Chapter 2

Adhesion Force Sensing and Activation of a Membrane-Bound Sensor to Activate Nisin Efflux Pumps in Staphylococcus aureus Under Mechanical and Chemical Stresses

Vera Carniello, Akshay K. Harapanahalli, Henk J. Busscher and Henny C. van der Mei
J Colloid Interface Sci 2018; 512:14-20.

Reprinted with permission from Elsevier
ABSTRACT

Nisin-associated-sensitivity-response-regulator (NsaRS) in *Staphylococcus aureus* is important for its adhesion to surfaces and resistance against antibiotics, like nisin. NsaRS consists of an intra-membrane-located sensor NsaS and a cytoplasmatically-located response-regulator NsaR, which becomes activated upon receiving phosphate groups from the intra-membrane-located sensor.

*Hypothesis:* The intra-membrane location of the NsaS sensor leads us to hypothesize that the two-component NsaRS system not only senses "chemical" (nisin) but also "mechanical" (adhesion) stresses to modulate efflux of antibiotics from the cytoplasm.

*Experiments:* NsaS sensor and NsaAB efflux pump transcript levels in *S. aureus* SH1000 adhering to surfaces exerting different adhesion forces were compared, in presence and absence of nisin. Adhesion forces were measured using single-bacterial contact probe atomic force microscopy.

*Findings:* Gene expression became largest when staphylococci experienced strong adhesion forces combined with nisin-presence and the two-component NsaRS response to antibiotics was enhanced at a stronger adhesion force. This confirms that the intra-membrane-located sensor NsaS senses both chemical and mechanical stresses to modulate antibiotic clearance through the NsaAB efflux pump. This finding creates better understanding of the antibiotic resistance of bacteria adhering to surfaces and, in the fight against antibiotic-resistant pathogens, may aid development of advanced biomaterials on which bacterial efflux pumps are not activated.
INTRODUCTION

Staphylococci possess an extensive infection capacity in a plethora of ecological niches within the host, including the surfaces of biomaterial implants and devices. An infection related to the presence of a biomaterial implant surface starts with the reversible adhesion of bacteria to the implant surface, after which adhering bacteria embed themselves in a matrix of extracellular polymeric substances (EPS) to yield a transition to irreversible adhesion and biofilm growth commences. The EPS matrix protects biofilm inhabitants against biological, mechanical and chemical stresses, such as the host immune response, fluid shear and antibiotic treatment [1]. Different biomaterials used in the clinical practice have different tendencies to become colonized and cause infection [2]. Moreover, antibiotic resistance of Staphylococcus epidermidis biofilms is related to the biomaterial used [3]. This indicates that adhering bacteria can sense properties of the biomaterial they adhere to.

Staphylococcus aureus interacts with its surrounding through a wide range of environmental sensors, often intra-membrane located, to regulate gene expression in response to environmental stimuli. Bacteria, including S. aureus, can either use one- or two-component systems to process these stimuli [4–6]. Nisin associated sensitivity response regulator (NsaRS) is a recently discovered two-component system in S. aureus, consisting of an intra-membrane bound histidine kinase NsaS and a cytoplasmatically located response regulator NsaR that becomes activated upon receiving phosphate groups from the NsaS sensor [7]. Intra-membrane located NsaS can sense environmental stimuli, such as chemical stress due to antibiotics or antimicrobial peptides targeting the cell wall, like nisin [8], and reprograms bacterial gene expression to reduce stress (see Fig 1). The cytoplasmatically located response regulator NsaR then activates or represses the target gene expression [9]. One of the target genes is nsaA, which upregulates the NsaAB efflux pump (Figure 1) to detoxify the cell and promote survival in the presence of a chemical stress, such as antibiotics [8].

