Novel Chimeric Lysin with High-Level Antimicrobial Activity against Methicillin-Resistant *Staphylococcus aureus* *In Vitro* and *In Vivo*

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The treatment of infections caused by methicillin-resistant *Staphylococcus aureus* (MRSA) is a challenge worldwide. In our search for novel antimicrobial agents against MRSA, we constructed a chimeric lysin (named as ClyH) by fusing the catalytic domain of Ply187 (Pc) with the non-SH3b-like cell wall binding domain of phiNM3 lysin. Herein, the antimicrobial activity of ClyH against MRSA strains *in vitro* and *in vivo* was studied. Our results showed that ClyH could kill all of the tested clinical isolates of MRSA with higher efficacy than lysostaphin as well as its parental enzyme. The MICs of ClyH against clinical *S. aureus* strains were found to be as low as 0.05 to 1.61 mg/liter. In a mouse model, a single intraperitoneal administration of ClyH protected mice from death caused by MRSA, without obvious harmful effects. The present data suggest that ClyH has the potential to be an alternative therapeutic agent for the treatment of infections caused by MRSA.

Staphylococcus aureus is a common pathogen with ability to develop resistance to virtually all classes of antibiotics (1–3). Infections caused by *S. aureus*, especially, methicillin-resistant *S. aureus* (MRSA) (4, 5), are becoming a serious problem worldwide; therefore, there is an urgent need to develop effective therapeutic agents against MRSA (6).

Among many new antimicrobial agents against *S. aureus*, bacteriophage lysins have been found promising because of their narrow spectra of activity, rapid antibacterial activity, and their target organism's low probability for developing resistance (7–11). Currently, a few lysins identified directly from genomes of bacteriophages have been studied for controlling infections caused by MRSA both *in vitro* and *in vivo* (10, 12, 13). However, production of a perfect lysin directly from phage genomes remains difficult, because of the poor solubility of the natural lysins when overexpressed in *Escherichia coli* (14).

To circumvent these problems, chimeric lysins have been introduced by shuffling the domains—i.e., the cell wall binding domains (CBDs) and the catalytic domains (CDs)—from natural lysins (15–19). Many chimeric lysins have chosen a CBD homologous to SH3b-like domains, similar to that of lysostaphin (see Table S1 in the supplemental material). However, it has been reported that the staphylococcal SH3b domains were not always staphylococcus specific (20). More importantly, the bacteria might have a chance, although low, to develop potential resistance to the lysins containing SH3b-like domains due to small alterations within the peptide cross-bridges of the bacterial cell wall as they did to lysostaphin (21). A few CDs—mainly cysteine and histidine-dependent aminopeptidase/hydrolase (CHAP) (17) and endopeptidase (19, 22)—have been used as the CDs of chimeric lysins. Among all of the CDs, we noted that the CD from lysin Ply187 (Pc, which consists of its N-terminal 157 amino acids) was special. It has been reported that the Pc has a much higher amidase activity than the whole lysin (23), and its activity could be further enhanced by adding a known SH3b CBD (24).

In the present work, as an effort to find novel chimeric lysins for controlling MRSA, Pc was fused with a CBD not homologous to SH3b domains, to generate a novel chimeric lysin, named ClyH. Various tests, including its lytic activity against clinical MRSA isolates *in vitro* and *in vivo*, were done to show the antimicrobial

efficacy of ClyH. These results supported the potential of ClyH as a novel therapeutic agent for treatment of infections caused by multidrug-resistant *S. aureus*.

MATERIALS AND METHODS

Bacterial strains. Bacterial strains (see Table S2 in the supplemental material) used in this work were routinely grown at 37°C. All of the staphylococcal strains were grown in Trypticase soy broth (TSB) medium. Clinical isolates of *S. aureus* with different genetic backgrounds were collected from various sources in China in order to cover all SCCmec types. MRSA strains were determined by PCR against *mecA* and *femB*, as described previously (25), with primers *MecA-F* (5'-GTAGAAATGACTGAACGTCCGATAA-3') and *MecA-R* (5'-CCAATTCACATTGTTTCGGTCTA A-3') and *FemB-F* (5'-TTACAGAGTTAACTGTTACC-3') and *FemB-R* (5'-ATACAAATCCAGCAGCTCT-3'), respectively. Once confirmed, their SCCmec types were further determined by multiplex PCR, as described previously (26). The Panton-Valentine leucocidin gene (*lukF/lukS-PV*) was determined by PCR according to the method described previously (27).

