Attachment mechanisms and properties of bacterial biofilms on non-living surfaces

Marko Kolari

Division of Microbiology
Department of Applied Chemistry and Microbiology
University of Helsinki
Finland

Academic Dissertation in Microbiology

To be presented, with the permission of the Faculty of Agriculture and Forestry of the University of Helsinki, for public criticism in the auditorium 1041 at Viikki Biocenter of University of Helsinki (Viikinkaari 5) on May 16th, at 12 o'clock noon.

Helsinki 2003

Supervisor: Professor Mirja Salkinoja-Salonen
Division of Microbiology
Department of Applied Chemistry and Microbiology
University of Helsinki, Finland

Reviewers: Professor Peter Neubauer
Bioprocess Engineering Laboratory
Department of Process and Environmental Engineering
University of Oulu, Finland

Dr. Kaarina Lähteenmäki
Division of General Microbiology
Department of Biosciences
University of Helsinki, Finland

Opponent: Professor Ulrich Szewzyk
Department of Microbial Ecology
Institute of Environmental Technology
Technical University of Berlin, Germany

ISSN 1239-9469
ISBN 952-10-0344-8
ISBN 952-10-0345-6 (pdf)

Electronic publication available at http://ethesis.helsinki.fi

Yliopistopaino 2003
Helsinki, Finland

: Marko.Kolari@helsinki.fi

Front cover: The four images (in clockwise order) illustrate biofilm development on paper machine surfaces. FESEM image of *Deinococcus geothermalis* cells attached to a stainless steel surface ⇒ FESEM image of interconnected cells of *D. geothermalis* within a cell cluster ⇒ Stereomicroscope image of biofilm microcolonies ⇒ Photograph of mature biofilms adhering to stainless steel surfaces in the wet-end of a fine paper machine.

Contents

List of original publications 5
The author's contribution 5
Abbreviations 6

1. INTRODUCTION 7

1.1. Current consensus model of bacterial biofilms 9
1.2. Biofilm mode of growth 10
1.3. Initial adhesion of bacteria 11
1.4. Bacterial behavior on surfaces 12
1.5. Effect of surfaces on attachment 12
1.6. Eukaryotic organisms in biofilms 13
1.7. Bacterial cell-to-cell communication 14
1.8. Cell-to-cell signalling in bacterial biofilms 14
1.9. Microbes in paper machines 15
1.10. Prevention of biofilms in paper machines 16
1.11. Resistance of biofilms to antimicrobials 17
1.12. Current methodology in biofilm studies 18
1.12.1. Atomic force microscopy (AFM) 18
1.12.2. Confocal laser scanning microscopy 20
1.12.3. Fluorescence in situ hybridization (FISH) 23

2. AIMS OF THE STUDY 25

3. MATERIALS AND METHODS 26

3.1. Methods used in this thesis 26
3.2. Methods other than those described in the papers I - IV 27
3.2.1. Tapping mode AFM of biofilm bacteria 27
3.2.2. Assays of cell-to-cell attachment 27
3.2.2.1. Lectin staining of biofilms 27
3.2.2.2. Alcian blue staining of biofilms 27
3.2.2.3. Inhibition of intercellular adhesion by carbohydrates 27
3.2.2.4. Enzymatic removal of biofilms 28
3.2.3. *In situ* -hybridization with DgeoF-probe 29
3.2.4. Analyses of cell-to-cell signalling molecules 30

4. RESULTS AND DISCUSSION 31

4.1. Structure of *Deinococcus geothermalis* biofilms 31
4.2. Adhesiveness of *D. geothermalis* cells 37
4.2.1. Firm attachment of *D. geothermalis* on surfaces 37
4.2.2. Firm but slippery attachment 38

4.3. Cell-to-cell attachment in *D. geothermalis* biofilms 40
4.3.1. Lectin assay of the adhesive structures 40
4.3.2. Alcian blue -staining of acidic polysaccharides 42
4.3.3. Inhibition of intercellular adhesion with carbohydrates 42
4.3.4. Effect of enzymes on biofilms 43
4.3.5. Summary on the assays of cell-to-cell attachment 44
4.4. Biofilm-forming bacteria in paper machines 44
4.4.1. Primary biofilm-formers 45
4.4.1.1. *Deinococcus geothermalis* 45
4.4.1.2. *Meiothermus silvanus* 48
4.4.1.3. *Thermomonas* spp. 49
4.4.1.4. Novel genus of "*Rhodobacter*-like" alpha-*Proteobacteria* 49
4.4.1.5. *Burkholderia* spp. 49
4.4.1.6. Other primary biofilm-formers 50
4.4.2. Does every machine have unique primary-biofilm-formers? 51
4.5. *Bacillus* species in paper machine biofilms 51
4.5.1. "Plate-count-agar illusion" 52
4.5.2. Interactive biofilm formation in paper machines 53
4.6. Other species in paper machine biofilms 54
4.7. Effect of biocides on the biofilms 55
4.8. Selection between the biofilm and planktonic mode of growth 58
4.8.1. Effects of nutrients and bacteriostatic compounds 58
4.8.2. Effects of flow, pH and ionic strength 60
4.9. AHL-mediated cell-to-cell signalling in paper machine biofilms 61
4.10. Structure of biofilms on ennobled stainless steel 62
4.11. Methods developed in this study 63
4.11.1. Improved procedure for isolation of primary-biofilm formers 63
4.11.2. The BF-assay 63
4.11.3. FISH protocol for *D. geothermalis* 64
4.11.4. Fluorescent stains for microscopy of biofilms 65
4.12. Deposited sequences and bacterial cultures 66
5. MAIN CONCLUSIONS 68
6. ABSTRACT 70
7. ACKNOWLEDGEMENTS 71
8. REFERENCES 73

List of original publications

This thesis is based on the following original articles, which are referred to in the text by their Roman numerals. In addition, some unpublished data are presented.

I **Kolari M**, J Nuutinen and MS Salkinoja-Salonen. 2001.
Mechanisms of biofilm formation in paper machine by *Bacillus* species: the role of *Deinococcus geothermalis*.
Journal of Industrial Microbiology & Biotechnology, 27: 343-351.

II **Kolari M**, U Schmidt, E Kuismanen and MS Salkinoja-Salonen. 2002.
Firm but slippery attachment of *Deinococcus geothermalis*.
Journal of Bacteriology, 184: 2473-2480.

III **Kolari M**, J Nuutinen, FA Rainey and MS Salkinoja-Salonen. 2003.
Colored moderately thermophilic bacteria in paper-machine biofilms.
Journal of Industrial Microbiology & Biotechnology, in press.

IV **Kolari M**, K Mattila, R Mikkola and MS Salkinoja-Salonen. 1998.
Community structure of biofilms on ennobled stainless steel in Baltic Sea water.
Journal of Industrial Microbiology & Biotechnology, 21: 261-274.

The original articles were reproduced with kind permission of the copyright holders

The author's contribution

Paper I:
Marko Kolari designed the experiments, interpreted the results, wrote the paper, and is the corresponding author. He carried out all the experimental work except for the isolation and characterization of the strains, and a part of the microtiter plate assays for biofilm formation.

Paper II:
Marko Kolari designed the experiments, interpreted the results, and wrote the paper. He performed all the experimental work under supervision of the AFM and micromanipulation specialists.

Paper III:
Marko Kolari designed the experiments, interpreted the results, wrote the paper, and is the corresponding author. He carried out all the experimental work except for the isolation and characterization of two out of the 95 strains, a part of the fatty acid analyses, and the 16S rRNA gene sequencing.

Paper IV:
Marko Kolari interpreted the results, wrote the paper, and is the corresponding author. He carried out the experimental work except for the propagation of biofilms, FISH, scanning electron microscopy, and the analysis of partition coefficients.

Abbreviations

A_{595}	Absorbance at wavelength 595 nm, measured in the BF-assay
AHL	*N*-Acylhomoserine lactone, an extracellular signalling molecule of gram-negative bacteria
AFM	Atomic force microscopy
BF-assay	Microtiter plate assay for biofilm formation
CFA	Clear filtrate agar, one type of paper-machine-water based growth media
CFU	Colony-forming units
CLSM	Confocal laser scanning microscopy
ConA	Concanavalin A lectin
CTYE	Castenholz trypticase yeast extract agar
DBNPA	2,2-dibromo-3-nitrilopropionamide, a biocide
DLVO	Theory of physico-chemistry of microbial adhesion by Derjaguin, Landau, Vervey & Overbeek
ECL	Equivalent chain length, in FAME analysis
EMBL	European Molecular Biology Laboratory, maintains nucleotide sequence databases
EPS	Extracellular polymeric substances
EtBr	Ethidium bromide, a fluorescent stain for nucleic acids
FAME	Whole-cell fatty acid methyl esters
FESEM	Field-emission scanning electron microscopy
FISH	Fluorescence *in situ* hybridization
FITC	Fluorescein isothiocyanate
HAMBI	Culture Collection of the Faculty of Agriculture and Forestry, University of Helsinki
HSL	Homoserine lactone (see also AHL)
log K_{ow}	Logarithm of the partition coefficient n-octanol:water
MBT	Methylene bisthiocyanate, a biocide
MIC	Microbially influenced corrosion
PA	Pulp agar, a growth medium containing chemical pulp, yeast extract and peptone
PIA	Polysaccharide intercellular adhesin of *Staphylococcus epidermidis*
rRNA	Ribosomal RNA
RDP	Ribosomal Database Project, refers to the public rRNA database maintained by RDP
RT	Room temperature
S.D.	Standard deviation
SDS	Sodium dodecyl sulfate
T_{max}	Maximum growth temperature
TRITC	Tetramethylrhodamine isothiocyanate
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TSBA	Trypticase soy broth agar (TSA with additional dextrose)
WW	White water, a general term for paper machine waters which have a white, cloudy appearance due to fine dispersion of fibres, sizing agents, fillers etc., and are reused in the papermaking. In this study WW refers to water that was collected from wire pits of the paper machines, sterilized, and used in bacterial cultivation as broth or solidified with agar (WWA).

1. INTRODUCTION

In natural aquatic environments bacteria are predominantly not free floating but grow as multi-species communities attached to submerged surfaces (O'Toole *et al*. 2000; Stoodley *et al*. 2002). A biofilm can be defined as a surface-attached (sessile) community of micro-organisms growing embedded in a self-produced matrix of extracellular polymeric substances (EPS).

Bacteria growing in a biofilm on a surface are generally more resistant to many antimicrobial agents than the same bacteria growing in a free-swimming (planktonic) state (Costerton *et al*. 1999; Donlan & Costerton 2002; Dunne 2002). The resistant characteristic of biofilms leads to persistent infections in the human body, as well as to troublesome biofilms in industrial processes. Biofilms including pathogenic bacteria growing inside the human body, e.g. in lungs or on implant surfaces (Donlan & Costerton 2002; Dunne 2002), or in drinking-water distribution systems can threaten human health (Szewzyk *et al*. 2000). In industrial processes biofilms cause malfunction of equipments, lower the efficiency of heat exchangers, and lower the end-product quality or safety in food industry (Carpentier & Cerf 1993; Flemming 2002).

Paper machines contain warm water rich in cellulose and various biodegradable additives, thus providing a good environment for the growth of many kinds of microbes (Blanco *et al*. 1996; Raaska *et al*. 1996; Salzburger 1996; Väisänen *et al*. 1998). Microbes can cause spoilage of raw materials and additives (pulp, mechanical pulp, recycled paper, starch, filler and pigment dispersions). Intense growth of spore-forming bacteria can cause problems with the hygienic quality of the end products in manufacturing of food-quality packaging paper and board. Volatile, malodorous metabolic products of microbes may enter the end products. According to Salzburger (1996) the most important economic loss caused by microbes in paper machines arises from the growth of biofilms, i.e., slime layers on the machine surfaces that are mostly made of stainless steel (Fig 1). The paper-machine biofilms are usually composed of bacteria, EPS produced by the bacteria, wood fibers, and miscellaneous paper making additives from the process. When the slimes grow thick they can reduce water flow in pipelines and block filters, wires, valves and nozzles. Fractions of the thick slime deposits can rip off, and these detached slime lumps can break the paper web or cause defects such as holes or colored spots in the end product.

Unless microbial growth is controlled, e.g. with biocides, these tiny organisms can bring the large, modern, computer-controlled, hi-tech paper machines – up to 9 meters wide and 200 m long – to a standstill (Salzburger 1996). Intensive use of biocides, however, can create resistant bacteria and most importantly, has limited effects, as the biocides are generally more effective against planktonic cells than against bacteria in biofilms (Czechowski & Stoodley 2002). Unfortunately, bacteria growing in biofilms seem to be much more problematic than planktonic bacteria for the runability of the paper machines. The fact that smoothly operating machines with a total count of planktonic bacteria of over 10^6 CFU ml^{-1} exists suggests that all bacterial species in paper machines are not problematic. Certainly some biocides are required to preserve the high quality of the raw materials in papermaking, but we believe that good runability could be achieved by targeted control of the most harmful biofilm bacteria (best biofilm-formers and filamentous bacteria) instead of the current approaches that aim at killing all bacteria. Complete eradication of all bacteria from a paper machine is difficult or even impossible. Maintaining the process

in a biological balance with harmless planktonic bacteria up to a certain threshold level, while only combating against the real troublemakers in biofilms, may actually be a more reasonable approach for the microbe control in paper machines. For that kind of process control a better understanding is needed of the initial attachment of microbes to surfaces and of the development of biofilms in connection with the responsible species. This was the field of study in Papers I-III.

Bacterial metabolism in biofilms may produce aggressive substances such as

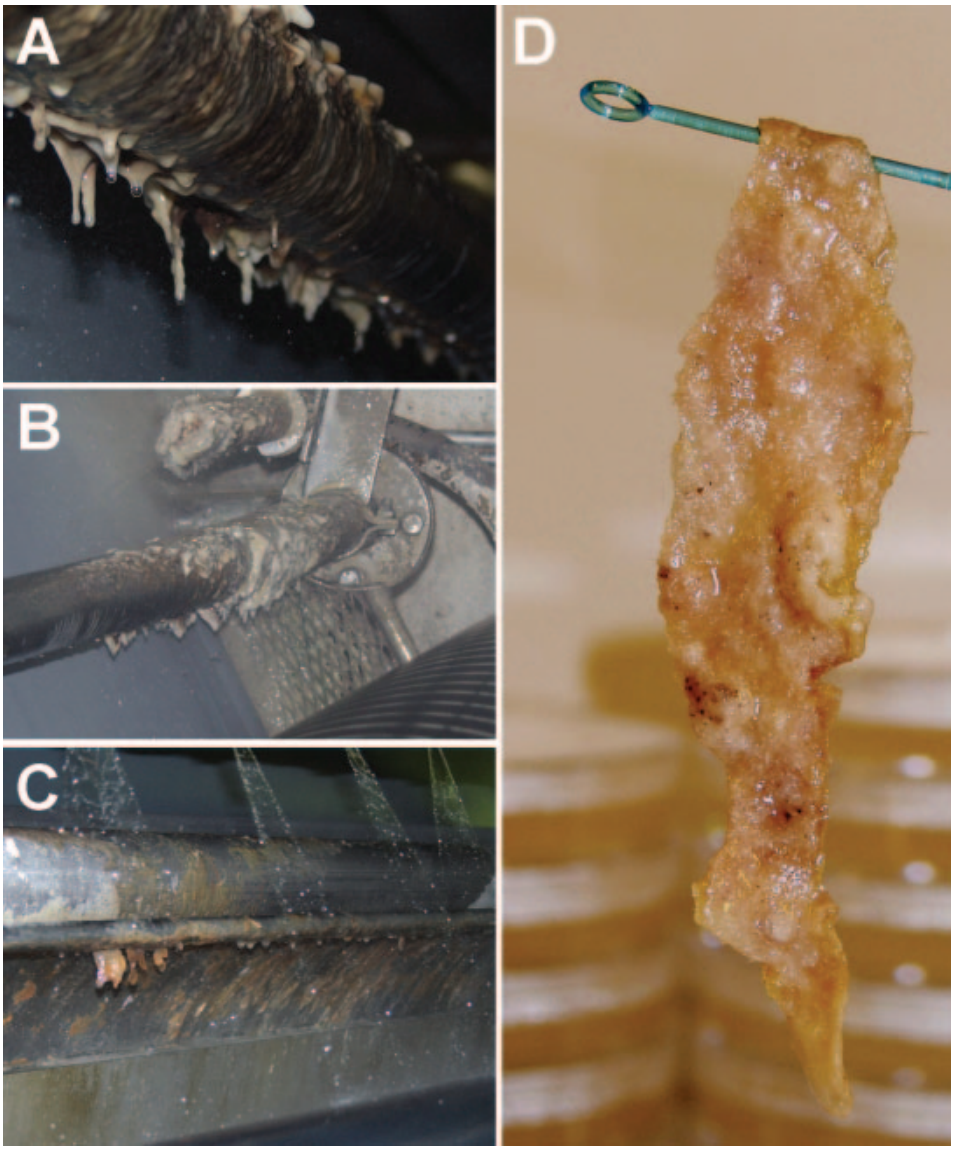


Figure 1. Photographs of biofilms (microbe slimes) that have grown on surfaces in the splash areas of the wet-end part of a paper machine (A-C). Areas that are constantly wet due to mist (B) or water sprays (C) are prone to be biofouled. Microbes can form coherent sheets of slime (D) that are likely to cause serious process disturbances if they detach from the machine surfaces.

acids, and the patchy nature of biofilms may generate regions with different oxygen content on the underlying steel surface. Both of these properties may promote corrosion (Borenstein 1994). This type of corrosion is called microbially influenced corrosion (MIC). It is not a "new" mechanism of corrosion, but merely indicates that bacterial activities have created conditions for "normal" corrosion to proceed. In natural waters corrosion of stainless steel is preceded by ennoblement, i.e. potential shift towards the noble direction (Mattila 2002). This ennoblement occurs during growth of biofilms, whose structure on stainless steel was examined (Paper IV).

1.1. Current consensus model of bacterial biofilms

Bacteria colonizing a surface produce EPS that will glue the cells to the surface and eventually form the biofilm matrix. Generally EPS are composed of polysaccharides, but may also contain proteins, nucleic acids and polymeric lipophilic compounds. In the terms of weight and volume EPS represent the major structural component of biofilms, being responsible for the interaction of microbes with each other as well as with interfaces (Flemming 2002; Neu *et al*. 2001).

As late as 1987, the biofilms were regarded as surface-attached microbes embedded in their EPS, growing in a selfish manner and producing unorganized slime layers on the surfaces (Stoodley *et al*. 2002). The current opinion is that biofilm formation is a much more complicated and also a well-controlled phenomenon (Fig 2). Increasing evidence exists that bacteria possess different growth modes and that cells in biofilms differ profoundly from planktonically growing cells of the same species (Kuchma & O'Toole 2000; Stoodley *et al*. 2002).

Stoodley *et al*. (2002) reviewed that biofilm formation can occur by at least three mechanisms: by the redistribution of surface-attached but motile cells, by the multiplication of attached cells, and by recruiting cells from the bulk fluid. The relative contribution of these mechanisms depends on the organisms involved, on the substratum, and on the environmental conditions. The maturation of a biofilm, resulting in the complex architecture with water channels, is influenced by a number of biological factors and by hydrodynamic features (Stoodley *et al*. 2002). The biological factors include cell-to-cell signalling between the biofilm bacteria, growth rates of the bacteria, extent of EPS production, motility of the biofilm bacteria as well as possible competition or cooperation between the bacteria.

A mature biofilm with its complex architecture provides niches with distinct physicochemical conditions, differing e.g. in oxygen availability, in concentration of diffusible substrates and metabolic side products, in pH, and in the cell density. Consequently, cells in different regions of a biofilm can exhibit different patterns of gene expression (Costerton *et al*. 1999). Mixed-species biofilms can contain niches with distinct groups of bacteria having metabolic cooperation (Kuchma & O'Toole 2000). The EPS matrix of a biofilm community can also contain microzones with different charge and hydrophobicity (Wolfaardt *et al*. 1998). Watnick and Kolter (2000) summarized that a mixed species biofilm is a dynamic community harboring bacteria that stay and leave with purpose, compete and cooperate, share their genetic material, and fill distinct niches within the biofilm. They stated, "The natural biofilm is a complex, highly differentiated, multicultural community much like our own city".

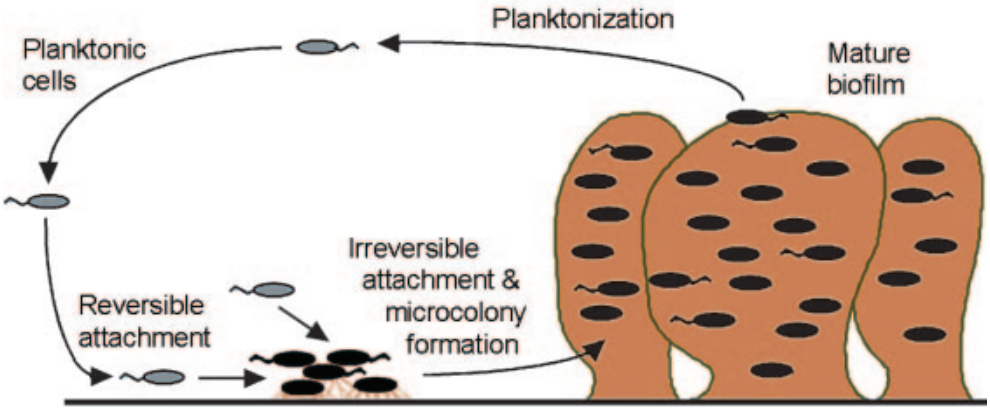


Figure 2. Model of biofilm development as a part of bacterial life cycle (O'Toole *et al*. 2000). Individual planktonic (free-swimming) cells can reach the surface by active or passive means. The initial cell-to-surface contact is often reversible, but if environmental cues and possible signalling molecules favor surface-attached growth, then the cells attach irreversibly with the aid of excreted EPS. Division of cells and growth of the population, while keeping cell-to-cell contacts, result in the formation of microcolonies. Mature biofilms often possess a hallmark architecture where microcolonies are surrounded by a network of water channels allowing the flow of nutrients into the interior of the biofilm. Hydrodynamics as well as cell-to-cell signalling molecules play a regulatory role in the development of the biofilm architecture (Stoodley *et al*. 2002). Stoodley *et al*. (2002) speculated that also the maintenance of open water channels in multi-species biofilms requires interspecies signalling to inhibit growth and EPS production into the channels. Some cells can be released to a planktonic lifestyle ensuring the occupation of new niches. The release of cells occurs due to physical detachment caused by shear forces, or due to a programmed set of events controlled by signalling molecules and leading to a local hydrolysis of EPS matrix, e.g. *P. aeruginosa* can cleave its own EPS by alginate lyase enzyme (image modified from O'Toole *et al*. 2000).

1.2. Biofilm mode of growth

Bacteria are capable of sensing surfaces (O'Toole *et al*. 2000). Contacting the surface initiates a complex differentiation program resulting in e.g. synthesis of alginate. Genes essential for production of this major EPS component of *Pseudomonas aeruginosa* were shown to be up-regulated already 15 min after attachment (Dunne 2002). Numerous changes in gene regulation cause the biofilm cells to become phenotypically and metabolically different from their planktonic counterparts (Kuchma & O'Toole 2000; Stoodley *et al*. 2002; Watnick & Kolter 2000; Whiteley *et al*. 2001). This difference has been persuasively shown for e.g. *Escherichia coli*, *P. aeruginosa*, *Pseudomonas putida*, and *Bacillus cereus* (Oosthuizen *et al*. 2002; O'Toole *et al*. 2000; Stoodley *et al*. 2002). *B. cereus* is a well-known food-poisoning organism (Granum & Lund 1997; Pirttijärvi 2000) that produces biofilms on stainless steel in protein-rich media such as milk (Lindsay *et al*. 2000; Peng *et al*. 2001). Oosthuizen *et al*. (2002) extracted all proteins expressed by planktonic cells of a *B. cereus* dairy isolate, and compared these to the protein composition of biofilm cells by 2D gel electrophoresis. The protein composition of cells that were harvested 2 h after

their attachment indicated expression of 15 unique proteins, supposedly required in the initial stages of biofilm development. The planktonic cells had high levels of 11 proteins that were absent in 2- and 18-h-old biofilms. Only 10% of the proteins were present in all growth phases and at the same level (no up- or down-regulation). Cells from the 18-h-old biofilm contained 7 unique proteins, whose identity suggested for e.g. fermentative metabolism in the biofilm (Oosthuizen *et al*. 2002). Similarly by 2D electrophoresis, *P. aeruginosa* has been shown to display at least three phenotypes with different protein profiles: a planktonic, a mature biofilm, and a dispersing biofilm phenotype (Stoodley *et al*. 2002). Whiteley *et al*. (2001) demonstrated by DNA microarray technology that in mature biofilms of *P. aeruginosa* genes e.g. for synthesis of flagella and the sigma factor RpoS were repressed, whereas genes encoding proteins for e.g. temperate bacteriophage, urea metabolism, membrane transport, translation and gene regulation were up-expressed.

