinetics of antibiotics (12, 34). For MRSA infections, which are not initially recognized, β-lactams not only are ineffective in treatment but also possibly contribute to poor outcomes by enhancing the pathogenicity of MRSA. Nonetheless, the underlying mechanisms remain obscure (6). In addition to antimicrobial activity, signal induction may be implemented by subinhibitory concentrations of β-lactams, which actively promote S. aureus biofilm formation (10), induce PBP2a to reduce peptidoglycan cross-linking in MRSA (3), and enhance virulence factors, such as alpha-toxins, PVL, SpA, and enterotoxins (9, 35–37).

In this study, we showed that a three-gene constituent lpl cluster in the MRSA genome was upregulated in response to β-lactam induction. This lpl cluster was widely distributed among the major prevalent MRSA clones (see Table S5 in the supplemental material). Lpls could be upregulated after treatment with nearly all β-lactam antibiotics (Fig. 1A). β-Lactams can induce PVL expression in S. aureus by interfering with PBP1 and triggering SarA and Rot global regulators (9). Our results showed that deletion of SarA (N315ΔsarA) failed to upregulate lpl expression under OXA treatment (Fig. 2F), whereas N315ΔagrA and USA300ΔagrA showed lpl expression comparable to their wild-type strains, indicating that β-lactam-induced Lpl expression in MRSA is probably SarA controlled via an agr-independent pathway. EMSA data revealed the direct regulation of SarA during Lpl expression (Fig. 2B). However, further investigations should be performed to clarify how β-lactams trigger SarA expression.

In contrast to β-lactam-induced SpA and PVL, which exhibit a controversial pathogenic role in S. aureus (9), some Lpps of S. aureus are crucial players in alerting the host immune system by recognizing TLR2/TLR1 or TLR2/TLR6 receptors (38, 39). Proinflammatory cytokines were not induced by purified lipidated SA2275-his proteins in BMDM (Fig. S3A), whereas Lpl induced IL-6 and TNF-α production in S. aureus by interfering with PBP1 and triggering SarA and Rot global regulators (9). Our results showed that deletion of SarA (N315ΔsarA) failed to upregulate lpl expression under OXA treatment (Fig. 2E), whereas N315ΔagrA and USA300ΔagrA showed lpl expression comparable to their wild-type strains, indicating that β-lactam-induced Lpl expression in MRSA is probably SarA controlled via an agr-independent pathway. EMSA data revealed the direct regulation of SarA during Lpl expression (Fig. 2B). However, further investigations should be performed to clarify how β-lactams trigger SarA expression.

Our study also demonstrated that increasing MRSA pathogenicity was attributed to β-lactam-induced Lpls (Fig. 5A, B, and D; Fig. S3B and C). A possible explanation is that the higher levels of IL-6 and TNF-α in mice induced by β-lactam-induced Lpls promoted exuberant, systemic inflammatory responses, thereby facilitating the pathogenicity of MRSA. Schmaler et al. (32) found that TLR2−/− or MyD88−/− mice showed more weight loss and higher bacterial loads in kidneys and spleens after infection with S. aureus than C57BL/6 mice. Our results also revealed that N315 and N315Δlpl strains caused more pronounced skin lesions in TLR2−/− mice than in C57BL/6 mice (Fig. 5E and F; Fig. S4B and C). This may be attributed to the low levels of proinflammatory cytokines induced in TLR2−/− mice, facilitating MRSA colonization and infection.

In conclusion, this work focused on the function and regulation of an lpl cluster in response to the induction of subinhibitory concentrations of β-lactams. β-Lactam-induced MRSA lpl expression is SarA dependent, and upregulation of lpl after β-lactam treatment is directly controlled by the global regulator SarA. We demonstrated that the increased Lpls in MRSA significantly promote TLR2-dependent signaling pathway activation and result in inflammatory response by triggering IL-6 and TNF-α levels in vitro and in vivo, thereby possibly contributing to bacterial pathogenicity by inducing host immune responses and promoting bacterial colonization. Our data support the recommendation to clinicians regarding the prudent usage of β-lactams, which possibly aggravate the clinical outcomes of MRSA infections.
MATERIALS AND METHODS

Ethics statement. BALB/c mice were purchased from the Laboratory Animal Center of Army Medical University. C57BL/6 TL2C−/− mice were provided as a gift by Yuzhang Wu at the Department of Immunology, Army Medical University. All animal experiments were approved by the Institutional Animal Care and Use Committee of Army Medical University (protocol no. SYXK-PLA-20120031). All animal experimental procedures were performed in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals approved by the State Council of the People’s Republic of China. Cervical dislocation was used as the euthanasia method for all experimental mice.