Bacterial interactions with their environment are not only stimulated by chemical stresses, but mechanical stresses exerted by adhesion forces between a substratum surface and adhering bacteria, constitute another mechanism of environmental sensing by bacteria [10]. In Escherichia coli for instance, initial surface adhesion causes mechanical stress to the cell envelope and activates the Cpx pathway for biofilm formation [6]. Similarly, Vibrio parahaemolyticus uses the ScrABC pathway [11] for surface sensing and biofilm formation. Adhesion forces arising from substratum surfaces exerted on adhering staphylococci have been shown to impact cell wall deformation [12], ica expression and associated production of the extracellular matrix component
poly-N-acetylglycosamine [13]. Although in *S. aureus*, NsaRS activation is clearly associated with chemical stress [7,8], the intra-membrane location of the NsaS sensor also suggests its possible activation by membrane deformation as a result of mechanical stress, i.e. the adhesion forces experienced. Therefore, we here hypothesize that the NsaRS system can sense both chemical and mechanical stresses and therewith plays an important role in biomaterial induced modulation of antibiotic resistance via the NsaAB efflux pump.

Here, we report on the verification of the above hypothesis by investigating differences in transcript levels of the NsaS sensor and NsaAB efflux pump during early *S. aureus*

![Figure 1](image.png)

**Figure 1.** Schematic presentation of the NsaRS two-component system regulating the downstream transporter NsaAB to remove possible antibiotics such as nisin, from the cytoplasm. The NsaS intra-membrane bound histidine kinase sensor rearranges itself upon sensing an environmental, chemical stress (1) and subsequently autophosphorylates to transfer phosphate to the NsaR response regulator (2). The NsaR activates or represses target gene expression (3), such as *nsaA*, which leads to upregulation of the NsaAB efflux pump (4). The NsaAB efflux pump modulates antibiotic resistance by pumping chemicals out of the bacterial cells [7,8].
biofilm formation in the presence and absence of nisin on two common biomaterials (stainless steel (SS) and polyethylene (PE)) exerting different adhesion forces on *S. aureus*. Bacterial adhesion forces are measured using single-bacterial contact probe atomic force microscopy (AFM) as an indicator of mechanical stress, while the presence of nisin constitutes a chemical stress.

**MATERIALS AND METHODS**

**Bacterial strains and culture conditions**
The bacterial strain *S. aureus* SH1000 was used in this study and kindly provided by Dr. Lindsey N. Shaw, Department of Cell Biology, Microbiology and Molecular Biology, University of South Florida, Tampa, FL, USA. The strain was cultured aerobically at 37 °C on blood agar. One colony was inoculated in 10 mL Tryptone Soya Broth (TSB, OXOID, Basingstoke, UK) and grown for 24 h at 37 °C. The pre-culture was used to inoculate a main culture, by suspending staphylococci in 10 mL TSB (1:100) and culturing for 16 h. Subsequent processing of the main culture was done as described under the different experimental sections.

**Materials and preparation**
SS and PE were prepared to possess a comparable surface roughness in the micron-range, i.e. 1.8 ± 0.2 μm and 1.0 ± 0.2 μm for SS and PE, respectively [13]. Briefly, SS was polished using 1200 grit SiC paper followed by MetaDi 3 μm diamond suspension (Buehler, Lake bluff, IL, USA) on a polishing mat for 15 min, using a mechanical polisher Phoenix Beta, fitted with Vector™ power head (Buehler, Dusseldorf, Germany). PE was used as received from the manufacturer (Goodfellow Cambridge Ltd, Huntingdon, England). Coupons were made to fit into a 6-wells plate with a total surface area of 7.5 cm², sterilized with ethanol (96 %), washed with sterile PBS and stored in sterile demineralized water until use.

**Water contact angles measurements**
Water contact angles were measured on SS and PE surfaces at 25 °C using the sessile drop technique with a home-made contour monitor. Droplets of 1.5–2 μL ultra-pure water were put on clean surfaces and contours measured between 5–10 s after placing a droplet, from which contact angles were subsequently calculated after grey-value thresholding. Contact angles were measured in triplicate on each of three different coupons for each material.
**Adhesion force measurements**