1.3. Initial adhesion of bacteria

Initial adhesion between bacteria and non-living surfaces is usually mediated by non-specific (e.g. hydrophobic) interactions, whereas adhesion to living surfaces is usually accomplished through specific molecular docking mechanisms (Dunne 2002). Later on, during the biofilm growth, also on non-living surfaces cell-to-cell adhesion can be mediated by specific adhesins such as the polysaccharide intercellular adhesin (PIA) of *Staphylococcus epidermidis* (Dunne 2002; Rupp *et al*. 2001). Coaggregation of genetically distinct bacteria via specific molecules has been shown common in the development of oral mixed-species biofilms (Rickard *et al*. 2003).

An important phenomenon in the initial adhesion of bacteria to non-living surfaces, e.g. to stainless steel, is surface conditioning (Carpentier & Cerf 1993; Korber *et al*. 1995). It means that when a pristine surface is immersed, interactions between the surface and the liquid phase begin immediately. The surface will be modified by the adsorption of inorganic salts, proteins, glycoproteins, and humic compounds etc., depending on the environment. The conditioning film is formed rapidly, as significant organic deposits have been detected after only 15 min. Film thicknesses ranging from 30 to 80 nm have been measured (Korber *et al*. 1995). In practise, most bacteria moving from the bulk aqueous phase towards a surface have their primary contact with a conditioned surface. Once a surface has been conditioned, its properties such as hydrophobicity are often permanently altered, so that the affinity of an organism for a native and a conditioned surface can be quite different (Carpentier & Cerf 1993; Dunne 2002).

The interaction between the cell and the conditioned surface is dictated by physicochemical variables, which are explained by two different theories (Korber *et al*. 1995). The "wetting" or the "surface free energy/hydrophobicity" theory is based on surface thermodynamics. If the total free energy of the system is reduced by cell contact with a surface, then adsorption will occur. This theory relies on determining critical surface tension of the bacteria and substratum, and is not taking electrostatic interactions into account (Carpentier & Cerf 1993). The extended DLVO theory equates repulsive and attractive forces acting on an adhering particle (Busscher *et al*. 1998; Korber *et al*. 1995; Poortinga *et al*. 2001). Microbial adhesion is described as a balance between attractive Van der Waals forces, electrostatic forces (often repulsive as most bacteria and conditioned surfaces are negatively charged), short range Lewis acid-base interactions and Brownian motion forces.

Magnitudes of these forces are affected by the distance of the bacterium from the surface (long-range and short-range forces) and by ionic strength. The theory predicts that there are two regions where attraction may occur (primary and secondary minimum, <1 nm and 5-10 nm from the substratum). Between these two positions there is a repulsive energy barrier. Generally it is assumed that bacteria adhere reversibly to the "secondary minimum" and irreversibly to the "primary minimum" with the aid of cell surface appendages or EPS that can pierce the repulsive energy barrier. Poortinga *et al*. (2001) showed that bacteria forced to adhere in the primary minimum by application of a high positive electrode potential could hardly be stimulated to desorb, indicating strong irreversible adhesion.

1.4. Bacterial behavior on surfaces

Once in initial contact with a surface, microbes develop different types of attachment behaviours (Korber *et al*. 1995). Motile attachment behaviour of *P. fluorescens* allows the flagellated cells to move along surfaces in a semi-attached condition within the hydrodynamic boundary layer, independent of the flow direction (Korber *et al*. 1995). Reversible adhesion of *E. coli* cells with residence times of over 2 min on a surface has been described as "near-surface swimming" (Vigeant & Ford 1997).

The expression "irreversible attachment" is used when microbes can no longer move perpendicularly away from the surface (Busscher *et al*. 1998). It is assumed that most microbes become irreversibly attached only after a period of unstable, reversible adhesion, during which the cells can show motion such as the rotating motion of apically adherent cells of *P. fluorescens* (Korber *et al*. 1995). *Vibrio cholerae* and *E. coli* first utilize the flagella to spread across the surface, and then anchor onto the surface with pili and possibly outer membrane proteins (O'Toole *et al*. 2000). *P. aeruginosa* requires type IV pili for twitching motion on a surface and for the subsequent build-up of stagnant microcolonies (O'Toole *et al*. 2000).

Microbes can attach irreversibly, while retaining active motility by mechanisms known as gliding, swarming, twitching, swimming, darting and sliding. Uropathogenic *E. coli* cells were shown to attach irreversibly and yet actively migrate along solid surfaces (Harkes *et al*. 1992). It was generally assumed that the biofilm structures in mature biofilms are rigidly "cemented" to their place on a surface until they age and detach. This view was challenged when time-lapse microscopy showed downstream migration of ripple-like biofilm structures of mixed species growing in turbulent flow (Stoodley *et al*. 1999). Tolker-Nielsen *et al*. (2000) showed that cells of *Pseudomonas* sp. move actively by their flagella inside and between the microcolonies of a developing biofilm.

1.5. Effect of surfaces on attachment

A multitude of studies exist where bacterial attachment has been studied with different bacterial species, to different surfaces, and in different ionic strength, flow or nutrient conditions. For example the adhesion of *Bacillus* spp. has been studied extensively due to their deleterious impact in the dairy industry. *Bacillus* spores adhered as monolayers on many kinds of surfaces, hydrophobic spores of *B. cereus* being the most adhesive (Husmark 1993).

The ultimate goal in the attachment studies has been to invent colonization resistant surfaces. While surfaces or surface coatings that retard bacterial adhesion have been described, none has been developed that totally prevent it (Dunne 2002). In 1995 Costerton and Lappin-Scott made an apt remark: ”For the past 15 years the corridors of Academe have echoed to the exuberance of scientists who are confident that they have discovered a material whose surface is inherently resistant to bacterial colonization. Laboratory tests have been uniformly encouraging and practical tests have been equally uniformly disappointing, and we believe that we can now conclude that bacteria will eventually adhere to and colonize the surface of any man-made material.”

There are reasons for this pessimism. Most importantly, bacteria are not constant inanimate particles, but living organisms that can adapt to the requirements set by their changing environment and alter their surface composition. Bacterial cell surfaces are generally hydrophobic, but the cells can possess hydrophilic appendages. The net charge of most bacteria is negative, but e.g. *Stenotrophomonas maltophilia* has positive surface charge at physiological pH (Dunne 2002). The cells can also have polarity in their charge. Poortinga *et al*. (2000) showed that in a field of direct current the rod-shaped cells of *Actinomyces naeslundii* adhered flat on the indium tin oxide surface, and in an alternating current field the cells adhered parallel to the field. Adhesiveness of bacteria also varies with different growth phases (Flint *et al*. 2001; Husmark 1993; Peng *et al*. 2001). And eventually, the surface properties of any novel “colonization-resistant” surface will be gradually altered by adsorption of salts, proteins, glycoproteins and other molecules from the environment.

1.6. Eukaryotic organisms in biofilms

In addition to bacteria, other organisms such as fungi, diatoms and other algae, amoebae and ciliates can be found in biofilms, but their quantity depends on the specific environment. Within biofilms in drinking-water distribution systems *Legionella* spp. may be enclosed in cysts of amoebae and thereby be protected from chlorination (Szewzyk *et al*. 2000). Except for the clinically important species, fungal biofilms have received little attention. The fungal species most commonly isolated from medical devices (catheters, heart valves and voice prostheses) is the dimorphic yeast *Candida albicans* (Kumamoto 2002). It forms biofilms composed of a layer of cells in the yeast form adhering to the substratum and, above this, a layer of cells in the hyphal form surrounded by an extensive EPS matrix. The most notable phenotypic attribute of the fungal cells within a biofilm is their increased resistance to antibiotics, strikingly similar to the biofilm phenotype of bacteria. The resistance may arise from contact-dependent expression of drug efflux genes not expressed in planktonic cells (Kumamoto 2002).

Biofouling of industrial processes is frequently caused by mixed-species biofilms. Reports exist on the presence of fungi in these biofilms, but only a few detailed studies have been performed despite the fact that fungi are excellent colonizers of surfaces (Jones 1995). Elvers *et al*. (1998 & 2002) showed that strains of *Fusarium oxysporum*, *F. solani* and *Rhodotorula glutinis* isolated from photo-processing industry were capable of forming biofilm as pure cultures and in mixed-cultures with bacteria. Mixed fungal–bacterial biofilms offered greater protection against isothiazolone biocide compared to single species biofilms.

1.7. Bacterial cell-to-cell communication

Bacteria are often considered as simple unicellular organisms, but research has recently shown that many bacteria possess ability to communicate with one another and to organize into communal groups with characteristics not exhibited by individual cells (Greenberg 1999). Bacteria produce diffusible extracellular signalling molecules, e.g., acylated homoserine lactones (AHLs; gram-negative bacteria) and oligopeptides (gram-positive bacteria), to monitor their own population density and to coordinate expression of specific sets of genes in response to the cell density. This type of cell-density-dependent gene regulation is termed quorum sensing. The AHL compounds of gram-negative bacterial species differ from another in length and substitutions on their acyl side chain. AHLs are constitutively synthesized in minute amounts and their concentration in the cell surroundings is monitored. At low cell density the AHLs are diluted in the medium, but at sufficient population densities these signalling molecules reach the threshold concentration required for gene activation.

It has been found that AHLs are involved in regulation of a wide range of cell functions, including bioluminescence in *Vibrio* species, conjugal transfer of Ti plasmid in *Agrobacterium tumefaciens*, production of virulence factors in *P. aeruginosa* and several other species, fruiting-body formation in *Myxococcus xanthus*, swarming motility in *Serratia liquefaciens*, and production of extracellular hydrolytic enzymes in many species (Dong *et al*. 2002; Greenberg 1999; Hentzer *et al*. 2002; O'Toole *et al*. 2000; Riedel *et al*. 2001). Unlike AHLs, which are mostly considered species-specific, a recently discovered boron-containing quorum-sensing molecule is produced by a large number of bacterial species and proposed to serve as a "universal" signal for inter-species communication (Chen *et al*. 2002).

What do bacteria benefit from the quorum sensing? They can limit production of a set of molecules to situations when these molecules are really required. This is beneficial e.g. for pathogenic bacteria because they do not need to reveal their weapons (exotoxins) before they have massively colonized the host and can thus overwhelm the host defenses (Greenberg 1999).

At times it may be useful for one group of bacteria to disrupt quorum sensing of a second competing group of bacteria, thereby gaining a competitive advantage in the environment. All of the tested strains of *Bacillus thuringiensis* and the closely related species *B. cereus* and *B. mycoides* produced a lactonase that inactivates AHL activity by hydrolysing the ester bond of the homoserine lactone ring (Dong *et al*. 2002). Transgenic plants expressing AHL lactonase quenched cell-to-cell signalling of pathogenic bacteria and showed enhanced resistance to infection (Dong *et al*. 2001).

1.8. Cell-to-cell signalling in bacterial biofilms

Davies *et al*. (1998) published the first study that showed a role for quorum sensing in the formation of biofilms, and launched a period of active research of cell-to-cell signalling in biofilms. Davies *et al*. (1998) showed that *lasI*$^-$ mutant cells of *P. aeruginosa* that were unable to synthesize $3OC_{12}$-HSL (3-oxododecanoylhomoserine lactone) were able to attach and initiate the biofilm formation similar to the wild type cells, but the mature biofilms were continuous sheets lacking the differentiated architecture with microcolonies and water channels. The biofilms were also sensitive to SDS in contrast to the wild type

biofilms. When $3OC_{12}$-HSL was added the *lasI*$^-$ mutant cells formed biofilms that resisted the detergent wash and had architecture similar to the wild type biofilms. Similarly, biofilm development of *Aeromonas hydrophila* and *Burkholderia cepacia* involved AHL-mediated signalling (Conway *et al*. 2002; Lynch *et al*. 2002). It has been shown that addition of 7,8-cis-tetradecenoyl-HSL to aggregates of *Rhodobacter sphaeroides* mutant cells caused cells to disperse and to grow as individual cells in suspension (Greenberg 1999). Similarly, AHLs and/or another factor present in stationary-phase culture supernatants mediated a reduction of *P. fluorescens* biofilm and loss of EPS (O'Toole *et al*. 2000). These studies suggest that AHL signals may be involved in biofilm dispersal as well. In mixed-species biofilms AHLs have been shown to mediate interspecies communication (Riedel *et al*. 2001).

The Australian macroalga *Delisea pulchra* produces halogenated furanone compounds that interfere with the AHL-mediated quorum sensing, and in this way protect the macroalga from bacterial fouling (Hentzer *et al*. 2002). Addition of a synthetic furanone compound made *P. aeruginosa* biofilms thinner and less virulent, and enhanced bacterial detachment. The furanones are attractive candidates for biofilm control in the future.

1.9. Microbes in paper machines

Blanco *et al*. (1996) reviewed the diverse microflora that has been observed in paper industry. It includes aerobic spore-forming bacteria (*Bacillus*), aerobic non-sporulating bacteria (e.g. *Acinetobacter, Alcaligenes, Klebsiella, Flavobacterium, Leptothrix, Micrococcus, Pseudomonas,* and *Staphylococcus*), anaerobic bacteria (e.g. *Desulfovibrio*), moulds (e.g. *Aspergillus*), yeasts and occasionally also algae.

Väisänen *et al*. (1998) performed a thorough examination of microbial communities of a printing paper machine. They isolated 390 strains of aerobic bacteria representing at least 34 species, thus demonstrating the large number of bacteria living in a single paper machine. The most frequent contaminants of the machine wet-end were species of genera *Bacillus, Burkholderia, Pantoea, Ralstonia,* and *Thermomonas*. Chaudhary *et al*. (1997) reported that *B. alvei* and *Aerobacter aerogenes* were the most prevalent contaminants in an Indian paper mill. In a recent study Oppong *et al*. (2000) isolated pink-pigmented bacteria *Deinococcus grandis*, *Flectobacillus* sp., *Methylobacterium zatmanii*, *Micrococcus* sp., and *Roseomonas* sp. from slime deposits of American paper machines. Harju-Jeanty & Väätänen (1984) showed that the filamentous bacterium *Sphaerotilus natans* can produce slimy aggregates in groundwater pulp (1%) at 28°C, but it was not growing above 40°C (Pellegrin *et al*. 1999), making it an unlikely biofilm-former in modern paper machines with operation temperatures near 50°C (see e.g. Table 4.4). Raaska *et al*. (2002) observed that contamination of starch-based glues with spore-forming bacteria (e.g. *Bacillus* and *Paenibacillus*) and enterobacteria (e.g. *Citrobacter* and *Enterobacter*) was the most important factor threatening the process hygiene and product safety in one machine manufacturing refined paper products for food packaging. Generally, the major contaminants of the dry paper products seem to be limited to three genera of spore-forming bacteria: *Bacillus*, *Brevibacillus* and *Paenibacillus* (Hughes-van Kregten 1988; Pirttijärvi *et al*. 1996; Raaska *et al*. 2002; Suominen *et al*. 1997; Väisänen *et al*. 1998).

1.10. Prevention of biofilms in paper machines

Detrimental microbial growth in paper machines is controlled by dispensing broad-spectrum biocides into the process. Total sales of slime prevention chemicals (slimicides) in Finland were over 1400 tons in the year 2001 (Fig 3). The growth was over 240% since the year 1994. Especially the use of organic bromine compounds has increased being almost 600 tons in year 2001 (Fig 3). Consumption of glutaraldehyde as well as "other compounds" including peracetic acid and hydrogen peroxide has also increased.

Biocides possess different mechanisms of antimicrobial activity. Glutaraldehyde reacts with amino and thiol groups in proteins causing irreversible cross-links in the cellular constituents (Paulus 1993). In gram-negative bacteria glutaraldehyde interacts principally with outer components of the cells, notably lipoproteins (Maillard 2002). High degree of cross-linking means that the cells are unable to perform their essential functions, resulting in bactericidal effect. Oxidizing biocides used are e.g. peracetic acid, hydrogen peroxide, and BCDMH (1-bromo-3-chloro-5,5-dimethylhydantoin). BCDHM is not biologically active as such, but its rapid hydrolysis yields hypobromic and hypochloric acids (Kemira Chemicals Oy, 2003). Glutaraldehyde and oxidizing biocides are effective also against bacterial spores (Paulus 1993). Methylene bisthiocyanate (MBT) chelates Fe^{3+} ions essential for the microbial growth (McCoy 1983). Isothiazolones (5-chloro-2-methyl-4-isothiazolin-3-one & 2-methyl-4-isothiazolin-3-one in a mixture) and 2,2-dibromo-3-nitrilopropionamide (DBNPA) are electrophilic active compounds reacting with cytoplasmic constituents such as thiol groups of proteins, thus inhibiting cellular metabolism (Millard 2002; Paulus 1993). Bronopol (2-bromo-2-nitro-propane-1,3-diol) also contains an active halogen group, but can release formaldehyde as well (Paulus 1993). Dazomet (3,5-dimethyl-1,3,5-2H-tetrahydrothiadiazine-2-tion) is rapidly hydrolyzed in water to methylene isothiocyanate (Kemira Chemicals Oy, 2003), but can also release formaldehyde (Paulus 1993).

Biocides are generally toxic with low biological selectivity. Some like DBNPA, isothiazolone mixtures, glutaraldehyde and MBT are also sensitizing (Kemira Chemicals Oy 2003; Paulus 1993; Pirttijärvi 2000). Many of the currently used biocides such as peracetic acid, hydrogen peroxide, BCDMH, DBNPA, glutaraldehyde or isothiazolone mixtures are reactive molecules that are rapidly (bio)degraded to non-toxic molecules. Thus these biocides are not detrimental for the biological wastewater treatment processes and are not persistent (bioconcentrated) in the environment (Kemira Chemicals Oy 2003; Paulus 1993).

Supplementary to the biocides different surface-active agents, non-toxic biodispersants, hydrolytic enzymes, or chelating agents are applied for controlling slime deposits (Blanco *et al.* 1996; Johnsrud 1997). Enzymes hydrolysing EPS to lower-molecular-mass soluble forms can eliminate biofilms directly by releasing cells from the slime matrix, and indirectly by increasing exposure of bacteria to biocides. In practise the enzymes have not performed sufficiently well to provide a full-scale alternative to the biocides (Flemming 2002; Johnsrud 1997). The non-ionic biodispersants can inhibit or reduce biofilm formation, and improve penetration of biocides into the biofilm (Blanco *et al.* 1996). Recently an electrochemical method was developed for the prevention of biofilms on stainless steel surfaces (Savcor Process Oy, 2003).

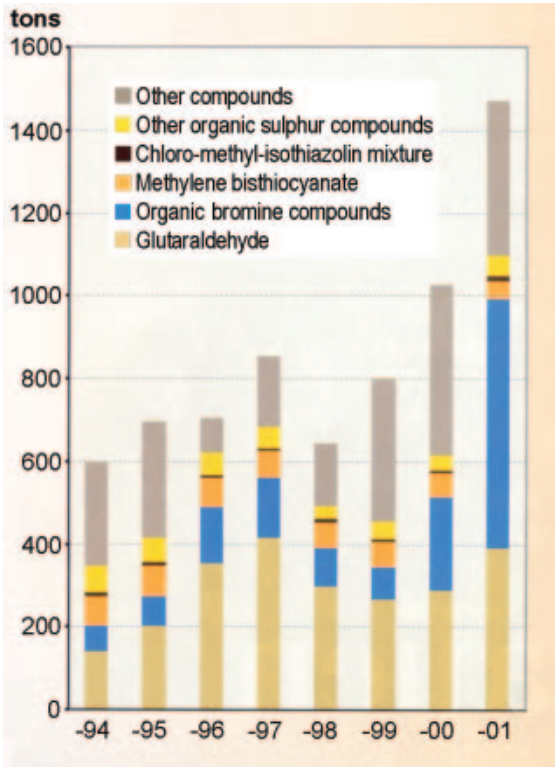


Figure 3. Total sales of slime prevention chemicals in Finland from the year 1994 to the year 2001. The group "other compounds" includes non-halogenated organic compounds, peracetic acid, and hydrogen peroxide (modified from Finnish Environment Institute, 2002).

1.11. Resistance of biofilms to antimicrobials

Bacteria in a mature biofilm are far more resistant to antimicrobials (biocides and antibiotics) than freely swimming cells. Different mechanisms have been proposed to account for this increased resistance that is most likely multifactorial (Costerton *et al*. 1999; Czechowski & Stoodley 2002; Donlan & Costerton 2002; Dunne 2002; McBain *et al*. 2002; Stoodley *et al*. 2002; Watnick & Kolter 2000; Whiteley *et al*. 2001). EPS may form

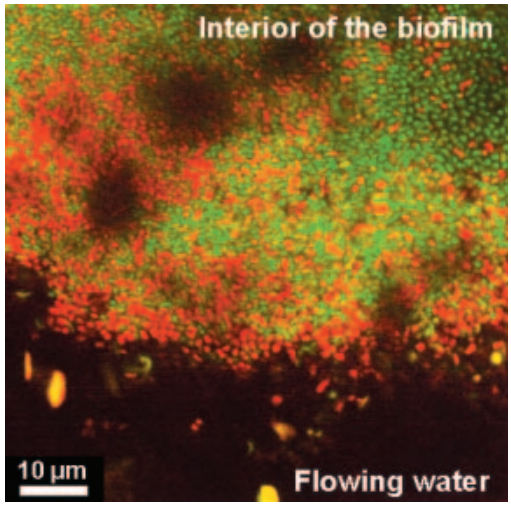


Figure 4. CLSM image of a biofilm grown in 5 d on stainless steel surface in the shower water line of a fine paper machine. The biofilm was stained with LIVE/DEAD® bacterial viability stains *in situ* on a stainless steel test piece to examine the spatial distribution of viable and dead cells (green and red fluorescence, respectively). Biocides applied in the paper machine had not prevented the biofilm formation. Biocides had killed most of the cells in interface of the biofilm and the flowing process water, but in interior of the biofilm viable cells were abundant (modified from Mattila *et al*. 2002).

permeability barriers or make complexes with the antimicrobials thus interfering with the antimicrobial action. Reactive oxidants may be deactivated in the outer layers of EPS faster than they diffuse. Extracellular enzymatic activity inside the biofilm may be high enough to destroy the antimicrobials. Different microenvironments existing at the deepest biofilm layers with altered pH, pCO_2, pO_2, cation concentrations etc., may affect the activity of antimicrobials. Bacterial cells of the biofilm phenotype may have reduced susceptibility because of altered cellular permeability, metabolism or growth rate. Das *et al*. (1998) reported changes in susceptibility that were greater than twofold and occurred immediately on attachment, and also in the presence of biocide concentrations which exceeded the minimum inhibitory concentrations for the planktonic cells.

Grobe *et al*. (2002) quantified the degree of biofilm resistance by calculating a resistance factor that compared killing times for biofilm and planktonic cells in response to the same concentration of biocides. The resistance factors averaged 29 for DBNPA, 34 for glutaraldehyde, 120 for chlorine and 1900 for a quaternary ammonium compound. This indicates that data on antimicrobial efficacy obtained with planktonic bacteria are not reliable indicators of biocide performance against biofilms. A part of the biofilm resistance could be attributed to incomplete penetration of chlorine, glutaraldehyde and DBNPA due to neutralizing reactions with EPS. The results also implicated presence of a tolerant subpopulation for the quaternary ammonium compound.

Resistance of biofilms to biocides is also observed in paper machines (Fig 4; Mattila *et al*. 2002). Due to the biofouling paper and board machines require regular operation breaks, during which sprays of pressurized water and alkaline washing-solutions are applied to remove the persistent slime-deposits formed on the machine surfaces during the run.

1.12. Current methodology in biofilm studies

Lawrence *et al*. (2002b) reviewed extensively the methods applicable to studying biofilms. Therefore only three of the methods used in this study are described in detail below.

