The Production of Extracellular Proteins Is Regulated by Ribonuclease III via Two Different Pathways in Staphylococcus aureus

Yu Liu, Jie Dong, Na Wu, Yaping Gao, Xin Zhang, Chunhua Mu, Ningsheng Shao, Ming Fan, Guang Yang
Beijing Institute of Basic Medical Sciences, Beijing, People’s Republic of China

Abstract

Staphylococcus aureus ribonuclease III belongs to the enzyme family known to degrade double-stranded RNAs. It has previously been reported that RNase III cannot influence cell growth but regulates virulence gene expression in S. aureus. Here we constructed an RNase III inactivation mutant (Δrnc) from S. aureus 8325-4. It was found that the extracellular proteins of Δrnc were decreased. Furthermore, we explored how RNase III regulated the production of the extracellular proteins in S. aureus. We found during the lag phase of the bacterial growth cycle RNase III could influence the extracellular protein secretion via regulating the expression of secY2, one component of accessory secretory (sec) pathway. After S. aureus cells grew to exponential phase, RNase III can regulate the expression of extracellular proteins by affecting the level of RNAIII. Further investigation showed that the mRNA stability of secY2 and RNAIII was affected by RNase III. Our results suggest that RNase III could regulate the pathogenicity of S. aureus by influencing the level of extracellular proteins via two different ways respectively at different growth phases.

Introduction

Ribonuclease III (RNase III) is a double-stranded endoribonuclease, which has been classified into three main groups on the basis of their domain organization [1]. Bacterial RNase III belongs to Group I family, which contains only one characteristic ribonuclease domain and one dsRNA-binding domain (dsRBD) [1,2].

RNase III has been thought to be important in Escherichia coli (E. coli) because it is involved in the process of both 16s and 23s rRNAs from a 30s precursor [3]. Further, it is found that RNase III has an additional function to degrade mRNA with the mediation of trans-acting antisense RNA in E. coli [4,5]. Although Staphylococcus aureus (S. aureus) RNase III seems to play a minor role in the formation of 30S rRNA [6], it is reported that RNase III can induce mRNA degradation mediated by RNAIII, which is an important regulator of the quorum sensing system (agr) [7,8]. It is reported that RNAIII generally acts by an antisense base pairing mechanism [7,8], and regulates many target genes via its control of a repressor protein gene called not, a member of the sarA family of transcriptional regulators [8,9,10]. RNase III can degrade the target mRNAs of RNAIII but not hydrolyze RNAIII [7,8]. It suggests that RNase III should be essential for virulence gene regulation in S. aureus. But the biological function of RNase III of S. aureus is still unclear.

The secreted proteins play an important role for the pathogenicity of S. aureus [11]. The majority of exported proteins are transported from the cytoplasm via the general secretory (sec) pathway including secA/Y/E/G in gram positive bacteria [12]. In addition, S. aureus contains an accessory Sec pathway involving the SecA2 and SecY2 proteins [12,13,14]. In contrast with the general sec pathway, SecY2 and SecA2 are not involved in the viability of S. aureus [12,14]. In some pathogenic gram-positive bacteria, SecY2 is required for the transport of certain proteins related to virulence [12,15,16,17]. However, SecY2-related secretomes have yet to be studied extensively [14].

We hereby tried to investigate the biological function of RNase III in S. aureus by constructing an RNase III inactivation mutant (Δrnc). Compared with its parent strain, both the extracellular proteins and the pathogenicity of Δrnc were reduced. In this report, we show that RNase III could influence the production of extracellular proteins of S. aureus by separately regulating the expression level of secY2 and RNAIII via respective mechanisms at the different phases.

Results

Inactivation of RNase III did not influence the growth of S. aureus

We constructed an RNase III inactivation strain (Δrnc) in 8325-4 with allelic homologous recombination. Then the mutant was
verified by RT-PCR (figure 1A) using the primers (rncV1 and rncV2) as listed in the Table 1. To further observe the phenotype of Dmc, the growth curves of Dmc and its parent strain were measured. The result showed that there were no obvious differences observed between the wild type and mutation strains (figure 1B). In previous reports, RNase III could degrade the target mRNAs (spa) of RNAIII [7], so we tested the mRNA level of spa by real-time quantitative PCR. Compared with its parent strain, the level of spa significantly increased in Dmc (figure 1C).

