SCIENCE CHINA
Life Sciences

• RESEARCH PAPERS •

September 2011 Vol.54 No.9: 863–869
doi: 10.1007/s11427-011-4195-5

The staphylococcal nuclease prevents biofilm formation in *Staphylococcus aureus* and other biofilm-forming bacteria

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Received March 18, 2011; accepted May 30, 2011; published online July 21, 2011

The staphylococcal nuclease, encoded by the *nuc*1 gene, is an important virulence factor of *Staphylococcus aureus*. However, the physiological role of the nuclease has not been fully characterized. The current study observed that biofilm development could be prevented in staphylococcal nuclease-producing strains of *S. aureus*; however, when the *nuc*1 gene was knocked out, the ability to form a biofilm significantly increased. Scanning electron and confocal scanning laser microscopy were used to evaluate the role of the *nuc*1 gene in biofilm formation. Moreover, the *nuc*1 gene product, staphylococcal nuclease, and recombinant NUC1 protein were found to have a visible effect on other biofilm-forming bacteria, such as *Pseudomonas aeruginosa*, *Actinobacillus pleuropneumoniae*, and *Haemophilus parasuis*. The current study showed a direct relationship between staphylococcal nuclease production and the prevention of biofilm development. The findings from this study underscore the important role of staphylococcal nuclease activity to prevent biofilm formation in *S. aureus.* They also provided evidence for the biological role of staphylococcal nucleases in other organisms.

biofilm formation, *nuc*1 gene, staphylococcal nuclease, *Staphylococcus aureus*

Citation: Tang J N, Kang M S, Chen H C, *et al*. The staphylococcal nuclease prevents biofilm formation in *Staphylococcus aureus* and other biofilm-forming bacteria. Sci China Life Sci, 2011, 54: 863–869, doi: 10.1007/s11427-011-4195-5

Bacterial biofilms are structured communities of cells enclosed in a self-produced hydrated polymeric matrix adherent to an inert or living surface [1]. Formation of these sessile communities and their inherent resistance to antibiotics and host immune attack are the basis of many persistent, often chronic, bacterial infections [2]. *Staphylococcus aureus* has evolved into highly adaptable human pathogens responsible for many infections ranging from folliculitis and foodborne intoxications to severe endocarditis, osteomyelitis, or septicemia [3]. Besides the production of exotoxins and surface proteins, the formation of highly organized multicellular complexes-biofilms is increasingly being recognized as an important virulence factor in *Staphylococcus* spp. [4]. Most *Staphylococcus* spp. have the ability to adhere and form biofilms, which exhibit a distinct phenotype and present a serious clinical problem [5]. Colonization alone by this bacterium does not usually lead to adverse events; however, once the epithelial and mucosal surfaces have been breached, serious disease can result [6]. Furthermore, inside the biofilm, *S. aureus* becomes more resistant to antibiotic treatments and the actions of the immune system [7]. Consequently, staphylococcal-biofilm-associated infections of this type are difficult to eradicate, and most can be eliminated only by the removal and substitution of the contaminated implant [3].

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Many studies have dedicated great effort to identifying factors involved in *S. aureus* biofilm development [8,9]. These studies have shown that staphylococcal biofilms are multifactorial and influenced profoundly by the infection milieu [6]. Until now, many *S. aureus* genes that contribute to its biofilm-forming ability have been reported, including *agr*, *sar*A, *sig*B, *ica*, *rbf*, *tca*R, *arl*RS, *als*SD, and surface adhesions genes such as *bap*, *spa*, *fnb*PA, *fnb*PB, and *sas*G [8,10–12]. Although the expression of the *ica* operon is of central importance in generating biofilm, the roles of other factors and co-factors have yet to be determined [13]. Of particular importance is the observation that most *S. aureus* clinical isolates possess and express the *ica* operon under various growth conditions, but many are incapable of biofilm development under the same conditions [6,13]. These findings indicate that our understanding of the molecular mechanisms contributing to biofilm formation is incomplete, and underscores the existence of other factors.

