An 11-year linezolid-resistant *Staphylococcus capitis* clone dissemination with a similar *cfr*-carrying plasmid in China

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**Highlights**
- An eleven-year persistence of nosocomial LRSC clone dissemination
- Similar vector backbones of *cfr*-carrying plasmids in evolution
- Instability of *cfr*-plasmids in MRSA
- Fitness cost of the carriage of the *cfr* gene on *Staphylococcus capitis*

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An 11-year linezolid-resistant Staphylococcus capitis clone dissemination with a similar cfr-carrying plasmid in China

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SUMMARY
Linezolid resistance has represented a global concern with its wide dissemination among nosocomial pathogens in recent years. One hundred and two linezolid-resistant Staphylococcus capitis (LRSC) were constantly isolated from 2011 to 2021, which demonstrated single clonal dissemination in a Chinese tertiary hospital. A structurally similar cfr-carrying plasmid was identified among 90 isolates. A chromosomal cfr was located beside a Tn4001-like transposon and ISEnfa4 in one strain (LR95). The loss of cfr-carrying plasmid was observed in 11 isolates and the in vitro passage experiments. Conjugation experiments demonstrated the horizontal transferability of the cfr-carrying plasmid into Staphylococcus aureus RN4220. Both cfr-positive LRSC and S. aureus showed no significant differences in growth rates, while only the former displayed competition defect, suggesting this plasmid imposed a certain fitness cost on LRSC. Hence, ongoing measurements are supposed to be adopted to control the spread of these antimicrobial-resistant bacteria.

INTRODUCTION
Linezolid, the first member of the oxazolidinone class of antibiotics, possessed the activity to Gram-positive bacteria and was regarded as the last resort for the treatment of serious infections by multidrug-resistant (MDR) strains, including methicillin-resistant Staphylococcus aureus (MRSA) and vancomycin-resistant enterococci (VRE). However, linezolid-resistant staphylococci emerged shortly after this antibiotic was approved for clinical practice in 2000, with the G2576T mutation in domain V of the 23S rRNA, which was considered to be the major mechanism contributing to the linezolid resistance. Besides, a variety of additional mutant positions in 23S rRNA and alterations in genes encoding the 50S ribosome proteins L3, L4, and L22 have been reported to knit together with the decreased susceptibility to linezolid as well. Nowadays, the 23S rRNA modifications are the main linezolid-resistance mechanism in staphylococci and are generally responsible for the high levels of linezolid resistance.

Another significant mechanism involving the resistance to linezolid amounts to the acquisition of a plasmidborne gene cfr, which encodes a methyltransferase and modifies adenosine at A2503 in the 23S rRNA, resulting in the cross-resistance phenotype to phenicols, lincomycin, oxazolidinones, pleuromutilins, and streptogramin A. Previous studies have spawned the genetic environment of the cfr gene and demonstrated that the transposases and mobilization proteins encoded by cfr-flanking regions such as IS256-like elements were responsible for the potential interchange of resistance loci among bacteria communities and transposon-mediated dissemination. To date, several cfr-harboring plasmids have been recognized both in linezolid-resistant Staphylococcus aureus (LRSA) and linezolid-resistant coagulase-negative staphylococci (LRCoNS), which led to the multifocal outbreaks at home and abroad. Though the increased annual trend of the presence of cfr was not observed from 2011 to 2016 with a prevalence of 0.02% in S. aureus, 62.5% S. aureus with decreased linezolid susceptibility and 21.9% LRCoNS carried this transferable gene.

In recent years, concern about LRCoNS is mounting. According to surveillance data provided by the LEADER (linezolid experience and accurate determination of resistance) program, the percentage of isolates nonsusceptible to linezolid in CoNS has increased more than 7-fold from 2004 to 2009 (0.2% and 1.47%, respectively), and a meta-analysis in 2020 illustrated the higher resistance rate to this antibiotic...
for CoNS (0.3%) when compared to MRSA (0.1%). The most frequently identified linezolid-resistant CoNS were reported to be *Staphylococcus epidermidis* and *S. capitis*, the latter of which is predominantly parasitic on the scalp and skin as an opportunistic pathogen related to the neonates with sepsis as well as severe infections like bloodstream infection (BSI) for adults. Linezolid-resistant *S. capitis* (LRSC) had yet to emerge domestically until 2012 when we reported the *cfr*-positive CoNS isolate for the first time. Following that, several nosocomial infections with *cfr*-harboring LRSC isolates were detected in several hospitals in China, Japan, and Europe. In 2015, we reported the emergence of *cfr*-carrying MRSA in our hospital, and the horizontal transfer of this plasmid among bacteria was confirmed by conjugation experiments.

