Cell wall deformation and Staphylococcus aureus surface sensing
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Chapter 4

Influence of Adhesion Force on icaA and cidA Gene Expression and Production of Matrix Components in Staphylococcus aureus Biofilms

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ABSTRACT

The majority of human infections are caused by biofilms. The biofilm mode of growth enhances the pathogenicity of *Staphylococcus* spp. considerably, because once adhering staphylococci embed themselves in a protective, self-produced matrix of extracellular-polymeric-substances (EPS). The aim of this study is to investigate the influence of staphylococcal adhesion forces to different biomaterials on *icaA* (regulating production of EPS matrix components) and *cidA* (associated with cell lysis and eDNA release) gene expression in *Staphylococcus aureus* biofilms. Experiments were performed with *S. aureus ATCC12600* and its isogenic mutant *S. aureus ATCC12600Δpbp4*, deficient in peptidoglycan cross-linking. Deletion of *pbp4* was associated with greater cell-wall deformability, while not affecting planktonic growth rate, biofilm formation or cell-surface-hydrophobicity or zeta-potential of the strains. Adhesion forces of *S. aureus ATCC12600* were strongest on polyethylene (4.9 ± 0.5 nN), intermediate on polymethylmethacrylate (3.1 ± 0.7 nN) and weakest on stainless steel (1.3 ± 0.2 nN). Production of poly-N-acetylglucosamine, eDNA presence and expression of *icaA* genes decreased with increasing adhesion forces. However, no relation between adhesion forces and *cidA* expression was observed. Adhesion forces of the isogenic mutant *S. aureus ATCC12600Δpbp4* (deficient in peptidoglycan cross-linking) were much weaker than of the parent strain and did not show any correlation with the production of poly-N-acetylglucosamine, eDNA nor the expression of *icaA* and *cidA* genes. This suggests that adhesion forces modulate the production of matrix molecules poly-N-acetylglucosamine, eDNA and *icaA* gene expression by inducing nanoscale cell-wall deformation with a pivotal role of cross-linked peptidoglycan layers in this adhesion force sensing.
INTRODUCTION

*Staphylococcus spp.* present an important group of potentially pathogenic strains and species. According to estimates by The National Institutes of Health, about 80% of all human infections are caused by biofilms (1). The biofilm mode of growth enhances the pathogenicity of *Staphylococcus spp.* considerably when formed on the surfaces of biomaterial implants and devices, such as total knee or hip arthroplasties or pacemakers (2). Biofilm formation starts with the adhesion of individual organisms to a substratum surface. Initially, adhesion is reversible but the bond between an adhering organism and a substratum surface rapidly matures over time to become stronger and eventually adhesion is irreversible (3). Adhesion is further enforced through the production of a matrix consisting of Extracellular Polymeric Substances (EPS) by the adhering organisms in which they grow and find shelter against the host immune system and antibiotic treatment. EPS composition largely depends on bacterial strains and environmental conditions, but major components of EPS across different species are polysaccharides, proteins and extracellular DNA (4).

It is difficult to envision how adhering bacteria regulate EPS production in response to their adhesion to different surfaces. Recently, we have proposed that the bacterial response to adhesion is dictated by the magnitude of the force by which a bacterium adheres to a surface (5) and distinguished three regimes of adhesion forces (Figure 1). In the planktonic regime, bacteria adhere weakly and accordingly cannot realize that they are on a surface and retain their planktonic phenotype. The opposite regime is called the lethal regime, where strong adhesion forces lead to high cell-wall stresses, retarded growth and finally cell death. Both the planktonic regime as well as the lethal regime occur mostly after application of coatings, like highly hydrated and hydrophilic polymer brush coatings or positively charged quaternary ammonium coatings exerting strong, attractive electrostatic forces on adhering bacteria, which are usually negatively charged under physiological conditions (6). Most biomaterials used for implants and devices however, exert intermediate adhesion forces on adhering bacteria and this regime is called the interaction regime. In the interaction regime, bacteria were hypothesized to respond to the adhesion forces exerted by a surface through production of various matrix components. Clinically indeed, biofilms of the same strain can have different pathogenicity when formed on different biomaterials (7). For example in abdominal wall surgery, hydrophobic surgical meshes made of polytetrafluoroethylene are more susceptible to infection than meshes of less hydrophobic polypropylene (8). On orthopedic biomaterials, icaA expression by *Staphylococcus*
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epidermidis and EPS production were higher on polyethylene than on polymethylmethacrylate. Moreover, biofilms on polyethylene showed lower susceptibility to gentamicin relative to biofilms on polymethylmethacrylate (9).

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**Figure 1** Regimented scheme for the interaction of bacteria with substratum surfaces. Weakly adhering bacteria remain to have a planktonic phenotype, while strongly adhering ones die upon contact. In the interaction regime bacteria are hypothesized to respond to their adhering state with differential gene expression according to the adhesion force value they experience (5).