1.12.1. Atomic force microscopy (AFM)

Electron microscopy allows high-resolution imaging of cell surfaces, but requires fixing, staining, and drying of the cells, and then imaging under vacuum. These manipulations may induce irreversible changes in soft biomaterials (Dufrene 2002). AFM is the only technique that can provide 3D images of surface ultrastructures with molecular resolution, in real time, under physiological conditions, and with minimal sample preparation (Dufrene 2002). Single proteins can be observed at a resolution better than 1 nm (Fotiadis *et al*. 2002). AFM is also more than just a surface-imaging tool, as it allows force measurements revealing physical properties of the specimen surface, such as hydrophobicity, charge and mechanical properties (Dufrene 2002).

The key element of an AFM is a very sharp probe mounted at the end of a flexible cantilever (Fig 5). When the probe is brought close to the specimen, interaction forces between the probe and the specimen surface cause deflection of the cantilever. The larger the deflection, the higher is the force experienced by the probe. The probe is scanned in *x* and *y* directions over the sample surface, and the cantilever deflections measured using a laser beam and a photodiode, yielding an AFM topography image of the specimen surface.

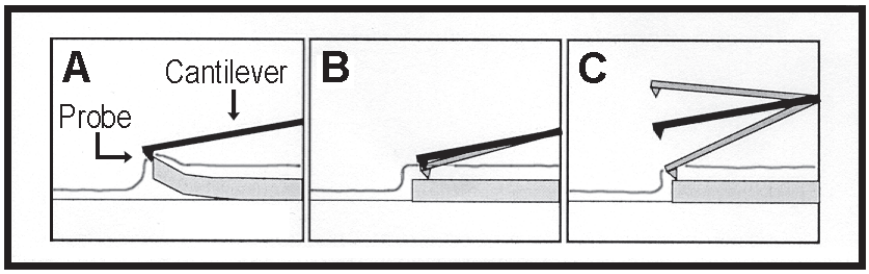


Figure 5. AFM imaging modes and their effects on the specimen surface. (A) In the contact mode imaging the probe (tip) is in intimate contact with the specimen surface and due to frictional forces the contact mode has tendency to damage or sweep away soft or weakly immobilized objects. In the non-contact mode (B) an oscillating probe is held a small distance above the surface and Van der Waals forces acting between the tip and the specimen are detected. In the tapping mode imaging (C) an oscillating probe is only intermittently contacting (tapping) the surface, and thus *x*-*y* movements of the probe in scanning produce less lateral shear forces to the specimen than the other scanning modes (image modified from Prater *et al*.).

AFM imaging modes can be classified as "contact" and "non-contact" depending on the distance between the probe and the specimen surface (Fig 5). In the traditional "contact mode AFM" the sharp tip is brought so close to the surface that repulsive forces between the sample and the tip dominate (Beech 1996a). The tip is scanned over the sample while maintaining a constant interatomic force between the tip and the sample surface, and the movement of the tip in *z*-direction is converted to a topography image. The contact mode imaging exerts forces large enough to distort delicate biological specimens (Fotiadis *et al*. 2002).

In the "non-contact AFM" the probe is oscillated vertically above the surface at a distance where the attractive forces are dominant (Beech 1996a). Oscillation of the probe at resonance frequency is produced acoustically or magnetically. During scanning over the specimen the proximity of the specimen surface is revealed by changes in the cantilever amplitude, phase or frequency. The cantilever moves in *z*-direction in order to keep amplitude reduction constant, resulting in a topography image of the specimen. Amplitude images are resulting from the feedback loop required to keep this amplitude reduction constant. The phase images show the phase shift occurring between the free oscillation of the tip and the reduced oscillation in the vicinity of the surface, the latter depending on the viscoelastic properties of the sample surface and on the adhesive potential between the sample and the tip. Oscillating the scanning probe during imaging reduces the destructive forces and enhances reproducibility (Han *et al*. 1996), but the resolution is lower than that achieved by the "contact mode AFM" (Fotiadis *et al*. 2002). In the "tapping mode AFM" the oscillating probe is intermittently brought in contact with the surface ("tapping") to provide high resolution, and then lifted off the surface to avoid dragging the tip across the surface, therefore the tapping mode produces less lateral shear forces to the specimen than the other imaging modes (Fotiadis *et al*. 2002).

Different cell surface structures (S-layers, purple membranes etc.) have been imaged at sub-molecular resolution as reconstituted monolayers on flat surfaces (Dufrene 2002). Surface structures of living fungi, e.g., rodlets on the surface of *Aspergillus oryzae*

spores and their changes upon germination, have been imaged at near-molecular resolution, but achieving such resolution with soft cells of yeast and bacteria remains challenging due to sample deformations (Dufrene 2002). Unlike many other biological specimens, AFM studies of bacterial biofilms do not require specimen immobilization, because the EPS facilitate attachment to the substratum (Beech 1996a). While information on biofilms in the hydrated state has not been obtained at molecular resolution due to high sample softness, direct visualization of attached bacteria and their EPS has been reported (Bremer *et al*. 1992; Dufrene 2002; Telegdi *et al*. 1998). Grantham and Dove (1996) used AFM in fluid tapping mode to investigate the interactions of *Shewanella putrefaciens* with iron coatings on silica glass surfaces. On uncoated silica glass the adherent bacteria failed to withstand the lowest imaging forces in the fluid tapping mode and detached, whereas they were more strongly attached to the Fe-coated silica glass and successfully imaged.

1.12.2. Confocal laser scanning microscopy

Epifluorescence microscopy and confocal laser scanning microscopy (CLSM) are both based on the usage of fluorescent stains that are excited by light and the resulting fluorescence emission is detected. Thickness of the specimens often limits the suitability of the epifluorescence microscopy for biofilm studies. When focusing the light beam inside a biofilm specimen with a standard epifluorescence microscope, fluorescent objects below and above the focal plane are also excited and their emission is detected as unsharp objects yielding a blurred image. Unlike the epifluorescence microscope, the confocal microscope only records information coming from the selected focal plane. Unsharp information from the other planes is removed by a pinhole (a small diaphragm) allowing the fluorescence only from the focal plane to enter into the detector (Lawrence *et al*. 2002a). The operation principle of the CLSM is illustrated in Figure 6 and effect of the pinhole is demonstrated in Figure 7.

The benefits of CLSM in comparison to the other light microscopic methods (demonstrated in Figure 8) have made it a popular tool in the study of biofilms (Donlan and Costerton 2002; Dunne 2002; Lawrence *et al*. 2002a & 2000b; Mattila 2002; Neu *et al*. 2001; Tolker-Nielsen *et al*. 2000). Stoodley *et al*. (2002) defined that the modern biofilm era began when the first CLSM images of living biofilms showed the presence of open water channels between the discrete microcolonies in which bacterial cells grew embedded in a matrix of EPS (Lawrence *et al*. 1991). This revolutionized the understanding of biofilms leading to the famous “mushroom model” of biofilm architecture (Costerton *et al*. 1994) depicted in the Figure 2.

Figure 7. (Opposite page, lower image) Example on the effect of the confocal pinhole. Two optical sections were recorded from the same spot of a packaging board specimen stained with acridine orange. Image A was recorded with the pinhole open and for image B the pinhole was optimally closed to record fluorescence from the focal plane only. Contaminant bacteria between the fibers were visible only when the pinhole was closed to cut-off the out-of-focus fluorescence.

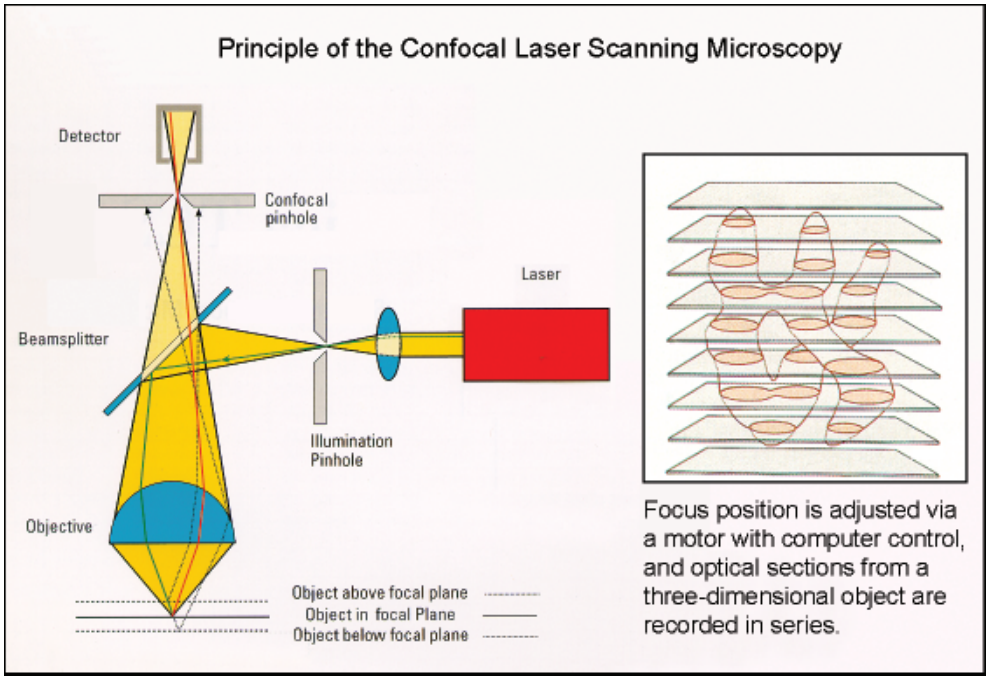
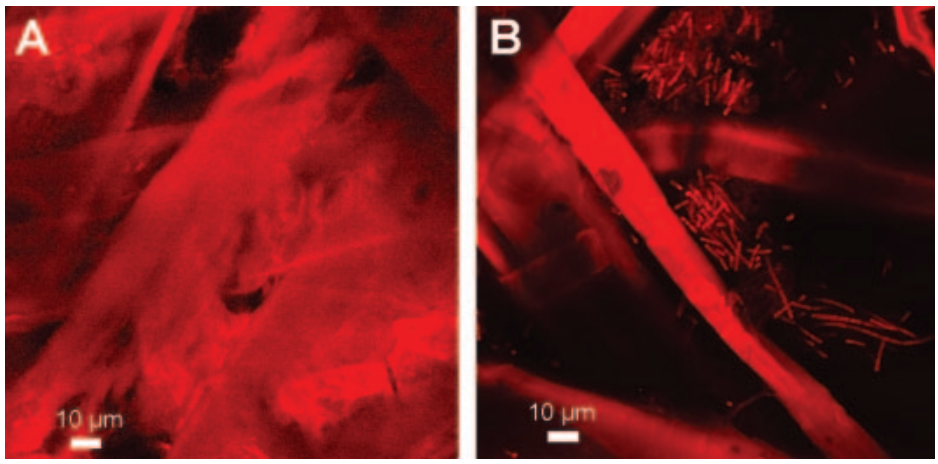


Figure 6. Principle of the confocal microscopy. The excitation laser beam is directed to the specimen through an illumination pinhole to create an illuminated point in the specimen. With motorized scanning mirrors the laser beam is scanned over the specimen pixel-by-pixel. Fluorescence emitted from the sample is directed to the detector via a pinhole allowing fluorescence only from the focal plane to be recorded (fluorescence from the focal plane is indicated by a red line). Optical sections at constant intervals can be recorded from a 3D object by adjusting the focus via a motor with computer control. The data set of optical section in series can be exploited in many ways. The simplest way of visualizing 3D information is a gallery of optical sections. The entire data set can also be viewed as a sum projection (extended depth of focus image). Also orthogonal sections, stereo images or rotary animations can be calculated (images modified from Zeiss Information with Jena Review 5/95 and from Leica TCS SP information brochure 1998).



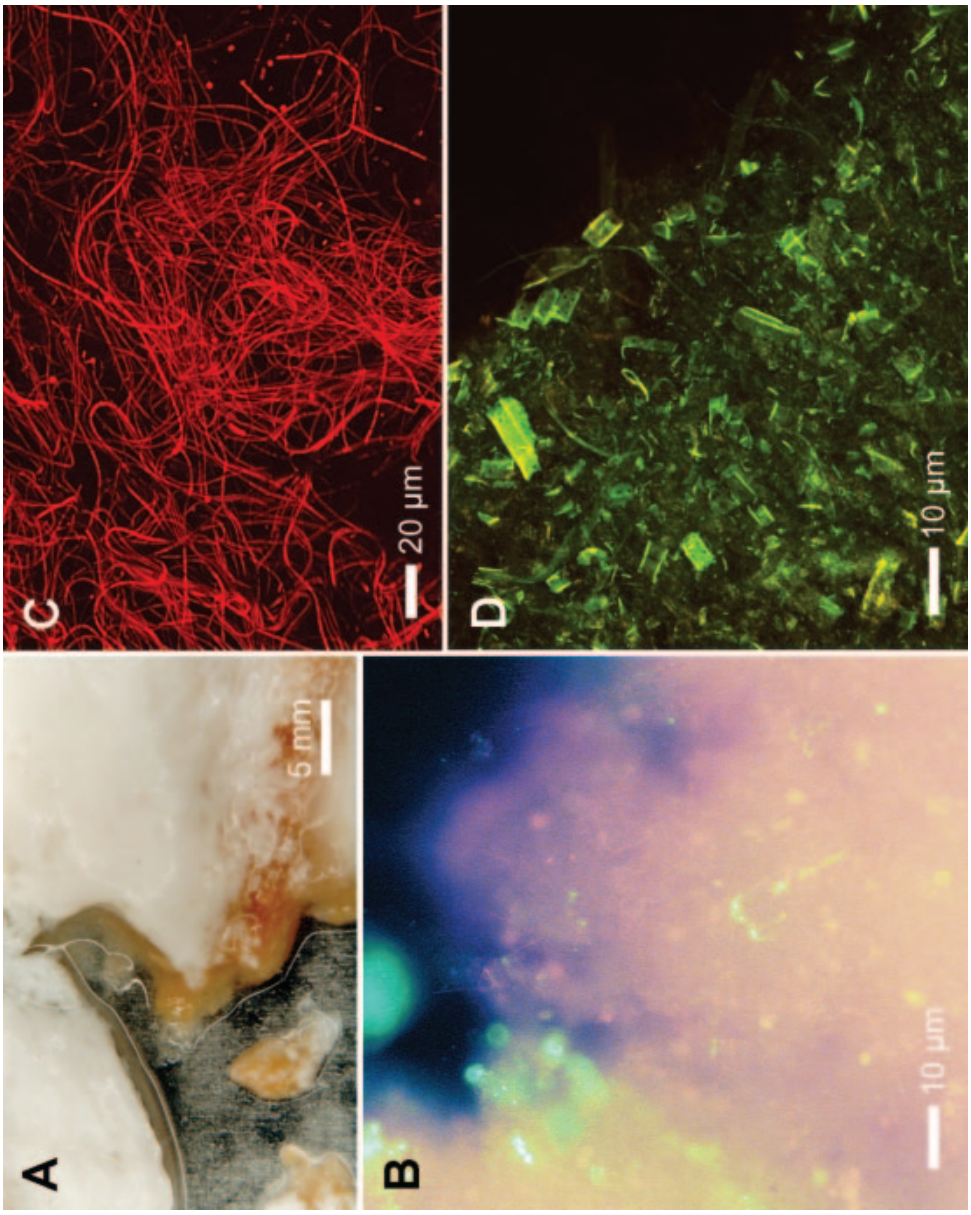

A
5 mm
B
10 µm
C
20 µm
D
10 µm

Figure 8. (Opposite page) The four pictures illustrate the major benefit of CLSM in the study of biofilms from paper industry. Stereomicroscope image (Panel A) shows the layered structure of an orange-colored slime that grew on a stainless steel test piece immersed in the flow of clear filtrate in a fine paper machine. Epifluorescence microscope image (Panel B) obtained from such a thick slime is unclear; focusing the objective into the interior does not resolve the objects because fluorescence emission from the whole thick specimen is masking all details. The slime was stained with the nucleic acid stain EtBr resulting into the pale red fluorescence; the greenish fluorescence is caused by autofluorescent material in the slime. By CLSM the sample can be sectioned optically and inner details can be seen also from a biofilm over 100 µm in thickness. CLSM image (Panel C) clearly shows the abundance of filamentous bacteria inside a thick slime grown on a polyethylene test plate during a 6-week immersion in white water line of a paper machine. The image shows the sum of five thin optical sections (z sum = 8 µm) from the interior of the slime deposit (stained with EtBr, excited by laser light at 488 nm). By CLSM it is also easy to detect whether a deposit is of microbiological origin or is a chemical deposition – a question that is often difficult to answer by other microscopic methods. As an example Panel D shows an optical section (z = 1.5 µm) from a deposit that contained only a few bacteria but was rich in process fines and fillers. This deposit was stained with the nucleic acid stain acridine orange and excited at 488 nm.

1.12.3. Fluorescence *in situ* hybridization (FISH)

Traditional cultivation methods are time-consuming and the choice of substrates for cultivation will inevitably cause selection. Frequently only a minority of the bacteria present in the sample will grow (Amann 2000; Amann *et al*. 2001). Molecular biology has evidenced that the cultivation dependent methods sometimes fail to disclose even the most abundant microbes in a given ecosystem (Amann 2000). Amann *et al*. (1995) reviewed that the percentage of culturable bacteria in comparison with total cell counts (microscopic) varied in different habitats, ranging e.g. in samples from a mesotrophic lake from 0.1% to 1%. Therefore a culture-independent method for rapid and specific identification of microbe cells within their natural environment was long awaited.

Fluorescence *in situ* hybridization (FISH) with rRNA-targeted (ribosomal RNA) oligonucleotide probes, "phylogenetic stains", is now a well-established technique for the direct, cultivation-independent identification of individual bacterial cells (Amann *et al*. 2001). In this technique the target organisms in a sample are specifically stained (hybridized with specific oligonucleotide probes with fluorescent label) and subsequently detected by fluorescent microscopy (epifluorescence microscopy or CLSM), providing information both on the number and spatial distribution of the target microorganisms within a few hours, independent of whether or not they can be cultured. FISH is not the only molecular biological method for cultivation-independent identification of microbes, but the other techniques do not provide information on the spatial distribution of the microbes in the sample, which is of importance e.g. in biofilm studies.

rRNA molecules are good target molecules for FISH as they exist in all living cells, and include both variable and highly conserved sequence domains (Amann *et al*. 2001). Analysis of the 16S rRNA gene sequence and its comparison to the large databases of sequence information existing is nowadays the basis of bacterial classification (Ludwig & Klenk 2001). Due to the variation in the sequence, oligonucleotide probes can be designed for a chosen group of microbes, ranging from a general probe for the eubacteria to a probe specific for a single species. In active microorganisms rRNAs occur in high copy numbers (several thousand per each cell), and thus plenty of probe molecules can bind to a single cell, which is essential for the FISH as one fluorescent molecule bound per cell is not sufficient for microscopic detection.

A typical FISH protocol includes four steps: (1) fixation and permeabilization of the cells (thus the protocol kills the cells) to guarantee free access for the probe; (2) hybridization; (3) washing to remove unbound probe; and (4) microscopic detection.

Gram-negative and gram-positive bacteria may require different degrees of permeabilization, but excess should be avoided so that the cells do not release their rRNAs. There is also strong variation in the accessibility of different target regions to the probe, caused by the 3D-structure of the 16S rRNA molecule (Fuchs *et al*. 1998). Low cellular rRNA content of dormant cells can cause fluorescent signal insufficient to be detected. The FISH technique is not suitable for high sample throughput because the results are read by microscopy. It is also worth to remember that only a small proportion of the microbial diversity in the world is known and represented by rRNA sequences in the databases (Amann 2000). Probes are only as good as the database used for their design, and because the databases continue growing steadily, there is a constant need for re-evaluation of the probe specificity (Amann *et al*. 2001). The present number of bacterial 16S rRNA gene sequences in the public database of RDP is over 65800 (http://rdp.cme.msu.edu/html/analyses.html; 31.3.2003).

Several studies have applied FISH to investigate diverse microbial communities in complex samples such as marine sediments, activated sludge, soil, or marine bacterioplankton, just to name a few examples of the multiple applications (Amann *et al*. 2001; Amann & Schleifer 2001). Applying FISH to the studies of biofilms growing in water distribution networks revealed the predominance of *Aquabacterium* spp. in several European drinking-water systems (Szewzyk *et al*. 2000). FISH in conjunction with CLSM has allowed defining the three-dimensional distribution of microbial populations in mixed-species biofilms of different environments (Lawrence *et al*. 2002b; Manz *et al*. 1999; Møller *et al*. 1996).

2. AIMS OF THE STUDY

Earlier studies of paper-machine slimes have shown that mature biofilms contain diverse microflora, but the studies have failed to answer the question that how these heterogeneous biofilms develop. Väisänen *et al.* (1998) showed that *Deinococcus geothermalis* strain E50051 was capable of producing thick biofilms on stainless steel surfaces as a pure culture. This was one starting point for this study, giving us a model bacterium to study mechanisms of biofilm formation in paper machines.

1. We aimed to reveal the attachment mechanisms of *D. geothermalis* to different non-living surfaces and to understand how the cells of *D. geothermalis* attach with each other in the growing biofilm.

2. Hundreds of microbial species are present in the paper machines. We aimed at identifying the most prevalent bacteria that are capable of independently initiating biofouling on clean surfaces.

3. Colored process slimes are deleterious for the end-product quality, but species responsible for the pink, yellow, orange or red pigments were inadequately known. We aimed to identify colored bacteria present in paper-machine slimes.

4. It is now known that many bacteria possess two distinct growth modes: planktonic and biofilm mode of growth. We aimed to understand which conditions regulate the choice of *D. geothermalis* between these growth modes.

5. In paper industry the efficacy of biocides are frequently evaluated based on measurements of their effects on the free-swimming bacteria. We aimed at designing and applying a simple assay for evaluating the performance of the biocides against biofilm growth in paper-machine environment.

6. We asked the question whether paper-machine biofilms could be controlled by influencing the bacterial cell-to-cell communication, thereby preventing biofilm formation, or making the biofilm more susceptible, or planktonizing an existing biofilm. As a first step we assessed the role of AHL signalling molecules in paper machine biofilms.

Better understanding of the microbial species that initiate the biofouling, of the selection of the growth mode, of the attachment mechanisms and of the bacterial communication in the biofilms, could all contribute to enhanced control of biofouling in paper machines.

In addition to these this study aimed:

7. To develop methods for studying biofilms.

8. To resolve the structure of biofilms growing on stainless steel surfaces during ennoblement.

3. MATERIALS AND METHODS

3.1. Methods used in this thesis

Experimental procedures used in this study are compiled in Table 3.1. One of the essential methods in this study was the microtiter plate assay for biofilm formation (BF-assay), which is illustrated in detail in Figure 9.

Table 3.1. Experimental procedures used in this study. Detailed descriptions of the materials and the methods are found in the original publications, referred to by their Roman numerals, or in the Chapter 3.2.

Experimental procedure	**Publication**			
Propagation of biofilms on stainless steel	I	II	III	IV
Microtiter plate assay for biofilm formation	I	II	III	
Durability test for biofilms		II		
Analysis for biocide susceptibility			III	
Analysis for susceptibility to bacterial extracts	I			
Quantitative fluorometry	I			
Stereomicroscopy	I		III	
Epifluorescence microscopy	I		III	
Confocal laser scanning microscopy	I		III	IV
Fluorescent staining of living biofilms (stains for nucleic acids, gram-reaction and viability)	I		III	IV
Carbohydrate analysis by fluorescent lectins *				IV
Inhibition of intercellular adhesion with carbohydrates *				
Alcian blue -staining *				
Enzymatic removal of biofilms *				
Detection of cell-to-cell signalling molecules *				
Permeability assay with fluorescent latex beads			III	IV
In situ -hybridization *				IV
Scanning electron microscopy		II		IV
Atomic force microscopy *		II		
Micromanipulation		II		
Image analysis	I	II	III	IV
Isolation of bacteria	I		III	
Gram-staining	I		III	
Analysis of catalase and hydrolytic activities			III	
Whole cell fatty acid analysis	I		III	
Analysis of maximum growth temperature (T_{max})			III	
Analysis of bacteriochlorophyll *a*			III	
Motility analysis for bacteria		II		
16S rRNA gene sequence analysis			III	
Bioinformatics			III	IV

* see Chapter 3.2.