In order to measure adhesion forces between the *S. aureus* SH1000 and SS and PE surfaces, staphylococci from a main culture were suspended to a low (pivotal to create single-bacterial contact AFM probes) concentration of $3 \times 10^6$ bacteria mL$^{-1}$ in 10 mM potassium phosphate buffer, pH 7.0, and immobilized on a tipless cantilever (NPO, Bruker AFM Probes, Camarillo, CA, USA) for AFM via electrostatic interaction with poly-L-lysine (PLL; Sigma-Aldrich, USA) using a micromanipulator (Narishige Groups, Tokyo, Japan). The far end of the cantilever was dipped in a droplet of PLL for 1 min and dried in air (2 min), followed by 1 min immersion in a droplet of bacterial suspension to let one bacterium adhere. To prove that we had a single-bacterial contact probe (see Figure 2), a calibration grid (HS-20MG BudgetSensors, Innovative Solutions Bulgaria Ltd., Sofia, Bulgaria) was imaged with a bacterial probe, a tipless cantilever and a regular AFM tip (NP; Bruker AFM Probes, Camarillo, CA, USA; length and width at the top 3 and 1 µm respectively, with a half angle at the tip end of 18 degrees), as described in detail before [14].

Freshly prepared bacterial probes were directly used for adhesion force measurements. Adhesion force measurements were performed at room temperature in phosphate buffered saline (PBS; 10 mM potassium phosphate, 0.15 M NaCl, pH 7.0) using a Dimension 3100 system (Nanoscope V, Digital Instruments, Woodbury, NY, USA). For each bacterial probe, force-distance curves were measured without surface delay at a 2 nN trigger threshold. Bacterial probes were prepared out of three different cultures. Three bacterial probes, prepared from three separately grown bacterial cultures, were used. With each probe, three different locations were measured on one coupon of each material, recording 10 force curves on each location. The spring constant of each cantilever was determined using the thermal method [15]. The integrity of a bacterial probe was monitored before and after the onset of each ten adhesion force measurements by comparing adhesion forces measured on a clean glass surface. Whenever this adhesion force had a difference $> 0.5$ nN, data obtained last with that probe were discarded and a new bacterial probe was made.

**nsaS and nsaA gene expression**

The main culture was diluted (1:5) and 3 mL was used to grow 3 h biofilms on coupons made of SS and PE in a 6 wells plate at 37 °C aerobic under static conditions in the presence and absence of 2 µg mL$^{-1}$ nisin, a sub-minimal inhibitory concentration (MIC for *S. aureus* SH1000 is 4 µg mL$^{-1}$ [7]). Biofilms were harvested by transferring the coupons with biofilms to a new 6 wells plate, washing the coupons twice with PBS and resuspending the staphylococci in 1 mL PBS by repeated pipetting. The
suspended biofilm was centrifuged at 4000 x g for 10 min, the supernatant was removed and the pellets were stored at -20 °C until RNA isolation.

Total RNA was isolated using RiboPure™-Bacteria Kit (Ambion, Invitrogen, Foster City, CA) according to the manufacturer’s instructions. Traces of genomic DNA were removed using DNAfree™ kit (Ambion, Applied biosystems, Foster City, CA) and the absence of genomic DNA contamination was verified by real-time PCR prior to cDNA synthesis. The amount and quality of extracted RNA was based on the 260/280 nm ratio measured by NanoDrop ND-1000 (NanoDrop technologies LLC, ThermoFisher Scientific, Wilmington, DE). A ratio of around 2.0 (± 10%) was accepted as “pure” for RNA. 200 ng of RNA was used for cDNA synthesis, 4 μl 5x iScript Reaction Mix, 1 μl iScript Reverse Transcriptase, in a total volume of 20 μl (Iscript, Biorad, Hercules, CA) according to manufacturer’s instructions. Real time RT-qPCR was performed in a 384-well plate HSP-3905 (Bio-RAD, Laboratories, Foster city, CA, USA) with the primer sets for 16S rRNA, nsaS and nsaA (Table 1). The following thermal conditions were used for all qPCR reactions: 95 °C for 15 min and 40 cycles of 95 °C for 15 s and 59 °C for 20 s. The mRNA levels were quantified in relation to endogenous control gene 16s rRNA. The nsaS and nsaA expression levels in the biofilms were normalized to S. aureus SH1000 planktonic culture. Gene expression was assessed in triplicate experiments with separately grown staphylococcal cultures.