The extracellular proteins in the supernatant of Dmc decreased significantly

The extracellular proteins play an important role for the pathogenicity of S. aureus [11]. We compared the profiles of the extracellular proteins from the same number of cells between Dmc and its parent strain at the different growth phases. According to the growth curve, S. aureus cultured for 1.5 h, 6 h and 12 h is at the lag phase, exponential phase and stationary phase respectively. The proteins in the supernatant at different time points (1.5 h, 6 h, and 12 h) were extracted as described in Material and methods and the profile of the extracellular proteins in the supernatant was determined by SDS-PAGE. It was surprising that the extracellular proteins of Dmc decreased significantly compared with its parent strain at three time points (figure 2). At the same time, we compared the total proteins of whole-cell between Dmc and its parent strain. However, we did not find obvious changes in the total proteins (figure 2).

A lower level of RNAIII in Dmc led to reduction of extracellular proteins at 6 h and 12 h

As RNAIII is a positive regulator of extracellular virulence [18] and RNase III can mediate the interaction between RNAIII and its target mRNAs [7,8], we checked the level of RNAIII in Dmc by Northern blot. Compared with its parent strain, the expression of RNAIII in Dmc decreased at 6 h and 12 h (figure 3A). In order to avoid the unintended mutation in agr system during we constructed the Dmc, we analyzed the sequence of agrA and agrC of Dmc. No mutated nucleotide was observed in the genome of Dmc strain (data not shown).

In the further study, we wondered whether the decrease of the extracellular proteins was due to the reduction of the RNAIII in Dmc. The profile of the extracellular proteins of the RNAIII (RNAIII deletion mutant) and its parent strain was tested at different time points. It was found that the extracellular proteins of RNAIII decreased when compared with its parent strain at 6 h and 12 h (figure 3B). In the further investigation, the plasmid of pOS1-RNAIII was constructed and transferred into Dmc to generate the strain of RNAIIIr, in which the RNAIII level was recovered. It was found that the extracellular proteins increased after the RNAIII level was recovered in Dmc at 6 h and 12 h (figure 3C). The RNase III inactivated mutant was also constructed from RNAIIIr, named as Dmc/RNAIII. The profile of the extracellular proteins from Dmc/RNAIII was the same as that from RNAIII (figure 3C). Meanwhile, the levels of RNAIII and spa mRNA in the different strains were detected by RT-PCR (figure 3C). This results suggested that the

Figure 1. Detection of RNase III inactive mutant. A: Verification of RNase III inactive mutant by RT-PCR. Total RNA of cells was extract and used as the template to amplify the rnc gene. In Dmc strain, the rnc mRNA could not be detected like WT and mcr because the kana gene was inserted into the rnc gene of Dmc genome. 16s rRNA was used as the internal control. WT (wild type, S. aureus 8325-4), Dmc (an RNase III inactivation mutant from 8325-4) and mcr (the restoration of RNase III activity in Dmc). B: The growth curves of S. aureus strains. There is no significant difference between WT and Dmc. WT: wild type, S. aureus 8325-4; Dmc: an RNase III inactivation mutant from 8325-4. The experiment has been repeated for three times. C: qRT-PCR quantification of the expression level of spa. The total RNA of the cells cultured for 6 h was extracted and the mRNA level of spa was detected by qRT-PCR. In the Dmc strain, the level of spa mRNA was significantly increased compared with WT. WT: wild type, S. aureus 8325-4; Dmc: an RNase III inactivation mutant from 8325-4; mcr: the restoration of RNase III activity in Dmc. (**: P<0.01). doi:10.1371/journal.pone.0020554.g001
lower level of RNAIII in Drnc was responsible for the reduction of extracellular protein at 6 h and 12 h.

The secretion of the proteins in Drnc was inhibited at 1.5 h

In above result there was no significant difference observed in the extracellular proteins production between RNAIII and its parent strain at 1.5 h (figure 3B). The reason should be that RNAIII was a cell density-dependent regulator [19] and the level of RNAIII was too low to regulate its targets at the lag phase. However, the extracellular proteins in Drnc decreased at 1.5 h (figure 2). It suggested that the reduction of extracellular proteins in Drnc was not completely due to the RNAIII level decreasing. And the reduction of extracellular proteins in Drnc at 1.5 h should have no relation with RNAIII. To discover other factors involved in this process, we chose Efb (extracellular fibrinogen binding protein) as the indicator of extracellular proteins [20,21,22] because the expression of Efb was not influenced by RNAIII (figure 4A) and the level of Efb in Drnc supernatant decreased at 1.5 h (figure 4B). Then we analyzed the mRNA level, translation and secretion of Efb to discover the mechanism of reducing the extracellular proteins in Drnc at 1.5 h.