3.2. Methods other than those described in the papers I - IV

3.2.1. Tapping mode AFM of biofilm bacteria

Biofilms for the TappingMode™ AFM were grown as described in Paper II, except that the incubation times ranged from 6 to 24 h. The biofilms were submerged in sterilized tap water and transported to the microscope. Biofilms were rinsed with distilled water and imaged with a NanoScope Multimode™ SPM with Nanoscope IIIa SPM Controller unit (Digital Instruments, Santa Barbara, CA, USA) in distilled water, or allowed to air-dry for 15 min and imaged in air. Air-dry biofilms were imaged with silicon cantilevers driven at a resonance frequency of 65 kHz. In water magnetic field was used to drive silicon nitride cantilevers at a resonance frequency of ~8 kHz.

3.2.2. Assays of cell-to-cell attachment

3.2.2.1. Lectin staining of biofilms

Biofilms of *D. geothermalis* E50051 were grown on polished test pieces of AISI 316 stainless steel in WW-medium as described in Paper I. To avoid artificial lectin binding fresh biofilms with no fixation were used. Live biofilms were rinsed with lectin buffer (0.05 M Tris-HCl buffered saline with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, pH 7.6) and stained with 0.5 µM fluor-conjugated lectins in lectin buffer (~125 µL cm^{-2}) for 40 min in dark at RT. The fluorescent lectins were purchased from Sigma (Sigma Chemical Co., St Louis, MO, USA) except for WGA and Con A (Molecular Probes Inc., Eugene, OR, USA). Binding specificities of the lectins are shown in Table 4.3. For testing the binding specificity a set of biofilm samples was stained in the presence of an excess of the target monosaccharide (8 - 23 mM). Stained biofilms were rinsed twice for 5 min with lectin buffer and counterstained for 10 min with the nucleic acid stain EtBr (100 µg ml^{-1}) or SYTO 16 (10 µM), or with Aldehyde-sulphate FluoSpheres® (0.02 µm in diameter, 3×10^{12} beads ml^{-1}). All counterstains were purchased from Molecular Probes Inc. The aldehyde-sulphate modified fluorescent latex beads bind to hydrophobic surfaces and molecules (Haugland 2001). Stained biofilms were examined with CLSM as described in Paper I. Voltages of the photomultiplier tubes were kept constant to qualitatively rank (from - to ++++) the binding of different lectins to the biofilms.

3.2.2.2. Alcian blue staining of biofilms

Alcian blue staining of acidic polysaccharides was performed as described previously (Murray *et al*. 1994).

3.2.2.3. Inhibition of intercellular adhesion by carbohydrates

BF-assays were performed in neutral WW amended with high carbohydrate or glycoprotein concentrations. Stock solutions were sterilized by filtration through syringe filters (0.2 µm in diameter) or by autoclaving (112°C and 15 min for carbohydrates, 121°C and 15 min for others). The BF-assays were performed as described in Paper III with the following exceptions. An over-night TSB-culture of *D. geothermalis* was spun down, the cells were re-suspended in one-fifth volume of sterilized tap water, and 10 µl of this concentrated inoculum was pipetted into each well of the polystyrene plates. The plates were incubated for 3 h with agitation.

3.2.2.4. Enzymatic removal of biofilms

Efficacy of different enzymes against *D. geothermalis* E50051 biofilms was evaluated with the BF-assay or in sterile tubes made of glass, polypropylene or polystyrene. Biofilms were pre-grown in WW and the used medium replaced with the enzyme in a suitable buffer. The plates or the tubes were incubated for 2.5 to 4 h at 28 or 37°C (depending on the enzyme) with an agitation of 160 rpm, rinsed with tap water, and stained with crystal violet or safranin. Alternatively, the cells of inoculum were exposed to the enzyme-buffer solution for 2 h prior inoculating the WW. The enzymes used were:

- Pectinase, Sigma P-4716, Poly[1,4-α–D-galacturonide] glycanohydrolase, used in concentrations of 1-45 U ml^{-1} in 0.1 M acetate buffer at pH 4.0 at 28°C for 4 h.
- Chitinase, Fluka 22725, Poly[1,4-β–(2-acetamido-2-deoxy-D-glucoside)] glycanohydrolase, used in concentrations of 0.4-4 U ml^{-1} in 0.1 M acetate buffer at pH 5.0 at 37°C for 3 h.
- Pronase, Sigma P-5147, a broad-spectrum protease from *Streptomyces griseus*, used in concentrations of 0.1-1 mg ml^{-1} in 0.1 M phosphate buffered saline at pH 7.3 at 37°C for 2.5 h.

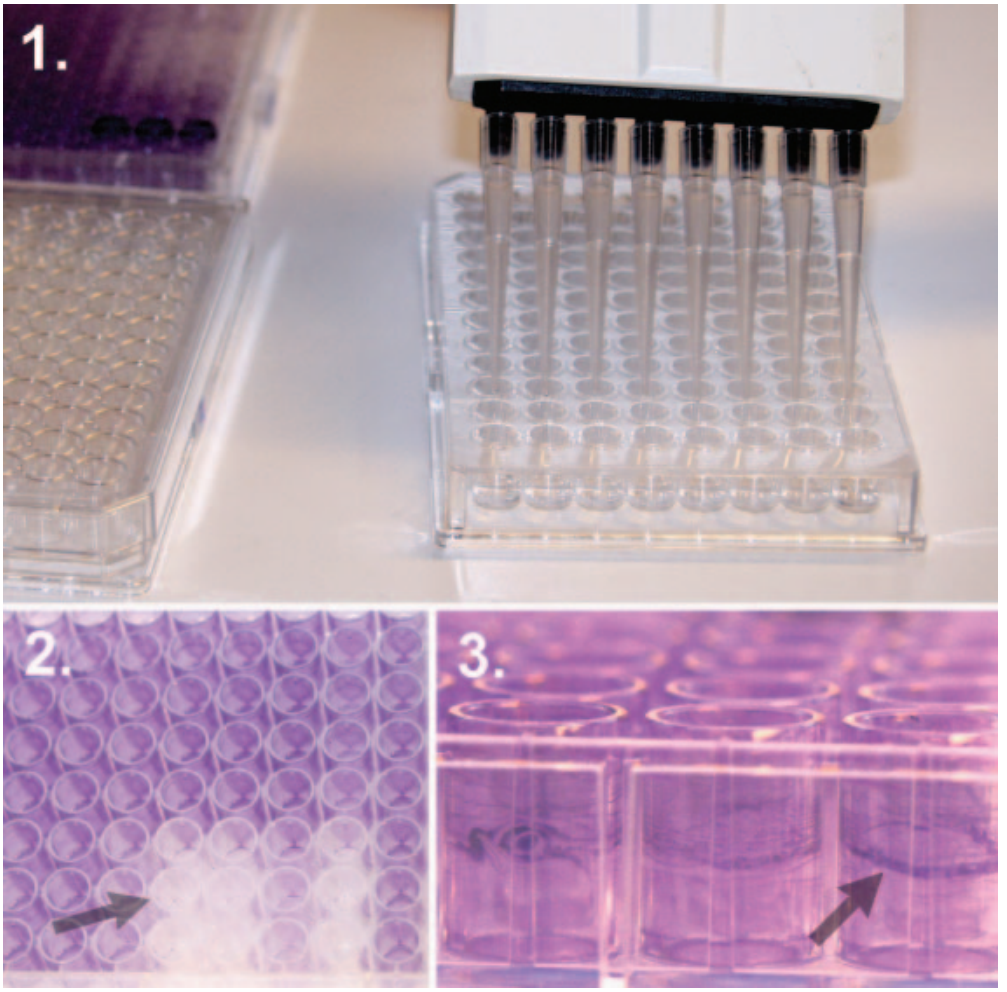


Figure 9. (Opposite page) The three pictures illustrate the microtiter plate assay for biofilm formation (BF). Microtiter plates of different sizes and properties can be used. In this study the BF-assays were performed in 6-well or in 96-well plates of regular polystyrene or of tissue-culture-treated polystyrene with more hydrophilic surfaces. **1.** Each well was filled with medium and a bacterial inoculum. The media used were R2-broth, different paper machine water media (WW; pH 5, 7 or 8), TSB or 10× diluted TSB. Effects of active compounds, e.g., biocides, were studied by dosing different concentrations of the substances in the wells. The plates were covered to prevent drying and placed on a rotary shaker (typically 160 rpm, 45°C, 1-2 d). After incubation planktonic growth in the wells was visually scored based on the turbidity and color of the wells. **2.** The wells were emptied, stained with crystal violet for 3 min, washed three times under a running water tap to remove non adhered biomass, and allowed to air-dry. Already at this point the wells with no biofilm (arrow) were distinguishable with the naked eye from those with biofilm. In this particular plate the *D. geothermalis* biofilms were inhibited in nine wells by substances extracted from Finnish wild-growing plants (Kolari *et al*. 2002). **3.** Different bacteria showed different patterns in the biofilm formation. A few bacteria formed biofilms preferably on the bottom of the wells. *D. geothermalis* (in the picture) formed biofilm on all surfaces of the well, but the thickest biofilms located in the air-water interface area (arrow). Biomass in the biofilm was quantified by leaching the biofilm-bound stain in ethanol for 1 h, after which A_{595} of the dissolved stain was measured with a plate reader. A_{595} readings between 0 and 4.0 correlated linearly with the CFU in the biofilm, and the lower detection limit of the BF-assay was ca. 5×10^3 CFU cm^{-2} (shown in Paper I). The threshold for evident biofilm formation was set to A_{595} value of 0.3 corresponding to ca. 4×10^4 CFU cm^{-2}.

3.2.3. *In situ* -hybridization with DgeoF-probe

DgeoF-probe (Kolari *et al*. 2003) was designed for detecting *D. geothermalis*. The probe was synthesized and labelled with Cy3-label to the 5'-end by MWG Biotech (Ebersberg, Germany). We used microscopy slides where a hydrophobic coating separates glass surface windows (Teflon printed slides, 12-well, 5 mm in diameter, Menzel, Braunschweig, Germany) and thus prevents mixing of probes applied to adjacent wells on the slide. Before use the slides were coated with gelatin by dipping them in a warm (70°C) solution of 0.1% gelatin and 0.01% $CrK(SO_4)_2 \times 12\ H_2O$ in distilled water (Amann 1995), and air-dried in vertical position. Fresh slime samples were used, or the slimes were activated prior to hybridization in R2-broth or in yeast extract (2 g l^{-1}) for 1 h at 50°C to increase the rRNA content of the cells. Approximately 10 µl of the slime sample was pipetted per each well on the gelatin-coated slides. The smears were allowed to air-dry and then dehydrated by successive passages through 50%, 80% and abs. ethanol (3 min each). Slides were stored dry in dark at RT until hybridization.

FISH: 8 µl of hybridization buffer (20 mM Tris-HCl pH 7.2, 0.01% SDS, 0.9 M NaCl, 20-40% formamide) containing 60-200 ng of DgeoF-probe (Cy3-labeled to the 5'-end, MWG Biotech, Germany) was pipetted in each well of the slide. Occasionally also 60-200 ng of FITC-labeled EUB338-probe (general probe that binds to most eubacteria) was added. The cells were hybridized for 2 h at 46°C. After this the slides were rinsed for 5 s with warm (46°C) hybridization buffer without the probe(s) and transferred to the washing buffer (20 mM Tris-HCl pH 7.2, 0.01% SDS, 5 mM EDTA, 20-120 mM NaCl) at 48°C for 20 min. The slides were rinsed with distilled water and air-dried. The results were read under an epifluorescence microscope (Nikon Eclipse E800, Tokyo, Japan) using a 100× oil immersion objective (Nikon Plan Fluor, NA 1.3).

Reference strains used for adjusting the stringency of the hybridization conditions were: *Burkholderia multivorans* F28L1, *Bacillus amyloliquefaciens* TSP55, *B. coagulans* E50L1, *B. licheniformis* D50141, *B. megaterium* HAMBI696, *B. pumilus* TSP66, *D. proteolyticus* DSM20540, *Micrococcus luteus* HAMBI1399, *P. aeruginosa* ATCC10145, *P. fluorescens* HAMBI27, *Staphylococcus aureus* HAMBI66, *S. epidermidis* O-47, *Thermomonas* sp. 41007 and *Thermomonas* sp. 11306. Cultures in active growth phase in TSB-broth were centrifuged and washed twice with buffer (100 mM Tris-HCl pH 7.7, 5 mM EDTA). Mixtures of these bacterial cultures were air-dried on the gelatin-coated slides (10 µl in each well), dehydrated and hybridized as explained above. Blocking solution consisted of unlabeled oligonucleotide (5'-GCA CAC CCA CCT CTC AGC GG-3') 12,5 µM in the hybridization buffer with 40% formamide. To reduce unspecific binding of DgeoF-probe to the slime EPS, the slides were pre-treated with this blocking solution (10 µl per well, 10 min, 46°C) and dried with blotting paper before starting FISH.

3.2.4. Analyses of cell-to-cell signalling molecules

AHL molecules were detected using reporter strains *Agrobacterium tumefaciens* NTL4 (pZLR4), *Chromobacterium violaceum* CV026 and *Escherichia coli* MT102 (pSB403). The strains were kindly donated to us by prof. Stephen Farrand, University of Illinois, USA (*A. tumefaciens*) and by Otto Geisenberger, Munich Technical University, Germany (*C. violaceum* and *E. coli*). These mutant strains are incapable of producing their own AHL signals, but when supplied with exogenous AHLs, they display a measurable reaction.

In the presence of AHL molecules *A. tumefaciens* NTL4(pZLR4) produces β–galactosidase that can hydrolyze 5-bromo-3-indolyl-β–D-galactopyranoside (X-Gal) in the medium, yielding a blue colour reaction (Cha *et al*. 1998). *C. violaceum* CV026 strain produces a violet pigment called violacein (McClean *et al*. 1997). *E. coli* MT102 (pSB403) responds by expressing the *lux*-genes and the bioluminescence can be measured (Winson *et al*. 1998). When the *E. coli* sensor was streaked on a plate parallel with the test strains, bioluminescence of the sensor was visually detected. When broth cultures of the *E. coli* sensor were amended with culture extracts, bioluminescence was detected by a luminometer (model 1253, BioOrbit, Turku, Finland). To isolate AHLs, bacteria were grown in 500 ml of half-strength TSB-broth (1 d, 37°C, 150 rpm) or in WW (5 d, 45°C, 150 rpm), cells were removed by centrifugation, and the cell-free spent broth was extracted with dichloromethane or with ethyl acetate acidified with glacial acetic acid at 0.1 ml l^{-1}. The extracts were dried over anhydrous magnesium sulphate, filtered, and evaporated to dryness. Residues were dissolved in methanol. The presence of AHL compounds in these extracts was tested with the biosensors (for details see: Cha *et al*. 1998; McClean *et al*. 1997; McLean *et al*. 1997; Winson *et al*. 1998).

The BF-assay (WW, 37°C, 150 rpm) was used to study effects of 16 different AHLs on the biofilms of *B. multivorans* F28L1 and *Thermomonas* spp. strains JN 11306, JN 11003 and JN 41007. Synthetic AHLs were applied at concentrations of 4 µM for the time of the biofilm formation (30 h), or applied for 4 h and 17 h after pre-growing the biofilms on the microtiter plates. The synthetic AHLs 3-oxobutanoyl-HSL, 3-oxohexanoyl-HSL, 3-oxooctanoyl-HSL, 3-undecanoyl-HSL, 3-dodecanoyl-HSL, butanoyl-HSL, hexanoyl-HSL, heptanoyl-HSL, octanoyl-HSL, decanoyl-HSL, dodecanoyl-HSL, 2-bromooctanoyl-HSL, 2-bromododecanoyl-HSL, 2-octenoyl-HSL, 2-decenoyl-HSL, and 2-octynoyl-HSL were a gift from Anatol Eberhard, Ithaca College, NY, USA.

4. RESULTS AND DISCUSSION

4.1. Structure of *Deinococcus geothermalis* biofilms

We selected *Deinococcus geothermalis* strain E50051 of paper machine origin as the model organism to study biofilm formation by paper machine bacteria. *D. geothermalis* E50051 was found to adhere to many different surface materials. In sterilized paper machine white water it formed biofilms on AISI 316 stainless steels polished to grades ranging from 320 to 1000 grit (Fig 10, Fig 2 in Paper I, and Fig 1 in Paper II), on surfaces of regular (Fig 1 in Paper I) and tissue-culture-treated polystyrene (Fig 1 in Paper I), HDPE (the biofilm formation vessels in Paper I), and glass (Fig 11). On these surfaces the cells of *D. geothermalis* E50051 grew as compact colonies forming patchy biofilms such as shown in Figure 11. The formed biofilms were always thickest near the air-water interface suggesting that on the continuously submerged surface regions the availability of oxygen may have limited biofilm formation of this aerobic bacterium.

Fluorescent latex beads in combination with nucleic acid stains were used as tools to study the architecture of *D. geothermalis* biofilms. Figure 12 shows that biofilms of *D. geothermalis* consisted of cell clusters with diameters of 5 to 100 µm depending on their age. The cells inside the clusters were in intimate contact. The small-sized, only 20 nm in diameter, hydrophobic microspheres did not penetrate into the interior of the large cell clusters, indicating a lack of voids and illustrating the compact nature of the clusters. The small (young) cell clusters were similarly impermeable to the beads. The obtained evidence indicates that the cell clusters of *D. geothermalis* were compact in nature already at an early stage of their growth.

Field-emission scanning electron microscopy (FESEM) of *D. geothermalis* E50051 biofilms revealed thin (15 to 60 nm) adhesion-threads connecting the cells to the polished (1000 grit) stainless steel surface (Fig 10, and Fig 1 in Paper II). Similar adhesion-threads mediated cell-to-cell attachment in the growing biofilm (Paper II). EPS are known to play an important role in the build-up of biofilms (Costerton *et al*. 1999; Korber *et al*. 1995; O'Toole *et al*. 2000; Watnick & Kolter 2000). In comparison to many biofilm studies showing cells embedded in an extensive slime matrix (Auerbach *et al*. 2000; Beech *et al*. 1996b; Bremer *et al*. 1992; Flemming 2002; Hussain *et al*. 1993; Steele *et al*. 1994; Telegdi *et al*. 1998), a smaller amount of EPS mediated the attachment of *D. geothermalis* E50051.

The FESEM technique requires fixing, dehydrating and coating of the biofilm as pre-treatment, and vacuum during the imaging. The atomic force microscopy (AFM) imaging requires only a short air-drying for 15 min to achieve images such as shown in Figures 13 and 14. Bacteria of the genus *Deinococcus* are known to be highly resistant to desiccation (Makarova *et al*. 2001), therefore it is likely that the *D. geothermalis* E50051 cells remained viable throughout the AFM imaging procedures. The adhesion-threads in Figure 13 were thicker than observed by the FESEM, indicating that the adhesion-threads had undergone an extensive shrinkage during the dehydration preceding FESEM. AFM showed that adherent cells on glass slides incubated for 24 h (an example is shown in Fig 14a) had longer and thicker adhesive threads than adherent cells on slides incubated for 6 h (Fig 14b). This suggests that the initial adhesion of the cells required only a small amount of the adhesive threads that became more bulky after the initial attachment had occurred.

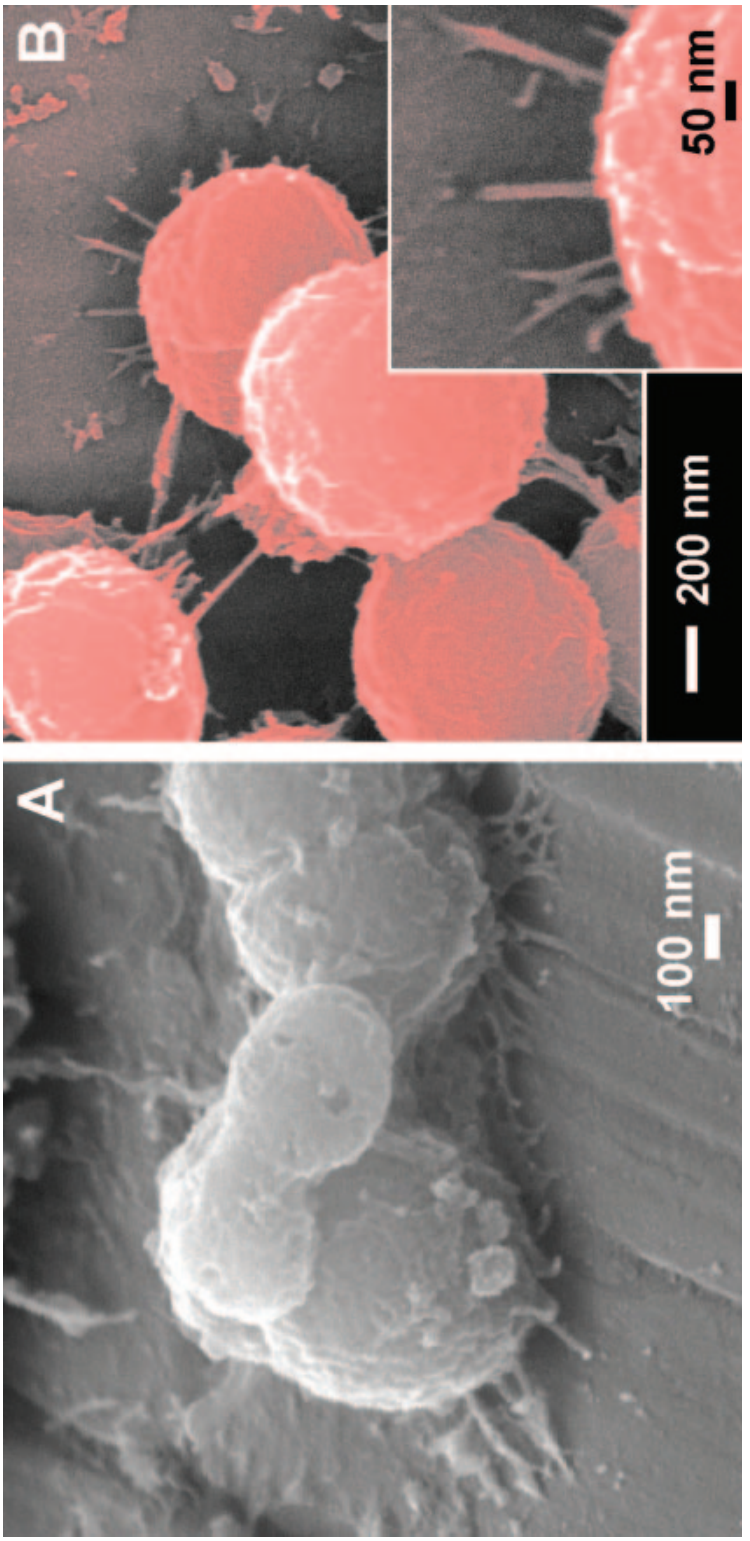


Figure 10. Field-emission scanning electron microscopy (FESEM) images of *Deinococcus geothermalis* E50051 biofilms grown on stainless steel surfaces. Laboratory biofilms were grown in sterilized paper machine circulating water (1 d, 45°C, 160 rpm). Thin adhesion-threads connected the cells to the polished (1000 grit) stainless steel surface. The FESEM image in Panel A was selected by the editor of Journal of Bacteriology as the journal cover in the May 2002 issue. FESEM yields greyscale images; the pink tone in Panel B is artificial, introduced to remind of the pink-pigmentation of *D. geothermalis*.

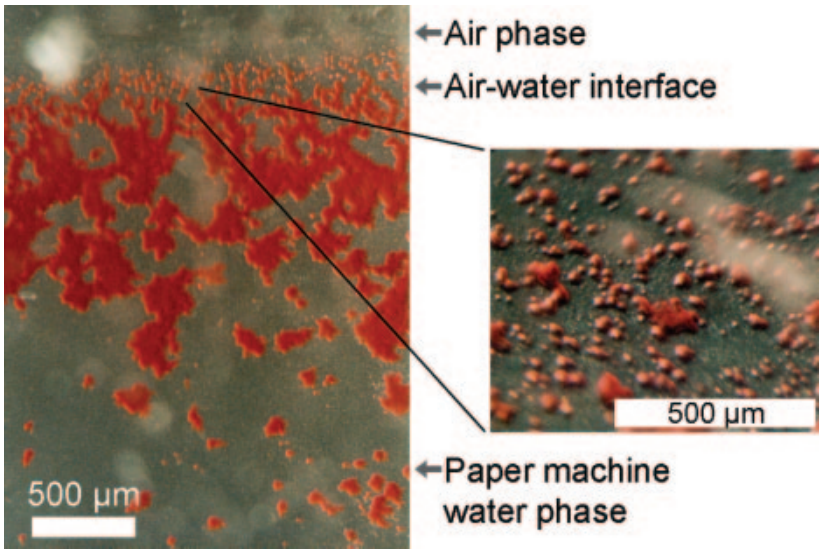


Figure 11. Stereomicroscope images of *Deinococcus geothermalis* E50051 biofilm grown on glass surface. Laboratory biofilm was grown for 1 d at +45°C in sterilized paper machine circulating water (amended with 100 mg l^{-1} of yeast extract, pH 7) on a rotary shaker (160 rpm), and stained with safranin. *D. geothermalis* grew as compact colonies forming a patchy biofilm on the glass surface. Richest biofilms formed in the air-water interface region.