Because some lysins (28) were reported to be active against both *S. aureus* and streptococcal strains, *Streptococcus thermophilus*, *Streptococcus sobrius*, *Streptococcus pyogenes*, and *Streptococcus suis* were tested to evaluate the specificity of ClyH. Other strains used include *Lactobacillus acidophilus*, *Bifidobacterium dentium*, *Enterococcus faecalis*, *Enterococcus faecium*, *Enterobacter sakazakii*, *Salmonella enterica*, *Listeria monocytogenes*, *Pseudomonas aeruginosa*, and *Xanthomonas oryzae*. All of these strains were cultured in brain heart infusion (BHI) medium. *Bacillus cereus* was tested as well but cultivated in Luria-Bertani (LB) medium. *Escherichia coli* BL21(DE3) was used for the cloning and expression of recombinant proteins.

Construction of gene-expressing plasmids. The chimeric lysin ClyH was constructed by fusing the N-terminal 157 amino acids of Ply187 (Pc)

Received 19 August 2013 Returned for modification 8 September 2013

Accepted 1 November 2013

Published ahead of print 4 November 2013

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AAC.01793-13>.

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doi:10.1128/AAC.01793-13

with the C-terminal 97 amino acids of phiNM3 lysin. To do this, the DNA fragment encoding the chimeric lysin was chemically synthesized by Songon Biotech (Shanghai, China). The resulting gene, corresponding to *clpH*, was cloned into pBAD24 vector with forward (-F) and reverse (-R) primers ClyH-F (5'-AAAAGAATTCATGGCACTGCCTAAACGGGTA AAC-3') and ClyH-R (5'-AAACTCGAGTTAAACACTTCTTTCACAA TC-3') for expression of untagged ClyH and into pET28a(+) vector with primers pH-F (5'-TTAACCATGGGCATGGCACTGCCTAAACG-3') and pH-R (5'-TTAACTCGAGAAACACTTCTTTCACAATCAATC-3') for expression of His-tagged ClyH (ClyH-His), respectively. To express the His-tagged parental CD (Pc-His), the gene fragment corresponding to Pc was cloned into pET28a(+) vector with primers PC-F (5'-AATTCCA TGGGCATGGCACTGCCTAAACG-3') and PC-R (5'-TTAACTCGAG TGGTGGTGTAGGTTTCGGTTC-3'). After confirmation by sequencing, the correct plasmids were transformed into *E. coli* BL21(DE3) for expression.

Purification of recombinant proteins. The recombinant proteins were expressed by the *E. coli* BL21(DE3) strain in standard LB medium and purified following procedures described previously (24, 29), with minor modifications. ClyH was induced overnight in BL21(DE3) cells with L-arabinose in a final concentration of 0.2% at 20°C. Briefly, cells were harvested by centrifugation and resuspended in 20 mM phosphate buffer (pH 7.4). After sonication, the supernatant was collected by centrifugation at $10,000 \times g$ for 30 min at 4°C. The supernatant was applied to a HiTrap Q Sepharose FF column (GE Healthcare) and then bound to a HiTrap SP Sepharose FF column (GE Healthcare) and eluted in a linear gradient from 0.02 M to 1 M NaCl solution. For the purification of ClyH-His as well as Pc-His, protein was expressed by inducing the bacteria with 1 mM isopropyl β -D-thiogalactoside (IPTG) when an optical density (OD) of 0.6 to 0.8 was reached. After induction, the bacteria were incubated overnight at 16°C to allow expression. Purification was achieved through a His₆ tag by using a nickel nitrilotriacetic acid column, by washing and elution with 60 and 265 mM imidazole solutions, respectively. Active fractions were pooled and dialyzed against 1× phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·H₂O, 1.4 mM KH₂PO₄ [pH 7.4]). After quantitation by the Bradford assay, the purified proteins were stored at -80°C until use.