Bacterial strains, plasmids, and primers. Bacterial strains and plasmids used in this study are listed in Table S4 in the supplemental material. All primers used are listed in Table S6.

Antibiotic susceptibility tests. Antibiotic susceptibility was determined using broth microdilution methods according to the protocols recommended by the Clinical and Laboratory Standards Institute (CLSI) (40). The antibiotic susceptibility results for all strains are listed in Table S2.

Preparation of recombinant lipitated SA2275-his, unlipidated SA2275-his (-sp), and SarA-his proteins. The lipidated SA2275-his proteins were isolated from the membrane fraction of MRSA N315Δlpl carrying expression vector pXK-sa2275-his (Table S4) as previously described (21). The endotoxin contamination in the purified lipidated SA2275-his stock was determined by a Tachypleus amebocyte lysate test (Horseshoe Crab Reagent Manufactury Co. Ltd., China), and a concentration of less than 0.08 endotoxin units (EU) was approved to be used for the stimulation of cytokine production by macrophages and animals (41).

pET28a-sa2275 and pET28a-sarA were transformed into Escherichia coli BL21(DE3) for the expression of SA2275-his (-sp) and SarA-his fusion proteins (42), which were purified by Ni-NTA affinity chromatography and identified by Western blotting.

Preparation of polyclonal antibodies against recombinant proteins. Female BALB/c mice (6 to 8 weeks) were immunized subcutaneously with SA2275-his (-sp) or SarA-his recombinant proteins to prepare polyclonal antibodies (42).

Preparation of total bacterial proteins and culture supernatant proteins. The overnight culture of S. aureus strain was diluted 1:100 in brain heart infusion (BHI) medium with or without the addition of β-lactam antibiotics and cultivated at 37°C to an OD600 of 2.0. Then, bacterial cells in 3 ml culture were harvested, washed twice with PBS, and resuspended in 1 ml of cold PBS supplemented with 1% (mass/vol) β-mercaptoethanol (Sigma, USA) and 1 mM phenylmethylsulfonyl fluoride (PMSF) (Beyotime, China) on ice. Cells were broken by addition of 0.1-mm-diameter zirconia-silica beads with shaking on the Minibeadbeater 16 instrument (Biospec, USA). Proteins in 1ml of the culture supernatant were precipitated with 7.5% (vol/vol) trichloroacetic acid (TCA)-0.2% (vol/vol) deoxycholic acid solution (43). The protein concentration was determined using the Bradford protein assay kit (Beyotime, China).

Protein identification. LC-MS/MS was performed to identify proteins induced by β-lactam antibiotics as previously described (42). The antibiotic-induced protein band was excised and analyzed through LC-MS/MS by using an UltiMate3000 RSLCnano-liquid chromatography/Bruker Maxis 4G Q-TOF instrument. The resulting peptide mass fingerprints were compared against the open reading frames (ORFs) of N315 by using Mascot and Mascot Daemon software (Matrix Science).

RT-PCR and RT-qPCR. Total RNA of MRSA N315 was extracted as previously described (44). RT-PCR was used to determine whether sa2275, sa2274, and sa2273 were cotranscribed. RT-qPCR was performed to detect the expression levels of lpl genes (sa2275, sa2274, and sa2273) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad, USA). The relative expression level of all tested genes was normalized to that of the 16S rRNA gene.

