MRSA epidemic linked to a quickly spreading colonization and virulence determinant

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The molecular processes underlying epidemic waves of methicillin-resistant Staphylococcus aureus (MRSA) infection are poorly understood1. Although a major role has been attributed to the acquisition of virulence determinants by horizontal gene transfer2, there are insufficient epidemiological and functional data supporting that concept. We here report the spread of clones containing a previously extremely rare3,4 mobile genetic element–encoded gene, sasX. We demonstrate that sasX has a key role in MRSA colonization and pathogenesis, substantially enhancing nasal colonization, lung disease and abscess formation and promoting mechanisms of immune evasion. Moreover, we observed the recent spread of sasX from sequence type 239 (ST239) to invasive clones belonging to other sequence types. Our study identifies sasX as a quickly spreading crucial determinant of MRSA pathogenic success and a promising target for therapeutic interference. Our results provide proof of principle that horizontal gene transfer of key virulence determinants drives MRSA epidemic waves.

S. aureus is a dangerous pathogen, causing a multitude of serious and sometimes life-threatening diseases around the globe. This is mostly due to widespread antibiotic resistance among S. aureus isolates, which considerably complicates the treatment of S. aureus infections5. MRSA strains in particular represent a major problem for public health systems, as they combine resistance to methicillin and other β-lactam antibiotics with relatively minor fitness costs, resulting in high infiltration of hospital and community settings6. Remarkably, in the US, the estimated number of deaths due to MRSA infections exceeds that due to HIV/AIDS7. Although molecular typing and genome sequencing have provided better insight into MRSA epidemiology in recent years8, there is a severe lack of knowledge regarding the mechanisms by which specific molecular determinants cause the spread and pathogenic success of MRSA clones. Only a very limited number of MRSA clones, belonging to five clonal complexes, are responsible for the majority of MRSA infections worldwide1. Specific sequence types are characteristic for a given geographical location. In China, as in most Asian countries, ST239 is predominant8. Recently, genome sequencing of an ST239 strain revealed a new gene of unknown function (SATW20_21850) that is present in only three of the 43 global ST239 strains that have been sequenced3,4,10 and absent from all other known S. aureus genomes3. On the basis of its amino acid sequence, which contains a signal peptide and an LPXTG surface-anchoring motif, this gene encodes a secreted, surface-anchored protein of 15 kDa in its processed form. It is located at the 3′ end region of a 127.2-kb ΦSpβ-like prophage, which lacks other virulence-associated genes9. There is no similarity of the surface protein to other proteins found in the NCBI databases except for the highly similar, ΦSpβ-like encoded Sesl of S. epidermidis RP62A11. We termed the gene sasX and showed that it is absent from other major global MRSA strains by analyzing a series of strains from divergent clonal and geographical backgrounds (Supplementary Data).

Prompted by the epidemiological connection of sasX to MRSA ST239, we wanted to evaluate the hypothesis that sasX represents a key factor determining the spread of MRSA in China9. To that end, we determined sequence type distribution and frequency of sasX-positive clones among >800 randomly selected S. aureus isolates from patients with S. aureus infections at three large teaching hospitals located in eastern China between 2003 and 2011 (Fig. 1a). The situation in China in terms of MRSA infection rate and the dominance of the ST239 (Brazil/Hungary) clone is largely characteristic of that in the Asian and Australian region, except Japan, South Korea and Australia8,12–15. Accordingly, ST239 clones were also predominant among the isolates investigated in our study, with ST5 being the second most frequently isolated sequence type (Supplementary Fig. 1). Notably, the frequency of sasX-positive invasive S. aureus clones increased significantly from 2003 to 2011 (from 19% to 31%, P = 0.0026). Among MRSA isolates, the frequency of sasX increased from 21% to 39% (P = 0.0028), whereas the increase among methicillin-sensitive S. aureus (MSSA) was not significant (Fig. 1b). Furthermore, sasX was found almost exclusively among ST239 strains in 2003–2005, whereas its frequency among isolates of several other sequence types considerably increased since then (increase from 5% to 28% of non-ST239

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clones among sasX-positive S. aureus between 2003–2005 and 2009–2011, P = 0.0048; Fig. 1c). Moreover, sasX was extremely rare (only one positive clone among 169 tested) among a collection of community isolates from healthy individuals in the same areas (Supplementary Data), indicating that sasX-positive clones spread predominantly within the hospital setting. Finally, we did not detect ST239 clones among community isolates (Supplementary Data), indicating that this sequence type is linked to hospital infections. Analysis of genes surrounding sasX in randomly selected clones of different sequence types showed that the horizontal transfer of sasX always occurs linked to the ΦSPβ-like prophage with a conserved phage insertion site, although occasional deletions within the phage were observed (Supplementary Table 1). Together, these findings demonstrate an epidemic wave of hospital-associated sasX-positive MRSA in China. Furthermore, they indicate events of horizontal gene transfer resulting in the spread of sasX from ST239 to other sequence types and a causative link between the presence of sasX and invasiveness.