Quantification of ClyH activity. Lytic activity was measured as previously described (8), with some modifications. Briefly, *S. aureus* strain CCTCC AB91118 (also called AB918 for short) was grown to an optical density at 600 nm (OD₆₀₀) of 0.2 to 0.3, centrifuged, and resuspended in PBS (pH 7.4) to a final OD₆₀₀ of 1.0. Next, 100 μ l of the purified ClyH in 2-fold serial dilutions was mixed with 100 μ l of the bacterial suspension in 96-well plates (Perkin-Elmer), respectively. The drop in OD₆₀₀ was monitored by a microplate reader (Synergy H1; BioTek) for 60 min at 37°C. A unit of ClyH activity was defined as the highest dilution that decreased the absorbance by 50% within 15 min (8). The lytic activities of ClyH at different pH values were also measured using a universal buffer described before (30). The buffer was prepared by mixing equal parts of 20 mM boric acid and 20 mM phosphoric acid, followed by titration with sodium hydroxide from pH 2 to 12.

After 1 U of ClyH was mixed with the suspension of *S. aureus* strain AB918, the decrease in viable cells corresponding to the loss of turbidity was also tested by plating the aliquots from the lytic assay at various time points (5, 15, 30, and 60 min) to TSB agar for counting of CFU. The action of ClyH on the cell wall was monitored by thin-section transmission electron microscopy (Tecnai G2 20 TWIN transmission electron microscope; FEI, Hillsboro, OR). The bacterial suspensions were incubated with 1 U of ClyH at 37°C for 3, 5, and 10 min, respectively, and then the reaction was terminated by addition of 2.5% glutaraldehyde before the transmission electron microscopy (TEM) analysis.

To compare the activity of ClyH with those of lysostaphin and Pc-His, mid-log-phase cultures of randomly selected *S. aureus* strains were pelleted and resuspended in PBS to a final OD₆₀₀ of 1.0, respectively. One hundred microliters of ClyH or lysostaphin at the same concentration

(0.16 μ M) was added to the bacterial suspension (100 μ l), respectively. The decrease in OD₆₀₀ was monitored by the spectrophotometer. To minimize the effect of His tag on the enzymatic activity, ClyH-His (1.2 μ M) was used for comparison with Pc-His at the same concentration.

To determine the specificity of ClyH, the lytic activities of ClyH to various bacterial strains were measured as the drop in milli-OD₆₀₀ per minute ($-mOD_{600}/min$) in the first 15 min, as described elsewhere (31).

All of the experiments described above were performed in triplicate, and bacterial cells treated with PBS were used as the blank controls.

MIC determinations. MICs of antibiotics (penicillin, gentamicin, vancomycin, and oxacillin) and ClyH were determined by microtiter broth dilution as described by the Clinical and Laboratory Standards Institute (CLSI) (32). The MIC was defined as the lowest concentration of antibiotic producing inhibition of visible growth.

Immunological neutralization test. The neutralization effect of ClyH-specific antibodies to the activity of ClyH was tested by using a standard immunological protocol, as described previously (10). ClyH (200-U) solutions were injected into the peritoneal cavities of mice 3 times, with a 10-day interval between injections. Mouse sera were sampled 15 days after the last injection, and the serum titers were checked by enzyme-linked immunosorbent assay (ELISA) using horseradish peroxidase-conjugated goat anti-mouse IgG. The detailed procedure of the ELISA was performed following the instructions of the manufacturer of a commercial ELISA kit (QF-Bio, Shanghai, China). Before the neutralization test, ClyH (about 0.5 U) was reacted with 80 μ l of the ClyH-immunized mouse serum at 37°C for 15 min, using nonimmunized mouse serum and PBS as the controls. The assay to determine the neutralization effect then was performed immediately by testing the lytic activity of each mixture for *S. aureus* strain AM025 by the same procedure as the lytic activity assay described above.

Mouse protection experiments. All mouse experiments were conducted with the approval of the Animal Experiments Committee of Wuhan Institute of Virology, Chinese Academy of Sciences (WIVA17201203). Female BALB/c mice (6 to 8 weeks old) were injected intraperitoneally with different concentrations of MRSA strain AM025 to determine the minimal lethal dose (MLD) that caused 100% mortality within 2 days. In the mouse protection assay, mice were inoculated intraperitoneally with $2 \times$ the MLD of AM025 cells and then divided into 3 groups randomly. Three hours after the challenge, two groups (6 each) received 180 U and 360 U (900 μ g) of ClyH intraperitoneally, respectively, and the other group ($n = 8$) was injected with PBS buffer. Another group ($n = 6$) without MRSA infection received 540 U of ClyH only. The survival rates of all the groups were observed for 10 days after the infection. To check the toxicity of ClyH, 5 mice without injection of the bacteria were given the ClyH solution for 7 days (200 U/injection, one injection/day, for a total dose of 1,400 U), and the survival rate, their body weights, and activities were observed for 10 days after the last injection.