Though the outbreaks for LRSC have been just reported sporadically, the increasing antibiotic resistance trend and the potential role as the reservoir for MDR genes may pose a challenge to clinical treatment and public health. During the past 11 years, LRSC were constantly isolated in our hospital. In this study, we investigated the molecular epidemiology of 102 LRSC collected from 2011 to 2021 and the evolution of *cfr*-carrying plasmids. Further, the transferability of *cfr*-carrying plasmids to *S. aureus* and the fitness cost for MRSA possessing the *cfr*-carrying plasmid were evaluated.

### RESULTS

#### Similar antimicrobial susceptibility profile and resistance determinants carriage for LRSC

The antimicrobial susceptibility results of LRSC were illustrated in Table 1. Ninety-one *cfr*-positive LRSC isolates shared a similar susceptibility profile with a linezolid minimal inhibitory concentration (MIC) of >256 mg/L (except strain LR4) and showed high-level resistance to chloramphenicol and clindamycin, while 11 *cfr*-negative LRSC exhibited relatively lower MICs of linezolid and chloramphenicol (16 mg/L to 32 mg/L) and inducible clindamycin resistance compared with that of *cfr*-positive isolates. Other than strain LR4, the 101 LRSC isolates displayed an MDR phenotype being resistant to erythromycin, ciprofloxacin, penicillin G, and oxacillin but remained susceptible to tetracycline, vancomycin, and rifampicin (except strain LR88, which was resistant to rifampicin at a MIC of >32 mg/L). For 80 LRSC isolates, resistance to gentamicin was observed with MICs of 16 to >32 mg/L, while 15 of them demonstrated either gentamicin-intermediate or gentamicin susceptibility.

The analysis for resistance determinants based on the whole-genome sequencing (WGS) data yielded the presence of multiple antimicrobial resistance genes in the tested strains (Figure 1), conferring resistance to β-lactams (*blaZ*), methicillin (* mecA*), erythromycin (*erm(A)*), and aminoglycosides (*aac(6’)-aph(2’)*), *aadD*, and *ant(9)-Ia*), with high positive rates of 98.16% (*n* = 107), 100% (*n* = 109), 93.58% (*n* = 102), 98.17% (*n* = 107), 89.91% (*n* = 98), and 95.8% (*n* = 102), respectively. *cfr* gene was detected in 91 LRSC isolates and conferred higher MICs for linezolid, chloramphenicol, and clindamycin when compared to other LRSC, indicating that the presence of the *cfr* gene contributed to higher levels of antibiotic resistance to a certain extent. According to our previous study, G2576T and C2104T mutations were detected in ten initial

| Antimicrobial agents | *cfr*-positive LRSC strains (n = 91) | *cfr*-negative LRSC strains (n = 11) |
|----------------------|-------------------------------------|------------------------------------|
|                      | MIC50 | MIC90 | Range        | MIC50 | MIC90 | Range        |
| Linezolid            | >256  | >256  | 4 to >256    | 32    | 32    | 16 to 32     |
| Chloramphenicol      | >64   | >64   | >32 to >64   | 32    | 32    | 16 to 32     |
| Erythromycin         | >32   | >32   | ≤0.5 to >32  | >32   | >32   | >32          |
| Clindamycin          | >32   | >32   | 8 to >32     | 0.5   | 0.5   | 0.5          |
| Gentamicin           | >32   | >32   | ≤1 to >32    | >32   | >32   | ≤1 to 32     |
| Ciprofloxacin        | >32   | >32   | 8 to >32     | >32   | >32   | >32          |
| Penicillin G         | >32   | >32   | >32          | >32   | >32   | >32          |
| Oxacillin            | >8    | >8    | >8           | >8    | >8    | >8           |
| Tetracycline         | ≤1    | ≤1    | ≤1           | ≤1    | ≤1    | ≤1           |
| Vancomycin           | ≤1    | ≤1    | ≤1           | ≤1    | ≤1    | ≤1           |
| Rifampicin           | ≤0.25 | ≤0.25 | ≤0.25 to >32 | ≤0.25 | ≤0.25 | ≤0.25        |
strains with high MICs for linezolid except for strain LR4, and these mutations were also recovered in all of the additional LRSC strains in our study (Figure 1). The lack of mutation in 23S rRNA and the existence of the cfr gene make sense for the low MIC of linezolid for strain LR4, implying that the high-level resistance against this antibiotic was the causality for the combination of both resistance mechanisms.