Little is known however, on the exact role of adhesion forces on the complex response of adhering bacteria in the interaction regime. A likely hypothesis is that the adhesion forces cause nanoscale cell-wall deformations and membrane stresses that act as a signaling mechanism for an organism to its adhering state. Therefore, the response of bacteria to their adhering state will not only differ on different biomaterials but will also depend on the rigidity of the cell-wall itself as maintained in Gram-positive strains by a relatively thick layer of cross-linked peptidoglycan. Measuring nanoscale cell wall deformation upon bacterial adhesion to a surface is extremely difficult. Recently a new, highly sensitive method has been proposed based on surface enhanced fluorescence that measures cell-wall deformation over a large number of adhering bacteria under the influence of the naturally occurring adhesion forces arising from a substratum surface (10). Surface enhanced fluorescence is the phenomenon of increased fluorescence when fluorophores come closer to a reflecting metal surface. It was first described for fluorescent proteins (11) and ranges over a distance of 30 nm beyond which it decreases exponentially with separation distance between the fluorophore and the reflecting surface. This relationship between
surface enhanced fluorescence and separation distance was validated using fluorescent proteins attached to polymeric spacers of varying lengths (12) and forms the basis for the interpretation of surface enhanced fluorescence of adhering fluorescent bacteria in terms of deformation of their cell wall. This method has a drawback that it can only be applied on reflecting metal surfaces, but bears as advantages with respect to atomic force microscopy e.g., that there are no external forces applied on an adhering bacterium, while it also measures a large number of adhering bacteria simultaneously.

The aim of this study is to investigate the influence of adhesion to different common biomaterials on icaA and cidA gene expression in Staphylococcus aureus biofilms. To this end, we first measure staphylococcal adhesion forces to different biomaterials and relate these adhesion forces with the expression of icaA and cidA genes. The ica operon is present in S. aureus and is mainly involved in production of capsular polysaccharides upon activation (13). Recently, it has also been reported that the ica locus is also required for colonization and immunoprotection during colonizing the host (13, 14). IcaA and icaD synthesize poly-N-acetylglucosamine (PNAG) which supports cell-cell and cell-surface interactions (15). cidA expression is associated with cell lysis and the release of eDNA during planktonic growth to facilitate adhesion and biofilm formation (16). Therefore, eDNA is known to act as an essential glue to maintain the integrity of both the EPS matrix and biofilms as a whole (16, 17). All experiments were performed with S. aureus ATCC12600 and its isogenic mutant S. aureus ATCC12600Δpbp4, deficient in peptidoglycan cross-linking. Higher deformability of the S. aureus ATCC12600Δpbp4 cell-wall with respect to the wild-type strain was demonstrated using surface enhanced fluorescence.
MATERIALS AND METHODS

Bacterial strains and culture conditions

Bacterial strains *S. aureus* ATCC12600 and *S. aureus* ATCC12600Δ*pbp4* were used throughout this study. All the strains were stored at -80°C in Tryptone Soya Broth (TSB, OXOID, Basingstoke, UK) containing 15% glycerol. Bacteria were cultured aerobically at 37°C on blood agar or TSB-agar plates with 10 μg ml\(^{-1}\) tetracycline. One colony was inoculated in 10 ml TSB and grown for 24 h at 37°C. The pre-culture was then inoculated in 10 ml fresh TSB (1:100) and cultured for 16 h. The main culture (1:100) was used for 24 h biofilm growth, while for other experiments staphylococci were suspended in TSB or phosphate-buffered saline (PBS; 10 mM potassium phosphate, 0.15 M NaCl, pH 7.0) to the desired density, as determined either by OD\(_{578}\) nm (Genesys™ 20 visible spectrophotometer, Beun de Ronde, Abcoude, The Netherlands) or enumeration of the number of bacteria per ml using a Bürker-Türk counting chamber. A stable chromosomal mutation in *S. aureus* ATCC12600Δ*pbp4* was obtained by transfecting the temperature sensitive pMAD-\(pbp4\) plasmid, as previously described (18). pMAD-\(pbp4\) plasmid was obtained from Dr. M. G. Pinho, Laboratory of Bacterial Cell Biology, and Dr. S. R. Filipe, Laboratory of Bacterial Cell Surfaces and Pathogenesis, Instituto de Tecnologia Quimica e Biológica, Universidade Nova de Lisboa.

To confirm that *pbp4* deletion had an influence on cell-wall deformation using surface enhanced fluorescence, GFP expressing variants (*S. aureus* ATCC12600-\(GFP\) and *S. aureus* ATCC12600Δ*pbp4*-\(GFP\)) were made by introducing the plasmid PMV158 into the staphylococci, as controlled by the MalP promoter using electroporation and selected on 10 μg ml\(^{-1}\) tetracycline TSB-agar plates.

