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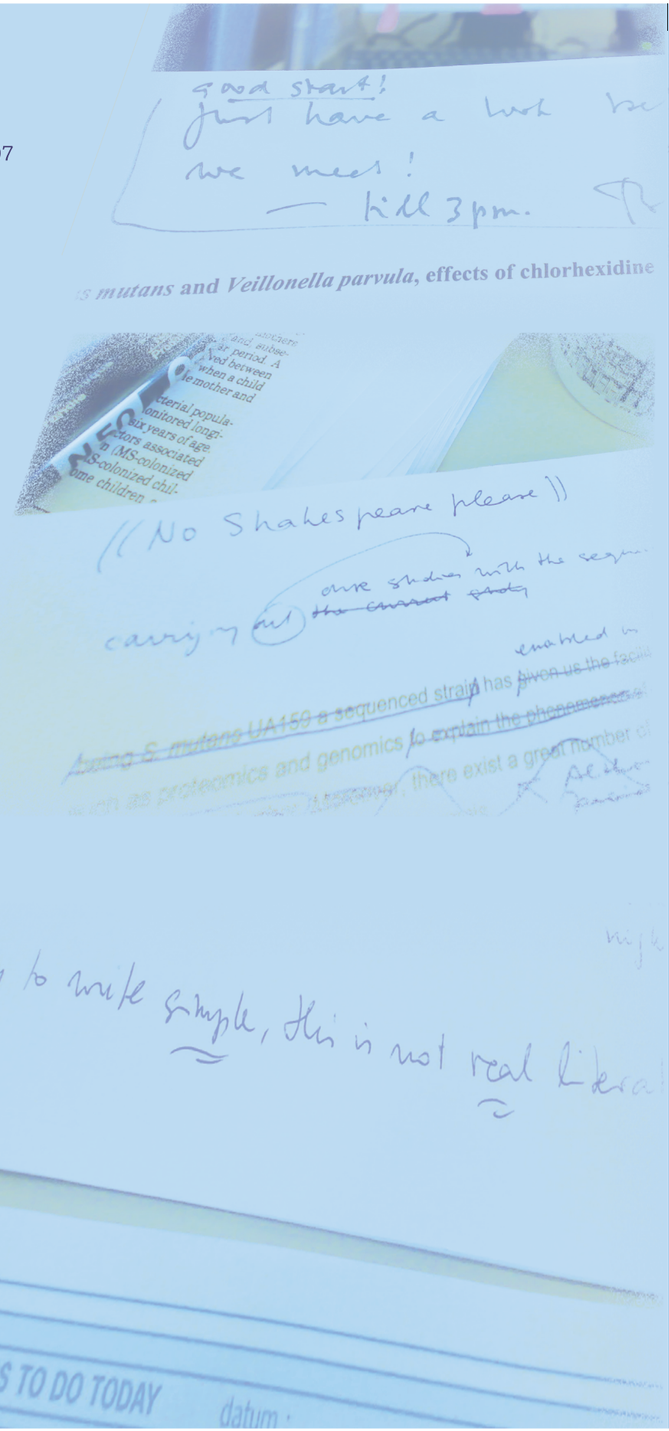

Duygu Kara

Interactions between oral biofilm bacteria

2007

Duygu Kara

Interactions between oral biofilm bacteria

Streptococcus mutans
&
Veillonella parvula

Invitation

to attend the public
thesis defence of

Duygu Kara

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Interactions between oral biofilm bacteria

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INTERACTIONS BETWEEN ORAL BIOFILM BACTERIA, *Streptococcus mutans* and *Veillonella parvula*

Duygu Kara

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INTERACTIONS BETWEEN ORAL BIOFILM BACTERIA, *Streptococcus mutans* and *Veillonella parvula*

ACADEMISCH PROEFSCHRIFT

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door

Duygu Kara

geboren te Ankara, Turkije

Promotor

Prof. Dr. J.M. ten Cate

Co-promotor

Dr. Ir. S.B.I. Luppens

Faculteit der Tandheelkunde

ON LIVING

Living is no laughing matter
You should live with great seriousness,
like a squirrel, for example-
I mean, without looking for something beyond and above living,
I mean, living must be your whole occupation

You should take living seriously,
So much, so and to such a degree
that, for example, your hands tied behind your back,
your back to the wall,
Or else, in your white coat and safety glasses in a laboratory
You will be able to die for people,
Even for people whose faces you have never seen,
Even though nobody has forced you to do so,
Even though you know living
is the most real, most beautiful thing..

I mean, you shall take living so seriously,
That even at seventy for example, you'll plant olive trees-
And not for your children either
But because although you fear death, you don't believe it,
Because living, I mean, weighs heavier
...

Nazim Hikmet (1902-1963)

CONTENTS

Chapter 1

General Introduction

What is 'biofilm'?

In October 1678, Antonie van Leeuwenhoek (1632-1723), who was the first to describe bacteria under the microscope, wrote in his letter to the Royal Society of London '…and I must say, for my part, that no more pleasant sight has ever yet come before my eye than these many thousand of living creatures, seen all alive in a little drop of water, moving among one another, each creature having its own proper motion:…' (Boortsin, 1991). Biofilms have been described in many systems since van Leeuwenhoek examined the plaque bacteria from his own teeth in the seventeenth century, but the general theory of biofilms has not started to be used until the late 1970s. Today, biofilm is defined as a microbially derived sessile community characterized by cells that are irreversibly attached to a substratum or interface or to each other, are embedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription (Donlan & Costerton, 2002). There is a high level of interest in the properties of biofilms across all sectors of industrial, environmental and medical microbiology (Marsh, 2004). Biofilms are important in human disease as 65% of microbial infections are caused by bacteria growing on surfaces rather than in the free planktonic state. According to the US National Institutes of Health, biofilms account for over 80% of microbial infections in the human body. Native valve endocarditis, otitis media, chronic bacterial prostatitis, cystic fibrosis and oral diseases are among the human pathologies involving biofilms (Donlan & Costerton, 2002).

Dental plaque as a biofilm

Dental plaque has been defined as the diverse community of microorganisms grown on the tooth surface as a biofilm, embedded in an extracellular matrix of polymers of host and microbial origin (Marsh, 2004). With new molecular techniques, more than 700 species have been isolated from human oral biofilm (Aas *et al*., 2005). These species together form a unique natural habitat rather than a random pattern; some have the ability to adhere with specific adhesins to the tooth surfaces as 'early colonizers'. The structural development continues while other bacteria are attached to neighboring bacteria through specific molecular interactions such as adhesins and their corresponding receptors. This balance between the host and the biofilm bacteria is continuously reinstalled after each mechanical cleaning of the tooth surface. Oral diseases (periodontal diseases and dental caries) arise as a result of environmental perturbations to the habitat of oral biofilms. Environmental perturbation of the biofilm can lead to an overgrowth of virulent species that form a part of the natural polymicrobial microflora of oral biofilm. Unlike most infectious diseases affecting humans, caries is suggested to be the result of an imbalance of the indigenous oral biota rather than a non-indigenous, exogenous pathogen (Caufield *et al*., 2005).

Oral biofilm bacteria; *Streptococcus mutans* and *Veillonella parvula*

Dental caries is an infectious and transmissible disease that is caused by bacteria colonizing the tooth surfaces (Caufield *et al*., 2005). *Streptococcus mutans*, an 'obligate' biofilm-forming bacterium (Wen & Burne, 2002), is the principal etiological agent of dental caries (Loesche, 1986) which was first described by Clarke in 1924. *Streptococcus mutans* is a Gram-positive, facultative anaerobic bacterium (Schleifer, 1986) and gained significant attention in the 1960s when it was demonstrated that caries could be experimentally induced and transmitted in animals after oral inoculation with this organism. The name 'mutans' results from the frequent transition from the coccal to the coccobacillary phase. Currently seven distinct species of human and animal mutans streptococci and eight serotypes (*a-h*) are recognized, based on the antigenic specificity of cell-wall carbohydrates. The term *Streptococcus mutans* is limited to human isolates belonging to three serotypes (*c, e* and *f*) (Samaranayake, 2002).

Infants do not harbor this organism until some time after the teeth emerge: mutans streptococci require the presence of a hard, non-desquamating surface for their colonization (Carlsson & Griffith, 1974; Berkowitz *et al*., 1975; Catalanotto *et al*., 1975). The major source from whom infants acquire mutans streptococci has been shown to be their caretakers; the evidence for this comes from several studies reporting that isolates of mutans streptococci harbored by mothers and their children exhibit similar or identical bacteriocin profiles (Berkowitz *et al*., 1975; Davey & Rogers, 1984) and identical plasmid or chromosomal DNA patterns (Caufield *et al*., 1982, 1985, 1988; Caufield & Walker, 1989; Hagan *et al*., 1989; Kulkarni *et al*., 1989).

Biochemical approaches identified three main unique properties of *S. mutans* that are responsible for its cariogenic potential and that are important in caries

initiation (Harper & Loesche, 1984; Macpherson *et al*., 1992; Biswas & Biswas, 2006):

- rapid metabolization of many dietary carbohydrates to acids (acidogenicity),
- continued production of acid in a low pH environment (aciduricity),
- extracellular polysaccharide synthesis from sucrose that can promote bacterial adherence to enamel or oral biofilm (sucrose dependent colonization).

Much attention has been focused on the mechanisms by which *S. mutans* colonizes the tooth surfaces. Both biochemical and genetic evidence suggest that this occurs via a two-step process: a sugar independent attachment to the tooth pellicle modulated by *S. mutans* adhesins, ionic or hydrophobic interactions, as well as impaction of the crevices on the tooth surface, followed by a sucrose-dependent enhancement of tooth colonization (Kuramitsu, 2001). A number of laboratories have isolated and characterized the gene for an adhesin termed SpaP, antigen I/II or Pac, which appears to modulate the initial attachment of *S. mutans* to saliva-coated tooth surfaces (Kuramitsu, 2000).

S. mutans counts ($>10^6$ CFU/ml) in saliva and/or plaque samples have been described as caries –risk predictor in many studies for the identification of high-caries-risk subjects (Kingman *et al*., 1988; Klock *et al*., 1989; Wilson & Ashley; 1989; Alaluusua *et al*., 1990; Russell *et al*., 1991; van Houte, 1993). It has been reported that elevated levels of mutans streptococci, both in plaque or saliva of caries-free children, appears to be associated with a considerable increase in caries risk (Thenisch *et al*., 2006). Plaque associated with incipient caries ('white spot') in caries-positive subjects usually contain high levels of mutans streptococci, whereas plaque associated with tooth surfaces which are and remain sound, whether in caries-positive or caries-free subjects, contain usually much lower levels of mutans streptococci. Cavitated coronal or root-surface lesions often also contain high levels of mutans streptococci (van Houte, 1993).

Veillonella parvula is a Gram-negative, anaerobic coccus that is part of the normal flora of the mouth, gastrointestinal tract and vagina in humans (Fisher &

Denison, 1996). *V. parvula* exists in the oral biofilm samples of both caries-free and caries-active individuals (Dent & Marsh, 1981; Corby *et al*., 2005). *V. parvula* can not ferment or incorporate glucose and most other sugars, but metabolizes lactic acid produced by streptococci, into weaker acids, such as propionic and acetic acid (Distler & Kröncke, 1981; Schleifer, 1986) with a reduced ability to solubilize enamel (Samaranayake, 2002). *V. parvula* has been suggested to be the responsible organism for reducing the cariogenic potential of *S. mutans*, since *V. parvula* decreases the lactate concentrations generated by *S. mutans* (van der Hoeven *et al*., 1978). The symbiotic metabolic interaction between these species; *S. mutans* as producer and *V. parvula* as consumer; has been studied in planktonic culture models (Coulter & Russell, 1974; Mikx & van der Hoeven, 1975; van der Hoeven *et al*., 1978). However, as stated before, bacteria grown in a biofilm exhibit an altered phenotype compared to planktonic phase bacteria. For instance, biofilm bacteria are more resistant to antimicrobials compared with planktonic cells. Oral biofilm bacteria display increased resistance to antimicrobial agents, including those used in dentifrices and mouth rinses (Marsh & Bradshaw, 1993; Kinniment *et al*., 1996; Wilson, 1996; Pratten & Wilson, 1999). Comparative proteome analysis of *S. mutans* grown in planktonic culture and grown in a biofilm also indicates significant differences mainly for enzymes associated with general metabolic functions such as glycolysis and alternative acid production (Rathsam *et al*., 2005a). As a result, a significant increase is observed in the last few years, in the development and use of *in vitro* oral biofilm models, rather than planktonic cell cultures, to study interspecies interactions.

Interactions among bacterial species can have a profound influence on the structure and physiology of microbial communities. Interspecies microbial interactions influence a biofilm during the initial stages of formation, bacterial attachment and surface colonization, and continue to influence the structure and physiology of the biofilm as it develops (James *et al*., 1995).

Therefore, we chose to study the interactions between *S. mutans* and *V. parvula* using a biofilm model, to obtain a better understanding of the oral biofilm physiology.

Methods to study bacterial interactions in oral biofilm

Confocal Laser Scanning Microscopy

Resolving important biofilm questions concerning the mutual interactions between bacteria depends on the application of methods to detect microscale spatial patterns within biofilms (Xu *et al*., 2000). Detection and visualization of microstructure of biofilms have been carried out using various techniques such as, scanning electron microscopy (SEM), environmental SEM, transmission electron microscopy (TEM), confocal laser scanning microscopy (CLSM) and episcopic differential interference contrast (EDIC), epifluorescence microscopy, a variety of microsensors, optical coherence tomography, and acoustic microscopy (Sutton *et al*., 1994; Davey & O'Toole, 2000; Perilli *et al*., 2000; Keevil, 2003). In particular, microbial ecology has advanced through novel techniques combining fluorescence microscopy with molecular methods that make it possible to detect and identify microorganisms directly in their natural habitats and to explore their physiological properties.

Fluorescent probes that target the different cellular functions of biofilm bacteria have been developed (Joux & Lebaron, 2000). Based on the previous inventions, Netuschil (1983) introduced vital fluorescence staining technique into dental biofilm research. With the combination of vital fluorescence staining and confocal laser scanning microscopy, it is possible to determine the structure of intact human dental biofilms and the spatial distribution of living and dead bacteria in the different biofilm layers in situ (Netuschil *et al*., 1998; Zaura-Arite *et al*., 2001; Auschill *et al*., 2001). Combination of these techniques has also been applied to *in vitro* oral biofilms (Guggenheim *et al*., 2001).

The confocal laser scanning microscope (CLSM) produces optical sections by scanning the specimen point by point with a laser beam focused within the specimen and using a spatial filter, usually a pinhole, to remove unwanted fluorescence from

above and below the focal plane of interest. The power of the confocal approach lies in the ability to image structures at discrete levels within an intact biological specimen (Wilson & Walker, 2005). Recently developed softwares add capabilities for visually exploring three dimensional data contained in confocal image stacks and thus greatly facilitate the understanding of complex spatial structures of biofilms (Daims *et al*., 2006).

Proteomics and transcriptomics

The genome is the full complement of DNA from an organism and carries all the information needed to specify the structure of every protein the cell can produce. The realization that DNA lies behind all of the cell's activities led to the development of what is termed molecular biology. Rather than a discrete area of biosciences, molecular biology is now accepted as a very important means of understanding and describing complex biological processes.

The DNA microarray is an assay that can be used to measure the level of expression in a collection of cells for thousands of genes. The phenotypic differences among different cell types are determined by differences in the level of expression of the genes. Consequently, an assay that can measure the level of expression of thousands of genes simultaneously provides genome-wide insight into the working of cells (Simon *et al*., 2002). Microarrays entered the scene of molecular biological research in the mid-1990s (Schena *et al*., 1995), and have quickly been established as an essential tool for gene expression profiling in relation to physiology and development. The primary application of microarrays has been in the identification of the sets of the genes that respond in an extreme manner to some treatment, or that differentiate two or more samples. Recently, genome-wide surveys of transcription have gained impact in biofilm research (Korem *et al*., 2005; Moorthy & Watnick, 2005; Hasona *et al*., 2007). Microarrays are now generally used to characterize genetic differences among isolates and closely related species (Gibson, 2002).

In order to completely understand how a cell works, it is necessary to understand the function (role) of single proteins in that cell and how they cooperate. The analysis of any specific disease will also require us to understand what changes have taken place in the protein component of the cell, so that we can use this information to understand the molecular basis of the disease, and thus design appropriate drug therapies and develop diagnostic methods. Almost every therapeutic drug that is currently in use has a protein as its target (Wilson & Walker, 2005). The term proteomics refers to the analysis of all proteins in a living system, including the description of co- and post-translationally modified proteins. This includes their covalent and non-covalent associations, spatial and temporal distributions within cells, and how all these are affected by changes in the extracellular and intracellular conditions. Mass spectrometry provides the key tools for the analysis and identification of proteins. Developments of technology and methodology in the field of mass spectrometry and proteomics have been rapid over the past five years and are providing improved and novel strategies for global understanding of cellular function (Guerrera & Kleiner, 2005).

Dental caries, although generally not life threatening, is of significant importance in human health. An understanding of the molecular nature of this disease could aid the development of novel methods of prevention and control, and increase our knowledge of its etiology (Kuramitsu, 2001). In caries research, sequencing the genome of a serotype *c* strain of *S. mutans*, has started a new era (Ajdic *et al*., 2002) as it allowed this cariogenic pathogen to be investigated by proteomics and transcriptomics. However, the effects of other oral biofilm bacteria on the physiology of *S. mutans* at the translational or transcriptional level have, according to our knowledge, never been studied before.

Objective of the thesis

The aim of this thesis was to elucidate the physiological interactions between a non-pathogenic oral bacterium (*V. parvula*) and a cariogenic oral pathogen (*S. mutans*) using a combined biochemical, microstructural, proteomic and transcriptomic approach.

Outline of the thesis

In Chapter 2, the growth and acidogenicity of *S. mutans* and *V. parvula* species grown together in a biofilm (dual-species biofilm) were compared with when these species are grown alone (single-species biofilms). Also, the effects of chlorhexidine on the viability of single and dual-species biofilms of these bacteria were assessed comparatively.

In Chapter 3, the microstructure of single and dual-species biofilms of *S. mutans* and/or *V. parvula* was studied before and after exposure to chlorhexidine using confocal laser scanning microscopy. Clustering of biofilm bacteria was statistically evaluated using spatial arrangement analysis.

In Chapter 4, protein expression in single and/or dual-species biofilms of *S. mutans* and/or *V. parvula* was assessed using two dimensional difference gel electrophoresis.

In Chapter 5, susceptibility of single and/or dual-species biofilms of *S. mutans* and/or *V. parvula* to different antimicrobials was investigated. Antimicrobials used with different mechanisms of action were cetylpyridinium chloride, amine chloride, zinc chloride and erythromycin. In addition, the effect of *V. parvula* on gene expression of *S. mutans* was studied using microarrays.

In Chapter 6, the main findings of this thesis are discussed briefly. Concluding remarks and possibilities for future research are presented.

Chapter 2

Differences between single- and dual-species biofilms of *Streptococcus mutans* and *Veillonella parvula* in growth, acidogenicity and resistance to chlorhexidine

This chapter has been published as:
Kara D, Luppens SBI, ten Cate JM (2006). Differences between single and dual-species biofilms of *Streptococcus mutans* and *Veillonella parvula* in growth, acidogenicity and resistance to chlorhexidine. *European Journal of Oral Sciences* 114: 58-63.

Abstract

Streptococcus mutans, considered the primary pathogen in dental caries, thrives in dental plaque which is a multispecies biofilm. Metabolic interactions between *S. mutans* and *Veillonella parvula* have been suggested. In this study we developed a biofilm model to quantify single-species (*S. mutans* or *V. parvula*) and dual-species (*S. mutans* and *V. parvula*) biofilm formation, and we identified the differences between the respective biofilms in terms of growth, acid formation and the response to chlorhexidine. Polystyrene 96-well microtiter plates were used for biofilm formation. These biofilms were exposed to various chlorhexidine concentrations (0.025 - 0.4 mg ml^{-1}) and treatment conditions. Growth of the biofilms and the effect of chlorhexidine were evaluated by viable counts. Viability of the two species in all biofilm types was similar ($\approx 10^8$ colony-forming units per well) after 72 h. Lactic acid accumulation of dual-species biofilms was significantly lower at 48 and 72 h than single-species biofilms of *S. mutans*. Dual-species biofilms were less susceptible to chlorhexidine than single-species biofilms when a neutralization step was included. These results indicate that bacteria in dual-species biofilms have different properties from bacteria in single-species biofilms.

Introduction

Biofilm can be described as a layer of microorganisms, attached to a surface, typically encased in an extracellular matrix (Costerton *et al.*, 1994). Dental plaque is a multi-species biofilm. More than 500 species of bacteria have been isolated from dental plaque (Paster *et al.*, 2001). These bacteria interact with each other in many ways, such as using the metabolic end products that other species produce or communicating by releasing specific molecules. Unraveling the interaction between species would lead to a better understanding of this complexity.

Streptococci and Veillonellae are members of the resident oral microflora and reported as early colonizers of dental biofilm (Marsh, 2000; Kolenbrander *et al.*, 2002). *Streptococcus mutans* has been the focal point of caries research for decades mainly as a result of its acidogenicity (Gibbons & Fitzgerald, 1969; Mikx *et al.*,1972). *Veillonella parvula* is considered a 'benevolent organism' in relation to dental caries as it metabolizes lactic acid produced by Streptococci into weaker acids, such as propionic and acetic acid, with a reduced ability to solubilize enamel (Samaranayake, 2002). The long and confusing history of Veillonellae taxonomy has made it difficult to extract information about the physiology of *V. parvula*. As pointed out earlier, investigators have generally designated any Veillonellae isolate as *V. alcalescens* (Rogosa, 1965; Ng & Hamilton, 1971). However, from recent studies there is much evidence for the existence of *V. parvula* in dental biofilms (Kamma *et al.*, 2000; Mager *et al.*, 2003).