LRSC belonged to one clone through phylogenetic analysis

To confirm the relatedness of the LRSC and linezolid-susceptible S. capitis (LSSC) strains, we constructed a core genome-based phylogenetic analysis using the draft genome sequences (Figure 1). The phylogenetic tree revealed that all the strains were clustered into two major clades; all of the LRSC (except strain LR4) and LS3 clustered into one single clade while the LSSC strains (except strain LS3) belonged to another one, confirming that LRSC phylogenetically belonged to one clone with the maximum of 115 SNPs between the strains, even including the 11 cfr-negative LRSC. It was notable that S. capitis LR4 was genetically close to the LSSC strains. S. capitis LR4 showed a minimum of 17 SNPs compared to strain LS1 whereas showed 10,002-10,058 SNPs versus the genomes of LRSC. Further, we also collected four available genome assemblies of cfr-carrying S. capitis in the public genome database from other regions in China with a geographical expanse, including strain LN2Z1-1 from Harbin (GenBank accession no. JGYJ00000000.1), strain XZ03 from Jiangsu (GenBank accession no. CP086659), and strain 12-498 together with strain 18-127 from Shanghai (GenBank accession no. JABBLC00000000000 and JABBMO00000000000). SNP analysis showed that these four strains were also closely related to our LRSC clone within 99 SNPs, suggesting that the clone dissemination was representative of the epidemic situation of LRSC in China. We also constructed an S. capitis core genome-based phylogenetic tree with the sequences of neonatal sepsis-associated S. capitis clone (NRCS-A, BioProject Accession number PRJNA493527) and 142 sequences of this species worldwide on the NCBI database. It was shown that our LRSC clone represented an individual clone with the four aforementioned isolates and discriminated from other clades including the strains elsewhere (Figure S1).

A similar cfr-carrying plasmid shared by LRSC strains and a chromosomal cfr gene in strain LR95 analyzed by molecular analysis

To investigate the evolution of cfr-carrying plasmids from 2011 to 2021, we performed a comparison for the plasmids and employed pLRSA417 as a reference. Contigs with a nucleotide sequence identity of >90% to plasmid pLRSA417 were utilized to establish the backbones of the plasmids, and three pairs of primers were designed to include the putative gaps that fell on the cfr-flanking regions. Except for strain LR95, the percentage of the coverage varied within a narrow range, indicating the high degree of similarity between the backbones of pLRSA417 and those of the remaining isolates. This yielded the full sequence of cfr-carrying vectors, and the representative plasmid pLR96 exhibited a size of 39,504 bp, which shared 99.17% identity with pLRSA417. Similarly, cfr-carrying plasmids in this study belonged to an unknown Inc-type. Meanwhile, a missense mutation C442A (leading to Gln148Lys alteration) in the cfr gene was detected in strain LR28 which was isolated in August 2012, together with most of the strains isolated after that (n = 61, Table S1).

Previous mapping of short reads of strain LR95 to reference vector pLRSA417 demonstrated minor similarity with a very small percentage of coverage, and it was assumed that the cfr locus was integrated into another plasmid backbone. Unexpectedly, the complete genome of strain LR95 obtained by hybrid assembly of short and long reads showed that the cfr locus was located on the chromosome as an insertion context. Further sequence alignment confirmed that the environment of cfr was consistent with that on pLRSA417. We have claimed the transposable unit comprising a Tn4001-like transposon, cfr, orf1, and ISEnfa4-like pLRSA417 in an earlier study, and the resembled genetic background in strain LR95 indicated that the chromosomal cfr was attributed to the mobile elements of the context and the mobilization of the transposon rendered this transposition event.
The transferability of cfr-carrying plasmids by conjugation experiments

The cfr gene could be conjugated into rifampicin-resistant S. aureus RN4220 from strains LR4, LR12, and LR96 but failed in strain LR95 even after several attempts. This observation further confirmed the chromosomal insertion of cfr gene for strain LR95. Acquisition of the cfr gene for transconjugants was verified by PCR, and decreased susceptibility to linezolid and chloramphenicol was obtained (Table 2). Similar conjugation efficiency was observed in the transconjugants of strains LR4, LR12, and LR96 at 9.62 × 10^7, 4.75 × 10^8, and 1.04 × 10^8, respectively. Further, the same linezolid MIC values of the transconjugants of S. capitis LR4 (carrying the wild-type cfr) and S. capitis LR96 (carrying the mutated cfr) were observed, indicating that the C442A mutations in the cfr gene did not contribute to a difference in the resistance level of linezolid.

