SUMMARY

Planktonic life is dangerous for most bacterial strains and species and adhesion to surfaces is often considered a survival mechanism. Once adhering, a cascade of events is triggered that involves amongst others, the production of an EPS-matrix leading to the formation of what is generally called a "biofilm". **Chapter 1** reviews the events occurring during the transition from bacterial adhesion to EPS-matrix production and biofilm formation from a physico-chemical perspective, offering new concepts like bacterial adhesion force-sensing and cell wall deformation as a trigger for the development of sessile bacterial phenotypes. Surface enhanced fluorescence has convincingly demonstrated the existence of minor cell wall deformation upon adhesion that can act as a trigger for an adhering bacterium to start EPS-matrix production, as a hallmark of the transition between planktonic and sessile phenotypes. For staphylococci, EPS-matrix production appears related with the magnitude of the adhesion forces felt. The importance of the EPS-matrix is ubiquitously present throughout the entire process of biofilm development, from facilitating initial bacterial adhesion to maintaining biofilm structural integrity during growth and offering "back up" resources in case of nutrient depletion during biofilm aging. During the entire biofilm life cycle, EPS protects the biofilm against chemical attacks such as from antimicrobial treatment and, through its viscoelastic properties, against mechanical stresses.

Hitherto, the majority of *in vitro* studies on antibiotic efficacy has been performed on planktonic bacteria, thus neglecting the protection offered by the biofilm-mode of growth occurring when bacteria are adhering to a surface, and the nanoscopic deformations of the bacterial cell wall arising from the adhesion forces between bacteria and the surfaces to which they adhere. Therefore, further research is needed into effects of mechanical stress on bacteria adhering to a surface, in addition to the chemical stress arising from antibiotic treatment. Thus, the aim of this thesis is to gain insight into the response(s) of *Staphylococcus aureus* strains to mechanical and chemical stresses, as governed by the physico-chemical properties of the substratum surfaces to which they adhere, grow and form a biofilm on. *S. aureus* is one of the most frequently occurring pathogens in many types of infections and multi-drug resistant *S. aureus* isolates are notoriously hard to handle currently.

Besides EPS-matrix production, other bacterial mechanisms can effectively protect bacteria from chemical and mechanical stresses. For instance, the nisin-associated-sensitivity-response-regulator (NsaRS) in *S. aureus* is important for its adhesion to surfaces and resistance against antibiotics, like nisin (**Chapter 2)**. 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. The intra-membrane location of the NsaS sensor led 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. 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. 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 confirmed that the intra-membrane-located sensor NsaS senses both chemical and mechanical stresses to modulate antibiotic clearance through the NsaAB efflux pump.

These findings then raised the question how nanoscopic cell wall deformation defines bacterial responses to environmental conditions and how it is influenced by antibiotics. In **Chapter 3**, staphylococcal cell wall deformation upon exposure to cell wall active and non-active antibiotics or their combinations, were compared for two green-fluorescent (GFP) isogenic *S. aureus* strains adhering to a gold surface, of which one lacks peptidoglycan cross-linking. Exposure to cell wall active antibiotics caused greater cell wall deformation than a buffer control in the GFP parent and in the Δ*pbp4*^{GFP} isogenic mutant, as measured by surface enhanced fluorescence. Cell wall non-active antibiotics only yielded greater deformation than a buffer control in the parent strain, while combinations of cell wall active and non-active antibiotics did not cause greater cell wall deformation. 3D-analysis of the impact of adhesion forces and Young's moduli of the cell wall, as both measured using atomic force microscopy, yielded the conclusion that increased deformation was mainly due to cell wall weakening and not due to effects of antibiotics on adhesion forces.

Nanoscopic cell wall deformations alter the tension in the cytoplasmic membrane, and in **Chapter 4** we hypothesized that adhesion forces and cell wall deformation stimulate gating of mechanosensitive channels located in bacterial membranes. Mechanosensitive channels open or close in response to changes in membrane tension invoked by changes in environmental osmolarity to allow water flow across the channel, and they have also been suggested to play a role in drug delivery and efflux. We related adhesion forces to different substratum surfaces measured using single-bacterial contact probe atomic force microscopy of a parent *S. aureus* strain and its isogenic Δ*mscL* mutant with the gating of mechanosensitive channels upon their adhesion. The percentage planktonic staphylococci or staphylococci adhering to each surface becoming fluorescent due to uptake of a fluorescent, negatively-charged molecule (calcein), increased exponentially with adhesion force and

was higher in the parent strain (66 %) than the Δ*mscL* mutant (40 %), suggesting its uptake through both large and small channels. However, these uptake levels were achieved at different critical adhesion forces of 4.1 and 1.2 nN for the parent strain and the Δ*mscL* mutant, respectively. Uptake of similarly-sized but positively-charged dihydrostreptomycin, monitored by staphylococcal killing upon exposure to dihydrostreptomycin, occurred in the parent strain by a reduction of 2.4 log-units CFUs, at a critical force of 3.6 nN, but remained low (reduction 1.0 log-unit CFU) in the mutant strain, independent of adhesion force. This suggests that due to attractive electrostatic charge interaction between the antibiotic and membrane channel proteins, the positively-charged antibiotic may be considered as a large molecule analogue. Accordingly, these observations prove that adhesion to surfaces plays a role in staphylococcal channel gating.

