Bacitracin resistance and enhanced virulence of *Streptococcus suis* via a novel efflux pump

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**Abstract**

**Background:** *Streptococcus suis* is a prominent pathogen causing septicemia and meningitis in swine and humans. Bacitracin is used widely as a growth promoter in animal feed and to control the spread of necrotic enteritis in most developing countries. This study aimed to characterize a novel membrane transporter module Sst comprising SstE, SstF, and SstG for bacitracin resistance.

**Results:** Comparative genomics and protein homology analysis found a potential efflux pump SstFEG encoded upstream of well-known bacitracin-resistance genes bceAB and bceRS. A four-fold decrease in bacitracin susceptibility was observed in sstFEG deletion mutant comparing with S. suis wildtype strain CZ130302. Further studies indicated that the bacitracin tolerance mediated by SstFEG is not only independent of the BceAB transporter, but also regulated by the two-component system BceSR. Given that SstFEG are harbored by almost all virulent strains, but not in the avirulent strains, we managed to explore its potential role in bacterial pathogenicity. Indeed, our results showed that SstFEG is involved in S. suis colonization and virulence in animal infection model by its potential competitive survival advantage against host bactericidal effect.

**Conclusion:** To our knowledge, this is the first study to functionally characterize the bacitracin efflux pump in S. suis to provide evidence regarding the important roles of the novel ABC transporter system SstFEG with respect to drug resistance and virulence.

**Keywords:** Bacitracin, Virulence, Serotype Chz, *Streptococcus suis*, SstFEG, Efflux pump

**Background**

*Streptococcus suis* is an emerging zoonotic pathogen imposing a serious burden on the porcine industry as well as a severe public health concern [1, 2]. It is found extensively in porcine breeding environments, which are considered as a potential natural reservoir of resistance genes among several bacteria [3–5]. Bacitracin produced by *Bacillus licheniformis* is a type of narrow-spectrum peptide acting on Gram-positive bacteria [6]. Bacitracin was previously used as a growth-promoting supplement in animal feed [7]. Furthermore, bacitracin can effectively control necrotic enteritis [8], which is used as a preparation in numerous countries. Prolonged use of bacitracin in animals increases resistance genes in microorganisms. Some molecular mechanisms underlying bacitracin resistance in bacteria have been reported [9–11].

In one such pathway, the Bce system comprising a two-component system (TCS) BceSR and the membrane transporter BceAB to export bacitracin [12]. Similar pathways have been identified in *Bacillus subtilis* [6], *Streptococcus mutans* [13], *Enterococcus faecalis* [9], and *Clostridium perfringens* [14]. Furthermore, *Escherichia coli* and *Staphylococcus aureus* highly express undecaprenol kinase to convert undecaprenol pyrophosphate to undecaprenol phosphate. Some organisms produce exopolysaccharides, such as *Streptococcus mutans*, or membrane-bound cell-surface phospholipids, such as *Xanthomonas campestris* [15], which could bind bacitracin to remain extracellular. Bce systems are widely

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distributed in bacteria as a bacitracin efflux pump. The Bce system functions as an importer, as revealed through structural studies. In Bce systems, the RS regulon functions as the key bacitracin-resistance locus; the other module AB pumps out bacitracin via a transmembrane channel. The present study aimed to characterize a novel membrane ABC transporter module Sst comprising three components SstF, SstE, and SstG, and their role in the regulation of bacitracin resistance. We generated a series of mutant strains to compare their differences in bacitracin resistance with the wild-type strain through a drug sensitivity test and qRT-PCR analysis for bceRS, bceAB, and sstFEG genes. Mice challenge experiments testing bacterial colonization and survival rate were further performed to assess the pathogenicity of the Sst FEG modules in strain CZ130302.

Results
A potential efflux pump for drug-resistance encoded by sstFEG
Recently, we reported a highly virulent S. suis strain CZ130302 designated as the new serotype Chz causing acute meningitis in piglets. Unexpectedly, CZ130302 also displayed a more extensive drug resistance pattern than S. suis serotype 2 (SS2) reference strains P1/7 and HA9801 in the MIC test, including bacitracin (Table 1).