In vitro low fitness cost of cfr-carrying plasmids

Growth curves and competition assays were performed to evaluate the biological fitness cost for the acquisition of the cfr gene. For S. aureus, similar growth rates were observed (p > 0.05) between the transconjugants and recipient, which also held true for strain LRSA417 and its filial strains without the cfr gene after serial passages (Figure 2A). Besides, the competition assays also suggested that no apparent fitness effects of cfr-carrying plasmids were imposed on S. aureus (Figure 2C). By contrast, the competitive index for cfr-carrying LRSC was relatively lower with a downtrend over time (Figure 2D), indicating that carriage of the cfr gene resulted in a certain burden on the hosts and incur fitness cost, though there was also no significant difference (p > 0.05) in growth rates among LRSC isolates (Figure 2B). Further, the co-culture competing between strains LR95 against LR96 showed no obvious competitive defect (Figure 2D), indicating that the chromosomal insertion of cfr locus would not increase the fitness burden on LRSC when compared to the plasmid-borne one.

Instability of cfr-carrying plasmids on MRSA

The stability of cfr-carrying plasmids was roughly estimated by the frequency of stable plasmids during the serial passages under minimized selection pressure. After 15 passages (300 generations), over 80% of cells of LRSC strains retained the cfr-carrying plasmid (Figure 2E). Conversely, the loss of cfr-carrying plasmids occurred more frequently in S. aureus and was observed on day three (Figure 2E), following which the frequency of plasmid-hold cells demonstrated a downtrend to about 5% after 12 passages (240 generations), indicating the instability of this plasmid for MRSA.

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Table 2. Susceptibility profile of S. aureus RN4220, LRSA417, and their derivate strains

| Strains    | MIC (mg/L) | Characteristics                                      |
|------------|------------|-----------------------------------------------------|
|            | Linezolid  | Chloramphenicol                                     |                         |
| TLR4-1     | 4          | 8                                                   | S. aureus transconjugants with donor S. capitis LR4 |
| TLR4-2     | 4          | 16                                                  | S. aureus transconjugants with donor S. capitis LR4 |
| TLR12-1    | 4          | 8                                                   | S. aureus transconjugants with donor S. capitis LR12 |
| TLR12-2    | 4          | 8                                                   | S. aureus transconjugants with donor S. capitis LR12 |
| TLR96-1    | 4          | 8                                                   | S. aureus transconjugants with donor S. capitis LR96 |
| TLR96-2    | 4          | 8                                                   | S. aureus transconjugants with donor S. capitis LR96 |
| RN4220     | ≤1         | 2                                                   | N/A                     |
| LRSA417    | 8          |                                                      | MRSA with cfr-carrying plasmid                         |
| LRSA417(−) | ≤1         | 4                                                   | LRSA417 derivates that loss the cfr-carrying plasmid   |
Figure 2. Fitness cost of cfr-carrying plasmid for S. aureus and LRSC

(A) Growth curves of cfr-positive and cfr-negative S. aureus. S. aureus TLR4-1, TLR4-2, TLR12-1, TLR12-2, TLR96-1, and TLR96-2 were obtained by filter mating using as donors S. capitis LR4, LR12, and LR96, respectively. S. aureus LRSA417 (–) was the cfr-negative derivative of S. aureus LRSA417 after serial passages.

(B) The dynamic of competition index for cfr-positive S. aureus. Competitor/tester strains were shown as the legend indicated.

(C) The growth curves of cfr-positive LRSC.

(D) The dynamic of competition index for cfr-positive LRSC. S. capitis LR12, LR18, LR96, and LR102 with plasmid-borne cfr as well as strain LR95 with chromosomal cfr were performed as the competitors, while cfr-negative strain LR51 and LR98 were employed as the tester strains. Competitor/tester strains were shown as the legend indicated.

(E) The dynamic of the frequency of plasmid-carrying LRSC and S. aureus cells.

Error bars represent the standard deviation (SD) of the mean (n = 3). Data are represented as mean ± SD.
DISCUSSION

*S. capitis* were classically reported as a broadly antibiotic-susceptible CoNS; however, with the extensive use of linezolid in clinical treatment, the emergence of LRCoNS has attracted widespread attention and made the clinical treatment of Gram-positive cocci infections problematic.

