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Bacterial Adhesion-force Sensing in Oral Biofilms

Wang, Can

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Bacterial Adhesion-force Sensing in Oral Biofilms

Can Wang

Bacterial Adhesion-force Sensing in Oral Biofilms

University Medical Center Groningen, University of Groningen
Groningen, The Netherlands

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PhD thesis

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the decision by the College of Deans.

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by

Can Wang

born on 1 July 1990

in Henan, China

Supervisors:

Prof. Y. Ren

Prof. H. J. Busscher

Prof. H. C. van der Mei

Assessment Committee:

Prof. L. W. M. van der Sluis

Prof. A. Visser

Prof. Y. Li

To all the people I treasure

Paranimfen:

Hao Wei

Abigail Mensah Forson

Table of Contents

Chapter 1.1	General Introduction Emergent Heterogeneous Microenvironments in Biofilms: Substratum Surface Heterogeneity and Bacterial Adhesion Force-sensing Y. Ren, C. Wang, Z. Chen, E. Allan, H. C. van der Mei, and H. J. Busscher (FEMS Microbiology Reviews 2018; 42: 259-272)	1
Chapter 1.2	Aim of This Thesis	27
Chapter 2	Bacterial Density and Biofilm Structure Determined by Optical Coherence Tomography J. Hou [#] , C. Wang [#] , R. T. Rozenbaum, N. Gusnaniar, E. D. de Jong, W. Woudstra, G. I. Geertsema-Doornbusch, J. Atema-Smit, J. Sjollem, Y. Ren, H. J. Busscher, and H. C. van der Mei (Scientific Reports 2019; 9: 9794)	31
Chapter 3	Emergent Properties in <i>Streptococcus mutans</i> Biofilms are Controlled through Adhesion Force Sensing by Initial Colonizers C. Wang, J. Hou, H. C. van der Mei, H. J. Busscher, and Y. Ren (mBio 2019; 10: e01908-19)	49
Chapter 4	<i>Streptococcus mutans</i> Adhesion Force Sensing in Multi-species Oral Biofilms C. Wang, H. C. van der Mei, H. J. Busscher, and Y. Ren (npj Biofilms and Microbiomes 2020; 6:25.)	75
Chapter 5	Adhesion-force Induced Gene Expression in <i>Streptococcus mutans</i> Biofilm at the Border between Two Surfaces with Different Hydrophobicities C. Wang, H. C. van der Mei, H. J. Busscher, and Y. Ren	99
Chapter 6	General Discussion	107
	SUMMARY	113
	SAMENVATTING	117
	ACKNOWLEDGEMENTS	121

Chapter 1.1

Emergent Heterogeneous Micro-environments in Biofilms: Substratum
Surface Heterogeneity and Bacterial Adhesion Force-sensing

Yijin Ren, Can Wang, Zhi Chen, Elaine Allan,
Henny C. van der Mei, Henk J. Busscher

FEMS Microbiology Reviews 2018; 42: 259-272.

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ABSTRACT

Phenotypically heterogeneous microenvironments emerge as biofilms mature across different environments. Phenotypic heterogeneity in biofilm sub-populations not obeying quorum sensing-dictated, collective group behavior, may be considered as a strategy allowing non-conformists to survive hostile conditions. Heterogeneous phenotype development has been amply studied with respect to gene expression and genotypic changes, but “biofilm genes” responsible for preprogrammed development of heterogeneous microenvironments in biofilms have never been discovered. Moreover, the question of what triggers the development of phenotypically heterogeneous micro-environments has never been addressed. The definition of biofilms as “surface-adhering and surface-adapted” microbial communities contains the word “surface” twice. This leads us to hypothesize that phenotypically heterogeneous microenvironments in biofilms develop as an adaptive response of initial colonizers to their adhering state, governed by the forces through which they adhere to a substratum surface. No surface is entirely homogeneous, while adhering bacteria can substantially contribute to stochastically occurring surface heterogeneity. Accordingly, bacterial adhesion forces sensed by initial colonizers differ across a substratum surface, leading to differential mechanical deformation of the cell wall and membrane, where many environmental sensors are located. Bacteria directly adhering to heterogeneous substratum domains therewith formulate their own local responses to their adhering state and command non-conformist behavior, leading to phenotypically heterogeneous microenvironments in biofilms.

ABBREVIATIONS

AFM	atomic force microscopy
DDS	dichlorodimethylsilane
eDNA	extracellular DNA
EPS	extracellular polymeric substances
HA	hydroxyapatite
PE	polyethylene
PEG	polyethylene glycol
PEO	poly(ethylene) oxide
PET	polyethylene terephthalate
PIA	polysaccharide intercellular adhesin
PDMS	polydimethylsiloxane
PMMA	polymethyl methacrylate
PS	polystyrene
QA	quaternary ammonium
SS	stainless steel
SR	silicone rubber
WCA	water contact angle

INTRODUCTION

Bacterial adhesion and biofilm formation

Bacteria adhere to surfaces in most industrial and natural environments, regardless of whether the surfaces are of synthetic or biological origin, and the latter includes the surfaces of prokaryotic and eukaryotic cells. Bacterial adhesion clearly marks the start of “biofilm” formation, but it still remains a challenge to define the end of biofilm formation. Biofilms are defined as surface-adhering and surface-adapted communities of microorganisms (1), which grow embedded in their self-produced matrix of extracellular polymeric substances (EPS: see Text Box 1) (2). Note that this definition includes cell-to-cell adhesion and therefore also encompasses planktonic aggregates (3).

Text Box 1. Extracellular polymeric substances

Polymers, such as polysaccharides, proteins, extracellular DNA (eDNA) or nucleic acids, secreted by bacteria and forming a “glue” that holds a biofilm together, possibly serving other functions like nutrient trapping and protection against antimicrobial challenges (2).

Emergent biofilm properties

The biofilm phenotype of bacteria is distinguished from the planktonic state by emergent properties (“localized gradients, sorption and retention, cooperation and competition, tolerance and resistance”: see Text Box 2) (4).

Text Box 2. Emergent biofilm properties

New properties which emerge in a biofilm that are not predictable from the properties of free-living bacterial cells (4).

Biofilm phenotypes do not emerge homogeneously across a biofilm. Heterogeneous microenvironments with different microbial composition, pH, live-dead ratios of bacteria, EPS-production, including eDNA-rich or -poor domains, differential penetrability, density, water content and channelization have been observed in biofilms using fluorescent probes (5) or optical coherence tomography (6). Phenotypically heterogeneous microenvironments are present in biofilms of both Gram-negative and Gram-positive species in different environments (Fig. 1), where non-conformists represent a bacterial sub-population that does not obey quorum-sensing commands (see Text Box 3), generally thought to coordinate a homogeneous response in an entire biofilm (7). Possession of heterogeneous microenvironments can be considered as a deliberate strategy of biofilm inhabitants, with the potential of offering multiple mechanisms to combat hostile conditions and therewith facilitate survival of non-conformists.

Text Box 3. Quorum-sensing

Intra- and interspecific bacterial communication by producing, releasing and detecting small, diffusible molecular auto-inducers. When auto-inducers reach a threshold concentration, it is commonly accepted that a whole population collectively obeys with homogeneous gene expression. Non-conformists represent a bacterial sub-population that does not obey quorum-sensing commands (7).

Phenotypically heterogeneous, emergent micro-environments

Heterogeneous gene expression or genotypic changes form the basis for the development of phenotypically heterogeneous microenvironments in biofilms. Gene expression is traditionally studied as an average behavioral property in a bacterial population. However, phenotypic heterogeneity occurs also already at the single-bacterium level (14) and it could be argued that phenotypic heterogeneities at the single-bacterium level form the basis of heterogeneously emerging properties in biofilms. The development of heterogeneous phenotypes at the level of biofilm communities, as well as at the level of single bacteria, has been amply studied and reviewed with respect to gene expression and genotypic changes in planktonic bacterial aggregates and biofilms grown in well plates or on agar (15). However, the question of what actually triggers the emergence of heterogeneous microenvironments in biofilms remains unanswered.

FIG 1 Examples of heterogeneously developing microenvironments in biofilms.

(A) Red-fluorescent patches of EPS in a *Streptococcus mutans* (green-fluorescent) biofilm on saliva-coated HA (8). Reprinted with permission from Elsevier Ltd. (B) Scattered red-fluorescent patches corresponding to EPS in 24 h *Staphylococcus epidermidis* (green-fluorescent) biofilm grown on saliva-coated HA discs with orthogonal distribution of catalytic nano particles (white) (8). Reprinted with permission from Elsevier Ltd. (C) Live (green-fluorescent) and dead (red-fluorescent) *Mycobacterium smegmatis* scattered through a biofilm on a hydrophobic PS surface after 72 h exposure to ciprofloxacin (9), indicating differential susceptibility to ciprofloxacin and presumably reflecting a variation in physiological state. Reprinted with permission from BioMed Central. (D) Distribution of bacteria and EPS after live-dead staining in a multispecies oral biofilm with *S. mutans*, *Streptococcus sanguinis*, and *Streptococcus gordonii*, formed on a dental adhesive surface (10). Reprinted with permission from MDPI. (E) Evolution of spatially-segregated communities in *Burkholderia cenocepacia* biofilms on PS, with different colony morphotypes showing differently colored fluorescence (11). Reprinted with permission from the Nature Publishing group. Three distinct colony morphotypes reproducibly emerged within biofilms inoculated with a single ancestor. (F) Uneven pattern of penetration and accumulation of Nile-red loaded micelles into a staphylococcal biofilm grown on glass (12). Reprinted with permission from American Chemical Society. The micelle carriers have a poly(ethylene)glycol shell and are biologically invisible allowing them to enter a biofilm, where they acquire a cationic

charge at low pH to interact electrostatically with the bacterial cell surface. Thus, the observed distribution of Nile-red likely demonstrates heterogeneity with respect to channelization and possibly low pH micro-environments within the biofilm. (G) In vitro grown *S. mutans* biofilm on HA, with green-fluorescent bacteria and blue-fluorescent EPS patches occurring unevenly across the biofilm (13). Reprinted with permission from Elsevier Ltd.

Hypothesis on the development of phenotypically heterogeneous, emergent micro-environments

Despite their frequent observation, heterogeneous microenvironments are usually taken for granted, without wondering why one only sees patches of EPS (16), polysaccharide intercellular adhesin (PIA) (17) or other compounds (18) appear in a microscopic image, why isolated regions of dead bacteria occur (9), why pH varies across a biofilm (19), why penetrability varies at different locations in a biofilm (12), or why some adhering bacteria develop motility while others remain non-motile (20)? Are these heterogeneous responses that emerge stochastically distributed by coincidence, are they a transient state in a kinetic process, are they a response to an environmental trigger or do they develop as a genetically preprogrammed, deterministic property in the transition from an adhering bacterium to a mature biofilm?

Since “biofilm genes” responsible for preprogrammed development of heterogeneous microenvironments in mature biofilms have consistently not been discovered (21), emergent phenotypic heterogeneity in biofilms is likely governed by environmental triggers (22) and physical cues (19, 24). However, the precise nature of the actual trigger or physical cue has not been addressed. The word “surface” occurs twice in the definition of biofilms by Tolker-Nielsen: “surface-adhering” and “surface-adapted” communities of microorganisms (1). This leads us to hypothesize that phenotypically heterogeneous, emergent microenvironments in biofilms develop as a response of bacteria to their adhering state and are governed by the local properties of the substratum surface.

Aim of this review

In this review, we summarize the events that stimulate different emergent phenotypes during biofilm formation on different non-biological materials with the aim of identifying substratum surface-associated triggers for the development of phenotypically heterogeneous, emergent microenvironments in a biofilm.

MICRO-ENVIRONMENTS IN BIOFILMS ON DIFFERENT SUBSTRATUM SURFACES

In Table 1, we summarize events stimulating emergent phenotypes across a wide variety of different bacterial strains and species and on different substrata. Data in the table are literature-derived without the intention of representing a complete overview of the literature. Instead, the table serves to identify substratum surface-associated triggers for emergent phenotypes, as discussed below. Opposite to the discussion below which is phenomenologically organized, the table is organized alphabetically for different strains.

TABLE 1 Summary of observations involving the emergence of different phenotypes across a wide variety of different bacterial strains and species and on different substrata.

Strain	Substrata	Observations	Relevant details	References
Single species studies				
<i>Caulobacter crescentus</i>	glass	bacteria made multiple surface contact before transitioning from reversible to irreversible adhesion.	WCA < 30°; microfluidic flow conditions	(25)
<i>Escherichia coli</i>	micron-scale patterned PDMS	surface appendages enable bacteria to overcome unfavorable surface patterns	static conditions	(26)
<i>E. coli</i>	PS well plates	pH heterogeneity within biofilms	type of polystyrene and WCA not reported; shaking conditions (30 rpm)	(19)
<i>E. coli</i>	hydrophobic glass beads	Cpx pathway regulates adhesion-induced gene expression		(27)
<i>Lactobacillus plantarum</i>	lectin monolayer and hydrophobic coatings	time-dependent binding to lectin layers; fast, time-independent binding to hydrophobic coatings		(28)
<i>Mycobacteria</i>	hydrophobic slides	biofilm viability and structure affected by antibiotic presence	30 min initial adhesion; orbital shaking (80 rpm)	(9)
<i>Pseudomonas aeruginosa</i>	glass, SS, PET, hydrophobic SS, hydrophilic PET	flagella increase adhesion on hydrophobic surfaces; straight and long flagella on PET and SS; curved and short flagella on glass	WCA and surface roughness provided for all surfaces	(29)
<i>Staphylococcus aureus</i>	PE, SS	adhesion force and nisin efflux pump efficacy was highest on hydrophobic PE surfaces	WCA for PE 85° and for SS 35°; static conditions	(30)
<i>S. aureus</i>	PE, SS, Ti-6Al-4V alloy, HA	adhesion forces, bacterial retention and viability are substratum related	WCA for SS 49°, for PE 82°, for Ti-6Al-4V 69° and for HA 95°	(31)
<i>S. aureus</i>	PE, SS, PMMA	matrix production and <i>icaA</i> gene expression is inversely related with adhesion forces	WCA for SS 33°, for PMMA 69° and for PE 84°; Submicron roughness	(32)
<i>S. aureus</i>	glass	cell wall deformation and long-range adhesion forces are related		(33)

TABLE 1 (Continued.)

Strain	Substrata	Observations	Relevant details	References
Single species studies				
<i>S. aureus</i>	glass	heterogeneous pattern of penetration and accumulation of Nile-red loaded micelles into biofilms		(12)
<i>Staphylococcus epidermidis</i> , <i>S. epidermidis</i>	QA-coatings	strong adhesion forces cause bacterial death	surfaces carry a positive charge	(34)
	SS, PMMA, PE	substratum dependent EPS production and gentamicin susceptibility		(16)
<i>Streptococcus sobrinus</i>	DDS coatings	substratum hydrophobicity determines bacterial retention, with less impact on adhesion	WCA for DDS coatings 90° and glass 20°	(35)
Multiple species studies				
<i>S. aureus</i> <i>E. coli</i>	nanoporous or nanopillared, hydrophobized aluminum oxide	adhesion to hydrophobic, nanopillared surfaces smaller than to hydrophilic or nanoporous surfaces	WCA varies from 0 - 162°; static and flow conditions	(36)
<i>S. aureus</i> <i>P. aeruginosa</i>	plasma etched black silicon	smaller, more densely packed pillars exhibited the greatest bactericidal activity	WCA varies from 8 - 160°; pillar heights of 212, 475 to 610 nm	(37)
<i>S. aureus</i> <i>S. epidermidis</i>	nanopillared-Si wafers	nanopatterning stimulates EPS-production and yields bacterial killing	regular patterning with sharply pointed pillars; flow conditions	(38)
<i>P. aeruginosa</i> <i>S. aureus</i>	graphene nanosheets	graphene nanosheets creates pores in bacterial cell walls, causing bacterial death.	roughness of the graphene sheets varies between 19 - 44 nm.	(39)
<i>Branhamella catarrhalis</i> , <i>Bacillus subtilis</i> , <i>E. coli</i> , <i>P. aeruginosa</i> , <i>Pseudomonas fluorescens</i> , <i>Pseudomonas maritimus</i> , <i>S. aureus</i> ,	cicada wing, nanopatterned surfaces	nanopatterning kills only Gram-negative bacteria		(40)

TABLE 1 (Continued.)

Strain	Substrata	Observations	Relevant details	References
Multiple species studies				
<i>Asticcacaulis biprosthecum</i> , <i>Agrobacterium tumefaciens</i> , <i>C. crescentus</i>	glass	reversible attachment of bacterial cells is mediated by motile cells bearing pili triggering adhesion production.		(41)
<i>S. aureus</i> , <i>S. epidermidis</i> , <i>P. aeruginosa</i>	SR; SR with Pluronic brush	adhesion forces dictated the transition from a planktonic to a biofilm mode of growth	flow conditions; WCA for SR 110°	(42)
<i>Actinomyces naeslundii</i> , <i>Lactobacillus acidophilus</i> , <i>Streptococcus mitis</i> , <i>Streptococcus mutans</i> , <i>Streptococcus oralis</i> , <i>Streptococcus sanguinis</i> , <i>S. sobrinus</i>	SS, bovine enamel	salivary conditioning films reduce adhesion forces	salivary films reduced WCA of SS to 23° and of enamel to 26°; sub-micron roughness	(43)
<i>S. aureus</i> , <i>S. epidermidis</i>	various substrata	staphylococcal biofilms show four distinct states, growing aerobically, growing fermentatively, dead, and dormant, contributing to their tolerance to antimicrobials	different reactor systems	(44)
<i>P. aeruginosa</i> , <i>S. epidermidis</i>	PEO-coatings	PEO-brush coating reduced adhesion of all strains and species	flow conditions	(45)
<i>Marinobacter hydrocarbonoclasticus</i> , <i>Psychrobacter</i> sp., <i>Halomonas pacifica</i>	glass	dissolved organic carbon alters surface properties with an impact on adhesion	flow conditions; surfaces conditioned with natural seawater	(46)

Relevant experimental details are included, when available in the references used.

Phenotypic drug tolerance and resistance

Phenotypic heterogeneity with respect to drug tolerance and resistance has been observed frequently in bacterial bulk cultures. Correct mechanistic distinction between tolerance and resistance is difficult (see Text Box 4). Phenotypic resistance is thought to be mainly due to environmentally

triggered changes in bacterial cell wall permeability impeding drug access, activation of efflux pumps and release of drug-deactivating enzymes (47). Examples of environmentally triggered events are the reversible change in porin expression levels in enteric bacteria in response to high osmolarity or temperature (48) or the reduced antibiotic sensitivity of *Enterobacter aerogenes* which results from reduced porin expression under antibiotic pressure (49). Phenotypic tolerance on the other hand, involves an environmental trigger of bacterial dormancy, persistence, differentiation and biofilm formation, including EPS production (47, 50). Although the mechanisms of phenotypic heterogeneity with respect to tolerance and resistance likely unite in a biofilm, the role of the substratum surface and its specific properties as an environmental trigger for the development of biofilm heterogeneity has not been considered (51, 52).

Text Box 4. Resistance and tolerance

Antibiotic resistance generally means an increase in the minimum inhibitory concentration (MIC) of an antibacterial agent due to a permanent change in the bacterium, e.g. by mutation or through horizontal gene transfer. Antibiotic tolerance is the ability of bacteria to survive the effect of an antibiotic due to a reversible phenotypic state. Two main forms of tolerance have been identified: “tolerance by slow growth” (occurs at steady state) and “tolerance by lag” (a transient state that is induced by starvation or stress) (51, 52).

S. epidermidis and *S. aureus* biofilms grown on polycarbonate filters on agar possessed at least four distinct phenotypes: bacteria growing either aerobically or fermentatively, dead or dormant (44). Multiple strains of *S. epidermidis* containing the *ica* locus, which encodes for PIA, were found to produce biofilms on hydrophobic polyethylene (PE) surfaces (water contact angle [WCA] of 84°) which contained large patches of EPS. Alternatively, on more hydrophilic acrylic and stainless steel surfaces (WCA of 69° and 33°, respectively), heterogeneously occurring EPS production was less and concurrently, *ica*-gene expression was low in these biofilms as compared with biofilms on PE (16). Similarly, EPS production in biofilms of *S. aureus* and *S. epidermidis* on hydrophobic silicone rubber (SR) surfaces (WCA of 110°) was massive and yielded resistance to gentamicin, whereas on hydrophilic polyethylene glycol (PEG), polymer-brush-coated SR (WCA of around 40°), EPS production was absent and bacteria remained susceptible to gentamicin. To a lesser extent, such differences were also observed in biofilms of the Gram-negative bacterium, *P. aeruginosa* (42, 45). Expression of the membrane located sensor, NsaS and the NsaA two-component efflux pump in *S. aureus* SH1000, responsible for nisin resistance in the planktonic state, was enhanced when the organism was adhering to a substratum surface. Moreover, adhesion to a hydrophobic polyethylene surface triggered a greater expression of *nsaS* and *nsaA* than adhesion to a more hydrophilic stainless steel surface (30). Despite the influence that the specific properties of the substratum surface have on emergent biofilm properties, most experiments are reported in the literature without reference to the substratum material. In many cases, biofilm assays are performed in multi-well polystyrene (PS) plates and the type of polystyrene is not specified even though this will affect surface properties: for example, bacterial-grade PS is more hydrophobic in the absence of surface treatment (WCA 78°) than tissue culture-grade PS after physical treatment (WCA 43°), and these differences may severely impact on

bacterial adaptive behavior. Moreover, often conclusions on surface adaptation are extrapolated from results obtained in biofilms grown on aqueous agar, which may not accurately reflect the conditions encountered on solid substratum surfaces.

Collectively, these examples demonstrate that the substratum surface, most notably its hydrophobicity or hydrophilicity (Text Box 5), provides an environmental trigger for the development of antibiotic resistance and tolerance in biofilms. Importantly, in most of these examples, a uniform response of the entire biofilm has been inferred without evidence that the biofilm is homogeneous over its entire volume. However, where available, closer inspection of micrographs in the published literature (Fig. 1 for specific examples), clearly shows stochastically occurring non-conformists, providing clear evidence of heterogeneity.

Text Box 5. Surface hydrophobicity

“Surface hydrophobicity” and its opposite “surface hydrophilicity” literally indicate the “fear” or “love” of a surface for water. Surface hydrophobicity can be quantitated by placing a small water droplet on a surface and measuring its degree of spreading, full spreading being characterized by a 0° WCA (hydrophilic surface). On super-hydrophobic materials, such as nanostructured hydrophobic surfaces, air can become entrapped and water has an almost 180° WCA (36), making it behave like a mercury droplet.

Swarming behavior

Swarming is another drug-resistance mechanism allowing bacteria to explore and subsequently escape an antibiotic-laden or otherwise hostile environment (53), and also enables bacteria to actively search for nutrients (54). Swarming phenotypes are often characterized by being hyperflagellated, elongated, multinucleate (55) and antibiotic-resistant. In *Paenibacillus vortex* biofilms, antibiotic-refractory, swarming phenotypes function to explore the environment for antibiotic-laden regions that should be avoided by the “builders” of the biofilm community (56).

Swarming bacteria either reside in (i) bulk suspension, where they are unlikely to experience any effects from a substratum surface, (ii) surface-constrained, near the surface but still in suspension and experiencing hydrodynamic shear or (iii) in direct interaction with the substratum (57). Swarming in the surface-constrained regime requires reversible adhesion on the one hand, but in order to prevent detachment back into the bulk suspension, bacteria must have a means to rapidly transit between reversible and irreversible adhesion. Indeed, studies on single cells of *C. crescentus* demonstrated that transitioning from reversible to irreversible adhesion is not a single event and most cells reversibly contact a surface multiple times before a final transition to irreversible adhesion takes place, with pili playing an important role in this transition (25).

Bacteria can sense the presence of a surface by obstruction of surface appendages such as flagella, pili or fimbriae (26, 58) and subsequent activation of membrane located sensors (59). In *C. crescentus*, arrest of flagellum rotation and concurrent stimulation of “just-in-time” polysaccharide adhesive occurs to maximize adhesion and prevent untimely detachment back into suspension (41). The presence of *P. aeruginosa* flagella and type IV pili increased bacterial adhesion to highly hydrophobic substratum surfaces (29), suggesting a role for substratum surface properties on development

of bacterial swarming phenotypes.

HOW BACTERIA DIFFERENTIATE BETWEEN DIFFERENT SUBSTRATUM SURFACES

Adhesion forces between bacteria and substratum surfaces

The observations that bacteria adapt differently to adhesion on different substratum surfaces, immediately raises the question of how bacteria sense that they are on a surface, and more importantly, how they tailor their adaptive response to the characteristic properties of the surface they adhere to. Adhesion, whether arising from specific, molecular ligand-receptor or non-specific interactions (60), is an interplay between ever present attractive Lifshitz-Van der Waals forces, attractive or repulsive acid-base interactions as a generalized form of hydrogen bonding, electrostatic forces with a magnitude depending on pH and ionic strength of the fluid environment and Brownian motion forces. The attractive Lifshitz-Van der Waals forces are the most long-ranged ones, acting over distances of up to 1 μm and becoming increasingly stronger when the interacting surfaces become closer. The sum total of these different forces determines the force by which a bacterium adheres to a substratum surface and this varies on different surfaces (31), while at close approach Lifshitz-Van der Waals forces are usually able to overcome electrostatic barriers (61, 62).

Text Box 6. Bacterial adhesion force measurement

Bacterial adhesion can be measured using atomic force microscopy (AFM). In bacterial probe AFM, a bacterium is attached to a highly flexible cantilever and brought into contact with a substratum surface, allowing contact between the bacterium and the surface for a defined time period and applied loading force. Upon retraction of the cantilever from the surface, the force required to break the bond between the bacterium and the substratum surface is recorded from the bending of the flexible cantilever. In this way, bacterial adhesion forces to biological and non-biological surfaces in the picoNewton (pN) to nanoNewton (nN) range have been measured (63).

Text Box 7. On the magnitude of bacterial adhesion forces to surfaces

Most forces by which bacteria adhere to surfaces are reportedly in the nN-range (28, 64-66), which is large compared to the gravity force experienced by bacteria. In air, the gravity force experienced by a bacterium is around 10^{-6} nN, while due to buoyancy, this force reduces in an aqueous suspension to around 10^{-8} nN. Assuming an adhesion force of around 1 nN, this implies that the forces by which bacteria adhere to a substratum surface are 10^6 - 10^8 fold higher than the gravity forces they experience.