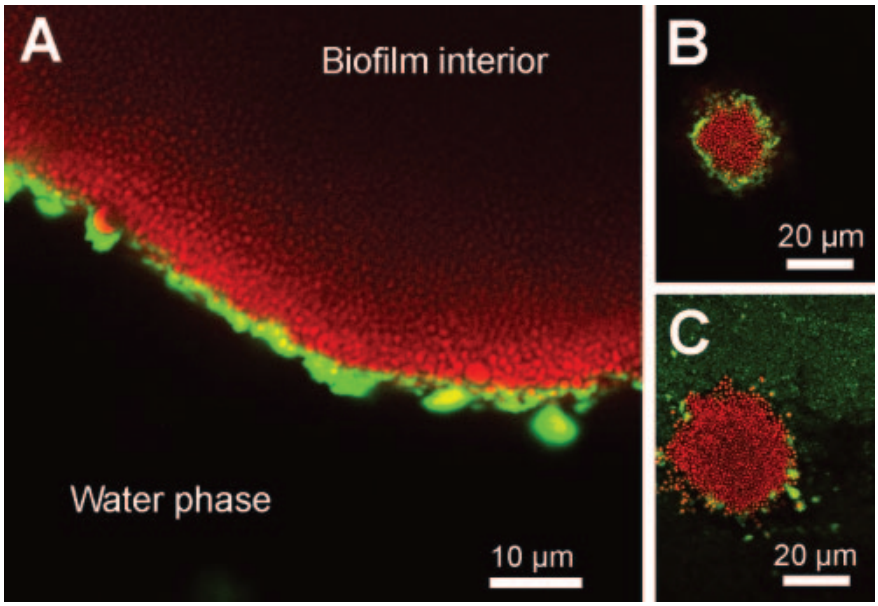


Figure 12. CLSM images of biofilm colonies formed by *D. geothermalis* E50051 on stainless steel test pieces in WW at +45°C in 1 d. The biofilm was stained with the nucleic acid stain EtBr (red fluorescence) and aldehyde-sulphate modified microspheres of 0.02 µm in diameter (green). Panel A is an optical section (z = 1 µm) of a large cell cluster. Panels B and C are optical sections (z = 1 µm, interval 8 µm) from a small, i.e., younger cell cluster; Panel B from almost the top of the cluster and Panel C from the metal surface. More CLSM images of biofilms are shown at http://www.honeybee.helsinki.fi/users/mkolari

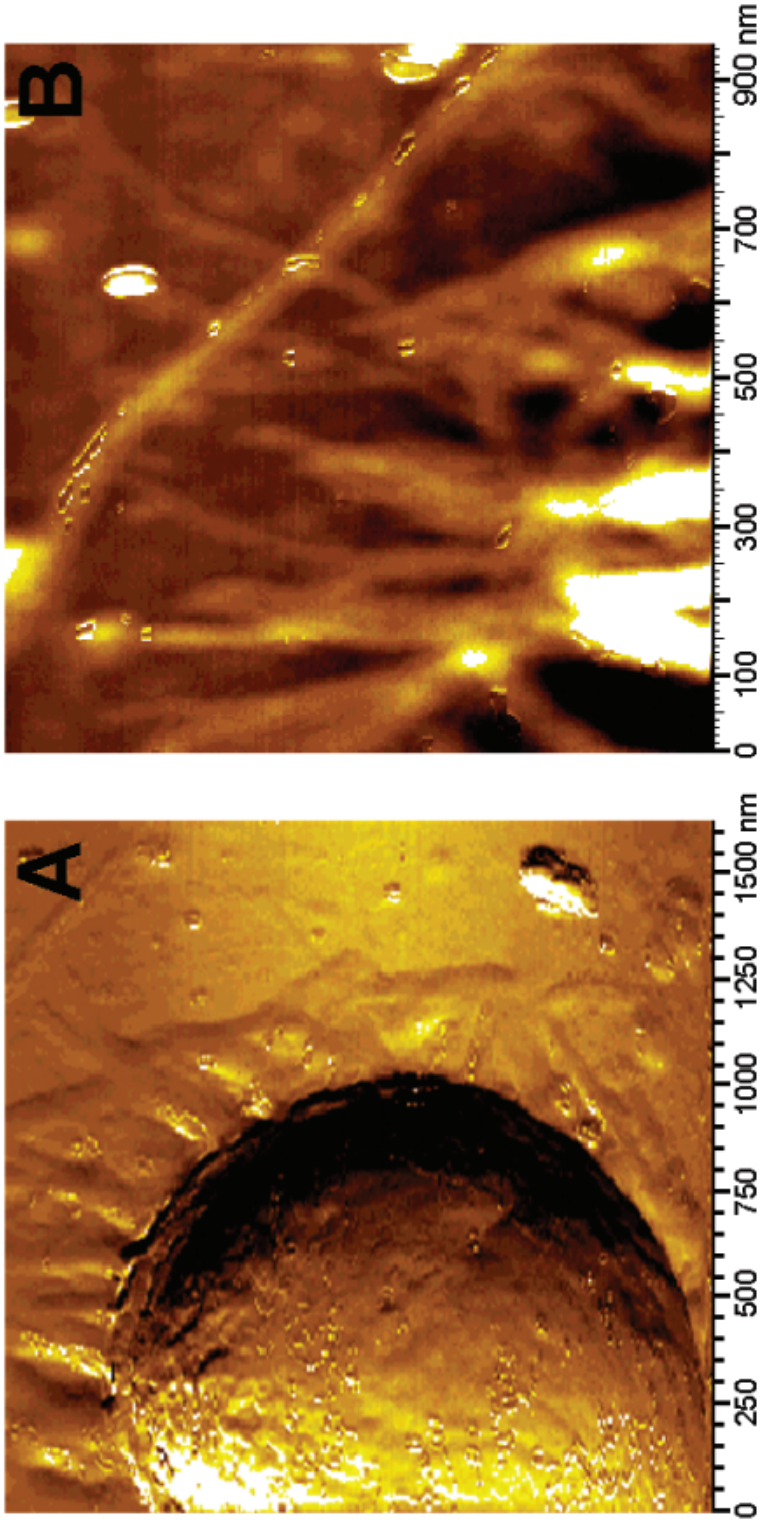


Figure 13. AFM images of the adhesive threads of *D. geothermalis* E50051 cells. The cells were allowed to attach to a glass surface in R2-broth for 1 d (45°C, 160 rpm), transferred into sterilized tap water, transported to the microscope, rinsed with distilled water, air-dried for 15 min, and imaged in "non-contact mode". Panel A shows the adhesive threads of a single attached cell, Panel B is a close-up image from the threads (both are amplitude images).

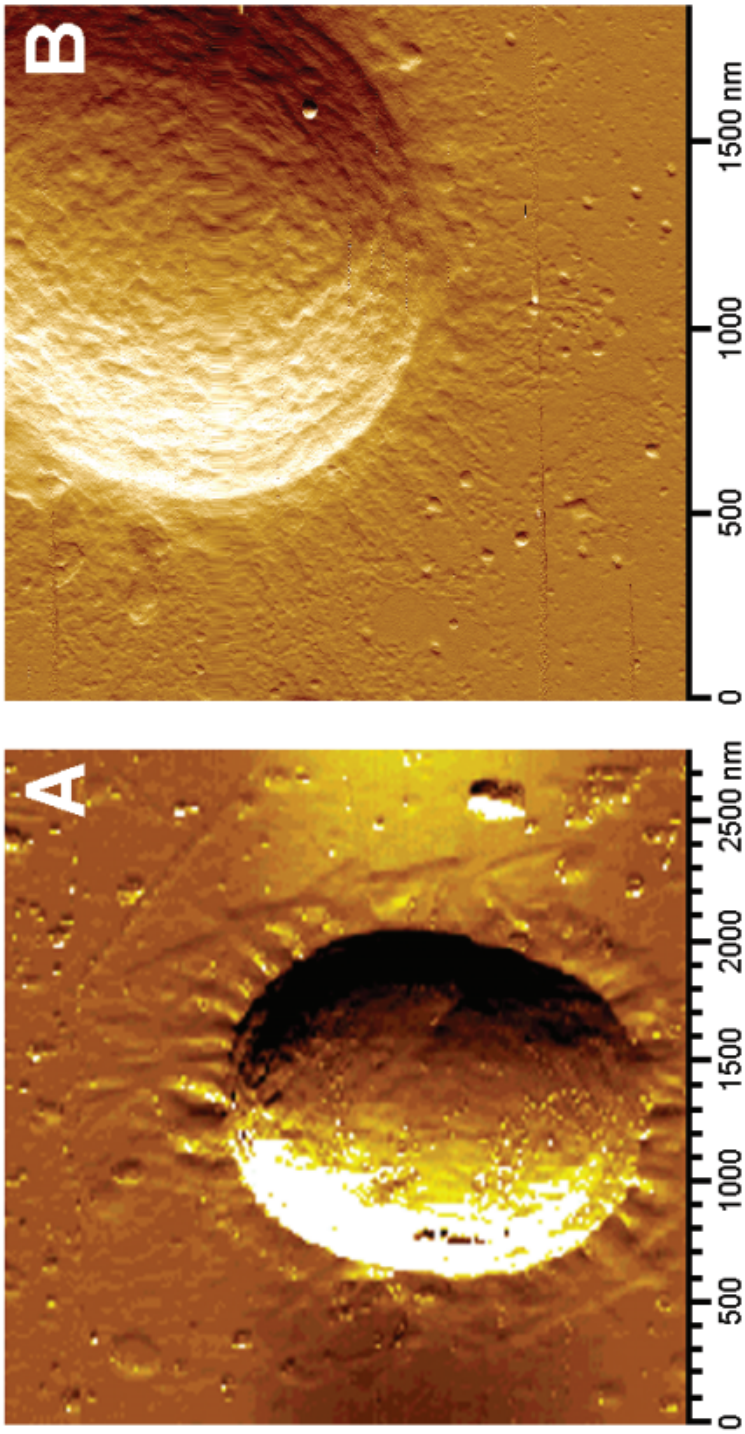


Figure 14. AFM images of *D. geothermalis* E50051 cells attached to glass surface. The glass slides were submerged in R2-broth inoculated with *D. geothermalis*, incubated for 6-24 h (45°C, 160 rpm), transferred into sterilized tap water, transported to the microscope, allowed to air dry for 15 min, and imaged in "non-contact mode" (Panel A, 24 h) or in "tapping mode" (Panel B, 6 h). Both are amplitude images.

When the attached cells were imaged in non-contact mode and in tapping mode (Panels A and B in Fig 14), the two different operation principles of AFM (Fig 5), the cell surfaces appeared slightly different. The non-contact mode displayed heterogeneity on the cell surfaces, "rough" and "smooth" areas (Figs 13a and 14a; and Fig 2 in Paper II), whereas in the tapping mode the cell surfaces looked uniformly wrinkled (Fig 14b). This shows that different scanning probe techniques can reveal different aspects of soft biomaterials.

The "rough" and "smooth" regions on the surface of cells (Figs 13a and 14a; amplitude images) differed in topography (Fig 2 in Paper II): the "rough" regions corresponded to regions 1 to 4 nm lower than the "smooth" regions. The AFM phase images (Fig 2 in Paper II) showed that these regions had a large phase difference also, indicating that the cell surfaces had regions differing in viscoelasticity of the cell surface material. The large phase difference corresponding to the height difference suggests that different surface layers of *D. geothermalis* were patchily exposed.

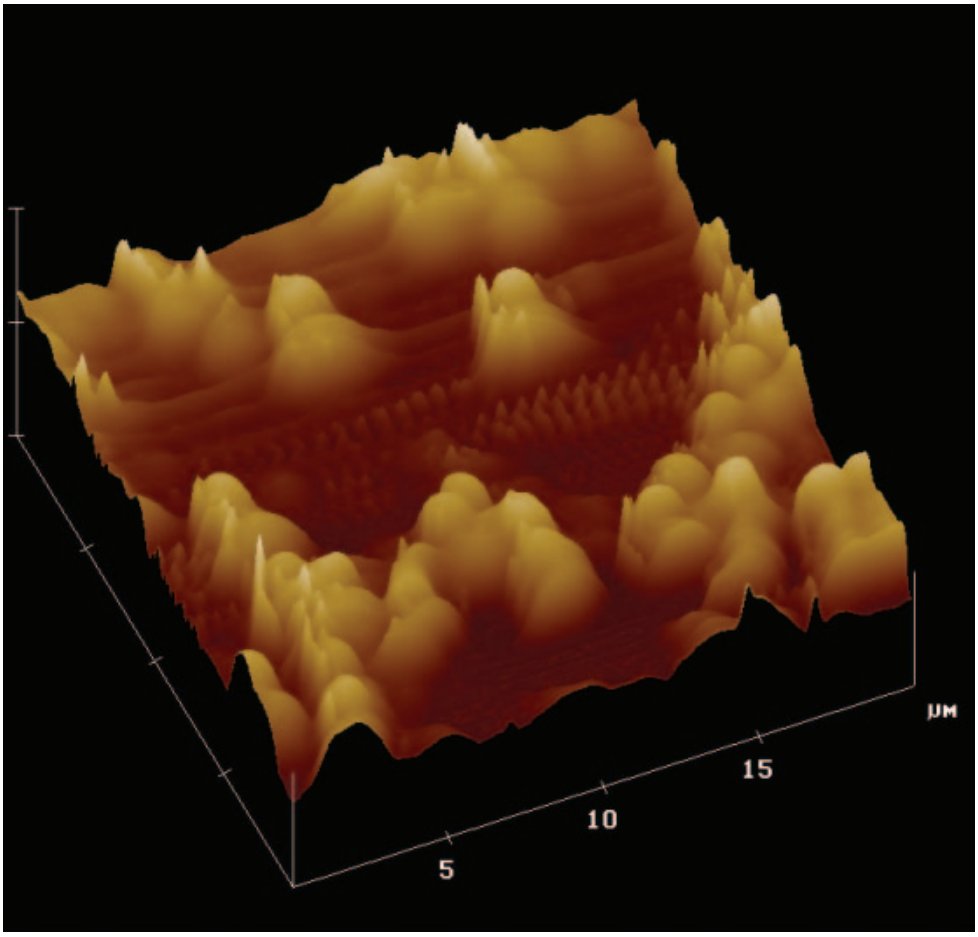


Figure 15. Tapping mode AFM image of *D. geothermalis* E50051 cells attached to glass. The monolayer of cells had attached during incubation of 6 h in R2-broth (45°C, 160 rpm). Prior to imaging the cells were rinsed with distilled water and allowed to air dry for 15 min. The image shows that scanning over a cell often aroused peak-like structures in the image, which were interpreted to be resulting from the adhesion of material on the cell surfaces to the scanning probe, thus altering the topography image.

4.2. Adhesiveness of *D. geothermalis* cells

Adhesion forces of the different surface regions of *D. geothermalis* cells were probed with AFM by applying amplitude-versus-distance spectroscopy (Fig 3 in Paper II). This revealed that the "rough" areas in amplitude images (lower areas in topography images, black areas in the phase images) were highly adhesive towards the silicon tip of the AFM. The "smooth" areas showed no adhesion towards the silicon tip used. It remains as an interesting subject for future work to study the adhesion forces of the different surface regions towards AFM tips with surface properties different from those of silicon.

The high adhesiveness of the *D. geothermalis* cells was also observed when the biofilms were inspected by AFM (Fig 15). Scanning over an attached cell often aroused peak-like structures in the image. This was interpreted as being caused by material on the cell surface that was adhesive towards the scanning tip, thus altering the topography image. The presence of regions with different adhesive properties on the surface of a single *D. geothermalis* cell (Fig 3 in Paper II) may explain why this bacterium is capable of initiating biofilm formation on surfaces with different surface properties (stainless steel, plastic and glass).

4.2.1. Firm attachment of *D. geothermalis* on surfaces

Durability of one-day-old biofilms of *D. geothermalis* and three other biofilm-forming bacteria were compared by growing biofilms of these bacteria in wells of microtiter plates, and subsequently exposing the biofilms to different washing procedures (Paper II). *Staphylococcus epidermidis* O-47 was chosen as a reference because it is a well-studied gram-positive biofilm-forming strain isolated from a hospital patient (Rupp *et al*. 2001). *Burkholderia multivorans* F28L1, a paper machine biofilm strain (formerly *B. cepacia*, Väisänen *et al*. 1998), was selected as a representative of the group *B. cepacia / multivorans*, which contaminates paper machines (Väisänen *et al*. 1994 & 1998). *D. radiodurans* strain DSM 20539^{T} was chosen to represent other *Deinococcus* species.

Results in Table 1 in Paper II show that the biofilms formed by *D. geothermalis* E50051 were not detectably removed from the polystyrene surfaces by one-hour-washing with 0.2% sodium dodecyl sulphate (SDS) or 0.2% NaOH. This proved the firm attachment of *D. geothermalis* to a non-living surface as well as the firm cell-to-cell attachment within the biofilms. The results in Table 1 in Paper II also show that *D. geothermalis* and the related species *D. radiodurans* shared the ability to generate biofilms that were much more durable towards washing with NaOH or anionic surfactant than those of *B. multivorans* or of *S. epidermidis*. Lack of efficacy of the one-hour-washing with 0.2% NaOH at 37°C to remove any biofilm of *D. geothermalis* E50051 indicates that the alkaline washing routinely used in paper machines may fail to clean surfaces biofouled with *D. geothermalis* E50051.

To test the durability of *D. geothermalis* biofilms further, effects of acid washing were studied in a similar experiment. Results in Table 4.2 show that the biofilms formed by *D. geothermalis* E50051 in R2-broth were not removed from polystyrene surfaces by one-hour-washing even with 1 M HCl, indicating tenacious attachment to the surfaces. It was also observed that under the otherwise identical conditions *D. geothermalis* formed more biofilm on surfaces of tissue-culture-treated polystyrene (A_{595} 3.15) than on regular polystyrene (A_{595} 1.80). This shows that increased hydrophilicity of the substratum favoured

adhesion of *D. geothermalis* E50051. Interestingly, in the WW medium no such difference was detected between these two grades of polystyrene (Fig 1 in Paper I). The conditioning layers formed by the components of the WW medium may have equalized the surface properties of these two polystyrenes grades. The A_{595} values of the acid-washed wells were higher than those of the reference wells, suggesting that the acid wash increased binding of the crystal violet into the biofilm EPS.

Table 4.2. Durability of *D. geothermalis* E50051 biofilms towards one-hour washing with hydrochloric acid[1]

HCl (mM)	pH	Quantity of biofilm (measured as A_{595})[2] persisting in the wells of microtiter plates of	
		Regular polystyrene	Tissue-culture-treated polystyrene
1	6.0	2.34 ± 0.36	3.73 ± 0.13
5	2.3	3.46 ± 0.24	4.20 ± 0.10
10	1.9	3.10 ± 0.37	4.29 ± 0.07
100	~ 1	2.80 ± 0.66	4.35 ± 0.10
1000	~ 0	1.83 ± 0.25	4.25 ± 0.10
tap water only	6.5	1.80 ± 0.26	3.15 ± 0.19

[1] Biofilms were pre-grown for 2 d in R2-broth in the wells of regular and tissue-culture-treated polystyrene plates (45°C, 160 rpm). Before the wash, the wells were emptied, rinsed twice with tap water, and then filled with HCl (0.001 M - 1 M). The plates were incubated for 1 h (45°C, 160 rpm), emptied, rinsed three times with tap water and the biofilms persisting in the wells were stained with crystal violet.

[2] The biofilm-bound crystal violet was dissolved in ethanol and the absorbance of the ethanolic solution measured with a microtiter plate reader. Values show the average A_{595} ± SD of the 16 parallel wells. Background values from clean wells were subtracted before calculations.

4.2.2. Firm but slippery attachment

AFM imaging of surface-attached cells of *D. geothermalis* E50051 in water (Figure 4 in Paper II) resulted in repositioning of the cells, but caused no detachment of the cells from the surface. The scanning probe created forces causing sliding of the cells along the surface, even when the "non-contact mode AFM" was used (Paper II). Surface-attached cells of *D. geothermalis* E50051 in water were imaged also with the "Tapping mode AFM" that in theory should cause less lateral shear forces than the non-contact mode AFM. When lonely surface-attached cells were imaged in water, they slid along the surface similar to the non-contact mode AFM. However, when several surface-attached cells were in close proximity to each other, they were possible to image as long as the tapping forces were kept to minimum (data not shown), suggesting that the lateral forces between the neighbouring cells stabilized the cells to such an extent that they resisted the low lateral shear forces caused by the tapping.

Air-drying abolished this flexibility of the attachment in a reversible manner: it was restored after re-immersion in water. The mild dehydration may have caused the adhesive threads to shrink and lose flexibility, locking the threads onto structural heterogeneities of the non-living substratum, resulting into immobilization. Repositioning of

cells during AFM occurred similarly on glass and stainless steel surfaces in water, indicating that the repositioning was not substratum dependent. When surface-attached cells of *D. geothermalis* E50051 were pushed with a glass capillary under an optical microscope (Fig 16), the irreversibly attached cells did not detach, but slid along the surface similarly to that observed during AFM.

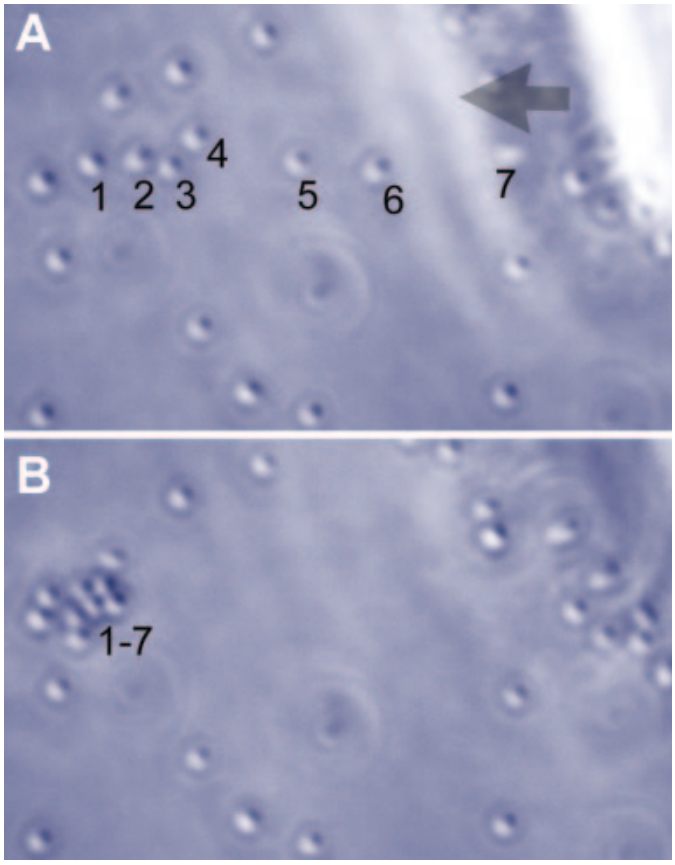


Figure 16. Phase contrast microscope images of *D. geothermalis* E50051 cells attached to a glass surface. Panel A shows positions of seven numbered surface-attached cells. The arrow points to a shadow of a glass capillary of a microinjector. Panel B shows new positions of the same cells (1-7) after being mechanically pushed with the tip of the glass capillary (pushing direction indicated by the arrow). All seven pushed cells repositioned with no detachment from the surface, indicating a mechanism of slippery attachment. All untouched cells retained their positions. More images demonstrating the firm but slippery attachment are shown at http://www.honeybee.helsinki.fi/users/mkolari

This study showed that *D. geothermalis* E50051 possesses a firm, but laterally slippery attachment, permitting the cells to escape external pushing forces by sliding along the surface. This kind of attachment has not been reported before for a non-motile species. *D. geothermalis* possesses no flagella or pili for active motility or attachment (Paper II and Ferreira *et al*. 1997). Before this study attachment of non-motile bacteria such as deinococci was thought to involve a short residence time on the target surface, followed by irreversible anchoring to one location on the surface (Korber *et al*. 1995). After this irreversible event the cells were thought to permanently dock in their place unless the anchoring polymers are mechanically or enzymatically cleaved. We observed that *D. geothermalis* E50051 attached irreversibly to abiotic surfaces by a firm, but slippery mechanism mediated by adhesion-threads. Friction between the exocellular adhesive material and the substratum was low enough to let the cells laterally escape external pushing forces. We proposed to call this behaviour on surfaces as slippery attachment. Our results conform to those of Busscher *et al*. (1998) who, based on the DLVO theory, reasoned that microbes attach irreversibly due to the perpendicular interactions between the microbe and a surface, but

on an ideally homogenous surface the microbe is never laterally immobilized. In reality, chemical and structural heterogeneities of the surface increase lateral interactions between the adhering microbes and yield immobilization (Busscher *et al*. 1998). Our results open new viewpoints to the attachment and behaviour of non-motile bacteria in biofilms. It is possible that external forces can cause surface repositioning of irreversibly attached, non-motile cells also other than *D. geothermalis*. The firm attachment mechanism may be a selective advantage for the deinococcal biofilms when biofouled surfaces of paper machines are cleaned. The laterally slippery attachment may be advantageous for the cells when pressure washers are used for cleaning splash area biofilms, as the cells may slide along the steel surface away from the region experiencing the strongest flushing forces created by the spray of compressed water.