Recent developments have revealed that the biofilm matrix is a complex mixture of macromolecules, including poly-N-acetylglucosamine (PNAG), proteins, and DNA [14,15]. Cynthia *et al*. [2] found that extracellular DNA was required for the initial establishment of *P. aeruginosa* biofilms. It was also shown that bacterial extracellular genomic DNA was an important structural component of the *S. aureus* biofilm matrix [11,15]. Given this information, compounds capable of dissolving matrix components (proteases, DNases, or glycoside hydrolases) can prevent the formation of biofilms or disrupt established biofilms.

S. aureus has the ability to produce a wide variety of exoenzymes, including nucleases, proteases, lipases, hyaluronidase, and collagenase [16], and staphylococcal nuclease is known to be an important marker unique to *S. aureus* and used as an indicator of *S. aureus* contamination [17]. This nuclease has the ability to degrade nucleic acids by hydrolyzing the phosphodiester bonds found in DNA and RNA yielding 3′-mononucleotides [18]. Our previous work has shown that two thermostable nucleases are present in *S. aureus*: The *nuc*1 gene was shown to encode the staphylococcal nuclease, and the *nuc*2 gene encoded the thermonuclease. The activity of the staphylococcal nuclease was found to be much higher than that of the thermonuclease in *S. aureus* [16]. Therefore, we postulated that the staphylococcal nuclease could play an important role in the prevention of biofilm development and formation in nuclease-producing *S. aureus*. The current study investigated the potential role of staphylococcal nucleases in biofilm formation in *S. aureus* and other biofilm-forming bacteria.

1 Materials and methods

1.1 Bacterial strains, media, and culture conditions

S. aureus strain RN4220 was kindly donated by Dr. Karen Battista (International Association for Food Protection, Kraft Foods North America). *S. aureus* RNΔ*nuc*1 and RNΔ*nuc*1-complement were constructed previously [16]. The strains and sources of biofilm formation bacteria are as follows: *Pseudomonas aeruginosa* CCTTCC AB93066 obtained from the China Center for Type Culture Collection, Wuhan University; *Actinobacillus pleuropneumoniae* (APP) Hubei 0504 and *Haemophilus parasuis* (HPS) 0322 provided by Huazhong Agricultural University. All bacteria were initiated using fresh overnight cultures grown at 37°C on tryptic soy agar (TSA) or in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI, USA).

1.2 Expression of recombinant NUC1 and preparation of culture supernatant

Recombinant plasmid, pET-17nuc1, constructed previously [16], was used to transform *Escherichia coli* BL21(DE3). The expression of the target protein was induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG). The supernatants from *S. aureus* strain RN4220 cultures were harvested by centrifugation. The enzymatic activities of the recombinant protein NUC1 and the culture supernatant of *S. aureus*, including staphylococcal nuclease, were tested on toluidine blue-DNA plates.

1.3 Glass tube biofilm assay

The ability to form biofilms was tested using a method previously described by Jin *et al*. [19]. Briefly, borosilicate glass tubes (13 mm×100 mm) filled with 1 mL of TSB were inoculated with 20 μL of inocula. After 18 h incubation at 37°C with circular agitation (150 r min^{-1}), the contents of each tube were removed using an injector. The tubes were stained with 1.5 mL of 1% Hucker crystal violet solution at room temperature for 5 min. The dye solution was then removed from the tubes, the tubes were rinsed under tap water for 3–5 min until no color was observed in the rinse water and excess water was then removed from the tubes.

1.4 Microtiter plate assay for biofilm formation

Quantification of biofilm formation on abiotic surfaces was assessed as previously described [20,21]. The wells of sterile 96-well flat-bottomed polystyrene microplates were filled with 100 μL TSB and 10 μL of overnight inocula was added to each well before the plates were covered and incubated aerobically at 37°C for 36 h. Each bacterium was tested in triplicate. To visualize biofilms, the contents of the wells were aspirated and the wells washed three times with 200 μL sterile phosphate-buffered saline (PBS) to remove loosely adherent cells. The remaining attached bacteria were fixed with 100 μL methanol for 15 min. After drying in air, the wells were stained with 100 μL 1% crystal violet solution for 5 min at room temperature. Excess stain was rinsed