Whole genome retrieval of CZ130302 (CP024974.1) identified a gene cluster encoding the well-known bacitracin transporter BceAB and the related TCS BceSR (Fig. 1a). Further genetic annotation and prediction revealed a novel ABC transporter located at the upstream of bceAB, which was predicted as a potential efflux pump for drug-resistance. Thus, three genes, CVO91_06470, CVO91_06465, and CVO91_06460, were designated as sstFEG for further studies.

SstFEG mediates bacitracin tolerance in S. suis
Subsequently, a series of deletion mutants were constructed via homologous replacement with a spectinomycin (Spc) resistance expression cassette [16]. Construction of the mutant strains was confirmed via PCR (Additional file 1). The bacterial growth of CZ13-ΔsstFEG showed 1 h delay in log-phase compared with that of wild-type strain (Additional file 2), but no significant difference in the last stage. It should be noted that the bacitracin sensitivity of different mutant strains was increased at varying degrees in comparison with the wild-type strain (Fig. 2a). The mutant strain CZ13-ΔsstFEG (MIC, 16 μg/mL) was approximately 4-fold more sensitive to bacitracin than the wild-type CZ130302 strain (MIC, 64 μg/mL), concurrent with the colony growth of CZ13-ΔsstFEG on the culture media supplemented with bacitracin at different concentrations (Fig. 2b). Otherwise, CZ13-ΔbceAB and CZ13-ΔbceRS (MIC, 4 μg/mL) were more sensitive to bacitracin than CZ13-ΔsstFEG was (MIC, 16 μg/mL). Based on the MIC, bacterial growth inhibition at 8 h was compared upon bacitracin supplementation at high concentrations in the log-phase. Obviously, the mutant strain CZ13-ΔbceAB displayed the greatest reduction in bacterial survival at each time point (Fig. 2c). In summary, both BceAB and SstFEG transporters co-regulate bacitracin resistance in strain CZ130302, but their functional correlation in this process is unclear.

| Antibiotics   | MIC(μg/mL) | Antibiotics   | MIC(μg/mL) |
|--------------|-----------|--------------|-----------|
| Penicillin G | 16        | Tetracycline | 128       |
| Ampicillin   | 64        | Doxycycline  | 32        |
| Streptomycin | 256       | Erythromycin | 512       |
| Gentamicin   | 256       | Lincomycin   | 512       |
| Kanamycin    | >512      | Bacitracin   | 64        |
| Spectinomycin| 32        | Vancomycin   | 256       |
| Amikacin     | 128       | Nisin        | 256       |
| Neomycin     | >512      | Ciprofloxacin| 64        |
| Chloramphenicol| 4       | Norfloxacin  | 128       |

*The Streptococcus pneumoniae ATCC 49619 was used as control strain here
inhibited the expression of \textit{sstFEG} (Fig. 3c), suggesting that the deficiency of bacitracin resistance caused by \textit{bceAB} deletion may be related with BceAB inactivation and \textit{sstFEG} downregulation together. These observations, together with the results shown in Fig. 2, further implied that SstFEG is an efflux pump for bacitracin and can transport bacitracin independently.

\textbf{BceSR regulates SstFEG in response to bacitracin resistance}

Here, we explored why the deletion of \textit{bceAB} inhibited the expression of \textit{sstFEG} (Fig. 3c), and found that \textit{bceRS} is downregulated by approximately 50\% in the \textit{\triangle bceAB} mutant. As revealed from the KEGG Pathway Database (Additional file 3) and previous studies [11], BceAB transporter serves as an efflux pump along with the BceSR TCS to regulate bacitracin perception. Thus, it is reasonable to speculate that BceSR also is the important regulator of \textit{sstFEG} operon. Indeed, the deletion of \textit{bceRS} caused significant downregulation of \textit{sstFEG} and \textit{bceAB} more than 50-fold (Fig. 3d), which almost lost the transcriptional activation of these genes in response to bacitracin stress under the 2 ng/\mu L concentration, indicating that BceSR is the major regulator for bacitracin.
resistance in this case. These results suggested that the downregulation of \textit{bceRS} caused by the deletion of \textit{bceAB} may be the primary reason of \textit{sstFEG} transcriptional inhibition, and \textit{sstFEG} regulation by BceSR.