Distinguishing three adhesion force regimes (67), it was proposed that extremely weakly adhering bacteria (adhesion forces less than 1 nN) do not realize they are in an adhering state and therefore do not show any adaptive response to a substratum surface. Alternatively, when adhering very strongly (proposed adhesion forces above 10 nN) as on quaternary-ammonium coated surfaces (42), cell wall damage is inferred resulting in bacterial cell death (34, 68). The intermediate regime comprising adhesion forces between 1 and 10-15 nN as occurs on most common substratum surfaces across a wide variety of bacterial strains and species (28, 64-66), invokes bacterial adaptation with production of EPS according to the magnitude of the adhesion forces experienced (32).

The ability to measure bacterial adhesion forces using the AFM (see Text Box 6) creates an awareness of the enormous magnitude of bacterial adhesion forces as compared with the gravitational forces they experience (see Text Box 7). Thus, it is not surprising that a lethal regime exists in which bacteria die due to cell wall damage as result of experiencing adhesion forces that are 10^6 – 10^8 fold higher than the gravitational force they experience. It has been argued that bacterial cell walls are rigid to resist large internal pressures, but remarkably plastic in order to adapt to a wide range of external forces (69), including adhesion forces. In fact, it has been demonstrated using AFM (33) and surface enhanced fluorescence (see Text Box 8), that the bacterial cell wall deforms under the influence of the relatively large adhesion forces arising from a substratum surface (Fig. 2), despite the rigidity provided to bacteria by their peptidoglycan layer. Also, AFM imaging of *S. epidermidis* trapped in a filter has shown structural and mechanical deformation of the cell wall (70).

Text Box 8. Surface enhanced bacterial fluorescence

Surface-enhanced fluorescence is the phenomenon that fluorophores within 20-30 nm from a metal surface show a stronger fluorescence intensity than expected for the same fluorophore in solution (71). Surface-enhanced bacterial fluorescence of fluorescent bacteria adhering to metallic surfaces can be exploited to demonstrate bacterial cell wall deformation, because more of the fluorescent, intracellular content of a bacterium is brought into the close vicinity of the surface upon adhesion and subsequent cell wall deformation, and therewith subject to surface enhanced fluorescence (72).

FIG 2 Bacterial cell wall deformation under the influence of adhesion forces arising from a substratum surface (33). An undeformed bacterium with a radius R approaching a substratum surface comes under the influence of the adhesion forces arising from the substratum. It gradually deforms, which brings more molecules (solid red region) under the influence of the adhesion forces, stimulating further adhesion until opposing forces arising from the rigid bacterial cell wall and increased intracellular pressure fully counteract the adhesion force.

Cell wall deformation and surface adaptation

The role of cell wall deformation in triggering bacterial responses is difficult to demonstrate experimentally, as bacterial cell wall deformation is small due to the rigidity provided by the bacterial pep-

peptidoglycan layer surrounding the membrane. In mammalian cells however, lacking a rigid cell wall, the influence of substratum hydrophobicity is more obvious and many different types of tissue cells remained “cauliflower” shaped on hydrophobic substratum surfaces while deforming to a “pancake” shape on hydrophilic ones (73). Also, in mammalian cells, sensors located in the cell membrane have been described which control the subsequent differentiation of stem cells in a substratum-dependent fashion (74).

Deliberate compression of bacteria between AFM cantilevers and substratum surfaces, has demonstrated that the bacterial cell wall deforms in a viscoelastic way (75, 76), although it should be noted that deformation under such conditions is not exactly the same as “spontaneous” deformation under the influence of adhesion forces arising from a substratum surface. *E. coli* and *B. subtilis* behaved like elastic rods when subjected to external forces, but deformed permanently in the plastic regime of viscoelastic deformation when cell wall synthesis occurred while the force was applied (69). Moreover, the offspring of plastically deformed bacteria always recovered their shape, but this required conditions allowing cell wall synthesis (69, 77) over several generations (78). Bacterial cell wall deformation changes the pressure profile across the lipid membrane (79) which is laden with environmental sensors that can become activated by such changes (80) through gating of mechanosensitive channels (81) or directly by conformational changes in membrane-located receptors (27). Thus adhesion-force sensing and subsequent cell wall deformation provide an important mechanism for adhering bacteria to realize they are on a surface and begin the process of surface adaptation. The role of rigid bacterial peptidoglycan layers in adhesion force-sensing and subsequent cell wall deformation is probably large, since a *S. aureus* $\Delta pbp4$ mutant, which lacks peptidoglycan cross-linking, seemed unable to adapt its response in line with the adhesion forces arising from a substratum surface (32).

HETEROGENEOUS SURFACES AND BACTERIAL INTERACTIONS

Surface heterogeneity due to protein adsorption

All naturally occurring and synthetic surfaces are heterogeneous, either on a micro- or nanoscopic scale and will exert different local adhesion forces on adhering bacteria to trigger different adaptive responses. Dental enamel is an excellent example of a naturally occurring heterogeneous surface with distinct crystalline hydroxyapatite (HA) structures comprised in an organic matrix, that in the oral cavity become covered within seconds with a conditioning film of adsorbed salivary proteins forming a network structure over the enamel surface (82, 83). Although the network structure of adsorbed proteins is a heterogeneous surface structure in itself, saliva contains many different proteins (84) that adsorb and displace each other in succession which further contributes to surface heterogeneity. In the oral cavity, formation and composition of salivary conditioning films varies on different surfaces (85) and precedes adhesion of bacteria and subsequently influences bacterial adhesion forces and biofilm detachment (86). A similar succession of protein adsorption and desorption occurs on cellular and synthetic graft surfaces exposed to blood (87). Note that, in the marine and other aqueous environments, conditioning films are often described as adsorbed films composed of dissolved organic carbon (46). Since bacteria diffuse more slowly than proteins, bacteria mostly adhere to such heterogeneous, adsorbed conditioning films, regardless of whether in the oral cavity or in any other environ-

ment.

Surface charge heterogeneity

Strong electrostatic attraction between positively charged QA-coated surfaces and negatively charged bacterial cell surfaces are reported to cause cell wall damage and subsequent cell death (34). Charge heterogeneity on glass surfaces, often thought to be homogeneous, became evident by repetitively allowing negatively charged, 1 μm diameter polystyrene particles to adhere to the same glass surface. Under low ionic strength conditions, particles always adhered first to the same, previously occupied microscopic location through strong, local electrostatic attraction (88), demonstrating the existence of positively charged heterogeneities on an overall negatively charged glass surface.

Heterogeneity in surface hydrophobicity and roughness

Heterogeneity in surface hydrophobicity and roughness at the sub-micrometer scale are easily demonstrable by the measurement of WCA hysteresis on material surfaces (see Text Box 9). Large differences between advancing and receding contact angles on “smooth” surfaces with a roughness less than 0.1 μm indicate regions with a large difference in surface hydrophobicity. Roughened, hydrophobic surfaces may appear as “superhydrophobic”, while roughened, hydrophilic surfaces possess smaller WCA than expected based on the hydrophobicity, respectively the hydrophilicity of their smooth counterparts.

Text Box 9. Contact angle hysteresis

When a water droplet advances over a perfectly smooth surface, it can be stopped by a small, more hydrophobic heterogeneity or rugosity, which causes the contact angle to be higher than when the droplet is in an equilibrium state. Equally so, when receding over an already wetted surface, water tends to remain behind on a hydrophilic heterogeneity and the contact angle appears smaller than in an equilibrium state. The difference in advancing and receding contact angles is called “contact angle hysteresis” (89). Only perfectly smooth and chemically homogeneous surfaces have a 0° contact angle hysteresis, which makes the measurement of contact angle hysteresis suitable for the measurement of surface heterogeneity in general at a sub-micrometer scale.

Bacteria themselves are in fact also ideal to demonstrate heterogeneity in substratum surface hydrophobicity due to differential interaction with hydrophobic and hydrophilic regions on a substratum surface. Micro-patterned substratum surfaces consisting of hydrophobic lines separated by wide hydrophilic spacings, for instance, attracted equal numbers of streptococci over its entire surface, but when challenged with a detachment force, streptococci were retained only on the hydrophobic lines (35), suggesting that the strength of bacterial adhesion is higher to hydrophobic regions. Adhesion force measurement using AFM on a patterned substratum consisting of square arrays of non-adhesive PEG hydrogels comparable in size to a bacterial cell on a hydrophobic, silanized glass surface showed that *S. aureus* adhesion was decreased at the hydrogel spacings as these presumably impeded contact between the bacterial cell and the hydrophobic surface (90).

Nanoscopically heterogeneous substratum surfaces

Nanotechnological advances have enabled the production of nanoscopically heterogeneous surfaces, that are often bioinspired (91) most notably by the so-called “lotus effect” (92). Such plant leaves, and also certain insect wings, remain free of bacteria through self-cleaning and antibacterial properties, thought to be mediated by nanopillared arrays (40) that inherently represent a nanoscopically heterogeneous substratum surface. Electron micrographs have clearly demonstrated that the bacterial cell wall can locally severely deform under the influence of the adhesion forces arising from extruding random (93) and periodic (38) nanostructures to yield pressure-induced EPS production and even bacterial cell death in Gram-positive staphylococci. This is supported by observations that killing of *P. aeruginosa* and *S. aureus* on graphene nanosheets related with density of the edges of the graphene (39). Approximately 98% of *P. aeruginosa* cells and 97% of *S. aureus* cells were killed on superhydrophilic and superhydrophobic black silicon surfaces with well-defined surface geometries and wettability, smaller, more densely packed pillars exhibiting the greatest bactericidal activity (37). It is speculated that the bactericidal activity is due to irreversible membrane bulging. In antibiotic-challenged *E. coli*, pores in the peptidoglycan network with a critical radius of around 20 nm, the typical distance between neighboring peptides and glycan strands, are required to cause bulging of the cytoplasmic membrane out through the pore. This bulging is irreversible and leading to loss of cell viability (94).

SUBSTRATUM SURFACE HETEROGENEITIES INDUCED BY ADHERING BACTERIA

During adhesion, bacteria can create heterogeneities as a means of communication (Fig. 3) to allow localized positive or negative cooperation in colonizing a substratum surface, that is, stimulate or discourage adhesion of other bacteria in their immediate surroundings (95). In a broader sense, bacteria have been suggested to leave “footprints” when adhering to and detaching from a substratum surface (96) that will contribute to substratum surface heterogeneity.

Localized cooperative phenomena and biosurfactant release

Biosurfactants (see Text Box 10), by their amphiphilic nature, are ideal molecules to be transported over large distances to reach remote areas of a substratum surface as a means to interact with other initial colonizers (Fig. 3A). *S. mitis* strains excrete biosurfactants that modify their immediate surroundings to make it less attractive for their competitors to adhere (97, 98) and the spreading of oral biosurfactants excreted by initial colonizers such as *S. mitis* over dental enamel surfaces reduced the adhesion forces of other colonizers (99). Lactobacilli also claim substratum surface area by excretion of biosurfactants that discourage adhesion of enterococci and other uropathogens (100).

Quorum-sensing controlled expression of phenol-soluble modulins in *S. aureus* (101) and rhamnolipids in *P. aeruginosa* (102) biofilms has been shown to mediate biofilm structuring and detachment. For *P. aeruginosa*, siderophores, eDNA and biosurfactants play multiple roles in the interaction between different sub-populations in a biofilm and influence its structural development, as related to biosurfactants concentration and composition (103).

Text Box 10. Biosurfactants

Biosurfactants are amphiphilic compounds produced by living organisms, mostly microorganisms, and excreted extracellularly, that contain hydrophobic and hydrophilic moieties, accumulating at an

interface and reducing interfacial tensions versus air, a liquid surrounding or another material (104, 105).

FIG 3 Bacterially-induced substratum surface heterogeneities as a means of communication and interaction between initially adhering bacteria. (A) Certain strains of bacteria excrete biosurfactants that spread over the substratum surface, modifying the immediate surrounding surface so that it is less favorable (red colored) for adherence by other bacteria. (B) Positive cooperativity is the mechanism by which an adhering bacterium changes the conformation of adsorbed proteins in its immediate surroundings or produces adhesive EPS, generating a more favorable surface (green colored) for adherence by other bacteria.

Bacterially-induced changes in adsorbed protein conformation and positive cooperativity

Bacteria also have other means to modify their immediate surroundings on a substratum surface to exert positive cooperativity (106, 107): several initial colonizers of protein-conditioned surfaces have the ability to induce conformational changes in the adsorbed protein film that surrounds them (Fig. 3B), making the film more attractive for their peers to adhere. Initial colonizers of oral surfaces *in vivo* have slightly stronger adhesion forces with salivary conditioning films than later colonizers (43), that may be underlying their ability to induce conformational changes in the adsorbed proteins to which they adhere. Since clinically, the relative prevalence of initially colonizing strains on a surface depends on the forces by which specific bacterial strains are attracted to their substratum surface (108), local induced changes in the conformation of adsorbed proteins may yield biofilm regions with a different bacterial composition.

Cooperativity through EPS production

EPS production can be considered as another cooperative phenomenon offering advantages in adhesion to neighboring bacteria by creating local surface heterogeneity around an adhering organism (see also Fig. 3B) (109) but, like for positive cooperativity in general, at the obvious expense of impairing dispersal of adhering bacteria to new locations. Psl for instance, is a cell wall anchored polysaccharide in *P. aeruginosa* (110) promoting aggregate formation between neighboring bacteria in microenvironments of a biofilm, that does not occur and subsequently yields less biofilm in strains lacking Psl (111). Mixed species oral biofilms on saliva-coated surfaces possess acidic niches in their

EPS matrix that selectively stimulate the localized growth of pathogenic *S. mutans* (112, 113).

THE COMMANDING ROLE OF INITIAL COLONIZERS IN BIOFILM FORMATION

Bacterial responses to prevailing environmental conditions is virtually always a survival strategy to maintain their adhering state in competition with others or under mechanical attack, while the production of EPS as an adaptive response embeds adhering bacteria in a matrix that also offers protection against chemical attacks (114, 115). Initially adhering bacteria have various ways to influence the development of microenvironments in the biofilm that grows on top of them, in which adhesion force-sensing plays a crucial role.

Adhesion force-sensing and biofilm composition

In the sequence of events that lead to a full-grown biofilm with heterogeneously occurring microenvironments, the initially adhering bacteria firstly have various ways to induce local heterogeneities on a substratum surface to which they adhere. Newcomers can recognize these heterogeneities by the strength of the local adhesion forces they experience and interpret them as signs to “stay away” or “welcome, adhere here”. This in turn, will create microenvironments in a biofilm with different microbial composition. Therewith the basis of cooperation, and possible conflicts, in a mature biofilm (116) is commanded by the initially adhering bacteria.

Adhesion force-sensing and EPS production

Emergent EPS production follows initial adhesion in the sequence of events leading to a mature biofilm, and is arguably one of the most important adaptive responses within a biofilm. Adhesion force-sensing constitutes an environmental trigger for EPS production. The production of the matrix molecule, poly-N-acetylglucosamine and the secretion of eDNA decreases with increasing adhesion force, suggesting that adhering staphylococci adjust their adaptive response to environmental need (32) to prevent unnecessary costs to their fitness (117). Similarly, EPS production by bacteria adhering under fluid shear conditions is more extensive than under stagnant conditions, suggesting that its expression is induced only when required (118, 119). Since the effective range of adhesion forces is limited to maximally 1 μm , it is impossible for bacteria other than the initial colonizers to directly sense a substratum, while their immediate neighbors reside at distances between 1-3 μm and are embedded in an EPS matrix (120). Accordingly, only initially adhering bacteria are able to sense and adapt to the adhesion forces exerted by a substratum surface and in fact, the majority of bacteria in a biofilm have never contacted the substratum surface (121). Since the same will be true for the bacteria in emergent heterogeneous microenvironments, this leads to the conclusion that initially adhering bacteria command the development of emerging heterogeneous microenvironments by sensing and adapting to the substratum and communicating with neighboring bacteria information about that surface (see Fig. 4). Stochastically occurring environmental triggers have been suggested before as being causative to phenotypic heterogeneity (122), but have never been associated with triggers derived from stochastically occurring substratum surface heterogeneity.

FIG 4 The commanding role in adaptive responses of initial colonizers in a biofilm. Initially adhering bacteria sense different local adhesion forces which triggers different adaptive responses that spread through the biofilm by diffusion of quorum-sensing molecules until their concentration is below a detectable threshold and the commands given are lost, limiting heterogeneous microenvironment development in space and time. Microenvironments, including the adhesion forces that trigger differential responses, the commanding organisms and obeying inhabitants of the micro-environment are indicated by different colors.

Text Box 11. Surface adaptation

Bacterial surface adaptation comprises the particular response of a bacterium to the surface properties of the substratum to which it adheres.

The surface adaptation (Text Box 11) of initial colonizers in response to direct contact with a substratum surface likely do not disappear with the first generation of later colonizers, not in direct contact with the surface, but will most probably disappear only after a number of generations (78) and the progeny returns to a more planktonic phenotype. Return to a planktonic phenotype does not necessarily imply bacterial return back into suspension, but may also occur in a biofilm, where bacteria are “suspended” or “free floating” in an EPS matrix at average distances of 1-3 μm from neighboring organisms (120), i.e. more specifically formulated, outside the influence of adhesion forces exerted by their neighbors.

Adhesion force-sensing and quorum-sensing

Identifying initial colonizers that are in direct contact with a substratum surface as “commanding” bacteria, implies that there must be a communication means available within a biofilm to pass information derived from adhesion force-sensing to bacteria that are not in direct contact with the substratum enabling them to indirectly sense the surface. The initially adhering bacteria likely pass substratum information by producing and releasing auto-inducing molecules to which later biofilms colonizers respond. Since the distance over which auto-transducers can be transported and remain detectable is limited by diffusion (122), quorum-sensing is eventually quenched which restricts the adaptive response to microenvironments in a biofilm, although “calling distances” between Gram-negative bacteria extending up to 78 μm have been reported (123). However, most effective calling distances for producing and releasing, sensing and responding to auto-transducer gradients are suggested to be between 4-5 μm (123, 124) and bacteria can optimize the use of auto-inducers by being in each

other's close vicinity. *Myxococcus xanthus*, *E. coli*, *B. subtilis* and lactobacilli for instance, use contact-dependent signaling for communication (125). Direct physical contact between bacteria in a biofilm is generally absent, unless co-adhering bacterial pairs are involved, that occur mostly in the oral cavity (126).

SUMMARY

In summary, all surfaces are heterogeneous with respect to hydrophobicity, charge and/or the possession of micro- or nanoscopic structures. Such stochastically occurring heterogeneities exert different adhesion forces upon adhering bacteria. Bacteria sense these adhesion forces through cell wall deformation, which subsequently activates membrane located sensors to stimulate phenotypic responses in initially adhering bacteria in direct contact with the surface. The local adaptive response of initial colonizers is conveyed to other biofilm inhabitants through diffusion of auto-inducers produced by the initial colonizers and their first generations progeny. Later generation progeny will lose the surface-adapted phenotype of the initial colonizers, while diffusion of auto-inducers occurs only over limited distances. This puts initial colonizers in command of the development of localized, stochastically occurring heterogeneous domains in a biofilm.

The role of adhesion force sensing in cell wall deformation as local triggers for the development of heterogeneous microenvironments in biofilms, puts a strong emphasis on the substratum surface on which biofilms are grown. Hitherto, in research on adaptive responses of bacteria to environmental triggers, conclusions are frequently extrapolated from agar-grown "biofilms" and biofilms on undefined well-plate materials to biofilms in general. Realization of the role of substratum properties in localized, adaptive responses of adhering bacteria and subsequent properties of a biofilm may accelerate development of much needed insight in the mechanisms of heterogeneous microenvironment development in biofilms.

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Chapter 1.2

Aim of This Thesis

AIM OF THIS THESIS

In **Chapter 1.1**, the events that stimulate different emergent phenotypes during biofilm formation were summarized. Adhesion-force sensing appeared as a new appealing environmental stimulus that externally triggers the development of emergent properties of bacteria in a biofilm mode of growth. In the oral cavity, many different soft (mucosa) and hard (teeth, dental implants, dentures, orthodontic appliances) substratum surfaces exist. Each of these surfaces possess their own typical surface heterogeneity, charge and hydrophobicity. Moreover, in the oral cavity substrata are coated with a salivary conditioning film, also known as the “acquired pellicle” that alters the substratum surface properties and contributes to further surface heterogeneity.

Biofilm formation in the oral cavity is a very ordered process and starts with the initial colonizers adhering to salivary conditioning film coated surfaces, followed by adhesion of later colonizers, amongst which *Streptococcus mutans* is the most prevailing cariogenic strain. As a further complication in oral biofilm formation, the oral cavity accommodates up to more than 700 bacterial strains and species. Accordingly, oral biofilm possesses many different strains and species. Co-adhesion between pairs of strains potentially interferes with adhesion-force sensing.

Based on this, the question arises whether adhesion-force sensing by initial colonizers of oral surfaces persists in multi-species biofilms and in the presence of a salivary conditioning film.

Therefore, the aim of this thesis was to investigate bacterial adhesion-force sensing by the important oral pathogen *S. mutans* in mono-species *S. mutans* biofilms and in multispecies biofilms comprising amongst others *S. mutans*. Experiments were conducted on different bare and salivary conditioning film coated surfaces, with different surface heterogeneity, charge and hydrophobicity.

Chapter 2

Bacterial Density and Biofilm Structure Determined by Optical Coherence Tomography

Jiapeng Hou#, Can Wang#, René t. Rozenbaum, Niar Gusnaniar, Ed D. de Jong, Willem Woudstra,
Gésinda I. Geertsema-Doornbusch, Jelly Atema-Smit, Jelmer Sjollema, Yijin Ren, Henk J. Busscher,
Henny C. van der Mei

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#These authors contributed equally to this work

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ABSTRACT

Optical-coherence-tomography (OCT) is a non-destructive tool for biofilm imaging, not requiring staining, and used to measure biofilm thickness and putative comparison of biofilm structure based on signal intensity distributions in OCT-images. Quantitative comparison of biofilm signal intensities in OCT-images, is difficult due to the auto-scaling applied in OCT-instruments to ensure optimal quality of individual images. Here, we developed a method to eliminate the influence of auto-scaling in order to allow quantitative comparison of biofilm densities in different images. Auto- and re-scaled signal intensities could be qualitatively interpreted in line with biofilm characteristics for single and multi-species biofilms of different strains and species (cocci and rod-shaped organisms), demonstrating qualitative validity of auto- and re-scaling analyses. However, specific features of *Pseudomonas* and oral multi-species biofilms were more prominently expressed after re-scaling. Quantitative validation was obtained by relating average auto- and re-scaled signal intensities across biofilm images with volumetric-bacterial-densities in biofilms, independently obtained using enumeration of bacterial numbers per unit biofilm volume. The signal intensities in auto-scaled biofilm images did not significantly relate with volumetric-bacterial-densities, whereas re-scaled intensities in images of biofilms of widely different strains and species increased linearly with independently determined volumetric-bacterial-densities in the biofilms. Herewith, the proposed re-scaling of signal intensity distributions in OCT-images significantly enhances the possibilities of biofilm imaging using OCT.

INTRODUCTION

Biofilms mostly grow on surfaces in aqueous environments, that range from marine and industrial to biomedical environments (1, 2). Biofilms have complicated, heterogeneous structures that influence their resistance to mechanical or chemical challenges (3-5), such as fluid shear or detergents and other antimicrobials. However, microscopic methods available for non-destructive analysis of biofilm structure, while not requiring staining, are scarce and frequently only provide low resolution images covering a small field of view. By consequence, these methods yield simple morphological characteristics, such as substratum surface coverage or thickness (6, 7). Low load compression testing and analysis of stress relaxation over time has also been suggested as a means to evaluate biofilm structure over a large surface area (8), but does not provide detailed structural information. Magnetic resonance imaging (MRI) or magnetic resonance microscopy can track free water molecules in biofilm structures on non-metal surfaces with a limited resolution (9), as opposed to bound, interfacial water present on any surface (10).

Optical coherence tomography (OCT) is a rapid, real-time, in situ and non-destructive imaging method, not requiring any staining, and is widely used in biofilm research to measure biofilm thickness and morphology (11-16). OCT is based on light scattering by a substratum surface, including biofilms grown on these surfaces. Accordingly, objects with higher light scattering will yield higher signal intensities, than objects with lower scattering, yielding a signal intensity distribution in OCT images of a biofilm, usually visualized as different shades of white-black in OCT images. In order to measure biofilm thickness from an OCT image, the whiteness of the biofilm reflecting high signal intensities has to be distinguished from the relatively black background of its aqueous environment, which can be done by proper thresholding (17) to define the exact position of the biofilm surface. However, since the biofilms itself also contains water, relatively black pixels reflecting low signal intensities are left inside the biofilm which have been tentatively ascribed to water-filled pores (18). In addition, limited attention has been given to relatively white pixels in biofilm images with a signal intensity above the threshold and how they possibly reflect different biomass components, such as bacteria and aggregates of water-insoluble extracellular polymeric substances (EPS) (19). Haisch and Niessner (20) were the first to introduce a whiteness scale bar in OCT biofilm imaging, representing the signal intensity of the scattered light and suggested signal intensities to increase with volumetric bacterial density in a biofilm, but without supporting data. Signal intensity increases in OCT images of biofilms have been observed after antimicrobial treatment of a biofilm, without rigorous interpretation (21). Signal intensities and comparisons of OCT images may be influenced by the auto-scaling applied in OCT instruments to ensure that each image contains an intensity distribution that covers the entire signal intensity range available. Auto-scaling of signal intensities in biofilm images has been applied to staphylococcal biofilms on stainless steel surfaces, but only yielded a qualitative relation between volumetric bacterial density in a biofilm and biofilm intensity in OCT images (22), presumably due to differences in intensity distribution across different individual images created by the auto-scaling.

The objective of this study was to develop a method to analyze and quantitatively compare the intensity distribution in different OCT images of biofilms from which biofilm structure and volumetric bacterial density can be derived. A re-scaling method was developed that effectively removes the in-

fluence of auto-scaling across different images and allows to quantitatively relate volumetric bacterial density with biofilm signal intensity in OCT images. Auto- and re-scaling methods, will be applied on single- and multi-species (or sometimes called “mixed-species” or “co-culture”) biofilms composed of Gram-positive and Gram-negative strains (cocci and rod-shaped organisms) with known properties, such as EPS production and ability to (co-)aggregate, as grown on different substratum surfaces under widely different conditions (static, under flow or in a constant depth film fermenter). Correspondence between expectations based on known properties of the biofilm forming strains with conclusions drawn from the auto- or re-scaling methods and enumeration of the number of bacteria in a biofilm, will be taken as a validation for the respective methods (see Table 1 for an overview of the biofilm cases involved).