RESULTS

Characteristics of ClyH. The purified ClyH, ClyH-His, and Pc-His displayed high purities (>90%) in 12% SDS-PAGE gels (Fig. 1A and B). As shown in Fig. 1C, after addition of ClyH, the OD₆₀₀ of the *S. aureus* AB918 suspension decreased rapidly with reaction time, while the OD₆₀₀ of the *S. aureus* suspension without ClyH had small changes. Figure 1C also showed that the loss of turbidity correlated with the decrease in viable cells.

The influence of pH, temperature, and ionic strength on the activity of ClyH was also studied. As shown in Fig. 1D, ClyH retained a high level of activity against AB918 cells in a broad pH range from pH 5 to 10 and reached its maximum activity at pH 6. The temperature had a significant effect on the lytic activity of ClyH. High-level lytic activities were observed at temperatures between 35°C and 45°C (see Fig. S1A in the supplemental mate-

FIG 1 Characteristics of ClyH activity. (A) SDS-PAGE of purified ClyH and Pc-His. (B) SDS-PAGE of purified ClyH-His and Pc-His. M, protein molecular mass markers; ClyH-his, His-tagged ClyH; Pc-his, the catalytic domain of lysin Ply187 fused with a His tag. (C) Lytic activity against *S. aureus* AB918 *in vitro*. The decrease in OD₆₀₀ was monitored after addition of ClyH (solid squares) with PBS as a control (open circles). Viability of treated cells measured as log CFU/ml was determined by serial dilution and plating to TSB agar plates (asterisks). (D) The relative activities of ClyH against AB918 cells in buffers at different pHs. (E to G) TEM images of AB918 cells exposed to ClyH. ClyH causes cell wall deformation (E), extrusion and loss of cytoplasmic contents either partly or totally (F), and ultimately formation of a cell "ghost" (G). Bar sizes, 200 nm.

rial). Ionic strength (ranging from 137 mM to 500 mM NaCl) had no significant effect on ClyH activity (see Fig. S1B).

We also tested the stability of ClyH at 4°C (see Fig. S2 in the supplemental material) and found that ClyH retained 63.7% and 21.2% lytic activity in terms of the initial lytic activity after being stored for 4 and 8 weeks, respectively (see Fig. S2B).

The TEM analysis showed that AB918 cells exposed to ClyH suffered a process from deformation to extrusion and then disruption in cell wall at single or multiple sites, which was quite consistent with the typical phenomenon of lysin-mediated cell lysis. The weakening and rupture of the cell wall resulted in the loss of cytoplasmic contents partly or totally (Fig. 1E and F) and formation of a cell "ghost" (Fig. 1G).

The specificity of ClyH. As shown in Fig. 2, ClyH had an effective lytic activity against staphylococci strains, including the

methicillin-sensitive *S. aureus* (MSSA) and MRSA strains tested (see Table S2 in the supplemental material), but not the other species tested, except *S. sobrinus*. This observation was quite consistent with an early report indicating that the CBD of phiNM3 was highly specific to staphylococci (19). Moreover, the lytic velocities were quite fast for all of the clinical isolate MRSA strains, regardless of their SCCmec types.

Comparison of the lytic activity of ClyH with those of other antimicrobials. To compare the activity of ClyH with those of other antimicrobial agents against *S. aureus*, the antimicrobial activity of ClyH was tested together with those of lysostaphin, Pc-His, and several antibiotics. As shown in Fig. 3A, ClyH displayed a higher activity than lysostaphin. Furthermore, ClyH could even lyse two strains (AM016 and AM045) that lysostaphin could not lyse. We also observed an obvious strain-to-strain variation of

FIG 2 Lytic activity of ClyH (0.5 U) against different strains *in vitro*. The activity of lysis is defined as the initial velocity of the decrease in OD₆₀₀ over time. Error bars show the standard errors of three independent assays.