Over the 11 years from 2011 to 2021, 102 LRSC were isolated and collected for our study, and the core-genome SNP analysis revealed that they were closely related, which strongly suggested the presence of long-term persistence and single clonal dissemination in our hospital. Recently, a study also reported a clone spread of LRSC in another tertiary hospital in Hangzhou and defined this clone as the L clone, which behaved as an individual clade among the hundreds of *S. capitis* genomes available on public repositories and showed discrimination with the common population like NRSC-A clone (a single multidrug-resistant *S. capitis* clone which is responsible for sepsis in preterm infants in neonatal intensive care units worldwide) in the representative phylogenetic tree. Based on that (Figure S1), we conferred that the phylogenetic background of the L clone was also of the same manner as the representative LRSC clone in our study, and the single clonal dissemination represented the epidemic situation of LRSC in China. Gu et al. have reported that significantly more LRCoNS were associated with outbreaks and 50% of those documented studies that analyzed LRCoNS involved clonal LRCoNS dissemination across healthcare settings, and the widespread diffusion of a single clone of LRSC was also reported in several countries. The aforementioned epidemic clone becomes successful in the hospital environment, especially in ICUs with the high linezolid use as empirical therapy for severe infections caused by MRSA and CoNS. Although yet to be proved in vitro, one of the reasons for the clonal establishment and increasing resistance in CoNS is that they tend to more readily develop resistance following linezolid exposure. Another hypothesis that could not be excluded is an intrinsic ability of the clone to acquire (following dissemination) resistance to linezolid due to hypermutator features, which means the linezolid dependence of this clone constitutes a selective advantage and facilitates its wide dissemination as predominant strains. Even though our data was not sufficient to support these hypotheses, the long-term endemic situation of LRSC clone in our hospital was still alarming and a great number of reports concerning the establishment of linezolid-resistant clones in hospitals preceded by increasing utilization rate for linezolid were not supposed to be ignored.

Our study also revealed the evolution of cfr-carrying plasmids as well as the propensity of acquisition and loss for their host in the cfr-mediated LRSC clone dissemination. Molecular analysis demonstrated that those plasmids of LRSC were similar without any significant alterations on the vector backbones during the 11 years. The acquisition of this plasmid via horizontal transfer was also confirmed in our study, and similar scenarios with the respective conjugative cfr plasmids were described elsewhere. The transferability of cfr-carrying plasmid may give a reason for the surprising relatedness of LSSC strains with LR4, which showed a low-level linezolid resistance mediated by the acquisition of cfr, and the same resistance gene carriage with LS1 also conformed to this presumption. However, the horizontal transfer of this plasmid was not only restricted between the same species but also took place beyond the biological border to other staphylococci including MRSA with a low fitness cost. In 2015, we have reported the clonal spread of cfr-positive MRSA in China; however, such strains had no longer been detected anymore in our hospital. Given the plasmid loss frequency and minor in-host plasmid stability for *S. aureus* without linezolid selective pressure we observed, it is conceivable that there was a tendency to lose the cfr-carrying plasmids during the procedure of clonal spread of these MRSA, which accounted for the presence of 11 cfr-negative isolates in the LRSC clonal dissemination as well. Generally, high fitness cost is the cause of plasmid instability. However, in our study, cfr-carrying plasmid imposed no obvious fitness cost on *S. aureus* but showed instability in this genus. The exact mechanism involving the frequent loss and instability of cfr-carrying plasmids in MRSA remains unclear. An extra pattern for the loss of cfr-carrying plasmid was also noticed and investigated in strain LR95 which was carrying the same cfr-insertion context but on its chromosome credited to the mobile elements in the cfr-flanking region. Though rare, the chromosomal cfr has also occurred in MRSA and *Staphylococcus sciuri* previously. However, to our best knowledge, it is the first report for the chromosomal cfr gene in clinical *S. capitis*.

In conclusion, our study systematically exhibited an 11-year single LRSC clone dissemination in our hospital with mutations in the 23S rRNA and cfr gene carriage, both of which mediated the high-level resistance to linezolid. Although during the spread of these strains, cfr-carrying plasmids were prone to possible loss and it also occasionally integrated into the chromosome, the horizontal transferability of cfr-carrying plasmids to *S. aureus* with a low fitness cost was still worrisome. Thus, judicious use of linezolid and implementing
and maintaining effective measures to prevent and control the spread of this resistance mechanism are needed.