The precise interactions between *S. mutans* and *V. parvula* in dental biofilm is not understood. Metabolic interactions between these species were studied using planktonic cells (Coulter & Russell, 1974). However, bacteria in a planktonic phase do not represent the behavior of the same bacteria in a biofilm (Mah & O'Toole, 2001; Stewart, 2003). This difference has been explained by several mechanisms, including expressing a specific biofilm phenotype through activation or repression of specific genes (Kuchma & O'Toole, 2000), quorum sensing (Li *et al.*, 2002), and general stress

response (Brown & Barker, 1999). Therefore, studying the interaction between *S. mutans* and *V. parvula* biofilms would give more realistic results than studying planktonic cell models.

A simple model commonly used for biofilm research is to grow biofilms in microtiter plates (Deighton & Balkau, 1990; Miyake *et al.*, 1992; Stepanovic *et al.*, 2000; Ramage *et al.*, 2001; Pitts *et al.*, 2003). One disadvantage of this is that the surface for biofilm growth is polystyrene. However, a recent study by our group has shown that *S. mutans* biofilm development on polystyrene surfaces was similar to that on hydroxyl-apatite with respect to viability and protein expression (Luppens & ten Cate, 2005).

In this study, our aim was, first, to develop a model to grow single-species (*S. mutans* or *V. parvula*) and dual-species (*S. mutans* and *V. parvula*) biofilms and second, to identify any differences between single-species (*S. mutans* or *V. parvula*) and dual-species (*S. mutans* and *V. parvula*) biofilms, in terms of growth, acidogenicity and response to chlorhexidine.

Materials and methods

Bacterial strains, inoculation procedure and growth of biofilms

The bacteria investigated were *Streptococcus mutans* C180-2 (de Stoppelaar *et al.*, 1969) and *Veillonella parvula* DSM 2008 (DSMZ, Braunschweig, Germany). Freezer stocks (overnight culture + 30% glycerol) were streaked onto separate blood agar plates (Tryptic Soy Broth; Difco, USA; containing 2% glucose and 5% sheep blood) and grown for 48 h (*S. mutans*) or 72 h (*V. parvula*). One colony of each bacterial strain was used to inoculate 10 ml brain heart infusion (BHI; Difco) and grown anaerobically for 16 h (*S. mutans*) or 64 h (*V. parvula*) to obtain the same number of cells [10^9 colony-forming units (CFU) ml^{-1}] for both species. Polystyrene, 96-well flat-bottomed microtiter plates (bio-one, Greiner, Frickenhausen, Germany) containing 0.2

ml of half-strength BHI supplemented with 50 mM lactic acid and 50 mM PIPES (medium) in each well, were inoculated with 2 x 10^6 CFU *S. mutans* and/or 10^7 CFU *V. parvula*. Single-species biofilms of *S. mutans* or *V. parvula*, and dual-species biofilms of *S. mutans* + *V. parvula*, were grown on separate microtiter plates for 24, 48 and 72 h. The culture medium was refreshed every 24 h. Following the removal of 0.2 ml of medium from each well, biofilm samples were harvested by suspention in 0.2 ml of cysteine peptone water (CPW) by vigorous pipetting, which dissociated chains and aggregates of bacteria; the bacteria were then quantified by colony counting on blood agar plates after incubation for 72 h. Medium removed from the wells (spent medium) was analyzed for the concentration of organic acids produced by the biofilms and to measure the pH. Sterile medium was also included to confirm the presence of 50 mM lactic acid. The lactic acid concentration in the medium was 49.3 ± 3.2 mM. Inoculation of bacteria, growth and chlorhexidine treatments of biofilms, and CFU assessments were performed at 37 ºC in an anaerobic chamber containing 80% N_2, 10% CO_2, and 10% H_2.

Treatment with chlorhexidine

A sterile stock solution of 6.4 mg ml^{-1} of chlorhexidine (diacetate salt, Sigma, St Louis, MO, USA) was freshly prepared on the day of use. Forty-eight-hour single and dual-species biofilms were exposed for 10 min to 0.2 ml of various chlorhexidine concentrations (0.025, 0.1, and 0.4 mg ml^{-1}) in each of three separate treatment sequences (a - c, below) (Fig. 1);

a) After chlorhexidine application and removal, the biofilm was washed once with sterile water, fresh medium was added and regrowth was evaluated after 24 h by viable counts (unneutralized regrowth).
b) After chlorhexidine application and removal, neutralizer was applied for 5 min to inactivate chlorhexidine (Russel *et al.*, 1992) and survival was directly evaluated by viable counts (neutralized killing). The neutralizer contained 3 g l^{-1} lecithin, 30 g l^{-1} polysorbate 80, and 0.25 mol l^{-1} phosphate buffer (NNI, 1997).

c) After the consecutive applications and removal of chlorhexidine and neutralizer, fresh medium was added for biofilm regrowth. Regrowth was evaluated after 24 h by viable counts (neutralized regrowth).

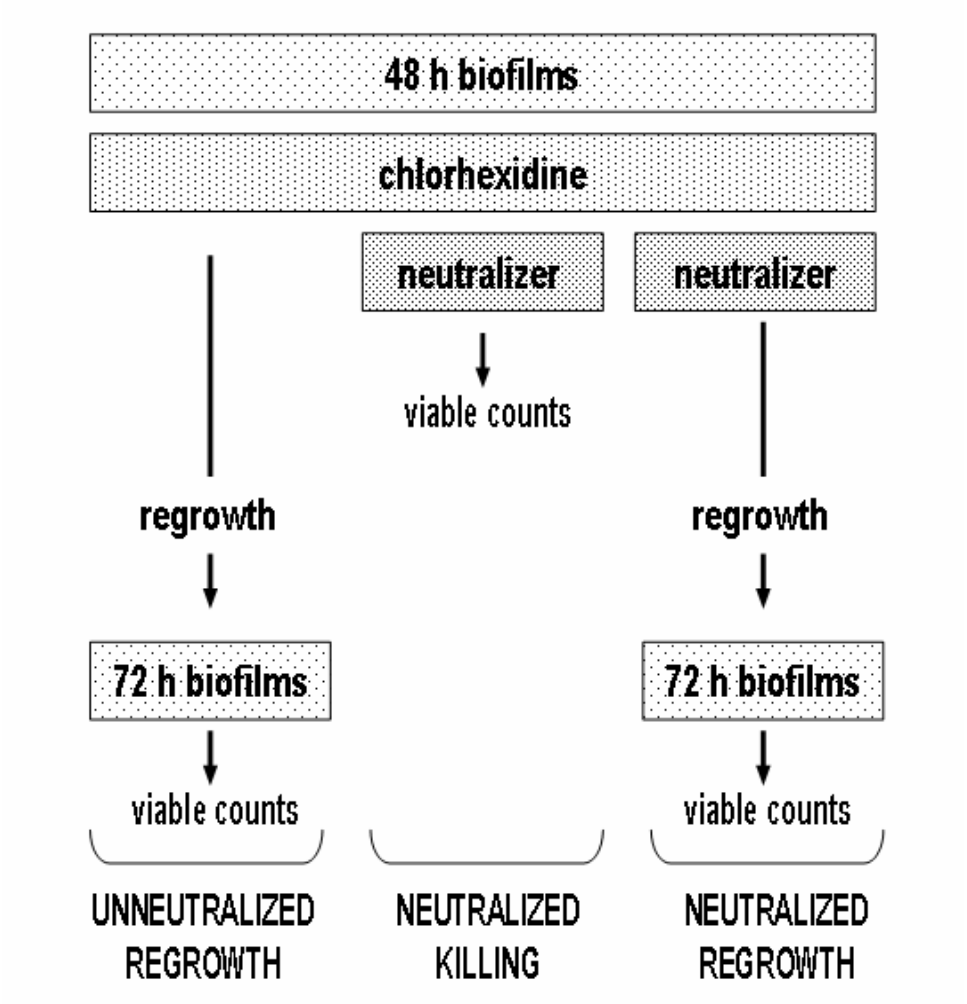

Fig. 1. Experimental design; see text for explanation.

As controls for the neutralized killing group, 48-h growth data were used. For the two other treatment groups, 72-h growth data were used. All experiments were repeated on three separate days, and on each day in triplicate.

Organic acid analysis

Organic acids were determined as their anions by capillary electrophoresis (Waters Capillary Ion Analyzer, Milford, MA, USA). Duplicate samples were run, and Millenium Chromatography Manager Software, version 3.05, was used for data analysis. Peak identification and peak area integration were manually corrected if necessary. Sodium salts of formic, acetic, propionic, butyric, succinic and lactic acid (Sigma) were used to prepare single and mixed standard solutions in deionized water. Calibration curves were made for each acid separately. As internal standard, 0.12 mM $NaNO_3$ and 0.1mM oxalic acid were included in all samples. Lactic, propionic, acetic, formic, butyric and succinic acid concentrations were determined.

Statistics

We compared the viable counts of control samples and chlorhexidine-treated samples, and we compared viable counts of the same species grown in single-species biofilms and dual-species biofilms that were not treated and that were exposed to chlorhexidine, using SPSS 10.0.7 statistical software (SPSS, Inc.) with univariate analysis of variance, and by using t-test; statistically significant differences were set at a p-value of < 0.05. Univariate analysis of variance, *t*-test and posthoc tests were used for statistical comparisons of between-days data for acid concentration, for single- and dual-species biofilms, and for lactic acid data between biofilms.

Results

Growth and viability of single- and dual-species biofilms

We cultured and analyzed single- and dual-species biofilms of *S. mutans* and *V. parvula* in microtiter plates. Biofilm microstructures established viable count levels ≈ 10^8 CFU per well at all time-points, except single-species biofilms of *V. parvula* at 24 h (Fig. 2). At this time point, *V. parvula* growth was significantly lower in single-species biofilms (≈ 10^6 CFU per well) than in dual-species biofilm (≈ 10^8 CFU per well). Viable counts of *S. mutans* in dual-species biofilms were higher than in single-species biofilms at all time-points and at 48 h; this difference was statistically significant ($p < 0.05$).

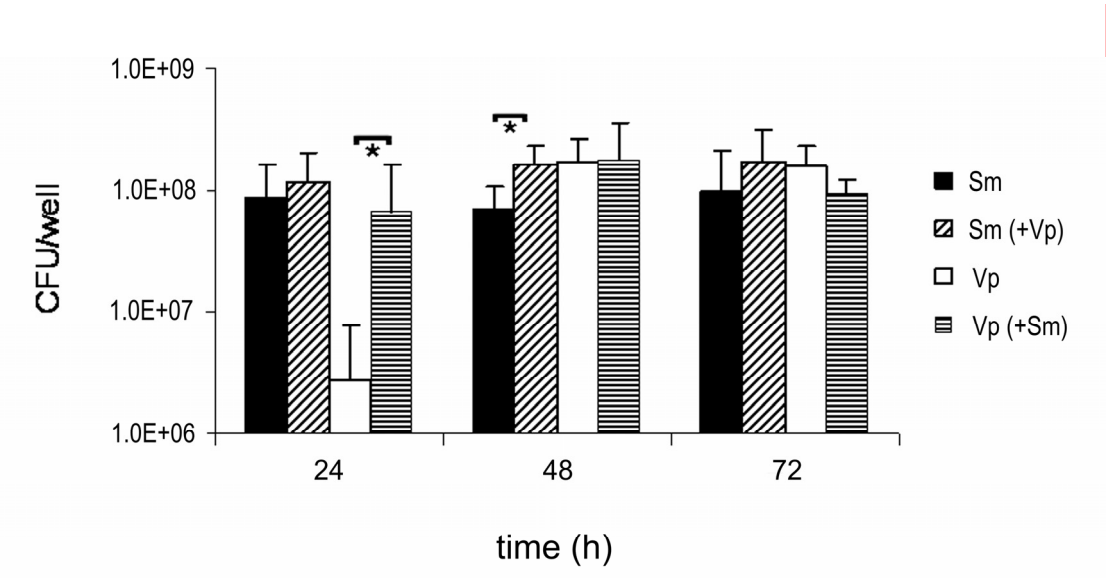

Fig. 2. Growth of single- and dual-species biofilm in time. Sm, *Streptococcus mutans*; Sm (+Vp), *S. mutans* in dual-species biofilm; Vp, *Veillonella parvula*; Vp (+Sm), *V. parvula* in dual-species biofilm. (*) denotes a significant difference ($p < 0.05$) within single- and dual-species biofilm counts of the same species at the same time point.

Acidogenicity of single- and dual-species biofilms

The pH of fresh medium and spent medium of all types of biofilms was 7.2 at all time points, indicating that the buffer was sufficiently strong to prevent pH changes.

Single-species biofilms of *S. mutans* produced low concentrations of formic, succinic, acetic, and butyric acids (Table 1). Lactic acid was added to the medium to 50 mM. Lactic acid was found to be the predominant acid present and its concentration was higher at 48 and 72 h than at 24 h. The concentration at 72 h (121 mM) was significantly higher than that at 24 and 48 h ($p < 0.001$). No propionic acid formation was observed at any time-point in *S. mutans* biofilms.

A substantial decrease in lactic acid concentration over time was detected in samples of single-species biofilms of *V. parvula* (Table 1). There was a significant correlation ($p < 0.05$) between decreasing concentrations of lactic acid and increasing concentrations of acetic acid. A significant, positive correlation ($p < 0.05$) was also detected between the concentrations of acetic and propionic acid produced. Formic acid was not detected. However, very low concentrations of butyric acid were formed at all time-points.

Lactic acid concentrations were not significantly different between the various time-points for dual-species biofilms of *S. mutans* and *V. parvula* (Table 1). Propionic and acetic acid concentrations were significantly higher at 48 and 72 h than at 24 h ($p < 0.05$). Low concentrations of formic, succinic, and butyric acid were also detected in dual-species biofilms.

At 24 h, lactic acid concentrations did not differ significantly between single-species biofilms of *S. mutans*, single-species biofilms of *V. parvula* or dual-species biofilms. These values were all in the range of the lactic acid concentration of the medium (50 mM). However, at 48 and 72 h, lactic acid concentrations in dual-species biofilm samples were significantly lower than lactic acid concentrations in single-species biofilms of *S. mutans* ($p < 0.05$).

Table 1

Concentrations of organic acids (mM) in samples of single- and dual-species biofilms during growth (#)

	24 h	48 h	72 h
Streptococcus mutans			
Acetic acid	1.7 (1.06)	2.9 (1.1)	4.7 (1.2)†
Lactic acid	47.1 (11.7)	62.2 (15.3)	121.0 (45.5)*†
Propionic acid	bdl	bdl	bdl
Formic acid	0.6 (0.7)	1.2 (0.7)	2.8 (0.7)
Succinic acid	0.02 (0.05)	0.3 (0.8)	2.4 (0.3)*†
Butyric acid	2.4 (0.4)	2.5 (0.7)	6.1 (2.4)*†
Veillonella parvula			
Acetic acid	3.7 (1.9)	15.4 (6.0)*	39.7 (13.6)*†
Lactic acid	37.9 (17.3)	31.6 (10.2)	14.6 (1.3)†
Propionic acid	4.2 (1.7)	13.7 (4.0)*	49.4 (17.7)*†
Formic acid	bdl	bdl	bdl
Succinic acid	1.0 (0.8)	1.5 (1.4)	6.5 (4.6)*†
Butyric acid	2.0 (0.7)	1.0 (0.5)*	1.3 (0.3)†
Streptococcus mutans and *Veillonella parvula*			
Acetic acid	10.8 (2.4)	28.1 (8.6)*	44.9 (18.9)*†
Lactic acid	40.8 (17.8)	29.4 (10.7)	35.2 (9.0)
Propionic acid	9.6 (3.1)	34.2 (10.3)*	51.5 (23.3)*†
Formic acid	1.1 (0.8)	1.9 (1.4)	3.2 (1.2)†
Succinic acid	0.8 (0.6)	2.4 (1.2)	6.1 (0.7)*†
Butyric acid	2.2 (0.6)	1.6 (0.5)	1.4 (1.6)

bdl: below detection limit (detection limit = 0.01 mM)

* indicates a significant difference from the previous time point.

† denotes a significant difference from the 24 h time point for each organic acid ($p < 0.05$) per biofilm type.

Every 24 h, the spent medium was removed for measurement and fresh medium containing 50 mM lactic acid was added.

Effects of chlorhexidine on the viability of single- and dual-species biofilms

Regardless of the concentration of chlorhexidine, the largest decrease in viable counts of all biofilms was detected when regrowth was evaluated 24 h after the application of chlorhexidine (unneutralized regrowth) (Fig. 3). Under this treatment condition, all concentrations of chlorhexidine caused a significant decrease in biofilm viable counts compared to non-treated controls ($p < 0.05$). For all biofilm types there was no significant difference between the efficacies of various chlorhexidine concentrations ($p > 0.05$).

Following the application of chlorhexidine and neutralizer on biofilms, when survival was evaluated directly by viable counts (neutralized killing), or when regrowth was evaluated after 24 h (neutralized regrowth), the dual-species biofilms were more resistant to the highest two concentrations of chlorhexidine (0.1 mg ml^{-1} and 0.4 mg ml^{-1}) than single-species biofilms of either strain (Fig. 3B, C). In these two chlorhexidine treatment sequences, a dose-effect of chlorhexidine was observed, with the highest concentration (0.4 mg ml^{-1}) generally having the highest efficacy on biofilms ($p < 0.05$).

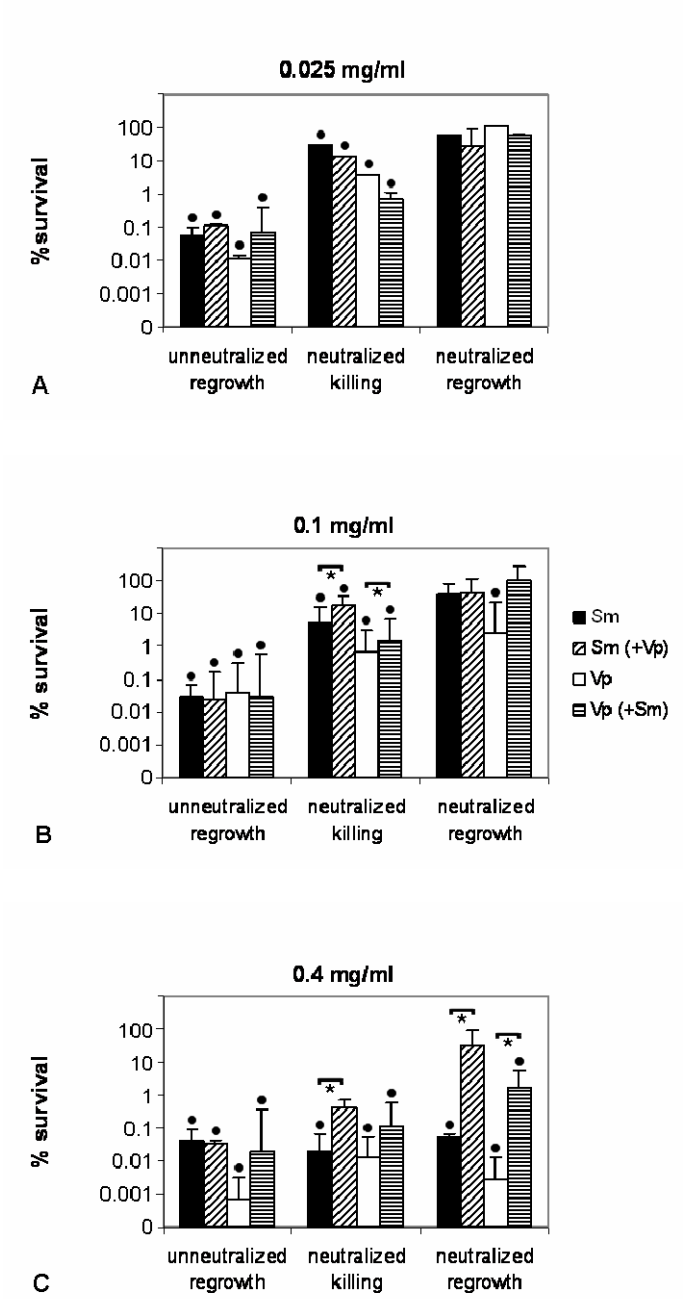

Fig. 3. Percentage survival of single- and dual-species biofilm bacteria after exposure to three different chlorhexidine concentrations (% survival = (CFU after chlorhexidine application/CFU control) x 100). Sm, *Streptococcus mutans*; Sm (+Vp), *S. mutans* in dual-species biofilm; Vp, *Veillonella parvula*; Vp (+Sm), *V. parvula* in dual-species biofilm. (●) denotes a significant decrease in the viable counts of each biofilm after related chlorhexidine treatment compared to the control, (*) denotes a significant difference between single- and dual-species biofilm viable counts of the same bacterium ($p < 0.05$).

Discussion

In this study, we developed a model to analyze the properties of single- and dual species biofilms of *S. mutans* and *V. parvula.* Furthermore, we showed that significant differences exist between dual-species biofilms of *S. mutans* and *V. parvula* and single-species biofilms of the same bacteria in growth, acidogenicity and antimicrobial resistance.

Growth of single-species biofilms of *S. mutans* in our model was similar to that found in another study (Luppens & ten Cate, 2005) with *S. mutans* biofilms grown on polystyrene surfaces of culture plates. The growth of *S. mutans* was better in dual-species biofilms than in single-species biofilms. Recently, it has been suggested that *Veillonella atypica* produces a signal that causes *Streptococcus gordonii* to increase expression of an α-amylase gene, which may enhance the carbohydrate fermenting capacity of Streptococci in dual-species biofilms (Egland *et al.*, 2004). Higher viable counts of *S. mutans* in dual-species biofilms, in comparison to single-species biofilms at all time points in our study might indicate such a signaling transaction between *S. mutans* and *V. parvula* which would justify further studies.