**Nisin susceptibility of S. aureus SH1000 biofilms**

In order to determine the nisin susceptibility of S. aureus SH1000 biofilms, the efficacy of the NsaS sensor and NsaAB efflux pump system was evaluated in early S. aureus biofilms. First, biofilms were grown on PE and SS coupons as described before in the presence and absence of increasing concentrations of nisin above the

**Table 1.** Primer sequences for RT-qPCR used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>nsaS-forward</td>
<td>GCAACATGGCATGCACCTC</td>
</tr>
<tr>
<td>nsaS-reverse</td>
<td>AGGTTATAATGGCCAGCGCC</td>
</tr>
<tr>
<td>nsaA-forward</td>
<td>TGCATGCCATGTTGCT</td>
</tr>
<tr>
<td>nsaA-reverse</td>
<td>TTCACCAGCTTCAACT</td>
</tr>
<tr>
<td>16S-forward</td>
<td>TACGGGAGGCAGACGAG</td>
</tr>
<tr>
<td>16S-reverse</td>
<td>ATTACCAGCGGCTGCTGG</td>
</tr>
</tbody>
</table>
nisin-MIC of *S. aureus* SH1000. Bacteria were then collected by repeated pipetting, serially diluted, plated on TSB agar and incubated overnight at 37 °C for colony forming units (CFU) counting. Experiments were performed in triplicate with separately grown staphylococcal cultures.

**Statistical analysis**

GraphPad Prism, version 5 (San Diego, CA) was employed for statistical analysis. Statistical significance was analyzed with a one-way analysis of variance (ANOVA), followed by Tukey HSD post-hoc test considering a confidence interval of 95 % (p < 0.05).

**RESULTS AND DISCUSSION**

**Staphylococcal adhesion forces to SS and PE surfaces**

Adhesion forces for *S. aureus* SH1000 were measured using single-bacterial contact probe AFM [16]. Single-bacterial contact was verified in separate experiments as demonstrated in Figure 2, in which images of a calibration grid (Figure 2A) are compared for a single-bacterial contact probe prepared on a tipless cantilever, a tipless cantilever without bacteria and a silicon nitride AFM tip. The bacterial probe measured the width of the AFM calibration grid equally well as the AFM tip, while the cantilever itself without a bacterium yielded a greatly distorted image of the grid with wrong grid dimensions (Figure 2C). Note that the bacterium should be located at the end of the cantilever in order to ensure contact between the bacterium and the substratum surface only (Figure 2B), as only this position yields an undistorted image of the grid. Thus, prepared probes were used to obtained force-distance curves (Figure 3A) between *S. aureus* SH1000 and SS and PE coupon surfaces, yielding adhesion forces between 3.5 ± 0.2 nN and 6.2 ± 0.2 nN, respectively (Figure 3B). These force values are in the same order of magnitude [17] or smaller [18] than staphylococcal adhesion forces obtained using other methods to prepare single-contact bacterial AFM probes, confirming our adhesion forces represent single-bacterial contact.

Bacterial adhesion forces were significantly stronger (p < 0.005) on PE (6.2 ± 0.2 nN) than on SS (3.5 ± 0.2 nN) surfaces. Such differences in adhesion forces can arise from several factors, including the physico-chemical properties of the SS and PE. Bacteria experience in general stronger adhesion forces on hydrophobic surfaces than on hydrophilic surfaces. Thewes *et al.* [19] have shown that *Staphylococcus carnosus* adhered two times stronger to hydrophobic silicon wafers than to hydrophilic ones, suggesting that short-range, non-specific hydrophobic interactions present between bacterial cell wall proteins and substratum surfaces enhance the adhesion forces.
forces. Our results show a similar trend with significantly stronger adhesion forces to hydrophobic PE (water contact angle 85 ± 2 degrees) compared to hydrophilic SS (water contact angle 35 ± 3 degrees) for *S. aureus* SH1000.