Firstly, the mRNA level of the efb gene was determined by qRT-PCR. It showed that the mRNA level of efb in Drnc did not alter at 1.5 h (figure 4C). Secondly, we constructed the lacZ fusion vectors to analyze the translation of Efb in Drnc and its parent strains (figure 4D). The upstream region of efb containing its promoter, 5'UTR and the signal Table 1. Sequences of forward and reverse primers used in this study.

| Primer/sequence | Oligonucleotide sequence (5' to 3') |
|-----------------|-----------------------------------|
| Up-rnc F-EcoRI  | CATCCGGAAATCTAGTCTAAACAAAAAGAA |
| Up-rnc R-KpnI   | AAACAAAAGACGTACCAGTGTAGATT      |
| Down-rnc F-KpnI | AATCTACCACGCCTGTCACCTCATTGTTT  |
| Down-rnc R-Sall | ACACCGTCCAGCTACGCTACCTGACAGC    |
| rs-rncF         | GACTACGTGAATTCGACCGTAGGTGA      |
| rs-rncR         | CATGCCTACTGACCTTTAATTGGT       |
| rs-mV1          | CATCCGGAAATCTCGAGTTTTAATTAAT   |
| rs-mV2          | TTTAGCCATTTCCGAGC               |
| 16s RT primerF  | GCCTAATACAGCAGGAGG             |
| 16s RT primerR  | CATGTTATAGGCCTAATTAG           |
| spa RT primerF  | AAGAAGATGAGTAAACGGGTA          |
| spa RT primerR  | GTGTACCGAGGAATTGA               |
| RNAl RT primerF | CCTAGATCACAGAGATGAGGTA         |
| RNAl RT primerR | AATGATAGCAGTGCAGATT             |
| efb RT primerF  | AACATTAGCGCAATAGG              |
| efb RT primerR  | TTTCGCTGCTGTGTTTT             |
| Uefb-lacZF      | GATATGCGATATTCTGATGATAT        |
| Uefb-lacZR      | ATCATGCGGATGCTCCCTCTCTTCTTGTCCTGATGTAATT | TACCTCTGAAATTATTTTCCAAATATT |
| UefbSP-lacZR    | CCAAGAGAAAAAGAGGAGGATACCGGATGAT |

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Figure 2. Detection of the protein profile from different phases of WT and Drnc. Equal number of S. aureus cells was harvested at the indicated time points. The total proteins of whole-cell and supernatant proteins were extracted. The results showed that the supernatant proteins of the Drnc were decreased significantly compared with WT, while the total proteins of whole-cell did not show the same change as the supernatant proteins. The experiment has been repeated for three times. 1,4,7: WT, wild type, S. aureus 8325-4; 2,5,8: Drnc; 3,6,9: rncR.

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The secretion of the proteins in Drnc was inhibited at 1.5 h

In above result there was no significant difference observed in the extracellular proteins production between ARNAIII and its parent strain at 1.5 h (figure 3B). The reason should be that RNAIII was a cell density-dependent regulator [19] and the level of RNAIII was too low to regulate its targets at the lag phase. However, the extracellular proteins in Drnc decreased at 1.5 h (figure 2). It suggested that the reduction of extracellular proteins in Drnc was not completely due to the RNAIII level decreasing. And the reduction of extracellular proteins in Drnc at 1.5 h should have no relation with RNAIII. To discover other factors involved in this process, we chose Efb (extracellular fibrinogen binding protein) as the indicator of extracellular proteins [20,21,22] because the expression of Efb was not influenced by RNAIII (figure 4A) and the level of Efb in Drnc supernatant decreased at 1.5 h (figure 4B). Then we analyzed the mRNA level, translation and secretion of Efb to discover the mechanism of reducing the extracellular proteins in Drnc at 1.5 h. Firstly, the mRNA level of the efb gene was determined by qRT-PCR. It showed that the mRNA level of efb in Drnc did not alter at 1.5 h (figure 4C). Secondly, we constructed the lacZ fusion vectors to analyze the translation of Efb in Drnc and its parent strains (figure 4D). The upstream region of efb containing its promoter and 5'UTR was fused with lacZ (named as Uefb::lacZ). The constructed vector was transformed to Drnc and its parent strain respectively. The results of β-galactosidase activity detection showed that Drnc did not exhibit significant difference comparing with its parent strain (figure 4E). This suggested that the inactivation of RNase III did not influence the transcription and translation of Efb. And thirdly, we checked if the secretion of Efb was affected in Drnc. The upstream of efb containing its promoter, 5'UTR and the signal
peptide fused with lacZ was termed as UefbSP::lacZ (figure 4D). The β-galactosidase activity of cultured medium in the two different strains was detected. Our results showed that the β-galactosidase activity of the cultured medium from Δrnc was significantly lower than that from its parent strain (figure 4F). It suggested that the reduction of extracellular proteins of Δrnc at 1.5 h was because the secretion of extracellular proteins was suppressed.