TABLE 1 Summary of the different biofilm cases involved in this study, specifying the strains and substratum materials, growth conditions and media as well as the characteristics of the biofilms, expected on the basis of literature.

Case	Strain	Material	Growth condition	Growth medium	Expected biofilm characteristics
Case 1	<i>S. epidermidis</i> 252	stainless steel	static	TSB	higher EPS production by <i>S. epidermidis</i> ATCC 35984 than <i>S. epidermidis</i> 252 (22, 23)
	<i>S. epidermidis</i> ATCC 35984				
Case 2	<i>S. mutans</i> UA159	polystyrene	static	BHI + 0.5% sucrose BHI + 1.0% sucrose	more water-filled channels upon higher sucrose levels (24)
Case 3	<i>P. aeruginosa</i> ATCC 39324	stainless steel	constant depth film fermenter	LB ASM	higher EPS production for biofilms grown in ASM than in LB (25)
Case 4	<i>S. oralis</i> J22	glass	flow	BHI ⁺	more compact multi-species biofilms due to co-aggregation (26) as compared with mono-species biofilms
	<i>A. naeslundii</i> T14V-J1			BHI ⁺	
	Multi-species <i>S. oralis</i> J22 and <i>A. naeslundii</i> T14V-J1			BHI ⁺	

MATERIALS AND METHODS

Bacterial strains and growth conditions. Non-EPS producing *Staphylococcus epidermidis* 252 and EPS producing *Staphylococcus epidermidis* ATCC 35984 were isolated from the stool of a patient and a catheter-associated sepsis of a patient, respectively (27). *Streptococcus mutans* UA159 (ATCC 700610) was isolated from a patient with active dental caries (28), while also *Streptococcus oralis* J22 and *Actinomyces naeslundii* T14V-J1 (rod-shaped) were both isolated from the human oral cavity

(29). *Pseudomonas aeruginosa* ATCC 39324 (rod-shaped) was isolated from sputum from a cystic fibrosis patient (30). Each strain was inoculated from a single colony taken from a blood agar plate, in a 10 mL pre-culture and grown for 24 h at 37°C. This pre-culture was inoculated in a 200 mL main culture, grown for 16 h at 37°C. The culture medium was Tryptone Soya Broth (TSB, Oxoid, Basingstoke, UK) supplemented with 0.25% D(+)-glucose ($C_6H_{12}O_6$, Merck, Darmstadt, Germany) and 0.5% NaCl (Merck) for *S. epidermidis* 252 and *S. epidermidis* ATCC 35984; Brain Heart Infusion (BHI, Oxoid, Basingstoke, UK) supplemented with 0.5% or 1% (w/v) sucrose for *S. mutans* UA159; TSB for *P. aeruginosa* ATCC 39324; Todd Hewitt broth (THB, Oxoid) for *S. oralis* J22; BHI⁺ (BHI supplemented with 1 g L⁻¹ yeast extract, 50 mg L⁻¹ hemin and 1 mg L⁻¹ menadion) for *A. naeslundii* T14V-J1. *A. naeslundii* T14V-J1 was cultured in an anaerobic cabinet, *S. mutans* UA159 in 5% CO₂ while all other strains were cultured in ambient air. Bacteria were harvested from their main cultures by centrifugation at 5000 g, 10°C, and washed twice with buffer, after which bacteria were enumerated using a Bürker-Türk counting chamber.

Biofilm formation. Biofilms of the different strains were grown on different substrata according to previously used protocols that will be briefly repeated for clarity for the different cases (see also Table 1). *S. epidermidis* ATCC 35984 and *S. epidermidis* 252 biofilms were grown on sterile, stainless steel 304 (SS) surfaces (15 x 15 x 1 mm) coated with 10% fetal bovine serum (FBS) under static conditions (22). After allowing bacterial adhesion for 1 h from a 1×10^9 mL⁻¹ bacterial suspension in TSB, the suspension was replaced by medium without staphylococci and biofilms were grown for 48 h at 37°C, after which biofilms were washed with reduced transport fluid (31) for subsequent experiments.

For *S. mutans* UA159 biofilms, a buffer suspension (1 mM CaCl₂, 2 mM potassium phosphate, 50 mM KCl, pH 6.8, bacterial concentration of 3×10^8 mL⁻¹) was added in a 24 wells polystyrene plate (Greiner Bio-One GmbH, Frickenhausen, Germany) under static conditions for 2 h at 37°C under 5% CO₂ to allow streptococci to adhere. After adhesion, the buffer was removed and carefully washed with buffer, 1 mL BHI with 0.5% or 1% sucrose (w/v) was added and the plate was incubated for 24 h under static conditions after which biofilms were washed with buffer for subsequent OCT experiments.

Biofilms of *P. aeruginosa* ATCC 39324 were grown on SS disks in a constant depth film fermenter (CDFF) (25) at 37°C. Sample holders were recessed to a well-depth of 100 µm and placed into so-called pans (5 sample holders per pan), of which 15 pans could be placed in the CDFF turntable. Two hundred mL bacterial (5×10^7 mL⁻¹) suspension in TSB was drop-wise added on the turntable during 1 h, while the turntable was rotating at 3 revolutions per minute and bacterial suspension was distributed over the sample holders in the various pans by a Teflon scraper-blade. After 1 h, rotation was stopped for 30 min to allow bacteria to adhere to the stainless steel surfaces. Next, rotation was continued and Luria Bertani (LB, Sigma-Aldrich, St Louis, MO, USA) broth or artificial sputum medium (32) (ASM, per liter: 4 g DNA, 5 g mucin, 5 mL egg yolk emulsion, 5 g NaCl, 2.2 g KCl, 5 g amino acids, pH 7.0) was drop-wise added at a flow rate of 15 mL h⁻¹ for 18 h, while the scraper blade removed biofilm growing above the wells in order to grow constant depth biofilms with 100 µm thickness. After 18 h, the stainless disks with biofilms were washed and submerged in phosphate buffered saline (PBS, 10 mM potassium phosphate, 150 mM NaCl, pH 7.0) for subsequent experiments.

Multi-species biofilms of the coccal *S. oralis* J22 and rod-shaped *A. naeslundii* T14V-J1 co-adhering pair and each single strain were grown on a salivary conditioning film covered glass surface, constituting the bottom plate of a parallel plate flow chamber (17.5 x 1.7 x 0.075 cm) at 37°C (33). *A. naeslundii* T14V-J1 was suspended in buffer (1 mM CaCl₂, 2 mM potassium phosphate, 50 mM KCl, pH 6.8) supplemented with 2% BHI⁺ to a concentration of 1×10^8 mL⁻¹, while *S. oralis* J22 was suspended to a concentration of 3×10^8 mL⁻¹. Briefly, for single strain biofilms, bacterial suspension was perfused through the flow chamber at 10 s⁻¹ for 2 h after which the buffer was replaced by growth medium for overnight growth at 3 s⁻¹. For biofilms of the co-adhering pair, *A. naeslundii* T14V-J1 was adhered first for 15 min at 10 s⁻¹, followed by rinsing and co-adhesion of *S. oralis* J22 for 2 h and buffer was replaced by 50% diluted BHI⁺ in buffer for 16 h growth at 3 s⁻¹. After growth, the flow chamber was rinsed with buffer and biofilms were imaged using the OCT while in the flow chamber and subsequently removed for further experiments.

OCT measurements and signal intensity analysis of OCT 2D images. Biofilms were imaged using an OCT Ganymede II (Thorlabs Ganymede, Newton, NJ, USA) with a 930 nm center wavelength white light beam and a Thorlabs LSM03 objective scan lens, able to provide a maximum scan area of 100 mm². Imaging frequency was 30 kHz with a sensitivity of 101 dB and the refractive index of biofilm was set as 1.33, equal to the one of water. 2D images had fixed 5000 pixels with variable pixel size, depending on magnification in the horizontal direction, while containing a variable number of pixels with 2.68 µm pixel size in the vertical direction. Images were created by the OCT software (ThorImage OCT 4.1) using 32 bit data, but methods outlined are equally applicable to 8 or 16 bit images. The back-scattered light from a sample was captured by a CCD camera and the analogue voltage output of the camera was set such that the average light signal intensity over an entire 2D image was zero. Next, signal intensity was expressed in decibel units with respect to an internal reference signal intensity, generated in the OCT. Decibel units digitized over a discrete intensity scale ranging from 0 a.u. (arbitrary units, with 0 a.u. reflecting the darkest pixel, i.e. the lowest signal intensity in the image) to 255 a.u. (whitest pixel, i.e. highest signal intensity in the image) in order to generate a 2D “auto-scaled” image. Thus, obtained signal intensities associated with each pixel were collected from ten randomly chosen 2D images of each biofilm (Labview 2014, National Instrument, Austin, Texas, USA) and computer-stored for further analysis. The thickness of the biofilms was calculated after Otsu (17) thresholding of a biofilm image, while averaging biofilm thicknesses obtained over ten images.

In addition to imaging biofilms, bacterial suspensions of the different strains in an aqueous medium over a wide range of bacterial concentrations in a 24 wells plate without a cover, were also imaged using OCT. Bacterial concentrations were quantitated by their optical density OD_{600nm} in polystyrene cuvettes after appropriate dilution to different concentrations. PBS was used as a control in OCT (no bacteria) and its optical density subtracted from all densities measured on a bacterial suspension. The bacterial concentration in aqueous medium was subsequently related with signal intensities in OCT images.

Calculation of volumetric bacterial density. For the determination of the volumetric bacterial density in the different biofilm cases, biofilms were removed from the different sample surfaces with a

sterile disposable cell scraper (Merck, Darmstadt, Germany), after which bacteria were dispersed in buffer by vortexing. The dispersed biofilms were sonicated 3 times on ice at 30 W (Vibra Cell Model 375, Sonics and Materials Inc., Danbury, CT, USA) for 10 s each time with 30 s interval to obtain single bacteria. Then, the bacterial concentrations in the suspensions were enumerated in a Bürker–Türk counting chamber and the volumetric bacterial density in the biofilms was calculated by dividing the total number of bacteria on a sample by the total biofilm volume, i.e. the biofilm covered substratum surface area multiplied by the biofilm thickness, as determined using OCT.

RESULTS AND DISCUSSION

OCT imaging. First, 2D OCT images were made of the biofilms (Fig. 1), representing the four different cases summarized in Table 1. All four cases showed distinctly different features within 2D cross-sectional OCT images of the different biofilms. For statically grown staphylococcal biofilms, EPS producing *S. epidermidis* ATCC 35984 possessed a smoother biofilm surface than its non-EPS producing counterpart. Statically grown *S. mutans* biofilm surfaces appeared quite rough and had clearly enhanced porosity when grown at the higher sucrose concentration. Both CDFP grown *P. aeruginosa* biofilms had the same thickness and were relatively smooth at their surfaces, due to the action of the CDFP scraper.

FIG 1 2D cross-sectional OCT images of the four case biofilms evaluated. The scale bars indicate 100 µm.

The *S. oralis* single-species biofilm grown under flow, appeared smoother than the *S. mutans* biofilms grown statically, while single-species biofilms of rod-shaped *A. naeslundii* and *A. naeslundii*

containing multi-species biofilms looked rougher despite also being grown under flow.

FIG 2 Influence of the OCT focus point on the signal intensity distribution across a 2D cross-sectional image of a homogeneous agar layer. OCT images represent the cross-sectional view of an MRS agar layer at different focus points, indicated by the arrows. For each image, the average signal intensity is presented as a function of the height in the agar layer. Scale bars equal 200 μm . a.u. denotes “arbitrary unit”.

Most noticeably, whereas the aqueous environments above the different case biofilms appeared relatively black in OCT images (auto-scaled signal intensity of the blackest pixel 0 a.u.), the substratum surfaces, i.e. either stainless steel, polystyrene or glass, appeared as the most intense signal region (auto-scaled signal intensity of the whitest pixel 255 a.u.). Despite the auto-scaling, the image examples in Fig. 1 represent different signal intensities for water above the biofilms, with the water above the multi-species biofilms composed of cocci and rod-shaped organisms, yielding the highest signal intensity (case 4). Although there evidently is water above all biofilms, the region above the biofilms appears to yield the lowest signal intensity above the examples of staphylococcal biofilms (case 1). This represents the main problem in comparing OCT images of different biofilms or on different substrata based on signal intensities and associated whiteness scale bars. The first reason that water yields a different signal intensity above different biofilms is due to the occurrence of single, extremely black pixels, invisible by the naked eye in an image due to their small size, but determinant

for the auto-scaling. A second reason is that proper focusing is extremely important. According to the Thorlab OCT manual, the focus point should be set just below the surface of the object to be imaged, which is not trivial for a biofilm surface. This is illustrated in Fig. 2, showing the effect of slightly miss-focusing too high above, or too low inside a 300 μm thick homogeneous MRS agar layer. A constant signal intensity across the entire thickness of the agar with a relatively black background (signal intensity around 28 a.u.) is obtained for the “correct” focus point just below the agar surface. Purposely focusing too high or too low, not only led to signal intensities that varied over the thickness of the homogeneous agar layer, but also to a different signal intensity of the water above the agar, similar to the differences in signal intensities seen above the different biofilm cases in Fig. 1. The constant intensity over the depth of a 300 μm thick homogeneous MRS agar layer, also implies that signal intensity analysis along the depth of a biofilm is not influenced by the decline of the signal-to-noise ratio (also called “drop-off”) (16) along the optical axis of the OCT device over the thickness of the biofilms studied.

FIG 3 Average intensity (auto-scaled) in OCT images of bacterial suspensions in PBS as a function of the optical density, OD_{600} , of the suspensions. Data involve different concentrations of widely different strains and species (*S. epidermidis* 252, *S. epidermidis* ATCC 35984, *P. aeruginosa* 39324, *S. mutans* UA159 or *S. oralis* J22). Different strains are indicated by different symbols, but not further specified due to overlap of data points at similar optical densities. Drawn lines represent the best fit to an assumed linear relation with correlation coefficient R^2 and significance of the slope P indicated.

Re-scaling of the signal intensity distribution. Since OCT measures the intensity of back-scattered light from a sample, large objects like bacterial aggregates will scatter more strongly light than a single bacterium or EPS molecules and thus bacterial aggregates will yield a higher signal intensity than EPS molecules or water. This is demonstrated in Fig. 3, in which the OCT signal intensity taken of bacterial suspensions in an aqueous medium, is plotted as a function of the bacterial concentration in suspension, expressed as an optical density. Clearly, signal intensity increases linearly with increasing bacterial concentrations regardless of the strain or species involved. Therefore, the intensity distribution within a biofilm image can be expected to reflect bacterial presence, or the presence of (non-water-soluble) EPS molecules. In order to allow quantitative comparison of different biofilm images, the influence of the auto-scaling (see Fig. 1), needs to be eliminated to which end we developed a new, re-scaling method, as explained in Fig. 4. Note that in the re-scaling method proposed,

the average signal intensity in an image of a biofilm-free substratum is used as the new maximal signal intensity and adjusted to a value of 255 a.u. The biofilm-free substratum is preferred to this end, as it is much easier to focus upon than on a biofilm-covered substratum, especially when covered by a thin biofilm. For the widely different materials used in this study, average substratum signal intensities in absence of biofilm prior to re-scaling were 84, 85 and 83 a.u. for SS, glass, and polystyrene, respectively. The rescaled intensity of pixel i (I_i) is

$$I_i = \frac{(I_0 - I_w) \times 255}{I_s} \quad (1)$$

where I_0 is the original intensity associated with a pixel before rescaling, I_w is the average intensity of pixels representing water above the biofilm excluding the potential pixels that are regarded by Otsu thresholding as floating bacteria or bacterial clusters, I_s is the intensity of the biofilm-free substratum.

FIG 4 Auto- and re-scaling based analyses of 2D cross-sectional OCT images. (A) The back-scattered light from the measured sample is collected by the OCT camera and its intensity outputted as analogue voltage. Panel A showed an example of the output voltage in the Z-direction perpendicular to the substrate. Note the OCT adjust the level of zero voltage to ensure zero average output over an entire image. (B) In the subsequent auto-scaling process, voltage is firstly expressed in decibel units with respect to a reference intensity provided by the instruments after which the decibel scale is

digitized in 256 discrete values from 0 and 255 a.u. (panel B1) to yield the image provided in panel B2. Intensity distribution in auto-scaled images is created by the OCT instrument to ensure optimal quality of an individual image. (C) Next, Otsu thresholding (17) is applied on the OCT image to determine the biofilm surface (green line), while the substratum surface is visually identified based on the abrupt increase in intensity as compared with the biofilm interior. In order to avoid a potential impact of the intensity of the substratum material on the intensity of the biofilm, for calculational purposes the substratum surface was positioned 3 μm above the visually identified surface (red line). (D) In the proposed re-scaling process of OCT images, the average auto-scaled signal intensity above the biofilm surface, as identified by Otsu thresholding, is given a new intensity value of 0 a.u., while the separately measured, average intensity of a biofilm-free substratum is used and adjusted to an intensity value of 255 a.u. (panel D1). A new OCT image is subsequently generated with the re-scaled signal intensity distribution (panel D2).

Comparison of auto- and rescaling methods for analysis of biofilm structure: Signal intensity as a function of height in the biofilm. The auto- and re-scaling based analyses were applied on OCT images of the four biofilm cases (see Table 1, Fig. 1). In order to determine the validity of both methods, results will first be compared with the expected structural properties of each of the biofilm cases.

In order to evaluate the merits of the different methods with regards to EPS production in biofilms, *S. epidermidis* 252 (non-EPS producing) and *S. epidermidis* ATCC 35984 (EPS producing) were included (Fig. 1, case 1). Under the static growth conditions applied, both staphylococcal strains grew biofilms with a similar thickness of 50 μm (*S. epidermidis* 252) to 54 μm (*S. epidermidis* ATCC 35984). EPS producing *S. epidermidis* ATCC 35984 had slightly lower bacterial density (0.16 bacteria μm^{-3}) than non-EPS producing *S. epidermidis* 252 (0.20 bacteria μm^{-3}). These structural features are schematically summarized in Fig. 5A. Both auto- and re-scaling analyses (Fig. 5B and C, respectively) showed a significantly (one-tailed and paired student t-test with $P < 0.05$) higher average signal intensity in biofilm images across the height of EPS producing *S. epidermidis* ATCC 35984 than of non-EPS producing *S. epidermidis* 252, although significance was not observed when comparing intensities at a given height. Volumetric bacterial density of the EPS producing *S. epidermidis* ATCC 35984 was probably lowest due to volume occupation by EPS.

Higher amounts of sucrose during growth of *S. mutans* biofilms yields biofilms with more water-filled channels (see Table 1) (24). In our study, biofilms grown with 1% sucrose added were found to be twice as thick (119 μm), but with lower bacterial density (0.08 bacteria μm^{-3}) than biofilm grown with 0.5% sucrose added. The above characteristics are shown schematically in Fig. 6A. Accordingly, OCT images of streptococcal biofilms grown with a higher amount of sucrose appeared on average significantly (one-tailed and paired student t-test with $P < 0.05$) with a lower signal intensity across the entire depth of the biofilms both in auto- and re-scaling analyses (Fig. 6B and C, respectively) due to the possession of more water-filled voids and pores. In addition, intensity analyses indicated that especially at heights in the middle of the biofilms, significantly more water-filled voids and pores are present.

Case 1

FIG 5 Signal intensity analysis of OCT images: biofilms of non-EPS producing *S. epidermidis* 252 and EPS producing *S. epidermidis* ATCC 35984. (A) Schematic presentation of non-EPS producing *S. epidermidis* 252 and EPS producing *S. epidermidis* ATCC 35984 biofilms (see Table 1) and their measured thickness (derived using Otsu thresholding of auto-scaled OCT images) and volumetric bacterial density, calculated from the biofilm thickness and enumeration of the number of bacteria in the biofilm. (B) Intensity as a function of biofilm height (%) for *S. epidermidis* 252 and *S. epidermidis* ATCC 35984 biofilms in auto-scaling analysis. (C) Same as (B), but now in re-scaling analysis. Error bars indicate standard deviations over triplicate experiments with separate bacterial cultures.

Case 2

FIG 6 Signal intensity analysis of OCT images: *S. mutans* UA159 biofilms grown in medium with

0.5% or 1% sucrose added. (A) Schematic presentation of *S. mutans* biofilms grown in medium with 0.5% or 1% sucrose added (see Table 1) and measured thickness and volumetric bacterial density. (B) Intensity as a function of biofilm height (%) for *S. mutans* biofilms grown with 0.5% or 1% sucrose added in auto-scaling analysis. (C) Same as (B), but now in re-scaling analysis. Error bars indicate standard deviations over triplicate experiments with separate bacterial cultures.

Biofilms of *P. aeruginosa* strains (case 3) were grown in a CDFF to yield a thickness of 100 μm in LB and ASM medium. Biofilms grown in LB had similar bacterial density (0.21 bacteria μm^{-3}) as in ASM (0.22 bacteria μm^{-3}). Growth in ASM medium was expected to yield a more EPS-rich matrix than growth in LB medium (25), as schematically indicated in Fig. 7A. However, auto-scaling analysis did not show any significant difference between intensity distribution across images of LB and ASM grown biofilms (Fig. 7B). Re-scaling analysis on the other hand (Fig. 7C), showed pseudomonas regions in biofilm images with significantly higher signal intensities (one-tailed and paired student t-test with $P < 0.05$) near to the substratum surfaces when grown in ASM than when grown in LB medium. This presents a clear advantage over re-scaling analyses compared to auto-scaling analyses. Potentially, scraper action has dragged EPS out of the outer regions of the biofilms.

FIG 7 Signal intensity analysis of OCT images of *P. aeruginosa* ATCC 39324 biofilms grown in LB and ASM medium. (A) Schematic presentation of *P. aeruginosa* biofilms grown in LB and ASM medium (see Table 1) and measured thickness and volumetric bacterial density. Note that due to growth in a CDFF, thicknesses are identical. (B) Intensity as a function of biofilm height (%) for *P. aeruginosa* biofilms grown in LB or ASM medium in auto-scaling analysis. (C) Same as (B), but now in re-scaling analysis. Error bars indicate standard deviations over triplicate experiments with separate bacterial cultures.

Single-species and multi-species oral biofilms of co-aggregating pairs were grown, giving rise to

variations in thickness, but most notably to a higher volumetric bacterial density in multi-species biofilms (see Fig. 8A for schematics) due to co-aggregation of *S. oralis* J22 with *A. naeslundii* T14V-J1 (see also Table 1), yielding less water voids or channels (26). Image intensities across the depth of the biofilms obtained by auto-scaling analyses did not reveal any significant difference between single-species biofilms nor between single- and multi-species biofilms (Fig. 8B). On average across the depth of the biofilms, the re-scaling analyses showed the expected higher signal intensities in images of multi-species biofilms as compared with *S. oralis* J22 ($P < 0.05$, one-tailed and paired student t-test), but not with respect to *A. naeslundii* T14V-J1 probably because volumetric bacterial densities were similar for *A. naeslundii* and multi-species biofilms (Fig. 8C). Higher signal intensity in images of multi-species biofilms after re-scaling analyses was especially evident nearest to the substratum. This suggest a gradient in bacterial composition in multi-species biofilms across the depth of the biofilms, created because *A. naeslundii* was first seeded on the substratum surface after which the streptococci were allowed to co-adhere and grow in concert with the adhering actinomyces.

Case 4

FIG 8 Signal intensity analysis of OCT images of single-species *S. oralis* J22 and *A. naeslundii* T14V-J1 biofilms and multi-species biofilms. (A) Schematic presentation of the biofilms for both single-species and the more compact multi-species biofilms (see Table 1) and measured thickness and volumetric bacterial density. (B) Intensity as a function of biofilm height (%) for both single-species and multi-species biofilms, in auto-scaling analysis. (C) Same as (B) but now for re-scaling analysis. Error bars indicate standard deviations over triplicate experiments with separate bacterial cultures.

Comparison of auto- and rescaling methods for analysis of biofilm structure: Relation between signal intensity and volumetric bacterial density in biofilms. In order to make a comparison between auto- and re-scaling methods of OCT images, volumetric bacterial densities of all biofilms are presented as a function of the average signal intensity over each OCT image of the biofilms, as calculated from auto- (Fig. 9A) and re-scaling (Fig. 9B) analyses. Eliminating auto-scaling by our proposed re-scaling method, yielded a significant linear relation between signal intensities

and volumetric bacterial density in a biofilm with signal intensity increasing with increasing density. Auto-scaling of OCT images clearly does not yield statistically reliable, quantitative conclusions to be drawn across different biofilms and different substratum surfaces regarding volumetric bacterial density. Thus, it can be concluded that the proposed re-scaling removed the impediment associated with auto-scaling to quantitatively compare individual images. Accordingly, the wide variety of different strains and species involved in the relation between signal intensity and volumetric bacterial density (Fig. 9B) enables to derive volumetric bacterial densities in biofilms from the re-scaled OCT intensity in biofilm images, without need to culture.

FIG 9 Average signal intensity in OCT images of biofilms as a function of the volumetric bacterial density for all individual biofilms grown of the different cases in auto- (panel A) and re-scaling analysis (panel B). Drawn lines represent the best fit to an assumed linear relation with correlation coefficient R^2 and significance of the slope P indicated. Dotted lines represent 95% confidence intervals.

Within the current collection of biofilms, volumetric bacterial densities of the different biofilms varied by a factor of 3, which covers the range of density variation for different biofilms in the literature (34-36). Since distances between biofilm inhabitants have been reported to range between 1 and 3 μm (37), volumetric bacterial densities in biofilms are generally low (38), as compared e.g. with the closest hexagonal packing of a 1 μm diameter sphere yielding a volumetric density of $1.5 \mu\text{m}^{-3}$. This confirms that most volume in biofilms is occupied by water with or without dissolved EPS.

The relation between signal intensity and volumetric bacterial density presented in Fig. 9B is obtained using averaged signal intensities over the entire biofilm height. This was needed in order to relate signal intensity with numbers of bacteria, which could not be determined as a function of height in the biofilm. In most biofilms, volumetric bacterial density decreases towards the top of a biofilm, where a more open structure prevails. Structural differences across the depth of a biofilm are strain-dependent and furthermore arise from different nutrient availability, physical stresses and many other environmental factors. However, based on Fig. 9B, local volumetric bacterial densities across the depth of a biofilm can now be derived from signal intensity distributions. This may be a major asset in biofilm research, particularly since antimicrobial exposure of biofilm has been described to be accompanied by an increase in signal intensity in OCT images (21) that can now be more solidly interpreted.