S. suis colonization and virulence requires bacitracin tolerance

The prevalence of \textit{sstFEG}, \textit{bceAB}, and \textit{bceSR} genes in 35 whole genomes of \textit{S. suis} from NCBI database were investigated, which showed that all strains encode \textit{bceAB} and \textit{bceRS} genes, but only 19 strains (18 highly virulent or virulent strains except for T15) encode \textit{sstFEG} genes, and almost all avirulent strains are \textit{sstFEG} negative (Table 2). In particular, serotype 3 strain ST3, isolated from a pig with pneumonia in Hubei province in 2009 [43], only harbors \textit{sstF}, but not \textit{sstE} and \textit{sstG}. These results suggested that SstFEG may be involved in the pathogenicity of \textit{S. suis}.

The colonization of \textit{S. suis} requires to antagonize mucosal microflora and host immune clearance. Many bacterial species from mucosal microflora can secrete bacitracin to achieve competitive advantage for optimal survival. Furthermore, bacitracin was previously used as a growth-promoting supplement in animal feed. Here, we managed to test whether SstFEG efflux pump is required for \textit{S. suis} original colonization. Indeed, the inactivation of SstFEG

| Antibiotic | \text{MIC (\text{\mu}g/mL)} | \text{CZ130302} | \text{CZ13-\text{\Delta}sstFEG} | \text{CZ13-\text{\Delta}bceAB} | \text{CZ13-\text{\Delta}bceRS} |
|------------|-----------------|---------------|-----------------|-----------------|-----------------|
| Bacitracin  | 64              | 16            | 4               | 4               |

Fig. 2 Bacitracin sensitivity of the mutant strains. \textbf{a} Comparison of MICs to bacitracin between wild-type and mutant strains. \textbf{b} The bacterial colony growth in THB plate with bacitracin resistance. Bacterial cells in the logarithmic phase were subjected to serial dilution and 10 \muL of bacterial cultures were in THB plates supplemented with bacitracin at different concentrations (0, 2, 4, 8, 16, 32, and 64 \muL/mL). \textbf{c} The bacterial survival of wild-type and mutant strains incubated with high bacitracin concentration. The bacterial cells were eliminated with 128 \muL/mL bacitracin. We monitored and recorded the bacterial loads incubated after 8 h with 128 \muL/mL bacitracin in the logarithmic phase. The CFU values of all mutants was reduced to a greater extent in comparison with the wild-type strain at each hour.
resulted in a 2- to 3-fold reduction in the bacterial loads compared to the wild-type strain in blood after intraperitoneal injection for 10 h (Fig. 4a), indicating that SstFEG mediating bacitracin tolerance are essential for S. suis to rapidly infect the host. As shown in Fig. 4b, mice infected with the wild-type strain CZ130302 had severe clinical symptoms and displayed 100% mortality on the fifth day after challenge. In contrast, the group infected with the mutant CZ13-ΔsstFEG strain displayed a 50% greater survival rate (5/11).

**Discussion**

Thus far, the underlying mechanism of bacitracin resistance has been reported in B. subtilis, S. mutans, and S. pneumoniae, but not in S. suis. The present study shows that the network of bacitracin resistance in Streptococcus partially comprises TCS and ABC transporters, as revealed from the KEGG Pathway Database (Additional file 3). BceSR (TCS) and BceAB (ABC transporter) are ubiquitous and significantly influence the bacitracin resistance of Streptococcus spp. In this study, we identified a novel ABC transport module (CVO91_06470, 06465, and 06460) mediating bacitracin resistance and virulence in S. suis. The CVO91_06470 gene encodes an ABC transporter ATP-binding protein that shares approximately 42% protein homology with the BcrA system, contributing to bacitracin resistance in B. subtilis.