ClyH activity, similar to those observed for other lysins (19, 28, 31, 33). It has been reported that the cell wall thickening is associated with adaptive resistance to antibiotics in MRSA clinical isolates (34), which may contribute to the observed variability of ClyH activity. To minimize the effect of His tag on the enzymatic activity, we expressed a His-tagged ClyH (ClyH-His) (Fig. 1B) and compared its lytic activity with that of Pc-His. The results illustrated that the lytic activity of ClyH-His was quite close to that of ClyH (see Fig. S3 in the supplemental material) and higher than that of Pc-His, improving 3.7- to 13.6-fold for the strains tested (Fig. 3B).

MIC tests (Table 1) showed that all of the isolates tested were highly resistant to penicillin, with minimum inhibition concentration (MIC) values higher than 319.4 mg/liter, except for strain AM058. MRSA strains displayed a relatively higher resistance to gentamicin than MSSA strains; however, all of the strains were highly sensitive to vancomycin and ClyH, with MIC values ranging from 0.53 to 1.99 mg/liter and 0.05 to 1.61 mg/liter, respectively.

Elimination of MRSA by ClyH in a mouse model. The *in vivo* protective efficacy of ClyH was tested in a mouse model. As shown in Fig. 4A, administration of 180 U of ClyH at 3 h after challenge with 4×10^9 CFU AM025/mouse protected 66.7% of mice against

lethality over the 10-day course of experiments. The protective efficacy was improved to 100% when the dose of ClyH increased to 360 U. In the group receiving no injection of ClyH, all mice were dead within 24 h after the challenge. Further tests showed that a single administration of higher doses (540 U) and the repeated administration (total dose of 1,400 U in 7 days) of ClyH alone neither influenced the survival rate nor produced adverse effects to the mice in terms of body weight and activity. However, as shown in Fig. 4B, the ELISAs demonstrated that repeated injection of ClyH (200 U) could induce an immune response in the mice (the antibody titers were over 4×10^5). Fortunately, the immunized serum showed no obvious neutralization effect on the lytic activity of ClyH (Fig. 4C).

DISCUSSION

The modular structure of lysin makes it possible to swap different catalytic domains and binding domains to create novel chimeric lysins, which not only may retain the binding specificity and/or lytic activity of the original lysins (35, 36) but also have better antimicrobial properties. As shown in Table S1 in the supplemental material, besides ClyH, several other chimeric lysins have been reported previously with activity against *S. aureus* (17, 19, 22, 24). The difference between ClyH and other chimeric lysins is its

FIG 3 Comparison of the activity of ClyH/ClyH-His with that of lysostaphin and Pc-His, respectively. (A) Lytic activity of ClyH (0.16 μ M) in comparison with that of lysostaphin at the same concentration. (B) Lytic activity of ClyH-His (1.2 μ M) in comparison with that of Pc-His at the same concentration. Error bars represent three independent assays.

TABLE 1 MICs for the *S. aureus* isolates in this study

Strain	MIC (mg/liter)				
	Penicillin	Gentamicin	Oxacillin	Vancomycin	ClyH
MSSA					
AB918	>319.42	160.00	1.00	0.53	0.19
AM058	>159.71	80.00	0.50	1.99	0.09
AM061	>319.42	20.00	0.50	1.99	0.09
AM062	>319.42	10.00	0.50	1.99	0.19
AM065	>319.42	20.00	0.50	1.99	0.09
AM066	>319.42	10.00	0.50	1.99	0.09
AM067	>319.42	20.00	0.50	1.99	0.19
AM068	>319.42	5.00	1.00	1.99	0.09
MRSA					
AM054	>319.42	160.00	80.01	1.99	0.09
AM044	>319.42	>319.98	>320.31	1.99	0.38
AM052	>319.42	80.00	>320.31	1.00	0.20
AM022	>319.42	80.00	>320.31	1.99	0.20
AM032	>319.42	319.98	>320.31	1.99	1.61
AM031	>319.42	40.00	>320.31	1.00	1.61
AM041	>319.42	160.00	320.31	1.99	0.05
AM012	>319.42	40.00	>320.31	1.00	0.20
AM036	>319.42	40.00	>320.31	1.99	1.61
AM001	>319.42	319.98	4.01	1.00	0.20
AM016	>319.42	40.00	>320.31	1.00	0.05
AM008	>319.42	10.00	8.01	1.00	0.82