The decrease of secY2 resulted in the inhibition of extracellular protein secretion in Δrnc at 1.5 h

The general secretory (sec) pathway is the most commonly used one for bacterial protein transport [12]. In addition, S. aureus contains an accessory Sec2 pathway involving the SecA2 and SecY2 proteins [13,14]. However, there were few reports on the sec pathway of S. aureus [12]. We analyzed the genome of S. aureus and detected the mRNA level of the genes which were involved in the general and accessory sec pathway (secA1, secY1, secA2 and secY2) by qRT-PCR. It was found that only the expression of secY2 decreased significantly in Δrnc at 1.5 h (figure 5A). Then the decline of secY2 mRNA level was confirmed by Northern blot (figure 5A). In addition, the production of extracellular proteins of ΔsecY2 (SecY2 inactivation mutant) was decreased at 1.5 h compared with its parent strain (figure 5B). In the further study, we constructed the plasmid of pOS1-secY2 using the promoter of ssrA, which is a tmRNA in S. aureus. It was found that the level of ssrA in Δrnc was not altered (data not shown). And then the plasmid was transferred to Δrnc to recover the expression level of secY2. The result showed the extracellular proteins increased after the expression level of secY2 recovered in Δrnc (figure 5B). At the same time, the level of Ebf in the supernatant was correspondingly restored (figure 5B). In the further investigation, the RNase III inactivated strain was constructed from the ΔsecY2 strain, named as Δrnc/ΔsecY2, it was found that the profile of extracellular proteins of the Δrnc/ΔsecY2 was the same as that of ΔsecY2 (figure 5B).

The mRNA stability of secY2 and RNAIII was decreased in Δrnc

In order to investigate how RNase III influences the expression level of secY2 and RNAIII, we tested the mRNA stability of secY2 and RNAIII. The transcriptions of secY2 and RNAIII were inhibited by addition of rifampicin when the cells had been cultured at 37°C for 1.5 h or 6 h individually. Then the RNA stability was tested by Northern blot. It was found that RNA stability of secY2 mRNA and RNAIII was decreased in Δrnc compared with its parent strain (figure 6).

The Δrnc was less pathogenic compared with its parent strain

In the further investigation, we compared the cytotoxicity induced by the supernatant between Δrnc and its parent strain. The supernatant of the cultured cells at 6 h was collected and incubated with MDBK cells. And then the cytotoxicity of the supernatant was tested with flow cytometric analysis. It was found that the percentage of apoptosis and necrosis induced by the Δrnc
supernatant was significantly lower compared with its parent strain (figure 7A). At the same time, we also detected the cytotoxicity induced by the heat-stable toxins in the supernatant using MTT assay. In line with expectations, the heat-stable toxins of \( D_{rnc} \) were decreased (figure 7B). Then the pathogenicity of \( D_{rnc} \) was assessed in a murine peritonitis model. The same numbers of cells of \( D_{rnc} \) and its parent strain were delivered intraperitoneally to mice. As shown in figure 7C, the survival rate of the mice in the \( D_{rnc} \) group was significantly higher than that of its parent strain group at the different time points (8 h, 16 h, and 24 h), which was in accordance with the cell toxicity results. It suggested that the RNase III played an important role in the pathogenicity of \( S. aureus \).

**Discussion**

As we know, the extracellular proteins play an important role in the infection caused by \( S. aureus \) [11]. In our study, we find that the extracellular proteins in the supernatant of \( D_{rnc} \) decreased significantly at three different growth phases compared with its parent strain. Most of the extracellular virulence factors in \( S. aureus \) is positively regulated by a regulatory RNA molecule — RNAIII.
Figure 5. The decrease of secY2 resulted in the inhibition of extracellular protein secretion in Δrnc at 1.5 h. A: Detection of the mRNA level of secA1, secY1, secA2 and secY2. The mRNA levels of secA1, secY1, secA2 and secY2 were detected at 1.5 h by qRT-PCR. The results showed that the level of secY2 was decreased in Δrnc \( (**: P < 0.01) \). Then the decrease of secY2 mRNA was confirmed by Northern blot. 16s rRNA was used as the internal control. WT: wild type, S. aureus 8325-4; Δrnc: an RNase III inactivation mutant from 8325-4; mR: the restoration of RNase III activity in Δrnc. B: Detection of the profile of extracellular proteins and the expression of Efb in the different strains. The pOS1-secY2 plasmid was constructed and transferred to Δrnc to recover the level of SecY2. At the same time, the double mutant Δrnc/secY2 was constructed. And then the extracellular proteins were extracted. The results showed that the production of the extracellular proteins was significantly increased at 1.5 h after the recovery of the level of secY2. The mRNA level of secY2 was measured by RT-PCR. 16s rRNA was used as the internal control. At the same time, the expression of Efb was determined by Western blot. The result showed that Efb was restored after the level of secY2 was recovered in Δrnc. 1: wild type; 2: Δrnc; 3: secY2r (the Δrnc strain transferred with the plasmid pOS1-secY2); 4, Δrnc/secY2; 5, ΔsecY2.