Interestingly, these three genes (designated as sstFEG) are coincidentally located upstream of the four genes encoding well-known BceAB and BceSR homologs (Fig. 1a). Bacitracin sensing depends on the BceRS signal transduction system and the BceAB transporter as a co-sensor. Jing Ouyang et al [12] reported that phosphorylated BceR upregulates the positively regulated expression of the bceABRS operon. In this study, we proposed that BceAB, BceRS, and SstFEG may constitute a seven-component system to regulate the bacitracin efflux optimally. Our results indicated that the bacitracin tolerance mediated by SstFEG is not only independent of the BceAB transporter, but also regulated by the TCS BceSR. Furthermore, sstFEG and bceAB are controlled by different operons, but the deletion of bceAB caused significant downregulation of bceRS, which decreased the bacitracin resistance mediated by SstFEG by inhibiting the transcriptional activation. In contrast, the deletion of sstFEG could not regulate the BceAB and BceSR systems for sensing bacitracin. A model for this regulatory pathway of bacitracin resistance in S. suis is proposed (Fig. 5).
Except for the bacitracin resistance, antibiotic susceptibility testing indicated that strain CZ130302 exhibits a more extensive drug resistance pattern than that of other S. suis strains. Comparative genomics studies have reported that the genomes of newly isolated CZ130302 harbor at least 45 ISs, including the novel 82-kb integrative conjugative element ICE

\[ \text{Ssu} \]

CZ130302 [17], which may increase the potential horizontal gene transfer from different bacterial species or genera. Further studies need to explore how and why strain CZ130302 acquired numerous exogenous antibiotic resistance genes and integrated into a chromosome, especially for newly identified SstFEG efflux pump.

Given that the SstFEG never been reported in any other opportunistic or commensal bacteria species, we speculated that its encoding genes may be transferred from pathogenic bacterial strains. To further examine the correlation between the virulence of S. suis strains and SstFEG, genomic data of 34 S. suis strains from GenBank were investigated. Consequently, SstFEG are harbored by

### Table 2 The distribution of seven bacitracin resistance genes in Streptococcus suis

| Strains   | Serotype\(^a\) | Virulence | host | Symptoms | BceB/A | BceS/R | sstFEG | Reference |
|-----------|----------------|-----------|------|----------|--------|--------|--------|-----------|
| CZ130302  | Chz            | Highly virulent | piglet | Meningitis | +      | +      | +      | [18]      |
| BM407     | 2              | Highly virulent | human | STSS     | +      | +      | +      | [19]      |
| 05ZYH33   | 2              | Highly virulent | human | STSS     | +      | +      | +      | [20]      |
| 98HAH33   | 2              | Highly virulent | human | STSS     | +      | +      | +      | [20]      |
| G21       | 2              | Highly virulent | human | STSS     | +      | +      | +      | [21]      |
| SC84      | 2              | Highly virulent | human | STSS     | +      | +      | +      | [19]      |
| SC19      | 2              | Highly virulent | piglet | Meningitis | +      | +      | +      | [22]      |
| 05ZY719   | 2              | Highly virulent | piglet | Septicemia | +      | +      | +      | [23]      |
| SC070731  | 2              | Highly virulent | piglet | Meningitis | +      | +      | +      | [24]      |
| P1/7      | 2              | Highly virulent | pig | Septicemia | +      | +      | +      | [25]      |
| S735      | 2              | Highly virulent | pig | Septicemia | +      | +      | +      | [26]      |
| A7        | 2              | Highly virulent | pig | Septicemia | +      | +      | +      | [27]      |
| CS100322  | 2              | Virulent      | pig | lung | +      | +      | +      | [28]      |
| SS2–1     | 2              | Virulent      | pig | diseased | +      | +      | +      | [29]      |
| T15       | 2              | Avirulent     | pig | Septicemia | +      | +      | +      | [30]      |
| SS12      | 1/2            | Virulent      | pig | lung | +      | +      | +      | [27]      |
| JS14      | 14             | Highly virulent | pig | lung | +      | +      | +      | [31]      |
| G20565    | 9              | Highly virulent | pig | Septicemia | +      | +      | +      | [31]      |
| LSM102    | –              | Highly virulent | pig | Septicemia | +      | +      | +      | [32]      |
| ST3       | 3              | Virulent      | pig | Septicemia | +      | +      | +/−    | [21]      |
| AH681     | Chz            | Avirulent     | pig | Healthy | +      | +      | −      | [18]      |
| HNI36     | Chz            | Avirulent     | pig | Healthy | +      | +      | −      | [18]      |
| ST1       | 1              | Avirulent     | pig | Healthy | +      | +      | −      | [27]      |
| HA0609    | 2              | Avirulent     | pig | Healthy | +      | +      | −      | [28]      |
| NSUI002   | 2              | Avirulent     | pig | Healthy | +      | +      | −      | [33]      |
| OSHA568   | 2              | Avirulent     | pig | Healthy | +      | +      | −      | [34]      |
| NSUI060   | 2              | Avirulent     | pig | Healthy | +      | +      | −      | [35]      |
| YB51      | 3              | Avirulent     | pig | Healthy | +      | +      | −      | [36]      |
| 6407      | 4              | Avirulent     | pig | Healthy | +      | +      | −      | [37]      |
| D9        | 7              | Avirulent     | pig | Healthy | +      | +      | −      | [27]      |
| D12       | 9              | Avirulent     | pig | Healthy | +      | +      | −      | [38]      |
| TL13      | 16             | Avirulent     | pig | Healthy | +      | +      | −      | [39]      |
| LS9N      | –              | Avirulent     | + | Healthy | +      | +      | −      | [40]      |
| 90–1330   | –              | Avirulent     | + | Healthy | +      | +      | −      | [41]      |
| DN13      | 9              | Avirulent     | pig | Healthy | +      | +      | −      | [42]      |
almost all virulent strains isolated from the patients and diseased pigs, but not in the avirulent strains isolated from healthy pigs. To further explore the potential roles of SstFEG in S. suis virulence, BALB/c mouse infection model was employed for challenge tests [44]. Indeed, the pathogenicity of the mutant strain deleted sstFEG was significantly decreased.