To understand the link between sasX and infection, we set out to elucidate the biological role of sasX. We first demonstrated that the SasX protein is expressed at the S. aureus cell surface by immunofluorescence and immunoblot detection (Fig. 2a,b). Surface proteins in S. aureus fulfill a variety of functions that include first and foremost adhesion to host tissue16. In particular, adhesion to nasal epithelial cells is of major importance for the colonization, spread and virulence potential of S. aureus strains, inasmuch as the nare

Figure 1 Spread of sasX-positive clones in China. (a) A total of 807 isolates from three teaching hospitals in eastern China were analyzed for sequence type, methicillin resistance and presence of sasX. (b) Percentage of sasX-positive among MSSA and MRSA isolates. Statistical analysis is by χ² test of all MRSA isolates (average curve, shown in black). The P value including all (MRSA and MSSA) isolates is 0.0026. (c) Percentage of sasX-positive isolates in sequence types other than ST239. Statistical analysis is by Fisher’s exact test of all isolates (average curve, shown in black).

are the predominant location of S. aureus colonization in the human body17 and there is an epidemiological link between nasal carriage and infection18. However, the molecular basis of nasal colonization is incompletely understood, and there are only a few molecular factors of S. aureus that have been identified as potentially responsible for nasal colonization, such as teichoic acids19 and specific surface proteins20–22. To measure the impact of sasX on nasal colonization, we constructed isogenic sasX deletion mutants in representative (Supplementary Fig. 2) ST239 and ST5 strains. Adhesion of the sasX mutant strains to human nasal epithelial cells in vitro was significantly impaired compared to the wild-type strains, and adhesion was restored in sasX-complemented mutant strains (Fig. 2c). Furthermore, recombinant SasX protein blocked adhesion of S. aureus to human nasal epithelial cells (Fig. 2d and Supplementary Fig. 3). Moreover, sasX had a significant impact on in vivo nasal colonization in separate and competitive colonization models (Fig. 2e). These data demonstrate that the SasX surface protein promotes nasal colonization.

In addition to facilitating adhesion to host tissue, S. aureus surface proteins may cause intercellular bacterial aggregation, a phenotype with multiple consequences such as increased biofilm formation and immune evasion capacity23–25. Indeed, we found that sasX facilitated the formation of large bacterial aggregates (Fig. 3a and Supplementary Fig. 4) and promoted biofilm formation (Fig. 3b).

Figure 2 The SasX surface protein facilitates nasal colonization. (a) Detection of SasX with SasX-specific polyclonal antiserum in an indirect immunofluorescence assay. (b) Immunoblot detection of SasX in different subcellular fractions. Black arrows point to the putative SasX protein. Note that surface proteins in this assay run higher than their predicted molecular weights in the processed forms, owing to the linkage to remaining peptidoglycan parts. W, whole cell preparation; S, surface protein fraction; C, cytoplasmic fraction; E, extracellular fraction. (c) Adherence of wild-type, isogenic sasX-mutant, sasX-complemented and control strains to human nasal epithelial cells in vitro. (d) Blocking of adherence to nasal epithelial cells by recombinant GST-SasX fusion protein. Purified GST protein alone and denatured GST-SasX fusion protein were used as controls. (e) Nasal colonization model. Female imprinting control region (ICR) mice (n = 10 per group in the separate model; n = 15 per group in the competition model) were inoculated intranasally with 1 x 10⁷ colony-forming units (CFUs) of wild-type or isogenic sasX-mutant strains, and bacteria were counted in mice that were killed at 7 d after inoculation. ST239 HS770 was used for the separate and ST239 HS663 for the competition model. Equal amounts of wild-type and deletion mutant bacteria were mixed for the competition model inoculum. Statistical analyses in c–e are by unpaired t tests. All error bars show s.e.m.
Figure 3 SasX promotes bacterial aggregation and mechanisms of immune evasion. (a) Microscopic images of 16-h cultures of ST239 wild-type, isogenic sasX-mutant, sasX-complemented and control strains. (b) Biofilm formation. Cultures were grown for 24 h in microtiter plates. Afterward, biofilms were measured using crystal violet staining. TBS, Tris-buffered saline (buffer control). (c) Primary attachment. The same experiment was performed as in b, but with only 1 h growth. (d) Phagocytosis assay. Phagocytosis by human neutrophils of ST239 or the isogenic sasX-mutant strain was examined using microscopy. (e) Microscopic examination of phagocytosis. Slides were stained using a modified Wright-Giemsa stain. At t = 90 min, lysed neutrophils were occasionally detected in the ST239 wild-type samples. The 90-min ST239 image shows such a lysed neutrophil with adherent S. aureus bacteria. (f) Survival in human blood at t = 90 min. (g) Neutrophil killing. Bacteria from mid-logarithmic growth phase were washed and incubated with human neutrophils at a 100:1 ratio. Neutrophil lysis was determined by measuring release of lactate dehydrogenase (LDH). Statistical analyses in b–d, f, g are by unpaired t tests. All error bars show s.e.m.