4.3. Cell-to-cell attachment in *D. geothermalis* biofilms

Several methods were used attempting to understand the chemistry of the firm cell-to-cell attachment in the *D. geothermalis* biofilms.

4.3.1. Lectin assay of the adhesive structures

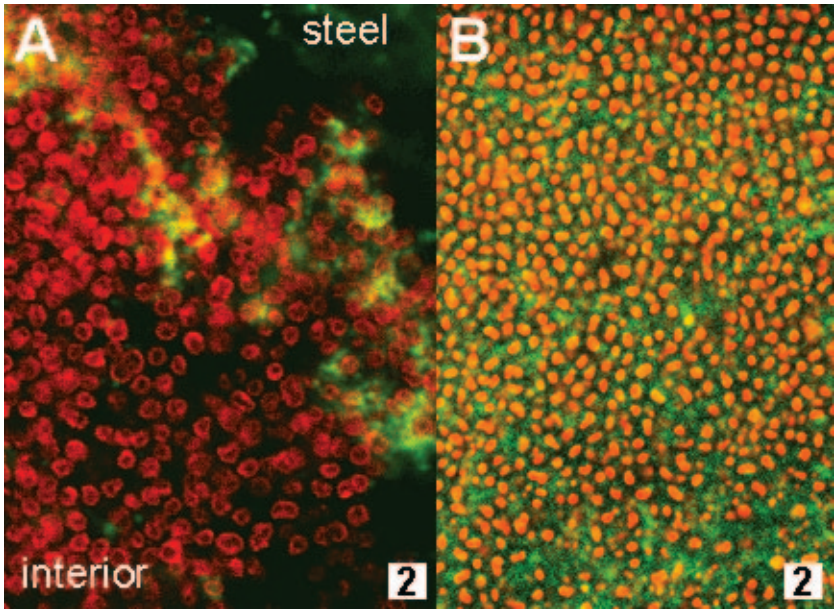


Figure 17. CLSM analysis of three-day-old biofilms of *D. geothermalis* E50051 grown on stainless steel and stained with fluorescently labelled lectins. Panel A shows a single optical section (z = 1 µm) through a deinococcal microcolony displaying cells stained with WGA lectin (red fluorescence). Green fluorescence indicates the sites to which aldehyde-sulphate-modified fluorescent latex beads (diameter 20 nm) adhered. Such sites were found on the steel surface and on the outer surface of the microcolony. Panel B shows a single section (z = 1 µm) through a microcolony stained with LPA lectin (green fluorescence) and counterstained with nucleic acid stain EtBr (red) to reveal positioning of the cells. Each side of a scale box is 2 µm.

Table 4.3. Binding of fluorescently labelled lectins into the *D. geothermalis* E50051 biofilms analyzed by CLSM.

Lectin source	Lectins with fluorescent label: Name	Binding specificity[1]	Label	Binding into the biofilm
Triticum vulgaris (Wheat germ)	WGA	N-acetyl-D-glucosamine, N-acetyl-neuraminic acid (sialic acid)	Texas Red	++++
Abrus precatorius (Jequirity bean)	Abrin	D-galactose (Galα or Galα-Galβ)	FITC[2]	++
Limulus polyphemus (Horseshoe crab)	LPA	fetuin[3], D-glucuronic acid, sialic acid	FITC	++
Canavalia ensiformis (Jack Bean)	ConA	terminal α-D-mannose or glucose (best oligomannose)	TRITC[4]	-
Glycine max (Soya bean)	SBA	N-acetyl-D-galactosamine, faintly D-galactose	TRITC	-/+
Ulex europaeus type I	UEA I	α-L-fucose	FITC	-

[1] according to the manufacturer of the lectin [2] fluorescein isothiocyanate
[3] a serum glycoprotein; contains galactose, mannose, N-acetylglucosamine, N-acetylgalactosamine and sialic acid (Sharon 1975)
[4] tetramethylrhodamine isothiocyanate

Lectins are proteins with high carbohydrate specificity and can be used for studying carbohydrate composition of biofilm EPS (Neu *et al*. 2001; Wolfaardt *et al*. 1998). Biofilms of *D. geothermalis* E50051 were stained with a set of fluorescently labelled lectins and examined with CLSM. In comparison to conventional chemical bulk analyses of extracted EPS, the lectin approach gives information also on the spatial arrangements of the biofilm components, thus easing the identification of the true cell-to-cell attachment molecules.

Results in Table 4.3 and Figure 17a show that biofilms of *D. geothermalis* reacted intensively with wheat germ agglutinin (WGA). This lectin stained the close proximity of each cell. The staining patterns of LPA (Fig 17b) and Abrin differed from that of WGA: both of these lectins were mostly reacting with substances that located in the intercellular space. Hence, it is probable that WGA reacted with the deinococcal cell wall, whereas LPA and Abrin stained the actual polymers mediating the cell-to-cell attachment. The staining patterns in this lectin assay suggest the presence of extracellular polysaccharides containing D-galactose, and sialic or D-glucuronic acids in-between the biofilm cells of *D. geothermalis*, whereas fucose, terminal α–D-mannose and terminal α–D-glucose were absent.

To verify specificity of the lectin binding into the deinococcal biofilms, a set of biofilm samples was stained in the presence of a thousandfold excess of the target monosaccharide. Presence of D-galactose (for Abrin), D-glucuronic acid (for LPA), or sialic acid and N-acetyl-D-glucosamine (for WGA) inhibited staining of the biofilms only partially, thus some unspecific binding of the lectins into the biofilms cannot be ruled out. This is in accordance with Neu *et al*. (2001) who used fluor-conjugated lectins and CLSM for

studying river-water-grown biofilms, and reported that a relatively high concentration of the lectin's target monosaccharide generally yielded incomplete inhibition of the binding. Carbohydrate inhibition patterns for most of the lectins were also broader than indicated by the manufacturers. In addition, Neu *et al*. (2001) showed that the fluor-conjugate can influence the binding specificity, and FITC-conjugated lectins (in our study Abrin and LPA) had more specific binding characters than TRITC-conjugated lectins. They concluded that despite the above-mentioned uncertainties, the fluorescent lectins are a valuable (only) tool allowing three-dimensional examination of the glycoconjugate distribution in a fully hydrated biofilm.

4.3.2. Alcian blue -staining of acidic polysaccharides

Biofilms of *D. geothermalis* E50051 on objective glass were stained with Alcian blue. In this staining biofilm microcolonies turned blue indicating the presence of acidic polysaccharides (data not shown).

4.3.3. Inhibition of intercellular adhesion with carbohydrates

The lectin assay and the Alcian blue -staining both indicated the presence of extracellular polysaccharides in the biofilms of *D. geothermalis* E50051. To identify carbohydrates possibly mediating the cell-to-cell adhesion, BF-assays were performed using WW amended with carbohydrates or glycoproteins in high concentration. If carbohydrates specific for the cell-to-cell adhesion are present in excess, this may prevent the intercellular adhesion. Because many sugars can be used as nutrients also (see Chapter 4.8.1), the experimental time was set as short as 3 h. The wells were inoculated with high amount of cells (grown in TSB, washed and concentrated by centrifugation), thus visible biofilm formation required only cell-to-polystyrene and cell-to-cell attachment, but no actual multiplication of the cells.

Hyaluronic acid[1] ($\geq$0.4 g l^{-1}) and polygalacturonic acid (500-900 ppm) prevented biofilm formation of *D. geothermalis* E50051 in WW in 3 h. Biofilm formation was reduced in the presence of chondroitin sulphate A[2] ($\geq$4 g l^{-1}), galacturonic acid ($\geq$4.6 g l^{-1}), glucuronic acid ($\geq$5 g l^{-1}), heparin[3] ($\geq$1.8 g l^{-1}), or pectin[4] ($\geq$0.8 g l^{-1}). Biofilm formation was neither prevented nor reduced by alginic acid ($\leq$4 g l^{-1}), arabinose ($\leq$17 g l^{-1}), cellobiose ($\leq$7 g l^{-1}), chitin ($\leq$6 g l^{-1}), fructose ($\leq$17 g l^{-1}), fucose ($\leq$11 g l^{-1}), galactose ($\leq$22 g l^{-1}), glucose ($\leq$55 g l^{-1}), lactose ($\leq$7 g l^{-1}), mannose ($\leq$11 g l^{-1}), N-acetyl-D-glucosamine ($\leq$17 g l^{-1}), rhamnose ($\leq$17 g l^{-1}), sialic acid ($\leq$5 g l^{-1}), starch ($\leq$8 g l^{-1}), or xylose ($\leq$22 g l^{-1}).

Sialic acid and glucuronic acid both are targets for LPA, which bound into the *D. geothermalis* biofilm (Table 4.3), but of these two only glucuronic acid reduced biofilm formation. Based on the results the cell-to-cell attachment of *D. geothermalis* E50051 may be sugar-mediated, and uronic acids may be the essential carbohydrates. Only hyaluronic acid and polygalacturonic acid inhibited the cell-to-cell attachment in 500 ppm concentration.

[1] proteoglycan; copoly(β–glucuronic acid-[1-3]-β–N-acetylglucosamine-[1-4]) (Sharon 1975)

[2] proteoglycan; copoly(β–glucuronic acid-[1-3]-β–N-acetylgalactosamine-4-sulphate-[1-4]) (Sharon 1975)

[3] proteoglycan; contains glucuronic acid, glucosamine and iduronic acid (Sharon 1975)

[4] partial methyl ester of α–(1-4)-linked D-polygalacturonate sequences interrupted with (1-2)-L-rhamnose residues (Budavari 1996)

4.3.4. Effect of enzymes on biofilms

To gain more understanding on the chemistry of attachment we investigated the sensitivity of *D. geothermalis* biofilms towards enzymes that hydrolyze macromolecules expected to represent components of its biofilm matrix. Treatment with pronase – a broad-spectrum protease from *Streptomyces griseus* – for 2.5 h detached *D. geothermalis* biofilms from the surfaces of glass and polystyrene (Fig 18), and polypropylene. When the buffer solutions were plated on TSA agar after this 2.5 h treatment, a higher number of cells grew out of the enzyme-buffer solution than of the buffer with no enzyme (the negative reference). Light microscopy showed that in the enzyme-buffer solution there were single cells rather than cell clusters. We interpreted these findings to mean that protease treatment released intact living cells from the biofilm matrix, and that proteins are involved in the cell-to-cell attachment of *D. geothermalis* in biofilms. This view was strengthened by the finding that a 97 kDa protein was exposed as the major immunoreactive component on the outer cell surface when whole cells of *D. geothermalis* were used for raising antibodies (Jaro Nuutinen, personal communication).

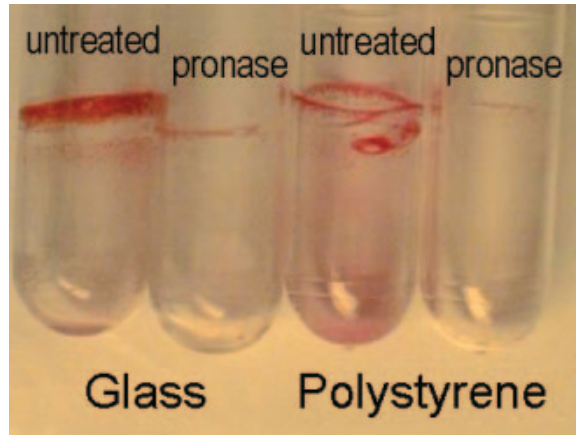


Figure 18. Removing biofilms of *D. geothermalis* E50051 by protease treatment. Biofilms were grown on glass and polystyrene surfaces in WW (3 d, 45°C, 160 rpm), the medium was replaced with pronase solution (1 mg ml^{-1} in 0.1M phosphate-buffered saline), and the tubes were incubated for 2.5 h with agitation. The remaining biofilm was stained with safranin.

Pectinase (poly[1,4-α–D-galacturonide] glycanohydrolase) did not disintegrate *D. geothermalis* biofilms. Alpha-1,4-linked polygalacturonates may therefore represent components not essential for consistency of mature biofilms, or they were inaccessible for the pectinase enzyme. A two-hour treatment of the cells with pectinase was done prior inoculation to strip off any polygalacturonate molecules present. This did not diminish biofilm formation in the BF-assay. This indicates that polygalacturonates were not essential for the attachment, or that these polymers were rapidly re-synthesized during the BF-assay. Similarly to our results, Claus & Müller (1996) and Wolfaardt *et al*. (1998) reported that 7-10 different polysaccharases selected for their specificity against residues identified from EPS by chemical or by lectin assay, had no effect on the structural integrity of the biofilm. Possibly the biofilm matrix composition is so complex that a partial hydrolysis caused by a single polysaccharase yields no visible disintegration of the biofilm.

Chitinase also did not disintegrate *D. geothermalis* biofilms supporting the observation that N-acetyl-D-glucosamine or chitin did not inhibit the cell-to-cell attachment (Chapter 4.3.3). This indicates that the cell-to-cell adhesion in *D. geothermalis* is not mediated by a molecule similar to PIA (linear β–1,6-linked N-acetyl-D-glucosaminyl polymer) described for *Staphylococcus epidermidis* (Dunne 2002). This was confirmed by a

negative reaction in immunolabeling of *D. geothermalis* biofilms with a PIA-specific antibody (Katrin Bartscht, University of Hamburg, personal communication).

4.3.5. Summary on the assays of cell-to-cell attachment

Treatment with protease (4.3.4) released cells from the biofilm matrix, indicating involvement of proteins in the cell-to-cell attachment of *D. geothermalis*. Combined results from the lectin assay (4.3.1), the Alcian blue -staining (4.3.2), and the attachment-inhibition assay (4.3.3) suggest that the cell-to-cell attachment of *D. geothermalis* involves also uronic-acid-containing polysaccharides. The lack of effect by the pectinase treatment (4.3.4) suggests that if uronic acids are involved, they are different from α–1,4-linked polygalacturonic acid.

4.4. Biofilm-forming bacteria in paper machines

A large number of microbial species live in paper machine waters and slimes (Blanco *et al*. 1996; Busse *et al*. 2002; Chaudhary *et al*. 1997; Claus & Müller 1996; Harju-Jeanty & Väätänen 1984; Hughes-van Kregten 1988; Oppong *et al*. 2000; Pellegrin *et al*. 1999; Raaska *et al*. 2002; Väisänen *et al*. 1994 & 1998; Salzburger 1996). Many of the earlier publications deal with "biofilm-forming bacteria from paper industry", but strictly speaking did not examine the isolated bacteria for their ability to form biofilm as pure cultures. Intensive slime formation on a rich laboratory medium was often used as an argument for stating that the isolate represented a biofilm former. To our knowledge, only two studies have shown evidence that the isolated paper machine strains really were capable of forming biofilm as pure cultures in paper machine water: Väisänen *et al*. (1998) showed the biofilm formation of *D. geothermalis* and *B. cepacia*, and Claus & Müller (1996) that of *Klebsiella terrigena* and a *Flavobacterium* sp. In this study the biofilm-forming bacteria of paper machines were thoroughly examined (Papers I & III).

The ability of bacterial isolates to form biofilms as pure cultures on clean surfaces was examined with the polystyrene plate assay for biofilm formation (BF-assay, Fig 9). There were many benefits in the use of the polystyrene plates instead of the stainless steel test pieces that were immersed in biofilm formation vessels, such as described in Paper I. The BF-assay was fast, allowed a large number of strains to be analyzed, and was not prone for contamination. We also showed that *D. geothermalis* and *B. cepacia* formed thick biofilms (Paper I and unpublished data), and different *Bacillus* species formed no biofilms (Paper I), similarly on surfaces of polystyrene and of stainless steel.

In this study altogether 214 selected strains originating mainly from Finnish and Swedish paper industry were examined with the BF-assay (Papers I, II & III, and unpublished data). A_{595nm} values of ≥0.6 in WW or ≥1.2 in R2-broth were considered as indicators of effective biofilm formation. Of the 214 strains 35% (74 strains) were effective biofilm formers on clean surfaces. Those strains are referred to as **the primary biofilm-formers**.

Several efforts were done to select for biofilm-formers during the isolation procedures: (1) biofilms, rather than circulating waters, were sampled; (2) rigorous washing was applied to enrich compact deposits (Paper III); and during the latest studies (3) biofilm formation was screened for already in the course of the isolation procedure and only biofilm-positive mixed cultures were selected for further purification. Despite of this selective

sampling, only 35% of the strains proved effective as biofilm-formers. These observations indicate that the primary biofilm-formers represent only a small proportion of the paper machine microbes. The plentitude of other bacteria present in mature biofilms cannot initiate the biofilm formation. These are referred to as **secondary biofilm bacteria**.

4.4.1. Primary biofilm-formers

Five groups of primary biofilm-formers common in Nordic paper machines were recognized: *Deinococcus geothermalis*, *Meiothermus silvanus*, a novel genus of "*Rhodobacter*-like" alpha-*Proteobacteria*, *Burkholderia* spp. and *Thermomonas* spp.

4.4.1.1. *Deinococcus geothermalis*

Table 4.4 summarizes the observations showing the presence of *Deinococcus* species in paper industry. Väisänen *et al*. (1998) isolated five strains of *D. geothermalis* and Oppong *et al*. (2000) one *D. grandis* strain from paper machines. We isolated altogether 43 strains of *D. geothermalis* from five different machines (Table 4.4). Based on the number of isolates, *D. geothermalis* is the major group of primary biofilm-formers in this study. In addition, epifluorescence microscopy of *in situ* -hybridized specimens showed the presence of *D. geothermalis* in three more mills (Table 4.4 and Fig 19), ranging from small proportions to ≤10% of all bacteria in the studied specimens. All this shows beyond doubt that *D. geothermalis* is common in the paper industry.

D. geothermalis was found from newsprint machines, magazine paper machines, one printing paper machine, one board machine, one tissue paper machine, and from one pulp-dryer (Table 4.4) independent on the usage of chemical or mechanical pulp. *D. geothermalis* was found both in acidic and neutral machines, most frequently in machines with pH around 5. This species was regularly found in slime deposits of the wire section

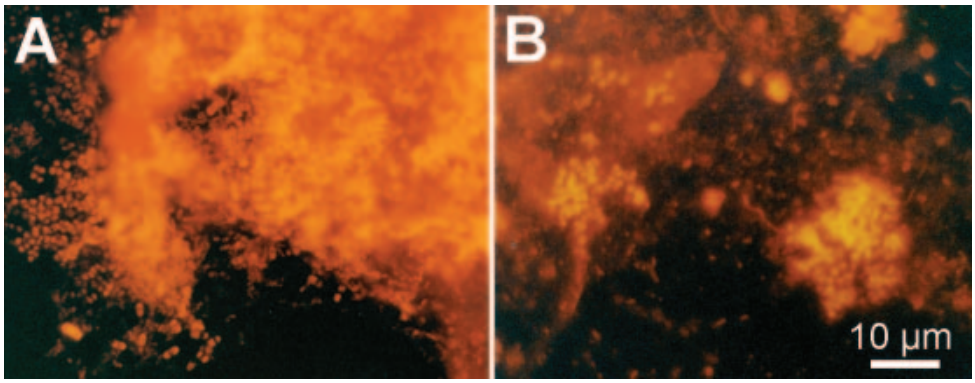


Figure 19. Epifluorescence microscopy images of biofilms *in situ* -hybridized with the Cy3-labeled DgeoF-probe. The selected images show the two slime samples with most abundant presence of *D. geothermalis* observed in this study. Panel A shows cells of *D. geothermalis* (intensive orange fluorescence) in abundance in a red slime sampled from the splash area of wire section of a newsprint machine (52°C, pH 5.3). *D. geothermalis* was counted to constitute ≤10% of the all bacteria in this particular slime sample. Panel B shows brightly fluorescent deinococcal microcolonies (5-15 µm in diameter) in a red-gray slime sampled from the vicinity of the first press cylinder of a magazine paper machine (52°C, pH 7.4).

Table 4.4. Observations on the presence of *Deinococcus* spp. in paper industry.

The machine studied: Main product, operation temperature, pH	Observation / origin and number of the isolates	Detection method	Reference
Printing paper, 45-50°C, pH 5.3	*D. geothermalis*. Pink slime formed on stainless steel test coupons in a wire water line. 6 isolates.	Isolation at 50°C on TGY agar.	Väisänen *et al.* 1998
Coated paper, 53°C, pH 5 (“machine B”)	*D. geothermalis*. Red slimes from wire section. Wet pulp and dried sheets of pulp. 10 isolates.	Isolation at 45°C on R2A (pH 5 or 7) or CTYE agar.	Paper III
	Many large cocci in red slimes from wire section.	CLSM of EtBr-stained slimes.	Paper III
Dry sheets of chemical pulp, 52-62°C, pH 6 (“machine C”)	*D. geothermalis*. Colored slimes from wire section and water wells. Circulating water. Machine felts. Dried sheets of chemical pulp. 24 isolates.	Isolation at 50°C on R2A (pH 5 or 7), PA (pH 5) or CTYE agar.	Paper III
	Intensive pink growth between fibers of the machine felts from the press section.	Stereomicroscopy of felt pieces after incubation (10 d, 50°C, R2A, pH 5).	This thesis
	Many large cocci in pulp sheets.	Epifluorescence microscopy of EtBr-stained pulp sheets.	This thesis
Uncoated newsprint, 52°C, pH 5.3 (“machine D”)	*D. geothermalis*. Red slimes in splash areas of the wire section. 2 isolates.	Isolation at 45°C on R2A (pH 5).	Paper III
	D. geothermalis microcolonies in red and yellow-red slimes from splash area of the wire section (<10% of all the cells) *	Epifluorescence microscopy of slimes *in situ* -hybridized with the DgeoF-probe.	This thesis
Uncoated newsprint, 54°C, pH 5.5	*D. geothermalis* microcolonies sporadically on test coupons that were immersed in the wire well. More on coupons immersed for 5 d than for 10 d.	Epifluorescence microscopy of slimes *in situ* -hybridized with the DgeoF-probe.	This thesis

Food-quality packaging board, 53°C, pH 6.5	*D. geothermalis*. Thin pink slime on stainless steel surface in splash area (38°C) of the well for wire water (53°). 2 isolates.	Isolation at 50°C on half-strength R2A prepared into WW, pH 6.5.	This thesis
Coated magazine paper, 52°C, pH 7.4 ("machine E")	*D. geothermalis* microcolonies in red-gray slime near the first press cylinder (≤2% of all the cells). *D. geothermalis* in small amounts (<<1% of all the cells) in pink slime from the wire section, on the test coupons immersed for 3 d in bright filtrate line, in red-yellow membranous slime from a disk-filter unit **	Epifluorescence microscopy of slimes *in situ* -hybridized with the DgeoF-probe.	This thesis
Newsprint, 50-55°C, pH 6.8-7.0, machine using no biocides	*D. geothermalis* in small amounts (<0.05%) in red-spotted gray slime from the wire alignment roll, in gray slimes from the stretch roll of pick-up felt and in gray slime from a starch dissolver ***	Epifluorescence microscopy of slimes *in situ* -hybridized with the DgeoF-probe.	This thesis
Coated magazine paper, 55°C, pH 4.8	*D. geothermalis*. Biofilms grown on stainless steel test coupons in 2-24 h in the white water line. 5 isolates ****	Isolation at 45°C on half-strength TSA.	Paper I
Unknown paper mill	*Deinococcus grandis*. Slime deposits ("not pink") from underfoil. One isolate.	Isolation at 25 or 30°C on plate count agar.	Oppong *et al*. 2000

* no *D. geothermalis* detected in brown-green slimes from the wire section.
** no *D. geothermalis* detected in yellow slimes from wire section, in yellow membranous slime from disk-filter unit and in red-white slime from air-water interface in a disk-filter unit.
*** no *D. geothermalis* detected in dark-gray slimes from head-box and in pink-spotted gray slime from thickener.
**** these test coupons were submerged in the white water line in September. No *D. geothermalis* was detected by *in situ* -hybridization when the stainless steel test coupons were submerged in the same site in December and in January.

and in felts of pressing cylinders, but less frequently also in wet pulp, in dried-pulp sheets and in circulating waters. In the study of colored slimes (Paper III), *D. geothermalis* was present in three of the six paper and board machines studied, and was involved in a troublesome red-slime out-break in a fine paper machine. The same study revealed *D. geothermalis* and *M. silvanus* as biofoulers of a pulp drying machine. These bacteria contaminated the otherwise clean chemical pulp, and the carry-over of the colored bacteria in the dry pulp likely triggered the outbreak of red slimes in the recipient paper machine. Our data showed that *D. geothermalis* is a frequent pink-slime-former in the paper industry rather than an accidental contaminant as suggested by other authors (Battista & Rainey 2001).