**nsaS and nsaA gene expression under mechanical stress**

Gene expression of *nsaS* and *nsaA* of *S. aureus* SH1000 upon mechanical stress, i.e. adhesion to SS and PE surfaces, was measured by isolating total RNA from biofilms grown on SS and PE surfaces (see Supplementary material Figure S1 for RT-qPCR amplification plots and melting curves of gene transcripts). The normalized level of *nsaS* and *nsaA* gene expression did not vary significantly on the two surfaces, despite exerting different adhesion forces (*p* > 0.05, Figure 3C and 3D). However, all normalized gene expression levels were above unity, representing the gene expression level measured for planktonic bacteria, although this was not statistically significant.

---

**Figure 2.** Use of a calibration grid to demonstrate single-bacterial contact probe AFM. (A) Schematic presentation of the dimensions of the AFM calibration grid used to check whether bacterial AFM probes yield single-bacterial contact. Multiple-bacterial contact of the AFM probe would not correctly reflect the dimensions of the grid and yield double contour lines in imaging. (B) Fluorescence image of LIVE/DEAD stained *S. aureus* SH1000 (Baclight viability stain; Molecular Probes Europe BV, Leiden, The Netherlands) on a tipless AFM cantilever. Viable bacteria are green fluorescent, scale bar represents 20 μm. (C) Height images (20 μm x 20 μm) of the topography of the calibration grid (see panel A) and corresponding height profiles obtained with - a regular AFM tip, - a tipless, PLL-coated cantilever without bacteria and - a cantilever with *S. aureus* SH1000. Note that double contour lines are equally absent when using the bacterial-probe as when using a cantilever with a regular AFM tip.
Surface sensing under mechanical stress occurs through deformation of the bacterial cell wall, and adhesion forces in the order of magnitude of 0.5 - 1 nN have been shown to yield substantial cell wall deformation in staphylococci, as demonstrated using surface fluorescence enhancement [13]. Whereas in S. aureus ATCC 12600 expression of icaA and poly-N-acetylglucosamine upon adhesion to different biomaterial surfaces increased with increasing adhesion forces [13], the levels of nsaS and nsA gene expressions of S. aureus SH1000 do not respond to increasing adhesion forces over the range of force values observed here, but seem to respond to the absence (planktonic state) or presence of adhesion forces (adhering state).

**nsaS and nsA gene expression under chemical and mechanical stresses**

In order to study the combination of a chemical and mechanical stress on nsaS and nsA gene expression, expression was also measured in the presence of nisin for S. aureus SH1000 biofilms grown on SS and PE. Normalized expression levels of both nsaS and nsA increased in the presence of nisin for biofilms on both surfaces, compared to normalized expression levels in biofilms grown in the absence of nisin. However, gene expression levels only increased significantly (p < 0.05) on PE (Figure 3C and 3D), exerting the strongest adhesion force (6.2 nN) on adhering S. aureus SH1000. On less hydrophobic SS exerting a smaller adhesion force (3.5 nN), no increased gene expression levels were found (Figure 3C and 3D).

Thus it can be concluded that the influence of mechanical stress, i.e. adhesion forces arising from a substratum surface, in S. aureus SH1000 only become manifest in combination with the presence of a chemical stress, such as provided here by nisin. Since increased gene expression levels were only observed upon combination of a chemical stress and a high mechanical stress, this suggests that a threshold mechanical stress exists above which it is likely that the intra-membrane located NsaS sensor triggers additional expression of nsaS and nsA.

**Nisin susceptibility of S. aureus SH1000 biofilms on SS and PE**

In order to demonstrate that a stronger adhesion force and increased expression of intra-membrane bound nsaS and nsA also lead to increased phosphorylation of the cytoplasmically located response regulator NsaR, required for a more effective two-component efflux pump system and nisin clearance from the cytoplasm, viability of S. aureus SH1000 biofilms grown on SS or PE during exposure to nisin were determined. In the absence of nisin, less staphylococci were found adhering in biofilms on PE than on SS (Figure 4), which matches the slightly lower levels of nsaS expression on PE compared with SS in the absence of nisin (Figure 3C). As soon as nisin was introduced in a concentration above the nisin MIC, staphylococci adhering
strongly to PE survived in higher numbers than those more weakly adhering on SS. Although this trend was not significant at lower nisin concentrations, the difference in survival between bacteria adhering to PE and SS coupons increased with increasing nisin concentrations, reaching a statistically significant 10-fold difference at a nisin concentration of 512 μg mL⁻¹, a value that approaches the nisin minimum biofilm eradication concentration [20]. Similarly, also biofilms grown on silicone rubber, exerting strong adhesion forces on bacteria, exhibited lower susceptibility to antibiotics compared to biofilms grown on polymer-brush coated silicone rubber, generating extremely low adhesion forces on adhering bacteria [21].