Notably, sasX also facilitated primary attachment to an abiotic surface (Fig. 3c), which is in keeping with our finding that sasX expression was highest during early growth and decreased afterward (Supplementary Fig. 5). Moreover, phagocytosis of the wild-type strain by human neutrophils was significantly lower than that of the sasX-mutant strain (Fig. 3d,e). Accordingly, survival in human blood (Fig. 3f) and lysis of human neutrophils as a sign of prolonged bacterial survival after phagocytosis (Fig. 3g) were significantly increased in the wild-type strain compared to the sasX deletion mutant. Notably, these effects are most likely caused predominantly by the aggregation phenotype and not by a gene regulatory effect, as sasX did not affect expression of the ica genes encoding biofilm exopolysaccharide biosynthesis, the biofilm and virulence regulator agr<sup>26</sup> or a series of cytolysins and other surface protein genes (Supplementary Fig. 2). Together, these data show that sasX facilitates intercellular aggregation, leading to a marked enhancement of S. aureus immune evasion mechanisms.

Figure 4 SasX is a key virulence determinant during MRSA skin and lung infection. (a) Skin infection model. Outbred, immune-competent, hairless mice were inoculated with 1 × 10<sup>8</sup> CFUs of the ST239 or ST5 wild-type and isogenic sasX-mutant strains. Developing abscess areas are shown (see representative images on the right). Differences between corresponding wild-type and mutant abscess sizes were statistically significant at every time point (unpaired t tests, P < 0.05). (b) Lung infection model. Female ICR mice were inoculated in the nose with 1 × 10<sup>8</sup> CFUs per 20 µl of the ST239 or ST5 wild-type or isogenic sasX-mutant strains. Mice were killed at day 5 after infection for subsequent analyses. Lung wet weight/ body weight ratios are shown. (c) Lung infection model, concentration of TNF-α in lung tissue samples. (d) Skin infection model, H&E-stained tissue samples. Note increased number of infiltrating inflammatory cells in wild-type sample images in d and extensive inflammation accompanied by hemorrhagic infiltration and disruption of pulmonary architecture in wild-type sample images in e. Statistical analyses in b,c are by unpaired t tests. All error bars show s.e.m.
S. aureus most frequently causes skin and respiratory infections\(^\text{27}\). Correspondingly, these were also the most abundant types of infection detected in our study (Supplementary Fig. 6). Among infections caused by sasX-positive clones, respiratory and pleural-cavity infections as well as abscesses were most prominent (significant difference by type of infection, \(P = 0.0139, \chi^2\) test). Therefore, to determine whether sasX is a virulence factor during S. aureus infection, we selected to perform mouse abscess and lung infection models (Fig. 4). Mice infected with the wild-type strains developed abscesses that were significantly larger than those in mice infected with the sasX-mutant strains (Fig. 4a). Furthermore, microscopic evaluation showed increased infiltration of inflammatory cells in the abscesses caused by the wild-type strain (Fig. 4d). Of note, the strong impact of sasX on abscess size is similar to that observed with potent S. aureus toxins such as \(\alpha\)-toxin\(^\text{28}\) or phenol-soluble modulins\(^\text{29}\). In addition, lung wet weight/body weight ratios, degree of inflammation in the lung as assessed by tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) concentration, and histological examination showed that sasX is also a major factor influencing S. aureus pathogenesis in the lungs (Figs. 4b,c,e). Thus, our data classify sasX as a key determinant of MRSA skin and lung infection.