Although the current study indicates that light scattering by bacteria is the overriding cause of sig-

nal intensity distributions in OCT images, else a relation as in Figure 9B would not develop, a disadvantage of OCT remains that also with the current methodology, the chemical composition of biofilm (matrix) cannot be directly determined.

CONCLUSIONS

A re-scaling method is presented that undoes the effects of auto-scaling in OCT images and therewith allows to compare signal intensity distributions in different OCT images of different biofilms, including single- and multi-species ones (coccal and rod-shaped) and on different substratum materials. Qualitatively, both auto- and re-scaled signal intensities in biofilm images as a function of depth in different biofilms could be interpreted in line with biofilm characteristics expected on the basis of literature for the different biofilms. However, specific features of *Pseudomonas* and oral multi-species biofilms were more prominently expressed after re-scaling. Average re-scaled signal intensities in OCT images of different biofilms increased linearly with independently determined volumetric bacterial densities in the biofilms, therewith quantitatively validating the re-scaling method proposed. Theoretically (16), the signal-to-noise ratio of the back-scattered light declines along the optical axis of the OCT device, which might create an impact of biofilm thickness on the measured light intensities. However, since signal intensity in our OCT images remained constant over the depth of 300 μm homogeneous agar layers (Fig. 2), we believe that our proposed method will be valid in biofilms of at least this thickness. Herewith the proposed re-scaling of the signal intensity distribution in OCT images of biofilms significantly enhances the usefulness of OCT biofilm imaging, as applicable on an entire biofilm image, but as can also be applied on image sections, representing e.g. high density bacterial clusters.

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Chapter 3

Emergent Properties in *Streptococcus mutans* Biofilms are Controlled
through Adhesion Force Sensing by Initial Colonizers

Can Wang, Jiapeng Hou, Henny C. van der Mei, Henk J. Busscher, Yijin Ren

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ABSTRACT

Bacterial adhesion is accompanied by altered gene expression, leading to “emergent” properties of biofilm bacteria that are alien to planktonic ones. With the aim of revealing the role of environmental adhesion forces in emergent biofilm properties, genes in *Streptococcus mutans* UA159 and a quorum-sensing-deficient mutant were identified that become expressed after adhesion to substratum surfaces. Using atomic force microscopy, adhesion forces of initial *S. mutans* colonizers on four different substrata were determined and related to gene expression. Adhesion forces upon initial contact were similarly low across different substrata, ranging between 0.2 and 1.2 nN regardless of the strain considered. Bond maturation required up to 21 s, depending on the strain and substratum surface involved, but stationary adhesion forces also were similar in the parent and in the mutant strain. However, stationary adhesion forces were largest on hydrophobic silicone rubber (19 to 20 nN), while being smallest on hydrophilic glass (3 to 4 nN). *brpA* gene expression in thin (34 to 48 μm) 5 h *S. mutans* UA159 biofilms was most sensitive to adhesion forces, while expression of *gbpB* and *comDE* expressions was weakly sensitive. *ftf*, *gtfB*, *vicR*, and *relA* expression was insensitive to adhesion forces. In thicker (98 to 151 μm) 24 h biofilms, adhesion-force-induced gene expression and emergent extracellular polymeric substance (EPS) production were limited to the first 20 to 30 μm above a substratum surface. In the quorum-sensing-deficient *S. mutans*, adhesion-force-controlled gene expression was absent in both 5 and 24 h biofilms. Thus, initial colonizers of substratum surfaces sense adhesion forces that externally trigger emergent biofilm properties over a limited distance above a substratum surface through quorum sensing.

IMPORTANCE

A new concept in biofilm science is introduced: “adhesion force sensitivity of genes”, defining the degree up to which expression of different genes in adhering bacteria is controlled by the environmental adhesion forces they experience. Analysis of gene expression as a function of height in a biofilm showed that the information about the substratum surface to which initially adhering bacteria adhere is passed up to a biofilm height of 20 to 30 μm above a substratum surface, highlighting the importance and limitations of cell-to-cell communication in a biofilm. Bacteria in a biofilm mode of growth, as opposed to planktonic growth, are responsible for the great majority of human infections, predicted to become the number one cause of death in 2050. The concept of adhesion force sensitivity of genes provides better understanding of bacterial adaptation in biofilms, direly needed for the design of improved therapeutic measures that evade the recalcitrance of biofilm bacteria to antimicrobials.

INTRODUCTION

Biofilms are surface-adhering and surface-adapted communities of microorganisms (1), in which adhesion to a substratum surface is the initial step. Two surfaces, including the surface of bacteria adhering on a substratum surface, can be attracted to each other by a combination of Lifshitz-Van der Waals, electrostatic double-layer, and acid-base forces (2). The sum total of these forces is generally called the “adhesion force”. The environmental adhesion forces by which a bacterium adheres to a surface are orders of magnitude larger than the gravitational forces bacteria experience and give rise to nanoscopic deformation of the cell wall (3, 4). Cell wall deformation in its turn causes changes in lipid membrane surface tension that provides a stimulus for the environmentally triggered expression of a great number of genes in adhering bacteria (5) to facilitate their surface adaptation. This leads to new, so-called “emergent” properties of adhering bacteria in their biofilm mode growth (6). Emergent properties reflect bacterial surface adaptation and arise only after bacteria have adhered to a surface. According to their definition (6), emergent properties of bacteria in biofilm mode growth are alien to their planktonic counterparts and cannot even be predicted on the basis of the properties of planktonic bacteria. The most prominent, landmark emergent property of adhering bacteria is the production of an extracellular polymeric matrix in which biofilm bacteria protect themselves against host defenses (7) and antimicrobial agents (8, 9) and through which they enforce their bond with a substratum surface (10).

Adhesion-force-induced surface adaptation in adhering bacteria has been observed in *Staphylococcus aureus* biofilms for the *icaA* gene, regulating production of extracellular polymeric substances (EPS). However, adhesion-force-induced surface adaptation was not observed for the *cidA* gene, which is associated with cell lysis and extracellular DNA (eDNA) release (11). Also, nisin clearance in staphylococci through the two-component NsaRS intramembrane-located sensor NsaS and NsaAB efflux pump (12) was enhanced when staphylococci adhered more strongly to a substratum surface (13). Hitherto, adhesion force sensing and associated cell wall deformation have appeared as an appealing concept to explain what environmental stimulus externally triggers the development of emergent properties of bacteria in biofilm mode growth. Yet, there still are many questions to be addressed, most urgently concerning the range over which adhesion force sensing operates in a biofilm. Typically, biofilms are much thicker than the range of the adhesion forces extending from a substratum surface. Adhesion forces can yield an attraction that can be sensed up to maximally 0.5 μm into a biofilm (2, 3). The exact magnitude and range of an adhesion force depend on the hydrophobicity and charge properties of the bacterial cell and substratum surfaces. Compared with the thickness of a biofilm, the range over which adhesion forces operate is relatively short. This suggests that quorum sensing plays a role in spreading the “news” that initial colonizers in a biofilm have “landed” on a substratum surface exerting a specific adhesion force. However, this suggestion has never been confirmed. Furthermore, adhesion force sensing has never been confirmed in other species than staphylococci.

Adhesion to surfaces is a survival mechanism for streptococci in the oral cavity (14). Accordingly, *Streptococcus mutans* has the ability to adhere to oral hard and soft tissues, abiotic restorative dental materials, and other bacteria in the oral cavity (15). Frequently studied genes involved in *S. mutans* initial adhesion and biofilm formation are summarized in Table 1. Based on the definition of “emergent”

properties as given by Flemming et al. (6) and literature description of gene functions, a hypothetical distinction is made between genes whose expression prepares planktonic bacteria for adhesion to a substratum surface and genes relevant for the development of emergent properties in adhering bacteria. For instance, genes that regulate synthesis of specific ligands of planktonic streptococci for optimal initial adhesion to saliva-coated surfaces, such as *ftf* and *gtfB* (16-19), are not considered to be involved in the development of emergent properties that arise by definition in already adhering bacteria. Also, genes regulating bacteriocin production, cell death, and chemical stress responses (*comDE*, *vicR*, *gbpB*, and *relA*), although vital in biofilm formation, may not bear direct relevance to EPS production, enforcing strong adhesion of biofilm inhabitants to a substratum surface (20–22). Autoinducer 2 in the *S. mutans luxS* quorum-sensing system (see also Table 1) coordinates communication in *S. mutans* biofilms (23) and may be expected to impact the extension of adhesion-force-sensitive genetic programming into a mature biofilm, as adhesion forces can only be directly sensed by initial colonizers (4).

TABLE 1 Summary of genes involved in *S. mutans* UA159 initial adhesion and subsequent processes occurring during biofilm formation.

Gene ^a	Function	References
Genes relevant to prepare initial adhesion in planktonic <i>S. mutans</i>		
<i>ftf</i>	Catalysis of sucrose cleavage to synthesize fructan to promote initial adhesion to salivary films	(16, 17)
<i>gtfB</i>	Synthesis of water-insoluble glucans (α 1,3-linked) to promote initial adhesion to saliva-coated tooth surfaces and establishment of microcolonies in biofilm	(18, 19)
Genes relevant to develop emergent properties in adhering <i>S. mutans</i>		
<i>brpA</i>	Regulation of cell wall stress responses, biofilm cohesiveness, and biofilm formation	(24, 33, 34)
<i>comDE</i>	Persister cell formation, bacteriocin production	(30)
<i>vicR</i>	Synthesis of EPS matrix components, regulation of bacteriocin production and cell death	(44, 45)
<i>gbpB</i>	Regulation of sensitivity to antibiotics, osmotic and oxidative stress, cell wall construction and maintenance, cell shape, hydrophobicity and sucrose-dependent biofilm formation	(28, 29)
<i>relA</i>	Regulation of stringent response, acid tolerance and biofilm formation	(46, 47)
<i>luxS</i>	Coordination of collective behaviors and cohesiveness in biofilms	(48, 49)

^aA hypothetical distinction has been made with respect to genes relevant to prepare initial adhesion in planktonic streptococci and genes involved in the development of emergent properties in adhering bacteria.

In order to further advance the concept of adhesion-force-induced gene expression in relation to emergent biofilm properties, the aim of this article is first to identify genes involved in biofilm formation by *S. mutans* and an isogenic, quorum-sensing-deficient mutant whose expression is controlled by environmental adhesion forces. This would confirm the hypothetical distinctions made in Table 1 between genes preparing planktonic bacteria for adhesion to a substratum surface and genes relevant for the development of emergent properties in adhering bacteria. To this end, biofilms of *S.*

mutans UA159 and its $\Delta luxS$ isogenic mutant were grown on four substratum surfaces with different hydrophobicities, and single-bacterial contact probe atomic force microscopy (AFM) was applied to measure the forces by which both strains adhere to each substratum surface. Gene expression was evaluated using RT-qPCR. Up- or downregulation of selected genes upon adhesion was related to the forces by which the streptococci adhere to yield a new concept of “adhesion force sensitivity of gene expression”. Uniquely, the extension of adhesion-force-induced genetic programming over the height of the biofilms above a substratum surface was investigated in cryosections of the biofilms taken at different heights above a substratum surface. Herewith it can be determined to what extent quorum sensing controls adhesion-force-induced gene expression in later biofilm inhabitants, residing further away from the substratum surface and not in direct contact with the substratum surface. Whiteness analyses of optical coherence tomography (OCT) images of biofilms was employed to support the conclusions regarding height-dependent gene expression taken from cryosections of the *S. mutans* biofilms.

RESULTS

Bacterial cell and substratum surface characteristics. First, it was established that *S. mutans* UA159 and its isogenic mutant UA159 $\Delta luxS$ exhibited comparable cell surface characteristics, despite exchange of the *luxS* gene using an erythromycin resistance determinant (24). Hydrophobicity and charge are both important physico-chemical bacterial cell surface characteristics involved in adhesion and in combination with comparable properties of the substratum surface define the magnitude of the adhesion forces (2). Cell surface hydrophobicity of bacteria is reflected among other characteristics by their removal from an aqueous phase by a hydrophobic ligand (see Fig. S1A in the supplemental material). Hydrophilic bacteria prefer to remain in the aqueous phase rather than being removed from it by adhesion to a hydrophobic ligand (25). Based on their equally low removal rates by hexadecane ($P > 0.05$, Mann-Whitney test), both strains can be classified as hydrophilic (Fig. S1B and C). In addition, streptococcal zeta potentials, reflecting surface charge, were slightly negative between -7 and -3 mV, with no significant differences between strains ($P > 0.05$, Mann-Whitney test). Like the hydrophobicity of the bacterial cell surfaces, the hydrophobicity of the substratum surfaces is also involved in bacterial adhesion and the forces by which bacteria adhere to a substratum surface. Water contact angles on substratum surfaces reflect the hydrophobicity of a material surface and were measured using the sessile drop technique (Fig. S1D). Water contact angles ranged from 11 to 103° for glass and silicone rubber surfaces, respectively, and differed significantly between all surfaces ($P < 0.05$, Mann-Whitney test). Also, hydrophobic, bacterial-grade and more hydrophilic, tissue-grade polystyrene surfaces (Fig. S1D) demonstrated a significant ($P < 0.05$, Mann-Whitney test) difference in water contact angles.

Bacterial adhesion forces. Streptococcal adhesion forces were measured on different substratum surfaces using single-bacterial-contact probe AFM (Fig. 1A). In single-bacterial-contact probe AFM, a bacterium attached to a flexible cantilever is brought into contact with a substratum surface and retracted after a specified time (the so-called “surface delay” or “bond maturation” time). Upon retraction, the cantilever bends until the bacterial bond with the substratum is disrupted. The force

at which this occurs is subsequently calculated from the cantilever bending and recorded as the adhesion force of the bacterium to the substratum surface. Adhesion forces increased with increasing bond maturation time between the bacterium and a substratum surface. (See Fig. 1B for examples of force-distance curves taken after different bond maturation times for the parent strain and its isogenic, quorum-sensing-deficient mutant.) Adhesion forces as a function of bond maturation time followed an exponential increase (Fig. 1B). Accordingly, adhesion forces as a function of bond maturation time were fitted to equation 1

$$F_t = F_0 + (F_{\text{stationary}} - F_0)[\exp(-\frac{t}{\tau})] \tag{1}$$

in which t denotes the surface delay time, F_0 is the initial adhesion force at 0 s surface delay time, F_t is the adhesion force after surface delay time t , and $F_{\text{stationary}}$ indicates the stationary adhesion force, while τ is the characteristic time constant for bond maturation. Initial adhesion forces, F_0 (Fig. 1C), were all in the sub-nN range on each substratum for the parent and the isogenic mutant strain ($P > 0.05$, one-way analysis of variance [ANOVA]). Bond maturation (compare τ values in Fig. 1C) occurred slower in the parent strain than in the isogenic mutant, especially on the silicone rubber. Like initial adhesion forces, stationary adhesion forces were similar in the parent strain and the isogenic mutant ($P > 0.05$, one-way ANOVA) when measured on the same material and increased for both strains with increasing hydrophobicity of the substratum surfaces. The difference between the two extremes in hydrophobicity on the glass and silicone rubber surfaces was significant within each strain ($P < 0.05$, one-way ANOVA).

FIG 1 Bacterial adhesion force characteristics of both streptococcal strains on four substratum surfaces with different hydrophobicities. (A) Schematics of single-bacterial-contact probe atomic force microscopy. A bacterium is attached to a tiplless AFM cantilever and brought to contact with a sub-

stratum surface, after which the cantilever is retracted following a surface delay that can be varied up to a maximum of 30 s. Upon retraction, the adhesion force by which the bacterium was attracted to the surface can be calculated from the cantilever bending. (B) Example of retraction force-distance curves taken after different surface delay times for *S. mutans* UA159 on a bacterial-grade polystyrene (PS) surface. (The arrow points to the force value, taken as the adhesion force.) Also included is a graph of streptococcal adhesion forces as a function of surface delay time for the parent strain and its quorum-sensing-deficient isogenic mutant. (C) Initial and stationary streptococcal adhesion forces F_0 and $F_{\text{stationary}}$, together with the characteristic bond maturation time constant τ on the different substratum surfaces. All data represent averages over 8 spots on 4 different surfaces of each substratum, measured with 4 different probes and bacteria from 4 different cultures, with $\pm$ signs representing standard deviation (SD) values over 32 measurements. Superscript letters in panel C indicate statistical significance as follows: a, statistically significant ($P < 0.05$, one-way ANOVA) differences from silicone rubber; b, statistically significant ($P < 0.05$, one-way ANOVA) differences between tissue-grade and bacterial-grade PS surfaces.

Streptococcal biofilm growth and gene expression. Streptococcal biofilms were grown, and their thicknesses were evaluated using optical coherence tomography (OCT) (see Fig. S2A in the supplementary material). Twenty-four-hour biofilms were all significantly ($P < 0.05$, Mann-Whitney test) thicker than 5 h biofilms. Five-hour biofilms showed thicknesses ranging from 34 to 48 μm for *S. mutans* UA159 and from 26 to 34 μm for its isogenic mutant, UA159 $\Delta luxS$ (Fig. S2B). Comparison within each substratum surface showed these differences between strains to be not statistically significant ($P > 0.05$, Mann-Whitney test).

Next, gene expression was evaluated in all streptococcal biofilms and normalized with respect to gene expression in planktonic streptococci of the corresponding strain (Fig. S3A). (Examples of amplification and melting curves are presented in Fig. S4). An example of a heat map for the different genes expressed on different substrata for *S. mutans* UA159 is given in Fig. S3B. Note, that all gene expressions were also normalized with respect to expression of the internal control gene *16S rRNA* and thus, different bacterial numbers will not affect the evaluation of gene expression. Gene expression as normalized with respect to planktonic streptococci, varied in each strain on the different substratum surfaces, both in 5 h and 24 h biofilms (Fig. S3C and D, respectively). Subsequently, normalized gene expressions on different substrata were plotted as a function of the environmental adhesion forces experienced by each of the two streptococcal strains (Fig. 2, see Fig. S5 and Fig. S6 in the supplementary material). In the parent strain, significant, linear relations (correlation coefficients 0.7 or higher, [Fig. 2]) were observed for three (*brpA*, *comDE* and *gbpB*) out of the seven genes evaluated in 5 h biofilms. However, in 5 h biofilms of the isogenic quorum-sensing-deficient mutant, none of the genes showed such linear relationships (correlation coefficients less than 0.7) and gene expression was considered not to be governed by adhesion forces. In cases where correlation coefficients were 0.7 or higher, the slopes in the graphs representing gene expression versus adhesion force can be interpreted as the sensitivity of a given gene to adhesion forces (Table 2). This renders expressions of *comDE* and *gbpB* genes as weakly sensitive to environmental adhesion forces, while externally triggered expression of *brpA* was strongly adhesion force sensitive in the parent strain.

Note that when evaluated over the entire thickness of the 3- to 4-fold-thicker 24 h biofilms, none of the genes showed adhesion-force-induced expression (Fig. S6), regardless of the strain involved.

FIG 2 Normalized fold gene expression with significant relationships to adhesion forces in *S. mutans* UA159 as a function of the stationary adhesion force to different substratum surfaces over the entire height of 5 h biofilms. Error bars denote SD values in fold gene expression over triplicate experiments, while the solid lines represent assumed linear relationships through the data points, with the correlation coefficient R^2 as presented. Dotted lines represent 95% confidence intervals.

TABLE 2 Adhesion force sensitivity of different genes over the entire height of 5 and 24 h *S. mutans* UA159 and UA159 $\Delta luxS$ biofilms^a.

Gene	<i>S. mutans</i> UA159				<i>S. mutans</i> UA159 $\Delta luxS$			
	Adhesion-force sen-		R^2		Adhesion-force sen-		R^2	
	5 h	24 h	5 h	24 h	5 h	24 h	5 h	24 h
<i>ftf</i>	-	-	0.3	0.4	-	-	< 0.1	0.4
<i>gtfB</i>	-	-	0.1	0.5	-	-	0.3	0.1
<i>brpA</i>	1.6	-	0.96	< 0.1	-	-	0.6	0.2
<i>comDE</i>	0.2	-	0.7	0.2	-	-	0.1	0.2
<i>vicR</i>	-	-	0.3	0.1	-	-	< 0.1	0.2
<i>gbpB</i>	0.1	-	0.7	0.1	-	-	0.6	0.2
<i>relA</i>	-	-	0.3	< 0.1	-	-	0.3	< 0.1

^aLinear relationships between gene expression and stationary adhesion force with a correlation coefficient of less than 0.7 were considered insignificant, and no sensitivity values were derived. Data in boldface are considered significant.

Extension of adhesion-force induced gene expression into a biofilm. In order to determine how far adhesion-force induced gene expression extended into a biofilm, gene expressions at different heights above a substratum surface (Fig. 3A) were evaluated in cryosectioned slices with a thickness of approximately 30 μm . Silicone rubber was chosen, because in 5 h biofilms grown on silicone rubber most genes studied were expressed most strongly (see Fig. S3C). Since 5 h biofilms were too thin for sectioning, sectioning was only done on 24 h biofilms. Setting gene expression normalized with respect to the internal *16S rRNA* control, and closest to the substratum surface at 100%, it can be seen in Fig. 3B that the adhesion-force induced expression of *brpA* and *comDE* was significantly decreased ($p < 0.05$, one-way ANOVA) in middle and top layers of the biofilm compared to 30 μm bottom layers, reducing to 30-70% in the top layer of the biofilm, depending on the gene considered.

FIG 3 Gene expression in different layers of 24 h *S. mutans* UA159 biofilm on a silicone rubber surface. (A) Schematics of biofilm cryosectioning and gene expression in three biofilm slices taken at different heights in the biofilm above the substratum surface. (B) Percentage of normalized (with respect to the internal *16S rRNA* control) adhesion-force-induced expression of selected genes at different heights above a silicone rubber surface in 24 h *S. mutans* UA159 biofilm, expressed relative to gene expression in the bottom layer of the biofilm closest to the substratum surface, set at 100%. Error bars denote SD values over triplicate experiments. *, statistically different at $P < 0.05$ by one-way ANOVA.

Extension of water- and EPS-filled pockets in streptococcal biofilms. OCT imaging of biofilms allows comparison of biofilm regions with different levels of back-scattering of incident light that can be associated with bacteria, insoluble EPS, and water- and soluble EPS-filled pockets (26). (See Fig. 4A for schematics.) Since bacteria are much larger than insoluble EPS molecules, most back-scattered light originates from bacterial presence, as confirmed recently for a wide variety of bacterial strains and species by a relationship between signal intensities in OCT images and volumetric bacterial densities (26). Using an artificial whiteness scale (white representing the highest signal intensity of back-scattered light), the average whiteness in images of 24 h *S. mutans* UA159 biofilms was significantly ($P < 0.05$, Mann-Whitney test) lower on all substratum surfaces than in biofilm images of *S. mutans* UA159 $\Delta luxS$ (Fig. 4B). This suggests that the great majority of individual bacteria in *S. mutans* UA159 biofilms were triggered to produce soluble EPS, while biofilm images of quorum-sensing-deficient *S. mutans* UA159 $\Delta luxS$ appeared much whiter in the absence of water- and soluble EPS-filled pockets. As a consequence of differential soluble EPS production, the volumetric density of bacteria in streptococcal biofilms (i.e., the number of bacteria per unit of biofilm volume, determined by

enumeration of the number of bacteria after biofilm dispersal from a defined substratum surface area, and subsequently divided by the biofilm volume) was lower ($P < 0.05$, Mann-Whitney test) for the parent strain than for the quorum-sensing-deficient mutant and related linearly to the average signal intensity in OCT images (Fig. 4C). Analysis of the local signal intensity in OCT images as a function of height above the substratum surfaces demonstrates that signal intensities of the *S. mutans* UA159 $\Delta luxS$ images (Fig. 4D) varied in a nearly identical fashion above both surfaces. However, in biofilm images of the parent strain, local signal intensities as a function of height above the surface suggest more extensive ($P < 0.05$, Student's *t* test) soluble EPS production on the hydrophobic silicone rubber surface than on the hydrophilic glass surface up to a height of 20 to 25 μm above the surfaces.

FIG 4 Analysis of OCT images of 24 h *S. mutans* UA159 and UA159 $\Delta luxS$ biofilms. (A) Schematics of signal intensity development by back-scattered light in OCT: based on an artificial whiteness scale, bacteria yield white regions (high signal intensity) due to back-scattering, while water- and soluble EPS-filled pockets do not back-scatter light and appear as black regions (low signal intensity). (B) Average signal intensity over an entire biofilm in 24 h streptococcal biofilms on the four different substratum surfaces. The superscript letter a in panel B indicates significant difference between *S. mutans* UA159 and UA159 $\Delta luxS$ ($P < 0.05$, Mann-Whitney test). (C) Average signal intensity over an entire biofilm as a function of the volumetric bacterial density for 24 h streptococcal biofilms of both strains on the four different substratum surfaces. Dotted lines represent 95% confidence intervals. (D) Local signal intensity in OCT images of 24 h streptococcal biofilms on glass and silicone rubber as a function of the biofilm height above the substratum surface. There are no statistically significant ($P > 0.05$, Mann-Whitney test) differences at corresponding heights for the mutant strain on hydrophobic silicone rubber and hydrophilic glass, while for the parent strain, signal intensities are lower on silicone rubber than on hydrophilic glass up to a thickness of 20 to 25 μm . Error bars indicate SD over different experiments with separately cultured bacteria ($n=3$).

DISCUSSION

S. mutans is an avid sugar consumer in the oral cavity, allowing it to produce acids that make it one of the world's most wide-spread pathogens, responsible for the decalcification of oral hard tissues. For its survival in the oral cavity, *S. mutans* needs to adhere (14). Once adhering, *S. mutans* enforces its adhesion to oral surfaces through the production of EPS (27) as a landmark, emergent biofilm property. In this article, we identified *gbpB*, *brpA*, and *comDE* as genes that became more strongly expressed upon adhesion of *S. mutans* UA159, compared with *ftf*, *gtfB*, *vicR*, and *relA*. This confirms our hypothetical distinction (Table 1) of *ftf* and *gtfB* genes being more relevant for the preparation of planktonic streptococci for their initial adhesion to surfaces. Also, it justifies the classification of the *gbpB*, *brpA*, and *comDE* genes as more relevant for the development of emergent properties in adhering streptococci. The *vicR* and *relA* genes play roles with respect to diverse processes occurring during biofilm formation (Table 1), but these are not exclusively involved in directly enforcing the initial adhesion of *S. mutans* to oral surfaces.