off by placing the plate under running tap water. Thereafter, the plates were dried in a 37°C incubator for approximately 30 min to ensure they were completely dry. After the adherent cells were resolubilized with 100 μL 33% (v/v) glacial acetic acid, the optical density (*A*) of each well was obtained at 630 nm using a Sunrise absorbance reader (Tecan, Maennedorf, Switzerland). All tests were performed three times and the values averaged. *A* values from the wells that had not been inoculated with bacteria were used as negative controls. The cut-off value (A_c) for determining a biofilm producer was defined as twice the negative control value. Based on *A* values, strains were classified as non-biofilm producers ($A \leqslant A_c$), weak biofilm producers ($A_c \leqslant A \leqslant 2 \times A_c$) or strong biofilm producers ($2 \times A_c \leqslant A$). For *P. aeruginosa* CCTTCC AB93066, *A. pleuropneumoniae* (APP) HuBei 0504, and *H. parasuis* (HPS) 0322, each bacterium was added to the supernatants of *S. aureus* including staphylococcal nuclease (20 μL), recombinant protein NUC1 (20 μL), and 10 U of DNase I per well into the culture for 12 h, and assayed after 36 h of growth. Mean values of three independent experiments were calculated and each bacterium was performed in triplicate.

1.5 Scanning electron microscopy (SEM)

Biofilms of the *S. aureus* strains were formed on 22 mm×22 mm glass coverslips (SAIL BRAND, China) and were placed at a 55° angle in a six-well polystyrene cell culture plate (FALCON, Becton Dickinson and Company, Franklin Lakes, USA) that had been filled with 4 mL of TSB with a 1:100 dilution of an overnight bacterial culture for 24, 36, or 72 h. SEM was performed as previously described [22]. Images were viewed using a JSM-6390/LV scanning electron microscope.

1.6 Confocal scanning laser microscopy

For observation of bacterial biofilm by confocal scanning laser microscopy (CSLM), the method described by Rice *et al.* [11] was used with slight modification. Briefly, biofilm formation of *S. aureus* and other bacteria were grown on 22 mm×22 mm glass coverslips (SAIL BRAND, China) and placed in six-well polystyrene microtiter plates (FALCON, Becton Dickinson and Company, Franklin Lakes USA) that had been filled with 4 mL of TSB with a 1∶100 dilution of an overnight bacterial culture. For incubations of 72 h, the coverslips were rinsed with distilled water to remove non-adherent bacteria and stained with LIVE/DEAD BacLightTm Bacterial Viability Kit solution. Samples were stored in physiological saline for a maximum of 15 min before being covered with distilled water and gently washed twice. Biofilm images were collected by CLSM using a LSM 510 META confocal scanning system (Zeiss, Germany). Fluorescence was detected by excitation at 488 nm and emission was collected with a 500–530 nm and >615 nm bandpass filter. All Z-sections were collected at 1-μm intervals by using a Zeiss Immersol 518F objective lens. Image acquisition and processing was performed by using a LSM Image Browser (Zeiss, Germany). Each strain was examined on at least three separate occasions and the average depth of the biofilm was determined for each strain using five independent Z-section measurements.

1.7 Statistical analysis

A values were collected from three independent experiments. Absorbance values from crystal violet staining assays were evaluated for statistically significant differences using Student's unpaired two-sided *t*-test. Bars in the figures indicate standard error from the mean.

2 Results

2.1 Staphylococcal nuclease affects *S. aureus* biofilm formation

Staphylococcal nuclease activity was determined for parental, mutant ($\triangle$ *nuc*1), and complement strains in our previous work [16]. Both the parent strain and complement strain exhibited strong nuclease activity, whereas the pink zone size of the mutant strain was significantly smaller than the parent and complement strains, showing that culture supernatants of the mutant strain (RNΔ*nuc*1) displayed the highest decrease in nuclease activity [16].