Cell-wall deformation

\(pbp4\) deletion was confirmed by PCR and its expression was quantified in both the staphylococcal strains using primer sets listed in Table 1. Main cultures were diluted 1:100 in 10 ml TSB and grown for 24 h under static conditions. Next, 1 ml of the resulting suspension was subjected to RNA isolation and cDNA synthesis procedures, as described below for icaA and cidA gene expression. To confirm that *pbp4* deletion had an influence on cell-wall deformation of the staphylococci, we applied a novel, highly sensitive method to demonstrate cell-wall deformation of bacteria adhering on reflecting metal surfaces based on
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surface enhanced fluorescence (19). Briefly, staphylococci suspended in PBS ($3 \times 10^8$ cells ml$^{-1}$) were allowed to sediment from a 0.075 cm high suspension volume above a stainless steel 316L (SS) substratum surface (7.6 x 1.6 cm) and the fluorescence radiance was measured as a function of time using a bio-optical imaging system (IVIS Lumina II, PerkinElmer, Inc., Hopkinton, MA, USA) at an excitation wavelength of 465 nm and emission wavelength between 515-575 nm. The IVIS was kept at 20°C with an exposure time of 5 s and images were taken from the entire SS substratum surface every 5 min over a period of 3 h. From three user defined regions of interest (1 cm$^2$) the average fluorescent radiance was determined with Living Image software package 3.1 (PerkinElmer Inc., USA). It was not necessary to correct the fluorescence enhancement for photobleaching because previously reported control experiments on glass showed negligible bleaching up to 5 h (19).

Staphylococcal sedimentation was monitored by direct observation and images of adhering bacteria were taken using a metallurgical microscope equipped with 40x objective (ULWD, CDPlan, 40PL, Olympus Co, Tokyo, Japan) connected to a CCD camera (Basler A101F, Basler AG, Germany). The images were analysed using an in-house developed software based on MATLAB to count the number of adhering bacteria in each image. Numbers of adhering bacteria over the entire substratum surface were subsequently expressed as a percentage with respect to the total number of bacteria present in the suspension volume ($0.912$ ml) above the substratum.

The increase of the fluorescence radiance due to sedimentation and adhesion of fluorescent staphylococci was measured relative to the fluorescence of suspended ones and expressed as a total fluorescence enhancement, $TFE(t)$, according to

$$TFE(t) = \frac{R(t) - R_0}{R(0) - R_0}$$

in which $R(t)$ denotes the fluorescence radiance at time $t$, while $R_0$ and $R(0)$ indicate the fluorescence radiance of a suspension in the absence of staphylococci and immediately prior to their sedimentation from suspension, respectively. Whereas total fluorescence enhancement is due to a combination of increasing numbers of sedimented staphylococci and their cell-wall deformation, increases in total fluorescence enhancement extending
beyond the time at which sedimentation is complete, are due to cell-wall deformation (19). Cell-wall deformation brings a larger volume of the bacterial cytoplasm closer to the surface and therewith more fluorophores inside the bacterium become subject to fluorescence enhancement, yielding a higher fluorescence signal. Fluorescence enhancement only occurs on reflecting substrata and accordingly effects of pbp4 deletion on cell-wall deformation were only examined on SS.

**Staphylococcal characteristics not-related to cell-wall deformation**

In order to verify that other characteristics relevant for the current study were not affected by pbp4 deletion, planktonic growth curves, biofilm formation, cell surface hydrophobicity and zeta potential of the bacterial cell surfaces were determined.

**Planktonic growth curves**

Planktonic growth curves of *S. aureus* ATCC12600 and *S. aureus* ATCC12600Δpbp4 were compared. Staphylococci were suspended in 10 ml TSB to an optical density OD₅₇₈nm of 0.05 and grown at 37°C under static conditions. Optical densities were subsequently measured as a function of time.

**Biofilm formation and quantitation**

Biofilms on SS, polymethylmethacrylate (PMMA) and polyethylene (PE) coupons were grown in triplicate in a 12-wells plate. After incubation for 6, 12 and 24 h at 37°C, the coupons with biofilms were carefully removed and placed into a new 12-wells plate and gently washed. The biofilms from three coupons of the same material were then suspended by repeated pipetting and pooled in 1 ml PBS. To measure the biofilm biomass, 1:10 dilutions of the pooled bacterial suspensions were prepared and optical densities OD₅₇₈nm were measured.

**Microbial Adhesion To Hydrocarbons (MATH)**

MATH was carried out in its kinetic mode (20) to reveal possible differences in adhesive cell surface properties between *S. aureus* ATCC12600 and its isogenic Δpbp4 mutant. To this end, staphylococci were suspended in phosphate buffer (10 mM potassium phosphate buffer, pH 7.0) to an optical density OD₅₇₈nm of 0.45-0.50 (A₀) and 150 μl hexadecane was added to
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3 ml bacterial suspension, and the two phase system was vortexed for 10 s (0.17 min) and allowed to settle for 10 min. The optical density ($A_t$) was measured and this procedure was repeated 5 more times (increasing vortexing times) and results were plotted as $\log (A_t / A_0 \times 100)$ against the vortexing time ($t$) to determine the rate of initial bacterial removal $R_0$ (min$^{-1}$) from the aqueous phase, i.e. their hydrophobicity as by the kinetic MATH assay, according to

$$R_0 = \lim_{t \to 0} \frac{d}{dt} \log \left( \frac{A_t}{A_0} \times 100 \right)$$

(Zeta potential) Bacterial suspensions of the wild-type and mutant strain were prepared as mentioned above. Main cultures were centrifuged at 4000 g for 10 min and washed 2 times in 10 ml PBS, pH 7.0. The washed pellets were resuspended in 10 ml PBS, pH 7.0 and zeta potentials were determined by particulate microelectrophoresis (Zetasizer nano-ZS; Malvern Instruments, Worcestershire, UK) at 25°C. The experiments were repeated three times and the data are presented as averages ± standard error of the mean.