In conclusion, we identified sasX as a crucial factor promoting nasal colonization, immune evasion and virulence and a probable main driving force of the Asian MRSA epidemic. Furthermore, our data provide strong support to the notion that acquisition of specific molecular determinants via horizontal gene transfer represents a major mechanism enhancing the pathogenic potential and epidemiological success of MRSA clones. Moreover, given our data, it is to be expected that the frequency of sasX among Asian and international MRSA clones will increase further. We thus propose drug development or vaccination efforts aimed at SasX to prevent MRSA colonization and infection.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.

Note: Supplementary information is available on the Nature Medicine website.

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AUTHOR CONTRIBUTIONS

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ONLINE METHODS

Bacterial isolates. We randomly selected 807 S. aureus isolates from inpatients with S. aureus infections at three different teaching hospitals in China: 100 each from 2004, 2007 and 2010 from Huashan Hospital, Fudan University, Shanghai; 107 from 2006–2008 and 79 from 2009–2010 from Shandong Provincial Hospital, Shandong University, Jinan; 104 from 2003–2005, 105 from 2006–2008 and 112 from 2009–2011 from the first affiliated hospital of Wenzhou Medical College, Wenzhou. We also collected 49 S. aureus isolates from nasal swabs among more than 500 healthy volunteers in Shanghai and 120 from nasal swabs among more than 1,000 healthy volunteers in Wenzhou as part of a population-based community prevalence study. All isolates were obtained in accordance with the protocols approved by the ethics committees of the First Affiliated Hospital of Wenzhou Medical College, Shandong Provincial Hospital and Huashan Hospital. All inpatients and healthy volunteers gave informed consent prior to donating S. aureus isolates. Characteristics of all isolates are listed in the Supplementary Data. Bacteria were identified as staphylococci by classic microbiological methods: Gram stain, catalase activity and coagulase activity on rabbit plasma. S. aureus strains were further categorized by biochemical characterization using the Api-Staph test (BioMérieux, Lyon, France). All strains and plasmids used in this study are listed in Supplementary Table 2. All oligonucleotides used for cloning or polymerase chain reaction procedures are listed in Supplementary Table 3.

Nasal colonization model. All mouse work was approved by the Ethics Committee of Fudan University. Female ICR mice were used for the nasal colonization model (ten mice per group). All mice were 6 to 8 weeks of age at the time of use and received drinking water containing penicillin at 100 µg ml−1. S. aureus strains were grown to mid-exponential growth phase, washed and resuspended in sterile PBS at 1 × 10⁸ CFUs per µl. Mice were anesthetized with isoflurane. The inoculum, which contained 1 × 10⁸ CFUs in 10 µl of PBS or PBS alone, was pipetted slowly into the nares of the anesthetized mice (n = 10 per group) without touching the nose. At 5 d after inoculation, mice were killed. The lungs from selected groups of mice were excised and washed with saline, and one lobe was fixed in 10% formalin (Sigma). Paraffin embedding and H&E staining were performed as previously described. The other lobe was homogenized in 0.5 ml of TSB; homogenized lung tissue and serum samples were used for TNF-α detection.

Skin abscess model. Female outbred, immune-competent, hairless mice were used for the abscess model. All mice were between 4 and 6 weeks of age at the time of use. S. aureus strains were grown to mid-exponential phase, washed once with sterile PBS and then resuspended in PBS at 1 × 10⁶ CFUs per 100 µl. Mice were anesthetized with isoflurane and inoculated with 100 µl PBS containing 1 × 10⁸ live S. aureus or saline alone in the right flank by subcutaneous injection. We examined test mice at 24-h intervals for a total of 10 d with a caliper. We applied length (L) and width (W) values to calculate the area of abscesses with the formula L × W. Abscesses as shown in Figure 4a developed into scarred lesions during the time of the experiment. The sizes of these lesions showed similar differences as the closed abscesses between the wild-type and sacX-mutant groups and remained until the endpoint of the experiment (Supplementary Fig. 7). However, only the sizes of closed abscesses were measured. All mice were killed after completion of the entire procedure.

Additional methods. Detailed methodology is described in the Supplementary Methods.

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