The present study showed that there was a close correlation between staphylococcal nuclease activity and biofilm formation and stability, where staphylococcal nuclease could disrupt or prevent the formation of *S. aureus* biofilms (Figures 1 and 2). The biofilms were washed, stained with crystal violet, and retained biomass was quantified by measuring the absorbance of each well at an absorbance of 630 nm. By measuring *A* values at the levels of biofilm formation, the *nuc*1 knock-out strain, RNΔ*nuc*1, was demonstrated to be a robust biofilm former. The *P*-value was 0.002351<0.0025 which represented the significant diversity of biofilm formation among *S. aureus* RNΔ*nuc*1, parental strain and its complemented strain (Figure 1A). The strain RNΔ*nuc*1 had an increased biofilm adherence. Accordingly, the formation ability of biofilm also varied considerably in the glass tube assay. The parental strain and its complemented strain showed weak biofilm-forming abilities ($A_c \leqslant A \leqslant 2 \times A_c$); however, the *nuc*1 mutant strain was significantly different with a strong biofilm-forming ability ($A \leqslant 2 \times A_c$) (Figure 1B). SEM was used to observe differences in the three-dimensional (3D) structures of biofilms formed by the three strains (Figure 2A). The RN4220 and RNΔ*nuc*1- complement strains were observed to have only several planktonic cells on the surface of coverslips, while the *nuc*1 knocked-out strain, RNΔ*nuc*1, was able to form

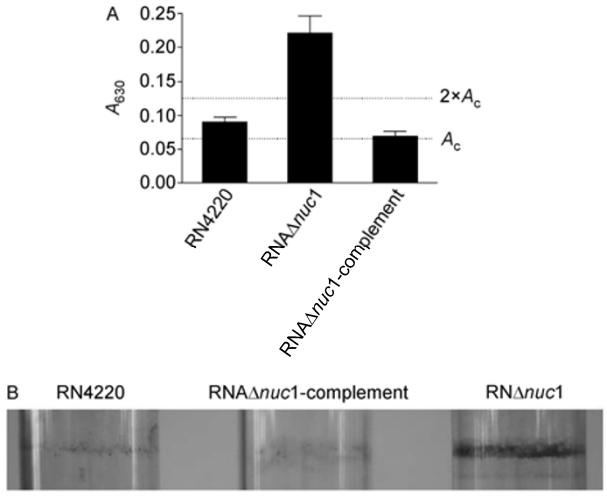

Figure 1 Quantitative analysis of biofilm formation. A, Microtiter plate assay for biofilm formation among *S. aureus nuc*1 mutant strain (RNΔ*nuc*1), parental strain (RN4220), and its complement strain (RNΔ*nuc*1-complement). Mean values from three independent experiments, each performed in triplicate, are shown. Bars indicate the standard error from the mean. B, Biofilm adherence assay among *S. aureus* RNΔ*nuc*1, RN4220, and RNΔ*nuc*1-complement after 18 h.

3D biofilm structures. Figure 2B shows the dynamic biofilm development and formation procedure of strain RNΔ*nuc*1 at 24, 36, and 72 h. The loss of staphylococcal nuclease activity as a result of the *nuc*1 knock-out significantly increased biofilm development (Figures 1 and 2). The presence of staphylococcal nuclease was shown to prevent biofilm development in the parental and RNΔ*nuc*1-complement strains. Furthermore, biofilm formation by the RN4220 *nuc*1 mutant and its complement strain were also assessed by CLSM. As shown in Figure 3, notable differences were observed among the *nuc*1 mutant, wild-type and complemented strains after staining with a viability stain for bacteria. Only the *nuc*1 mutant strain showed a high level of fluorescence indicating living cells on the surface of the coverslip with an average depth of (16±0.7) μm. Other strains showed red fluorescence indicating dead cells with an average depth of (2±0.4) μm. Interestingly, the *nuc*1 mutant strain with DNase I treatment produced similar results as the staphylococcal nuclease-producing strains with red fluorescence.

2.2 Staphylococcal nuclease affects biofilm formation by other bacteria

To further evaluate the influence of staphylococcal nuclease on biofilm formation by other bacteria, three biofilm-forming bacteria (*P. aeruginosa*, APP, and HPS) were assayed in this study. As shown in Figure 4, the culture supernatant containing staphylococcal nuclease and the recombinant NUC1 protein had different effects on the biofilm formation of the three different bacteria. There was a 2.5-fold decrease ($P<0.001$) in biofilm formation for the *P. aeruginosa* strain, while HPS ($P<0.05$) and APP ($P<0.05$) only had slightly decreased biofilm formation (Figure 4). Obvious differences in the dimensional biofilm structures were detected by CLSM (Figure 5). The staphylococcal nuclease and recombinant NUC1 protein were both shown to inhibit and decrease the development of biofilm formation for the three different bacteria, suggesting that *S. aureus* nucleases could possibly have profound effects on biofilm formation for other bacteria.