Preparation of bacterial AFM probes and adhesion force measurements In order to measure adhesion forces between the $S. aureus$ strains and different biomaterials, staphylococci were immobilized on a cantilever for atomic force microscopy (AFM), as described before (21). Bacteria were cultured as described above, with the difference that they were washed and suspended in demineralized water. Adhesion force measurements were performed at room temperature in PBS using a Dimension 3100 system (Nanoscope V, Digital Instruments, Woodbury, NY, USA). For each bacterial probe, force-distance curves were measured with no surface delay at a 2 nN trigger threshold. Using the same bacterial probe, fifteen force measurements were recorded and three different probes were used on three random locations on each material surface. Adhesion forces were determined from the cantilever deflection data which were converted to force values (nN) by multiplication with the cantilevers spring constant according to Hooke’s law

$$F = K_{sp} \times D$$

where $K_{sp}$ is the spring constant of the cantilever and D is the deflection of the cantilever. The spring constant of each cantilever was determined using the thermal method (22). The integrity of a bacterial probe was monitored before and after the onset of each adhesion cycle.
by comparing adhesion forces measured on a clean glass surface. Whenever this adhesion force differed more than 0.5 nN, data obtained last with that probe were discarded and a new bacterial probe was made.

**icaA and cidA gene expression**

Gene expression analysis was performed on 1 h, 3 h and 24 h old biofilms. Biofilms were grown by adding 2 ml of 1:100 diluted main culture with growth medium to each sample. Total RNA from the biofilms was isolated using RiboPure™-Bacteria Kit (Ambion, Invitrogen) according to the manufacturer’s instructions. Traces of genomic DNA was removed using DNAfree™ kit (Ambion, Applied biosystems, Foster City, CA) and absence of genomic DNA contamination was verified by real-time PCR prior to cDNA synthesis. cDNA synthesis was carried out using 200 ng of RNA, 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 triplicates in a 96-well plate AB0900 (Thermo Scientific, UK) with the primer sets for *gyrB*, *icaA* and *cidA* (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 60°C for 20 s. The mRNA levels were quantified in relation to endogenous control gene *gyrB*. Expression levels of *icaA* and *cidA* in all biofilms were expressed relative to biofilms grown on PE.
Production of matrix components in staphylococcal biofilms.

PNAG extraction and quantitation

Extraction of PNAG from *S. aureus* was performed as previously described (13). Briefly, 24 h staphylococcal biofilms grown on SS, PMMA and PE coupons as described above were suspended in 1 ml PBS for normalization, and diluted to an OD$_{578}$nm of 0.75 for slime extraction. The bacterial suspension was pelleted at 4000 g for 15 min, the supernatant was aspirated and the pellet was re-suspended in 50 μl 0.5 M EDTA (pH 8) and incubated 5 min at 100°C on a hot plate. Cell debris was pelleted at 8500 g for 5 min and 30 μl of the EPS containing supernatant was pipetted into fresh tubes. The samples were treated with 10 μl proteinase K (20 μg ml$^{-1}$) for 30 min at 37°C before quantitation. The concentrated EPS was diluted 1:100 with ultrapure water and 20 μl was blotted on nitrocellulose membrane using Bio-Dot® apparatus (Biorad, Hercules, CA). The nitrocellulose membrane was then blocked using 1% bovine serum albumin-Tris buffered saline (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 0.05% Tween20) for 1 h under mild shaking at room temperature. The membrane was subsequently incubated with the lectin (wheat germ agglutinin, Sigma-Aldrich, Saint Louis, USA) isolated from *Triticum vulgaris* that detects 1,4 β-N-acetyl-D-glucosamine, labeled with biotin as a primary antibody in a 1:1000 dilution for 1.5 h under mild shaking at room temperature. Finally, Streptavidin-Infra Red Dye® (LI-COR Biosciences, Leusden, The Netherlands) was added as a secondary antibody in 1:10,000 dilution for 30 min under mild shaking at room temperature. The membrane was washed 3 times, for 10 min each, with Tween20-Tris buffered saline and the amount of PNAG measured using an Odyssey Infrared Imaging System (LI-COR Biosciences).

eDNA extraction and quantitation. Extraction of eDNA was performed, as previously described (14), but with some minor modifications. Briefly, biofilms grown for 24 h on SS,
PMMA and PE coupons as described above were suspended in 1 ml 500 mM NaCl containing 10 mM EDTA and 50 mM Tris.HCl, pH 7.5 and transferred into chilled tubes. OD$_{578\text{nm}}$ of the suspensions were measured for normalization and staphylococci were centrifuged at 4000 g for 15 min to separate bacteria and eDNA. The supernatant was collected and subjected to DNA extraction twice with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated using 1/10 (v/v) of 3 M sodium acetate and 2/3 (v/v) of ice cold isopropanol. After centrifugation (15 min, 4°C, 8500 g), the pellet was washed with 100% ethanol and air dried. The dried DNA pellet was dissolved in 50 μl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 7.5). The amount of eDNA was quantified using CyQuant cell proliferation assay kit (Invitrogen, molecular probes, Eugene, Oregon, USA) based on a calibration curve of λDNA from 0 to 1000 ng ml$^{-1}$. The eDNA samples were processed according to the manufacturer's instructions and measured by a fluorescence plate reader at an excitation wavelength of 485 nm and emission wavelength of 520 nm.