Limitations of the study
This work presented two limitations. In our study, most of the LRSC strains were isolated from ICU patients (91/102), which were related to the higher exposure rate to linezolid as previously described; however, there is a bias that the isolating rates of this LRSC clone in other wards were potentially underestimated due to lack of routine antimicrobial susceptibility test when they were recovered from most of the samples as normal bacterial colonization. This neglected the existence of the LRSC clone in the circulation environment of the hospital and increased the potential risk for bloodstream infections and other severe infections for LRSC-colonized patients once admitted to the ICUs for invasive treatment. Another limitation was that this study was in a single center; thus, our data were unable to represent the scenarios in other healthcare settings, and this could be another case for further study.

STAR METHODS
Detailed methods are provided in the online version of this paper and include the following:

- KEY RESOURCES TABLE
- RESOURCE AVAILABILITY
  - Lead contact
  - Materials availability
  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
  - Ethics statements
- METHOD DETAILS
  - Bacterial strains
  - Antimicrobial susceptibility testing (AST)
  - Whole-genome sequencing (WGS) and genome analysis
  - The detection of 23S rRNA mutations
  - The transferability of cfr-carrying plasmids
  - The measurement of growth curves
  - Competition assays
  - Stability of cfr-carrying plasmids
- QUANTIFICATION AND STATISTICAL ANALYSIS

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.isci.2022.105644.

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AUTHOR CONTRIBUTIONS
J. Cai and R. Zhang conceived and designed the work. J. Cai provided samples and collected S. capitis isolates. W. Shen and J. Chen performed the whole-genome sequencing and sequence assembly. W. Shen and J. Cai analyzed the data and interpreted the results. W. Shen drafted the manuscript. All authors revised the manuscript and approved the final version.

DECLARATION OF INTERESTS
The authors declare no competing interests.
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## STAR METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|--------------------|--------|------------|
| **Bacterial and virus strains** |
| Staphylococcus capitis LR1 to LR102 | This study | N/A |
| Staphylococcus capitis LS1 to LS7 | This study | N/A |
| Staphylococcus aureus LRSA417 | (Cai et al. 11) | N/A |
| **Chemicals, peptides, and recombinant proteins** |
| Brain heart infusion agar | Oxoid | Cat# CM1135 |
| Brain heart infusion | Oxoid | Cat# CM1135 |
| TaKaRa Taq™ | TaKaRa | Cat# R001B |
| Yeast extract | Oxoid | Cat# LP0021 |
| Tryptone | Oxoid | Cat# LP0042 |
| Linezolid | Macklin | Cat# L830356 |
| Chloramphenicol | Sangon | Cat# A600118 |
| Rifampicin | Sangon | Cat# A600812 |
| **Critical commercial assays** |
| Hi pure bacterial DNA kit | Megan | Cat# D3146-03 |
| **Deposited data** |
| The complete sequences of the chromosome and plasmids of S. capitis LR95 | This paper | NCBI GenBank: CP092857, CP092857, and CP092859, respectively |
| The genome of S. capitis LR1 | This paper | NCBI GenBank: JAKTNG000000000 |
| The genome of S. capitis LR4 | This paper | NCBI GenBank: JAKTNF000000000 |
| The genome of S. capitis LR16 | This paper | NCBI GenBank: JAKTNE000000000 |
| The genome of S. capitis LR96 | This paper | NCBI GenBank: JAKTN000000000 |
| The genome of S. capitis LR102 | This paper | NCBI GenBank: JAKTN000000000 |
| The complete sequence of plasmid pLRSA417 | NCBI | GenBank: KJ922127.1 |
| The genome of S. capitis LNZR-1 | (Li et al. 25) | NCBI GenBank: JGYJ000000000.1 |
| The complete sequence of S. capitis XZ03 | (Jiang et al. 26) | NCBI GenBank: CP086659 |
| The genome of S. capitis 12-498 | (Ding et al. 22) | NCBI GenBank: JABBLZ000000000 |
| The genome of S. capitis 18-127 | (Ding et al. 22) | NCBI GenBank: JABBMO000000000 |
| The genome of neonatal sepsis-associated S. capitis clone (NRCS-A) | (Wirth et al. 28) | BioProject Accession number PRJNA493527 |
| **Oligonucleotides** |
| pLRSA417-pair1-forward: GCCTACAGTATTAAGAGGTGGG | This paper | Tsingke |
| pLRSA417-pair1-reverse: TGGAAGAAGCTAAAAAGGGAACACAACA | This paper | Tsingke |
| pLRSA417-pair2-forward: TCGCGAGCTTCTTCCAATACT | This paper | Tsingke |
| pLRSA417-pair2-reverse: TCTTATCAAGGTGTTCCTGC | This paper | Tsingke |
| **Software and Algorithms** |
| SPAdes v.3.13.1 | (Bankevich et al. 35) | https://github.com/ablab/spades |
| Unicycler v.0.4.4 | (Wick et al. 36) | https://github.com/rwick/Unicycler |