We found significantly higher viable counts of *V. parvula* in dual-species biofilm compared with the single-species biofilm of *V. parvula* at 24 h. This could be explained by more favorable growth conditions generated by utilization of the additional lactate produced by *S. mutans*. Furthermore, colonization of the polystyrene surface by *S. mutans* might have enhanced the growth of *V. parvula* in dual species biofilm. In an *in vivo* study it has been shown that 300-fold more *V. alcalescens* cells were bound to teeth populated with *S. mutans* than bound to teeth populated with a non-coaggregating *S. mutans* strain (McBride & van der Hoeven, 1981).

Our findings are in agreement with those of Noorda *et al.* (1988) who found that, using *S. mutans* and *V. alcalescens,* CFU values of mixed bacterial plaques were higher than of the monobacterial plaque. However, they referred to the total counts of

mixed-species dental plaque instead of specific viable counts of species in the mixed plaque.

The ability to produce, tolerate or utilize lactic acid is important in dental biofilms (van Houte, 1994; Filoche *et al.*, 2004). Streptococci produce lactate during growth on sugars, and Veillonellae can utilize short-chain organic acids, especially lactate, for growth. This metabolic interaction has been documented *in vitro* (van Houte, 1994) and *in vivo* in gnotobiotic rats (Mikx *et al.*, 1972; van der Hoeven *et al.*, 1978). However, in these studies, *V. alcalescens* was used instead of *V. parvula*.

Cariogenic plaques produce lactic acid as the predominant end-product in the presence of high sugar concentrations, and thus lactic acid is considered to be the main acid involved in caries formation (Carter *et al.*, 1956). According to our findings, *V. parvula* caused lactic acid concentrations to remain constant in time in dual-species biofilm, which may lead to a less cariogenic environment than when *S. mutans* grows alone. These results also indicate the shortcomings of using a single-species in dental microbiology work, in particular *S. mutans*, which has been the focal organism in caries research for decades.

Generally, 0.2% (2 mg ml^{-1}) chlorhexidine is preferred for clinical treatments. The chlorhexidine concentrations used in our study were lower than this. Chlorhexidine is diluted by saliva in the mouth, and the salivary concentration of chlorhexidine shows a logarithmic fall during the first hours of application (Bonesvoll *et al.*, 1974). Furthermore, in a study using a microtiter biofilm model, 0.2% chlorhexidine reduced the viability of a multispecies biofilm to zero (Guggenheim *et al.*, 2001).

Chlorhexidine binds to oral surfaces more strongly than other antimicrobial agents. This binding delays its clearance from the mouth, thereby prolonging its effectiveness (Dawes, 2004). Salivary proteins have the capacity to neutralize the activity of chlorhexidine. Therefore, in our study, chlorhexidine was applied in different modalities. The prolonged binding of the agent without the effect of saliva was studied in ‘unneutralized regrowth’ group. So, the recovery of bacteria in the

presence of residual chlorhexidine was tested. In other modalities, neutralizer was used to mimic saliva-induced clearance and inhibition after chlorhexidine application. In the 'neutralized regrowth' group, recovery of bacteria without the prolonged effect of chlorhexidine was measured. Also, the 'neutralized killing' group was designed to assess the immediate killing effect of chlorhexidine. This made it possible to compare the recovery in the two other modalities. Recovery of bacteria in all biofilm types was better when neutralizer was applied after chlorhexidine exposure, indicating that the residual chlorhexidine bound to the cells was enough to inhibit outgrowth.

For the first time, the results of this study convincingly show that bacteria in dual-species biofilms are more resistant to antimicrobial treatments than single-species biofilms. Bacteria in biofilms have already been shown to be more resistant to antibiotics, biocides and disinfectants when compared with planktonic cells (Hoyle & Costerton, 1991; Xu *et al.*, 2000). However, we are unaware of any reports of decreased susceptibility to chlorhexidine in dual-species biofilm cells compared with single-species biofilms. Several factors may account for this decreased susceptibility. For example, the increase in environmental heterogeneity of the biofilm might promote different levels of resistance throughout the community. It can also be hypothesized that more extracellular polysaccharide associated with increased viable numbers of *S. mutans* may reduce the susceptibility of dual-species biofilms to chlorhexidine. Further research is needed to clarify the mechanisms in dual-species biofilm cells that result in reduced susceptibility to antimicrobial agents.

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Chapter 3

Microstructural differences between single-species and dual-species biofilms of *Streptococcus mutans* and *Veillonella parvula*, before and after exposure to chlorhexidine

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Abstract

Dual-species biofilms of *Streptococcus mutans* and *Veillonella parvula* are less susceptible to antimicrobials than single-species biofilms of the same microorganisms. The microstructure of single and dual-species biofilms of *S. mutans* and/or *V. parvula* was visualized to find out whether spatial arrangement of bacteria in biofilms is related to survival strategies against antimicrobials. Biofilms were grown in glass-bottomed 96-well microtiter plates and exposed to chlorhexidine at 48 h. Fluorescent probes were used for staining. Microstructure of biofilms was analyzed by confocal laser scanning microscopy at 48, 96, 192, and 264 h. Spatial arrangement analysis was performed using DAIME software for 48 h biofilms. A decrease in viability and thickness in all types of biofilms was detected after chlorhexidine treatment in time. In untreated biofilms, clustering was observed. In chlorhexidine-treated single-species biofilms, bacteria were dispersed. However, the most prominent clustering was observed in chlorhexidine-treated dual-species biofilm bacteria which had a higher survival rate compared to chlorhexidine-treated single-species biofilms. Bacteria in dual-species biofilms establish a specific spatial arrangement, forming clusters within distances below 1.2 μm as a survival strategy against antimicrobials while the same bacteria lack this defensive construction in single-species biofilm.

Introduction

More than 700 bacterial species have been detected in the oral cavity. Although most of the species are located in specific sites, Veillonellae and Streptococci are commonly found in all sites investigated (Aas *et al.*, 2005). Veillonellae and Streptococci are early colonizers of dental biofilm and are resident members of the oral microflora (Marsh, 2000; Kolenbrander *et al.*, 2002). *Streptococcus mutans* has been the focal microorganism of caries research for decades, mainly as a result of its acidogenic and aciduric properties (Gibbons & Fitzgerald, 1969). *Streptococcus mutans* utilizes carbohydrates and produces organic acids, mainly lactic acid. *Veillonella parvula* is considered a 'benevolent organism' in relation to dental caries because it catabolizes lactate, produced by Streptococci, into shorter-chain-length acids such as propionic and acetic acid with a reduced ability to solubilize enamel (Samaranayake, 2002). Such metabolic cooperation among bacteria is shown to be crucial for the establishment of oral biofilms (Bradshaw *et al.*, 1994).

Attempts have been going on in dental research to attain a non-cariogenic oral flora considering that dental caries is a bacterial disease. Antimicrobial strategies are of importance in oral health in preventive and treatment measures. Chlorhexidine is a commonly used antimicrobial agent in the oral cavity. The authors' group has shown for the first time that both *S. mutans* and *V. parvula* are less susceptible to chlorhexidine in dual-species biofilm compared with single-species biofilms of each bacterium (Kara *et al.*, 2006). Still, the nature of the interaction between *S. mutans* and *V. parvula* responsible for this phenomenon is unknown. Unraveling the interaction between species would lead to a better understanding of the complexity of oral biofilm and allow for a rational approach in prevention and treatment of dental caries.

Substantial evidence indicates that biofilm microstructure represents a key determinant of biofilm growth, physiology, species diversity, and susceptibility to antimicrobial strategies (Singleton *et al.*, 1997). Methods for microscopic identification of oral microorganisms have been developed using fluorescence

detection with targeting either via antibody technology (Palmer *et al.*, 2006) or nucleic acid probes (Zaura-Arite *et al.*, 2001). In biofilm research, confocal laser scanning microscopy (CLSM) has been a preferred technique, as it allows a three-dimensional visualization of cells and the reconstruction of the biofilm structure without distortion.

In this study, it was aimed to investigate the differences in microstructure and viability between *S. mutans* and/or *V. parvula* single and dual-species biofilms, before and after exposure to chlorhexidine, to find a possible cause for the increased resistance of dual-species biofilms to chlorhexidine compared with single-species biofilms.

Materials and methods

Bacterial strains

In this study, *S. mutans* UA159 (kind gift from Dr. Marquis, Department of Microbiology & Immunology, University of Rochester Medical Center, Rochester, NY) and *V. parvula* DSM 2008 (DSMZ, Braunschweig, Germany) were used. Freezer stocks (overnight culture + 30% glycerol) of each strain were streaked onto separate blood agar plates (tryptic soy broth; Difco, Sparks, MD, USA; containing 2% glucose and 5% sheep blood) and grown for 48 h (*S. mutans*) or 72 h (*V. parvula*). One colony of each bacterial strain was used to inoculate 10 ml brain heart infusion (BHI; Difco) and grown anaerobically for 16 h (*S. mutans*) or 64 h (*V. parvula*) to obtain the same number of cells (10^9 CFU ml^{-1}) for both species.

Biofilm growth

Glass-bottomed 96-well microtiter plates (Bio-one; Greiner, Frickenhausen, Germany) containing 0.2 ml of half-strength BHI supplemented with 50 mM lactic acid and 50 mM PIPES (medium) in each well, were inoculated with 2 x 10^6 CFU *S. mutans* and/or 10^7 CFU *V. parvula.* Three types of biofilms (single-species biofilms of *S.*

mutans, single-species biofilms of *V. parvula*, and dual-species biofilms of *S. mutans* and *V. parvula*) were grown on separate microtiter plates for 48, 96, 192, and 264 h. The culture medium was refreshed every 24 h. Inoculation of bacteria, growth and chlorhexidine treatment of biofilms were performed at 37°C in an anaerobic chamber containing 80% N_2, 10% CO_2, and 10% H_2.

Chlorhexidine treatment

Following the removal of 0.2 ml of medium from each well, 48 h single and dual-species biofilms were exposed to 0.2 ml of 0.4 mg ml^{-1} of chlorhexidine (diacetate salt; Sigma, St Louis, MO, USA) for 5 min which was freshly prepared on the day of use. After chlorhexidine application and removal, neutralizer was applied for 5 min to inactivate chlorhexidine. The neutralizer contained 3 g l^{-1} lecithin, 30 g l^{-1} polysorbate 80, and 0.25 mol^{-1} phosphate buffer (Russel *et al.*, 1992). Untreated biofilms as controls were exposed to sterile water for 5 min and then to neutralizer as described above. After the removal of neutralizer, control and chlorhexidine-treated samples were subjected to staining and analysis. Fresh medium was added for biofilm growth for the rest of the samples to be evaluated at 96, 192 and 264 h.

Bacterial staining

ViaGram™ Red Bacterial Gram Stain and Viability Kit (Molecular Probes, Inc., USA) was used for staining the biofilms before each analysis according to manufacturer's instructions. The kit contains high-affinity SYTOX® Green nucleic acid stain (membrane-impermeant dye) for staining of bacteria with damaged membranes and 4',6-diamidino-2-phenylindole (DAPI) for staining of bacteria with intact cell membranes. Thus, live bacterial cells are fluorescent blue, while dead bacterial cells with damaged membranes are fluorescent green. The third component, Texas Red®-X dye labeled wheat germ agglutinin component, selectively binds to the surface of Gram-positive bacteria (e.g. *S. mutans*) and stains them fluorescently red, even in the

presence of viability stains. Therefore, *S. mutans* cells in our study, were stained with Texas Red® in dual-species biofilm samples to distinguish them from *V. parvula,* which is Gram-negative.

CLSM analysis

Stained chlorhexidine-treated and untreated control biofilms (n=3) were analyzed with a Confocal Laser Scanning Microscopy (CLSM; Leica SP2-AOBS confocal system and Leica DM IRB microscope, Wetzlar, Germany) at 48 h (right after the chlorhexidine treatment), 96 h, 192 h and 264 h. The respective absorption and emission wavelengths are 410/470 nm for DAPI, 500/540 nm for SYTOX® Green, and 590/640 nm for Texas Red®. The specimens were examined with Diode (561 nm), Argon-ion (488 nm) and coherent Argon-ion UV (405 nm) lasers according to the fluorescent dyes used for the individual specimen. The sequential line scan mode was used in order to avoid crosstalk. Texas Red® was imaged first followed by SYTOX® Green and DAPI detection respectively to avoid bleaching. A scan speed of 800 lines sec^{-1} was used to ensure a minimal dislocation due to movement of the cells. Images of the three colors were each adapted to the 8-bit range of the system. A HCX PL APO CS 63.0x1.32 OIL UV objective was used with an additional zoom of x 4 , resulting in 512 x 512 image with a pixel size of 0.12 µm. The pinhole size was set at 1 Airy disc. Series through the whole thickness of the biofilm were made with a stepsize of 0.3 µm. Three samples were processed for each of three experiments and one representative spot of each experiment is represented in 'Results'.

The thickness of the biofilms, percentage of live and dead bacteria and percentage of *S. mutans* and *V. parvula* in the biofilms were assessed through three-dimensional segmentation using DAIME; a recently developed image analysis program for microbial ecology and biofilm research (Daims *et al.*, 2006). Biofilm image stacks of 48 h were evaluated with the same program for spatial arrangement analysis.

Statistics

Comparison between the thickness of the chlorhexidine-treated and untreated biofilms in time and comparison between the thickness of single- and dual-species biofilms was assessed using SPSS 10.0.7 statistical software (SPSS, Chicago, IL, USA) by using one-way ANOVA and post-hoc tests (LSD); statistically significant differences were set at a p-value of < 0.001. Statistical evaluation of spatial distribution analysis was performed with DAIME software (Daims *et al.*, 2006).

Results

We visualized the single- and dual-species biofilms of *S. mutans* and/or *V. parvula* and the effect of chlorhexidine on these biofilms by CLSM. A set of examples for all biofilm types at 48 h is presented in Fig. 4.

Thickness of the biofilms

The thickness of all biofilms increased in time (Table 2). Control dual-species biofilms of *S. mutans* and *V. parvula* were significantly thicker than control single-species biofilms of either bacterium ($p < 0.001$) at 96, 192 and 264 h. However, chlorhexidine-treated samples of all biofilm types were thinner than their corresponding controls and this difference was statistically significant ($p < 0.001$) in most types of biofilms at most time points; the exceptions were single-species biofilms of *S. mutans* at 192 h, and single-species biofilms of *V. parvula* at 48 and 192 h.

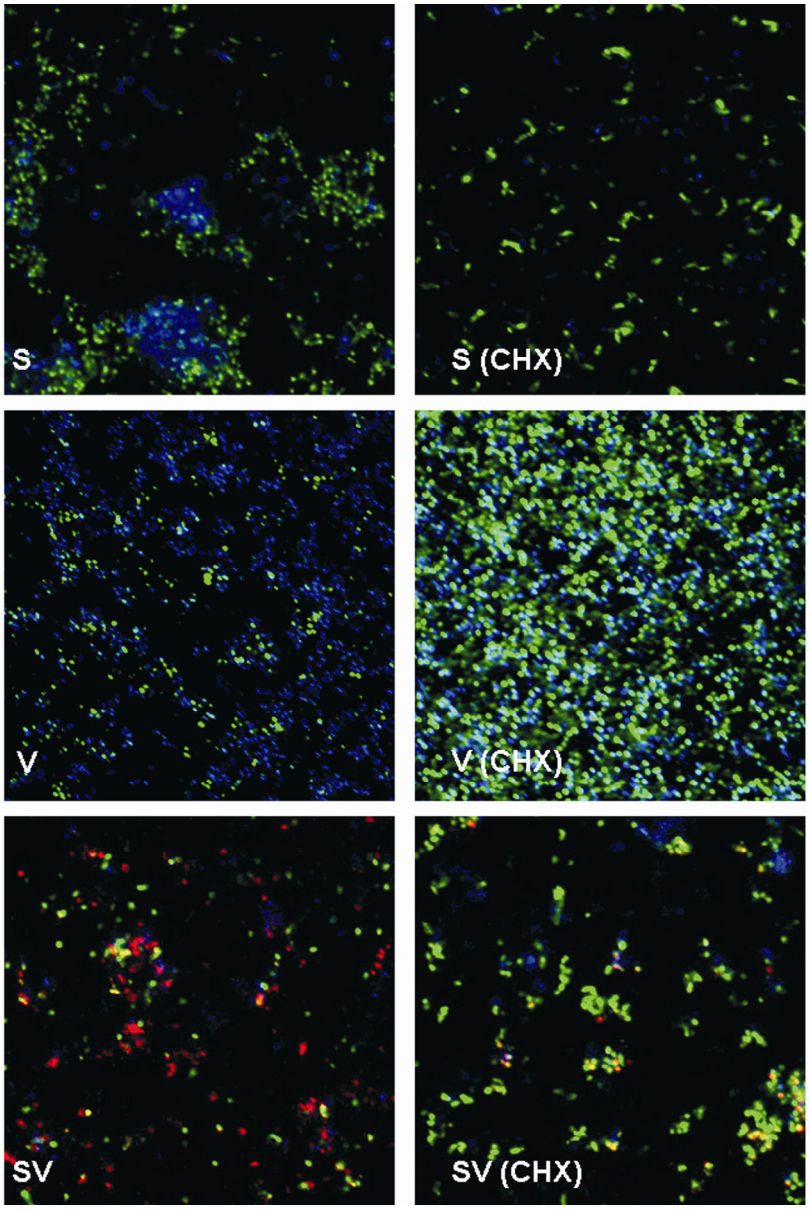

Fig. 4. CLSM images of all types of biofilms at 48 h.
Scanned image in XY axis is 60 x 60 μm.
(S: single-species biofilms of *Streptococcus mutans*; V: single-species biofilms of *Veillonella parvula*; SV: dual-species biofilms of *S. mutans* and *V. parvula*; (CHX): chlorhexidine-treated sample of the corresponding biofilm),
in S, S(CHX), V, V(CHX); blue staining indicates live bacteria; green staining indicates dead bacteria, in SV, SV(CHX); red staining indicates *S. mutans*; blue staining indicates live *V. parvula*; green staining indicates dead *V. parvula.*

Table 2

Thickness (μm) of control and chlorhexidine-treated single- and dual-species biofilms in time

	time (h)			
biofilm type	48	96	192	264
S	19.2 (0.3) †	20.1 (0.3) †	20.7 (0.6)	35.7 (0.6) *†
S (CHX)	18.0 (0.3)	18.3 (0.3)	20.1 (0.3) *	33 (0.9) *
V	12.9 (0.6)	18 (0.3) †	20.1 (0.9) *	26.1 (0.9) * †
V (CHX)	12.0 (0.6)	14.1 (0.3) *	19.8 (0.6) *	24.9 (0.3) *
SV	15 (0.3) †	24 (0.6) * †	24 (1.2) †	54 (1.2) * †
SV (CHX)	12 (0.3)	20.4 (0.6) *	22.2 (0.3) *	25.2 (0.3) *

* indicates a significant difference from the previous time point in a row

† denotes a significant difference from the chlorhexidine-treated sample of the corresponding biofilm type

(S: single-species biofilms of *Streptococcus mutans*; V: single-species biofilms of *Veillonella parvula*; SV: dual- species biofilms of *S. mutans* and *V. parvula*; (CHX): chlorhexidine-treated sample of the corresponding biofilm)

Percentage of live and dead bacteria, and Gram-positive and Gram-negative bacteria in biofilms

Single- and dual-species biofilms of *S. mutans* and/or *V. parvula* were subjected to chlorhexidine treatment at 48 h and survival of bacteria was measured in time resulting in regrowth of bacteria. Figure 2 shows the percentage of dead and live bacteria in untreated control biofilms and biofilms that had been exposed to chlorhexidine. Furthermore, it shows the percentage of *S. mutans* and *V. parvula* in dual-species biofilms.

The percentage of live bacteria in all control biofilm types at all time points (range 57-94%) was higher than the percentage of dead bacteria. The percentage of live bacteria in control dual-species biofilms (*c*. 60%) was less than that of control single-species biofilms at all time points. The ratio of *S. mutans* to *V. parvula* in control dual-species biofilms remained stable in time.

Chlorhexidine had a killing effect on all types of biofilms. However, the survival of bacteria (live %) in chlorhexidine-treated dual-species biofilms was higher than in chlorhexidine-treated single species biofilms of *S. mutans* and *V. parvula* at all time points.

The percentage of *S. mutans* was lower in chlorhexidine-treated dual-species biofilms compared with untreated controls at all time points (Fig. 5G and 5H). The lowest percentage of *S. mutans* in chlorhexidine-treated dual-species biofilms was detected at 96 h; however the ratio of *S. mutans* to *V. parvula* increased in time from this time-point.