**Substratum surfaces, contact angle and surface roughness measurements**

Substratum surfaces used in this study were SS, PMMA and PE. All substratum surfaces were prepared to possess a comparable surface roughness in the micron-range, 1-2 μm in order to rule out possible effects of surface roughness. SS was polished using 1200 grid SiC paper followed by MetaDi 3 μm diamond suspension (Buehler, Lake bluff, IL, USA) on a polishing mat for 20 min, while PMMA and PE surfaces were used as received. Circular coupons of 0.5 mm thickness with a surface area of 3.1 cm$^2$ were made to fit into a 12-wells plate, sterilized with methanol, washed with sterile PBS and stored in sterile demineralized water until use. Water contact angles were measured on all materials at 25°C using the sessile drop technique in combination with a home-made contour monitor. Surface roughness of the biomaterials was determined by AFM (Nanoscope IV DimensionTM 3100) using a silicon nitride tip (Mountain View, CA, USA; probe curvature radius of 18 nm).
RESULTS

Physico-chemical surface properties of biomaterials

Hydrophobicities of the biomaterials were evaluated using water contact angles. Water contact angles varied considerably over the three materials included in this study. SS was the least hydrophobic material with an average water contact angle of 33 ± 9 degrees, followed by PMMA 69 ± 6 degrees and PE 84 ± 1. Surface roughnesses measured with AFM of the materials were all in the micron-range and amounted 1.8 ± 0.2 µm, 2.0 ± 0.4 µm and 1.0 ± 0.2 µm for SS, PMMA and PE, respectively.

Effects of pbp4 deletion on S. aureus

Peptidoglycan cross-links provide cell-wall rigidity, therefore effects on cell-wall deformation were determined from total fluorescence enhancement of S. aureus sedimenting and adhering to SS. The initial linear increase (1–2 h) in total fluorescence enhancement for S. aureus ATCC12600-GFP and S. aureus ATCC12600Δpbp4-GFP is due in part to an increase in the number of sedimented bacteria (compare Figure 2a and Fig 2b), but the slow increase in total fluorescence enhancement after 3 h once all staphylococci from the suspension have sedimented on the surface, is fully due to cell-wall deformation. Accordingly, it can be seen that S. aureus ATCC12600Δpbp4-GFP deforms to a greater extent than does S. aureus ATCC12600-GFP due to the absence of pbp4 crosslinking.
Figure 2 Effects of pbp4 deletion on cell-wall deformation. (a) Cell-wall deformation of *S. aureus* ATCC12600-GFP and *S. aureus* ATCC12600Δpbp4-GFP upon adhesion to SS, as measured using surface enhanced fluorescence. As an adhering bacterium deforms, its fluorescent intracellular content gets closer to the reflecting metal surface yielding a surface enhanced fluorescence that increases with increasing deformation. Each point represents an average ± standard error of the mean over three individual experiments. All differences between *S. aureus* ATCC12600 and *S. aureus* ATCC12600Δpbp4 are statistically significant (p < 0.05). (b) The number of adhering *S. aureus* ATCC12600-GFP and *S. aureus* ATCC12600Δpbp4-GFP on SS surfaces as a function of sedimentation time, expressed as a percentage of bacteria adhering (nₐ) with respect to the total number of bacteria (nₜ𝑜𝑡) in the suspension volume above the substratum surface. Each point represents an average ± standard error of the mean over three individual experiments. All differences between *S. aureus* ATCC12600 and *S. aureus* ATCC12600Δpbp4 are statistically significant (p < 0.05).

In order to establish that pbp4 deletion solely affected the cell-wall deformability of *S. aureus* ATCC12600 and no other properties, planktonic growth (Figure 3a), biofilm formation (Figure 3d and 3e), cell surface hydrophobicities (Figure 3B) using the MATH-test in its kinetic mode (20) and zeta potentials (Figure 3c) were compared with the ones of *S. aureus* ATCC12600Δpbp4. Growth curves, zeta potentials and cell surface hydrophobicities (initial removal coefficients R₀ of 0.0002 min⁻¹) of both strains were identical. Generally, *S. aureus* ATCC12600Δpbp4 formed less biofilm than *S. aureus* ATCC12600. For both strains on SS, more biofilm is formed than on PMMA and PE for all time points measured (Figure 3d, 3e), although no statistically significant differences could be established in amount of biofilm on the three substratum surfaces after 24 h of growth.
**S. aureus adhesion forces to different biomaterials**

The adhesion forces of *S. aureus* ATCC12600 and *S. aureus* ATCC12600Δpbp4 were measured using AFM, equipped with a bacterial probe as recently advocated by Alsteens et al. (23). For *S. aureus* ATCC12600 (Figure 4a), strongest adhesion forces were observed on the PE surface (4.9 ± 0.5 nN) that decreased in a statistically significant manner (p < 0.05) toward more hydrophilic PMMA (3.1 ± 0.7 nN) and SS (1.3 ± 0.2 nN) surfaces. Adhesion forces of the Δpbp4 mutant were significantly smaller (p < 0.05) than of *S. aureus* ATCC12600 (Figure 4b).