Based on the differential expression of the *gbpB*, *brpA*, and *comDE* genes in streptococci adhering on different substratum surfaces and relating it to the adhesion forces experienced by adhering bacteria, a new concept of “adhesion force sensitivity of gene expression” is introduced. Adhesion force sensitivity reflects whether expression of a gene is more or less strongly influenced by the adhesion force sensed by bacteria upon their adhesion to a substratum surface. Among the three genes identified, *gbpB* had the weakest adhesion force sensitivity. However, *gbpB* is not only involved in enforcing initial streptococcal adhesion but also possesses an array of other pivotal functions in biofilm formation (Table 1) (28, 29). *comDE* is also weakly adhesion force sensitive and also possesses other functions than enforcing initial adhesion, including persister cell formation (30). However, persister cell formation usually involves bacteria closely associated with a substratum surface (31), and hence the weak control of adhesion forces over *comDE* expression as determined over the entire height of a biofilm is not surprising. Moreover, these weakly adhesion-force-sensitive genes as identified in this study have also been found to be upregulated in biofilm detached cells (32). Detachment is an important mechanism for bacterial survival, since it protects the biofilm from overpopulation, which is opposite from enforcing initial adhesion. Expression of *brpA* was by far several fold more sensitive to adhesion forces than *gbpB* and *comDE*, and its role in biofilm formation has been forcefully emphasized in the literature (24, 33, 34).

When averaged over the entire height of relatively thin, 5 h biofilms of *S. mutans* UA159, biofilms demonstrated adhesion-force-controlled gene expression, but this was not observed in thicker, 24 h biofilms (Table 2). In order to study the biofilm height above a substratum surface over which initially adhering streptococci in direct contact with a substratum surface can signal the news of being in an adhering state on a specific surface, 24 h biofilms on silicone rubber were sliced (Fig. 3A). Biofilm slices taken at different heights were examined for expression of the three adhesion-force-sensitive genes identified. In 24 h biofilms, slices taken closest to the substratum surface demonstrated higher expression of the three adhesion-force-sensitive genes than slices of biofilm taken more distant from the surface (Fig. 3B). Thus, adhesion-force-induced gene expression extended over at least half of the biofilm height above a surface, which represents a considerably larger distance than that over which adhesion forces arising from the substratum surface can range (2, 3). In addition to this, most

bacteria in a biofilm have never visited a substratum surface (35). This implies that quorum sensing must be responsible for the extension of adhesion-force-induced gene expression in biofilms. This conclusion is supported by the observation that adhesion-force-induced gene expression of quorum-sensing-deficient *S. mutans* UA159 $\Delta luxS$ was fully absent in both 5 and 24 h biofilms (Table 2).

Moreover, in quorum-sensing-deficient *S. mutans* UA159 $\Delta luxS$, EPS production reflected by local back-scattered light intensities (Fig. 4D) showed identical distributions of soluble EPS over the height of biofilms on silicone rubber and glass (Fig. 4D). Alternatively, in biofilms of *S. mutans* UA159 with the ability of quorum sensing, soluble EPS production on hydrophobic silicone rubber was higher than on hydrophilic glass up to a distance of around 20 to 25 μm above the substratum surface. Thus, it can be concluded based on height-dependent gene expression and local EPS production, that adhesion-force-induced expression of genes extends into a biofilm through quorum sensing over a height limited to 20 to 30 μm above the substratum surface, beyond which auto-inducer concentrations become below their threshold concentrations required to invoke a response. "Calling" distances over which bacteria can communicate through quorum sensing have been reported between 5 μm (36) and 200 μm (37), which indicates that our estimate of 20 to 30 μm as the calling distance in streptococcal biofilms is reasonable.

In summary, this work extends our understanding of emergent properties in streptococcal biofilms and the role of quorum sensing herein. Environmental adhesion forces have been identified to externally control expression of genes that are directly involved in the development of emergent biofilm properties in adhering *S. mutans*, leading to a new concept of "adhesion-force-induced gene expression in adhering bacteria". *brpA* was the most adhesion-force-sensitive gene, as well as the most strongly expressed gene in adhering streptococci. Extension of its expression decreased with height above the substratum surface. Adhesion-force-induced gene expression was fully absent in a quorum-sensing-deficient isogenic streptococcal mutant. The concept of adhesion-force-induced gene expression and its extension through a biofilm through quorum-sensing mechanisms advance our understanding of why biofilms of the same strain or species may possess different properties when grown on different substrata, which is relevant in all environmental, industrial, and biomedical applications where biofilms develop.

MATERIALS AND METHODS

Bacterial strains, growth conditions and harvesting. *S. mutans* UA159 and UA159 $\Delta luxS$ were cultured at 37°C in 5% CO₂ on blood agar for 24 h. One colony was inoculated in 10 mL brain heart infusion (BHI) broth (Oxoid, Basingstoke, United Kingdom) with 1% (wt/vol) sucrose added at 37°C in 5% CO₂ for 24 h. These precultures were used to inoculate the main cultures (1:20 dilution), which were grown for 16 h. For *S. mutans* UA159 $\Delta luxS$, 30 $\mu\text{g mL}^{-1}$ erythromycin was added to both precultures and main cultures. Bacteria were harvested by centrifugation (Beckman J2-MC centrifuge; Beckman Coulter, Inc., Pasadena, CA, USA) for 5 min at 5,000 $\times g$ and washed twice with freshly made buffer (1 mM CaCl₂, 2 mM potassium phosphate, 50 mM KCl, pH 6.8) and resuspended in buffer. In order to break streptococcal chains, bacterial suspensions were sonicated 3 times for 10 s each with 30 s intervals at 30 W (Vibra cell model 375; Sonics and Materials, Inc., Danbury, CT, USA), while cooling in an ice-water bath. The bacterial suspensions were diluted in buffer to a

concentration appropriate for the respective experiments, as determined by enumeration in a Bürker-Türk counting chamber or measurement of the optical density at 600 nm (OD_{600}).

Bacterial cell surface characterization. Microbial adhesion to hydrocarbons (MATH) (Fig. S1) was carried out in its kinetic mode (25) to reveal possible differences in adhesive cell surface properties between *S. mutans* UA159 and UA159 $\Delta luxS$. To this end, streptococci were suspended in buffer to an OD_{600} of between 0.4 and 0.6 (A_0), and 150 μ L hexadecane was added to 3 mL of bacterial suspension. The two-phase system was vortexed for 10 s and allowed to settle for 10 min. The optical density (A_t) was measured, this procedure was repeated 6 more times, and the 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 equation 2:

$$R_0 = \lim_{t \rightarrow 0} \frac{d}{dt} \log \left(\frac{A_t}{A_0} \times 100 \right) \quad (2)$$

Zeta potentials of both *S. mutans* strains ($3 \times 10^8 \text{ mL}^{-1}$) were determined in buffer by particulate microelectrophoresis (Zetasizer nano-ZS; Malvern Instruments, Worcestershire, United Kingdom) at 37°C. All bacterial cell surface characterizations were done in triplicate with different bacterial cultures, and data are presented as averages + standard deviations (SD) of the mean.

Substratum materials and characterization. Four different substratum materials were used in this study: glass (Thermo Scientific, Braunschweig, Germany), bacterial-grade polystyrene (Greiner Bio-One GmbH, Frickenhausen, Germany), tissue-grade polystyrene (Greiner Bio-One GmbH), and medical-grade silicone rubber (ATOS Medical B.V., Zoetermeer, The Netherlands). Polystyrene is a hydrophobic material, mostly applied in microbiology for well plates to keep bacteria in suspension. Therefore, the company also advocates it for use as “suspension culture plates” made of hydrophobic “bacterial-grade” polystyrene. In cell biology, a hydrophilically modified type of polystyrene is preferred, since cells grow on surfaces. These plates are called “tissue culture plates” made of relatively hydrophilic “tissue-grade” polystyrene. All materials were made to fit into a 24-well plate, allowing samples with a surface area of 1 cm^2 . Polystyrene surfaces were used as received, while glass and silicone rubber surfaces were cleaned first with 2% RBS (Rue Bollinckx, Brussels, Belgium) under sonication and rinsed with warm tap water, sterilized in ethanol (96%), and finally washed with sterilized buffer.

The hydrophobicities of the different substratum materials were determined through water contact angle measurements. Water contact angles were measured at 25°C using the sessile drop technique with a home-made contour monitor. Droplets of 1.5 -2 μ L ultra-pure water were put on the different surfaces and the contours of the droplet were 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 the four materials.

Adhesion-force measurement. Single-bacterial-contact probes were prepared by attaching streptococci to a tipless cantilever (NP-O10; Bruker AFM Probes, Camarillo, CA, USA) via electrostatic interaction with poly-L-lysine (PLL) (molecular weight, 70,000 to 150,000; Sigma-Aldrich, St. Louis,

MO, USA) adsorbed to the cantilever using a micromanipulator (Narishige Groups, Tokyo, Japan). Cantilevers were calibrated using the thermal method (38), yielding spring constants in the range of 0.03 to 0.12 N m⁻¹. Briefly, the far end of a tipless cantilever was dipped in a droplet of PLL for 1 min and dried in air for 2 min, followed by 2 min of immersion in a droplet of bacterial suspension (3×10^7 mL⁻¹ in buffer) to allow one bacterium to adhere to the cantilever. Attachment to the PLL-coated cantilever did not affect the viability of the bacteria (39, 40). Freshly prepared bacterial probes were directly used for adhesion force measurements. Adhesion force measurements (Fig. 1A) were performed at room temperature in buffer using a Dimension 3100 system (Nanoscope V; Digital Instruments, Woodbury, NY, USA). For each bacterial probe, force-distance curves were measured with 0, 2, 5, 10, and 30 s of surface delay at a 5 nN trigger threshold. In order to verify whether a measurement series had disrupted bacterial integrity, five force-distance curves at a loading force of 5 nN and surface delay of 0 s were measured at the beginning and end of each experiment on glass. When the adhesion forces measured differed more than 1 nN from the beginning to the end of an experiment, data were discarded and the probe was replaced by a new one.

Biofilm formation. Silicone rubber and glass samples were put in 24-well plates of either bacterial or tissue grade, and initial bacterial adhesion was allowed by adding 1 mL of streptococcal suspension (3×10^8 mL⁻¹) in buffer to each well under static conditions for 2 h at 37°C under 5% CO₂. In addition, initial adhesion was allowed on the bottom of 24-well plates of either bacterial or tissue grade. After 2 h, the bacterial suspension was removed, and each well was carefully washed once with 1 mL buffer, after which 1 mL BHI with 1% sucrose (wt/vol) was added to each well to allow biofilm growth under a static condition in 5% CO₂ at 37°C. After 5 or 24 h of growth, biofilms were carefully washed with buffer and then imaged with OCT (Thorlabs Ganymede, Newton, NJ, USA) to determine their thickness and whiteness distribution over the biofilm height above the substratum surface. Then streptococcal biofilms were carefully scraped off the surfaces and resuspended in buffer for gene expression or for bacterial enumeration in a Bürker-Türk counting chamber as described above in order to calculate volumetric bacterial densities in the biofilm, defined as the number of bacteria divided by the volume they occupy in a biofilm. Alternatively, intact biofilms were embedded in Tissue-Tek OCT compound (Sakura Finetek USA, Inc., Torrance, CA, USA) and stored at -80°C for later cryosectioning.

Gene expression of planktonic and biofilm-grown bacteria. (i) Gene expression in planktonic and resuspended biofilms. Planktonic as well as resuspended biofilm-grown streptococci were centrifuged at $6,500 \times g$ for 5 min, the supernatant was removed, and pellets were stored at -80°C until RNA isolation. In order to prevent possible alterations in gene expression during sample collection, resuspension, centrifugation, and freeze storage were done as fast as possible (less than 45 min). Total RNA was isolated using RiboPure bacterial kit (Ambion, Invitrogen, Foster City, CA) according to the manufacturer's instructions. Traces of genomic DNA were removed using the DNAfree kit (Ambion, Applied Biosystems, Foster City, CA). The amount and quality of extracted RNA were based on the 260/280-nm ratio measured using a NanoDrop ND-1000 (NanoDrop Technologies LLC, Thermo Fisher Scientific, Wilmington, DE). A ratio of A_{260}/A_{280} around 2.0% ($\pm 10\%$) was accepted as "pure" for RNA. A mixture of 200 ng RNA, 4 μ L 5 $\times$ iScript reaction mixture, and 1 μ L iScript reverse transcriptase, in a total volume of 20 μ L (iScript; Bio-Rad, Hercules, CA), was used for cDNA synthesis according to the manufacturer's instructions. Real-time reverse transcription-quantitative PCR (RT-qP-

CR) was performed in a 384-well plate (HSP-3905; Bio-Rad Laboratories, Foster City, CA, USA) with the primer sets for the selected genes (see Table S1 in the supplementary material). The following thermal conditions were used for all RT-qPCRs: 95°C for 3 min and 39 cycles of 95°C for 10 s and 59°C for 30 s. The mRNA levels were quantified in relation to endogenous control gene coding for *16S rRNA*. Gene expression levels in the biofilms were normalized to planktonic *S. mutans* UA159. Gene expression was assessed in triplicate experiments with separately grown cultures.

(ii) Gene expression in biofilm slices as a function of biofilm height above a substratum surface. Twenty-four-hour biofilms grown on silicone rubber surfaces were washed with freshly made buffer and removed from their 24-well plates. Tissue-Tek OCT compound (Sakura Finetek USA, Inc., Torrance, CA, USA) was applied to the biofilm surface, and thus embedded biofilms were subsequently stored at – 80°C. Embedded biofilms were sliced using a cryostat into 10-µm-thick slices taken parallel to the substratum surface. The top, middle, and bottom slices of biofilm (6 slices of 10 µm of the biofilm) were collected separately in 1.5 mL tubes and stored at – 80°C for further RNA isolation and analysis of the expression of selected genes, as described above. Finally, gene expression was normalized with respect to gene expression in the layer adjacent to the substratum surface (i.e., the bottom slices).

OCT imaging. Biofilms were imaged using an OCT Ganymede II (Thorlabs Ganymede, Newton, NJ, USA) with a 930-nm center wavelength white light beam and a Thorlabs LSM03 objective scan lens, providing a maximum scan area of 100 mm². The imaging frequency was 30 kHz, with a sensitivity of 101 dB, and the refractive index of biofilm was set as 1.33, equal to the one of water. Two-dimensional (2D) images had fixed 5,000 pixels with variable pixel size, depending on magnification in the horizontal direction, while containing a variable number of pixels with a 2.68 µm pixel size in the vertical direction. Images were created by the OCT software (ThorImage OCT 4.1) using 32 bit data and signal intensities of back scattered light were reflected by a whiteness distribution in OCT images (41). Biofilm thickness was subsequently determined from the OCT images after Otsu thresholding (42). To eliminate the influence of autoscaling by the instrument on signal intensities of back-scattered light, rescaling was applied (26, 43). Rescaled signal intensities have been demonstrated to reflect the absence or presence of water- and EPS-filled pockets in a biofilm and relate to the volumetric bacterial density in biofilms (26, 43).

Statistical analysis. GraphPad Prism, version 7 (San Diego, CA) was employed for statistical analysis. Significance among groups was assessed by one-way analysis of variance (ANOVA) followed by Dunn's multiple comparisons test. Alternatively, Mann-Whitney test was used to compare two sets of data at a time. For comparison of OCT signal intensities at different biofilm heights Student t-test was applied. Significance was adapted at $p < 0.05$.

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SUPPLEMENTARY MATERIAL

FIG S1 Bacterial cell and substratum surface characteristics involved in adhesion. (A) Microbial Adhesion to Hydrocarbons (MATH) in its kinetic mode (25) measures the removal of bacteria by a hydrophobic ligand from an aqueous phase as a function of vortexing time. Streptococci were suspended in buffer (1 mM CaCl_2 , 2 mM potassium phosphate, 50 mM KCl, pH 6.8) to an optical density $\text{OD}_{600\text{nm}}$ between 0.4 and 0.6 (A_0), and 150 μL hexadecane was added to 3 mL bacterial suspension. The two-phase system was vortexed for 10 s and allowed to settle for 10 min and the optical density (A_t) was measured. This procedure was repeated 6 more 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

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

Hydrophilic bacteria prefer to remain in the aqueous phase rather than be removed from it by adhesion to a hydrophobic ligand and have a low removal rate. (B) OD ratios $\log(A_t/A_0) \times 100$ of *S. mutans* UA159 and *S. mutans* UA159 $\Delta luxS$ grown in BHI with 1% sucrose added as a function of vortexing time. Error bars, indicating SD over triplicate experiments with different streptococcal cultures, are smaller than the data points. (C) Hydrophobicities of bacterial cell and substratum surfaces, expressed by initial removal rates in MATH and water contact angles (WCA), respectively together with the bacterial zeta potentials measured in buffer (3×10^8 bacteria mL^{-1}) using particulate

microelectrophoresis (Zetasizer nano-ZS; Malvern Instruments, Worcestershire, UK) at 37°C. Bacterial cell surface characteristics are averages over triplicate experiments with different streptococcal cultures. $\pm$ indicates SD values over three separate MATH assays, while in case of zeta potentials $\pm$ represents average SD values over a population of around 50 streptococci in a separate culture. (D) The hydrophobicities of the different substratum materials were determined through water contact angle measurements. Water contact angles were measured at 25°C using the sessile drop technique with a home-made contour monitor. Droplets of 1.5 -2 μ L ultra-pure water were put on the different surfaces, and the contours of the droplet were measured between 5 and 10 s after placing a droplet, from which contact angles were subsequently calculated after grey-value thresholding. Water contact angles are averages over triplicate experiments with different samples (with at least 3 spots were chosen on each sample), with $\pm$ indicating SD values. *PS is polystyrene. Superscript letters a to d in panel D indicate significant differences ($p < 0.05$, Mann-Whitney test) between four surfaces used. Shown is spreading of sessile water droplets on the different substratum surfaces used. Note that part of a droplet reflects in the surface, enabling the determination of the exact position of the surface relative to which the contact angle must be calculated.

3

FIG S2 Examples of OCT images of *S. mutans* UA159 and UA159 *ΔluxS* biofilms and their thicknesses. (A) OCT cross-sectional images of 5 and 24 h streptococcal biofilms on the different substratum surfaces involved in this study. The scale bar represents 100 μ m. (B) Thicknesses (μ m) of 5 and 24 h streptococcal biofilms on the different substratum surfaces, determined from the OCT images after Otsu thresholding (42). All 24 h biofilms are significantly ($P < 0.05$, Mann-Whitney test) thicker than corresponding 5 h biofilms. “ $\pm$ ” indicates SD over different experiments with separately cultured bacteria ($n=3$).

FIG S3 Biofilm gene expression in *S. mutans* UA159 and UA159 $\Delta luxS$ biofilms, grown on four different substratum surfaces, after 5 and 24 h. (A) Gene expression in biofilms was normalized to planktonic streptococci in each of the two strains used. (B) Heat map of gene expression in 5 h biofilms of *S. mutans* UA159 on the different substratum surfaces. The pseudocolor scale represents the level of expression of the respective genes. (C) Fold expression of selected genes in 5 h streptococcal biofilms on different substratum surfaces, normalized with respect to planktonic streptococci in each of the two strains used. (D) Same as panel C, now for 24 h biofilms.

FIG S4 Example of *brpA* amplification and melting curves in 5 h biofilms of both streptococcal strains on silicone rubber surfaces (results from duplicate wells). The green line indicates background fluorescence.

FIG S5 (to be continued.)

FIG S5 (continued.) Normalized fold gene expression with insignificant relationships with adhesion forces in *S. mutans* UA159 and its $\Delta luxS$ isogenic mutant as a function of the stationary adhesion force to different substratum surfaces over the entire height of 5 h biofilms. Error bars denote SD values in fold gene expression over triplicate experiments, while the solid lines represent assumed linear relationships through the data points, with the correlation coefficient R^2 as presented. Dotted lines represent 95% confidence intervals.

FIG S6 (to be continued.)

FIG S6 (continued.) Normalized fold gene expression with insignificant relationships to adhesion forces in *S. mutans* UA159 and its $\Delta luxS$ isogenic mutant as a function of the stationary adhesion force to different substratum surfaces over the entire height of 24 h biofilms. Error bars denote SD values in fold gene expression over triplicate experiments, while the solid lines represent assumed linear relationships through the data points, with the correlation coefficient R^2 as presented. Dotted lines represent 95% confidence intervals.

TABLE S1 Primer sequences for RT-qPCR used in this study.

Gene	Primer sequence (5' to 3')	
	Forward	Reverse
<i>16SrRNA</i>	CCTACGGGAGGCAGCAGTAG	CAACAGAGCTTTACGATCCGAAA
<i>ftf</i>	AAATATGAAGGCGGCTACAACG	CTTCACCAGTCTTAGCATCCTGAA
<i>gtfB</i>	AGCAATGCAGCCAATCTACAAAT	ACGAACTTTGCCGTTATTGTCA
<i>brpA</i>	GGAGGAGCTGCATCAGGATTC	AACTCCAGCACATCCAGCAAG
<i>comDE</i>	ACAATTCCTTGAGTTCCATCCAAG	TGGTCTGCTGCCTGTTGC
<i>vicR</i>	TGACACGATTACAGCCTTTGATG	CGTCTAGTTCTGGTAACATTAAGTCCAATA
<i>gbpB</i>	ATGGCGGTTATGGACACGTT	TTTGGCCACCTTGAACACCT
<i>relA</i>	ACAAAAAGGGTATCGTCCGTACAT	AATCACGCTTGGTATTGCTAATTG

Chapter 4

Streptococcus mutans Adhesion Force Sensing in Multi-species

Oral Biofilms

Can Wang, Henny C. van der Mei, Henk J. Busscher, Yijin Ren

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ABSTRACT

Bacteria utilize chemical and mechanical mechanisms to sense their environment in order to survive hostile conditions. In mechanical-sensing, intra-bilayer pressure profiles change due to deformation induced by the adhesion forces bacteria experience on a surface. Emergent properties in mono-species *Streptococcus mutans* biofilms, like extracellular-matrix production, depend on the adhesion forces that streptococci sense. Here, we determined whether and how salivary conditioning film (SCF)-adsorption and the multi-species nature of oral biofilm influence adhesion force sensing and associated gene-expression by *S. mutans*. Hereto, *Streptococcus oralis*, *Actinomyces naeslundii* and *S. mutans* were grown together on different surfaces in absence and presence of an adsorbed SCF. Atomic force microscopy and RT-qPCR were used to measure *S. mutans* adhesion forces and gene-expressions. Upon SCF-adsorption, stationary adhesion forces decreased on a hydrophobic and increased on a hydrophilic surface to around 8 nN. Optical coherence tomography showed that triple-species biofilms on SCF-coated surfaces with dead *S. oralis* adhered weakly and often detached as a contiguous sheet. Concurrently, *S. mutans* displayed no differential adhesion force sensing on SCF-coated surfaces in the triple-species biofilms with dead *S. oralis*, but once live *S. oralis* were present *S. mutans* adhesion force sensing and gene-expression ranked similar as on surfaces in absence of an adsorbed SCF. Concluding, live *S. oralis* may enzymatically degrade SCF-components to facilitate direct contact of biofilm inhabitants with surfaces and allow *S. mutans* adhesion force sensing of underlying surfaces to define its appropriate adaptive response. This represents a new function of initial colonizers in multi-species oral biofilms.

INTRODUCTION

Environmental sensing is of vital importance for bacteria to counter the many hostile conditions they may endure during their growth. Bacteria can sense and respond to chemical signals such as pH, ionic strength or auto-inducers involved in quorum sensing (1). However, bacteria also respond to a variety of mechanical signals (2), such as adhesion forces arising from a surface to which they adhere. Upon adhesion to a surface, bacterial cell surfaces slightly deform under the influence of the adhesion forces (3), which impacts the intra-bilayer pressure profile across their lipid membrane (4). These pressure profile changes can stimulate gene expressions in bacteria upon adhesion to a surface that are important for the adaptation of planktonically growing bacteria into a biofilm mode of growth. In a biofilm mode of growth, biofilm inhabitants often possess new, emergent properties alien to their planktonic counterparts (5). Increasing adhesion forces of *Staphylococcus aureus* on surfaces decreased expression of *icaA* genes and production of poly-N-acetylglucosamine and eDNA, both of direct relevance as a glue in the formation of the extracellular polymeric substance (EPS) matrix of a biofilm (6). Also, *NsaS* sensor and *NsaAB* efflux pump transcript levels in *S. aureus* were enhanced as a response to antibiotic presence when staphylococci were adhering to a surface (7). *Streptococcus mutans*, one of the main cariogenic strains in the oral cavity, has been demonstrated to possess three adhesion force sensitive genes (*brpA*, *gbpB*, *comDE*) responsible for emergent EPS production on silicone rubber, bacterial grade polystyrene, tissue-grade polystyrene and glass (8) (these surfaces are listed in the order of decreasing water contact angle ("hydrophobicity"), corresponding with a decrease in adhesion forces from 20 nN to 3 nN). Adhesion force induced gene-expression and emergent EPS production in *S. mutans* biofilms were limited to the first 20-30 μm above a substratum surface, but fully absent in a quorum-sensing deficient *S. mutans* strain. A distance of 20-30 μm above a substratum surface is within the "calling-distances" reported for producing, releasing, sensing and responding to auto-transducer gradients in quorum-sensing (9-11). Collectively, this suggests that the results of adhesion force sensing by streptococci directly in contact with a surface are carried into a biofilm through quorum-sensing. This highlights the interplay between chemical and mechanical, environmental sensing by adhering bacteria and their role in biofilm formation.

In oral biofilm formation, adsorption of salivary proteins precedes adhesion of initial bacterial colonizers to a surface, modulating bacterial adhesion to surfaces (12) and possibly adhesion force sensing and associated surface adaptation. Moreover, oral biofilms are composed of many different bacterial strains and species that are all involved in biofilm formation in a spatio-temporal sequence (13, 14). *Streptococcus oralis* and *Actinomyces naeslundii* have their own role in this sequence and are among the dominant initial colonizers that adhere to the adsorbed salivary conditioning film and form a co-aggregating pair. After adhesion of initial colonizers, later colonizers, such as *S. mutans* and other oral bacterial strains come into play, due to coaggregation (13) or local changes in the environment (15, 16), giving them a different position in the spatio-temporal sequence of oral biofilm formation. Oral bacteria not only adhere to tooth surfaces, but also to the surfaces of composite restorations, orthodontic appliances (17) and other oral bacterial cell surfaces. Accordingly, oral bacteria encounter many different surfaces, each with their own surface charge and hydrophobicity, to which they may adhere and adapt with different emergent properties (11).

