Adhesion force sensing and activation of a membrane-bound sensor to activate nisin efflux pumps in *Staphylococcus aureus* under mechanical and chemical stresses

Vera Carniello¹, Akshay K. Harapanahalli¹, Henk J. Busscher, Henny C. van der Mei

*University of Groningen and University Medical Center Groningen, Department of Biomedical Engineering, Groningen, The Netherlands*

**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 cytoplasmically-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
**1. 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 (Fig. 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 para-
haemoliticus 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 biofilm formation
in the presence and absence of nisin on two common biomaterials
(stainless steel (SS) and polyethylene (PE)) exerting different adhe-
sion 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.

2. Materials and methods

2.1. 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 Biol-
ogy, Microbiology and Molecular Biology, University of South Flor-
da, 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 suspend-
ing staphylococci in 10 ml TSB (1:100) and culturing for 16 h. Sub-
sequent processing of the main culture was done as described
under the different experimental sections.

2.2. 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).
Coupions 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.

2.3. 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 ultrapure water were put on clean
surfaces and contours measured between 5 and 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.

2.4. 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 ± 10³ 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 Fig. 2), a
 calibration grid (HS-20MG BVSensors, 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 respec-
tively, 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 adhe-
sion force measurements. Adhesion force measurements were per-
formed at room temperature in phosphate buffered saline (PBS;
10 mM potassium phosphate, 0.15 M NaCl, pH 7.0) using a Dimen-
sion 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. Bacte-
rial 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 bacte-
rial probe was monitored before and after the onset of each ten
adhesion force measurements by comparing adhesion force mea-
sured on a clean glass surface. Whenever this adhesion force had a
difference >0.5 nN, data obtained last with that probe were dis-
carded and a new bacterial probe was made.

2.5. 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 staphy-
lococci in 1 ml PBS by repeated pipetting. The suspended biofilm
was centrifuged at 4000g 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 instruc-
tions. Traces of genomic DNA were removed using DNA-
free™ 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
5× 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 HSD-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 16 s 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.

### 2.6. 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 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.

### 2.7. 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).

### 3. Results and discussion

#### 3.1. 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 Fig. 2, in which images of a calibration grid (Fig. 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 (Fig. 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 (Fig. 2B), as only this position yields an undistorted image of the grid. Thus prepared probes were used to obtained force-distance curves (Fig. 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 (Fig. 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. 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.

3.2. 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 Fig. 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, Fig. 3C and D). However, all normalized gene expression levels were above unity, representing the gene expression level measured for planktonic bacteria, although this was not statistically significant.

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 nsaA 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).

3.3. nsaS and nsaA gene expression under chemical and mechanical stresses

In order to study the combination of a chemical and mechanical stress on nsaS and nsaA 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 nsaA 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 (Fig. 3C and D), 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 (Fig. 3C and D).

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 nsaA.

3.4. 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 nsaA 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 absence of nisin, less staphylococci were found adhering in biofilms on PE than on SS, (Fig. 4) which matches the slightly lower levels of nsaS expression on PE compared with SS in the absence of nisin (Fig. 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].

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.

4. 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 surface. The enhanced expression of the nsaS and nsaA genes due to this increased sensitivity directly leads to increased phosphorylation of cytoplasmically 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).

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jcis.2017.10.024.

References