**Figure 3** Effects of expression of pbp4 in *S. aureus* ATCC12600 on strain characteristics not-related to cell-wall deformation. (a) Planktonic growth curves of *S. aureus* ATCC12600 and *S. aureus* ATCC12600Δpbp4 at 37°C (fully overlapping). (b) The optical density log (A_t/A_0 × 100) as a function of the vortexing time for the removal of *S. aureus* ATCC12600 and its isogenic mutant *S. aureus* ATCC12600Δpbp4.
ATCC12600Δpbp4 from an aqueous phase (10 mM potassium phosphate buffer, pH 7.0) by hexadecane. Absence of removal indicates a hydrophilic cell surface. Each point represents an average ± standard error of the mean over three individual experiments with separately grown staphylococcal cultures. None of the differences between S. aureus ATCC12600 and S. aureus ATCC12600Δpbp4 are statistically significant. (c) Zeta potentials of S. aureus ATCC12600 and S. aureus ATCC12600Δpbp4 in PBS, pH 7.0. Each point represents an average ± standard error of the mean over three individual experiments with separately grown staphylococcal cultures. None of the differences between S. aureus ATCC12600 and S. aureus ATCC12600Δpbp4 are statistically significant. (d) and (e) Biofilm formation of S. aureus ATCC12600 and S. aureus ATCC12600Δpbp4 expressed as OD$_{578}$ nm after 6, 12 and 24 h of growth on SS, PMMA and PE.

**Figure 4** S. aureus adhesion forces to different biomaterials. (a) Adhesion forces of S. aureus ATCC12600 to SS, PMMA and PE. (b) Similar as in (a), for S. aureus ATCC12600Δpbp4. Each bar represents an average of 135 adhesion force curves measured with 9 different bacterial probes taken from three separately grown staphylococcal cultures. Error bars represent the standard errors of the mean. * indicates significant differences (p < 0.05) in staphylococcal adhesion forces to different biomaterials (two tailed, two-sample equal variance Student’s t-test).
Production of matrix components and gene expression in relation with staphylococcal adhesion forces in 24 h old biofilms.

PNAG production normalized with respect to the amount of biofilm formed decreased with increasing adhesion force towards the more hydrophobic PE surface in a significant manner (p < 0.05) (Figure 5a). Normalized amounts of eDNA in 24 h *S. aureus* ATCC12600 biofilms decreased as well with increasing adhesion force (p < 0.05) (Figure 5b). However for 24 h *S. aureus* ATCC12600Δ*pbp4* biofilms, neither PNAG production nor eDNA presence relates in a significant way with its adhesion forces to different biomaterials (Figure 5c and 5d).

**Figure 5** *S. aureus* PNAG production and eDNA presence *versus* adhesion forces. (a) Normalized PNAG production in 24 h *S. aureus* ATCC12600 biofilms as a function of the adhesion force. (b) Normalized eDNA presence in 24 h *S. aureus* ATCC12600 biofilms as a function of the adhesion force. (c) Similar as in (a) for *S. aureus* ATCC12600Δ*pbp4*. (d)
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Similar as in (b) for *S. aureus* ATCC12600Δ*pbp4*. Linear regression analysis was performed in all graphs to analyse the correlation between PNAG production, eDNA presence and adhesion force. The drawn line represents the best fit to a linear function, while $r^2$ values represent the correlation coefficients. The dotted lines enclose the 95% confidence intervals. PNAG and eDNA were normalized to the amount of biofilm formed on each substratum and each point represents an average ± standard error of the mean over three individual experiments with separately grown staphylococcal cultures.

In Figure 6 we have plotted the staphylococcal adhesion forces on the different biomaterials *versus* their *icaA* and *cidA* gene expression in 24 h old biofilms, as responsible for the production of PNAG and eDNA respectively. In *S. aureus* ATCC12600, *icaA* gene expression decreased as adhesion forces increased (Figure 6a) in line with PNAG production. *cidA* gene expression did not follow a similar trend as that of *icaA* expression in 24 h old biofilms, but was equally expressed on all the biomaterials irrespective of the adhesion forces experienced over different biomaterials (Figure 6b). In *S. aureus* ATCC12600Δ*pbp4*, lacking peptidoglycan cross-linking, neither expression of *icaA* nor of *cidA* relates with its adhesion force to the different biomaterials (Figs. 6c and 6d).
Influence of Adhesion Force on Gene Expression