(Continued on next page)
RESOURCES AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jiachang Cai (caijiachang@zju.edu.cn).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- The complete sequences of the chromosome and plasmids from S. capitis LR95 have been deposited in GenBank under accession numbers CP092857, CP092857, and CP092859. The genomes of S. capitis LR1, S. capitis LR4, S. capitis LR16, S. capitis LR96, and S. capitis LR102 have also been deposited at NCBI GenBank under accession numbers JAKTG0000000000, JAKTNF0000000000, JAKTNE0000000000, JAKTND0000000000, and JAKTNC0000000000, respectively. Accession numbers are listed in the key resources table.

- This paper does not report original code.

- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics statements
This study was approved by the Ethics Committee of The Second Affiliated Hospital of Zhejiang University School of Medicine.

METHOD DETAILS

Bacterial strains
In a span of 11 years from 2011 to 2021, a total of 102 non-duplicate LRSC were obtained from the patients hospitalized in the neurology intensive care unit (NICU, n = 55), intensive care unit (ICU, n = 12), emergency intensive care unit (EICU, n = 8), surgical intensive care unit (SICU, n = 9), and other wards (n = 18) of the second Affiliated Hospital of Zhejiang University, which is a 3000-bed comprehensive tertiary care hospital in Hangzhou, China. The majority of these isolates were recovered from blood culture (92/102) while the others were isolated from diverse samples, including central vein pipe (5/102), cerebrospinal fluid (3/102), wound secretions (1/102), and pus (1/102) (Table S1). The species identification was performed by MALDI-TOF MS (Bruker Daltonik GmbH, Bremen, Germany). To investigate the relatedness between LRSC and linezolid-susceptible S. capitis (LSSC) in our hospital, we randomly selected seven LSSC strains derived from blood culture on ICUs for comparison and as controls for any phenotypes. Ten initial LRSC (strains LR1 to LR10) isolated from our hospital in 2011 were also included.

Antimicrobial susceptibility testing (AST)
The minimal inhibitory concentrations (MICs) of antimicrobial agents for 109 isolates were determined using the broth microdilution method according to the Clinical and Laboratory Standards Institute (CLSI) standard, with the following antibiotics being included: linezolid, chloramphenicol,
erythromycin, clindamycin, gentamicin, ciprofloxacin, penicillin G, oxacillin, tetracycline, vancomycin, and rifampicin. The susceptibilities of antimicrobial agents were interpreted according to CLSI recommendations.34

Whole-genome sequencing (WGS) and genome analysis
Genomic DNA extracted from S. capitis strains was subjected to WGS, which was carried out by using the Illumina NovaSeq 6000 platform, and the reads for each of the strains were de novo assembled into contigs by using the open-source assembler-pipeline SPAdes v.3.13.1.35 One of the strains, whose cfr-carrying plasmid could not be completely constructed according to the reference sequence on NCBI with Illumina short-read sequencing, was subjected to Oxford Nanopore PromethION 48 for further sequencing concurrently. Hybrid genome assembly of both the short and long reads was conducted with Unicycler v.0.4.4.36 The carriage of the antimicrobial resistance genes and the Inc-type of plasmids for the assembly scaffolds were identified with default settings by ResFinder 3.237 and PlasmidFinder 2.0,38 respectively at the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/). The Harvest suite was implemented for the multi-alignment of the core genomes of strains as well as single-nucleotide polymorphism (SNP) calling, and the maximum likelihood phylogeny was constructed using the parsnp39 script and subsequently visualized by an online tool iTOL (V3)40 for further annotation. The percentage of isolates a gene must be in to be considered as the core genome was set as 95%. The comparison of sequences of cfr-carrying plasmids was accomplished using the blastn program with that of pLRSA417 (GenBank accession no. KJ922127) as a reference, which was isolated from our hospital and reported in 2015. Based on that, a portion of contigs containing the segments for the target plasmid was partially determined and the putative gaps were filled by PCRs and Sanger sequencing to generate the closed-circle plasmids.