The aim of this article is to determine whether and how salivary conditioning film adsorption and

the presence of *S. oralis* and *A. naeslundii* influence adhesion force sensing and associated expression of *brpA*, *gbpB* and *comDE* by *S. mutans* in a triple-species, oral biofilm model. In order to evaluate possible interference by *S. oralis* or *A. naeslundii* on *S. mutans* adaptive gene expression, live and dead *S. oralis* and/or *A. naeslundii* were used to grow triple-species biofilms. Single bacterial contact probe atomic force microscopy (AFM) was applied to measure the adhesion forces that *S. mutans* senses on different surfaces in the absence and presence of an adsorbed conditioning film, while adaptive gene expressions in biofilms were evaluated using RT-qPCR. Genes were selected that all have a demonstrated role in adhesion and growth of *S. mutans*: *brpA* regulates cell wall stress responses, biofilm cohesiveness and biofilm formation, while *gbpB* regulates antibiotic sensitivity, osmotic and oxidative stresses, cell wall construction and maintenance, cell shape, hydrophobicity, and sucrose-dependent biofilm formation and *comDE* relates with persister cell formation and bacteriocin production (8).

RESULTS

Physical interactions in triple-species biofilms

(i) ***S. mutans* adhesion-forces.** *S. mutans* adhesion forces on different surfaces were measured using single bacterial contact probe AFM. The bacterial probe was brought to a surface and after different time periods retracted from the surface. During this bond maturation time period, bacterial cell surface appendages re-arrange, molecules change conformation and water is removed from in between the bacterial cell surface and a substratum surface to increase the adhesion force. These processes are all physico-chemical in nature and equally occur in bacteria as well as in inert polystyrene particles (18). All *S. mutans* adhesion forces increased with increasing bond maturation times (Fig. S1) between the bacterium and any surface, whether glass, silicone rubber or a bacterial cell surface. Accordingly, adhesion forces were plotted as a function of bond maturation time (Fig. S2) and fitted to an exponential function, yielding an initial adhesion force F_0 measured immediately upon contact, a characteristic bond maturation time τ and a stationary adhesion force, $F_{\text{stationary}}$ reached after complete bond maturation (see Supplementary material for calculational details). All initial adhesion forces were significantly lower than the stationary adhesion forces calculated (Table 1). Initial adhesion forces increased fastest on hydrophilic glass surfaces (τ is 4 s), and slowest on hydrophobic silicone rubber surfaces (τ equals 21 s). *S. mutans* adhesion forces with any of the other two strains increased with intermediate bond maturation times. Stationary adhesion forces on glass increased after adsorption of a salivary conditioning film ($P > 0.05$, Mann-Whitney test), while on silicone rubber stationary adhesion forces decreased ($P < 0.05$, Mann-Whitney test). Interestingly, both surfaces displayed similar stationary *S. mutans* adhesion forces after adsorption of a salivary conditioning film ($P > 0.05$, Mann-Whitney test). Stationary adhesion forces between *S. mutans* and *S. oralis* and *A. naeslundii* were all smaller than the adhesion forces arising from the substratum surfaces ($P > 0.05$, one-way ANOVA). Live and dead, heat-killed *S. oralis* and *A. naeslundii* presented the same stationary adhesion forces to *S. mutans*, indicating that heat-killing did not affect the adhesive surface properties of the strains. This was confirmed by zeta potential measurements, showing similarly negative zeta potentials for live and dead bacteria (Table S1, $P > 0.05$, Mann-Whitney test). Summarizing, *S. mutans* stationary adhesion forces differed greatly on silicone rubber and glass surfaces, but converged upon

adsorption of a salivary conditioning film to these surfaces. *S. mutans* stationary adhesion forces to *S. oralis* and *A. naeslundii* cells were smaller than to the materials surfaces, regardless whether these bacteria were dead or alive.

TABLE 1 Initial and stationary streptococcal adhesion forces F_0 and $F_{\text{stationary}}$ together with the characteristic bond maturation time constant τ for *S. mutans* UA159 with different substratum surfaces in the absence and presence of an adsorbed salivary conditioning film (SCF) and with *S. oralis* J22 and *A. naeslundii* T14V-J1. $\pm$ signs represent standard deviations (SD) over 35 force-distance curves. The force-distance curves were recorded using 3 separately prepared bacterial probes, taken out of 3 different bacterial cultures, each measuring adhesion forces on 7 different contact points and measuring 5 force-distance curves for on each contact point.

<i>S. mutans</i> UA159 adhesion to	F_0 (nN)	τ (s)	$F_{\text{stationary}}$ (nN)
glass ^a	0.7 ± 0.1^{bc}	4 ± 3^c	4.1 ± 1.3^c
SCF-coated glass	0.4 ± 0.1	8 ± 4	7.9 ± 4.3
silicone rubber ^a	1.2 ± 0.4^b	21 ± 11	19 ± 14^b
SCF-coated silicone rubber	0.3 ± 0.1^c	14 ± 9	7.8 ± 2.6
live <i>S. oralis</i>	0.4 ± 0.1	13 ± 3	2.8 ± 0.1
dead <i>S. oralis</i>	0.5 ± 0.2	13 ± 5	3.4 ± 0.9
live <i>A. naeslundii</i>	0.6 ± 0.3	11 ± 9	4.1 ± 1.5
dead <i>A. naeslundii</i>	0.8 ± 0.3	9 ± 4	5.5 ± 1.1

^a Data taken from Wang et al (8).

^b Significantly different from SCF-coated substrata ($P < 0.05$, Mann-Whitney test).

^c Significantly different from corresponding data on silicone rubber ($P < 0.05$, Mann-Whitney test).

(ii) (Co-)aggregation of different strain combinations. The adhesion forces measured using AFM occurred after forced contact between the interacting surfaces. In order to determine up to what extent the three strains can be expected to adhere to each other and sense adhesion forces exerted upon each other during adhesion and biofilm formation from suspension, a co-aggregation assay was carried out. Co-aggregation of the three bacterial strains was investigated using the well-established and generally-accepted, semi-quantitative assay proposed by Kolenbrander and Andersen (19). In the assay, equal volumes of one or more of the bacterial suspensions are mixed. Aggregation of any of the bacterial strains in mono-species suspension in absence of other strains did not occur (Table 2), regardless of whether bacteria were alive or dead. Also, *S. mutans* did not co-aggregate with *S. oralis* nor *A. naeslundii* in dual-species suspensions, also regardless of whether alive or dead. This implies that in triple-species biofilms, *S. mutans* is unlikely to sense any adhesion forces arising from either *S. oralis* or *A. naeslundii*. Both alive and dead *S. oralis* and *A. naeslundii* co-aggregated in dual-species

suspensions, while also triple-species suspensions demonstrated clear co-aggregation.

TABLE 2 (Co-)aggregation scores^a of different combinations of *S. mutans* UA 159, live or dead *S. oralis* J22 and *A. naeslundii* T14V-J1 in mono-, dual- and triple-species suspensions. Experiments were done in triplicate with separately cultured bacteria, yielding similar scores.

Mono-species suspensions			Score ^a
<i>S. mutans</i>	<i>S. oralis</i>	<i>A. naeslundii</i>	
live	-	-	0
-	live	-	0
-	dead	-	0
-	-	live	0
-	-	dead	0
Dual-species suspensions			Score ^a
<i>S. mutans</i>	<i>S. oralis</i>	<i>A. naeslundii</i>	
live	live	-	0
live	dead	-	0
live	-	live	0
live	-	dead	0
-	live	live	3
-	live	dead	2
-	dead	live	2
-	dead	dead	2
Triple-species suspensions			Score ^a
<i>S. mutans</i>	<i>S. oralis</i>	<i>A. naeslundii</i>	
live	live	live	3
live	live	dead	2
live	dead	live	2
live	dead	dead	2

^a Score 0: no change in turbidity and no visible co-aggregates; Score 1: weak coaggregation with dispersed aggregates in a turbid background; Score 2: clearly visible, small co-aggregates, not settling immediately; Score 3: large settling co-aggregates, leaving a slightly turbid suspension; Score 4: maximum coaggregation large co-aggregates settled immediately leaving a fully clear supernatant (19).

Thickness and composition of biofilms. *S. mutans* mono-species biofilms and different triple-species biofilms were grown on SCF-coated glass and silicone rubber surfaces for 5 h and 24 h. In order to determine their thickness, biofilms were imaged using optical coherence tomography (OCT) (Fig. 1). Thickness could only be measured in cases of bacteria initially adhering to substratum surfaces in a-

FIG 1 Examples of OCT images of *S. mutans* UA159 mono-species biofilm and different triple-species biofilms. The triple-species biofilms included live or dead *S. oralis* J22 and *A. naeslundii* T14V-J1

in addition to live *S. mutans* UA159 after 5 h and 24 h growth on different substratum surfaces. OCT images of *S. mutans* mono-species biofilms on glass and silicone rubber surfaces in the absence of an adsorbed salivary conditioning film (SCF) are taken from (8). Scale bars indicate 50 μm .

absence of a salivary conditioning film, and in cases where live *S. oralis* were present. All 24 h biofilms were thicker than 5 h biofilms.

Contiguous sheets of *S. mutans* mono-species biofilms as well as of triple-species biofilms in the absence of live *S. oralis* detached during handling of the biofilms from SCF-coated substrata, as caused by even the slightest fluid flow within the wells. This may well be due to the relatively small adhesion forces of the initial colonizers to salivary conditioning films as compared to bare substratum surfaces (20). Moreover, it indicates that only live *S. oralis* paves a way through the salivary conditioning film to ensure direct, strong contact of individual biofilm inhabitants and strong adhesion of an entire triple-species biofilm to a substratum surface.

TABLE 3 The number of CFU per unit substratum surface area ($10^7/\text{cm}^2$) of each strain in *S. mutans* UA159 mono-species and in different triple-species biofilms with live or dead *S. oralis* J22 and *A. naeslundii* T14V-J1 on salivary conditioning film (SCF)-coated glass and silicone rubber surfaces. Experiments were done in triplicate with separately cultured bacteria, $\pm$ signs represent standard deviations (SD).

Biofilm	Biofilm inhabitants	SCF-coated glass		SCF-coated silicone rubber	
		5 h	24 h	5 h	24 h
Mono-species	live <i>S. mutans</i>	4.3 $\pm$ 0.6	19.0 $\pm$ 6.3	9.4 $\pm$ 2.4	16.3 $\pm$ 6.6
Triple-species	live <i>S. mutans</i>	1.5 $\pm$ 0.3	4.6 $\pm$ 2.7	1.8 $\pm$ 0.3	6.3 $\pm$ 3.1
	live <i>S. oralis</i>	33.4 $\pm$ 7.2	0.1 $\pm$ 0.1	41.6 $\pm$ 0.3	0.3 $\pm$ 0.1
	live <i>A. naeslundii</i>	5.2 $\pm$ 1.9	0.1 $\pm$ 0.0	5.4 $\pm$ 3.3	0.1 $\pm$ 0.1
Triple-species	live <i>S. mutans</i>	4.1 $\pm$ 1.2	17.3 $\pm$ 3.5	3.5 $\pm$ 0.8	15.4 $\pm$ 3.2
	dead <i>S. oralis</i>	0	0	0	0
	dead <i>A. naeslundii</i>	0	0	0	0
Triple-species	live <i>S. mutans</i>	1.1 $\pm$ 0.5	0.5 $\pm$ 0.1	0.8 $\pm$ 0.3	0.3 $\pm$ 0.1
	live <i>S. oralis</i>	35.5 $\pm$ 5.8	0.1 $\pm$ 0.0	28.9 $\pm$ 13.4	0.1 $\pm$ 0.04
	dead <i>A. naeslundii</i>	0	0	0	0
Triple-species	live <i>S. mutans</i>	6.6 $\pm$ 1	10.0 $\pm$ 0.4	3.6 $\pm$ 1.1	10.0 $\pm$ 5.6
	dead <i>S. oralis</i>	0	0	0	0
	live <i>A. naeslundii</i>	2.8 $\pm$ 2.1	ND*	2.0 $\pm$ 1.2	ND*

*ND is not detectable CFU $< 10^3/\text{cm}^2$.

In order to monitor the composition of the triple-species biofilms grown as a function of time, biofilms were scraped off the surfaces, suspended in buffer and serial dilutions grown on different (selective) agar plates. Growth on blood agar allowed growth of all strains, while selective agar plates

only allowed growth of exclusively *S. mutans* or *A. naeslundii*. *S. oralis* counts were then determined by subtraction from the blood agar count. *S. mutans* mono-species biofilms contained between 4.3×10^7 CFU/cm² and 9.4×10^7 CFU/cm² after 5 h of growth on salivary conditioning films adsorbed on glass and silicone rubber surfaces, respectively. *S. mutans* numbers increased to 19.0×10^7 CFU/cm² and 16.3×10^7 CFU/cm² after 24 h of growth (Table 3).

FIG 2 The percentage composition of different triple-species biofilms containing *S. mutans* UA159, in combination with live or dead *S. oralis* J22 and *A. naeslundii* T14V-J1. The percentage composition was expressed relative to the total number of CFU/cm² in a biofilm. Error bars represent SD values over triplicate experiments with different bacterial cultures.

The percentage composition of each strain in different biofilms was calculated by dividing the number of CFU of each strain by the total number of CFUs in a biofilm, as displayed in Fig. 2. Biofilm compositions were similar on SCF-coated glass and SCF-coated silicone rubber. Whenever present as a live organism, *S. oralis* was the dominant strain in 5 h old triple-species biofilms, whereas in corresponding 24 h old biofilms *S. mutans* became the dominant strain. *A. naeslundii* presence remained low, except when grown in triple-species with dead *S. oralis*.

***S. mutans* gene expressions in different biofilms.** Adaptive gene expression of *brpA*, *gbpB* and *comDE* in *S. mutans* UA159 biofilms, previously demonstrated to be most adhesion force sensitive genes (8), was determined in planktonic and mono-, dual- and triple-species biofilms with RT-qPCR.

In the absence of an adsorbed salivary conditioning film, 5 h *S. mutans* mono-species biofilm displayed *brpA* gene expression that increased from a planktonic state to an adhering state. Expression of *brpA* gene was higher on hydrophobic silicone rubber than on hydrophilic glass (Fig. 3A). Once coated with a salivary conditioning film, differences in *brpA* gene expression in *S. mutans* biofilms on both substratum surfaces completely disappeared, but expression remained higher than for *S. mutans* in a planktonic state. This typical ranking of *brpA* gene expression observed in *S. mutans* mono-species biofilms in absence of an adsorbed salivary conditioning film was nearly fully restored in triple-species biofilms containing live *S. mutans* and *S. oralis* in combination with dead *A. naeslundii*. Triple-species biofilms in which all three strains were alive suggest the on-set of restoration of the ranking observed for *S. mutans* mono-species biofilms, but not as convincingly as when dead *A. naeslundii* were included in the biofilm.

FIG 3 Gene expression in *S. mutans* UA159 mono-species biofilms and different triple-species oral biofilms. Different triple-species biofilms were comprised of *S. mutans* UA159 (*S. m.*) in combination with live or dead *S. oralis* J22 (*S. o.*) and *A. naeslundii* T14V-J1 (*A. n.*) in the absence and presence of an adsorbed salivary conditioning film on different substratum surfaces. (A) *brpA* gene expression. (B) *gbpB* gene expression. (C) *comDE* gene expression. Error bars represent SD values over triplicate experiments with separately grown biofilms. Data on *brpA*, *gbpB* and *comDE* expressions in *S. mutans* UA159 mono-species biofilms on glass and silicone rubber surfaces in absence of an adsorbed salivary conditioning film were taken from (8).

Restoration of the ranking as observed for *brpA* gene expression in *S. mutans* mono-species biofilms in absence of an adsorbed salivary film on the different surfaces by live *S. oralis*, was confirmed in triple-species biofilms for *gbpB* (Fig. 3B) and *comDE* (Fig. 3C) gene expression on SCF-coated surfaces. Differences in gene expressions were minor in 24 h mono-species and triple-species oral biofilms (Fig. S3).

DISCUSSION

In this article we aimed to determine whether the principles of mechano-microbiology (2), most notably adhesion force sensing (21) with respect to environmental sensing by bacteria adhering to substratum surfaces, are preserved in multi-species biofilms in absence and presence of an adsorbed salivary conditioning film on the surfaces. The early dominance of *S. oralis* in 5 h old triple-species biofilms followed later by *S. mutans* dominance in 24 h old biofilms. In absence of the ability to co-aggregate with the two initial colonizers involved in this study (see Table 2), *S. mutans* appears later in spatio-temporal sequence of multi-species biofilm growth in vitro (22) and oral biofilm formation in general (13) due to the acid environment it needs to create for itself (15,16).

Hydrophobicity is an important substratum surface characteristic determining bacterial adhesion forces. Generally, bacterial adhesion forces are stronger on hydrophobic surfaces than on more hydrophilic surfaces (23,24). In an environment, laden with proteins, protein adsorption proceeds faster than bacterial adhesion because proteins diffuse faster towards a surface than bacteria (25,26). Hence in many environments, bacteria adhere to a protein film coated surface (27). Saliva is composed of a large variety of proteins that maintain surface thermodynamic homeostasis (28,29) and converge differences in the hydrophobicity of surfaces exposed to the oral cavity to a well-controlled, narrow range. Oral surface thermodynamic homeostasis is achieved by polar-apolar layering in SCF adsorption (28), i.e. hydrophilic surfaces, such as a glass surface, are made more hydrophobic by allowing protein adsorption in a conformation that presents hydrophobic groups to its outer surface, while on a hydrophobic surface the opposite occurs. Polar-apolar layering therewith explains why stationary adhesion forces sensed by *S. mutans* decreased on hydrophobic silicone rubber and increased on hydrophilic glass upon SCF-coating to a similar value (Table 1). Oral surface thermodynamic homeostasis therewith fully explains why *S. mutans* did not display differential adhesion force sensing in 5 h mono-species biofilms on SCF-coated surfaces (Fig. 3).

Having excluded differential adhesion force sensing on different SCF-coated materials surfaces, we now address the probability of *S. mutans* sensing of adhesion forces arising from other species in our triple species biofilms. The probability that *S. mutans* also senses adhesion forces arising from other bacteria is considered low, as co-aggregation between *S. mutans* and any of the two initial colonizers involved in this study, was absent (Table 2). Also, volumetric bacterial densities of different biofilms in absence of co-aggregating strains and species are generally less (30) than 0.3 bacteria μm^3 with distances between biofilm inhabitants between 1 and 3 μm (31). This is far beyond the range (< 500 nm) of adhesion forces between surfaces (32,33) and excludes interferences in *S. mutans* adhesion force sensing by direct contact with other bacteria in a multi-species biofilm.

The ranking of gene-expression by planktonic *S. mutans* and *S. mutans* adhering on substratum surfaces in the absence of a salivary conditioning film was only restored in triple-species biofilms with live *S. oralis* present (see also Fig. 3). *S. mutans* gene expression in the presence of live *S. oralis* was weaker in the additional presence of live *A. naeslundii* than in the presence of dead *A. naeslundii* on SCF-coated silicone rubber, presumably because both initial colonizers in a live state competed for real estate of a SCF-coated substratum. Having now excluded *S. mutans* sensing of adhesion forces arising from different SCF-coated materials and from other bacteria in our triple-species biofilms, this leaves the question how *S. mutans* adhesion force sensing on SCF-coated surfaces is re-

stored to the ranking observed on uncoated materials surfaces.

To answer this question, we turn to the sheet-detachment observed. Sheet-detachment of oral biofilm from SCF-coated substrata is a new phenomenon to us, that may have remained unnoticed, also in the literature, because the field of view of microscopic observation applied in most studies is much smaller than in optical coherence tomography (approximately 10 mm² in OCT as applied in this study versus 0.1 mm² e.g. in CLSM at 40 × magnification). Also, the search for beautiful images as common in microscopic observation may have biased the literature and prevented observation of sheet-detachment of biofilm. Sheet-detachment from SCF-coated surfaces of our *S. mutans* mono-species and *S. mutans* containing triple-species biofilms exclusively occurred in absence of live *S. oralis*. Oral bacteria adhere significantly with weaker forces to SCF-coated surfaces as compared with surfaces in the absence of salivary conditioning films (20,34). It may be considered a new, befitting role for initial colonizers to facilitate direct adhesion of other biofilm inhabitants to a substratum surface and therewith establish much stronger adhesion forces of bacteria with a substratum surface than established when the salivary conditioning film is in between. *S. oralis* with its high prevalence in triple-species biofilms as opposed to *A. naeslundii* (see Fig. 2), is ideally equipped for enzymatic degradation of salivary proteins. *S. oralis* is known to possess glycosidase and proteolytic activities to break down salivary conditioning film components into metabolizable fragments (35). Oppositely and in addition to its low prevalence, *A. naeslundii* is not able to degrade oral mucins, unless in combination with *Streptococcus mitis*, *Streptococcus gordonii* and *Streptococcus cristatus* (36). Collectively, these considerations lead to the conclusion that *S. oralis* clears the way through a salivary condition film for strong adhesion forces of a biofilm and direct contact of other biofilm inhabitants to directly contact a surface. This allows *S. mutans* surface sensing to define its appropriate response to SCF-coated substratum surfaces in multi-species biofilms.

Adhesion force sensing was not observed in 24 h triple-species biofilms, nor in mono-species *S. mutans* biofilms regardless of the absence or presence of an adsorbed salivary conditioning film on a substratum surface (Fig. S3). Previously, we attributed this to the limited calling distance of quorum-sensing (8). Analysis of gene-expression of cross-sections of mono-species *S. mutans* biofilms on substrata in absence of a salivary conditioning film demonstrated extension of adhesion force induced gene expressions up to a height of 20 – 30 µm above a surface, far above the thickness of our 24 h triple-species biofilms (Fig. 1).

In summary, bacteria cause infections by adhering to a variety of protein coated natural or synthetic surfaces in the human body (37,38). Most human infections are caused by bacteria in a biofilm-mode of growth, in which they surface-adapt (39). This study shows, that in multi-species oral biofilms adhering to SCF-coated surfaces, surface-adaptation by *S. mutans* to surfaces remains to exist. Based on available literature, this is suggested to occur through breaking down of the salivary conditioning film by initial colonizers, most notably *S. oralis*. The three genes considered in this article to establish this conclusion (gene *brpA*, *gbpB* and *comDE*) are all relevant for the production of the *S. mutans* protective extracellular matrix (40-42). Since *S. mutans* is a prominent cariogenic pathogen (43) whose colonization of the oral cavity may initiate serious organ infection (44) including endocarditis (45), the importance of this conclusion extends far beyond oral health.

METHODS

Substratum materials and salivary conditioning film adsorption. Glass (Thermo Scientific, Braunschweig, Germany) and medical-grade silicone rubber (ATOS Medical B.V., Zoetermeer, The Netherlands) were used as substrata, as they have been previously shown to represent two ends of the scale with respect to hydrophobicity (water contact angles 11 and 103 degrees, respectively) and *S. mutans* gene expression (*brpA* gene expression was maximal on hydrophobic medical-grade silicone rubber surface and minimal on hydrophilic glass surface) in mono-species biofilms (8). All materials were made to fit into a 24 well-plate, allowing samples with a surface area of 1 cm², cleaned with 2% RBS 35 detergent (Omnilabo International BV, The Netherlands) under sonication for 5 min, rinsed with warm tap water, sterilized with 70% ethanol, and finally washed with sterilized demineralized water.

Depending on the experiment, cleaned surfaces were coated with a salivary conditioning film. To this end, human whole saliva was collected and prepared, as previously described (27,46). Briefly, human whole saliva from 20 healthy volunteers of both sexes was collected into ice-chilled Erlenmeyer flasks after stimulation by chewing Parafilm®. Saliva was collected with the informed consent of the voluntary saliva-donors, in accordance with the rules set out by the Ethics Committee at the University Medical Center Groningen. After collection, the salivary samples were pooled and centrifuged twice (10,000 × g, 15 min, 4°C), and phenylmethylsulfonyl fluoride was added to a final concentration of 1 mM as a protease inhibitor. Afterwards, the solution was centrifuged again, dialyzed (24 h, 4°C) against demineralized water, and freeze-dried for storage. For salivary conditioning film adsorption, freeze-dried saliva was dissolved in a calcium-phosphate buffer (1 mM CaCl₂, 2 mM potassium phosphate, 50 mM KCl, pH 6.8) at a concentration of 1.5 mg mL⁻¹ and samples were immersed in 1 mL of reconstituted human whole saliva for overnight adsorption. After salivary conditioning film adsorption, samples were immediately used for further experiments.

Bacterial strains, growth conditions and harvesting. All bacterial strains used were cultured on blood agar (Mediaproduits BV, Groningen, The Netherlands) plates from a frozen stock. *S. mutans* UA159 was grown at 37°C in 5% CO₂ for 24 h, *S. oralis* J22 at 37°C in ambient air for 24 h and *A. naeslundii* T14V-J1 at 37°C under anaerobic conditions for 48 h. Next, one colony of each strain was inoculated in 10 mL brain heart infusion (BHI) broth (Oxoid, Basingstoke, United Kingdom) with 1% (wt/vol) sucrose added and cultured at 37°C in 5% CO₂ for 24 h. These pre-cultures were used to inoculate main cultures (1:20 dilution), which were grown for 16 h. Bacteria were harvested by centrifugation (Beckman J2-MC centrifuge; Beckman Coulter, Inc., Pasadena, CA, USA) for 5 min at 5000 × g and washed twice in buffer. In order to break streptococcal chains, bacterial suspensions were sonicated once for 30 s at 130 W (Vibra cell model VCX130, Newtown Connecticut USA) while cooling in an ice-water bath. The bacterial suspensions were diluted in buffer to a concentration appropriate for the respective experiments, as determined by enumeration in a Bürker-Türk counting chamber. Non-viable *S. oralis* J22 and *A. naeslundii* T14V-J1 suspension were prepared by heat-killing of the suspended bacteria in a water bath, kept at 60°C for 30 min. Heat-killing was confirmed using plate counting (see below).

Bacterial cell surface characterization. Zeta potentials are an important characteristic of bacteria, relevant for initial adhesion (32) and were determined in bacterial suspensions ($3 \times 10^8 \text{ mL}^{-1}$) in the above described calcium-phosphate buffer using particulate microelectrophoresis (Zetasizer nano-ZS; Malvern Instruments, Worcestershire, United Kingdom) at 37°C . Zeta potentials of all strains were measured in triplicate with different bacterial cultures, and data are presented as averages $\pm$ standard deviations (SD) of the mean.

Co-aggregation assay. Pairwise co-aggregation properties of the three bacterial strains involved in the biofilm model applied were measured in order to determine whether or not bacteria in different combinations had direct adhesive contact with each other. For co-aggregation measurements, we have used the well-established and generally-accepted, semi-quantitative Kolenbrander assay (19). Co-aggregation was assayed by mixing equal volumes (0.5 mL) of one or more bacterial suspensions ($1 \times 10^9 \text{ mL}^{-1}$) in buffer during 120 min and subsequently monitoring the decrease in turbidity of the suspension due to co-aggregation. After mixing, co-aggregation was scored on a scale from 0 (evenly turbid suspension) to 4 (fully clear suspension) (19). A full description of all scores is given in the footnote to Table 2 and visual images of each score are presented in Fig. S4.