**Figure 6** *S. aureus* icaA and cidA gene expressions *versus* adhesion forces in 24 h old biofilms. (a) Normalized icaA expression in 24 h *S. aureus* ATCC12600 biofilms as a function of the adhesion force. (b) Normalized cidA expressions in 24 h *S. aureus* ATCC12600 biofilms as a function of the adhesion force. (c) Similar as in (a) for *S. aureus* ATCC12600Δpbp4. (d) Similar as in (b) for *S. aureus* ATCC12600Δpbp4. Linear regression analysis was performed in all graphs to analyse the correlation between gene expression and adhesion force. The drawn line represents the best fit to a linear function, while $r^2$ values represent the correlation coefficients. The dotted lines enclose the 95% confidence intervals. IcaA and cidA expression were normalized to gyrB and presented as normalized fold expression with respect to PE. Each point represents an average ± standard error of the mean over three individual experiments with separately grown staphylococcal cultures.
icaA gene expression in relation with staphylococcal adhesion forces in 1 and 3 h old biofilms of *S. aureus* ATCC12600

In order to assess the speed at which gene expression is regulated by the adhesion forces an adhering bacterium experiences, icaA gene expression was also assessed in 1 h and 3 h old biofilms of *S. aureus* ATCC12600 and plotted against adhesion forces (Figure 7). In 1 h old biofilms, icaA gene expression did not show any relation with adhesion force (Figure 7a), but in 3 h old biofilms (Figure 7b) a similar relation with adhesion force was observed as in 24 h old biofilms (compare Figure 7b and Figure 6a).

**Figure 7** icaA gene expression *versus* adhesion forces in 1 h and 3 h old biofilms of *S. aureus* ATCC12600.(a) Normalized icaA expression in 1 h old *S. aureus* biofilms as a function of the adhesion force. (b) Similar as in (a) in 3 h old *S. aureus* biofilms. Linear regression analysis was performed to analyse the correlation between icaA gene expression and adhesion force. The drawn line represents the best fit to a linear function, while $r^2$ values represent the correlation coefficients. The dotted lines enclose the 95% confidence intervals. IcaA expression was normalized to *gyrB* and presented as normalized fold expression with respect to PE. Each point represents an average ± standard error of the mean over three individual experiments with separately grown staphylococcal cultures.
DISCUSSION

In this study, we hypothesized that adhesion forces sensed by *S. aureus* upon adhesion to different biomaterials regulate the expression of two important genes *icaA* and *cidA*, known to contribute in the formation of their self-produced EPS matrix. Over the range of adhesion forces between 1 and 5 nN, *icaA* gene expression decreased with increasing adhesion forces in 3 h and 24 h old biofilms but not in 1 h old ones, while for *cidA* gene expression no influence of adhesion forces was found. Moreover, production of the EPS matrix components PNAG and eDNA decreased with increasing adhesion forces experienced by *S. aureus* ATCC12600 on different biomaterials, making it unlikely that *cidA* expression solely regulates eDNA release. The differences in eDNA presence in biofilms grown on SS, PMMA and PE can be caused by autolysin *atl* gene. This gene produces two functional proteins responsible for regulating growth, cell lysis and biofilm formation (24). The expression of the *alt* gene occurs under several external stress conditions (25) including adhesion as a potential trigger for DNA release. Since matrix components (PNAG and eDNA) provide an important means through which bacteria can evade the host immune response and antibiotic attack, we can speculate from the results in this study that pathogenicity of *S. aureus* biofilms is regulated in part by the adhesion forces arising from the substratum to which they adhere.

Bacterial behavior has been found to be extremely sensitive to minor differences in adhesion forces. In *S. aureus*, invasive isolates exhibited higher mean adhesion forces to a fibronectin-coated substratum by 0.28 nN than non-invasive control isolates (26). Moreover, strains of *Listeria monocytogenes* with adhesion forces to the silicon nitride tip of an AFM cantilever stronger than 0.38 nN were found more pathogenic than strains with smaller adhesion forces (27), coinciding with our conclusion on the impact of adhesion forces on *S. aureus* gene expression and associated pathogenicity. In the current study, we measured adhesion forces between *S. aureus* and different biomaterial surfaces with bacterial probe AFM. This method has been applied more often, but raises concerns as to whether contact is established by a single organism or multiple ones. In the past (28), we have noticed that multiple contacts seldom or never happen because bacteria attached to the cantilever are unlikely to be equidistant to the substratum surface within the small distance range of interaction forces. In addition, the bacterial probe is contacting the surface at an angle of 15 degrees which makes it less probable for multiple contacts. Multiple contact points however, would become evident from double contour lines when a bacterial probe is used for imaging.
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Routine checks on probes have never yielded double contour lines and hence it is safe to assume that our bacterial probes do not yield multiple contact points.