Conclusions
This study reports a novel membrane transporter module SstFEG, which functions as not only an efflux pump for bacitracin resistance, but also a virulence-related protein involved in S. suis pathogenicity.

Methods
Bacterial strains, plasmids, and culture conditions
S. suis strain CZ130302 from the novel serotype Chz was isolated from a diseased piglet in Changzhou, China [18, 45]. Plasmid pSET-4S with Spc resistance gene was generously provided by Professor Daisuke Takamatsu. Plasmid pUC19 was maintained in the OIE Reference Laboratory for Swine Streptococcosis. S. suis were cultured in Todd Hewitt Broth (THB, BD) or agar comprising 6% (v/v) sheep blood at 37 °C and 5% CO₂. E. coli strains were cultured in Luria-Bertani (LB, BD) medium at 37 °C supplemented with 100 μg/mL Spc (Sigma) per requirement for S. suis and 50 μg/mL Spc or 100 μg/mL ampicillin (Amp, Sigma) for E. coli. Different types of antibiotics, especially bacitracin (Bac, Sigma), were used to determine the minimum inhibitory concentration. In total, 100 μg/mL lysozyme (Lzm, Sigma) and 1 μg/mL vancomycin (Van, Sigma) were used for phagocytosis assays.

Antimicrobial susceptibility assays
In accordance with the standardized methods per the Clinical and Laboratory Standards Institute (CLSI) guidelines (2015), the minimum inhibitory concentrations (MICs) of different types of antibiotics, including beta-lactams, aminoglycosides, tetracyclines, amide alcohols, macrolides, lincosamides, polypeptides, and fluoroquinolone against S. suis CZ130302 were determined.
brief, the strains were diluted 1000-fold into Cation Adjusted Muller Hinton Broth (CAMHB) with lysed horse serum (2.5% v/v) and cultured to an optical density at 600 nm (OD 600) of approximately 0.5; thereafter, 180 μL of the culture was inoculated into the first vertical well, while 100 μL was inoculated in the other wells. Different initial antibiotic concentrations in 20 μL were placed in the first well and mixed, and 100 μL of the mixture from the first well was transferred to the subsequent well. This was repeated and as an analogy until the last well. Subsequently, cultures were incubated at 37 °C and 5% CO2 for 20 h. CZ130302 was tested through a serial dilution from 10^{-1} CFU to 10^{-7} CFU, and the THB plates with a 2-fold dilution of bacitracin, initiating at 64 μg/mL. All experiments were performed in triplicate. The Streptococcus pneumoniae strain ATCC 49619 was used as a control to ensure the reliability of tested data.