EMSA. The predicted lpl cluster promoter, an AT-rich motif fragment (56 bp), was synthesized using primer pairs (EMSA-lpl fwd/EMSA-lplP rev) as described previously (45). The corresponding mutated GC-rich motif fragment was also synthesized by primer pair EMSA-lplP rev/EMSA-lplP fwd and served as controls. Ten picomoles of DNA fragment was incubated with a variable amount of recombinant SarA-his (0 to 240 pM) in a 20-μl reaction mixture containing 10 mM HEPES (pH 7.6), 1 mM EDTA, 2 mM dithiothreitol, 50 mM KCl, 0.05% (vol/vol) Triton X-100, and 5% (vol/vol) glycerol. Binding reaction mixtures were equilibrated for 20 min at room temperature before electrophoresis. Reaction mixtures were separated on 6% (mass/vol) native polyacrylamide gel electrophoresis in 0.5× TBE (Tris-boric acid-EDTA) buffer at 90 V for 2 h at 4°C. Gels were stained by GelRed dye (Biotium, USA) and observed under UV light.

Construction of gene deletion mutant and overexpression strains. The lpl cluster markerless deletion mutant was constructed using homologous recombinant strategy described previously (44). Briefly, pYT3-Δlpl and pBT2-Δlpl plasmids were used to construct lpl cluster markerless deletion mutant. The deletion of lpl cluster was confirmed by PCR and DNA sequencing. Similar strategies were used to construct N315ΔsarA, N315ΔagrA, USA300ΔagrA, and USA300Δlpl/pΔlpl mutant strains.

The pLI-lpl plasmid was electroporated into N315Δlpl and USA300Δlpl to generate lpl overexpression strains N315Δlpl/pLI-lpl and USA300Δlpl/pLI-lpl, respectively. A similar strategy was used to construct N315ΔsarA/pLI-sarA. The empty pLI50 plasmid-transformed N315Δlpl, N315ΔsarA, and USA300Δlpl strains served as controls.

Cytokine determination. For stimulation experiment, RAW 264.7 cells (105/well) were infected with MRSA strains (multiplicity of infection [MOI] of 30) in a 24-well microtiter plate for 6 h as previously described (46). Then, the supernatant was collected, and the levels of IL-6 and TNF-α were determined with an ELISA kit according to the manufacturer’s instructions (R&D Systems, USA).

BMDMs were isolated from 12-week-old female wild-type C57BL/6 or C57BL/6 TL2C−/− mice as described previously (47). Briefly, bone marrow cells were obtained from femurs by flushing with β-Lactams Stimulate MRSA Pathogenicity

May/June 2019 Volume 10 Issue 3 e00880-19 mbio.asm.org 11
complete RPMI 1640 medium (HyClone). After removed of red blood cells, the cells were cultured on 6-well plates with RPMI 1640 containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS), 20 ng/ml macrophage colony-stimulating factor (M-CSF), 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C to a fluent monolayer. Cells were identified by the BD FACS Canto II flow cytometer (BD Biosciences, USA) (48). BMDMs were seeded at a density of 5 × 10^5 cells/well in 24-well plates and allowed to adhere overnight followed by stimulation with lipidated SA2275-his proteins for 6 h, and the levels of IL-6 and TNF-α were measured by ELISA.

Female BALB/c mice were infected via tail vein injection with 1 × 10^7 CFU of the MRSA strain of interest for 6 h. To detect the cytokine induction capacity of purified lipidated SA2275-his proteins in vivo, female C57BL/6 and C57BL/6 TLR2−/− mice were challenged via tail vein injection with 30, 100, and 500 ng recombinant SA2275-his proteins for 6 h, respectively. Blood samples were collected 6 h postinjection, and the levels of IL-6 and TNF-α in mouse sera were determined by ELISA.

Animal experiments. BALB/c mice were randomly divided into two groups and infected via tail vein injection with 1 × 10^7 CFU of the GFP expression plasmid (pGFP)-transformed N315 or N315Δipl and sacrificed 5 days after infection. Mouse organs (i.e., heart, lung, liver, spleen, and kidney) were isolated and subjected to the determination of GFP fluorescence efficiency in organs with the IVIS Lumina LT system and analyzed by Living Image 4.4 Software. The bacterial loads in the infected kidneys were also counted via plate dilution assay as described previously (49).