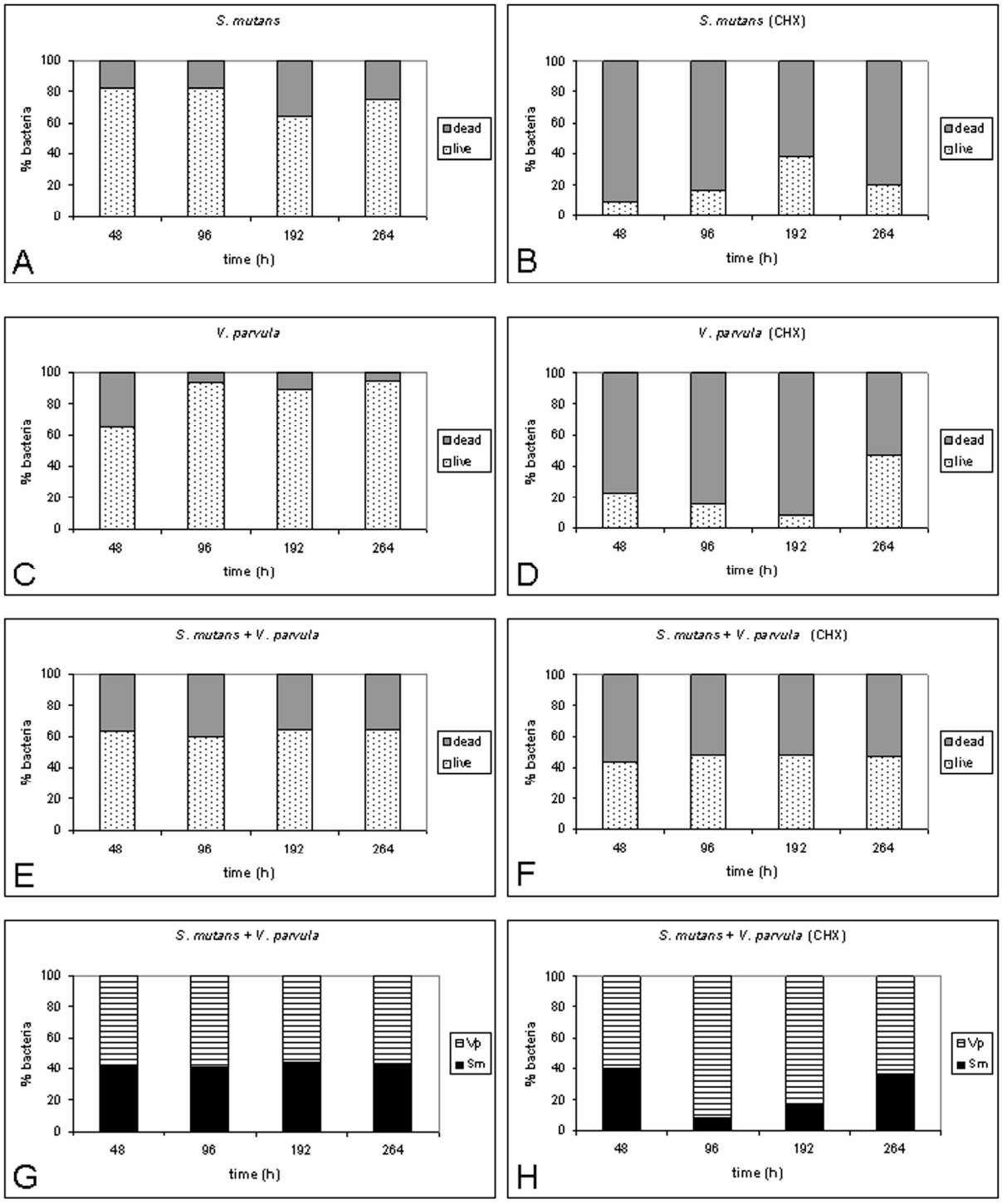

Fig. 5. A-F: The percentage of dead and live bacteria in biofilms.
G, H: The percentage of *Streptococcus mutans* and *Veillonella parvula* in dual-species biofilms.

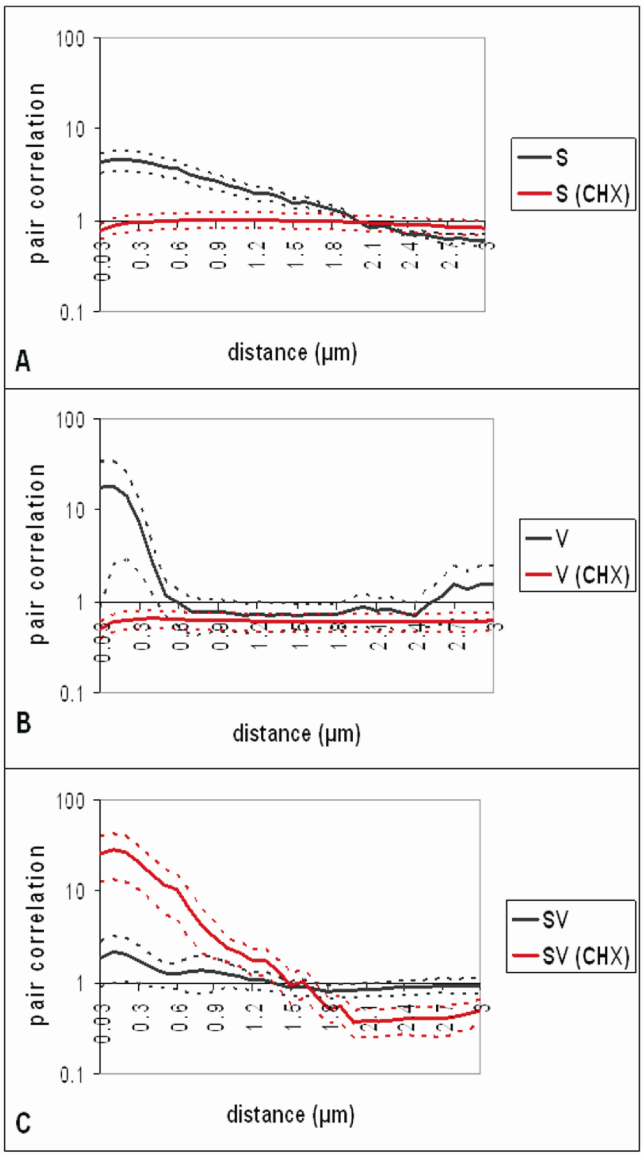

Fig. 6. Spatial arrangement analysis of 48 h biofilms.
Two populations; which is dead and live bacteria were analyzed for each biofilm type.
Graphs illustrate the mean pair cross-correlation function (continuous lines) and the 95% confidence limits (dotted lines) of the mean pair cross-correlation function plotted against the corresponding values of the analyzed distances [The mean pair-cross correlation function for a given distance is determined as described in Daims *et al.* (2006)]. Pair cross-correlation values >1 indicate clustering of the population in a given distance, values <1 indicate repulsion, and values (approximately) equal to 1 indicate random distribution. Number of bacteria that were "hit" during this analysis is more than 95% for each biofilm.

Spatial arrangement analysis

In control single-species biofilm samples of *S. mutans*, clustering was observed between live and dead bacteria at distances below 1.7 μm with a maximum at 0.03 μm (Fig. 6A). Clustering between live and dead bacteria in *V. parvula* control biofilms was observed at distances below 0.4 μm with a maximum at 0.1 μm (Fig. 6B). In dual-species control biofilm of *S. mutans* and *V. parvula,* dead and live bacteria were clustered at distance values below 1.2 μm between the populations with a maximum at 0.1 μm (Fig. 6C). In chlorhexidine-treated single-species biofilms of *S. mutans* and *V. parvula,* bacteria were randomly distributed, showing no signs of clustering (Fig. 6A and 6B, respectively). The most prominent clustering among all biofilm types was detected in chlorhexidine-treated dual-species biofilms with bacteria at distances below 1.2 μm with a maximum at 0.1 μm (Fig. 6C). The degree of clustering between live and dead bacteria in chlorhexidine-treated dual-species biofilm was significantly higher than in all other biofilm types (Fig. 6C). Statistical support for significant differences was provided by the non-overlapping confidence intervals over the distance ranges.

Control and chlorhexidine-treated dual-species biofilms were also analyzed to quantify the spatial arrangement of live and dead bacteria of *V. parvula*, respectively, relative to *S. mutans* at 48 h (Fig. 7). Clustering between *S. mutans* and both live and dead *V. parvula* bacteria was observed at distances below 0.4 μm with a maximum at 0.03 μm in untreated dual-species biofilms. In chlorhexidine-treated dual-species biofilms, clustering occurred at distances below 1.2 μm with a maximum at 0.03 μm. Regardless of live or dead bacteria, clustering was more prominent between *S. mutans* and *V. parvula* after chlorhexidine treatment.

Discussion

In this study it was demonstrated that there are differences between microstructures of single- and dual-species biofilms of *S. mutans* and *V. parvula,* using fluorescent probes and CLSM. It was also shown that chlorhexidine exposure affects biofilm microstructure, and that the microstructural alteration caused by chlorhexidine in single-species biofilms is different from that observed in dual-species biofilms.

The ratio of *S. mutans* to *V. parvula* remained stable during growth of control dual-species biofilms, in agreement with a previous study (Kara *et al.*, 2006). The percentage of live and dead bacteria was also quantified in all types of biofilms. The percentage of dead bacteria was relatively higher in control dual-species biofilms than that in control single-species biofilms at all time points, possibly indicating a faster life cycle of bacteria depending on the metabolic interactions between *S. mutans* and *V. parvula*. After chlorhexidine treatment, the survival of bacteria (live %) was higher in dual-species biofilms than in single-species biofilms, confirming the previous results (Kara *et al.*, 2006) for 48 h. The findings of the current study indicate that, this better survival phenotype of bacteria in dual-species biofilms continues for another 216 h after chlorhexidine treatment. Furthermore, preferential killing of *S. mutans* by chlorhexidine was observed. In chlorhexidine-treated dual-species biofilms, the percentage of *V. parvula* was higher than of *S. mutans* at all time points. Also, killing effect of chlorhexidine was higher in single-species *S. mutans* biofilms than in *V. parvula* at 48 h. This result is in accord with the general finding that Gram-negative bacteria tend to be more resistant than Gram-positive bacteria against antimicrobials (McDonnell & Russell, 1999).

It was observed that thickness of all types of biofilms increased with time. Also, dual-species biofilms were thicker than single-species biofilms at 96, 192, and 264 h. These data are in agreement with the study of Siebel and Characklis (1991) showing that biofilm thickness is affected by the number of component organisms even though they could not identify any metabolic interaction between the species. According to

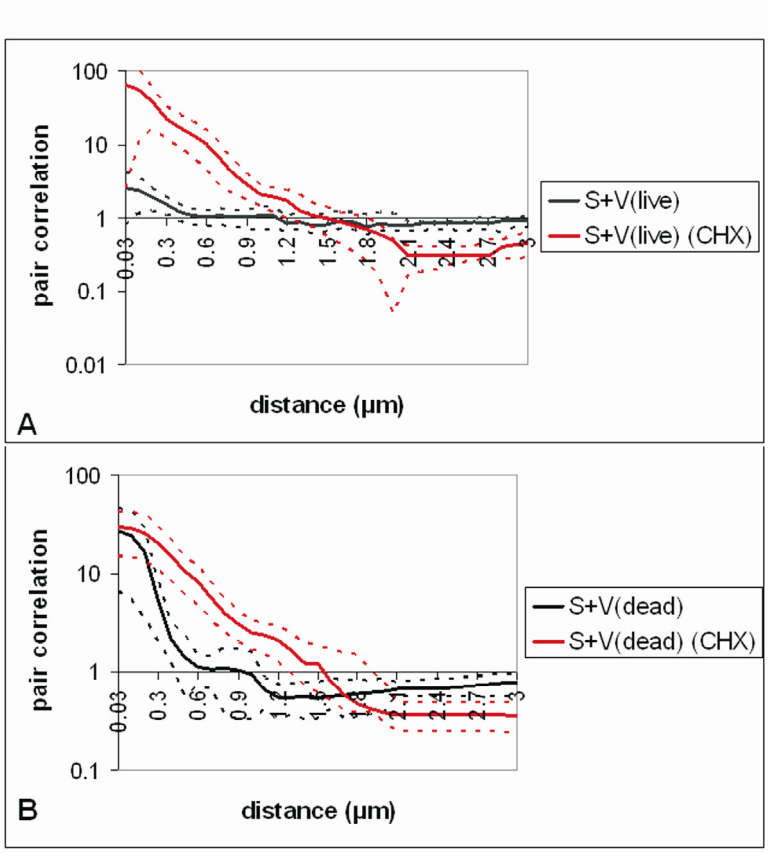

Fig. 7. Spatial arrangement analysis of 48 h dual-species biofilms.

Live and dead *Veillonella parvula* relative to *Streptococcus mutans* were analyzed in untreated (black line) and chlorhexidine-treated (red line) dual-species biofilms.

A: Pair correlation between live *V. parvula* and total *S. mutans* and effect of chlorhexidine.

B: Pair correlation between dead *V. parvula* and total *S. mutans* and effect of chlorhexidine.

For explanation of pair correlation function, see text and Fig. 6.

the present results, chlorhexidine treatment at 48 h caused a decrease in the thickness of the biofilms, and this effect lasted during the recovery phase; at 264 h, the thickness of all chlorhexidine-treated biofilms was significantly less than that of controls ($p < 0.001$). Contraction of *in vitro* multi-species biofilms of oral microorganisms due to chlorhexidine treatment has been reported (Hope & Wilson, 2004). Ionic interactions between the positively charged chlorhexidine molecules and negatively charged extracellular polysaccharides, which comprise the bulk of the volume of the biofilm, were suggested to create a shift toward neutral of the net charge of the matrix, reducing the repulsive forces, allowing closer associations between polymeric strands, and reducing the volume occupied by the biofilm.

The spatial arrangement of bacterial populations is considered an important structural feature of biofilms leading to biofilm specific physiological properties and phenotypically adapted bacteria. Mutualistic and antagonistic relationships between bacteria may be reflected in spatial clustering or repulsion. Differences in spatial arrangement could also be responsible for the better survival of dual-species biofilm bacteria after exposure to chlorhexidine. However, simple visual observation whether two populations really cluster together might give biased results when not supported by statistical analysis. Therefore, recently developed software DAIME (Daims *et al.*, 2006), was used to quantify the spatial arrangement patterns of probe-labeled microorganisms in 48 h biofilms.

In all types of untreated biofilms, live and dead cells were clustered. This finding is not surprising because *S. mutans* cells are embedded in extracellular polysaccharide matrix which might stimulate the cluster formation in the natural bacterial growth within biofilms. Clustering in dual-species biofilms of *S. mutans* and *V. parvula* might also be a result of their mutual relationship. In dual-species biofilms, clustering was observed at distances below 1.2 μm. Beyond 1.2 μm distance, the localization pattern switched to repulsive distribution, probably indicating that metabolic interactions between the species did not influence their localization over larger distances. In addition, if bacteria preferentially cluster, fewer bacteria will be

found at large distances than would be in a random distribution, resulting in negative pair correlation values. Similar to the present results, in a study where interspecies communication in dual-species biofilms of *Streptococcus gordonii* and *Veillonella atypica* was studied, close proximity between these species during biofilm growth was reported (Egland *et al.*, 2004). The present data also agree with those of Diaz *et al.* (2006), showing that *Streptococcus* species were seen in small multispecies clusters of cells in early undisturbed *in situ* plaque samples. However, a difference was observed between single- and dual-species biofilms after exposure to chlorhexidine; cells were randomly or repulsively distributed in single-species biofilms whereas dual-species bacteria were clustered even more than they had been before the chlorhexidine application. This more prominent clustering was observed between live and dead cells as well as *S. mutans* and *V. parvula*. Therefore, it is suggested that bacteria in dual-species biofilm form a specific spatial arrangement different from bacteria in single-species biofilm after exposure to chlorhexidine as a survival strategy. A protective effect of spatial distribution as an adaptive response to antimicrobials in multi-species biofilms was suggested earlier (Leriche *et al.*, 2003). However, in the cited study, evaluation of spatial distribution was carried out subjectively by scanning electron microscopy and the antimicrobial was applied during the biofilm growth for longer periods. In conclusion, besides the metabolic interaction between *S. mutans* and *V. parvula*, enhanced cluster formation in dual-species biofilms could be responsible for increased resistance to chlorhexidine.

Chapter 4

Effect of *Veillonella parvula* on the protein expression by the oral pathogen *Streptococcus mutans*, in a dual-species biofilm

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[*]These two authors contributed equally to the paper

Abstract

Streptococcus mutans is considered the major pathogen involved in dental caries. In previous studies, we found that *S. mutans* and another oral bacterium, *Veillonella parvula* in a biofilm become less susceptible to antimicrobials when grown in each others presence than when grown separately. The aim of the current study was to identify changes in protein expression of *S. mutans* and *V. parvula*, to find a possible cause for this decreased susceptibility. Single and dual-species biofilms of *S. mutans* and/or *V. parvula* were grown on polystyrene for 48 h. Protein extraction and two dimensional difference gel electrophoresis of the three types of biofilms were performed. Gels were analyzed using Decyder 6.5 software package (GE Healthcare). Differential expression of proteins was compared using specialized mathematical and statistical analysis. Significantly differentially expressed proteins were excised from the gels and analyzed with mass spectrometry. We found that 42 out of the 455 proteins detected were significantly differentially expressed in dual-species biofilms compared to single-species biofilms ($p < 0.05$). Of these 42 proteins, sixteen were identified. The expression of proteins responsible for protein synthesis, protein folding and protein breakdown was upregulated in *S. mutans* when grown with *V. parvula*. The majority of these proteins were previously shown to be related to bacterial adaptation to and survival of antimicrobial exposure, and are therefore good candidates to explain the observed increase in resistance.

Introduction

Biofilm is a layer of microorganisms attached to a surface, typically encased in an extracellular matrix (Costerton *et al.*, 1994). Oral biofilm (dental plaque) is one of the most extensively studied biofilms due to easy accessibility and crucial role in etiology of dental caries. Oral biofilm harbors more than 700 bacterial species. These species mutually interact in many ways, such as using metabolic end products that other species produce or communicating by releasing specific molecules.

Streptococci and Veillonellae are members of the resident oral microflora and early colonizers of oral biofilm (Marsh, 2000; Kolenbrander *et al.*, 2002). *Streptococcus mutans* has been the primary pathogen in dental caries etiology in the last decades, mainly due to its strong acidogenic and aciduric properties (Loesche, 1986; van Houte, 1994). *Veillonella parvula* is considered a 'benevolent organism' in relation to dental caries as it metabolizes lactic acid produced by Streptococci into weaker acids such as propionic and acetic acid with a reduced ability to solubilize enamel (Samaranayake, 2002). Such metabolic cooperation among bacteria is shown to be crucial for the establishment of oral biofilms (Bradshaw *et al.*, 1994).

Attempts have been going on in dental research to attain a non-cariogenic oral flora considering that dental caries is a bacterial disease. Antimicrobial strategies are of importance in oral health in preventive and treatment measures. Chlorhexidine is an antimicrobial agent commonly used in the oral cavity. Our group has shown for the first time that both *S. mutans* and *V. parvula* are less susceptible to chlorhexidine in dual-species biofilm compared to single-species biofilms of each bacterium (Kara *et al.*, 2006). Still, the nature of the interaction between *S. mutans* and *V. parvula* responsible for this phenomenon is unknown. Unraveling the interaction between species would lead to a better understanding of the complexity of oral biofilm and allow for a rational approach in prevention and treatment of dental caries.

Proteomic approaches are widely used to investigate the mechanisms involved in acid-tolerant growth of planktonic cells of *S. mutans* (Wilkins *et al.*, 2002; Len *et*

al., 2004a, 2004b; Nakano *et al.*, 2005). Although these studies provide a valuable indication for planktonic cell physiology, *S. mutans* resides in oral ecology in a biofilm state in close association with other bacteria. Protein expression by *S. mutans* grown in biofilms has been of interest in recent years (Luppens & ten Cate, 2005; Rathsam *et al.*, 2005a, 2005b; Welin *et al.*, 2003; Welin *et al.*, 2004). However, interaction of *S. mutans* with other oral biofilm species has never been studied with the help of proteomics, probably mainly due to the lack of adequate techniques of statistical analysis. Statistical analysis programs provided so far are only suitable for finding differences in protein expression patterns between single-species samples. Indeed, the need for improved statistical analysis has been emphasized lately in *Journal of Proteome Research* (Jan 2007 vol: 6) with a call to address the fundamental problems of statistical data analysis that are important to proteomic research (www.ushupo.org).

In proteome research, two dimensional difference gel electrophoresis (2D-DIGE) is used to overcome difficulties with biological and experimental errors associated with the reproducibility of conventional 2D approach (Rathsam *et al.*, 2005b).

Aim of this study was to identify differences in protein expression between single and dual-species biofilms of *S. mutans* and/or *V. parvula* to find a possible explanation for the increased resistance of dual-species biofilms to chlorhexidine compared to single-species biofilms. To this end a new statistical analysis approach was developed.

Materials and methods

Bacterial strains

The bacterial strains investigated were *S. mutans* UA159 (kind gift from Dr. Marquis, University of Rochester Medical Center, Rochester, NY) and *V. parvula* DSM 2008 (DSMZ, Braunschweig, Germany). Freezer stocks (overnight culture + 30% glycerol) of each strain were streaked onto separate blood agar plates (Tryptic Soy Broth; Difco, Sparks, MD, USA; containing 2% glucose and 5% sheep blood) and grown for 48 h (*S. mutans*) or 72 h (*V. parvula*). One colony of each bacterial strain was used to inoculate 15 ml brain heart infusion (BHI; Difco) and grown anaerobically for 16 h (*S. mutans*) or 64 h (*V. parvula*) to obtain the same number of cells [10^9 colony-forming units (CFU) ml^{-1}] for both species.

Biofilm growth

Polystyrene culture plates (Ø 14 cm, Bibby Sterilin Ltd, Stone, Staffordshire, United Kingdom) containing 100 ml of half-strength BHI supplemented with 50 mM lactic acid and 50 mM pH 7.2 PIPES (medium), were inoculated with 2 x 10^6 CFU *S. mutans* and/or 10^7 CFU *V. parvula.* Three types of biofilms (single-species biofilms of *S. mutans*, single-species biofilms of *V. parvula*, and dual-species biofilms of *S. mutans* and *V. parvula*) were grown in separate culture plates for 48 h. The culture media were refreshed at 24 h. Inoculation of bacteria and growth of biofilms were performed at 37°C in an anaerobic atmosphere containing 80% N_2, 10% CO_2, and 10% H_2.