Surface adhesion alone is not sufficient to form a biofilm, but several steps need to occur before initially adhering bacteria can build the complex community architecture of a biofilm. Surface sensing, creating bacterial awareness of their adhering state on the surface, is essential to initiate the phenotypic and genotypic changes that characterize the transition from initial bacterial adhesion to a biofilm. Physico-chemistry has been frequently applied to explain initial bacterial adhesion phenomena, including bacterial mass transport, role of surface properties in initial adhesion and the transition from reversible to irreversible adhesion. However, recent advances show that also emergent biofilm properties such as EPS production originate from biofilm growth that is programmed by the properties of surfaces to which initial adhesion of individual bacteria occurs. **Chapter 5** presented a comprehensive description of the role of physical-chemistry from initial bacterial adhesion to surface-programmed biofilm growth. To this end, biofilm formation was described in four distinct steps: (1) bacterial transport towards a surface, (2) reversible bacterial adhesion and (3) transition to an irreversible state and finally (4) cell wall deformation and associated emergent properties. Bacterial mass transport mostly occurs from sedimentation or convective-diffusion, while the initial state of bacterial adhesion is described by surface thermodynamic and Derjaguin–Landau–Verwey–Overbeek (DLVO)-analyses. These approaches consider bacteria as inert, colloidal particles and, as a result, surface thermodynamic and DLVO-theories have failed to yield a generalized mechanism of bacterial mass transport and adhesion. Application of DLVO-theories is hampered by the presence of cell surface tethers, creating a multiscale roughness of the cell surface that impedes proper definition of the interaction distance in DLVO-theories. Application of surface thermodynamics is also difficult, because initial bacterial adhesion is only an equilibrium phenomenon for a short

period of time, when bacteria are attached to a substratum surface by a single cell surface tether. Bond-strengthening over time occurs in several minutes leading to irreversible adhesion due to progressive removal of interfacial water, conformational changes in cell surface proteins, re-orientation of bacteria on a surface and the progressive involvement of more tethers in adhesion. This is clearly physico-chemistry without any relation to bacterial metabolic processes such as EPS production, as highly similar observations have been made with inert, non-biological colloidal particles. After these initial bond-strengthening processes, adhesion forces arising from a substratum surface cause deformation of the bacterial cell wall against the elasticity of the rigid peptidoglycan layer and the intracellular pressure of the cytoplasm. Cell wall deformation not only increases the contact area with a substratum surface, presenting another physico-chemical bond-strengthening mechanism, but is also accompanied by membrane surface tension changes to which membrane-located sensor molecules react to control emergent phenotypic and genotypic properties in biofilms, most notably adhesion-associated ones like EPS production. However, also bacterial efflux pump systems may be activated or mechano-sensitive channels may be opened upon cell wall deformation arising from bacterial adhesion to a substratum surface. The physico-chemical properties of the substratum surface thus control the response of initially adhering bacteria and through excretion of autoinducer molecules extend the awareness of their adhering state to other biofilm inhabitants who subsequently respond in a similar way until the autoinducer concentration becomes too low. Herewith, physico-chemistry is involved in the initial and final stages of biofilm formation, as programmed by the substratum surface. This conclusion is pivotal for the development of new strategies to control biofilm formation on surfaces, that have hitherto been confined to the initial adhesion stages.

In this perspective, future studies should aim at further elucidating the role of adhesion force sensing, ranging from single adhering bacteria to mature biofilms. In single bacteria, the role of mechanosensitive channels gating in adhesion force sensing should be investigated by determining whether a minimum level of gating is required to provoke phenotypic and genotypic changes typical of the biofilm mode of growth. Slicing mature biofilms parallel to a substratum surface would enable to analyze depth-dependent gene expression in order to explain the role of surface properties in modulating emergent biofilm properties. Also, future studies should encompass more strains and species, other than *S. aureus*. Finally, comparing Gram-positive to Gram-negative bacterial strains, strong EPS producers to non-EPS producers, and bacteria with surface appendages as opposed to their bald counterparts, would yield a more comprehensive physico-chemical model of biofilm formation.

Summarizing, this thesis provides new insight into the responses of *S. aureus* on mechanical and chemical stresses, as governed by the physico-chemical properties of the substratum surfaces to which they adhere, grow and form a biofilm on. It is the first description of the role of physico-chemistry in explaining biofilm formation from initial adhesion to emergent surface-programmed properties of a biofilm.