Figure 3.
(A) Force-distance retraction curves for S. aureus SH1000 on SS (red line) and PE (green line).
(B) Adhesion forces of S. aureus SH1000 on SS (red column) and PE (green column). Each column represents an average ± standard deviation over 90 AFM force distance curves, obtained using three bacterial probes, prepared from three separately grown bacterial cultures and using each probe to measure adhesion forces on three different locations on one coupon of each material, recording 10 force curves on each location.
(C) nsaS expression normalized to 16s rRNA in S. aureus SH1000 adhering to stainless steel (SS) and polyethylene (PE) under mechanical and chemical (2 μg mL⁻¹ nisin) stress. For control, gene expression in a planktonic state was taken as a reference and set to unity. SS⁺ and PE⁺ (red and green shaded, respectively) indicate the presence of nisin for the respective conditions. Each column represents an average ± standard error of the mean over three individual experiments with separately grown staphylococcal cultures.
(D) Same as (C), now for nsaA expression.
Connecting lines between bars show statistical significance (p<0.05).
Since it has been demonstrated in general that stronger adhesion forces are associated with larger nanoscale cell wall deformation, including deformation of the cytoplasmic membrane, activation of membrane located sensors may constitute the onset of increased survival of bacteria strongly adhering to a substratum surface. Bacterial cell wall deformation changes the pressure profile across the lipid membrane [22] with a possible impact on the membrane located proteins constituting the NsaS sensor.

CONCLUSIONS

In conclusion, while activation of NsaRS efflux pumps in *S. aureus* has been associated with chemical stress in planktonic staphylococci [7,8], we here demonstrate that adhesion to substratum surfaces constitutes a mechanical stress that plays an important role too in antibiotic clearance from the cytoplasm in *S. aureus* through membrane located efflux pumps. The intra-membrane located NsaS sensor was more sensitive to nisin when staphylococci adhered more strongly to a biomaterials

![Graph](image.png)

**Figure 4.** The number of CFU retrieved from *S. aureus* SH1000 biofilms grown on PE (green) or SS (red) as a function of the nisin concentration in the growth medium. MIC for nisin is 4 µg mL⁻¹ [7] and the minimum biofilm inhibitory and eradication concentrations are 64 µg mL⁻¹ [20] and > 256 µg mL⁻¹ [20], respectively. Each data point represents an average ± standard error of the mean over six coupons of each material prepared from three separately grown bacterial cultures.
surface. The enhanced expression of the *nsaS* and *nsaA* genes due to this increased sensitivity directly leads to increased phosphorylation of cytoplastically located NsaR sensor and therewith to stimulation of the two-component NsaRS efflux pump system, as evidenced by increased survival of staphylococci adhering more strongly to PE as compared to SS. These findings contribute to a better understanding of the influence of adhesion forces on antibiotic resistance in bacteria adhering to surfaces, and may lead to the development of advanced biomaterials on which bacterial efflux pump systems are not stimulated. Such developments are direly needed in the fight against increasing numbers of antibiotic-resistant bacterial pathogens.

**ACKNOWLEDGEMENTS**

This study was entirely funded by UMCG, Groningen, The Netherlands. H.J.B is also director of a consulting company SASA BV. The authors declare no potential conflicts of interest with respect to authorship and/or publication of this article. Opinions and assertions contained herein are those of the authors and are not construed as necessarily representing views of the funding organization or their respective employer(s).
REFERENCES


Figure S1.
(A) Real time RT-qPCR amplification plots for *nsaA* and *nsaS* and endogenous control gene *16s rRNA*.
(B) Melting curves of *16s rRNA* gene transcripts detected by real time RT-qPCR.
(C) Same as (B) for *nsaS*.
(D) Same as (B) for *nsaA*.