Isolation of Biofilm Cells

After 48 h of growth, culture medium was poured off the polystyrene plates, 15 ml fresh pre-heated medium (37°C) was added, and biofilm cells were removed from the surface by swabbing. Swabs and resulting suspensions were vortexed for 30 s and sampled to quantify biofilm growth. This was done by dilution in cysteine peptone

water (CPW) (8.5 g NaCl, 5 g Bacto yeast extract, 1 g Bacto peptone, 0.5 g cysteine HCl per liter, pH 7.2) and conventional plating on blood agar plates (Tryptic Soy Agar; Difco, Sparks, MD, USA; with 2% glucose and 5% sheep blood added). Colony forming units (CFU) were counted after 2 days. The remaining suspension was centrifuged (15 min, 3939 x g, 37°C), pooled and the pellets were stored at -80°C until further (protein) analysis. pH of the suspensions was measured with a PHM220 lab pH meter (Radiometer, Zoetermeer, the Netherlands). The three types of biofilms were harvested consecutively on the same day in triplicates and this was repeated on three separate days (n=9).

Protein extraction and 2D-DIGE

Cellular protein extraction and 2D-DIGE of the three types of biofilms were done as described previously (Luppens & ten Cate, 2005), with the following modifications: pH range of the 18 cm-IEF-strips was 4-7. Run time was adjusted to the strip length. Preparative 2D gels were prepared separately in the same way as for the DIGE experiment. Coomassie staining (poststaining) was used instead of Cye dyes (prelabelling).

Statistical analysis

Gels were analyzed using Decyder 6.5 software package (GE Healthcare). No filter settings were used, normalized volume data were exported for further statistical analysis and all spots with volumes smaller than 10^4 were discarded.

The fraction of each species in the dual-species biofilms was calculated by minimizing

$$\sum_{i=1}^{n}\left(SV_i^j - \left(\alpha^j \bar{S}_i + \left(1-\alpha^j\right)V_i\right)\right)^2 \text{ over } \alpha$$

i protein identifier

j experiment number

SV_i^j value for the i^{th} protein in the j^{th} dual-species experiment

$\overline{S}_i$ mean value for protein *i* from all *S. mutans* single-species experiments

$\overline{V}_i$ mean value for protein *i* from all *V. parvula* single-species experiments

α^j fraction of *S. mutans* in the j^{th} dual-species experiment

Then, for each spot, the spot volume in the dual-species biofilm was predicted from the values obtained from the single-species experiment and the appropriate α. This predicted value was divided by the measured value. The values from all spots were ranked, and the products of the three rank numbers (from the three repeats) were calculated for each spot. To determine the spots of which the spot volume had changed significantly these rank products were compared to rank products from simulated rankings as described by Breitling *et al.* (1994, 1995). In these simulations the spot volumes are given random ranks. The products of these random ranks are calculated. This is repeated a large number of times. The results of these simulated rank products are compared to the ones obtained from the experiment. If the rank product obtained from the experiment is in the tail of the distribution of the simulated ones it can be assumed that in the dual-species biofilm compared to the single species biofilms a significant change occurred. A significance level of 0.05 was used. With this statistical approach all problems concerning normality of the data and multiple testing are avoided.

Protein identification

Protein spots from the stained 2D gels were cut and digested with trypsin according to the in-gel method of Shevchenko *et al*. (1996). For peptide mass fingerprinting mass spectra were obtained on a TofSpec 2E MALDI (Waters, Manchester, UK) using a solution of α-Cyano-4-hydroxycinnamic acid as the matrix. If needed, MSMS information was acquired with a QTof1 (Waters, Manchester, UK) coupled with a nano LC system (LC Packings Dionex, Sunnyvale, CA, USA) or by using the MALDI MSMS capability of a Q-star XL (Applied Biosystems, Foster City, CA, USA). The

resulting peptide mass lists and MSMS information were used for identification in MASCOT using the MSDB database (http://www.matrixscience.com).

Results and Discussion

In a previous study we found that *S. mutans* and *V. parvula* in a biofilm are less susceptible to chlorhexidine when grown in each others presence than when grown separately (Kara *et al.*, 2006). Since these findings suggest that there are differences in physiology between bacteria grown in single-species biofilm and bacteria grown in dual-species biofilm, we studied their protein expression patterns with the help of two dimensional difference gel electrophoresis (2D-DIGE).

Analysis method

To find the proteins whose expression consistently changed over repeated experiments in dual-species biofilms compared to both types of single-species biofilms, we developed a customized method for the analysis of the 2D-DIGE gels. This was necessary, as the analysis methods developed so far are only suitable for finding differences in protein expression patterns between single-species samples. The developed method consists of four steps. First, the fraction of each bacterial species in the dual-species protein samples is calculated with the help of the data for all spots. This is necessary even when the fraction of each species in the biofilm is known, since the protein extraction efficiency for each species may differ. Then for each separate spot a prediction of the protein abundance is calculated from this fraction and the values of the single-species. After that, this predicted abundance of the protein is divided by the measured abundance in the dual-species biofilm sample. Then, the rank products method by Breitling *et al.* (2004, 2005), a non-parametric test, is used to determine the p-value for each protein. We used this method because the distribution of our data is unknown, and this test does not rely on any assumption on the data distribution. Furthermore, we used the method because it takes multiple testing into

account. In our case we tested the significance of 455 spots, which, at a significance level of 0.05, would lead to 23 falsely discovered significant spots when no correction for multiple testing would be used (see materials and methods section for more details about the method).

Biofilm growth

We have grown *S. mutans* and *V. parvula* separately in single-species biofilm and *S. mutans* together with *V. parvula* in dual-species biofilms for 48 h. All biofilms contained $3x10^8$-10^9 CFU/cm^2. The results are similar to those of our previous study (Kara *et al.*, 2006), where we grew the biofilms in microtiter plate wells, instead of in petri dishes.

Differences between protein expression of single- and dual-species biofilms

Using 2D-DIGE and the analysis method discussed above we found that 42 out of 455 spots were significantly differentially expressed in dual-species biofilms compared to single-species biofilms ($p < 0.05$), even though these cells were in stationary phase which may make it more difficult to detect the differences. Of these 42 spots, the relative expression of 16 spots increased and the expression of 26 spots decreased in dual-species biofilm compared to the weighed sum of the single-species (Fig. 8). Of the 16 upregulated spots, 13 could be identified by mass spectrometry and were produced by *S. mutans* (Table 3). Of the 26 downregulated spots, 3 were identified; 2 from *S. mutans* and 1 from *V. parvula* (Table 3).

Table 3

Proteins differentially expressed in dual-species biofilms of S. mutans *and* V. parvula

Protein name, definition, EC no	Spot ID[a] (oralgen)	Average fold change[b]	Regulation[c]	p-value
Amino acid biosynthesis; Serine family				
glyA (putative serine hydroxymethyltransferase) 2.1.2.1	SMu0990	1.3	up	0.030
Translation; Amino acyl tRNA synthetases and tRNA modification				
thrS (threonyl-tRNA synthetase) 6.1.1.3	SMu1441	1.4	up	0.003
asnS (asparaginyl-tRNA synthetase) 6.1.1.22	SMu1198	1.3	up	0.030
gatB (aspartyl/glutamyl-tRNA amidotransferase subunit B) 6.3.5.-	SMu1657	1.3	up	0.045
gltX (glutamyl-tRNA synthetase) 6.1.1.17	SMu0296	1.3	up	0.048
Translation; Protein synthesis; Ribosomal proteins: synthesis and modification				
rpsA (putative ribosomal protein S1; sequence specific DNA-binding protein) -	SMu1097	1.3	up	0.038
Translation; Protein fate; Degradation of proteins, peptides, and glycopeptides				
clpP (ATP-dependent Clp protease, proteolytic subunit) 3.4.21.92	SMu1524	1.3	up	0.033
Cellular processes; Chaperones				
clp (putative Clp-like ATP-dependent protease, ATP-binding subunit) -	SMu0868	1.3	up	0.016
Purines, pyrimidines, nucleosides, and nucleotides; Nucleotide and nucleoside interconversions				
adk (adenylate kinase) 2.7.4.3	SMu1821	1.4	up	0.009
Transcription; RNA; RNA synthesis				
rpoA (DNA-directed RNA polymerase, alpha subunit) 2.7.7.6	SMu1817	1.3	up	0.035
Energy metabolism; Glycolysis/gluconeogenesis				
gapN (NADP-dependent glyceraldehyde-3-phosphate dehydrogenase) 1.2.1.9	SMu0618	1.3	up	0.038
Energy metabolism; Anaerobic				
pfl (pyruvate formate-lyase) 2.3.1.54	SMu0363	1.4	up	0.009
Energy metabolism; Fermentation				
pta (putative phosphotransacetylase) 2.3.1.8	SMu0952	1.4	up	0.038
adhE (putative alcohol-acetaldehyde dehydrogenase) 1.2.1.10	SMu0131	1.4	down	0.006

(continued on following page)

(Table 3 – continued)

Fatty acid and phospholipid metabolism				
mmdA (methylmalonyl-CoA decarboxylase alpha chain) 4.1.1.41	**	1.4	down	0.003
Transcription; Transcription factors				
nusA (putative transcription factor NusA)	SMu0379	1.4	down	0.009

[a] Based on gene ID from the Oralgen website (http://www.oralgen.lanl.gov/).

[b] Average fold change in dual-species biofilm compared with both single-species biofilms.

[c] Up-regulated proteins were more expressed in dual-species biofilms than in single-species biofilms, down-regulated proteins were less expressed in dual-species biofilms than in single-species biofilms.

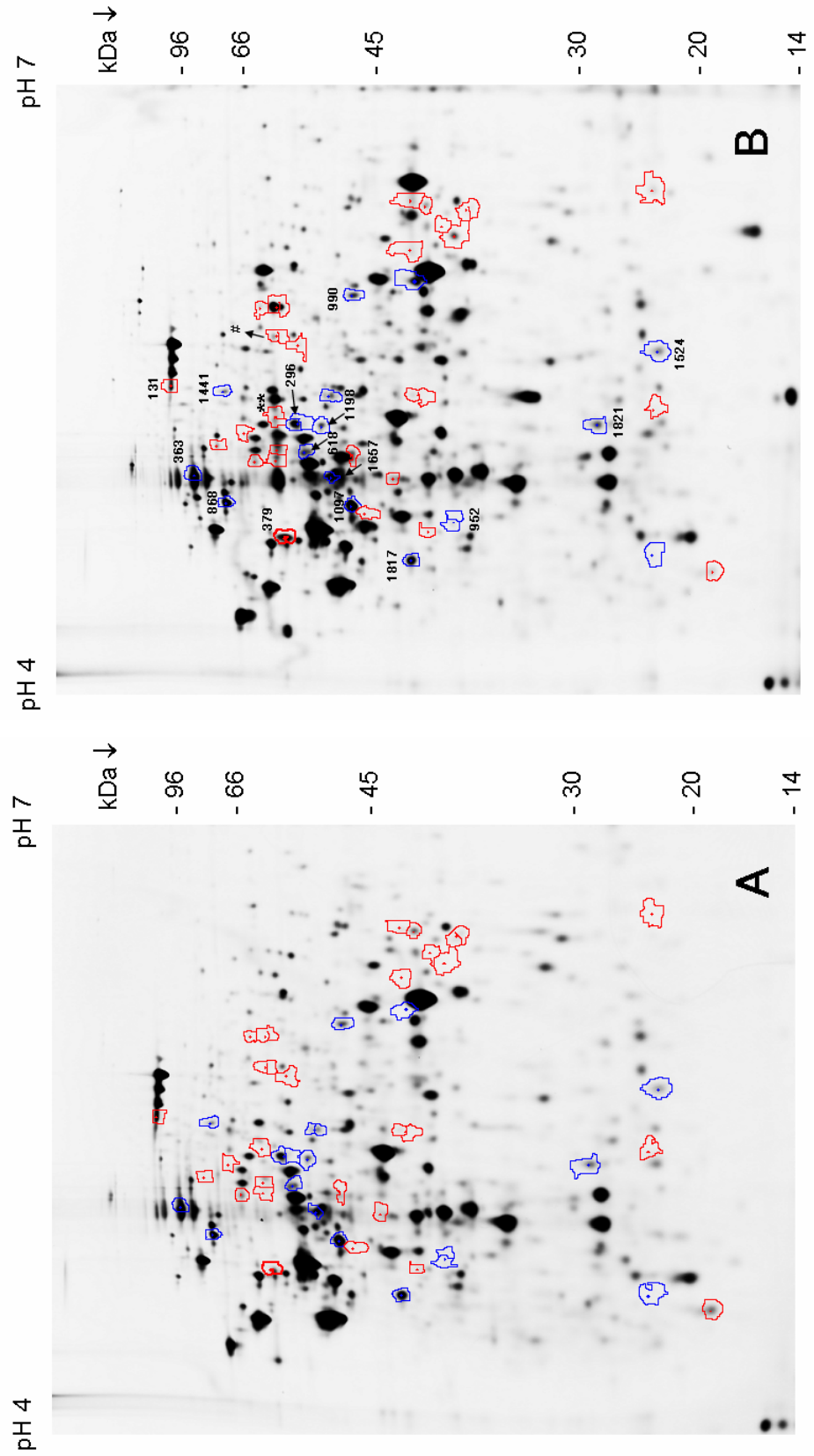
** marks the *V. parvula* protein mmdA.

S. mutans proteins

***S. mutans* amino acid metabolism, protein synthesis and protein processing:** One of the significantly upregulated proteins in *S. mutans* was putative serine hydroxymethyltransferase (**GlyA**) which intermediates the serine-glycine pathway during amino acid biosynthesis.

Six out of the 13 upregulated proteins in *S. mutans* are involved in translation, with functions ranging from protein synthesis to degradation of proteins. In translation, the incorporation of correctly encoded amino acids into proteins depends on the attachment of each amino acid to an appropriate tRNA by aminoacyl tRNA synthases (Cooper, 2000). Biofilm growth in presence of *V. parvula* resulted in the upregulation of threonyl-tRNA synthetase (**ThrS**), asparaginyl-tRNA synthetase (**AsnS**), and glutamyl-tRNA synthetase (**GltX**); as well as subunit B (**GatB**) of aspartyl/glutamyl-tRNA amidotransferase, which is required for the transamidation of misacylated Glu-$tRNA^{Gln}$ to form Gln-$tRNA^{Gln}$ in *S. mutans* (Curnow *et al.*, 1997).

In *S. mutans*, the 50S and 30S ribosomal subunits are composed of 51 proteins (Ajdic *et al.*, 2002). Of these, ribosomal protein **S1** was identified as being upregulated in *S. mutans* in dual-species biofilm in our study indicating an induction in protein synthesis. Protein S1 was also detected in other studies of *S. mutans* in batch cultures, at significant levels at pH 7.0 (Wilkins *et al.*, 2002; Len *et al.*, 2004a) and in biofilms (Welin *et al.*, 2004; Svensater *et al.*, 2001). Two additional *S. mutans* ribosomal proteins were identified (SMu0869 and SMu1847) in our study as upregulated (both 1.2 fold), though the differences were not statistically significant ($p = 0.19$ and $p = 0.09$, respectively). It is possible that the expression of other ribosomal proteins was also upregulated. However, we were not able to detect these proteins since their molecular weight is too small or their isoelectric point is too high to be present in our gels.


pH 7
kDa
- 96
- 66
- 45
- 30
- 20
- 14
pH 4
A
B
131
1441
363
868
879
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1817
990
962
1524
1821

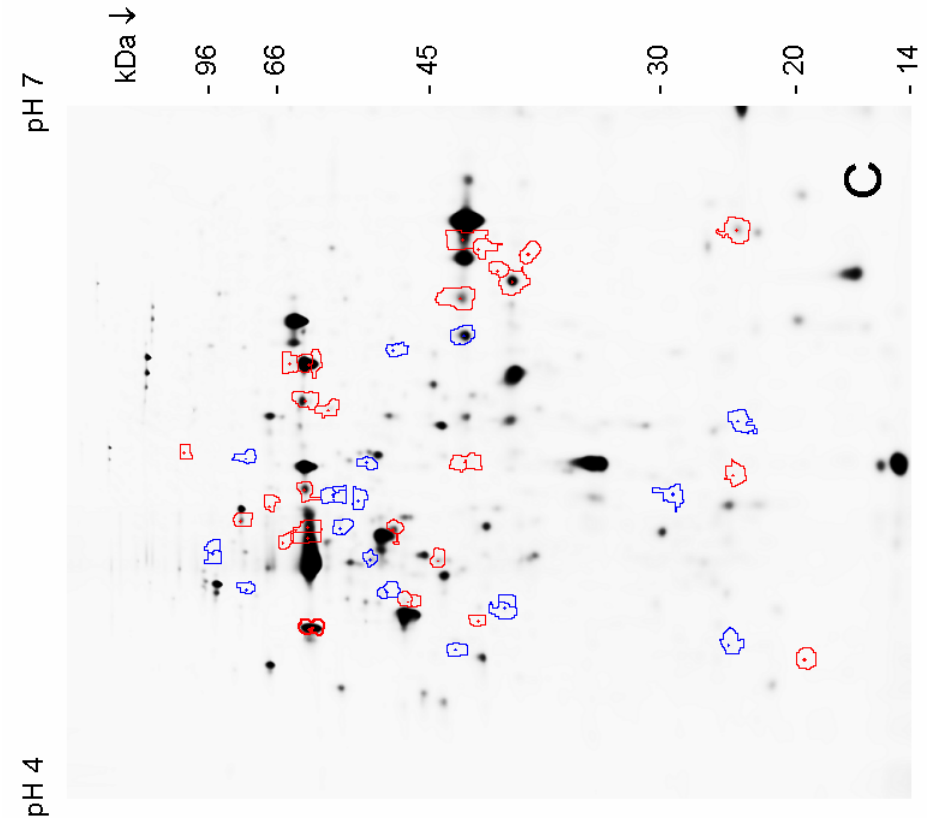

Fig. 8. Protein profile of
(A) single species biofilms of *S. mutans*,
(B) dual-species biofilms of *S. mutans* and *V. parvula*,
(C) single-species biofilms of *V. parvula.*
Protein spots with blue borders are upregulated in dual-species biofilms
(16 protein spots);
protein spots with red borders are downregulated in dual-species biofilms
(26 protein spots)
compared to single-species biofilms.
Identified proteins are marked with Oralgen ID number
(http://www.oralgen.lanl.gov.tr).
** is the identified protein of *V. parvula* (mmdA).
# is the most significantly downregulated protein in dual-species biofilms
(displayed in Fig. 9 in three-dimensional view).

Chaperones catalyze the correct folding of other proteins into their correct 3D structure within the cell (Ellis & Hartl, 2006). A chaperone, putative Clp-like ATP dependent protease, ATP-binding subunit (**Clp)** of *S. mutans* was upregulated according to our results in dual-species biofilms. Proteins which cannot be folded by molecular chaperones may be targeted for degradation, in order to recycle amino acids for *de novo* protein synthesis (Jenal & Hengge-Aronis, 2003). Among the proteins that can carry out such functions are ATP-dependent proteases of the Clp family, which possess a dual chaperone/protease role. Proteolysis by Clp requires a serine type peptidase, the **ClpP** subunit (Lemos & Burne, 2002) which is also upregulated in *S. mutans* in our dual-species biofilms.

***S. mutans* nucleotide and RNA synthesis:** Adenylate kinase, (**Adk)** which we found to be enhanced in *S. mutans* in dual-species biofilms is associated with purine nucleotide biosynthesis and is one of eight enzymes in the salvage pathway that interconverts ATP to GTP (Rathsam *et al.*, 2005b). Similarly, Adk was reported to be enhanced in *S. mutans* biofilm cells compared to their planktonic counterparts (Svensater *et al.*, 2001).

Growth of *S. mutans* in a biofilm in the presence of *V. parvula* led to upregulation of the alpha subunit of DNA-directed RNA polymerase, alpha subunit (**RpoA**). It can be suggested that proximity of *V. parvula* in a biofilm forms a stress condition for *S. mutans* and induces responses needed for the survival of the cell which requires the transcription of appropriate gene. RpoA was previously found to be upregulated in *S. mutans* biofilms compared to planktonic phase bacteria (Svensater *et al.*, 2001; Welin *et al.*, 2004; Rathsam *et al.*, 2005a) and acid stressed planktonic cells of *S. mutans* (Len *et al.*, 2004a; Nakano *et al.*, 2005).

In contrast, transcription factor, **NusA** was significantly downregulated in *S. mutans* cells in dual-species biofilm. NusA enables RNA polymerase in *Escherichia coli* to recognize a characteristic class of termination sites (Berg *et al.*, 2002). It binds to the transcription complex and modulates the rate of transcript elongation and the recognition of termination signals. NusA enhances pausing, which may be important

for keeping the elongation of the polymerase synchronous with the translation of the nascent mRNA (Richardson, 2001).

Interestingly, both downregulated proteins of *S. mutans* in dual-species biofilm in our study; AdhE and NusA were shown to be related to Mutacin I production in *S. mutans*. Dysfunctions of these factors have been reported to eliminate the production of Mutacin I mainly as a part of the global physiological stress rather than a specific phenomenon (Tsang *et al.*, 2005).

***S. mutans* energy metabolism:** NADP-dependent glyceraldehydes-3-phosphate dehydrogenase (**GapN**), pyruvate formate-lyase (**Pfl**) and phosphotransacetylase (**Pta**) are *S. mutans* proteins involved in carbon metabolism (Carlsson *et al.*, 1985) which were upregulated in dual-species biofilm in this study. GapN intermediates a pathway for the pyruvate production generated from glycolysis. Under anaerobic conditions and limited glucose, pyruvate is then converted in *S. mutans* by Pfl to a variety of end-products, including acetate and formate or ethanol; through the pyruvate formate-lyase pathway (Carlsson & Griffith, 1974; Yamada & Carlsson, 1975). In one branch of this pathway, Pta together with Pfl catalyzes acetate production (Rathsam *et al.*, 2005a). However, in another branch, putative alcohol-acetaldehyde dehydrogenase (**AdhE**) of *S. mutans*, which is involved in ethanol production, is downregulated in our study in dual-species biofilm. Therefore we suggest that *S. mutans* enhances the energy metabolism in favor of acetate while diminishing ethanol production in the presence of *V. parvula* when grown in a biofilm. This assumption is indeed confirmed by our previous findings showing that acetate production by dual-species biofilms at 48 h was significantly higher (28.1 ± 8.6 mM) compared to that by single-species biofilms of *S. mutans* and of *V. parvula* (2.9 ± 1.1 mM and 15.4 ± 6.0 mM, respectively) (Kara *et al.*, 2006).