unique fusion of the CD of Ply187 lysin with the CBD of phage phiNM3 lysin. Upon exposure of *S. aureus* AB918 cells to ClyH, the rapid loss of turbidity and the cell wall damage (Fig. 1) indicated that ClyH was highly active against *S. aureus*. The specific activity of ClyH was about 400 U/mg, which is about 2-fold higher than that of its closely related chimera, ClyS (19). Furthermore, unlike most lysins which are usually active only in a pH range from 5 to 8 (28, 37), ClyH retained a high lytic activity (above 30% of the maximum) under pHs of 5 to 10. Besides pH, ionic strength also had a minor effect on the activity of ClyH. These properties make ClyH suitable to work under some environmental conditions that render other lysins inactive.

In vitro tests showed that ClyH was a highly potent agent to kill *S. aureus*. Its capability to lyse all of the tested clinical MRSA isolates (Fig. 2), regardless of their SCCmec types, indicated that ClyH might be used to control all kinds of MRSA *in vitro*. The greatly improved lytic activity of ClyH (ranging from 3.7- to 13.6-fold) over that of Pc-His indicated that the non-SH3b CBD could add activity to the whole lysin, which is similar to that found in the chimera Ply187AN-KSH3b (where a lytic activity 10-fold higher than that of Pc-His was found after addition of an SH3b CBD) (24). Since Pc has been reported having a higher lytic activity than the whole lysin Ply187 (23), it is easy to conclude that ClyH has significantly improved lytic activity compared to Ply187. Moreover, ClyH displayed not only higher lytic activity than lysostaphin but also a broader lytic spectrum to the two clinical MRSA isolates (AM016 and AM045), which were resistant to lysostaphin (Fig. 3A). This may be due to the non-SH3b binding domain of ClyH, which is much more difficult to evoke resistance than the SH3b domain of lysostaphin (21).

The low MIC values of ClyH suggested that ClyH has the potential to be used as an antimicrobial agent for the treatment of infections caused by MRSA *in vivo*. Our initial study demon-

strated that a single intraperitoneal administration of a low dose of ClyH could greatly improve the survival rate of mice infected by a lethal dose of MRSA (Fig. 4). Importantly, an accumulated excessive dose of ClyH (up to 1400 U) showed no adverse effects on the body weights and activities of the mice, which indicated that ClyH did not have obvious toxicity. As a protein, ClyH could induce a humoral immune response, which might block its usage for treating repeated infections. Fortunately, our neutralization test showed that although repeated administration of ClyH did evoke an obvious immune response, the antibodies induced did not influence the activity of ClyH. All of these results suggested that ClyH might be systematically administered with safety to combat the increasing infections caused by multidrug-resistant *S. aureus*.

FIG 4 Protective effect of ClyH on mice from death caused by MRSA. (A) Curative effects in a mouse model of systemic MRSA infection. Three hours after infection, one group of mice was given 180 U of ClyH, the second group was given 360 U of ClyH, and the third group was given PBS buffer. Meanwhile, another group of mice without MRSA infection were given 540 U of ClyH to test its toxicity. (B) Titers of anti-ClyH antibody induced by repeated injection of ClyH. The control serum is nonimmunized mouse serum. (C) Effect of ClyH-immunized serum on the lytic activity of ClyH against AM025.

In conclusion, the novel chimeric lysin ClyH showed good antimicrobial activities against all clinical MRSA isolates tested and some improved properties over other lysins. Although more tests are needed, the present data strongly support the idea that ClyH offers great potential to be used as a novel agent for the treatment of infections caused by MRSA.

ACKNOWLEDGMENTS

This work was supported by the Basic Research Program of the Ministry of Science and Technology of China (2012CB721102 to H. P. Wei and J. P. Yu), the National Natural Science Foundation of China (21075131), the Post-Graduate Scientific and Technological Innovation Project of Chinese Academy of Sciences (Y204081YZ1), and the Key Laboratory on Emerging Infectious Diseases and Biosafety in Wuhan.

We thank Xiancai Rao from Third Military Medical University for providing the *S. aureus* strains and Yingliu Liu from Wuhan University for his kind help in collecting the *S. aureus* strains from hospitals in Wuhan.

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