3 Discussion

The opportunistic pathogen, *S. aureus*, and other *Staphylococcus* spp. are able to form biofilms on host tissues and implanted medical devices often causing chronic infections [12]. While some genes associated with biofilm development have been identified, the focus of the current study

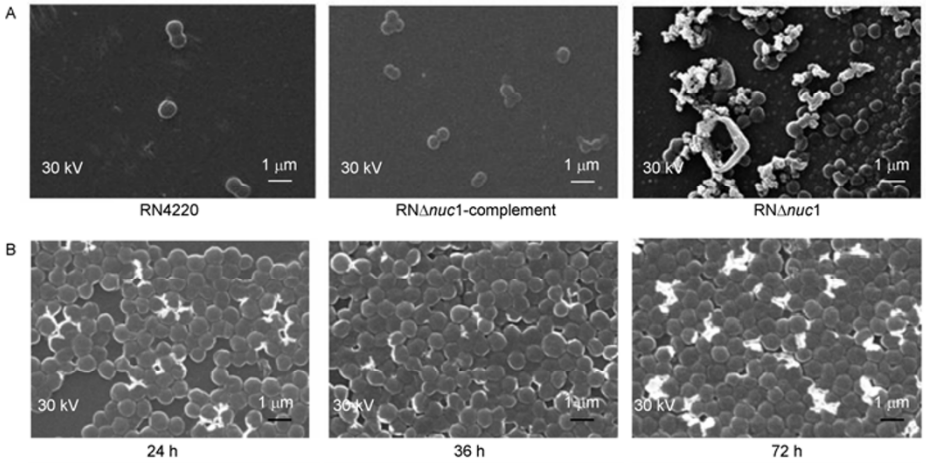

Figure 2 SEM analysis of biofilm formation. SEM of biofilms for *S. aureus nuc*1 mutant strain (RNΔ*nuc*1), parental strain (RN4220), and its complement strain (RNΔ*nuc*1-complement) at 72 h. 10000× magnification. B, SEM analysis for biofilm development of *nuc*1 mutant strain RNΔ*nuc*1 at different culture times (24, 36, and 72 h).

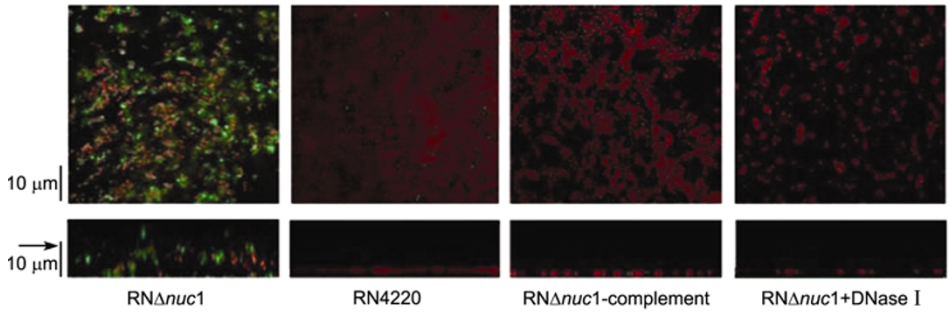

Figure 3 CLSM analysis of biofilms. Formation biofilms by *nuc*1 mutant strain RNΔ*nuc*1, parental strain RN4220, its complement strain RNΔ*nuc*1-complement and RNΔ*nuc*1 with DNase I treatment. All strains were grown for 72 h and stained with a viability stain. Representative orthogonal views are shown at 630× magnification with three independent experiments. Arrow indicates the top of the biofilms. Top panels show single sections through the *X*-*Y* plane, and the bottom panels are single sections through the *X*-*Z* plane.