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Figure 6. Stability of RNAs. Half-lives of secY2 mRNA and RNAIII were determined in the presence of rifampicin (500 μg ml\(^{-1}\)) in the WT and Δrnc strains. Percentage of RNA was calculated normalizing with 5s rRNA.

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which is thought as an antisense RNA regulating many genes expression (except \( \delta \)-hemolysin encoding). It is demonstrated that the duplex of mRNA and RNAIII can be degraded by RNase III in vitro [7,8].

We find that the expression of \( \text{spa} \), one target RNA of RNAIII which can be degraded by RNase III, increased in \( \Delta \text{rnc} \). It indicates that the degradation mediated by RNAIII is altered. In the further investigation, it shows that the level of RNAIII decreased in \( \Delta \text{rnc} \). These results suggest that the decline of RNAIII may be the reason for the reduction of extracellular proteins of \( \Delta \text{rnc} \). In our experiments, it is revealed that the extracellular protein of \( \text{ARNAIII} \) decreases comparing with its parent strain at 6 h and 12 h. After the level of RNAIII is recovered in \( \Delta \text{rnc} \), the extracellular proteins increased correspondingly. The results suggest that there should be some other proteins regulated by RNase III through the RNAIII-independent pathway. Two previous reports showed that the level of RNAIII was not altered in the \( \text{rnc} \) mutant strains from RN6390, which is not consistent with our results [7,8]. The occurrence of the different results might be due to the different strains used in the experiments. Our previous work also has shown that there are some difference between \( S. \text{aureus} \ 8325-4 \) and RN6390, although both of them are originated from \( S. \text{aureus} \ 8325 \) [25,26,27,28].

In the investigation, it is also found that the extracellular protein Efb decreases at 1.5 h in the supernatant of \( \Delta \text{rnc} \). Efb is indentified as the 15.6-kilodalton extracellular fibrinogen-binding protein [20,21,22]. Hence, we chose Efb as an indicator for the further study. Our results show that the transcription and translation of Efb are not altered in \( \Delta \text{rnc} \). However, the results of \( \beta \)-galactosidase activity assay show that the secretion of \( \beta \)-galactosidase decreases in \( \Delta \text{rnc} \) supernatant when \( \text{lacz} \) gene is fused with the Efb signal peptide. These results suggest that the transport of Efb is inhibited in \( \Delta \text{rnc} \).
The secretory (sec) pathway, which includes sec1 and sec2 in *S. aureus*, is thought to be responsible for secretion of the extracellular proteins of *S. aureus*, but there are few published data on the sec pathway of *S. aureus* [12]. So we checked the expression of the components of sec pathway by qRT-PCR. It is found that the level of secY2 significantly decreases in Δmrc. The further investigation shows that the recovery of secY2 in Δmrc can increase the production of extracellular proteins at 1.5 h. In several pathogenic gram-positive bacteria, the accessory sec pathway (SecA2 and SecY2) is required for the transport of certain proteins related to virulence[12,15,16,17]. In addition, it has been reported that SecY2 can interact with the Sec1 channel in *S. aureus* [13]. Our results show that Sec2 is the major pathway which is responsible for the extracellular proteins transport of *S. aureus* at 1.5 h. It is interesting that we do not observe significant difference of the extracellular proteins profile between ΔsecY2 and wild type at 6 h and 12 h (data not shown). Our data indicates that SecY2 might play an important role in protein secretion at lag phase but not at exponential and stationary phases. We also found that the stability of secY2 mRNA and RNAIII was decreased in Δmrc. It suggests that RNAIII is involved in the RNA degradation of both genes. RNase III is a kind of ribonuclease, so the decline of the RNA stability in Δmrc should be indirectly regulated by RNase III. We are trying to investigate the mechanism of this regulation.