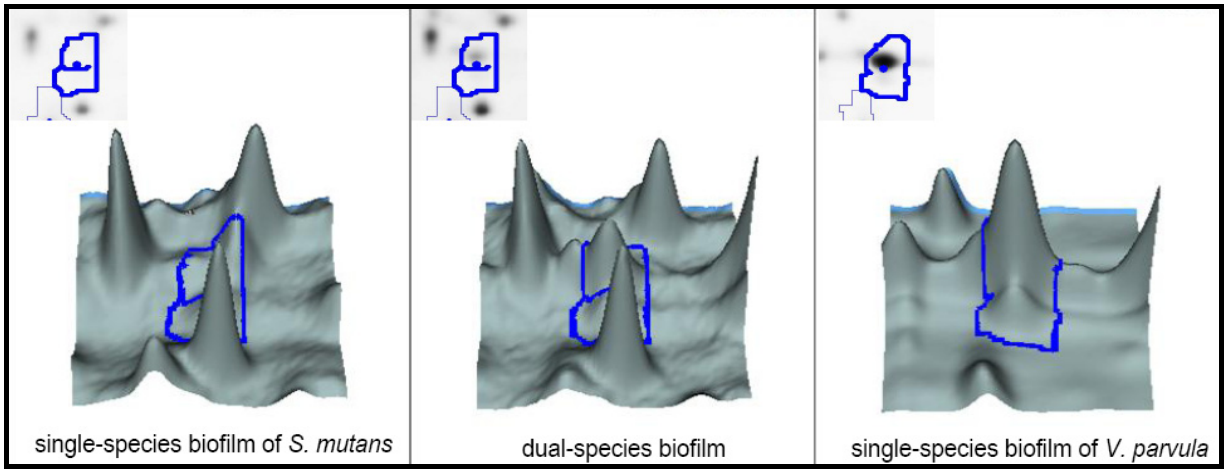

Fig. 9. Three-dimensional view of the most significantly downregulated dual-species biofilm protein (average fold change: 1.81; p = 0.0001)

V. parvula proteins

Of 26 significantly downregulated proteins in dual-species biofilm cells of *S. mutans* and *V. parvula*, we were able to identify 3 proteins. This was in part due to the difficulty of spot detection and absence of signals in mass spectrometry analysis as a result of low abundance of these proteins.

Other spots had good spectra but no matches to proteins in the databases. A reason for this could be that most of the significantly downregulated proteins in dual-species biofilm were produced by *V. parvula*. The genome of *V. parvula* has not been sequenced, which makes protein identification very difficult. Fifteen of the 23 unidentified down-regulated proteins had good spectra and the three-dimensional views of the protein spots in the three types of biofilms were similar to that of the protein spot shown in Fig. 9. Figure 9 shows the most significantly downregulated protein in dual-species biofilm cells (average fold change 1.81; p = 0.0001). What can be seen is that the protein is not present in *S. mutans* single-species biofilm but is present in *V. parvula* single-species biofilm and in dual-species biofilm. Together with a good spectrum without a match, this pattern is a strong indication that the protein is produced by *V. parvula*.

In this study, we also report the *V. parvula* 2D-DIGE protein map for the first time according to our knowledge (Fig. 8C). The identified *V. parvula* protein, which was significantly downregulated in dual-species biofilm, was the alpha chain of methylmalonyl-CoA decarboxylase (**MmdA**) (indicated with a ** in Table 3 and Fig. 8). Methylmalonyl-CoA decarboxylase is an enzyme in the fermentation pathway of lactate to propionate in *V. parvula* (Dimroth, 1987) which acts also as a sodium ion pump (Huder & Dimroth, 1993). In this way, the free energy yield of methylmalonyl-CoA decarboxylation in *V. parvula* is conserved by conversion into an electrochemical gradient of Na^+ ions (Hilpert & Dimroth; 1982).

General remarks

In this study we show that it is possible to determine which proteins are upregulated in a bacterium that is grown in the presence of another bacterial species when the right analysis method is used. Furthermore, we show that in the presence of *V. parvula*, the physiology of *S. mutans* grown in a biofilm changes. The expression of proteins involved in *S. mutans* energy metabolism, nucleotide and RNA synthesis enhances. The most striking difference is the upregulation of proteins involved in protein metabolism, which will give *S. mutans* an increased capacity for protein synthesis, a better ability to fold, process and stabilize proteins and an increased capacity to degrade dysfunctional proteins. In short: a more dynamic protein machinery.

Indeed, studies on *S. mutans* and related Gram-positive bacteria have shown that a more dynamic protein machinery helps to survive different forms of stress: In *Streptococcus pneumaniae* and *Bacillus subtilis* the relative transcript levels of the majority of ribosomal proteins increase after exposure to sublethal levels of various antibiotics (Dandliker *et al.*, 2003; Ng *et al.*, 2003). In planktonic cells of *S. mutans*, deletion of ClpP results in increased susceptibility to chlorhexidine and hydrogen peroxide (Deng *et al.*, 2006). Planktonic cells of *S. mutans* grown under acidic stress conditions (pH 5.0) show a similar response as *S. mutans* grown in the presence of *V. parvula*. Eight of 13 upregulated proteins of *S. mutans* in our current study,

translational proteins ThrS, GatB, RpsA, a chaperone Clp, transcriptional factor RpoA and energy metabolism factors GapN, Pfl and Pta, have previously been identified as being elevated during acid-tolerant growth in planktonic cells of *S. mutans* in these studies (Len *et al.*, 2004a; Nakano *et al.*, 2005).

Collectively the above evidence indicates that presence of *V. parvula*, acid exposure or sublethal concentrations of antimicrobials all induce a similar response, namely, a more dynamic protein synthesis and protein turnover machinery. With a more dynamic protein machinery, cells exposed to the antimicrobials will be able to react more quickly in synthesizing new proteins necessary for repair of the damage caused by the antimicrobials. Our hypothesis is that due to this response, *S. mutans* has an increased capacity to survive antimicrobial exposure when it grows together with *V. parvula*. This explanation for increased capacity of *S. mutans* to survive antimicrobial exposure in the presence of *V. parvula* needs to be confirmed with the help of physiological experiments in future studies.

In conclusion, growing in a biofilm together with *V. parvula* gives the dental pathogen *S. mutans* an advantage in surviving antimicrobial treatment, which (in part) is due to upregulation of the same proteins that are necessary to cope with different types of stress in *S. mutans* and other Gram-positives. More generically, we postulate that the presence of other (non-pathogenic) bacteria changes the phenotype of a pathogen and can concomitantly increase the resistance of oral biofilms and particularly inhabiting pathogens. This is an important finding to consider in the study of pathogen physiology, antimicrobial or antibiotic susceptibility testing of disease-causing bacteria and dental caries treatment.

Chapter 5

Effect of *Veillonella parvula* on the antimicrobial resistance and gene expression of *Streptococcus mutans* grown in a dual-species biofilm

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Luppens SBI[*], Kara D[*], Bandounas L, Jonker MJ, Wittink FRA, Bruning O, Breit TM, ten Cate JM, Crielaard W: The effect of *Veillonella parvula* on antimicrobial resistance and gene expression of *Streptococcus mutans* grown in dual-species biofilms
[*]These two authors contributed equally to the paper

Abstract

Introduction: Our previous studies showed that *Streptococcus mutans* and *Veillonella parvula* dual-species biofilms have a different acid production profile and a higher resistance to chlorhexidine than their single-species counterparts. The aim of the current study was to test whether the susceptibility of *S. mutans* grown in the presence of *V. parvula* is also decreased when it is exposed to various other antimicrobials. Furthermore, the aim was to identify other changes in the physiology of *S. mutans* when *V. parvula* was present, by transcriptomics. Methods: Susceptibility to antimicrobials was assessed by killing experiments. Transcript levels in *S. mutans* were measured with the help of *S. mutans* microarrays. Results: When *V. parvula* was present *S. mutans* showed an increase in survival after exposure to various antimicrobials. Furthermore, this co-existence altered the physiology of *S. mutans*; the expression of genes coding for proteins involved in amino acid synthesis, the SRP-translocation pathway, purine metabolism, intracellular polysaccharide synthesis and protein synthesis changed. Conclusion: Growing in a biofilm together with a non-pathogenic bacterium like *V. parvula* changes the physiology of *S. mutans*, and gives this bacterium an advantage in surviving antimicrobial treatment.

Thus, the study of pathogens implicated in polymicrobial diseases, such as caries and periodontitis, should be focused more on multi-species biofilms. In addition, the testing of susceptibility to currently used and new antimicrobials should be done on a multi-species microbial community rather than with single pathogens.

Introduction

Streptococcus mutans is considered as one of the primary cariogenic pathogens present in dental plaque. *S. mutans* has been studied extensively as a monoculture grown in suspension. In recent years, the majority of the studies on *S. mutans* have focused on monoculture biofilms, because *S. mutans*' natural habitat, dental plaque, is a biofilm. Dental plaque is a multi-species community from which hundreds of species have been isolated. Therefore, instead of studying monocultures, it would be more realistic to study the properties of *S. mutans* grown in the presence of other bacteria, especially because other studies have shown that the presence of another bacterium may influence the gene expression, virulence and other properties a bacterium (Duan *et al*., 2003; Egland *et al*., 2004; Ruiz-Perez *et al*., 2004).

A method that is frequently used to obtain an overview of the changes in physiology in bacteria after a certain stimulus is transcriptional profiling with the help of microarrays. Microarrays have been used to study the difference in gene expression between *S. mutans* grown in suspension and in biofilm (Shemesh *et al*., 2007) changes in gene expression in mutant strains (Merritt *et al*., 2005; Wen *et al*., 2006; Hasona *et al*., 2007) and for comparative genome hybridization of *S. mutans* strains (Waterhouse *et al*., 2007).

Up to now, *S. mutans* microarrays have not been used to study physiological changes in response to the presence of another bacterium. Some reasons for this could be that it is difficult to grow two species with different nutrient requirements together, that the results of the dual-species analyses have to be compared with those of the two single species which triples the amount of samples and that it needs to be verified that the sample of the one species does not bind to the microarray of the other species.

Veillonella parvula lives in the same complex multi-species plaque community as *S. mutans*. It can not ferment glucose and most other sugars (Distler & Kroncke, 1981; Krieg & Holt, 1986), but it metabolizes lactic acid, a waste product of *S. mutans*, and converts it into weaker acids, such as acetic and propionic acid which have a

reduced ability to solubilize enamel (Samaranayake, 2002). For this reason, it is considered a benevolent plaque organism.

Previously, we studied the effect of *V. parvula* on *S. mutans* grown in a biofilm. *V. parvula* influenced *S. mutans*: In the dual-species biofilm with *V. parvula* the acid production profile changed. Furthermore, both *V. parvula* and *S. mutans* grown in dual-species biofilm showed an increased survival (Kara *et al*., 2006, 2007) and were able to regrow faster than bacteria grown in single-species biofilms after exposure to the antimicrobial chlorhexidine (Kara *et al*., 2006). Therefore, we decided to study the interaction between these two plaque bacteria in more detail.

The first aim of the current study was to determine whether *V. parvula* and *S. mutans* grown together in dual-species biofilm are more resistant to other antimicrobials with a mode of action related to chlorhexidine and antimicrobials with a mode of action completely different from that of chlorhexidine. For this purpose the survival of bacteria in dual-species biofilm was compared to the survival of bacteria in single-species biofilm after exposure to five antimicrobials with different modes of action (Block, 2001; Schlunzen *et al*., 2001; Decker *et al*., 2003): cetylpyridinium chloride (CPC), zinc chloride, erythromycin, hydrogen peroxide and amine chloride (the chloride salt of Olaflur). The second aim was to determine what other changes in *S. mutans*' physiology occur in response to the presence of *V. parvula* by transcriptional profiling with the help of microarrays.

Methods

Bacterial strains and growth conditions

48 h single-species *S. mutans* UA159 biofilms, single-species *V. parvula* DSM 2008 (DSMZ) biofilms, and dual-species biofilms with both *S. mutans* and *V. parvula* were grown as described previously (Kara *et al*., 2006) on the bottom of 96 well polystyrene microtiter plates (Kara *et al*., 2006) (antimicrobial treatment) or on the bottom of

polystyrene culture plates (Luppens & ten Cate, 2005) (RNA isolation). Medium was refreshed after 24 h.

Antimicrobial treatment

The following antimicrobials were used: amine chloride (bis(hydroxyethyl)-aminopropyl-N-hydroxyethyl-octadecylamine dihydro-chloride; GABA International; 0.014 %), zinc chloride (0.1 mmol l^{-1}) and CPC (VWR International; 0.2 mmol l^{-1}), erythromycin (Sigma-Aldrich; 0.015 g l^{-1}) and hydrogen peroxide (Merck; 2.5 %). Killing experiments were done in triplicate as described previously ('neutralized killing') (Kara *et al*., 2006), with the following exceptions: Biofilms were exposed to antimicrobial for 5 min instead of 10 min and after exposure to amine chloride, zinc chloride and erythromycin cysteine peptone water (Kara *et al*., 2006) was used as a neutralizer. Percentage survival was calculated by dividing the viable counts of a treated sample by the average of the viable counts of the control samples. To test the hypothesis that the survival of *S. mutans* or *V. parvula* was not influenced when they co-existed with each other, we compared survival percentage of a species grown in single-species biofilms to the same species' survival percentage in dual-species biofilms. For this purpose a permutation test was used (Sokal & Rohlf, 1995) ($\alpha \leq 0.05$). We used this non-parametric test because normality could not be assumed.

RNA Isolation

48 h biofilms were harvested as follows: the medium was poured off the polystyrene plates, 15 ml of fresh warmed (37°C) medium was added and the biofilm cells were removed from the surface by swabbing. The resulting suspension was centrifuged once (15 min, 3939 × g, 37°C) and the liquid was removed. The pellets were resuspended in 1 ml RNAPro solution (FastRNA Pro Blue Kit, Q-Biogen), snap frozen in liquid nitrogen and stored at -80 °C until further use. Total RNA was extracted by bead beating and the subsequent steps described in the protocol of the FastRNA kit, and a

subsequent step using Trizol (Invitrogen) followed by a DNase treatment with RNase-Free DNase. Isolated total RNA was further purified using the Rneasy Mini kit (Qiagen). Quantification of total RNA was performed in a NanoDrop ND-100 UV/VIS spectrometer (Nanodrop Technologies). Validation of RNA quality was performed using the RNA 6000 NanoAssay on an Agilent 2100 BioAnalyzer (Agilent Technologies) and recording the rRNA ratios (28S/18S) and the RNA Integrity Number (RIN). For each biofilm type, five biological replicates were used to isolate total RNA and hybridize with microarrays.

Microarrays, target preparation and hybridization

Microarrays specific for *S. mutans* were obtained from the Pathogen Functional Resource Centre (TIGR). The libraries represent in total 1948 oligos and 500 controls printed in four fold. The oligonucelotide library was printed with a Lucidea Spotter (Amersham Pharmacia Biosciences) on commercial Epoxy slides (Scott Nexterion) and processed according to the manufacturer's instructions. Total RNA samples were hybridized, according to a common reference design without dye swap, with a pool of test samples as common reference (containing equal amounts of dual-species RNA, *S. mutans* single-species RNA and *V. parvula* single-species RNA). In a common reference design the fluorescence intensity of the experimental sample is compared to that of the common reference on the same slide, which makes the accurate comparison between the slides possible. From the total RNA samples with RIN-value >7, 15 µg (single-species samples) or 30 µg (dual-species samples and common reference sample) was labeled using a random primed procedure with SuperScript II Reverse Transcriptase (Invitrogen), dCTP Cy5 (experimental samples) and dCTP Cy3 (common reference) according to the manufacturer's instructions. The reason for the use of a double amount (30 µg) of RNA sample for the dual-species samples was that we assumed that only half of the RNA from these samples would bind to the slide, because *V. parvula* RNA hardly hybridized to the *S. mutans* microarray slide (see below) and because we assumed that approximately half of each dual-species sample

consisted of *S. mutans* mRNA. This last assumption was based on microscopic cell counts and confirmed by the fact that there were no major differences in overall intensity levels between the slides hybridized with dual-species samples and *S. mutans* single-species samples. We normalized for the modest difference in overall intensity between dual-species slides and *S. mutans* single-species slides using lowess-normalization (see below). Labeled cDNA was assessed for the amount of incorporated label with the NanoDrop ND-100 UV/VIS spectrometer. The microarrays were hybridized overnight with 200 µl hybridization mixture, consisting of 50 µl Cy3- and Cy5-labeled cDNA, 100 µl Formamide and 50 µl 4 x MicroArray Hybridization Buffer (Amersham Pharmacia Biosciences) at 37°C and washed in an Automated Slide Processor (Amersham Pharmacia Biosciences), and subsequently scanned in Agilent DNA MicroArray Scanner (Agilent Technologies).

Data Analysis and Statistics

Microarray spot intensities were quantified using Feature Extraction software (version 8.5). Data were processed further using R (version 2.2.1 (R Development Core Team, 2006) and Bioconductor (http://www.bioconductor.org) MAANOVA package (version 0.98.8). All slides were subjected to quality control checks. Quality checks included visual inspection of the scans, examining the consistency among the replicated samples by principal components analysis, testing against criteria for signal to noise ratios, for consistent performance of the labeling dyes, consistent pen performance, and visual inspection of pre- and post-normalized data with box plots and RI plots. The quality checks revealed that the RNA from *V. parvula* samples only resulted in background expression values caused by non-specific binding. Further analysis was therefore focused on the difference between samples from *S. mutans* biofilms and *S. mutans* + *V. parvula* biofilms. After log2 transformation, the data were normalized using a spatial lowess smoothing procedure and statistically analyzed using a two stage mixed ANOVA model (Kerr *et al*., 2000; Wolfinger *et al*., 2001). First, array, dye and array-by-dye effects were modeled globally. Subsequently, the residuals from this first

model were fed into the gene-specific model to fit biofilm and spot effects on a gene-by-gene basis using a mixed model ANOVA. These residuals are reported as expression values. To test the hypothesis that *S. mutans* genes were not influenced when *S. mutans* co-exists with *V. parvula*, a permutation based F1 test was applied, which allows relaxing the assumption that the transcript data is normally distributed (Cui & Churchill, 2003). To account for multiple testing, p-values from the permutation procedure were adjusted to represent a false discovery rate (FDR) of 5% (Benjamini & Hochberg, 1995). An overview of pathways (and protein complexes) that contained one or more of the differentially expressed genes was constructed with the help of database mining and gene ontology. The pathways complexes were related to differences in gene expression through gene set enrichment analysis on the F1-statistics (Mootha *et al*., 2003), using the gene-set-test facility in the bioconductor package Limma. Only the pathways that showed a significant change are mentioned in Results section. Microarray data have been deposited in the ArrayExpress database (http://www.ebi.ac.uk/aerep) with experiment accession number E-MEXP-1034.

Results

S. mutans UA159 and *V. parvula* were grown separately in single-species biofilm and together in dual-species biofilms for 48 h. All biofilms contained $3x10^8$-10^9 CFU cm^{-2}. In a previous study with *S. mutans* C180-2, we exposed these three types of biofilms to chlorhexidine, and found that the bacteria in the dual-species biofilms survived better than the bacteria in single-species biofilm (Kara *et al*., 2006). Similar results were found for *S. mutans* UA159 exposed to chlorhexidine (data not shown). In the present study the bacteria in the single-species biofilms and dual-species biofilms were exposed to five other antimicrobials with different modes of action.

Fig. 10 shows that *V. parvula* survived erythromycin treatment better when *S. mutans* was present. Results for survival of *V. parvula* after exposure to hydrogen peroxide are not reliable, since the counts were below the detection limit, and are therefore not shown. The average percentage of *S. mutans* that survived antimicrobial treatment was higher in dual-species biofilm than in single-species biofilm ($p = 0.05$) for all five antimicrobials used. Thus, *S. mutans* growing in these dual-species biofilms was better able to survive exposure to in total six antimicrobials than *S. mutans* growing in single-species biofilm.