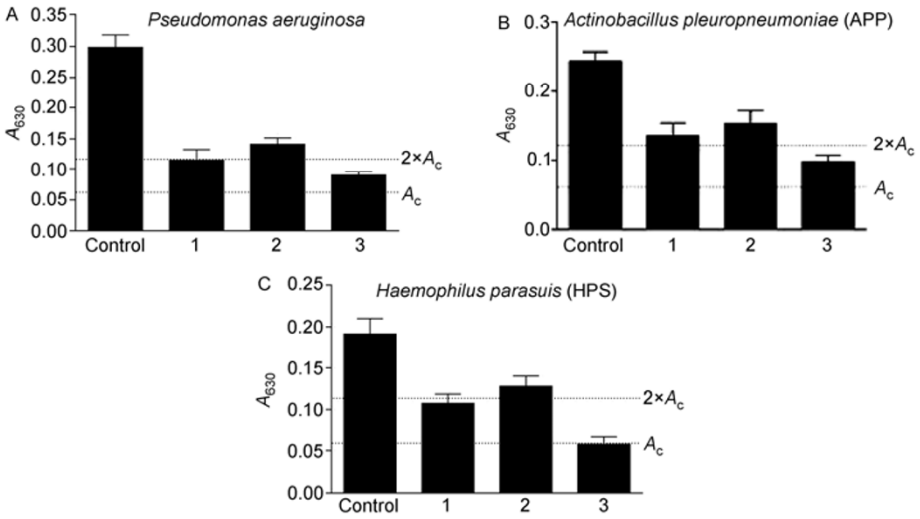

Figure 4 Microtiter plate assay for biofilms formation among *Pseudomonas aeruginosa* (A), *Actinobacillus pleuropneumoniae* (APP) (B) and *Haemophilus parasuis* (HPS) (C) with different treatments. All bacteria were respectively added to supernatants of *S. aureus* that contained staphylococcal nuclease (1), recombinant NUC1 protein (2), and DNase I (3) into the 12 h culture and assayed after 36 h of growth. Mean values of three independent experiments, each performed in triplicate, are shown. Bars represent the standard error from the mean.

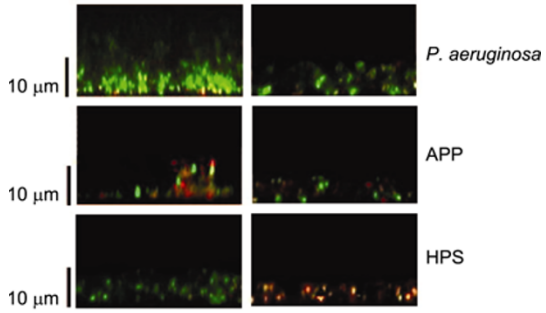

Figure 5 CLSM analysis of biofilms formation by *Pseudomonas aeruginosa*, *Actinobacillus pleuropneumoniae* (APP) and *Haemophilus parasuis* (HPS) after 72 h growth. Biofilms were stained with viability. Left panels show a control culture without any treatment and right panels show 12 h cultures treated with recombinant NUC1 protein.

was on the role of the staphylococcal nuclease in biofilm formation. The results of this study showed a new way to control biofilm formation. We examined and demonstrated the role of the *nuc*1 gene in *S. aureus* biofilm development and formation. Furthermore, we also provided evidence for the biological role of the staphylococcal nuclease in inhibiting biofilm formation of other bacteria.

In the present study, the analysis of the *S. aureus* RN4220 strain and its isogenic *nuc*1 mutant (RNΔ*nuc*1) in the context of biofilm formation led to the discovery that staphylococcal nuclease contributes to the prevention of biofilm formation. Mutation of the *nuc*1 gene resulted in significantly increased biofilm formations suggesting that biofilm formation could be prevented in staphylococcal nucleases-producing strains under normal conditions. These results emphasize the role of the staphylococcal nuclease in biofilm formation.

Our previous work has revealed that most of the *S. aureus* strains possess *nuc*1 and *nuc*2 genes that encode staphylococcal nuclease and thermonuclease, respectively. The

activity of staphylococcal nuclease is evidently higher than that of thermonuclease [16]. In the current study, *nuc*1 gene knock-out resulted in a notable increase in biofilm formation. CLSM and SEM were used to observe the diversity of different bacteria in the formation of biofilms. As expected, high levels of biofilm formation ability were observed in the mutant strain RNΔ*nuc*1, whereas biofilm formation was barely detectable for the parental strain, complemented strain, and the mutant strain with DNase I treatment. It is likely that extracellular genomic DNA, an important structural component of the *S. aureus* biofilm matrix [15,23,24], is broken down by the staphylococcal nuclease in staphylococcal nuclease-producing strains, preventing the adherence of cells for the formation of biofilm. Furthermore, staphylococcal nuclease likely prevents biofilm formation and promotes programmed cell death, resulting in the observation of many dead cells in biofilms. By contrast, living cells were present in biofilms produced by the *nuc*1 gene knock-out strain as a result of the loss of staphylococcal nuclease activities. In agreement with an earlier study [15], the staphylococcal thermonuclease may degrade extracellular genomic DNA (eDNA) as a means to promote biofilm dispersal.