In conclusion, our study reveals a novel biological function of RNAIII in *S. aureus*, which can regulate the production of extracellular proteins via two molecules respectively at the different growth phases. At the lag phase, RNAIII can positively regulate the level of secY2 to increase the secretion of the extracellular proteins. After *S. aureus* cells grow to a certain density, RNase III can regulate the expression of extracellular proteins by affecting the level of RNAIII. Since the extracellular proteins are essential for the infection caused by *S. aureus*, RNase III might be a potential target of anti-Staphylococcus aureus infection.

**Materials and Methods**

**Ethics Statement**

This study was carried out in strict accordance with the recommendations in the national guidelines for the use of animals in scientific research “Regulations for the Administration of Affairs Concerning Experimental Animals”. The protocol was also approved by the Animal Care and Use Committee of Beijing Institute of Basic Medical Sciences ( Permit Number BMS091008), and all efforts were made to minimize suffering.

**Bacterial strains and growth conditions**

The strains used in this study are listed in Table 2. Strains were grown in 5 ml of brain heart infusion (BHI) or Luria-Bertani (LB)-medium (BD) at 37°C for 12 h with shaking at 200 rpm in a 25-ml test tube. Cells from 1 ml of preculture were transferred to 100 ml of BHI or LB medium in a 500-ml flask and incubated at 37°C on a rotary shaker at 200 rpm. *S. aureus* strains were routinely grown in BHI and *E. coli* strains were grown in LB medium either without antibiotics, or with 20 μg ml⁻¹ erythromycin, 100 μg ml⁻¹ ampicillin and 100 μg ml⁻¹ kanamycin respectively.

**Construction of insertion mutant of RNase III (Δmrc)**

The mutant was constructed using the procedures described previously [29] with some modifications. In order to create an

| Table 2. Bacterial strains and plasmids. |
|------------------------------------------|
| **Strain or plasmid** | **Comments** | **Source or reference** |
| *S. aureus* | | |
| 8325-4 | Wild-type, rsbU | [25] |
| RN4220 | Restriction-negative strain, 8325 derivative | | |
| Δmrc | 8325-4 with a maseII-kan mutation | This study |
| ΔRNAIII | 8325-4 with a malleII-kan mutation | This study |
| ΔJRNAl | the restoration of RNase III activity in Δmrc | This study |
| ΔJRNAlR | the restoration of RNAIII activity in ΔRNAIII | This study |
| ΔJsecY2 | 8325-4 with a SecY2 inactive mutation | This study |
| Δmrc/secY2 | a secY2 and RNAIII double inactive mutation | This study |
| Δmrc/RNAIII | a RNAIII and RNAIII double inactive mutation | This study |
| E. coli | | |
| DH5α | A host strain for cloning | Transgene |
| **Plasmids** | | |
| pAUL-A | Temperature-sensitive *S. aureus* suicide vector; Em | [36] |
| pMD19T | *E. coli* cloning vector, amp | TaKaRa |
| pOS1 | *E. coli*-*S. aureus* shuttle vector, Cm | [37] |
| pOS1-RNAIII | pOS1 derivative for expression of RNAIII | This study |
| pOS1-secY2 | pOS1 derivative for expression of secY2 | This study |
| pOS1-lacZ | pOS1 contains a copy of lacZ encoding β-galactosidase without promoter and 5' UTR | This study |
| pOS1-Uefb-lacZ | UTR of efB-lacZ fusion(Uefb-lacZ) shuttle vector, a derivative of pOS1 | This study |
| pOS1-UefbSP-lacZ | UTR and signal peptide of efB-lacZ fusion(UefbSP-lacZ) shuttle vector, a derivative of pOS1 | This study |
insertion mutant of RNase III in S. aureus 8325-4, two regions of DNA flanking the *rnase III* gene were amplified by PCR using the primers (Up-*rn* F-EcoRI/Up-*rn* R-KpnI, Down- *rn* F-KpnI/Down- *rn* R-Sall) with restriction sites as listed in the Table 1. The upstream fragment (529 bp) was digested with EcoRI and KpnI, and the downstream fragment (232 bp) was digested with KpnI and Sall. The two fragments were cloned together into pMD19T digested with EcoRI and Sall. The resulting construct was digested with KpnI, and then a 1.6-kb kanamycin cassette which was amplified from the plasmid of pTZ-TRAP-kan provided by Dr. Balaban N was inserted. The resulting plasmid was digested with EcoRI and Sall, and a fragment harboring kanamycin resistance between the upstream and downstream fragments was ligated into pAUL-A digested with EcoRI and Sall to create plasmid pAUL-A*rn*. pAUL-A*rn* has a temperature-sensitive origin of replication that is active in *S. aureus* at 30°C but not at 42°C. The recombinant plasmid, initially isolated from *E. coli*, was introduced into *S. aureus* RN4220 by electroporation and colonies resistant to kanamycin and erythromycin were selected after growth at 30°C. The resistant clones were subjected to a temperature shift to 42°C to select for plasmid integration into the chromosome. Bacteria resistant to kanamycin but sensitive to erythromycin were selected. The mutation was confirmed by PCR, and followed by transduction into strains 8325-4 and 8325-4 and transformants were selected on tryptic soy agar plates containing 10 mg L⁻¹ chloramphenicol at 37°C. The supernatant was collected and filtered through a 0.22 µm filter to remove residual cells. Culture supernatant from equal numbers of cells was precipitated by adjusting filtered supernatants to 10% tricarboxylic acid (TCA) and incubated at 4°C for 4 h. After centrifugation (12,000 g, 10 min), precipitated proteins were washed twice in ice-cold 96% ethanol, air dried. The proteins were resolved in an appropriate volume of a solution containing 7 M urea, 2 M thiourea. The samples were then subjected to 15% SDS-PAGE and visualized by Coomassie blue G-250 staining.