Prediction and distribution of bacitracin-resistant genes in Streptococcus suis

The complete genome sequence of S. suis strain CZ130302 was obtained from NCBI (https://www.ncbi.nlm.nih.gov/, GenBank: CP024974.1). Antibiotic Resistance Genes Database (ARDB, http://ardb.cbcb.umd.edu/) was used to predict resistance genes in the genome of S. suis. KEGG PATHWAY Database (http://www.genome.jp/kegg/pathway.html) was used to extract previously reports pathways underlying bacterial bacitracin resistance and the distribution of resistance genes in S. suis exhibited in the NCBI database. In addition, Rockhopper software was used to predict the operon, transcription start site (TSS), and transcription termination sites (TTS) of CZ130302 in accordance with the distribution of reads in the genome.

Construction of gene deletion mutants

To investigate the contribution of predictable genes, a series of deletion mutants was constructed via natural DNA transformation instead of the traditional method with pSET-4 s because this method was not suitable for S. suis CZ130302. Primers used to construct and confirm the mutants are enlisted in Additional file 4. The construction strategy for sstFEG deletion mutant strain is shown in Fig. 1. In brief, fragment AB (574 bp) and CD (867 bp) were amplified from S. suis CZ130302, using primers sst-A (BamHI site at its 5′ end) and sst-C, and sst-D (Sal I site at its 3′ end) as upstream and downstream of sstFEG, respectively. The spp sequence (1133 bp) with the promoter was amplified from plasmid pSET-4 s by primers Spc-F and Spc-R. These three fragments were fused via PCR and ligated with pUC19 to form the recombinant plasmid pUC19-AB-Spc-CD. The CZ13-ΔsstFEG mutant was obtained via homologous recombination and resistance screening. Other mutant strains including CZ13-ΔbceAB and CZ13-ΔbceRS were constructed as previously described.

Anti-pressure analysis of bacitracin

sstFEG mutant strains were cultured up to an optical density of approximately 0.7 at 600 nm (OD_{600}). Thereafter, bacitracin was added at a high concentration. All bacteria were cultured for 8 h at 37 °C and 5% CO2.
continuously, followed by plating of serial ten-fold dilutions on THB agar to enumerate bacteria at each hour. The trial was performed in triplicate, and the results were determined using the following formula: CFUs of viable bacteria at each hour – CFUs of original bacteria.

qRT-PCR analysis
To analyze the expression levels of related genes and their interactions, total RNA was isolated from CZ130302 and mutant strains upon culturing to an optical density at 600 nm (OD600) of 0.6 in THB broth, with or without 2 μg/mL bacitracin, using RNAiso Plus (Takara, Japan) in accordance with the manufacturer’s instructions. When bacterial strains were cultured with bacitracin, the *Streptococcus pneumoniae* strain ATCC 49619 was used as control. The resulting RNA was then treated with gDNA Eraser to remove genomic DNA and further converted to cDNA via reverse transcription (RT), using PrimeScript™RT reagent Kit (Takara). cDNA samples were synthesized using RNA species harvested from three independent cultures of each strain. Real-time quantitative PCR assays were performed in triplicate with the method of SYBR Green detection. Primers for qRT-PCR analysis are enlisted in Additional file 4. The relative amount of target gene mRNA was normalized to the housekeeping gene *parC* [46]. The relative fold change was calculated by the threshold cycle \((2^{-\Delta\Delta Ct})\) method [47]. Each assay was performed in duplicate in three independent experiments.

Animal experiments
A competitive test was performed to compare the colonization ability between wild-type and mutant strains in mice. Prior to this experiment, all mice were fed with bacitracin in water to activate bacterial genes. Eighteen female 6-week-old BALB/c mice were equally segregated into three groups. The wild-type and mutant strains were cultured until the OD600 was approximately 0.8, and the density of each strain was adjusted to 1.3 × 10⁸ CFU in PBS. Thereafter, the wild-type and different mutant strains were respectively mixed in a 1:1 ratio and challenged intraperitoneally with 200 μL/mouse. After 10 h, blood was sampled from each mouse, followed by plating serial five-fold dilutions on THB agar, containing 50 μg/mL kanamycin with or without 100 μg/mL Spc to distinguish and enumerate bacteria. In addition, the virulence of mutant CZ13-△Sst FEG strain was assessed in the BALB/c mouse model of infection. Thirty-three female 6-week-old BALB/c mice were equally segregated into three groups and challenged intraperitoneally with 200 μL/mouse at approximately 2.6 × 10⁷ CFU (10 × LD₅₀) in PBS. Mice infected with the vehicle (PBS) were used as controls. Mortality was monitored every 8 h for 7 d. All experiments were performed in triplicate. All animals used in this study were humanely euthanized by carbon dioxide asphyxiation in an airtight box.