Adhesion-force measurements. For adhesion force measurements, single bacterial contact probes (47) were prepared as described before (7,48). First, *S. mutans* was attached to a tipless cantilever (NP-O10; Bruker AFM Probes, Camarillo, CA, USA) via electrostatic double-layer interaction with poly-L-lysine (PLL) (molecular weight 70,000 to 150,000; Sigma-Aldrich, St. Louis, MO, USA) adsorbed to the cantilever using a micromanipulator (Narishige Groups, Tokyo, Japan). Cantilevers were calibrated using the thermal method, yielding spring constants in the range of 0.03 to 0.12 N m^{-1} . Briefly, the far end of a tipless cantilever was dipped in a droplet of PLL for 1 min and dried in air for 2 min, followed by 2 min of immersion in a droplet of a streptococcal suspension at a relatively low concentration ($3 \times 10^7 \text{ mL}^{-1}$ in buffer) to allow one bacterium to adhere to the cantilever. Single bacterial adhesion from low concentration suspensions was demonstrated previously using fluorescence microscopy of LIVE/DEAD stained (BacLight viability stain; Molecular Probes Europe BV, Leiden, The Netherlands) staphylococci (7) and streptococci (48) attached to a PLL coated cantilever. Use of LIVE/DEAD staining furthermore allowed us to conclude that the viability of bacteria was not affected by adhesion to a PLL coated cantilever, as also demonstrated by others for *Escherichia coli* (49). For the inadvertent event that multiple bacteria might have adhered to a cantilever, bacterial probes were used for imaging. All bacterial probes made in the above way, correctly imaged and reflected the dimensions of AFM calibration grids (7,48) without the occurrence of double-contour lines indicative of double-contact. *S. mutans* probes in the current study were checked for single bacterial adhesion to the cantilever (Fig. S5A) and absence of double-contour lines generation by imaging streptococci adhering to a glass surface (Fig. S5B). Freshly prepared streptococcal probes were directly used for adhesion force measurements. Adhesion force measurements were performed at room temperature in the calcium-phosphate buffer using a Dimension 3100 system (Nanoscope V; Digital Instruments, Woodbury, NY, USA). Adhesion forces were measured on glass and silicone rubber surfaces with and without a salivary conditioning film and bacterial cell surfaces. Briefly, the

cantilever with an attached *S. mutans* was brought into contact with a substratum surface or a bacterial cell surface and retracted after a specified time (bond maturation time). Upon retraction, the cantilever bends until the bacterial bond with the surface under probing was disrupted. The force at which this occurred was subsequently calculated from the cantilever bending and recorded as the adhesion force of the bacterium to the surface probed. For each bacterial probe, force-distance curves were measured after 0, 2, 5, 10, and 30 s of bond maturation time at a 5 nN loading force. In order to verify whether a measurement series had disrupted bacterial integrity, five force-distance curves at 0 s bond maturation time were measured at the beginning and end of each experiment on glass. When the adhesion forces measured differed more than 1 nN from the beginning to the end of an experiment, data were discarded, and the bacterial probe was replaced by a new one.

Single bacterial probes were directly used on the different substratum surfaces to measure the adhesion force between the bacterium and the surface in the absence and presence of a salivary conditioning film. *S. mutans* probes were in addition used to measure its adhesion forces with *S. oralis* or *A. naeslundii*. To this end, *S. oralis* or *A. naeslundii* were immobilized on glass slides, in essence similarly as described above for the preparation of a streptococcal probe. In short, a droplet of PLL was adsorbed to a cleaned glass surface and dried in air, after which a droplet of a bacterial suspension ($3 \times 10^8 \text{ mL}^{-1}$) was pipetted onto the surfaces and dried in air for another 15 min. Surfaces were rinsed with demineralized water and dried in air for 15 min, resulting in partly dehydrated bacteria on the surface by the loss of free water from its surface (50), without removal of internally bound water. This type of drying did not affect bacterial viability (7,48), as can also be seen from the green fluorescence of the single LIVE/DEAD stained bacterium on the cantilever in Fig. S5A. Before adhesion force measurements, the surfaces were scanned using the AFM and zoomed in to ensure the contact between the *S. mutans* probe and a single target bacterium (either *S. oralis* or *A. naeslundii*) attached to the surface.

Triple-species biofilm formation. Bacterial suspensions of the three bacterial strains were mixed (1:1:1 volume) to provide an inoculum with a defined final concentration of *S. mutans* ($1 \times 10^8 \text{ mL}^{-1}$), *A. naeslundii* (either live or dead, $1 \times 10^9 \text{ mL}^{-1}$), and *S. oralis* (either live or dead, $1 \times 10^8 \text{ mL}^{-1}$).

To grow a triple-species biofilm, 1 mL of the mixed bacterial suspension was added to a 24-well plate containing a glass or silicone rubber sample with an adsorbed salivary conditioning film. Wells were kept under static conditions for 2 h at 37°C in 5% CO₂ to allow bacterial sedimentation and adhesion. After 2 h, the bacterial suspension was removed, and each well was carefully washed once with 1 mL buffer, after which 1 mL BHI complemented with 1% (wt/vol) sucrose was added to each well to allow biofilm growth under static conditions in 5% CO₂ at 37°C. After 5 or 24 h of growth, all biofilms were carefully washed with buffer and imaged using Optical Coherence Tomography (OCT) to determine their thicknesses (see below). Subsequently, biofilms were carefully scraped off the surfaces and resuspended in buffer for plate enumeration of colony forming units (CFU) or for RNA isolation and gene expression (see below).

Enumeration of colony forming units (CFU). For enumeration of the number of CFUs in different biofilms, bacteria were scraped off the different surfaces, suspended in buffer, ten-fold serially diluted and agar plated. Selective agar plates were used to differentiate between the different strains isolat-

ed from triple-species biofilms. Home-made TSY20B agar (51) plates were used for enumeration of *S. mutans*, commercially-bought blood agar plates were used for enumeration of *S. oralis* and home-made CFAT (52) agar plates for *A. naeslundii* enumeration. Briefly, 100 μ L of a bacterial dilution was pipetted onto the corresponding agar plate, spread using a glass swab and left to incubate under different conditions. Blood agar plates were kept in ambient air at 37°C for 24 h, TSY20B agar plates in 5% CO₂ at 37°C for 48 h and another 24 h in ambient air and CFAT agar plates were left at 37°C under anaerobic conditions for four days. Bacterial colonies were enumerated using a plate counter (New Brunswick Scientific Edison, N.J., USA) after incubation.

Optical coherence tomography imaging. Biofilm thicknesses were measured using OCT (Ganymede II, Thorlabs Ganymede, Newton, NJ, USA) equipped with a 930-nm center wavelength white light beam and a Thorlabs LSM03 objective scan lens. The imaging frequency was 30 kHz, with a sensitivity of 101 dB, and the refractive index was set as 1.33 for the biofilm, equal to the one of water. Two-dimensional (2D) images had fixed 5,000 pixels with variable pixel size while containing a variable number of pixels with a 2.68 μ m pixel size in the vertical direction. Images were created by the OCT software (Thor-Image OCT 4.1) using 32-bit data, and signal intensities of back-scattered light were reflected by a whiteness distribution in OCT images. Biofilm thickness was subsequently determined from the OCT images after thresholding as described by Otsu (53).

Gene expression of planktonic and biofilm-grown bacteria. Planktonic as well as resuspended biofilm bacteria were centrifuged at 6500 $\times$ g for 5 min, supernatants removed and pellets stored at -80°C until RNA isolation. Total RNA was isolated using RiboPure bacterial kit (Ambion, Invitrogen, Foster City, CA) according to the manufacturer's instructions. Traces of genomic DNA were removed using the DNFree kit (Ambion, Applied Biosystems, Foster City, CA, USA). The amount and quality of extracted RNA was based on the 260/280-nm ratio measured using a NanoDrop ND-1000 (NanoDrop Technologies LLC, Thermo Fisher Scientific, Wilmington, DE, USA). A ratio of A₂₆₀/A₂₈₀ absorbance of around 2.0 ($\pm$ 10%) was accepted as "pure" for RNA. A mixture of 200 ng RNA, 4 μ L 5 $\times$ iScript reaction mixture, and 1 μ L iScript reverse transcriptase, in a total volume of 20 μ L (iScript; Bio-Rad, Hercules, CA, USA), was used for cDNA synthesis according to the manufacturer's instructions. Real-time reverse transcription-quantitative PCR (RT-qPCR) was performed in a 384-well plate (HSP-3905; Bio-Rad Laboratories, Foster City, CA, USA) with the primer sets for the selected genes (Table S2). The following thermal conditions were used for all RT-qPCRs: 95°C for 3 min and 39 cycles of 95°C for 10 s and 59°C for 30 s. The mRNA levels were quantified in relation to endogenous control gene coding for *16S rRNA*. Gene expression levels in the biofilms were normalized to planktonic *S. mutans* UA159. Gene expression was assessed in triplicate experiments with separately grown cultures. Specific primers were chosen for analyzing gene expression of *S. mutans*. None of the primers presents cross-reaction between streptococci and actinomyces or between different streptococcal strains (54), as independently confirmed for our triple-species biofilms (data not shown).

Statistical analysis. GraphPad Prism, version 7 (San Diego, CA, USA), was employed for statistical analysis. Significant differences between groups were assessed by one-way analysis of variance

(ANOVA) followed by Dunn's multiple-comparison test. Alternatively, the Mann-Whitney test was used to compare two sets of data at a time. Significance was adapted at $P < 0.05$.

Data Availability

The datasets generated in this study are available from the corresponding author upon request.

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AURHOR CONTRIBUTIONS

C.W., H.v.d.M., H.J.B., Y.R. conceived and designed the experimental study. C.W. performed the experiments. All authors wrote and approved the manuscript.

COMPETING INTERESTS

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 their respective employers.

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SUPPLEMENTARY MATERIAL

TABLE S1 Zeta potentials measured in a calcium-phosphate buffer (1 mM CaCl₂, 2 mM potassium phosphate, 50 mM KCl, pH 6.8) of the three bacterial strains involved in this study, demonstrating similar zeta potentials of live and dead *S. oralis* J22 and *A. naeslundii* T14V-J1 (no significant difference at P > 0.05, Mann-Whitney test). ± signs stand for standard deviation (SD) over triplicate experiments.

Strains	Live/dead	Zeta potential (mV)
<i>S. mutans</i> UA159	live	-2.8 ± 0.3
<i>S. oralis</i> J22	live	-8.4 ± 0.2
	dead	-8.7 ± 0.5
<i>A. naeslundii</i> T14V-J1	live	-4.6 ± 0.3
	dead	-5.3 ± 0.5

TABLE S2 Primer sequences of the genes used in this study.

Gene	Primer sequence (5' to 3')	
	Forward	Reverse
<i>16SrRNA</i>	ACCAGAAAGGGACGGCTAAC	TAGCCTTTTACTCCAGACTTTCCTG
<i>brpA</i>	GGAGGAGCTGCATCAGGATTC	AACTCCAGCACATCCAGCAAG
<i>gbpB</i>	ATACGATTCAAGGACAAGTAAG	TGACCCAAAGTAGCAGAC
<i>comDE</i>	ACAATTCCTTGAGTTCCATCCAAG	TGGTCTGCTGCCTGTTGC

Calculation of initial adhesion forces, characteristic bond maturation times and stationary adhesion forces. Adhesion forces have been described (1) to increase with contact time between the interacting according to an exponential function

$$F_t = F_0 + (F_{stationary} - F_0)[\exp(-\frac{t}{\tau})]$$

in which F_t is the adhesion force after bond maturation time t , F_0 is the initial adhesion force at 0 s bond maturation time and $F_{stationary}$ indicates the stationary adhesion force reached after complete bond maturation, while τ is the characteristic time constant for bond maturation. Least-square fitting of F_0 , τ and $F_{stationary}$ to the adhesion forces measured as a function of time was applied for their calculation.

FIG S1 Examples of retraction force-distance curves taken after different bond maturation times for a *S. mutans* UA159 probe on SCF-coated glass (left) and SCF-coated silicone rubber (right) surfaces, on live *S. oralis* J22 and *A. naeslundii* T14V-J1 cell surfaces (left) and on dead *S. oralis* J22 and *A. naeslundii* T14V-J1 cell surfaces (right). The arrow points to the most-negative force value, taken as the adhesion force for a given curve.

FIG S2 (to be continued.)

FIG S2 (continued.) *S. mutans* UA159 adhesion forces as a function of bond maturation time on SCF-coated glass (left) and SCF-coated silicone rubber (right) surfaces, on live *S. oralis* J22 and *A. naeslundii* T14V-J1 cell surfaces (left) and on dead *S. oralis* J22 and *A. naeslundii* T14V-J1 cell surfaces (right). Error bars represent standard deviations (SD) over example measurements, each taken on 5 contact points with 1 *S. mutans* probe.

FIG S3 Gene expression in 24 h *S. mutans* UA159 (*S. m.*) mono-species biofilms and different triple-species oral biofilms. Biofilms comprised of *S. mutans* UA159 (*S. m.*) in combination with live or dead *S. oralis* J22 (*S. o.*) and *A. naeslundii* T14V-J1 (*A. n.*) on different substratum surfaces in the absence and presence of a salivary conditioning film. (A) *brpA* gene expression. (B) *gbpB* gene expression. (C) *comDE* gene expression. Error bars represent SD values over triplicate experiments with separately grown biofilms. Data on *brpA*, *gbpB* and *comDE* expressions in *S. mutans* UA159 mono-species biofilms on glass and silicone rubber surfaces in absence of a salivary conditioning film were taken from Wang et al (2).

FIG S4 Visual aggregation assay showing different coaggregation scores. Score 0: no change in turbidity and no visible co-aggregates; Score 1: weak coaggregation with dispersed aggregates in a turbid background; Score 2: clearly visible, small co-aggregates, not settling immediately; Score 3: large settling co-aggregates, leaving a slightly turbid suspension; Score 4: maximum coaggregation large co-aggregates settled immediately leaving a fully clear supernatant (3) (Reprinted with permission from Taylor & Francis Ltd., www.tandfonline.com).

FIG S5 Demonstration of single bacterial probe use. (A) Fluorescence image of a single LIVE/DEAD stained *S. mutans* UA159 bacterium adhering on a tipless AFM cantilever. Green fluorescence demonstrates bacterial viability. Scale bar represents 20 µm. (B) Verification of the absence of double-contour lines upon imaging *S. oralis* adhering to a glass surface, using a *S. mutans* probe. Scale bar represents 2 µm.

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Chapter 5

Adhesion-force Induced Gene Expression in *Streptococcus mutans* Biofilm
at the Border between Two Surfaces with Different Hydrophobicities

Can Wang, Henny C. van der Mei, Henk J. Busscher, Yijin Ren

ABSTRACT

Bacteria respond to adhesion forces arising from a surface to which they adhere through adaptation of gene expression. In previous chapters (**Chapters 3 and 4**), we found that emergent EPS-production in *S. mutans* biofilms was controlled through adhesion-force sensing and maintained in multi-species biofilms on salivary-conditioning film coated surfaces through the action of initial colonizers, most notably *S. oralis*. Adhesion-force induced gene-expressions were limited to the first 20-30 μm above a substratum surface. To further advance our understanding of the influence of surface heterogeneity on adhesion-force sensing and development of phenotypically heterogeneous micro-environments in biofilms, as discussed in **Chapter 1.1** of this thesis, *S. mutans* biofilms were grown at the border between hydrophobic silicone rubber and hydrophilic glass. To this end, after various other trials, clamping together of both substratum materials turned out to be the most suitable method to create such a border. Horizontal ranges of adhesion-force induced gene expression in biofilm samples taken at different spots at increasing distances from each side of the border were determined using RT-qPCR. Preliminary results of four pilot experiments were irreproducible, but suggest that gene expression in adhering bacteria around a border of hydrophilic and hydrophobic surfaces may be a stochastic process due to stochasticity in adhesion, growth and quorum-sensing processes. If this is the case, reproducible experiments are inherently impossible. However, this conclusion needs to be confirmed using more accurate experimental protocols, including e.g. better spot-size confined collection of biofilm.

INTRODUCTION

In the introductory chapter (**Chapter 1.1**) to this thesis, adhesion-force sensing by adhering bacteria was described as an important tool for bacteria to define their proper adaptation to their adhering state. Individual, adhering bacteria were hypothesized to adhere to intrinsic, stochastically distributed substratum surface heterogeneities yielding different adhesion forces over a substratum surface that trigger emergence of heterogeneous micro-environments in biofilms.

In the previous chapters (**Chapters 3 and 4**), we have demonstrated that *S. mutans* senses a different adhesion force on hydrophilic glass than on hydrophobic silicone rubber and adapts accordingly with different gene expression to both surfaces. Importantly, *S. mutans* surface sensing and adaptation was maintained in a multi-species oral biofilm and on salivary conditioning film covered surfaces in presence of live *S. oralis*, producing protein-degrading enzymes.

In the previous **Chapters 3 and 4**, it was assumed that the glass and silicone rubber surfaces were “homogeneous” in surface properties relevant for adhesion force sensing, although they likely possessed intrinsic heterogeneities.

In order to better verify the role of surface heterogeneity in adhesion force sensing and emergence of heterogeneous micro-environments in *S. mutans* biofilms, we here aim to study gene expression and surface adaptation of *S. mutans* at the border between hydrophilic glass and hydrophobic silicone rubber. Several concepts were considered to create such a border. Our initial concept was to fabricate a heterogeneous surface with hydrophilic and hydrophobic patches, each with water contact angles similar as on glass and silicone rubber surfaces, respectively. To this end, we modified a hydrophobic polydimethylsiloxane (PDMS) surface (water contact angle 107 degrees, similar as of silicone rubber) with a hydrophilic microgel (poly(N-isopropylacrylamide). However, AFM images showed that the distribution of the microgel was not homogeneous. Besides, water contact angles on the microgel coated hydrophilic patch were 35 degrees, failing to meet our expectation of a water contact angle similar to the one on glass (11 degrees). We also tried to coat PDMS surfaces with different concentrations of APTES (3-aminopropyl)triethoxysilane) in a longitudinal gradient manner. This method was discarded either because of instability of the water contact angles on the modified surface or limited difference in adhesion forces between both ends of the gradient. After these, we considered coating of hydrophilic glass surfaces with different silanes, including 0.05% APTES, 0.05% Bis(2-hydroxyethyl)aminopropyltriethoxysilane) and 0.05% PTS (N-propyltrichlorosilane). Whereas these modifications created the desired differences in hydrophobicity, they failed to yield to similar stationary adhesion forces on *S. mutans* as observed on silicone rubber and glass. Probably for this modification, surface charge played an important additional role above hydrophobicity in determining adhesion forces between bacteria and the surfaces (1), as streaming potentials demonstrated that APTES modification resulted in a coating with positively-charged amine groups ($-\text{NH}_2$), Bis modification yielded a negatively-charged coating ($-\text{OH}$), and PTS modification yielded an uncharged coating (see also Fig. 1). Finally we turned to mechanical means to create a border by pressing a glass and a silicone rubber sample as closely and evenly together as possible and studied the pattern of horizontal gene expression around the border for the most adhesion-force sensitive genes of *S. mutans*: *brpA*, *comDE* and *gbpB* (see **Chapter 3**).

FIG 1 Charged groups on a glass surface, coated with different silanes.

METHODS

Substratum materials, bacterial strains, growth conditions and harvesting, biofilm growth, Optical Coherence Tomography (OCT), gene expression and statistical analyses have all been described in **Chapter 3** and are therefore not repeated. Unique to this chapter is the preparation of the border between glass and silicone rubber and its characterization.

Glass (Thermo Scientific, Braunschweig, Germany) and medical-grade silicone rubber (ATOS Medical B.V., Zoetermeer, The Netherlands) were used as substrata, as they have been previously shown to represent two ends of the scale with respect to hydrophobicity (water contact angles 11 and 103 degrees, respectively). Moreover, *S. mutans brpA* gene expression was maximal on hydrophobic medical-grade silicone rubber surface and minimal on hydrophilic glass surface in mono-species biofilms (2) leading to “emergent” properties of biofilm bacteria that are alien to planktonic ones. With the aim of revealing the role of environmental adhesion forces in emergent biofilm properties, genes in *Streptococcus mutans* UA159 and a quorum-sensing-deficient mutant were identified that become expressed after adhesion to substratum surfaces. Using atomic force microscopy, adhesion forces of initial *S. mutans* colonizers on four different substrata were determined and related to gene expression. Adhesion forces upon initial contact were similarly low across different substrata, ranging between 0.2 and 1.2 nN regardless of the strain considered. Bond maturation required up to 21 s, depending on the strain and substratum surface involved, but stationary adhesion forces also were similar in the parent and in the mutant strain. However, stationary adhesion forces were largest on hydrophobic silicone rubber (19 to 20 nN). Glass and medical-grade silicone rubber surface were cut into 1 cm × 0.5 cm and placed in a PMMA mold as shown in Fig. 2, clamping both materials together as tightly as possible in view of elastic bolding of the silicone rubber. To compensate for differences in thickness between glass and silicone rubber surface, the mold had slightly different depths on both sides. This yielded a tightly clamped border with a maximal height difference between both sides of around 11 ± 7 μm, as measured using Sony magenscale LY-101.

The surface and the mold were cleaned with 2% RBS under sonication for 5 min, rinsed with warm tap water, sterilized with 70% ethanol, and finally washed with sterilized demineralized water.

FIG 2 Mold used to create a glass-silicone rubber border. (A) Side-view. (B) Top-view.

Importantly, after 5 h growth of *S. mutans* biofilms around the border as described before (**Chapter 3**), biofilms were picked up using the far end of a sterile disposable inoculation loop (Greiner Bio-One GmbH, Frickenhausen, Germany) at different spots away from the glass-silicone rubber border (Fig. 3) to determine gene-expressions. The end of the loop had a thickness of approximately 1 mm and by scraping along the width of samples, parallel to the border, biofilm was collected from an area of 1 mm × 10 mm for each spot (see Fig. 3). 5h old biofilms were chosen, because they yielded the best indication of adhesion-force induced gene expression on different surfaces (see **Chapter 3**).

FIG 3 Schematics of biofilm samples selection at three spots at different distances away from the border. (A) Inoculation loop with the end used for biofilm collection indicated in the orange square. (B) Spot 1, approximately 0.5 mm away from the border; spot 2, approximately 2.5 mm away from the border; spot 3, approximately 4.5 mm away from the border (Top view).

RESULTS AND DISCUSSION

Four pilot experiments were done with independent bacterial cultures and separately prepared borders, with the goal to see whether the method to create a hydrophilic-hydrophobic border and horizontal biofilm picking yielded results within expectation.

Expression of adhesion-force sensitive genes far away from either side of the border was expected to be the same as observed on “homogeneous” glass or silicone rubber surfaces. We did not have clear expectations about gene expression close to the border. However, it is within reason to expect that border gene-expression will gradually change away from either side of the border to the gene expression observed before on “homogeneous” glass and silicone rubber surfaces.

FIG 4 Horizontal pattern of normalized fold gene expressions in 5 h *S. mutans* biofilms around a glass-silicone rubber border. (A) Normalized gene expression with respect to the internal *16S rRNA* control, at different spots away from each side of the border in 5 h *S. mutans* UA159 biofilms. Gene expressions in 5 h biofilms on “homogeneous” glass or silicone rubber surfaces were included for comparison, i.e. as a control as taken from Chapter 3. (B) Quantitative presentation of the data in panel (A). Data represent a single experiment.

The first trial yielded fairly interpretable results. Gene expression on the glass side of the border were comparable within the limitations of a single pilot experiments as observed on “homogeneous” glass, especially for *brpA* and *gbpB* gene expression. Potentially, expression of *comDE* was somewhat lower. This similarity persisted to spot 1, closest to the border. Gene expression of *brpA* is greatly reduced on the silicone side of the border as compared to “homogeneous” silicone rubber. Possibly this might also be said for the other two, less adhesion force sensitive genes. Reduced gene expression at the silicone rubber side of the border might indicate that auto-inducers produced on the glass side of the border overrule adhesion force sensing close to the border, bearing similarity to collective oscillations in biofilm growth benefits to a community. These collective oscillations may result in sub-population conflicts (3), as potentially occurring at the border between hydrophilic and hydrophobic substratum surfaces. Reduced gene expression further away from the border at spots 2 and 3 is fully unexpected.

Three other pilot experiments could not confirm these tentative conclusions and moreover, did not yield a single pair or triplet of data with reproducible results within the four pilot experiments. Therefore, the reliability of these pilot experiments and the tentative conclusions drawn from the first pilot experiment must be cautiously treated. This leaves as a major point for discussion the reasons for our inability to reproduce. We like to list a number of potential reasons for this:

1. the numbers of bacteria collected from each spot was very low, despite the use of a relatively large size of the end of the collection loop and cDNA concentrations remained below detection. This

is evidenced by the fact that no melting curves were obtained after running PCR.

2. spot-size confined collection of biofilm at reproducible distances from the border was extremely difficult to do manually, particularly considering the size of the collection loop end.

3. the silicone rubber surface might not present itself with the same physico-chemical properties in a clamped state.

4. the behavior of bacteria at glass-silicone rubber borders in our study likely depends stochastically on whether more bacteria, or more metabolically active (we have only partly synchronized cultures) adhere first and on which side of the border and spread the news about their arrival with the help of the well-known quorum sensing system.

Amidst many other possible reasons, the first two reasons tend to explain the major obstacle in the interpretation of the first pilot experiment in a better way, i.e. why gene expression far away from the border on the silicone rubber side does not resemble gene expression observed on “homogeneous” silicone rubber.

Irreproducibility might be a result of our experimental protocol, but could also be inherently associated with quorum-sensing mechanisms as such. QS-controlled behavior is known to be unproductive when undertaken by a single bacterium and only becomes effective when performed in synchrony (4) by a large number of bacteria (5). Stochastically-governed adhesion around the border may therefore yield heterogeneous domains, each with their own unique evolution track (6).

Summarizing, these pilot studies tentatively show the study of gene expression in adhering bacteria around a border of hydrophilic and hydrophobic surfaces may be a stochastic process due to the stochasticity in adhesion, growth and quorum-sensing processes. If this is the case, reproducible experiments are inherently impossible. However, this conclusion needs to be confirmed using more accurate experimental protocols, particularly with respect to spot-size controlled biofilm sampling.