**Flow cytometric analysis of apoptosis and necrosis of MDBK cells**

The supernatant was collected according to the above method. For flow cytometric analysis, the MDBK cells were resuspended at a concentration of 1×10⁶ cells ml⁻¹ and added to a 12-well plate (1 ml/well). At 40–50% confluency (24 h post seeding), the cultivated cells were treated with medium alone or with supernatant of *S. aureus* strains for 12 h. Prior to harvesting, the cells were washed twice with PBS (Phosphate Buffered Saline), trypsinized, and pelleted. Then cells were resuspended at a concentration of 1×10⁵ cells ml⁻¹ in Binding Buffer (0.01 M HEPES/NaOH, pH 7.4, 14 mM NaCl, 0.25 mM CaCl₂). Aliquot cells (500 µl) were added into FACS tubes and mixed with 25 ng ml⁻¹ FITC-conjugated annexin V and 10 mg ml⁻¹ propidium iodide(PI), incubated for 15 min at room temperature in the dark. Then the apoptosis and necrosis were analyzed immediately by flow cytometry. The final data was reported as the mean ± SD for each of the three independent experiments.

**MTT assay of cellular toxicity**

For MTT assay, the supernatant was boiled for 10 min, and then centrifuged to remove the precipitate. The MDBK cells were cultured in RPMI-1640 medium (Gibco) with 100 U ml⁻¹ penicillin and 100 µg ml⁻¹ streptomycin at 37°C with 5% CO₂. Then they were detached using 0.25% trypsin/EDTA and counted by means of hemocytometer. The cells were resuspended and a total of 1×10⁵ in 0.1 ml culture medium was seeded into each well of a 96-well plate and cultured for 24 h. At 40–50% confluency (24 h post seeding), the cultivated cells were treated with medium alone or with the boiled supernatant of *S. aureus* strains. Then, MTT assay was performed 24 h after treatment. 10 µl of MTT (1 mg ml⁻¹, Sigma-Aldrich) was added into each well and the incubation was continued for 4 h at 37°C with 5% CO₂. After 4 h, 100 µl SDS buffer (10% SDS, 0.1 M HCl) was added to the wells. The absorbance of the wells was determined using a plate reader at a test wavelength of 595 nm after 8 h. The cell viability percentage was calculated as: Viability percentage (%) = (Absorption value of supernatant of treatment group)/(Absorption value of supernatant of control group) ×100%.

**Western blot**

The protein samples were subjected to 15% SDS-PAGE and the proteins were blotted onto Hybond-ECL nitrocellulose membrane (Amersham Biosciences). The membrane was blocked in 5% non-fat dry milk at 37°C for 2 h, probed with 1:500 diluted...
polyclonal rabbit anti-Efb antibodies (prepared by ourselves) for 1 h at room temperature, and washed twice with PBS with 0.5% Tween 20 (PBST). Then the membrane was incubated in a 1:5,000 solution of HRP-conjugated goat anti-rabbit secondary antibody at room temperature for 1 h. After further washing with PBST, the membrane was assayed by the enhanced chemiluminescence (ECL) western blotting detection system.