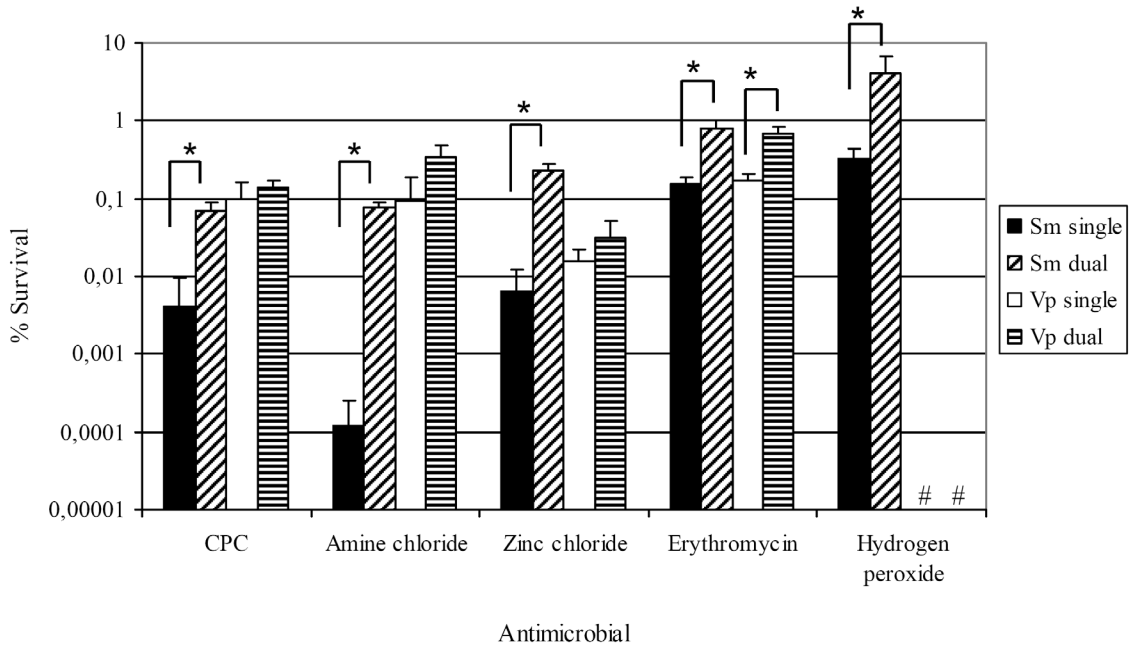


Fig. 10: Percentage survival of single- and dual-species biofilm bacteria after exposure to 0.2 mmol l^{-1} CPC, 0.014 % amine chloride, 0.1 mmol l^{-1} zinc chloride, 0.015 g l^{-1} erythromycin and 2.5% hydrogen peroxide. Sm single: *S. mutans* in single-species biofilm; Sm dual: *S. mutans* in dual-species biofilm; Vp single: *V. parvula* in single-species biofilm; Vp dual, *V. parvula* in dual-species biofilm. 100% survival values (average ± stdev) are $2.5\pm0.93\bullet10^8$ (Sm single), $2.8\pm0.55\bullet10^8$ (Sm dual), $3.9\pm2.2\bullet10^8$ (Vp single) and $4.1\pm1.3\bullet10^8$ CFU/well (Vp dual). *Significant difference between single and dual-species biofilm viable counts of the same bacterium ($p = 0.05$). #Value below the detection limit (Vp single: 0.08%, Vp dual: 0.11%). Error bars represent the standard deviation.

These findings suggested that there are differences in physiology between *S. mutans* grown in single-species biofilm and *S. mutans* grown in dual-species biofilm together with *V. parvula*. To gain more insight in these differences we compared, with the help of *S. mutans* microarrays, the transcript levels in *S. mutans* grown alone directly to those of *S. mutans* grown together with *V. parvula*. This direct comparison was possible because hybridization of the *V. parvula* samples to the *S. mutans* microarrays only resulted in background expression values caused by non-specific binding (Fig. 11). This figure shows that the distribution of the spot fluorescence intensities of *S. mutans* microarrays hybridized with a *V. parvula* sample was similar to that of spots on the microarrays that did not contain oligonucleotides (empty spots).

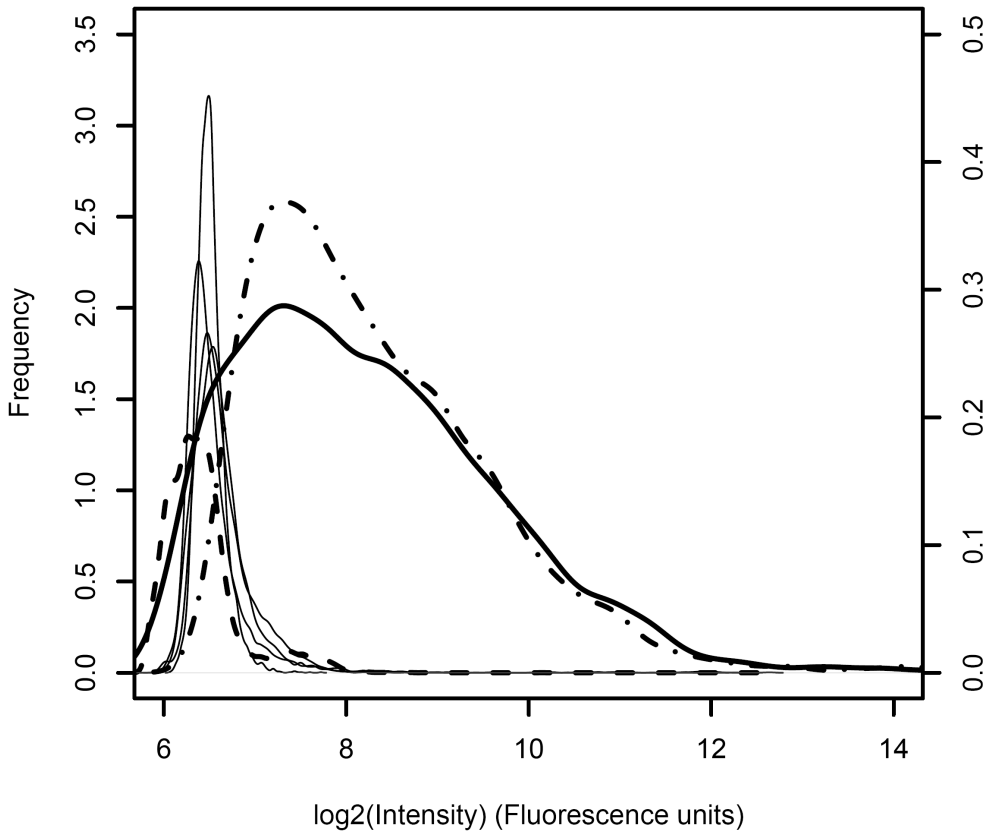


Fig. 11: Frequency plots of the intensities of the spots from an arbitrary microarray hybridized with *S. mutans* sample (black thick line, right y-axis), from an arbitrary microarray hybridized with *S. mutans* and *V. parvula* dual-species sample (dashed-dotted line, right y-axis) from empty control spots on the microarrays (dashed line, left y-axis), and from the spots from microarrays hybridized with *V. parvula* sample (black thin lines, left y-axis). Empty spots are spots that deliberately do not contain oligonucleotides. The x-axis indicates the log2 of the Cy5 fluorescence signal and the y-axes indicates the relative concentration of spots with the corresponding fluorescence intensity.

Table 4

Overview of gene transcripts upregulated in dual-species biofilms §

locus #	definition *	gene name	fold change	p-value
Translation; Ribosomal proteins: Synthesis and modification				
SMU.2017c	50S ribosomal protein L14	*rplN*	2.2	0.041
SMU.2018c	30S ribosomal protein S17	*rpsQ*	2	0.033
SMU.2019c	50S ribosomal protein L29	*rpmC*	1.9	0.045
SMU.2023c	30S ribosomal protein S19	*rpsS*	2.8	0.034
SMU.2024c	50S ribosomal protein L4	*rplD*	2.9	0.041
SMU.2025c	50S ribosomal protein L3	*rplC*	2.9	0.041
SMU.2026c	30S ribosomal protein S10	*rpsJ*	2.8	0.030
Cellular processes; Protein and peptide secretion				
SMU.91	peptidyl-prolyl isomerase, trigger factor	*ropA*	1.3	0.046
Purines, pyrimidines, nucleosides, and nucleotides; Purine ribonucleotide biosynthesis				
SMU.35	putative phosphoribosylglycinamide formyltransferase	*purN*	1.6	0.046
Unassigned				
SMU.641	oxidoreductase	*qor*	2.3	0.034
SMU.679	oxidoreductase, aldo/keto reductase family	*ycgG*	1.4	0.046
Unknown				
SMU.543	conserved hypothetical protein	-	1.4	0.027
Hypothetical				
SMU.33	hypothetical protein	-	1.7	0.048
SMU.642	hypothetical protein	-	2.3	0.034

§ Gene transcripts in *S. mutans* grown together with *V. parvula* were compared to gene transcripts of *S. mutans* grown in single-species biofilms (fold-change ≥ 1.3, $p < 0.05$).

Genbank locus tag

* http://www.oralgen.lanl.gov/

Table 5

Overview of gene transcripts downregulated in dual-species biofilms (§)

locus #	definition	gene name	fold change	p-value
Amino acid biosynthesis; Aspartate family				
SMU.70	threonine synthase	*thrC*	6.1	0.017
Amino acid biosynthesis; Glutamate family				
SMU.913	NADP-specific glutamate dehydrogenase	*gdhA*	4	0.042
Protein synthesis; tRNA aminoacylation				
SMU.158	cysteinyl-tRNA synthetase	*cysS*	4.2	0.030
SMU.773c	lysyl-tRNA synthetase	*lysS*	4.3	0.032
SMU.1311c	asparaginyl-tRNA synthetase	*asnS*	3.7	0.035
Protein synthesis; Protein modification				
SMU.466	cysteine aminopeptidase C	*pepC*	6.7	0.029
Protein synthesis; Translation factors				
SMU.608	peptide chain release factor 3	*prfC*	2	0.034
Protein fate; Protein and peptide secretion and trafficking				
SMU.744	cell division protein; signal recognition particle	*ftsY*	2.1	0.030
Transport and binding proteins; ABC Superfamily: ATP-binding protein				
SMU.803c	ABC transporter, ATP-binding protein	*ykhF*	2.1	0.041
SMU.1120c	sugar ABC transporter, ATP-binding protein	*psaA*	6.6	0.047
Energy metabolism; Sugars				
SMU.1538c	glucose-1-phosphate adenylyltransferase	*glgC*	1.6	0.046
SMU.1565c	4-alpha-glucanotransferase	*malM*	7.1	0.013
Energy metabolism; Fermentation				
SMU.1021	citrate lyase alpha chain	*citF*	3.4	0.032
Cellular processes; Cell division				
SMU.1003	glucose-inhibited division protein	*gidA*	6.6	0.020
Cellular processes; Adaptations to atypical conditions				
SMU.1060c	signal recognition particle	*ffh*	6.3	0.039
Cellular processes; Toxin production and resistance,Transport and binding proteins; Other				
SMU.71	MATE efflux family protein	-	3.5	0.046
Unassigned				
SMU.1693c	uncharacterized hemolysin	-	4.1	0.046
Unknown				
SMU.575c	conserved hypothetical protein	-	5.3	0.045
SMU.862	conserved hypothetical protein	-	2.4	0.034

§ Gene transcripts in *S. mutans* grown together with *V. parvula* were compared to gene transcripts of *S. mutans* grown in single-species biofilms (fold-change ≥ 1.3, $p < 0.05$). # Genbank locus tag

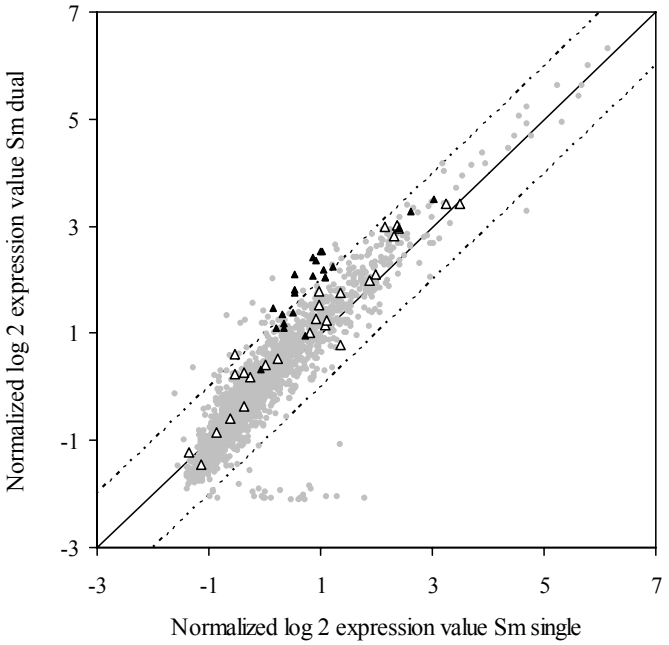
Analysis showed that in *S. mutans* the transcript levels of 15 genes were significantly ($p < 0.05$) higher in the presence of *V. parvula* than in the absence of *V. parvula* and 19 were lower (Table 4 and 5). Out of the 15 upregulated genes, seven were encoding ribosomal proteins and one was encoding trigger factor (*ropA*, SMU.91), a ribosome associated chaperone (Hesterkamp *et al.*, 1996; Kramer *et al*., 2002). The transcript levels of an additional 4 genes encoding ribosomal proteins were higher in *S. mutans* grown in dual-species biofilm than *S. mutans* grown in single-species biofilm at a lower significance level ($p < 0.10$). Gene set enrichment analysis showed that one specific group of 23 genes encoding for ribosomal proteins was upregulated ($p = 2\times10^{-5}$, data not shown). Furthermore, practically all (47 out of 51) ribosomal protein gene transcript levels were higher in *S. mutans* grown in dual-species biofilm than in *S. mutans* grown in single-species biofilm (data not shown).

Another upregulated gene codes for PurN (SMU.35), which is involved in purine synthesis. Gene set enrichment analysis showed that the expression of the genes of the entire purine synthesis pathway (SMU.29, SMU.30, SMU.32, SMU.34, SMU.35, SMU.37, SMU.48, SMU.50, SMU.51, SMU.59 and SMU.268) in which L-glutamine and PRPP are joined and converted to AMP increased ($p = 0.035$). SMU.33, a gene that encodes for a hypothetical protein and lies in between two of the genes coding for enzymes of the purine synthesis pathway, was also significantly upregulated.

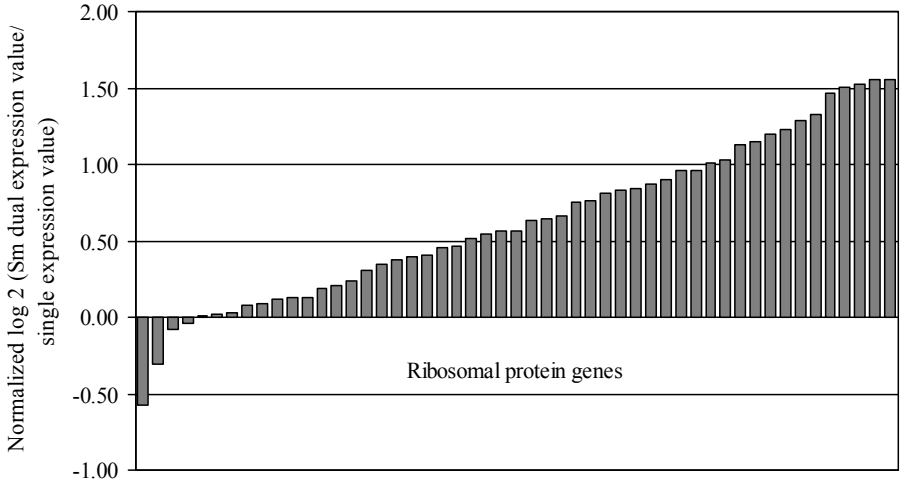
Two of the 19 downregulated genes are involved in amino acid metabolism: ThrC (SMU.70), which catalyzes the conversion of O-phospho-L-homoserine to L-threonine and GdhA (SMU.913), which converts L-glutamate to 2-oxoglutarate and ammonia. In addition, the transcript level of the gene encoding GlnA (SMU.364) the enzyme that converts L-glutamine to L-glutamate and ammonia, was down 10.3-fold ($p = 0.31$, data not shown).

In *S. mutans* grown in dual-species biofilm, expression of the peptide chain release factor 3 gene (*pfrC*, SMU.608) was significantly decreased. PfrC is involved in translation, but is a dispensable factor (Tate *et al*., 2001).

In *S. mutans,* glycogen is called intracellular polysaccharide (IPS). In addition to *glgC* (SMU.1538), which showed a significant decrease in expression, other genes involved in IPS metabolism (Spatafora *et al*., 1995) were also downregulated: SMU.1535 ($p = 0.126$), SMU.1536 ($p = 0.092$), SMU.1537 ($p = 0.062$), SMU.1539 ($p = 0.104$). Gene set enrichment analysis showed that the expression of SMU.1535-1539 together decreased significantly ($p = 0.0091$). SMU.1565, another significantly downregulated gene, codes for a putative 4-alpha-glucanotransferase (synonym of amylomaltase). In *S. mutans* it may be involved in IPS production from maltose (Wursch & Koellreutter, 1985; Simpson & Russell, 1998).



A



B

Fig. 12: (A) Normalized log2 expression values of transcripts in *Streptococcus mutans* grown in single-species biofilms (Sm single), compared to their respective values in *S. mutans* grown in dual-species biofilm (Sm dual). Filled triangles denote all ribosomal proteins between locus SMU.2000 and SMU.2026c, open triangles denote all other ribosomal proteins and closed grey circles denote all other genes. The dotted lines denote 1 fold change. (B) Log2 of fold-change values of all transcripts coding for ribosomal proteins in *S. mutans* (positive values denote a higher expression in *S. mutans* grown in dual-species biofilms).

Discussion

In a previous study we have shown that the susceptibility to chlorhexidine of *S. mutans* and *V. parvula* grown in biofilm decreases in each others presence. The current study shows that this is also true when these biofilms are exposed to other antimicrobials. *V. parvula* was able to survive erythromycin treatment better in dual-species biofilm than in single-species biofilm. Survival after exposure to the hydrogen peroxide concentration that was used to kill *S. mutans* was too low to be detected, as can be expected for a bacterium with a very low oxygen tolerance. *S. mutans* was better able to survive antimicrobial exposure when grown in dual-species biofilm than in single-species biofilm. This was not only true after exposure to antimicrobials with a mode of action related to chlorhexidine (CPC and amine chloride), something which may have been expected. But it was even true after exposure to antimicrobials with a completely different mode of action (hydrogen peroxide, erythromycin and zinc chloride). An increased resistance to antimicrobials was also found in other mixed-species biofilms compared to single-species biofilms (Skilman *et al*., 1997; Elvers *et al*., 2002; Burmolle *et al*., 2006).

Our results suggest that *V. parvula* changes the physiology of *S. mutans*. Recent studies on behavior of multi-species communities have shown that presence of one or more bacteria can change virulence and gene expression in other pathogens: Animal experiments with pathogenic *Pseudomonas aeruginosa* and avirulent oropharyngeal flora showed that avirulent strains enhance lung damage by *P. aeruginosa* (Duan *et al*., 2003). Strains of *Veillonella* and *Lactobacillus* were shown to decrease the expression of *aggR*, which codes for a global transcriptional regulator of enteroaggregative *Escherichia coli* virulence factors, whereas *Enterococcus faecalis* and *Clostridium innocua* were shown to increase the expression of this gene (Ruiz-Perez *et al*., 2004).

A study on the interaction between *Streptococcus gordonii* and *Veillonella atypica* showed that *S. gordonii* increases its expression of the α-amylase encoding gene *amyB* in the presence of *V. atypica* (Egland *et al*., 2004). In the current study, comparative

analysis of transcript levels in *S. mutans* growing in dual-species biofilm and in single-species biofilm showed that the physiology of this bacterium is indeed changed by the presence of *V. parvula*. We found quite some differences, even though these cells are in stationary phase, which may make it more difficult to detect differences. According to our results the most important changes that occurred when *V. parvula* was present were:

- The SRP-translocation pathway genes *ftsY* and *ffh* (Gutierrez *et al*., 1999) were downregulated. FtsY and Ffh are needed for normal growth at low pH in *S. mutans* (Gutierrez *et al*., 1999; Hasona *et al*., 2005). However, in our previous experiments we have shown that the overall pH of the bulk medium is constant for the three types of biofilms (Kara *et al*., 2006), due to the presence of a buffer. Apparently, *ftsY* and *ffh* can also be controlled by regulators that are independent of pH fluctuations.
- Changes occurred in the amino acid metabolism. We concluded this from the lower expression of enzymes involved in aspartate and glutamate/glutamine metabolism, and from the lower expression of *pepC* which shows 85% identity with the thiol aminopeptidase *pepC* from *Streptococcus thermophilus* (Chapot-Chartier *et al*., 1994). Aminopeptidases are believed to contribute to the final degradation of short peptides. An explanation for the change in amino acid metabolism could be that the amino acid concentration of the outside medium was higher because *V. parvula* breaks down proteins extracellularly to amino acids. *V. parvula* has previously been shown to degrade various proteins extracellularly (Wikstrom, 1983). *S. mutans* could take up these amino acids, perhaps even specifically glutamine and aspartate, and therefore would need to synthesize less amino acids by itself. This may also explain why *pepC* is expressed less: there would be less need for breakdown of small peptides in the cell, because they would already have been broken down to amino acids extracellularly. Glutamine and glutamate are also important for other cellular processes, such as amine metabolism, cell wall synthesis, pyrimidine and

purine synthesis and oxidation and reduction. However, there was no indication that a change in these processes took place when *V. parvula* was present, since the transcript levels of genes coding for the other enzymes involved in these pathways did not show a significant change or a change in ratio (data not shown). The only exception to this was the change in purine metabolism.

- Purine metabolism was upregulated, which would coincide nicely with the presumed higher concentration of glutamine in the cell, since this amino acid is necessary for the first step of purine synthesis.
- Intracellular polysaccharide metabolism was downregulated. *S. mutans* is known to produce intracellular polysaccharides (IPS) from sugars such as glucose and sucrose, which can serve as metabolic substrates for acid production during periods of carbohydrate limitation (Freedman & Coykendall, 1975; Spatafora *et al*., 1999). The downregulation of this metabolism means that either less IPS was made in dual-species biofilm in this stage of growth or less IPS was broken down. The latter probability seems the most plausible, since in stationary phase there probably is a sugar shortage, thus no synthesis of IPS. *S. mutans* growing in dual-species biofilm used less of the IPS, possibly because the cells needed less energy for synthesis of amino acids, and for transport of lactate out of the cell. *V. parvula* consumes the lactic acid that is produced by *S. mutans*, which results in lower lactic acid concentration present in the medium at t=48 h for dual-species biofilms than for single-species biofilms (Kara *et al*., 2006).
- The most prominent change that took place in dual-species biofilm was that expression of a large number of genes coding for ribosomal proteins and ropA, a ribosome associated chaperone (Hesterkamp *et al*., 1996; Kramer *et al*., 2002), were upregulated. This indicates the presence of more ribosomes. Similarly Len *et al.* (2004a) found that all detected ribosomal proteins were upregulated when planktonic *S. mutans* cells were grown under low pH stress. Studies on related Gram-positive bacteria have shown similar changes in gene

expression patterns in response to antimicrobial exposure. In *Streptococcus pneumoniae*, *Bacillus subtilis* and *Enterococcus faecalis* the relative transcript levels of the majority of the genes that code for ribosomal proteins increase in response to the presence of various antibiotics (Ng *et al*., 2003; Dandliker *et al*., 2003; Aakra *et al*., 2005). The decrease in transcript levels of the genes coding for amino acyl tRNA synthetases that was found in the current study also occurred in *E. faecalis* exposed to erythromycin and in *S. pneumoniae* and *B. subtilis* growing in the presence of various antibiotics (Ng *et al*., 2003; Dandliker *et al*., 2003; Aakra *et al*., 2005). Also in accordance with our results is the increase in relative transcript amounts of genes involved in purine metabolism in *S. pneumoniae* exposed to four antibiotics (Ng *et al*., 2003). Apparently, *S. mutans* responds to the presence of *V. parvula* in a manner similar to the response of other Gram-positive bacteria to antibiotics.