In *S. aureus*, biofilm formation has been shown to involve both *ica*-mediated biofilm and *ica*-independent biofilm development mechanisms [13]. Bacterial surface proteins, such as Aap and Bap, could mediate polysaccharide intercellular adhesin/PNAG-independent intercellular accumulation during biofilm development [13]. However, our study highlighted a novel role of staphylococcal nucleases in *ica*-independent biofilm mechanisms. Our research may also explain why the *ica* locus is maintained, expressed, and regulated in many staphylococcus clinical isolates incapable of biofilm formation. Staphylococcal nucleases should have a great potential for use in the removal of biofilms from device-related infections. Several studies have suggested that DNase I treatment disrupts *P. aeruginosa* biofilm grown *in vitro*; which has been used in combination with antibiotics to treat *P. aeruginosa* infections in cystic fibrosis patients [25,26]. Similarly, treatments of streptococcal biofilm with DNase I have a negative effect on biofilm adherence [11,27,28]. To further evaluate the relative efficacy of staphylococcal nuclease, we carried out additional analyses. The current study showed that staphylococcal nuclease and recombinant NUC1 protein were able to prevent biofilm formation by *P. aeruginosa*, APP, and HPS. Staphylococcal nuclease-producing *S. aureus* strains were also shown to have a greater ability to interfere with biofilm adherence of *S. aureus* or other biofilm-formation bacteria.

To date, the production of exotoxins and surface proteins, including staphylococcal nucleases, has been recognized as important virulence factors in *S. aureus* [29]. In this context, staphylococcal nuclease was seen to have a novel role preventing the generation of biofilms and, therefore, virulence. This could explain how biofilm formation is believed to have an important role in the pathogenesis of *S. epidermidis* infections, and might be related to the emergence of *S. epidermidis* as a new pathogen [4,30,31]. Before the advent of modern medicine, *S. epidermidis* was rarely pathogenic [29]. *S. epidermidis* infections were predominantly associated with medical devices, such as intravascular devices or implanted prosthetic devices. *S. epidermidis* and some *S. aureus* strains do not possess the *nuc*1 gene, which means that these bacteria do not have the ability to produce staphylococcal nucleases. Therefore, our results could explain why *S. epidermidis* and some *S. aureus* strains have the ability to form biofilms leading to device-related infections. Since the capacity of *S. epidermidis* and *S. aureus* to form biofilms is an important virulence factor in the development of device-related infections [13,31], we presume that the majority of biofilm-mediated device-related infections are caused by *S. epidermidis* and some of *S. aureus* that do not possess the *nuc*1 gene. These conclusions are likely to be the focus of future studies.

We have presented evidence for the biological role of staphylococcal nuclease in biofilm formation. Our study demonstrated the role staphylococcal nuclease has preventing biofilm development and formation by *S. aureus* and other biofilm-forming bacteria. This suggests that staphylococcal nucleases and DNase I treatment inhibit biofilm formation and might be beneficial as an early prophylactic measure to prevent the establishment of chronic biofilm-related infections. A combination of phenotypic and genotypic tests is recommended for future investigations of the influence of staphylococcal nucleases on biofilm formation in more clinically-related *S. aureus* strains.

This work was supported by the National Natural Science Foundation of China (Grant Nos. 31071515 and 31070113), the Special Foundation for Young Scientists of Sichuan Province, China (Grant No. 2011JQ0043), and the Fundamental Research Funds for the Central Universities, Southwest University for Nationalities (Grant No. 09NZYZJ04). We thank Dr. Karen Battista (International Association for Food Protection, Kraft Foods North America Inc., USA) for S. aureus RN4220. We also thank Yao Hang of the Laboratory of Huazhong Agricultural University for assistance with CLSM and Qin LiHong of Huazhong Agricultural University for assistance with SEM.

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