The biofilm formation potential of 36 paper machine strains of *D. geothermalis* was thoroughly examined with the BF-assay (Figs 5a & 5b in Paper III). *D. geothermalis* strains were primary biofilm-formers both in neutral and acidic paper machine water. In neutral WW over 80% of the *D. geothermalis* strains formed biofilms that in general were better prevented by 15 ppm of MBT than by DBNPA (Fig 5a in Paper III).

Slimes were sampled from the paper mills during years 1999-2002 in all seasons. Table 4.4 shows that large amounts of *D. geothermalis* were observed: in the red slimes of the wire section and in the wet pulp from the "machine B" (from late April to early June); in slimes and in felts from the pulp-drying "machine C" (October); in red slimes from the wire section of the newsprint "machine D" (June); and in red-gray slime vicinal to the first press cylinder of the magazine paper "machine E" (June). In general *D. geothermalis* was mostly observed between April and October, suggesting that their abundance may vary with the season although this was not systematically studied. A seasonal study on the variation in the primary-biofilm formers of a paper machine would be a relevant future topic.

4.4.1.2. *Meiothermus silvanus*

In this study 19 red-pigmented strains of *M. silvanus* were isolated from two mills in connection to a red-slime outbreak (Paper III). In addition to this, *M. silvanus* strain ox-13-pink was isolated from a used felt, removed because of lowered drainage properties, from the press section of a machine producing coated magazine paper (this thesis). These three machines operate at acidic pH, suggesting that paper machines with pH below 6 are most prone to biofouling by *M. silvanus*. This study was the first to show the involvement of *M. silvanus* in the red-slime formation in paper industry. In addition, *Meiothermus* sp. F-WWA-41005 was isolated from red slime of an alkaline board machine (Paper III). This suggests that *Meiothermus* species in general may play a role in red-slime outbreaks in paper industry.

The detected strains of *M. silvanus* were identified as primary biofilm-formers in the neutral and in the acidic WW (Figs 5a & 5b in Paper III). One half of the *M. silvanus* strains were inhibited in biofilm formation by 15 ppm of MBT, but not by DBNPA in neutral WW. Among the five groups of frequent primary biofilm-formers recognized in this study, *M. silvanus* strains had the highest maximum growth temperatures, displaying T_{max} of 59-63°C, and one strain even higher, displaying a T_{max} of 67°C. This finding suggests that red-slimes will not disappear from paper machines simply by raising the operating temperatures with a few degrees, as is the current trend. The cells of *M. silvanus* are rod-shaped, also capable of growing as filaments. Red slime pellets formed of these filaments were not disintegrated even in a five-minute sonication (Paper III). Such compact slime pellets with a diameter of 1-5 mm are likely to cause serious process disturbance and deteriorate the product quality, when detaching from the machine surfaces.

4.4.1.3. *Thermomonas* spp.

When bacteria were isolated from slime deposits and clear filtrate of a printing paper machine (Paper III), *Thermomonas* spp. were most frequent, representing 21% of all isolates. We isolated 28 strains from four different machines, and 86% of these from biofilms (Papers I & III, and unpublished data). The type strain of *T. haemolytica* originates from kaolin slurry used for paper production (Busse *et al*. 2002). These results imply that *Thermomonas* spp. are common in paper machines. The BF-assay revealed both biofilm-negative and biofilm-positive strains. When the BF-assay was carried out in WW or in R2-broth, *Thermomonas* sp. 11306 was the most efficient biofilm-former (A_{595} up to 2.4 in one day at 45°C). Biofilms of *Thermomonas* sp. 11306 were less sensitive towards substances extracted from Finnish wild-growing plants, or towards biocide MBT, than *D. geothermalis* E50051 or *M. silvanus* B-R2A50-3 (unpublished data). Our results suggest that some of the thermomonases are persistent primary-biofilm formers of paper machines.

Initially the genus *Thermomonas* contained only the species *T. haemolytica*, isolated from a man-made environment, a paper mill (Busse *et al*. 2002). Recently a second species *T. hydrothermalis* was isolated from a natural thermal environment (Alves *et al*. 2003). *T. hydrothermalis* SGM-6^T formed evident biofilm in BF-assay (R2, 45°C, 2 d), in contrast to *T. haemolytica* A50-7-3^T. *T. haemolytica* and *T. hydrothermalis* cannot be differentiated based on their fatty acid compositions (Alves *et al*. 2003), and therefore we cannot presently assign the species status of our 28 strains. We will examine in future studies if the biofilm-positive strains belong to one species, possibly *T. hydrothermalis.*

4.4.1.4. Novel genus of "*Rhodobacter*-like" alpha-*Proteobacteria*

Six strains of red-pigmented "*Rhodobacter*-like" bacteria were isolated from slimes in two paper machines and one pulp-dryer (Paper III). The strains formed thick biofilms in neutral, but not in acidic WW (Figs 5a & 5b in Paper III). They were isolated from three different machines in different seasons, suggesting that these bacteria are regular red-slime-formers in paper industry. The partial 16S rRNA gene sequences of the six strains were identical. The full 16S rRNA gene sequence of the strain A-col-R2A-4 had highest similarity of only 93.5% to phototrophic *Rhodobacter blasticus* ATCC33485^T. Thus these strains probably represent a novel genus within the *Rhodobacteraceae* family of α–*Proteobacteria*.

4.4.1.5. *Burkholderia* spp.

Väisänen *et al*. (1994) reported *Burkholderia* (at that time *Pseudomonas*) *cepacia* as a frequently found species in paper and board machine slimes. A further study of Väisänen *et al*. (1998) showed *B. cepacia* as one of the most frequent contaminants of the wet-end of an acidic fine paper machine, and one of the three dominant strains isolated from biofilms grown on stainless steel test pieces immersed in the wire water. They also showed that *B. cepacia* strains E28L3 and F28L1 were capable of forming biofilm on stainless steel surfaces as pure cultures in WW. Lindberg *et al*. (2001a) reported that three strains of *B. cepacia*, isolated by Väisänen *et al*. (1998), formed biofilm with 11-48 µg of biofilm sugar cm^{-2} on glass slides in plate count broth. Twelve strains of *Burkholderia* spp. were isolated from wire water of an acidic machine producing coated magazine paper (Jaro Nuutinen, personal communication). In this study *B. cepacia* was found from biofilms in an acidic newsprint machine only. These observations suggest that *Burkholderia* spp. are common in paper machines operating near pH 5. Their frequency in paper machines may relate to their ability to utilize different types of wood extractives (Lindberg *et al*. 2001b).

Biofilm formation ability of seven strains (incl. E28L3 and F28L1 from Väisänen *et al*. 1998) was studied with the BF-assay. All strains except B2842 formed thick biofilms both in R2-broth and WW (Table 1 in Paper II and unpublished data). Biofilms of *B. cepacia* are involved in the human lung disease cystic fibrosis pneumonia (Costerton *et al*. 1999). As a human pathogen *B. cepacia* is categorized in Hazard Group 2. Recently it was demonstrated that strains identified as *B. cepacia* represent at least nine phenotypically similar but genotypically distinct groups of strains (genomovars I-IX), together referred as the *B. cepacia* complex (Vermis *et al*. 2002). Partial sequencing of the 16S rRNA genes of five strains from Väisänen *et al*. 1998, and comparison to the nucleic acid databases more up-to-date showed that the strains E28L3, F28L1, F45L5 and F453dL1 were more similar to *B. multivorans* (99.5 - 99.8%) than to *B. cepacia* (99.3 - 99.5%). *B. multivorans* (adj. *multivorans* = digesting many compounds) also belongs to the *B. cepacia* complex (Vandamme *et al*. 1997). Strain B2842, the only strain not forming biofilm, showed highest similarity of 99.6% to *B. kururiensis*. This indicates that members of several *Burkholderia* species are present in paper machines, and suggests that of these species those belonging to the *B. cepacia* complex are primary-biofilm formers. Recently Conway *et al*. (2002) studied the biofilm formation in the *B. cepacia* complex reporting that strains of genomovars II (*B. multivorans*) and III-a elaborated the most abundant biofilms in Luria broth.

The innate resistance of *B. cepacia* against antimicrobial agents has been reported as a reason for its persistent infections of human lungs (Vermis *et al*. 2002). In conformity with this we observed that biofilm formation of *B. multivorans* F28L1 was more difficult to prevent by biocide MBT than that of the other common primary-biofilm-formers *D. geothermalis*, *M. silvanus*, the "*Rhodobacter*-like" alpha-*Proteobacteria* and *Thermomonas* spp. (unpublished data).

4.4.1.6. Other primary biofilm-formers

Orange-pigmented "*Porphyrobacter*-like" bacteria were found causative for the colored slime formation in one printing paper machine (Paper III). These "*Porphyrobacter*-like" bacteria likely represent a novel species within the α4-subgroup of the *Proteobacteria* (Paper III). They were capable of producing colored biofilms over 1 mm in thickness on the stainless steel surfaces in the alkaline clear filtrate of this machine, but these bacteria were not common in any of the other mills analyzed during this study. In the BF-assay they formed thick biofilms in neutral, but not in acidic WW (Group V in Figs 5a & 5b in Paper III). They seem to represent a group of primary biofilm-formers that is not as widespread as the five groups mentioned above.

Aspergillus fumigatus strain G-3.1 was also an effective biofilm-former. This single mold strain was isolated from a coherent, almost chewing-gum-like sheet of yellow slime (Fig 1d) that had grown on stainless steel surface in splash area of the wire section in a board machine. It grew at temperatures up to 52°C. In the BF-assay it formed slimy biofilms with the highest A_{595} values measured within this thesis study: A_{595} values up to 4.4 in R2-broth and up to 3.2 in WW (amended with yeast extract 0.1 g l^{-1} and starch 1 g l^{-1}). Similarly to this, flow conditions favoured copious production of EPS during biofilm formation of the dimorphic yeast *Candida albicans* (Kumamoto 2002). *A. fumigatus* G-3.1 formed very coherent biofilms, most probably due to bundling of the filaments and abundant EPS. Slimes formed by this mold are likely to cause serious process disturbances, if detaching from the machine surfaces.

4.4.2. Does every machine have unique primary-biofilm-formers?

When 95 pigmented strains from six different machines were studied for their biofilm formation, the results suggested that paper machines contain different bacteria as primary-biofilm formers depending on whether they are operated under neutral or acidic conditions (Paper III). A few strains of *D. geothermalis* were the only exception, as they formed biofilms both in neutral and acidic paper machine water. Paper I describes the isolation of five *D. geothermalis* strains and 11 strains of *Thermomona*s spp. from three different paper machines. Interestingly, *D. geothermalis* and *Thermomonas* were never found in the same machine. When orange-colored slimes from a printing paper machine (Paper III) were examined, biofilm-forming "*Porphyrobacter*-like" strains and one "*Rhodobacter*-like" strain of novel α*–Proteobacteria*, and strains of *Thermomonas* spp. were found from the same machine. All these results suggest that each paper machine has its own assortment of primary biofilm-formers.

A general observation in this thesis study was that only one to three groups of efficient biofilm-formers were found per one paper machine. This may indicate that the diversity of primary biofilm-formers in any one Nordic paper machine is low. It is probable that paper machines in different climate, e.g. in Brazil or in India, contain primary-biofilm formers other than those which we observed, but that has not been studied. Interesting questions remain for future studies: Do the primary biofilm-formers of a paper machine vary with season, or is the assortment of primary biofilm-formers in any specific machine regular through all the seasons? Do small changes in machine operation permanently alter the assortment of primary biofilm-formers? Knowledge on these issues is required if we aim to inhibit paper machine biofouling by targeted control of the primary biofilm-formers. Other studies of microbial ecology have shown that e.g. in drinking-water biofilms some seasonal fluctuations of the species composition occurred, but *Aquabacterium commune* was the dominant species involved in the initial biofilm formation in all seasons (Kalmbach *et al*. 2000).

4.5. *Bacillus* species in paper machine biofilms

Paper industry desires for paper processes with low numbers of *Bacillus*, *Brevibacillus* and *Paenibacillus* cells, because spores of these species can survive the heat in the drying-section of paper machines, and thus are deleterious for the end-product quality. *Bacillus* species, including *B. cereus*, have been frequently found from paper machine water and slimes (Blanco *et al*. 1996; Chaudhary *et al*. 1997; Hughes-van Kregten 1988; Pirttijärvi *et al*. 1999; Väisänen *et al*. 1989a, 1989b, 1991a, 1994 & 1998) as well as from pink slime in a cellulose factory (Salzburger 1996). Also in this study members of the genus *Bacillus* were found in high numbers in the biofilms of three paper machines (39% of the isolates in Paper I). The biofilms thus represent an important reservoir for these spore-forming bacteria. Bacteria can be continuously released from the growing biofilm to the bypassing process water. No studies exist on the quantity of such a biotransfer function in paper machine biofilms, but Flint *et al*. (2001) examined biotransfer in biofilms of *B. stearothermophilus* strain isolated from a dairy manufacturing plant. Biofilms up to 5×10^6 cells cm^{-2} grew on stainless steel in a continuously flowing experimental reactor, the cells had a doubling time of 25 min, and all daughter cells were released into milk passing over the biofilm, i.e. up to 1×10^7 cells h^{-1} from each square centimeter.

We examined biofilm formation of 40 *Bacillus* strains (7 species) with paper industry origin, and observed that none of the *Bacillus* strains was capable of forming biofilm on clean surfaces in paper machine water (Paper I). This indicates that although *Bacillus* species are frequently present in mature biofilms, they are not primary-biofilm formers themselves, thus other paper machine bacteria must initiate the biofouling of clean surfaces. In contrary to our findings *Bacillus* species have been reported to produce primary-biofilms on stainless steel surfaces in dairy environments (Flint *et al*. 1997 & 2001; Lindsay *et al*. 2000; Parkar *et al*. 2001) or in TSB (Peng *et al*. 2001).

Dunne (2002) claimed that bacterial species persisting in a fully planktonic state under all growth conditions would not exist. The difference between dairies and paper machines as a growth environment for bacteria is huge. It is possible that only in dairy conditions bacilli activate metabolic routes required for the initial adhesion and the primary-biofilm formation as pure cultures, whereas in paper machines the same bacteria may depend on other primary-biofilm forming species. It is also worth remembering that although same *Bacillus* species have been found in both environments, strains within one single species may differ in their phenotypic properties, such as the ability to form biofilm. Pirttijärvi *et al*. (1999) already showed that *B. cereus* strains originating from paper board industry had ribotypes predominantly different from strains of dairy and dairy product origin. They observed marked differences in the emetic toxin production also. Different flow velocities in the experimental setups can also partly explain the different results. In our experiments the estimated flow was 6 cm s^{-1} (Paper I), whereas in other attachment studies of dairy bacilli the flow of the milk medium was only ~10% of that (Flint *et al*. 2001) or there was no flow at all (Lindsay *et al*. 2000). One further explanation for the different surface behaviour could be the differences in the quality of the conditioning layer formed on the stainless steel surfaces in dairies and in paper machines. No studies were performed on this issue, but it is known that environmental conditions rule the composition of the conditioning layer that forms on pristine surfaces immediately after their immersion, and that the nature of such a conditioning layer affects the affinity of organisms for a surface (Dunne 2002). For example the attachment of vegetative cells of *B. stearothermophilus* to stainless steel increased 10-100 fold by the presence of milk fouling the stainless steel (Flint *et al*. 2001). However, in the case of *B. cereus* spores or cells in the late stationary phase, there were no significant differences in the adhesion to stainless steel in milk or in phosphate buffered saline (Peng *et al*. 2001). Furthermore, Peng *et al*. (2001) observed no adhesion of log phase vegetative cells of *B. cereus* to stainless steel, indicating that the growth phase of the cells is also affecting their attachment to surfaces.

4.5.1. ”Plate-count-agar illusion”

Cultivation-based approach to study a microbial population requires many choices to be taken by the researcher, all of which will affect on the population that eventually grows on the agar plates. A heavy selection of bacteria is already done via the choice of medium composition, growth atmosphere (aerobic/microaerophilic/anaerobic) and incubation temperature. A few fast-growing bacteria can mask the presence of others during the incubation. Amann *et al*. (2001) reported that even during a short incubation, the community structure of marine bacterioplankton shifted from the high natural abundances of members of the *Cytophaga-Flavobacterium* cluster towards rapidly growing α– and γ–*Proteobacteria*. Also choosing colonies for the further purification is quite a subjective working step.

Many bacilli grow very well on nutrient rich cultivation media such as plate count agar, TSA or nutrient agar that were used for bacterial isolation in many paper machine studies (Blanco *et al*. 1996; Chaudhary *et al*. 1997; Hughes-van Kregten 1988; Oppong *et al*. 2000; Raaska *et al*. 2002; Väisänen *et al*. 1989a, 1989b, 1991a, 1994 & 1998). These media with high peptone or monosaccharide concentrations are very diverse from the paper machine environment where the bacteria are really living in; the choice of these media is mostly reflecting their intensive use in the field of clinical or food microbiology. On the rich media the fast growing *Bacillus* species, many of which also produce bacteriocins (Zuber *et al*. 1993), may mask the presence of a multitude of other bacteria, and eventually lead to distorted picture of the bacterial flora in the biofilms analyzed. None of the 25 strains of *Bacillus* spp. that we isolated from biofilms using half-strength TSA plates was capable of forming primary-biofilm (Paper I). After noticing this we started to believe that due to biased isolation procedures many biofilm-formers may have been omitted, and the amounts of bacilli in paper machine biofilms have been overestimated, in our study (Paper I) as well as in many of the other studies mentioned above. Therefore in the later phase of this thesis study low nutrient media such as R2A agar were preferred in the isolation of paper machine bacteria.

Our observations resemble those reported on drinking-water biofilms (Szewzyk *et al*. 2000). Molecular techniques demonstrated that the bacteria frequently isolated by cultural methods (e.g. *Pseudomonas*, *Acinetobacter*, and *Bacillus* spp.) existed only in low numbers in the real biofilms, thus their high yield in cultivations was merely indicating that these bacteria were easily and quickly culturable organisms, rather than having major relevance in the drinking-water biofilm.

4.5.2. Interactive biofilm formation in paper machines

In the natural setting biofilms are generally composed of many coexisting species. Studies of biofilms in the oral cavity have revealed a fascinating universe of specific interspecies interactions (Rickard *et al*. 2003). Streptococci and actinomycetes are the major initial colonizers of the tooth surface, and the interactions between them and other bacteria establish the dental plaque community (Rickard *et al*. 2003). It has been demonstrated that coaggregation between different oral bacteria via specific molecules is a common phenomenon, and it may facilitate the development of niches of metabolically co-operating bacteria within multi-species biofilms. Recently coaggregation between different species from drinking water biofilms was demonstrated (Rickard *et al*. 2003).

In this study (Paper I) we observed that in paper machine biofilms *Bacillus* species were secondary, i.e. incapable of forming biofilm by themselves. We showed that when grown together with *D. geothermalis*, which is forming the primary biofilm, *Bacillus* cells joined the growing biofilm (Paper I and Fig 20). CLSM showed that a part of the *Bacillus* strains grew as truly mixed microcolonies with *D. geothermalis* on the stainless steel surface (Panels B & C in Fig 20), whereas other strains of *Bacillus* preferred attachment to the edges of the growing microcolonies of *D. geothermalis* (Panel D).

The species interaction varied from synergy to antagonism (Paper I). *B. licheniformis* D50141 was the strongest antagonist of *D. geothermalis*. After co-adhering with deinococci to steel or polystyrene surfaces, this *Bacillus* strain inhibited deinococcal growth almost completely by producing extracellular, heat-stable, methanol-soluble metabolites (Paper I). Our results suggest that the persistence of bacilli in paper machine biofilms

relates to their ability to conquer biofilms, whose formation is initiated by primary colonizing species such as *D. geothermalis*. To our knowledge this study of microbial ecology of paper machine biofilms (Paper I) was the first published study showing species interactions within paper machine biofilms.

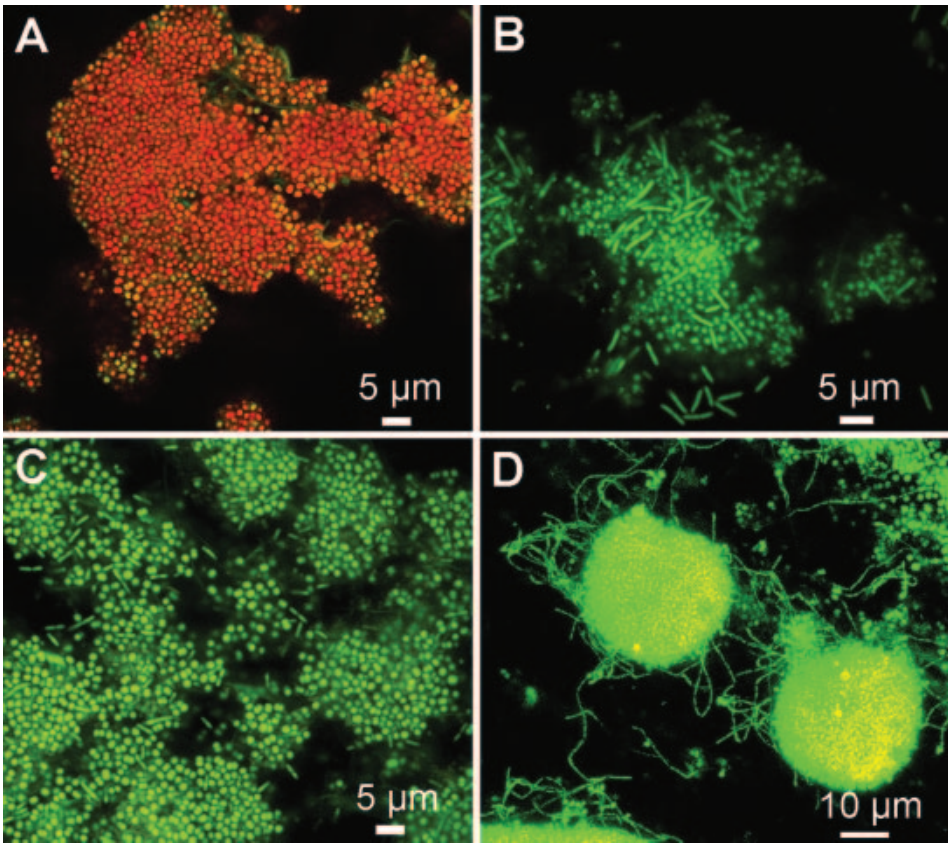


Figure 20. CLSM images of acridine orange stained biofilms of *D. geothermalis* E50051 and paper machine *Bacillus* species grown on disks of stainless steel. Panel **A** shows a typical biofilm microcolony formed by *D. geothermalis* E50051 in monoculture in WW medium (1 d, 45°C, 160 rpm). Panels B to D show mixed species biofilms formed by *D. geothermalis* E50051 in coculture with *B. cereus* BM-VIII P1 (**B**), *B. pumilus* TSP66 (**C**) or *B. coagulans* E50L1 (**D**) under similar conditions. Panels A, B & C show single optical sections lateral to the steel surface (dual channel detection: 515-525 nm and >580 nm). Panel D is a sum projection built using maximum intensity method from 10 optical sections lateral to the steel surface with *z*-steps of 1.0 µm (single channel detection only: 515-525 nm).