Statistical analyses
GraphPad Prism version 5 is used to analyze and plot the data. Student’s t-test (unpaired) was performed to determine differences between the means of the two samples. Differences with a \(P\) value of < 0.05 were considered significant, and a \(P\)-value of < 0.01 was considered greatly significant.

Ethics statement
Six-week-old female germfree BALB/c mice were purchased from the Comparative Medicine Center of Yangzhou University. Animal experiments were carried out in the Laboratory Animal Center of Nanjing Agricultural University and approved by Laboratory Animal Monitoring Committee of Jiangsu Province, China [Permit number: SYXK (SU) 2017-0007].

Supplementary information
Supplementary information accompanies this paper at https://doi.org/10.1186/s12917-019-2115-2.

### Additional file 1.
Identification of mutant strains via PCR.

### Additional file 2.
Comparison of growth curves between wild-type CZ130302 and mutant strain CZ13-△sstFEG.

### Additional file 3.
Genes involved in bacitracin transport.

### Additional file 4.
Primers used in this study.

### Abbreviations
CLSI: Clinical and Laboratory Standards Institute; LB: Luria-Bertani; MICs: The minimum inhibitory concentrations; SS: *Streptococcus suis*; TCS: Two-component system; THB: Todd Hewitt Broth; TSS: Transcription start site

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### Authors’ contributions
ZP conceived and designed the experiments. JM, JL, YZ and DW performed the experiments. GL contributed materials and analysis tools. JM, HY and ZP analyzed the data. JM and JL drafted the manuscript. RL, GL and ZP revised the manuscript. ZP coordinated the study. All authors have read and approved the final manuscript version.

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### Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.