For skin abscess formation, BALB/c, C57BL/6, and C57BL/6 TLR2−/− mice were fully anesthetized with 1% (mass/vol) pentobarbital sodium (50 mg/kg of body weight), and the back hair was depilated completely with 6% (mass/vol) sodium sulfide. Then, mice were subcutaneously inoculated with 5 × 10^8 CFU of MRSA N315 and N315Δipl on both flanks of the murine back as described previously (49) and then randomly divided into two groups. The mice of the treatment group were intraperitoneally injected with 1 μg of OXA per gram of weight twice a day for 14 days. The PBS-injected mice served as controls. The abscess area assessed by the maximal length by width of the developing ulcer was measured daily.

Statistical analysis. Statistical analysis was carried out using GraphPad Prism 6.0. Replicate numbers and statistical tests for each experiment are listed in the figure legends.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at https://doi.org/10.1128/mBio.00880-19.

FIG S1, TIF file, 2 MB.
FIG S2, TIF file, 0.4 MB.
FIG S3, TIF file, 1.8 MB.
FIG S4, TIF file, 2.8 MB.
TABLE S1, DOCX file, 0.1 MB.
TABLE S2, DOCX file, 0.02 MB.
TABLE S3, DOCX file, 0.04 MB.
TABLE S4, DOCX file, 0.03 MB.
TABLE S5, DOCX file, 0.02 MB.
TABLE S6, DOCX file, 0.03 MB.

ACKNOWLEDGMENTS
This work was supported by the National Key Biosafety Technology Research and Development Program of China (2017YFC1200404-4) and the National Natural Science Foundation of China (81672071 and 81701967).

REFERENCES
1. Lowy FD. 1998. *Staphylococcus aureus* infections. N Engl J Med 339:520–532. https://doi.org/10.1056/NEJM199808203390806.
2. Liu H, Shang W, Hu Z, Zheng Y, Yuan J, Hu Q, Peng H, Cai X, Tan L, Li S, Zhu J, Li M, Hu X, Zhou R, Rao X, Yang Y. 2018. A novel SigB(Q225P) mutation in *Staphylococcus aureus* retains virulence but promotes biofilm formation. Emerg Microbes Infect 7:72. https://doi.org/10.1038/s41424-018-0078-1.
3. Muller S, Wolf AJ, Iliev ID, Berg BL, Underhill DM, Liu GY. 2015. Poorly biotic resistance: the case of methicillin-resistant *Staphylococcus aureus*. Pharmacoeconomics 33:285–325. https://doi.org/10.1007/s40273-014-0242-y.
4. Antonanzas F, Lozano C, Torres C. 2015. Economic features of antibiotic resistance: the case of methicillin-resistant *Staphylococcus aureus*. J Antimicrob Chemother 52:1970–1979. https://doi.org/10.1093/jac/dkv577.
5. Barrios Lopez M, Gomez Gonzalez C, Orellana MA, Chaves F, Rojo P. 2013. *Staphylococcus aureus* abscesses: methicillin-resistance or Panton-Valentine leukocidin presence? Arch Dis Child 98:608–610. https://doi.org/10.1136/archdischild-2012-302695.
6. Watkins RR, David MZ, Salata RA. 2012. Current concepts on the virulence mechanisms of methicillin-resistant *Staphylococcus aureus*. J Med Microbiol 61:1179–1193. https://doi.org/10.1099/jmm.0.043513-0.
7. Paul M, Kariv G, Goldberg E, Raskin M, Shaked H, Hazzan R, Samra Z, Paghis D, Bishara J, Leibovici L. 2010. Importance of appropriate empirical antibiotic therapy for methicillin-resistant *Staphylococcus aureus* bacteraemia. J Antimicrob Chemother 65:2658–2665. https://doi.org/10.1093/jac/dkq373.
8. Kim SH, Park WB, Lee KD, Kang CL, Bang JW, Kim HB, Kim EC, Oh MD, Choe KW. 2010. Outcome of inappropriate initial antimicrobial treatment in patients with methicillin-resistant *Staphylococcus aureus* bacteraemia. J Antimicrob Chemother 54:489–497. https://doi.org/10.1093/jac/dkh366.
9. Dumitrescu O, Choudhury P, Boisset S, Badiou C, Bes M, Benito Y, Wolz C, Vandenesch F, Etienne J, Cheung AL, Bowden MG, Lina G. 2011. Beta-lactams interfering with PBP1 induce Panton-Valentine leukocidin...
expression by triggering sarA and rot global regulators of Staphylococcus aureus. Antimicrob Agents Chemother 55:3261–3271. https://doi.org/10.1128/AAC.01401-10.