Biofilm formation starts with adhesion of so-called “linking film” bacteria, which provide the groundwork for further biofilm growth. In essence, only these linking film bacteria are capable of sensing a substratum surface, since all organisms later appearing in a biofilm adhere to neighboring organisms. Yet we found that a similar relation between icaA gene expression in 3 h old biofilms of S. aureus ATCC12600 (see Figure 7b) as in 24 h old ones (compare Figure 7b and 6a), while in 1 h old biofilm this relation was still lacking (see Figure 7a) as bacteria may not have adapted within 1 h to the substratum to which they adhere. This shows that gene expression is a time-dependent process and stable expression only occurs after 3 h and lasts minimally during 24 h of biofilm growth. This raises the important question how organisms appearing later in a biofilm, either due to growth or progressive co-adhesion, sense the adhesion forces arising from a substratum. Clearly, the range of all attractive or repulsive forces arising from a substratum surface is limited to few tens of nm, making it impossible for later organisms to directly sense a surface. Much more, they will experience adhesion forces from neighboring organisms with which they co-adhere (29). This implies that there must be a communication means available within a biofilm through which substratum information is passed to bacteria in a biofilm that are not in direct contact with the substratum.

Expression of icaA, but not of cidA genes decreased with increasing adhesion forces experienced by adhering staphylococci. Adhesion forces arising from substratum surfaces have recently been demonstrated to induce nanoscopic cell-wall deformation, yielding membrane stresses (21). Deformation of lipid bilayers has been shown to result in opening of mechanosensitive channels involved in adhesion force sensing, as they transduce a mechanical force into chemical signals (30). Note that also for Pseudomonas aeruginosa, surface-associated organisms have been found to produce more pili than their planktonic counterparts, suggesting that a localized mechanical signal, i.e. cell-wall stress arising from surface-association, plays a pivotal role in regulating genes associated with surface adhesion (31). Cell-wall stress and resulting deformation are extremely difficult to measure due to the rigidity of the peptidoglycan layer and therefore we employed an isogenic mutant S. aureus ATCC12600Δpbp4-GFP lacking peptidoglycan cross-linking and confirmed the greater deformability of the isogenic mutant (Figure 2) using surface enhanced fluorescence (32). Surface enhanced fluorescence can only be measured on reflecting surfaces and was thus
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only performed on SS. Importantly, due to the extreme sensitivity of surface enhanced fluorescence measurements, also other wild-type strains have been shown to deform upon adhesion to a surface (19). As important aspects of surface enhanced fluorescence, the number of bacteria involved in a single analysis is much larger than can be obtained using more microscopic methods, like AFM, while secondly it measures deformation under the naturally occurring adhesion forces that is, not under an applied force as in AFM (21). Therefore, it can be anticipated that differences in adhesion forces between S. aureus and various substratum surfaces may actually induce different degrees of cell-wall deformation which supports our hypothesis that adhesion forces cause nanoscale cell-wall deformations and membrane stresses that act as a signaling mechanism for an organism to its adhering state.

cidA expression did not relate with adhesion forces, possibly because cidA membrane proteins program cell death based on the oxidation and reduction state of the cell membrane (33) rather than its deformation suggesting that other environmental conditions like pH, nutrient availability, biofilm age or antimicrobial stress influencing DNA release (34). The peptidoglycan layer, ensuring rigidity to the bacterial cell-wall, appears of pivotal importance in adhesion force sensing, as its deformation is directly transmitted to the membrane. In the isogenic mutant S. aureus ATCC12600Δpbp4, lacking cross-linked peptidoglycan and therewith possessing a softer cell-wall, adhesion force sensing appears to be ineffective as no relation was found between adhesion forces and gene expression.

Deletion of pbp4 from S. aureus ATCC12600 neither had an effect on planktonic growth, cell surface hydrophobicity or zeta potential, and had only a small effect on biofilm formation (see Figure 3). However, it may be considered strange, that the amount of biofilm of both strains formed on different materials bears no significant relation with the forces experienced by these linking film organisms. This can be explained by the fact that that bacteria will only adhere once they experience attractive forces that exceed the prevailing detachment forces in a given environment. The current experiments were carried out under static conditions rather than under flow, which implies a virtually zero detachment force operating during adhesion and making any adhesion force large enough for a bacterium to remain adhering. In this respect, it is not surprising that S. aureus ATCC12600Δpbp4 had a similar ability to form biofilm than its parent strains as both its cell surface hydrophobicity as well as its zeta potential are similar to the ones of the parent strain (see Figure 3). Importantly for the development of biofilms in the presence of weak adhesion forces, biofilms even form on highly hydrated, polymer-brush coatings, exerting very small
adhesion forces in the sub-nN range that were found insufficient for adhering bacteria to even realize they were in an adhering state (35).

Concluding, *S. aureus* reacts to its adhering state based on the magnitude of the adhesion forces it experiences as arising from the substratum surface to which it adheres. This response predominantly involves *icaA* gene expression and the production of EPS matrix components (PNAG and eDNA) that both decrease with increasing adhesion forces. Increasing adhesion forces bring an adhering organism closer to the “lethal” regime which might be a reason as to why less EPS is produced by organisms experiencing stronger adhesion forces. In addition, our data also suggest that mechanical properties of the cell-wall as provided by the peptidoglycan layer surrounding the cell membrane, serve as an important tool for the adhesion force sensing capacity in *S. aureus*.

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