### Ethics approval and consent to participate
All animal experiments in this study were carried out in the Laboratory Animal Center of Nanjing Agricultural University and approved by Laboratory Animal Monitoring Committee of Jiangsu Province, China [Permitted number: SYXK (SU) 2017-0007]. All efforts were made to minimize suffering.
Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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References
1. Palmieri C, Varaldo PE, Facinelli B. Streptococcus suis, an emerging drug-resistant animal and human pathogen. Front Microbiol. 2011;2:235.
2. Wertheim HF, Nghia HD, Taylor W, Schultsz C. Streptococcus suis: an emerging human pathogen. Clin Infect Dis. 2009;48(3):154–25.
3. Huang J, Shang K, Hu X, Liang Y, Li D, Wu Z, Chen L, Wang L. Genetic diversity of Streptococcus suis isolated from three pig farms of China obtained by acquiring antibiotic resistance genes. J Sci Food Agric. 2015;95(7):1454–60.
4. Huang J, Ma J, Shang K, Hu X, Liang Y, Li D, Wu Z, Dai L, Chen L, Wang L. Evolution and diversity of the antimicrobial resistance associated Mobiliome in Streptococcus suis: a probable Mobile genetic elements reservoir for other streptococci. Front Cell Infect Microbiol. 2016;6:118.
5. Bernard R, El Ghachi M, Mengin-Lecreulx D, Chippaux M, Denizot F. BcrC from Bacillus subtilis acts as an undecaprenyl pyrophosphate phosphate in bacitracin resistance. J Biol Chem. 2005;280(32):28852–7.
6. Eppelmann K, Doekel S, Marahiel MA. Engineered biosynthesis of the peptide antibiotic bacitracin in the surrogate host Bacillus subtilis. J Biol Chem. 2001;276(37):34824–31.
7. Wei S, Gutek A, Ulbrich M, Yu Z. Abundance of pathogens in the gut and litter of broiler chickens as affected by bacitracin and litter management. Vet Microbiol. 2013;166(4–5):595–601.
8. Collinder E, Cardona ME, Berge GN, Norin E, Stern S, Midveld T. Influence of zinc bacitracin and Bacillus licheniformis on microbial intestinal functions in weaned piglets. Vet Res Commun. 2002;27(7):513–26.
9. Marson JM, Keis S, Smith JM, Cook GM. Acquired bacitracin resistance in enterococcus faecalis is mediated by an ABC transporter and a novel regulatory protein, BcrT. Antimicrob Agents Chemother. 2004;48(10):3743–8.
10. Ohki R, Gyanto TK, Masuya W, Moriya S, Kobayashi K, Ogasawara N. The BceRS two-component regulatory system induces expression of the bacitracin transporter, BceAB, in Bacillus subtilis. Mol Microbiol. 2003;49(4):1135–45.
11. Radcek J, Gebhard S, Orchard PS, Kirchner M, Bauer S, Mascher T, Fritz G. Anatomy of the bacitracin resistance network in Bacillus subtilis. Mol Microbiol. 2016;100(6):607–20.
12. Ouyang J, Tian XL, Versey J, Wishart A, Li YH. The BceABRS four-component system regulates the bacitracin-induced cell envelope stress response in Streptococcus mutans. Antimicrob Agents Chemother. 2010;54(9):3895–906.
13. Tsuda H, Yamashita Y, Shibata Y, Nakano Y, Koga T. Genes involved in bacitracin resistance in Streptococcus mutans. Antimicrob Agents Chemother. 2002;46(12):3756–62.
14. Charlebois A, Jaillert LA, Harel J, Masson L, Archambault M. Characterization of genes encoding for acquired bacitracin resistance in Clostridium perfringens. PLoS One. 2012;7(10):e44449.
15. Rodrigues J, Casseti L. Incorporation of bacitracin in Langmuir films of phospholipids at the air-water interface. Thin Solid Films. 2017;622:95–103.
16. Zhu Y, Dong W, Ma J, Zhang Y, Pan Z, Yao H. Utilization of the ComRS system for the rapid markerless deletion of chromosomal genes in Streptococcus suis. Future Microbiol. 2019;14:207–22.
17. Pan Z, Liu J, Zhang Y, Chen S, Ma J, Dong W, Wu Z, Yao H. A novel integrative conjugative element mediates transfer of multi-drug resistance between Streptococcus suis strains of different serotypes. Vet Microbiol. 2019;229:110–6.
18. Pan Z, Ma J, Dong W, Song W, Wang K, Lu C, Yao H. Novel variant serotype of streptococcus suis isolated from piglets with meningitis. Appl Environ Microbiol. 2015;81(5):976–85.
42. Zheng H, Du P, Qiu X, Kerdsin A, Roy D, Bai X, Xu J, Vela AI, Gottschalk M. Genomic comparisons of Streptococcus suis serotype 9 strains recovered from diseased pigs in Spain and Canada. Vet Res. 2018;49(1):1.

43. Hu P, Yang M, Zhang A, Wu J, Chen B, Hua Y, Yu J, Chen H, Xiao J, Jin M. Complete genome sequence of Streptococcus suis serotype 3 strain ST3. J Bacteriol. 2011;193(13):3428–9.

44. Zhang Y, Lu P, Pan Z, Zhu Y, Ma J, Zhong X, Dong W, Lu C, Yao H. SspP1, a Streptococcus suis Fimbria-Like Protein Transported by the SecY2/A2 System, Contributes to Bacterial Virulence. Appl Environ Microbiol. 2018; 84(18)x0:1385-18.

45. Pan Z, Ma Y, Ma J, Dong W, Yao H. Acute meningitis of piglets and mice caused by co-infected with Streptococcus suis and Aerococcus viridans. Microb Pathog. 2017;106:60–4.

46. Wu Z, Wu C, Shao J, Zhu Z, Wang W, Zhang W, Tang M, Pei N, Fan H, Li J, et al. The Streptococcus suis transcriptional landscape reveals adaptation mechanisms in pig blood and cerebrospinal fluid. Rna. 2014;20(6):882–98.

47. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2−(ΔΔCT) method. Methods. 2001;25(4):402–8.

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