27. Liu Y, Mannan AC, Pan CH, Krikunov IA, Thiel DJ, Cheung AL, Zhang G. 2006. Structural and function analyses of the global regulatory protein SarA from Staphylococcus aureus. Proc Natl Acad Sci U S A 103: 2392–2397. https://doi.org/10.1073/pnas.0510493103.

28. Sterba KM, Mackintosh SG, Blevins JS, Hurlburt BK, Smeltzer MS. 2003. Characterization of Staphylococcus aureus SarA binding sites. J Bacteriol 185:4410–4417. https://doi.org/10.1128/JB.185.11.4410-4417.2003.

29. Tsompanidou E, Sibbalb MJ, Chlebowicz MA, Dreisbach A, Back JW, van Dijl JM, Buist G, Denham EL. 2011. Requirement of the agr locus for colony spreading of Staphylococcus aureus. J Bacteriol 193:1267–1272. https://doi.org/10.1128/JB.00276-10.

30. Rogero MM, Calder PC. 2018. Obesity, inflammation, toll-like receptor 4 and fatty acids. Nutrients 10:E432. https://doi.org/10.3390/nu10040432.

31. Nguyen MT, Uebele J, Kumari N, Nakayama H, Peter L, Ticha O, Wosching AK, Schmaler M, Khanna N, Dohmnae N, Lee BL, Bekedjyan-Ding I, Gof T. 2017. Lipid moieties on lipoproteins of commensal and non-commensal staphylococci induce differential immune responses. Nat Commun 8:2246. https://doi.org/10.1038/s41467-017-02234-4.

32. Schmaler M, Jann NJ, Ferraccio F, Landoldt LZ, Biswas I, Gotz F, Landmann R. 2009. Lipoproteins in Staphylococcus aureus mediate inflammation by Toll-like receptor-4 and iron-dependent growth in vivo. J Immunol 182:7110–7118. https://doi.org/10.4049/jimmunol.0804292.

33. Lim D, Strynadka NC. 2002. Structural basis for the beta-lactam resistance of PBPa from methicillin-resistant Staphylococcus aureus. Nat Struct Biol 9:870–876. https://doi.org/10.1038/nsb858.

34. Wushouer H, Tian Y, Guan XD, Han S, Shi LW. 2017. Trends and patterns of antibiotic consumption in China’s tertiary hospitals: based on a 5 year surveillance with sales records, 2011-2015. PLoS One 12:e0190314. https://doi.org/10.1371/journal.pone.0190314.

35. Ohsen K, Ziebuhr W, Koller KP, Hell W, WichelsaUH, Haker J. 1998. Effects of subinhibitory concentrations of antibiotics on alpha-toxin (hla) gene expression of methicillin-sensitive and methicillin-resistant Staphylococcus aureus isolates. Antimicrob Agents Chemother 42:2817–2823. https://doi.org/10.1128/AAC.42.11.2817.

36. Hashimoto H, Kuroda M, Cui L, HiramaT K. 2007. Subinhibitory concentrations of beta-lactam induce haemolytic activity in Staphylococcus aureus through the SaRS two-component system. FEMS Microbiol Lett 268: 98–105. https://doi.org/10.1111/j.1574-6968.2006.00568.x.

37. Subit N, Mesak LR, Davies J. 2011. Modulation of virulence gene expression by cell wall active antibiotics in Staphylococcus aureus. J Antimicrob Chemother 66:979–984. https://doi.org/10.1093/jac/dkr244.

38. Rock S, Murgueitio MS, Wolber G, Wendt G. 2016. Acute myeloid leukaemia-deranged Langerhans-like cells enhance Th1 polarization upon TLR2 engagement. Pharmacol Res 105:44–53. https://doi.org/10.1016/j.phr.2016.01.016.

39. Hashimoto M, Tawaratsumida K, Kariya H, Aoyama K, Tamura T, Suda Y. 2011. Lipoprotein is a predominant TLR2 ligand in bacterial species reveals a unit of evolution spanning two adjacent genes in Enterococcus faecalis. J Antimicrob Chemother 68:2453–2457. https://doi.org/10.1093/jac/dkt213.