Quantitative reverse transcription PCR (qRT-PCR)

Total bacterial RNA was extracted from S. aureus using Trizol (Invitrogen) as previously described [30]. DNase digestion of 80 μl of total RNA was performed with 10 U of RNase-free DNase I (Promega) and 10 μl of the 10× reaction buffers in a total reaction volume of 100 μl for 30 min at 37°C. For cDNA synthesis, 1 μg of total RNA was mixed with 500 ng of random hexamer (Promega). Samples were incubated at 65°C for 10 min with 5 μl of 5× first-strand buffer, 2 μl of 5 mM dNTP, 20 U of Rnasin (Takara), 1 μl of M-MLV reverse transcriptase (Promega) and distilled water to a total volume of 25 μl. The qRT-PCR reaction mixture contained 12.5 μl of 2×SYBR green PCR mix (GenePharma), 0.3 μM of gene-specific forward and reverse primers, and 1 μl of template, made up to a final volume of 25 μl with distilled water. The primers are shown in Table 1. Cycling parameters were set as follows: initial activation step at 95°C for 10 min, denaturation at 94°C for 30 s, annealing at 58°C for 30 s, and extension at 72°C for 40 s. Melting curve analysis was performed from 58°C to 95°C with stepwise fluorescence acquisition at every 1°C s⁻¹. Melting curves observed for each gene were confirmed to correspond to the correct amplicon size by agarose gel electrophoresis of the curves observed for each gene were confirmed to correspond to the correct amplicon size by agarose gel electrophoresis of the PCR products. The levels of gene expression were calculated by relative quantification using 16s rRNA as the endogenous reference gene. All samples were amplified in triplicate and the data analysis was carried out using the MxPro qRT-PCR system software (Stratagene).

Construction of lacZ reporter vector

The fragments (promoter-5'UTR and promoter-5'UTR-signal peptide of efb) was amplified by PCR from S. aureus B325-4 chromosomal DNA with primers Uefb-lacZF/Uefb-lacZR and Uefb-lacZF/UefbSP-lacZR (Table 1). The PCR products were digested with EcoRI and BamHI, and ligated into EcoRI and BamHI-digested pOS1-lacZ plasmid DNA, which contains a copy of lacZ without promoter and 5'UTR, resulting in the in-frame fusion of lacZ to the amplified fragments. The recombinant plasmids were transformed into DH5α, then electrotansfected to S. aureus RN4220. The plasmid was isolated from RN4220, then electrotransfected into S. aureus B325-4 and Anc.

β-Galactosidase assay

The cells were prepared for the assay as described before with some modification [33]. Cells were grown as described above, and 1 ml culture was centrifuged. Briefly, the pellet was washed in PBS, and then the cells were adjusted to an OD600nm of 1 in a volume of 500 μl. The cells were sedimented by centrifugation and the pellet was resuspended in 500 μl lysis buffer (0.01 M potassium phosphate buffer, pH 7.8, 0.015 M EDTA, 1% Triton X-100) containing lysostaphin at the final concentration of 20 μg ml⁻¹, and incubated at 37°C for 30 min, with gentle shaking. The culture was centrifuged at 20,000 g for 30 min. The supernatant was subjected to galactosidase assays according to the method described by Miller [34].

Northern Blot and RNA half-life determination

Total RNA was separated by electrophoresis on a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to nylon membrane. Hybridizations with the specific α-32P-labeled DNA probes were carried out to detect the secF2 mRNA or RNAIII. 16s or 3s rRNA was used as the internal control.

RNA half-lives were determined by treating cells with rifampicin (final concentration: 500 μg ml⁻¹) and isolation of RNA at 0, 30, 60, and 90 min after rifampicin addition. SecF2 mRNA stability was determined in lag phase (cultured for 1.5 h; OD600 = 0.5) and RNAIII stability was determined in stationary phase (cultured for 6 h; OD600 = 10).

Acute murine peritoneal infection model

Groups (n = 10) of 6- to 8-week-old, male Balb/c mice were injected intra-abdominally with 500 μl of Anc or its parent strain (containing 1×10⁶ CFU (colony forming units)). The survival number of mice was recorded at the different time points (3 h, 16 h and 24 h) post challenge. Survival outcomes in Anc or its parent strain groups were compared. The experiment was performed twice.

Statistical analysis

All quantitative data were analyzed using student t-tests. P<0.05 was considered statistically significant.

Author Contributions

Conceived and designed the experiments: GY. Performed the experiments: YL JD. Analyzed the data: NW YG XZ CM. Contributed reagents/materials/analysis tools: NS MF. Wrote the paper: GY.
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