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Chapter 6

General Discussion

Biofilms are defined as surface-adhering and surface-adapted communities of microorganisms (1). Bacterial attachment to a surface is the initial step in biofilm formation. Upon adhesion to a surface, bacterial cell surfaces slightly deform under the influence of the adhesion-forces experienced. This in turn, causes changes in lipid membrane surface tension that provides a stimulus for the environmentally triggered expression of a great number of genes in adhering bacteria (2) to facilitate their surface adaptation from planktonically growing bacteria into a biofilm mode of growth. Understanding bacterial adhesion, surface adaptation and emergent biofilm properties are critical in bacterial pathogenicity and control of biofilm-related infections. This thesis focuses on adhesion-force sensing, surface adaptation and emergent biofilm properties relevant in oral biofilm formation.

Relevance of adhesion-force sensing in the oral cavity

The oral cavity houses many different soft (mucosa, gingiva) and hard surfaces. The variety of hard surfaces is huge and encompasses teeth, dental implants or restorative materials, surfaces of dentures and denture liners and orthodontic appliances. All differ in chemical and physical properties. Hydrophobicities of common surfaces found in the oral cavity are listed in Table 1. As can be seen, water contact angles (WCA) on these surfaces vary between the two extreme hydrophobicities of glass and silicone rubber surfaces used in this PhD research. Adsorption of salivary conditioning films varies on different oral surfaces (3) and precedes adhesion of bacteria and subsequently influences bacterial adhesion forces and biofilm detachment (4). Based on the literature data compiled in Table 1, it can be observed that here too surface thermodynamic homeostasis occurs equalizing hydrophobicities upon salivary conditioning film formation (see **Chapter 4**).

TABLE 1 Water contact angle (WCA) on different surfaces occurring in the oral cavity with or without an adsorbed salivary conditioning film.

Surfaces	WCA (degrees)		References
	without SCF	with SCF	
Hydroxyapatite	30 ± 1	26 ± 1	(8)
Commercial CAD/CAM composite			
Cerasmart ^a	63 ± 2	95 ± 2	(9)
Katana Avencia ^b	64 ± 4	95 ± 2	
Lava Ultimate ^c	76 ± 4	95 ± 2	
Block HC ^d	65 ± 3	95 ± 2	
Solidex ^e	60 ± 2	95 ± 2	
Stainless steel	49 ± 2	23 ± 2	(8, 10)
Dental implants			
ZrO ₂	63 ± 3	7 ± 4	(11)
Ce- ZrO ₂ / Al ₂ O ₃ R	70 ± 3	10 ± 2	
Ti	57 ± 8	28 ± 10	
Ti-6Al-4V	69	56 ± 3	(10, 12)
Tongue	77 ± 4	51 ± 2	(13)
Gingiva	-	72 ± 10	(14)

composition of the CAD/CAM composites: ^a Bis-MEPP, UDMA, DMA based; SiO₂, Baglass filler (71 wt%); ^b UDMA based; SiO₂, Al₂O₃ filler (62 wt%); ^c Bis-GMA, UDMA based, Bis-EMA, TEGDMA based; SiO₂ ZrO₂ aggregated, ZrO₂/SiO₂ cluster filler (80 wt%); ^d UDMA, TEGDMA based; SiO₂ microfumed, SiO₂ zirconium silicate filler (61 wt%); ^e UDMA based; SiO₂, Al₂O₃ filler (53 wt%);

Biofilms form on all surfaces exposed to the oral cavity and can cause serious issues, such as dental caries, secondary caries, denture stomatitis, periodontitis, and dental implant failure (5). While some dental materials promote selective bacterial adhesion during early biofilm formation (6), other biomaterials may provide resistance to bacterial adhesion and biofilm formation (7). This is controlled to a large extent by the hydrophobicity of the surfaces.

Generally, bacterial adhesion forces are stronger on hydrophobic surfaces than on more hydrophilic surfaces (15). However, preference for surface hydrophobicity differs among different bacterial species and usually more hydrophobic strains prefer hydrophobic surfaces for adhesion and more hydrophilic bacteria have a preference to adhere to hydrophilic surfaces. Moreover, in the oral cavity, the constant presence of salivary conditioning films on surfaces can also greatly affect how hydrophobicity influences the interaction between oral bacteria and dental materials (16), either by physically blocking the direct contact between bacteria and the underlying surfaces, or by surface thermodynamic homeostasis phenomena (Table 1). *Streptococcus mutans*, the most prevailing cariogenic strain (17), experienced the lowest adhesion force on the most hydrophilic surface (water contact angle WCA 11 degrees) and the highest adhesion force on the most hydrophobic surface (silicone rubber WCA 103 degrees) (**Chapter 3**). Therewith, biofilms on different surfaces occurring in the oral cavity are not only different due to selective adhesion of bacterial strains from the enormous variety of strains and species in saliva (18) but biofilm inhabitants are also subject to different adhesion forces sensed on different surfaces. Based on the results of this thesis, even biofilms of the same composition found on different oral surfaces may be expected to carry different adaptive properties, that are particularly relevant for biofilms with *S. mutans* as an inhabitant due to its role in (secondary) caries formation.

Clinical relevance of bacterial surface adaption in oral biofilm

Bacteria tailor their adaptive response to the characteristic properties of the surface they adhere to (19). Bacterial adhesion force sensing constitutes an environmental trigger for EPS production, one of the most important adaptive responses within a biofilm. Previous research has shown that gene expression of *S. mutans* differed in biofilms formed on different dental surfaces (20). In this thesis, three genes related with biofilm formation and EPS production including *brpA*, *gbpB* and *comDE* in *S. mutans* were identified to become expressed upon adhesion to a substratum surface and displayed a linear relation with the magnitude of the adhesion force that is sensed when adhering to a surface (**Chapter 3**).

At a first glance, this may look like a highly scientific result, bearing little clinical relevance. Therefore, it is considered appropriate to speculate on its clinical relevance. If we accept the simple fact that bacteria will stick to every surface in absence of (sufficiently strong) removal forces (21), manipulation of their adaptive response by adjusting the hydrophobicity of the surfaces they adhere to may be the only option that is left to aid further improving oral health. All genes evaluated here for possible adhesion-force sensitivity have relevance for matrix production. The oral biofilm matrix prevents (22, 23) deep penetration in biofilms of oral antimicrobials from toothpastes or mouthrinses (24) and regulates much of the forces required to remove tenaciously adhering oral biofilms during toothbrushing (25) or by naturally occurring oral shear forces, as during eating, drinking or swallowing. Modification

of the properties of dental materials, including the tooth surface may be a worthwhile path to go in order to exploit the results of this thesis for the benefit of oral health (see Fig. 1) (26).

FIG 1 Non-disturbed, oral biofilm formation on a moderately hydrophobic tooth surface (left) and a highly hydrophobic PTFE (Teflon) surface (right) after nine days exposure to the oral cavity. The difference in oral biofilm formation shows the impact of naturally occurring shear forces upon the ease with which oral biofilm on different surfaces can be removed (27). Reprinted with permission from Journal of Dental Research. Note biofilm formation near the gingival margin where removal forces are small, also on hydrophobic PTFE.

Future research

At the on-set of this PhD research, it was decided to focus on adhesion-force sensing and adaptive responses in *S. mutans*, because *S. mutans* is a key cariogenic pathogen in the oral cavity. However, considering the new role attributed in this thesis (**Chapter 5**) to *S. oralis* as an initial colonizer of surfaces exposed to the oral cavity in paving the way for *S. mutans* to directly contact a surface (**Chapter 5**), it would be worthwhile to study whether this feature of *S. oralis* is also initiated by adhesion force sensing.

As another initial decision taken at the on-set of this PhD research, it was first established whether *S. mutans* adaptive gene expression was under control of adhesion forces. As a result, the topic of possible phenotypically heterogeneous domains in oral biofilms due to inherent surface heterogeneity of virtually all surfaces had remained being understudied. A pilot in vitro experiment on *S. mutans* gene expression at the border between a glass and a silicone rubber surface had been performed with inconclusive results mainly related to technical limitations to achieve reproducible, small spot-size confined biofilm collection. Possible future experiments to this end, may include laser capture scanning microscopy, allowing isolation of small groups of bacteria from defined locations within a biofilm, combined with multiplex quantitative real-time reverse transcriptase PCR (28). Also advanced optical coherence tomography as outlined in **Chapter 2** might be helpful for analysis of such localized responses.

Ultimately, in vivo experiments on oral biofilms, such as might be formed in and on a variety of container devices glued or otherwise attached by orthodontic means to the dentition to allow non-disturbed oral biofilm formation (29, 30), must be carried out to confirm the clinical relevance of the results from this thesis.

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SUMMARY

The aim of this thesis was to investigate bacterial adhesion-force sensing by the oral pathogen *Streptococcus mutans* in mono-species *S. mutans* biofilms and in multi-species biofilms comprising *S. mutans* amongst others. Therefore, we started with a review in **Chapter 1** on how substratum heterogeneity and bacterial adhesion-force sensing may influence heterogeneous microenvironments in biofilms. Phenotypically heterogeneous microenvironments emerge as biofilms mature across different environments. Phenotypic heterogeneity in biofilm sub-populations not obeying quorum sensing-dictated, collective group behavior may be considered as a strategy allowing non-conformists to survive hostile conditions. Heterogeneous phenotype development has been amply studied with respect to gene expression and genotypic changes, but “biofilm genes” responsible for preprogrammed development of heterogeneous microenvironments in biofilms have never been discovered. Moreover, the question of what triggers the development of phenotypically heterogeneous microenvironments has never been addressed. The definition of biofilms as “surface-adhering” and “surface-adapted” microbial communities contains the word “surface” twice. This leads us to hypothesize that phenotypically heterogeneous microenvironments in biofilms develop as an adaptive response of initial colonizers to their adhering state, governed by the forces through which they adhere to a substratum surface. No surface is entirely homogeneous, while adhering bacteria can substantially contribute to stochastically occurring surface heterogeneity. Accordingly, bacterial adhesion forces sensed by initial colonizers differ across a substratum surface, leading to differential mechanical deformation of the cell wall and membrane, where many environmental sensors are located. Bacteria directly adhering to heterogeneous substratum domains therewith formulate their own local responses to their adhering state and command non-conformist behavior, leading to phenotypically heterogeneous microenvironments in biofilms.

Biofilms have complicated and heterogeneous structures and microscopic methods available for non-destructive analysis of biofilm structure without requiring staining are scarce and frequently only provide low resolution images covering a small field of view. Optical-coherence-tomography (OCT) is a non-destructive tool for biofilm imaging, not requiring staining, and used to measure biofilm thickness and putative comparison of biofilm structure based on signal intensity distributions in OCT-images. Quantitative comparison of biofilm signal intensities in OCT-images, is difficult due to the auto-scaling applied in OCT-instruments to ensure optimal quality of individual images. In **Chapter 2**, a novel method was developed to eliminate the influence of auto-scaling in order to allow quantitative comparison of biofilm densities in different images. Auto- and re-scaled signal intensities could be qualitatively interpreted in line with biofilm characteristics for mono- and multi- species biofilms of different strains and species (cocci and rod-shaped organisms), demonstrating qualitative validity of auto- and re-scaling analyses. However, specific features of *Pseudomonas* and oral multi-species biofilms were more prominently expressed after re-scaling. Quantitative validation was obtained by relating average auto- and re-scaled signal intensities across biofilm images with volumetric-bacterial-densities in biofilms, independently obtained using enumeration of bacterial numbers per unit biofilm volume. The signal intensities in auto-scaled biofilm images did not significantly relate with volumetric-bacterial-densities, whereas re-scaled intensities in images of biofilms of widely different strains and species increased linearly with independently determined volumetric-bacterial-densities in the biofilms. Herewith, the proposed re-scaling of signal intensity distributions in OCT-images signifi-

cantly enhances the possibilities of imaging phenotypic micro-environments in biofilms.

Biofilm formation in the oral cavity is an ordered process and starts with the adhesion of initial colonizers to salivary conditioning film coated surfaces, followed by adhesion of later colonizers, amongst which *S. mutans* is the most prevailing cariogenic strain. As a further complication in oral biofilm formation, the oral cavity accommodates up to more than 700 bacterial strains and species. Accordingly, oral biofilm possesses many different strains and species. These different bacteria have all their own adhesion-force sensing with the different surfaces in the oral cavity. Co-adhesion between pairs of strains potentially interferes with adhesion-force sensing. The question is whether adhesion-force sensing by initial colonizers of oral surfaces persists in multi-species biofilms and in the presence of a salivary conditioning film. Therefore, it was investigated how bacterial adhesion-force sensing by the important oral pathogen *S. mutans* plays a role in mono-species *S. mutans* biofilms (**Chapter 3**) and in multi-species biofilms on different bare and salivary conditioning film coated surfaces (**Chapter 4**).

In **Chapter 3**, we aimed at revealing the role of environmental adhesion forces in emergent mono-species *S. mutans* biofilm properties. Genes in *S. mutans* UA159 and a quorum-sensing-deficient mutant were identified that become expressed after adhesion to substratum surfaces. Using atomic force microscopy, adhesion forces of initial *S. mutans* colonizers on four different substrata were determined and related to gene expression. Adhesion forces upon initial contact were similarly low across different substrata, ranging between 0.2 and 1.2 nN regardless of the strain considered. Bond maturation required up to 21 s, depending on the strain and substratum surface involved, but stationary adhesion forces also were similar in the parent and in the mutant strain. However, stationary adhesion forces were largest on hydrophobic silicone rubber (19 to 20 nN), while being smallest on hydrophilic glass (3 to 4 nN). *brpA* gene expression in thin (34 to 48 μm) 5 h *S. mutans* UA159 biofilms was most sensitive to adhesion forces, while expression of *gbpB* and *comDE* expressions was weakly sensitive. *ftf*, *gtfB*, *vicR*, and *relA* expression was insensitive to adhesion forces. In thicker (98 to 151 μm) 24 h biofilms, adhesion-force-induced gene expression and emergent extracellular polymeric substance (EPS) production were limited to the first 20 to 30 μm above a substratum surface. In the quorum-sensing-deficient *S. mutans*, adhesion-force-controlled gene expression was absent in both 5 h and 24 h biofilms. Thus, initial colonizers of substratum surfaces sense adhesion forces that externally trigger emergent biofilm properties over a limited distance above a substratum surface through quorum sensing. Herewith we introduced a new concept in biofilm science: “adhesion force sensitivity of genes”, defining the degree up to which expression of different genes in adhering bacteria is controlled by the environmental adhesion forces they experience. Analysis of gene expression as a function of height in a biofilm demonstrated that the information about the substratum surface to which initially adhering bacteria adhere is passed up to a biofilm height of 20 to 30 μm above a substratum surface, highlighting the importance and limitations of cell-to-cell communication in a biofilm.

In **Chapter 4**, it was determined whether and how salivary-conditioning-film (SCF)-adsorption and the multi-species nature of oral biofilms influence adhesion-force sensing and associated gene expression by *S. mutans*. Hereto, *Streptococcus oralis*, *Actinomyces naeslundii* and *S. mutans* were grown together on different surfaces in absence and presence of an adsorbed SCF. Atomic-force-microscopy and RT-qPCR were used to measure *S. mutans* adhesion-forces and gene-expressions. Upon SCF-adsorption, stationary adhesion-forces decreased on a hydrophobic and increased on a

hydrophilic surface to around 8 nN. OCT showed that triple-species biofilms on SCF-coated surfaces with dead *S. oralis* adhered weakly and often detached as a contiguous sheet. Concurrently, *S. mutans* displayed no differential adhesion-force sensing on SCF-coated surfaces in the triple-species biofilms with dead *S. oralis*, but once live *S. oralis* were present *S. mutans* adhesion-force sensing and gene-expression ranked similar as on surfaces in absence of an adsorbed SCF. Concluding, live *S. oralis* may enzymatically degrade SCF-components to facilitate direct contact of biofilm inhabitants with surfaces and allow *S. mutans* adhesion-force sensing of underlying surfaces to define its appropriate adaptive response. This represents a new function of initial colonizers in multi-species oral biofilms.

To further advance our understanding of the influence of surface heterogeneity on adhesion-force sensing and development of phenotypically heterogeneous micro-environments in biofilms, as discussed in **Chapter 1.1** of this thesis, in **Chapter 5** *S. mutans* biofilms were grown at the border between hydrophobic silicone rubber and hydrophilic glass. To this end, after various other trials, clamping together of both substratum materials turned out to be the most suitable method to create such a border. Horizontal ranges of adhesion-force induced gene expression in biofilm samples taken at different spots at increasing distances from each side of the border were determined using RT-qPCR. Preliminary results of four pilot experiments were irreproducible, but suggested that gene expression in adhering bacteria around a border of hydrophilic and hydrophobic surfaces may be a stochastic process due to the stochasticity in adhesion, growth and quorum-sensing processes. If this is the case, reproducible experiments are inherently impossible. However, this conclusion needs to be confirmed using more accurate experimental protocols, including better small spot-size confined collection of biofilm.

In the general discussion of this thesis (**Chapter 6**), an overview is given of the variety of different surfaces on which oral biofilm grows in the human oral cavity. Also, the wide range of hydrophobicities they represent is highlighted and the clinical relevance of adhesion-force sensing and surface adaptation is discussed. Future studies to further advance the results of this PhD thesis are given.

SAMENVATTING

In **Hoofdstuk 1** is een overzicht gegeven van de verschillende wijzen waarop de heterogeniteit van een oppervlak van invloed kan zijn op bacteriële hechtingskrachten en daarmee op de heterogeniteit van biofilms. Fenotypische verschillen tussen bacteriën in heterogene micro-omgevingen in biofilms worden deels verklaard door verschillen in hechtingskrachten over een oppervlak. Quorum-sensing is bepalend voor het groepsgedrag binnen een biofilm, maar mogelijk stelt fenotypische heterogeniteit van subpopulaties in een biofilm, die niet gehoorzamen aan het collectieve groepsgedrag, bacteriën in staat om specifieke, ongunstige omstandigheden te overleven. Hechting en hechtingskrachten kunnen hiervoor bepalend zijn.

De definitie van biofilms als "hechtend aan een oppervlak en aangepast aan het oppervlak" van microbiële gemeenschappen bevat het woord "oppervlak" twee keer. Dit leidde tot de hypothese dat fenotypisch heterogene micro-omgevingen in biofilms zich ontwikkelen als een reactie van initiële hechtende bacteriën aan een oppervlak en bepaald worden door de krachten waarmee ze hechten aan het oppervlak.

Het doel van dit proefschrift was te onderzoeken of de hechtingskrachten die worden gevoeld door de orale pathogeen *Streptococcus mutans* tijdens hechting op een oppervlak de genexpressie van de bacterie beïnvloeden.

Niet-destructieve microscopische methoden om de gecompliceerde en heterogene structuren van biofilms te analyseren zonder dat de biofilm gekleurd wordt, zijn schaars en geven vaak lage resolutiebeelden, die een klein gezichtsveld bestrijken. 'Optical-coherence-tomography' (OCT) is een niet-destructieve methode om de biofilm te visualiseren zonder kleuring en wordt gebruikt voor het meten van biofilm dikte en structuur op basis van de verandering van signaal intensiteiten in de OCT-beelden. Kwantitatieve vergelijking van signaalintensiteiten in OCT-beelden van een biofilm is moeilijk door de automatische schaling die in OCT-instrumenten wordt toegepast om een optimale kwaliteit van de individuele beelden te garanderen. In **Hoofdstuk 2** is een nieuwe methode ontwikkeld om de invloed van automatische schaling te elimineren zodat een kwantitatieve vergelijking van biofilm dichtheden in verschillende beelden mogelijk gemaakt wordt. Automatisch geschaalde en her-geschaalde signaalintensiteiten konden kwalitatief worden geïnterpreteerd conform de eigenschappen voor verschillende biofilms. Kwantitatieve validatie werd verkregen door het relateren van automatisch en her-geschaalde signaalintensiteiten aan bacterie dichtheden per volume-eenheid in deze biofilms. In tegenstelling tot automatisch geschaalde signaalintensiteiten, namen de her-geschaalde intensiteiten van verschillende biofilms lineair toe met bacteriële volume dichtheden in een biofilm. Derhalve worden met de opgestelde her-schalingsmethodiek, de interpretatie mogelijkheden van OCT-afbeeldingen sterk vergroot.

Biofilmvorming in de mond begint met de hechting van bacteriën aan een geadsorbeerde speeksel laag op orale oppervlakken. Na initieel hechtende bacteriën, volgen latere hechters, waaronder *S. mutans* (de meest voorkomende cariogene bacterie stam). Orale biofilmvorming is complex omdat de mondholte plaats biedt aan meer dan 700 bacterie stammen. Deze verschillende bacteriën voelen allemaal een eigen hechtingskracht met de verschillende oppervlakken in de mond. Hechting tussen verschillende bacteriën interfereert mogelijk met het waarnemen van de hechtingskrachten vanuit

een ander oppervlak. De vraag is of de hechtingskracht vanuit een oppervlak waargenomen door de initieel hechtende bacteriën van invloed blijft in biofilms met meerdere bacterie soorten en in de aanwezigheid van een speeksel laag op het oppervlak. Daarom werd onderzocht hoe de bacteriële hechtingskrachten zoals gevoeld door *S. mutans* een rol blijven spelen in biofilms bestaande uit alleen *S. mutans* (**Hoofdstuk 3**) en biofilms bestaande uit *S. mutans* en nog twee bacterie stammen op oppervlakken met en zonder een geadsorbeerde speeksel laag (**Hoofdstuk 4**).

In **Hoofdstuk 3** werden met behulp van atomaire kracht microscopie de hechtingskrachten van *S. mutans* op vier verschillende oppervlakken gemeten en gerelateerd aan de genexpressie. De hechtingskrachten bij het eerste contact waren laag voor de verschillende oppervlakken en varieerde tussen 0.2 en 1.2 nN. Tot 21 s werd de hechtingskracht steeds sterker, maar dit was afhankelijk van of de gebruikte *S. mutans* stam quorum-sensing eigenschappen had of niet. Stationaire hechtingskrachten waren vergelijkbaar voor beide stammen. Echter, stationaire hechtingskrachten waren het grootst op hydrofoob siliconenrubber (19 tot 20 nN), terwijl ze het kleinst waren op hydrofiel glas (3 tot 4 nN). De expressie van het *brpA* gen in dunne (34 tot 48 µm), 5 uur gegroeide *S. mutans* UA159 biofilms was het meest gevoelig voor hechtingskrachten, terwijl expressie van de genen *gbpB* en *comDE* niet erg gevoelig waren voor hechtingskrachten. De expressie van de genen *ftf*, *gtfB*, *vicR*, en *relA* waren ongevoelig voor hechtingskrachten. In dikkere (98 tot 151 µm), 24 uur gegroeide biofilms, waren de geïnduceerde genexpressies en extracellulaire polymere substantie (EPS) productie door de hechtingskracht beperkt tot de eerste 20 tot 30 µm boven een substraatoppervlak. In de quorum-sensing-deficiënte *S. mutans* was de hechtingskracht gecontroleerde genexpressie afwezig in zowel 5 uur als in 24 uur gegroeide biofilms. Dus initiële hechters voelen de hechtingskrachten van oppervlakken maar geven die informatie slechts over een beperkte afstand door aan later hechtende bacteriën. Hiermee hebben we een nieuw concept in de biofilm wetenschap geïntroduceerd: "hechtingskrachtgevoeligheid van genen". De hechtingskracht bepaalt de mate waarin de expressie van verschillende genen in hechtende bacteriën wordt gecontroleerd door de hechtingskrachten die ze ervaren.

In **Hoofdstuk 4** werd bepaald of en hoe een geadsorbeerde speeksel laag in een orale biofilm met meerdere bacterie stammen van invloed is op de hechtingskracht gevoeligheid en genexpressie door *S. mutans*. Hiertoe werden biofilms gegroeid van *Streptococcus oralis*, *Actinomyces naeslundii* en *S. mutans* op verschillende oppervlakken met en zonder een geadsorbeerde speeksel laag. Atomaire kracht microscopie en RT-qPCR werden gebruikt om de hechtingskrachten van *S. mutans* en genexpressies te relateren. De stationaire hechtingskrachten van *S. mutans* namen af in aanwezigheid van een geadsorbeerde speeksel laag op een hydrofoob oppervlak en namen toe op een hydrofiel oppervlak. OCT liet zien dat een biofilm met drie bacterie stammen op een oppervlak met speeksel laag zonder of met dode *S. oralis* zwak hechtte. In een biofilm met drie bacterie stammen waarin *S. oralis* levend was, vertoonde *S. mutans* een sterkere hechting en genexpressie die vergelijkbaar was met die op oppervlakken zonder speeksel laag. De conclusie werd getrokken dat *S. oralis* geadsorbeerde speeksel componenten afbreekt zodat *S. mutans* direct contact met een speeksel bedekt oppervlak kan maken, de krachten vanuit het oppervlak waarneemt en een vergelijkbare genexpressie kan vertonen als op een oppervlak zonder speeksel laag. Deze afbraak

functie geeft een nieuwe functie aan van initieel hechtende bacteriën in een orale biofilm, waarmee initiële hechters de biofilm beter kunnen aanpassen aan een oppervlak.

In **Hoofdstuk 5** werd een *S. mutans* biofilm gegroeid op de grens tussen hydrofoob siliconenrubber en hydrofiel glas. Hiertoe bleek het samenkleven van beide materialen de meest geschikte methode om een dergelijke grens te creëren. Genexpressie werd gemeten in biofilms die op verschillende afstand van de grens werden gesampled. Resultaten van de vier pilot-experimenten waren niet goed reproduceerbaar, maar suggereerden dat genexpressie in hechtende bacteriën rond de grens van hydrofiele en hydrofobe oppervlakken een stochastisch proces kan zijn als gevolg van de toevalligheden in hechting-, groei- en quorum-sensing processen. Als dit het geval is, zijn reproduceerbare experimenten inherent onmogelijk. Deze conclusie kan echter ook sterk beïnvloed zijn door de gebruikte sample methode en nauwkeuriger sampling van kleinere hoeveelheden biofilms moet deze conclusie bevestigen.

In de algemene discussie van dit proefschrift (**Hoofdstuk 6**) wordt een overzicht gegeven van de verscheidenheid aan verschillende oppervlakken waarop een orale biofilm groeit in de mond. Ook wordt het brede scala aan hydrofobiciteit van al deze oppervlakken belicht en wordt de klinische relevantie van het waarnemen van hechtingskrachten en gerelateerde genexpressie besproken. Toekomstige studies om de resultaten van dit proefschrift verder uit te bouwen worden gegeven.

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