We speculate that with a higher number of ribosomes present, cells exposed to antimicrobials may be better able to synthesize new proteins, a mechanism that is necessary for repair of the damage caused by the antimicrobials. A finding that could support this theory is the involvement of ropA in the resistance of *S. mutans* to hydrogen peroxide (Wen *et al*., 2005). However, whether the statistically significant 1.3 times upregulation of *ropA* also makes a physiologically significant difference is not clear. The proposed theory to explain the increased survival rate of *S. mutans* after antimicrobial exposure in the presence of *V. parvula* needs to be tested in future studies.

In conclusion, growing in a biofilm together with a non-pathogenic bacterium like *V. parvula* changes the physiology of *S. mutans*. The expression of genes coding for proteins involved in amino acid metabolism, IPS metabolism, the SRP-translocation pathway, purine metabolism and protein synthesis changed. Furthermore, the presence of *V. parvula* gives the cariogenic *S. mutans* an advantage in surviving antimicrobial treatments. These findings show that in dental plaque the presence of *V.*

parvula can not solely be considered as an advantage. Besides its beneficial property of lactate consumption and conversion to less cariogenic acids, *V. parvula* could have an adverse effect: It can increase the survival of *S. mutans* after exposure to antimicrobials that are intended to reduce *S. mutans* numbers in dental plaque.

This study shows that the presence of another bacterium can change the phenotype of a pathogen and can increase its resistance to antimicrobials. Thus, the study of pathogens implicated in polymicrobial diseases, such as caries and periodontitis, should be focused more on multi-species biofilms, and the testing of the susceptibility to the currently used and new antimicrobials should be done on a multi-species microbial community rather than with single pathogens.

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Chapter 6

Summary,
General discussion and Future Directions

It is proposed that oral biofilm communities form as a result of intimate interactions between cells of different species rather than clonal growth of genetically identical cells (Kolenbrander *et al*., 2005). Interspecies interactions have a major effect on the structure and physiology of oral biofilms during the initial stages of formation, bacterial attachment and surface colonization, and continue to influence the structure and physiology of the biofilm as it develops. Although the majority of the research on bacterial interactions has involved planktonic communities, the characteristics of biofilm growth suggest that interspecies interactions may be more significant in biofilms (James *et al.,* 1995). *Streptococcus mutans* and *Veillonella parvula* are early colonizers and resident members of oral flora (Kolenbrander *et al*., 2002). Both species become significantly more prevalent in oral biofilms in relation to progression of dental caries (Aas, 2006).

Mutual interactions between *S. mutans* and *V. parvula* grown in a biofilm were studied in this thesis using a biochemical, microstructural, proteomic and transcriptomic approach.

In **Chapter 2**, we first evaluated the growth of *S. mutans* and *V. parvula* in a biofilm alone (single-species) and in the presence of the other (dual-species). At 48 and 72h, both species developed similar viable counts ($\approx 10^8$ colony-forming units per well) indicating the establishment of a stable biofilm.

Second, we measured the acid production by single- and dual-species biofilms, as greater acid production might indicate a more cariogenic challenge. At 48 and 72 h, lactic acid production of dual-species biofilms (29.4 and 35.2 mM, respectively) was significantly less than that of *S. mutans* (62.2 and 121.0 mM, respectively). *V. parvula* apparently caused a significant decrease in lactic acid concentrations when the two bacterial species were grown together in a biofilm. However, the cariogenic potential of weaker acids such as propionic and acetic acid compared with the stronger lactic acid, is still a largely unsolved question in cariology (Featherstone & Rodgers, 1981). Higher concentrations of weaker acids in dual-species biofilms compared with those in

S. mutans single-species biofilms triggered us to question the so called 'benevolent role' of *V. parvula* in oral biofilms.

Third, we compared the response of single-species biofilms and dual-species biofilms, to the most commonly used antimicrobial agent in dentistry, chlorhexidine. Chlorhexidine was applied in three different treatment modalities to comparatively assess the survival of biofilms. We used a neutralizer which mimics the role of saliva after antimicrobial treatments. We also applied chlorhexidine in three different concentrations (0.025, 0.1 & 0.4 mg ml^{-1}) and observed a dose response in biofilms. The most striking result was that both species were less susceptible to chlorhexidine in dual-species biofilms when compared with single-species biofilms; in other words, *S. mutans* and *V. parvula* became more resistant to chlorhexidine when grown in a biofilm in each other's presence. In the latter chapters, possible mechanisms behind this synergism are investigated.

In **Chapter 2**, we used *S. mutans* strain C180-2. We also evaluated the effect of chlorhexidine on single- and dual-species biofilms of *S. mutans* and *V. parvula* using another strain of *S. mutans*, UA159. Similar to our previous findings, both species demonstrated a better survival after chlorhexidine exposure when grown in dual-species biofilms compared with single-species biofilms (data not shown). This result showed that better survival response of *S. mutans* to chlorhexidine in dual-species biofilms was not strain dependent. It also allowed us to study the interactions between these two species at the translational and transcriptional level (presented in **Chapter 4** and **Chapter 5**) as the genome of *S. mutans* UA159 strain has been sequenced (Ajdic *et al.,* 2002).

Better survival of dual-species biofilm bacteria compared with single-species against chlorhexidine prompted another research question: Was this increased survival specific to chlorhexidine? Or was it a general phenomenon, valid for various antimicrobials? To this end, we evaluated the effects of four other antimicrobials on single- and dual-species biofilms; cetylpyridinium chloride, amine chloride, zinc chloride and erythromycin, which have different mechanisms of action. Results of this

experiment are presented in **Chapter 5**, summarized in Fig. 10. The data clearly showed that in both species, a better survival response against antimicrobials was developed when bacteria are grown in dual-species biofilms, regardless of the antimicrobial applied.

In **Chapter 3**, the temporal aspects of microstructure of single- and dual-species biofilms, before and after exposure to chlorhexidine were assessed. For this purpose, we used fluorescent probes to stain the biofilm cells and confocal laser scanning microscopy (CLSM) to image the biofilms.

Single- and dual-species biofilms were grown for a total of 264 h. Biofilms were subjected to chlorhexidine at 48 h as described in **Chapter 2** ('neutralized killing'). Thickness, percentage of dead and live cells, and percentage of *S. mutans* and *V. parvula* in dual-species biofilms were assessed in chlorhexidine-treated and untreated control biofilms at 48 h immediately after the treatment, and at 96, 192 and 264 h to evaluate the lasting term effect of chlorhexidine. A decrease in the thickness and viability of single- and dual-species biofilms in time was observed after chlorhexidine treatment. Better survival of bacteria in dual-species biofilms was detected at 48 h confirming the findings in Chapter 2. This result indicates the agreement between CLSM assessment using molecular probes with a determination of the viability with conventional plating. Notably, better survival of dual-species biofilm bacteria was observed at all time points.

Next, in the same study, we evaluated the differences between the spatial arrangements of bacteria in single- and dual-species biofilms at 48 h, before and after exposure to chlorhexidine, to find out a possible link between microstructure and the better survival of bacteria in dual-species biofilms. Spatial arrangement analysis of biofilms was carried out using a recently developed software program, DAIME (Daims *et al.*, 2006). DAIME is a novel image analysis program for microbial ecology and biofilms research, which extracts quantitative data from image stacks, complements recent molecular techniques for analyzing structure-function relationships in biofilms and helps to characterize symbiotic interactions among organisms. A remarkable

feature of this program is that it determines the quantitative aspects of bacterial spatial arrangements.

According to the results of the spatial arrangement analysis, in all untreated control biofilms, significant clustering of bacteria was observed. However, after exposure to chlorhexidine, a more prominent clustering occurred between the bacteria in dual-species biofilms compared to controls, whereas bacteria in single-species biofilms were randomly or repulsively distributed. Proximity is an indication for metabolic interaction and a physiological state for biofilm growth. Proximity of cells within the clusters provides an ideal environment for creation of nutrient gradients, exchange of genes, and quorum sensing. Therefore we suggest that enhanced clustering after the application of chlorhexidine in dual-species biofilm bacteria (in contrast to dispersion and repulsion of bacteria in single-species biofilms) is a survival strategy.

In addition to the results presented in Chapter 3, we have performed similar experiments and microstructural spatial arrangement analysis using another strain of *S. mutans* (C180-2). The data were similar to aforementioned results (data not shown). This shows that the enhanced cluster formation between bacteria in dual-species biofilms after chlorhexidine application is not strain dependent.

A significantly decreased susceptibility of bacteria in dual-species biofilms against antimicrobials was detected at 48 h, as presented in **Chapter 2**, **Chapter 3** and **Chapter 5**. We therefore studied the effect of *V. parvula* on *S. mutans* in more detail. What was the effect of one bacterium on (the transcript and protein levels of) the other at that time point? If any, was there a link between these effects and decreased susceptibility?

In **Chapter 4**, we determined the differences in protein expression between single- and dual-species biofilms of *S. mutans* and/or *V. parvula*. Two-dimensional difference gel electrophoresis and specialized statistical analysis methods were used in this study. We found that 42 out of the 455 proteins detected, were significantly differentially expressed in dual-species biofilms compared with single-species biofilms

($p < 0.05$). Of these 42 spots, 16 were upregulated and 26 were downregulated. We have to remark at this point that, having no genome database of *V. parvula* made it difficult to identify *V. parvula* spots and therefore to get information about the effects of interspecies interactions on the physiology of this bacterium. Nevertheless, the vast majority of the upregulated proteins (13 in 16) in dual-species biofilms, were identified to originate from *S. mutans'*. Growing in a biofilm in the presence of *V. parvula* caused an enhancement in enzymes involved in *S. mutans*' energy metabolism, nucleotide and RNA synthesis, an increased capacity for protein synthesis, a better ability to fold, process and stabilize proteins and an increased capacity to degrade dysfunctional proteins.

In **Chapter 5**, we identified which gene transcript levels were changed in *S. mutans* when growing in co-existence with *V. parvula*. *S. mutans* microarrays were used to determine the differences in transcript levels between *S. mutans* growing in single-species biofilm and *S. mutans* growing in dual-species biofilm. Our analysis showed that in *S. mutans*, transcript levels of 15 genes were significantly ($p < 0.05$) higher in the presence of *V. parvula* than in the absence of *V. parvula* and 19 were lower. Out of the 15 upregulated genes, seven were ribosomal proteins. Other genes which had a significant increase in expression were related to protein synthesis and purine metabolism. It is also of importance to note that, to our knowledge, in the studies presented in **Chapter 4** and in **Chapter 5**, two dimensional difference gel electrophoresis and DNA microarrays were used for the first time to study the mutual physiological effects of two different species.

When the data of **Chapter 4** and **Chapter 5** are combined, the picture becomes more clear: growing in a biofilm together with *V. parvula* significantly alters the physiology of *S. mutans*. This alteration in *S. mutans* is characterized by a more dynamic protein synthesis and protein turnover machinery. We conclude that co-existence with *V. parvula* in a biofilm increases the survival of dental pathogen *S. mutans* against antimicrobials by altering the phenotype of this pathogen.

Conclusions

- *Streptococcus mutans* and *Veillonella parvula* become less susceptible to antimicrobial treatments when grown in a biofilm in each other's presence (dual-species) than when grown alone (single-species).

- Use of single-species biofilm models in antimicrobial susceptibility testing could give rise to overestimating the effects of antimicrobials.

- Basic structural unit of studied biofilms are clusters. However, antimicrobial treatments cause different responses in architecture of single- and dual-species biofilm bacteria. In dual-species biofilms, clustering enhances significantly whereas single-species biofilm bacteria become dispersed or repulsed, after exposure to antimicrobials. Enhanced clustering after antimicrobial exposure is a survival strategy for dual-species biofilm bacteria.

- Growing together in a biofilm with *V. parvula* significantly alters the physiology of the dental pathogen *S. mutans*, which can be seen by the changes in gene transcript and protein levels compared with when grown alone. Our hypothesis is that the more dynamic protein machinery in *S. mutans* grown in dual-species biofilms, leads to a better survival against antimicrobials.

Future directions

Dental caries is still a major health problem in most industrialized countries as it affects 60-90 % of school-aged children and the vast majority of adults. World Health Organization targets in 2020, to increase the proportion of caries free 6-year-olds, to reduce the 'decayed missing filled teeth' (DMFT) index, particularly D component at age 12 years, with special attention to high-risk groups and to reduce the number of teeth extracted due to dental caries (Hobdell *et al*., 2003). Decreasing the prevalence of dental caries among the world population is possible with a better understanding of etiology of the disease and effective prevention and treatment applications. Perturbations in the microbial composition of oral biofilms (dental plaque) in favor of cariogenic pathogens cause dental caries. Antimicrobials are commonly used both for prevention in high-caries risk groups and for treatment measures in caries active patients. This thesis explores the mutual interspecies interactions between the primary pathogen of dental caries, *Streptococcus mutans* and *Veillonella parvula*, which has to be taken into consideration in antimicrobial testing.

Molecular biology, the human genome project, transcriptome and proteome analysis have recently opened vast opportunities for the translation of basic science discoveries to oral health care. Education and training of the oral health professional community about the process of discovery, from basic discovery through clinical applications influencing and improving standards of oral health care, has not received sufficient emphasis until recently.

In this thesis, we have shown that the mutual interaction between two oral biofilm species; *S. mutans* and *V. parvula*, leads them to survive antimicrobial treatments better. We have also detected changes in phenotype of *S. mutans* at the translational and transcriptional level when grown in the presence of *V. parvula* in a biofilm. Whether these changes at the translational and transcriptional level are directly or

indirectly responsible for the better survival of *S. mutans* in dual-species biofilm, remains to be determined. It would be revealing to analyze the proteome and transcriptome of the same oral biofilm bacteria in single- and dual-species biofilms immediately after antimicrobial exposure. With respect to using microarrays to detect the effect of *V. parvula* on the gene expression of *S. mutans* in Chapter 5, real-time polymerase chain reaction (RT-PCR) should be performed on a selected set of genes from each functional group to confirm the microarray results.

In Chapter 4, we have analyzed the protein expression in single- and dual-species biofilms of *S. mutans* and *V. parvula*. In this study, however, no membrane proteins were identified. This is consistent with 2D-DIGE analysis of *S. mutans* as well as other bacteria, where membrane-proteins are notably under-represented (Molloy *et al*., 2000; Santoni *et al*., 2000; Len *et al*., 2003). This might be due to the low abundancy, poor solubility and inherent hydrophobicity of membrane proteins leading to self-aggregation during the first dimension. Another technique (Zuobi-Hasona *et al.*, 2005) can be applied in future studies for extraction of membrane proteins from *S. mutans* in order to enable their resolution using 2D-DIGE.

This thesis also indicates that the benevolent role of *V. parvula* in oral biofilms is ambiguous due to its capacity of increasing the potential virulence of *S. mutans*. For the same reason, the genome sequencing of *V. parvula* should be considered a priority to elucidate the physiology of this bacterium and its interactions with other species in oral biofilm. Oral microbiology has been energized by, and will continue to benefit from, the genome projects of its constituent organisms (Duncan, 2003). The genome sequences of refractory oral organisms will provide insights into their metabolism that will lead to the development of better prevention and treatment conditions.

Research on oral biofilms is proceeding on many fronts, with particular emphasis on the genes specifically expressed by biofilm-associated organisms, evaluation of

various control strategies for either preventing or remediating biofilm colonization of oral tissues, and development of new methods for assessment and improvement of the efficacy of antimicrobial treatments. Research should also focus on the role of biofilms and interspecies interactions in antimicrobial resistance, biofilm as a reservoir for pathogenic organisms. The key to success may hinge upon a more complete understanding of what makes the multi-species biofilm phenotype so different from the single-species biofilm phenotype.

Samenvatting

Een biofilm is een laag micro-organismen gehecht aan een oppervlak die omgeven wordt door een zelfgeproduceerde slijmlaag. Tandplak is een multi-species biofilm: uit deze biofilm zijn meer dan 700 verschillende soorten micro-organismen geïsoleerd die op de verschillende oppervlakken in de mond leven. Deze bacteriën werken op verschillende manieren op elkaar in. Ze gebruiken bijvoorbeeld de eindproducten van elkaars metabolisme en ze communiceren door het uitscheiden van specifieke moleculen. Het ontrafelen van de interactie tussen deze bacteriën zal leiden tot een beter begrip van deze complexe biofilm.

Streptococcus mutans en *Veillonella parvula* zijn twee bacteriën die als eerste de mond koloniseren en ze zijn een vast onderdeel van de mondflora. Allebei komen ze meer voor in tandplak naarmate tandcaries zich ontwikkelt.

Dit proefschrift beschrijft een studie naar de onderlinge interacties tussen *S. mutans* en *V. parvula*, die bestudeerd werden met behulp van proteomics, transcriptomics en door de microstructuur van de biofilm en de zuurproduktie door de biofilm te bekijken. De twee bacteriën werden allebei apart gekweekt in een *in vitro* biofilm en samen.

Als eerste hebben we de zuurproductie van deze biofilms gemeten. We hebben ook de gevoeligheid voor antimicrobiële middelen van deze biofilms gemeten. Daarvoor hebben we zes verschillende antimicrobiële middelen gebruikt, met verschillende werkingsmechanismen: chlorhexidine, cetylpyridinium chloride, anime chloride, zink chloride, waterstofperoxyde en erythromycine. Beide soorten bacteriën waren resistenter tegen antimicrobiële middelen als ze samen waren gekweekt dan wanneer ze apart waren gekweekt. Deze bevindingen worden beschreven in Hoofdstuk 2 en 5.

Waarom worden deze bacteriën resistenter tegen antimicrobiële middelen als ze samen groeien? Mogelijke antwoorden op die vraag worden beschreven in Hoofdstuk 3, 4 en 5.

Hoofdstuk 3 beschrijft de resultaten van het onderzoek naar de microstructuur van de biofilms vóór en na de behandeling met chlorhexidine. De bacteriën vormden celclusters in alle onbehandelde biofilms. De bacteriën die apart waren gekweekt waren verspreid direct na de behandeling. Bij de bacteriën die samen waren gekweekt nam de clustervorming echter toe na de blootstelling aan chlorhexidine. Dit wijst er op dat deze toename in clustering (en dus nabijheid van andere bacteriën) na de behandeling met een antimicrobieel middel een overlevingsstrategie is voor deze biofilm-bacteriën.

In Hoofdstuk 4 en 5 hebben we de verschillen in eiwit-expressie en in transcriptie-niveau's bepaald tussen de apart gekweekt bacteriën enerzijds en de samen gekweekte bacteriën anderzijds. Het bleek dat de fysiologie van *S. mutans* significant veranderde als deze bacterie samen met *V. parvula* in een biofilm groeide. Eén van de belangrijkste veranderingen in *S. mutans* was een toename in de expressie van eiwitten die belangrijk zijn voor eiwitsynthese, eiwitvouwing en hergebruik van eiwitten.

Onze conclusie is dat samenleven met *V. parvula* in een biofilm de overleving van de tandpathogeen *S. mutans* verhoogt na blootstelling aan antimicrobiële middelen, door het fenotype van deze pathogeen te veranderen.

Het onderzoek naar ziekteverwekkende bacteriën die betrokken zijn bij ziekten die door meerdere bacteriën tegelijk veroorzaakt worden, zoals caries en parodontitis, zou zich meer moeten richten op biofilms die meerdere bacteriën bevatten. Het testen van de gevoeligheid van deze ziekteverwekkende bacteriën voor de huidige en nieuwe antimicrobiële middelen, zou gedaan moeten worden met een biofilm die meerdere micro-organismen bevat in plaats van met een biofilm die maar één ziekteverwekker bevat.

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Curriculum Vitae

Duygu Kara was born on 23rd of September 1973, in Ankara, Turkey. She completed her primary and secondary education in her hometown, where she obtained her high school diploma from Private Yükseliş College of Ankara in 1991. The same year she began her studies in dentistry at the University of Ankara, Turkey, where she graduated in 1996 receiving a D.D.S. degree. In September 1996, she joined the Dental Faculty of University of Ankara as a clinical and research assistant at the Department of Pedodontics, for six years. During this period she also worked part-time as a dentist at a general dentistry practice in Ankara. In January 2002 she was awarded a grant in scope of the NATO Science Fellowships programme to study the wear characteristics of direct restoratives and human enamel in Dental Materials Science Department of Academic Centre for Dentistry Amsterdam, supervised by Prof. Albert Feilzer. After her return to Ankara in July 2002, she published her first doctoral dissertation titled Evaluation of Physical and Mechanical Properties of Resin Based Filling Materials, in Department of Pedodontics, Faculty of Dentistry, University of Ankara. In November 2002, she started her studies at the Department of Cariology Endodontology Pedodontology, Academic Centre for Dentistry Amsterdam, in the Netherlands. Five years of research have resulted in this thesis.

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…..

So, somehow it does not seem appropriate to say "Farewell", but rather it's time for a reincarnation and to move on...