The detection of 23S rRNA mutations
PCR amplification and Sanger sequencing were performed to detect the 23S rRNA mutations. The genomic DNA of each strain was extracted by the Hi pure bacterial DNA kit (Megan) and used as templates in PCR amplification. Domain V of the 23S rRNA genes was amplified using conditions described by Kehrenberg & Schwarz7 and Toh et al.31 The similarity of the sequences was analyzed by Blasts program (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and the S. capitis strain AYP1020 was used as the reference strain.

The transferability of cfr-carrying plasmids
Conjugation experiments were performed to evaluate the transferability of cfr-carrying plasmids with filter mating methods. Induced rifampicin-resistant S. aureus RN4220 was used as the recipient strain while cfr-positive isolates were selected as donors. The putative transconjugants that grew on the brain heart infusion (BHI) agar plates supplemented with 10 mg/L florfenicol and 100 mg/L rifampicin were identified by MALDI-TOF MS and screened for the acquisition of the cfr gene by PCR. The conjugation frequency equaled the number of transconjugants divided by the number of recipients.

The measurement of growth curves
To access the growth performance and the fitness cost of S. aureus in vitro, we plotted the growth curves for both S. aureus and S. capitis in triplicate when they were cultured alone. For all the strains involved, a total of 100 μl from each of their growing BHI broth with an optical density at 600 nm (OD600) of 1 were inoculated to the subculture with 10 ml of fresh medium and then incubated with shaking (200 rpm) at 37°C. OD600 measurements were recorded at intervals of 1 h and finally plotted as a growth curve using the GraphPad Prism 7.0 software. The growth curves were estimated with the one-way analysis of variance followed by Tukey tests and a p < 0.05 was considered statistically significant.

Competition assays
The competition assays were carried out using four groups of competitors and tester strains, including S. aureus transconjugants against S. aureus RN4220, S. aureus LRSA417 against its filial strains without the cfr gene, cfr-positive LRSC against cfr-negative LRSC strains, as well as S. capitis LR95 against LRSC with plasmid-borne cfr. Overnight cultures for each strain were diluted to a 0.5 McFarland standard, then the competitor and tester strains were mixed at a 1:1 ratio in 10 ml BHI broth and incubated at 37°C. After 24 h of growth, a volume of 50 μl mixture was transferred to 10 ml fresh BHI broth for further incubation, and the serial passage was conducted three times. The concentrations of the two strains were determined by plating serial dilutions onto antibiotic-free LB plates as well as selective LB plates.
containing 6 mg/L and 30 mg/L chloramphenicol for *S. aureus* and LRSC, respectively. As for the competition culture of *S. capitis* LR95 and LR96, their concentrations were calculated by the number of colonies on the antibiotic-free LB plates, and the colonies were able to distinguish by morphology. The competition index\(^4\) was defined as the ratio of the competitor and tester cells in each competing culture and expressed as the mean of three independent biological repeats.

**Stability of cfr-carrying plasmids**

Plasmid stability was estimated by the frequency of plasmid-stable cells occurring during the overnight culture in non-selective conditions. Both LRSC stains (LR12, LR16, LR96, and LR102) and *S. aureus* strain LRSA417 harboring the same cfr-plasmid in our previous study\(^1\) were employed as the hosts. The host strains were individually incubated in the BHI broth of 5 ml for overnight culture at 37°C. Then an amount of 50 μl of the culture was transferred to 5 ml of fresh BHI broth, and the serial passage was conducted every 24 h for 15 days, which corresponds to approximately 300 generations. To access the plasmid stability, every culture of passage was diluted and plated onto antibiotic-free LB plates and LB plates with 6 mg/L chloramphenicol. The frequency of the stable plasmids was calculated as the ratio of the number of colonies grown on the LB plates containing chloramphenicol to the number of colonies on antibiotic-free LB plates.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Details of statistical analysis are provided within the relevant figure legends, their legends, and associated detailed methods. The growth curves were estimated using the one-way analysis of variance (ANOVA) followed by Tukey tests with GraphPad Prism 9.0 (GraphPad Software, Inc., USA). An overall error at a 0.05 level (95% confidence interval) was considered statistical significance. Data are represented in the figures as mean ± SD.