40. Yuan J, Yang J, Hu Z, Peng H, Zheng Y, Shang W, Hu Q, Yang Y, Liu H, Jiang B, Wang Y, Li S, Hu X, Rao X. 2016. Positive feedback cycle of TNPalfa promotes staphylococcal enterotoxin B-induced Th1 cell apoptosis. Front Cell Microbiol 6:109. https://doi.org/10.3389/fcimb.2016.00109.

41. Yuan J, Yang J, Hu Z, Yang Y, Shang W, Hu Q, Zheng Y, Peng H, Zhang X, Cai X, Zhu J, Li M, Hu X, Zhou R, Rao X. 2018. Safe staphylococcal platform for the development of multivalent nanoscale vesicles against viral infections. Nano Lett 18:725–733. https://doi.org/10.1021/acs.nanolett.7b03895.

42. CLSI. 2017. Performance standards for antimicrobial susceptibility testing, 27th ed. CLSI, Wayne, PA.

43. Zhang X, Shang W, Yuan J, Hu Z, Peng H, Zhu J, Hu Q, Yang Y, Liu H, Jiang B, Wang Y, Li S, Hu X, Rao X. 2016. Positive feedback cycle of TNFpalpha promotes staphylococcal enterotoxin B-induced Th1 cell apoptosis. Front Cell Microbiol 6:109. https://doi.org/10.3389/fcimb.2016.00109.

44. Yuan J, Yang J, Hu Z, Yang Y, Shang W, Hu Q, Zheng Y, Peng H, Zhang X, Cai X, Zhu J, Li M, Hu X, Zhou R, Rao X. 2018. Safe staphylococcal platform for the development of multivalent nanoscale vesicles against viral infections. Nano Lett 18:725–733. https://doi.org/10.1021/acs.nanolett.7b03895.

45. Burls MA, Williams WA, DeBoer R, Missiakas DM. 2005. ExsA and ExsB are secreted by an ESAT-6-like system that is required for the pathogenesis of Staphylococcus aureus infections. Proc Natl Acad Sci U S A 102:1169–1174. https://doi.org/10.1073/pnas.0405620102.

46. Yuan W, Hu Q, Cheng H, Shang W, Liu N, Hua Z, Zhu J, Hu Z, Yuan J, Zhang X, Li S, Chen Z, Hu X, Fu J, Rao X. 2013. Cell wall thickening is associated with adaptive resistance to amikacin in methicillin-resistant Staphylococcus aureus clinical isolates. J Antimicrob Chemother 68:1096. https://doi.org/10.1093/jac/dkt282.

47. Correa EM, De Tulio L, Velez PS, MartinA CA, Aragona CE, Barra JL. 2013. Analysis of DNA structure and sequence requirements for Pseudomonas
46. Curry H, Alvarez GR, Zwilling BS, Lafuse WP. 2004. Toll-like receptor 2 stimulation decreases IFN-gamma receptor expression in mouse RAW264.7 macrophages. J Interferon Cytokine Res 24:699–710. https://doi.org/10.1089/jir.2004.24.699.

47. Kim HY, Baik JE, Ahn KB, Seo HS, Yun CH, Han SH. 2017. *Streptococcus gordonii* induces nitric oxide production through its lipoproteins stimulating Toll-like receptor 2 in murine macrophages. Mol Immunol 82:75–83. https://doi.org/10.1016/j.molimm.2016.12.016.

48. Bain CC, Hawley CA, Garner H, Scott CL, Schridde A, Steers NJ, Mack M, Joshi A, Guiliams M, Mowat AM, Geissmann F, Jenkins SJ. 2016. Long-lived self-renewing bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities. Nat Commun 7:ncomms11852. https://doi.org/10.1038/ncomms11852.

49. Wen W, Liu B, Xue L, Zhu Z, Niu L, Sun B. 2018. Autoregulation and virulence control by the toxin-antitoxin system SavRS in *Staphylococcus aureus*. Infect Immun 86:e00032-18. https://doi.org/10.1128/IAI.00032-18.