4.6. Other species in paper machine biofilms

During this study in total 261 bacterial cultures were isolated and characterized. In addition to the primary biofilm-formers and *Bacillus* species mentioned above, we gained new information on other taxa occurring in paper machine biofilms (Papers I & III, and unpublished data).

One example is the group of "*Roseomonas*-like" bacteria, comprising of 12 related isolates of pink or red-pigmented, rod-shaped, gram-negative bacteria that we found from slimes and circulating water of three machines (A, E & F in Paper III). These three machines differed in the raw materials, in the type of end-product and in the operation pH (ranging from 7 to 9). The results suggest that the "*Roseomonas*-like" bacteria are frequent in neutral and alkaline machines. However, in BF-assay these isolates did not form biofilm (Paper III), thus their role in biofilms is secondary. The partial 16S rRNA gene sequences of the strains F-WWA-41004 and A-cf-CFA-6 (from two different mills) had highest similarities of only 94.9-95.1% to *Roseomonas genomospecies* 4 (Paper III). Therefore the strains likely represent a novel genus of α–*Proteobacteria* related to the *Roseomonas*.

In our laboratory the common practice for preliminary identification of bacterial isolates includes analysis of the cell morphology, the gram-reaction and the whole-cell fatty-acid composition. A common result in this study was that these analyses gave no preliminary identification for the paper machine isolates, especially when R2A or WWA agars were used for isolation (Paper III and unpublished data). This indicates that when the microbe communities of paper machine biofilms were examined with media other than the conventional nutrient-rich media, we discovered a multitude of bacteria that are not included in the commercial fatty acid libraries. This suggests that paper machine biofilms contain a lot of hitherto unknown bacterial species.

4.7. Effect of biocides on the biofilms

In this study over 100 paper machine isolates were examined with the BF-assay for the effects of biocides on their biofilm formation (Paper III & unpublished data). Table 4.7 shows data for eight strains originating from biofilms as an example of the different biofilm behaviours exhibited by the bacteria in response to biocides in paper machine water. Five to fifteen ppm of methylene bisthiocyanate (MBT) or 2,2-dibromo-3-nitrilopropionamide (DBNPA) did not prevent biofilm formation of *D. geothermalis* E50051 completely, but 15 ppm of MBT reduced it 62 ±5% (Table 4.7). Of the five common primary biofilm-formers found in this study, *M. silvanus* B-R2A5-50-4 formed the thickest biofilms in WW at pH 7. It resisted 15 ppm of DBNPA, but was prevented by 15 ppm of MBT (96% reduction of A_{595}). *D. geothermalis* C-PA5-6c formed the thickest biofilms in acidic WW. Towards biocides it reacted similar to *D. geothermalis* E50051. "*Roseomonas*-like" A-col-R2A-3 formed no primary biofilms under any of the tested conditions. Biofilm formation of "*Porphyrobacter*-like" A-col-CFA-6 was prevented by as low as 5 ppm of MBT, but it resisted 15 ppm of DBNPA.

D. geothermalis C-PA5-8a formed no evident biofilm in absence of biocide, but the presence of 15 ppm of MBT increased its biofilm by 316 ±2% (from A_{595} of 0.15 to 0.64). "*Roseomonas*-like" A-col-CFA-5 formed no biofilm in absence of biocide, but in response to 5 or 15 ppm of MBT it initiated the biofilm formation. In this study we found over 40 strains that responded to the presence of biocide by increasing their biofilm formation 5-753% (Paper III & unpublished data). A few strains showed only planktic growth until biocide was introduced, initiating the production of biofilms. Low concentrations of DBNPA increased biofilm formation also when a fresh paper machine slime sample was directly used as inoculum (Table 4.11), indicating that induction of biofilm formation in response to biocide stress may be common among paper machine bacteria.

Table 4.7. Biofilm formation of selected strains in two days in paper machine water (pH 7, 45°C, 160 rpm), and effects of two biocides. The test was carried out on microtiter plates (BF-assay).

Strain (originating from paper industry biofilm)	Quantity of biofilm (A_{595nm} ± SD) in the presence of:									
	No biocide		Methylene bisthiocyanate (MBT)				2,2-dibromo-3-nitrilo-propionamide (DBNPA)			
			5 ppm		15 ppm		5 ppm		15 ppm	
Deinococcus geothermalis E50051	0,82	±0,17	0,75	±0,12	0,31	±0,04	0,56	±0,15	0,67	±0,11
Meiothermus silvanus B-R2A5-50-4	2,11	±0,02	0,84	±0,06	0,09	±0,01	2,15	±0,07	2,14	±0,06
D. geothermalis C-PA5-6c *	0,60	±0,02	0,60	±0,01	0,42	±0,01	0,55	±0,04	0,59	±0,04
"*Roseomonas*-like" A-col-R2A-3	0,05	±0,01	0,05	±0,00	0,03	±0,00	0,09	±0,04	0,06	±0,01
"*Porphyrobacter*-like" A-col-CFA-6	0,99	±0,10	0,10	±0,00	0,09	±0,01	1,16	±0,16	0,83	±0,02
D. geothermalis C-PA5-8a	0,15	±0,01	0,29	±0,02	0,64	±0,01	0,15	±0,01	0,19	±0,01
"*Roseomonas*-like" A-col-CFA-5	0,05	±0,01	0,46	±0,05	0,46	±0,02	0,09	±0,04	0,06	±0,01

* in WW at pH 5

Our results show that external stress factors such as the two biocides cited above, in concentrations commonly employed in paper industry, can activate the transition from the planktonic mode of growth to the biofilm mode of growth. Table 4.7 shows that this is not the case for all of the isolates as some were very sensitive towards biocides. But concerning the more resistant strains, our results suggest that concentrations of biocide inhibiting planktonic but not surface-attached cells may actually promote biofouling.

Our results are supported by those of Das *et al*. (1998) who reported that susceptibility of *Staphylococcus epidermidis* and of *E. coli* to biocides changed immediately upon attachment, and the growth of attached cells continued in biocide concentrations inhibiting growth of the planktonic cells. As a practical point of view Grobe *et al*. (2002) reported that improved killing of *P. aeruginosa* biofilms required concentrated dose of biocide (shock dosing) rather than prolonged doses of a lower concentration, as the latter can be insufficient to prevent biofilm formation. Prolonged exposure to sub-lethal concentration of a biocide may also provoke selection, adaptation or evolution of strains with increased biocide resistance (McBain *et al*. 2002).

A few selected strains were also examined for the effects of biocides on pre-existing biofilms, as in practise the paper machine surfaces are rarely biofilm-free when biocide dosing is started. In short, complete killing of pre-existing biofilms required more biocides than was required for preventing the biofilm formation, in agreement with the current literature on the intrinsic resistance of biofilms (Costerton *et al*. 1999; Dunne 2002). Studies have suggested that biofilms contain small subpopulations of "persister cells", which can survive the biocide treatment and are the basis for rapid biofilm regeneration (Costerton *et al*. 1999; Czechowski & Stoodley 2002; Grobe *et al*. 2002). Debris of killed cells remaining on the surfaces may also serve as a good foothold and nutrient source for new surface colonizers.

The inherent resistance of biofilms suggests that an efficient strategy for biofilm control should rather aim at preventing the biofilm formation instead of trying to get rid of the persistent biofilms after they have already matured. It is an interesting paradox that most of the antimicrobials currently in use were originally discovered and evaluated based on their efficacy against planktonic cultures, usually in pure culture (Czechowski & Stoodley 2002; Dunne 2002). In reality, they should be tested against multi-species cultures and biofilms.

One limitation in the BF-assay is that it requires inoculums of 2 to 4% (vol/vol). Therefore the cell density in this assay after inoculation was up to 5×10^7 CFU ml^{-1}. It corresponds to the highest cell densities occurring in the circulating waters, but is higher than the average. That may have enhanced biocide inactivation by the bacteria, making this BF-assay as a sensitive tool to show possible biocide resistance. Therefore, when applying the BF-assay results in practice, in paper machine water with lower cell density, it is plausible that using somewhat lower biocide concentrations than used in the BF-assay will execute the same biocidal action. In general, the biofilm-forming strains from paper machines were more susceptible to MBT than to DBNPA.

4.8. Selection between the biofilm and planktonic mode of growth

4.8.1. Effects of nutrients and bacteriostatic compounds

D. geothermalis E50051 was incubated in neutral WW amended with different carbohydrates, amino acids, peptones, inorganic compounds, nisin, and bacteriostatic compounds, to examine their effects on the selection of the growth mode. Sodium azide (1.8 g l^{-1}), erythromycin (20 ppm), chloramphenicol (200 ppm), and nisin (3 ppm) all prevented the planktic and the biofilm growth of *D. geothermalis* in WW, but did not prevent the inoculated cells from adhering. This indicates that the cells of *D. geothermalis* possessed surface molecules required for the initial adhesion already after overnight planktic growth in TSB.

Table 4.8.1 shows that many carbohydrates, such as cellobiose, glucose, and starch, promoted growth of thicker biofilms by *D. geothermalis* than WW only. *D. geothermalis* formed biofilms also in the presence of 55 g l^{-1} of glucose, proving that even in high concentrations the glucose added into WW did not convert all cells from biofilm mode of growth to planktonic growth mode. Lactic acid reduced biofilm formation, but this was likely caused by the resulting low pH, ~4. Amending the WW with galacturonic acid, glucuronic acid, glucuronic acid lactone, or polygalacturonic acid also reduced biofilm formation, but this was not a pH effect as these acids were neutralized before use, and after BF-assay the pH in the wells was ≥6. Short-time assays on the cell-to-cell attachment (Chapter 4.3.3) showed that uronic acids may play a role in the cell-to-cell attachment of *D. geothermalis.* Excess presence of these uronic acids may have inhibited cell-to-cell adhesion thus reducing biofilm formation in assays lasting for 15 to 21 h. In longer experiments (≥24 h) the uronic acids were probably degraded as the biofilm-reducing effect disappeared.

Several nitrogen-containing organic substances, glucosamine, glutamate, leucine, heparin and peptones (tryptone or TSB), enhanced planktonic growth and diminished biofilm formation (Table 4.8.1). In high concentrations leucine (>1.3 g l^{-1}) and peptones (>3.3 g l^{-1}) caused transition of all cells from biofilm mode of growth to the planktic growth. Similarly, high concentrations of ammonium (1.7 g l^{-1}), phosphate (1 g l^{-1}) or NaCl (1.4%) prevented biofilm formation. When the biofilms were allowed to pre-grow in the microtiter plates before the test compounds were added, 1.6 g l^{-1} of leucine or 9 g l^{-1} of tryptone planktonized the pre-grown biofilms of *D. geothermalis* E50051 in 6 h, whereas the other test compounds did not. Similarly, biofilms of *D. geothermalis* that were pre-grown in WW on stainless steel test pieces were planktonized by peptones in high concentration. The results thus suggest a major role for the availability of nitrogen, but not of e.g. carbohydrate, for the selection of the growth mode by *D. geothermalis*. Similarly to this, we observed that *B. multivorans* F28L1 cells grew surface-attached as well as planktonic in WW medium, but adding 4 g of tryptone l^{-1} induced the cells to go over in planktonic mode of growth.

The effects of nutritional content of the medium on the biofilm development vary among bacteria. *P. aeruginosa* and *P. fluorescens* initiate biofilm development in response to a nutrient-rich medium, and return to the planktonic mode of growth when they are nutrient deprived (O'Toole *et al*. 2000). This starvation response allows the cells to search for a fresh source of nutrients. Biofilm formation of many other species is initiated by starvation, and is assumed to be advantageous for bacteria in oligotrophic environments (Korber *et al*. 1995). Paper machine white water contains organic carbon in excess,

Table 4.8.1. Effects of selected carbohydrates and other substances on the biofilm formation of *D. geothermalis* E50051 in BF-assay in WW.

Compound	Concentration	Observed effect on *D. geothermalis* E50051	Experimental conditions
Arabinose	0.3 - 17 g l^{-1}	No effects	23 h, 37°C, 150 rpm
Cellobiose	0.1 - 7 g l^{-1}	Produced ≤82% more biofilm than in WW only	15 h, 37°C, 160 rpm
Fructose	0.3 - 17 g l^{-1}	Produced 8-33% more biofilm than in WW only	18 h, 45°C, 140 rpm
Galactose	0.3 - 22 g l^{-1}	Produced 3-26% more biofilm than in WW only	18 h, 45°C, 140 rpm
Galacturonic acid	8 g l^{-1}	Reduced biofilm formation	21 h, 37°C, 150 rpm
Glucose	0.6 - 55 g l^{-1}	Produced 26-56% more biofilm than in WW only	18 h, 45°C, 140 rpm
Glucuronic acid	8 g l^{-1}	Reduced biofilm formation by 53%	15 h, 37°C, 160 rpm
Glucuronic acid lactone	>0.9 g l^{-1}	Reduced biofilm formation	21 h, 37°C, 150 rpm
Lactic acid	200 ppm	Reduced biofilm formation by 91%	18 h, 37°C, 160 rpm (in R2-broth)
Lactose	0.2 - 7 g l^{-1}	No effects	23 h, 37°C, 150 rpm
Mannose	0.2 - 11 g l^{-1}	No effects	17 h, 37°C, 140 rpm
Polygalacturonic acid	100 ppm	Reduced biofilm formation	17 h, 37°C, 140 rpm
Rhamnose	0.3 - 17 g l^{-1}	No effects	23 h, 37°C, 150 rpm
Starch	0.1 - 8 g l^{-1}	Produced more biofilm than in WW only	15 h, 37°C, 160 rpm / 72 h, 45°C, 160 rpm
Xylose	0.3 - 17 g l^{-1}	Produced 0-36% more biofilm than in WW only	18 h, 45°C, 140 rpm
Glucosamine	8 g l^{-1}	Prevented biofilm formation	15 h, 37°C, 160 rpm
Glutamate	>1.6 g l^{-1}	Reduced biofilm formation	21 h, 37°C, 150 rpm
Heparin[1]	>1.8 g l^{-1}	Reduced biofilm formation	21 h, 37°C, 150 rpm
Leucine	1.3 g l^{-1}	Reduced biofilm formation by 50%	18 h, 45°C, 140 rpm
Tryptone	>4 g l^{-1}	Planktonic growth only	21 h, 37°C, 150 rpm
TSB[2]	1:6 diluted	Planktonic growth only	17 h, 37°C, 140 rpm
Urea	0.3 - 14 g l^{-1}	More biofilm than in WW only	15 h, 37°C, 160 rpm
NaCl	>1.4%	No biofilms formed	23 h, 37°C, 150 rpm
NH_4^-	1.7 g l^{-1}	No biofilms formed	15 h, 45°C, 160 rpm
PO_4^{3-}	≥0.6 g l^{-1}	Reduced biofilm formation	23 h, 37°C, 150 rpm

[1] a proteoglycan; contains glucuronic acid, glucosamine and iduronic acid (Sharon 1975)
[2] Tryptic Soy Broth (Difco): tryptone 15 g l^{-1}, soytone 5 g l^{-1} and NaCl 5 g l^{-1}

but only 1-18 mg l^{-1} of total nitrogen (Väisänen *et al*. 1994 & 1998), which can drive *D. geothermalis* to biofilm mode of growth. The control of biofilms by the limitation of nutrients has been suggested as an alternative for the application of biocides (Blanco *et al*. 1996). Our results suggest that this may actually favour for the biofilm mode of growth of *D. geothermalis*. However, *D. geothermalis* E50051 retained surface-attached growth also in nutrient-rich conditions, e.g. in the presence of 1 g l^{-1} of peptones or >10 g l^{-1} of monosaccharides, suggesting that the selection of the growth mode involves also factors other than the nutrient availability.

4.8.2. Effects of flow, pH and ionic strength

We examined *D. geothermalis* E50051 to understand what kind of environmental factors lead to biofilm formation or cause the preference for planktonic growth. Flowing conditions were observed as a prerequisite for the formation of firm biofilms. In the laboratory experiments a rotary shaker, creating a peripheral flow of approximately 6-34 cm s^{-1} in the biofilm formation vessels (depending on the diameter of the vessel), was sufficient for the deinococci to grow as a biofilm. In paper machines the flow can be high; biofilm formation has been reported even in a flow of 1.8 m s^{-1} (Mattila *et al*. 2002). We found biofilms of *D. geothermalis* in several machines (Table 4.4), indicating liking for flow conditions, but the maximum flow velocity allowing its biofilm formation is not known. Stoodley *et al*. (2002) reviewed that biofilms grown at higher fluid shear stresses are more rigid and stronger than those grown at low flow conditions.

The effect of pH on the selection of the growth mode was studied with the BF-assay with tissue-culture-treated polystyrene wells. In WW of a neutral paper machine (pH 7.0) no biofilm was formed when pH was adjusted (hydrochloric acid) below 6, but in contrary to this, Fig 5b in Paper III shows that *D. geothermalis* E50051 formed biofilms in WW of an acidic (pH 4.9) paper machine. Biofilms pre-grown in WW at pH 7 were removed from the polystyrene surfaces in 6 h when pH was set below 4 by adding 0.2 M HCl, whereas biofilms pre-grown in R2 broth at pH 7 were not removed in one-hour acid wash (Table 4.2). These results show that the role of pH in the selection of the growth mode of *D. geothermalis* is not simple.

Table 4.8.2 Effects of ionic strength on the biofilm formation of *D. geothermalis* E50051 in glass and polypropylene tubes (3 d, 45°C, 160 rpm).

Medium (w/vol %)	Conductivity[1] mS cm^{-1}	Biofilm formation[2]
Tryptic Soy Broth (TSB)	10.8	-
TSB 1:20 diluted with 0.9% NaCl	16.8	-
TSB 1:20 diluted with 0.45% NaCl	9.0	+
TSB 1:20 diluted with 0.1% NaCl	2.4	+
TSB 1:20 diluted with distilled H_2O	0.6	+++
Paper machine water medium (WW)	1-1.2	+++

[1] measured with a portable conductivity meter (Philips PW 9529)
[2] the tubes were emptied, stained with crystal violet for 3 min, rinsed with flowing water, and scored visually for the biofilm formation

The effect of ionic strength on the biofilm formation of *D. geothermalis* E50051 was examined by growing cells in glass and polypropylene tubes in WW, in TSB, and in TSB diluted with aqueous solutions of NaCl. Table 4.8.2 shows that thick biofilms only grew in media with conductivity of 600 to 1200 μS cm^{-1}. The results indicate that low ionic strength favours biofilm formation of *D. geothermalis* E50051. The biofilm reduction induced by phosphate or ammonium ions shown in Table 4.8.1 may thus be partially due to an increased ionic strength, and not the nutrient effect alone.

4.9. *N*-Acylhomoserine-lactone-mediated cell-to-cell signalling in paper machine biofilms

The ultimate aim in our study of the signalling molecules was to find the possible signals regulating the choice between the biofilm and the planktonic modes of growth of paper machine bacteria. McLean *et al*. (1997) showed the first evidence of acylhomoserine lactone (AHL) activity in biofilms. They collected stones covered by river-water-grown biofilms, and incubated these stones on agar plates in the presence of a reporter strain. We used the same approach, collected slimes from paper machines, and set them directly on agar plates inoculated with three different sensor strains. None of the 32 studied slime specimens from three different paper machines induced violacein production by the sensor strain *Chromobacterium violaceum* CV026 or β–galactosidase production by *Agrobacterium tumefaciens* NTL4(pZLR4). The reference strain *P. aeruginosa* ATCC10145, known for producing AHLs, growing on the same agar plates induced the biosensors yielding colour reactions on the agar plates.

As the next step, 21 bacterial isolates from paper machines were analyzed as pure cultures by inoculating them as streaks parallel to the sensor strains on agar plates. None of the isolates induced any of the biosensors, whereas the reference strains *P. aeruginosa* ATCC10145 and *A. tumefaciens* NT1(pTiC58Δ*accR*) did induce colour reactions (*C. violaceum* and *A. tumefaciens*) or bioluminescence (*E. coli*) on the plates.

Cultures of 19 paper machine isolates and the two AHL-positive reference strains were extracted with dichloromethane or with ethyl acetate, the respective extracts concentrated, and assayed for the presence of AHL compounds using the biosensor strains. None of the extracts from the tested 19 paper machine isolates induced the sensor strains excepting for a weak induction of the *E. coli* sensor by *Pseudomonas* sp. strain JN 31102. The amount of bioluminescence was only 21% of that of the *P. aeruginosa* reference strain tested under identical conditions.

The BF-assay was used in exploring the effects of known signalling molecules on the biofilms of *B. multivorans* F28L1 and three *Thermomonas* spp. strains, all originating from paper machine slimes. Sixteen synthetic AHL compounds were tested at the concentration of 4 μM, but none of these detectably affected the quantity or quality (based on visual observations) of the growing biofilms nor planktonized pre-grown biofilms.

Several strategies were thus applied for detecting AHL-mediated cell-to-cell signalling in the paper machine biofilms, but the results were uniformly negative. This suggests that the known AHL signalling molecules play no role in the cell-to-cell communication of gram-negative bacteria in paper machine biofilms. The result was surprising, as AHLs have been detected in many gram-negative bacteria and in biofilms grown in different environments (Bachofen & Schenk 1998; Davies *et al*. 1998; Greenberg 1999;

McLean *et al*. 1997). *P. aeruginosa* and *B. cepacia* are among the most studied AHL-producing bacteria (Conway *et al*. 2002; Stoodley *et al*. 2002). In this study AHLs were not detected in any of the 21 paper machine isolates studied, of which four belonged to the genera *Pseudomonas* or *Burkholderia*.

In all our experiments the positive reference strains known for AHL production induced the sensor strains. An ethyl acetate extract was also prepared from a culture of *B. multivorans* F28L1 that had produced thick biofilms in R2-broth in 5 d. This extract was analyzed by electro-spray mass spectrometry, but no evidence for any AHLs was detected (Anatol Eberhard, personal communication). These results show that the negative results were not caused by an experimental failure. Recently Conway *et al*. (2002) studied species of the *B. cepacia* complex and observed that only 3 of 8 studied *B. multivorans* strains synthesized AHLs, although all of them demonstrated capacity for biofilm formation. This result is in accordance with our results suggesting that AHLs may not be involved in the biofilm formation of all *B. multivorans* strains.

In summary, our results suggest that the known AHLs play no role in the paper machine biofilms. It is possible that the paper machine bacteria, of which many are moderately thermophilic and inadequately studied, produced different signalling molecules not detectable with the biosensor strains used. The knowledge on bacterial signalling molecules is as yet limited (Greenberg 1999; Stoodley *et al*. 2002). AHLs have not been reported in paper machine biofilms except for Martinelli *et al*. (2002). They reported that an extract made of a sample from newsprint machine induced violacein production of the *C. violaceum* CV026 sensor strain, but the origin of the sample or the extraction procedure were not specified.

Several *Bacillus* species have been shown to produce AHL lactonases (Dong *et al*. 2002). It is attempting to think that the frequent presence of bacilli in paper machine biofilms may relate to their ability to inactivate quorum sensing of other bacteria in biofilms. The presence of AHL lactonases in the slimes would also explain why no AHLs were detected in the slime specimens by the biosensor strains. Recently, *Ralstonia* sp. isolates from a mixed-species biofilm were shown to degrade AHLs by an aminoacylase (Lin *et al*. 2003) and use the acyl side chain of the AHL molecules as the sole energy source for growth. Interestingly, the signal-inactivation encoding gene *aiiD* of *Ralstonia* sp. shared 52% identity with a deduced protein of undemonstrated function from the genome of *Deinococcus radiodurans* (Lin *et al*. 2003).

4.10. Structure of biofilms on ennobled stainless steel

Corrosion of stainless steel in natural waters is preceded by ennoblement, i.e. an increase of the open circuit potential by 100-400 mV, and this ennobling is coupled to the growth of biofilms on the stainless steel surfaces (Mattila 2002). A minor part of this thesis study concerned the architecture of biofilms emerging on ennobling stainless steel in Baltic Sea water (Paper IV). The post-ennoblement biofilms on stainless steel were composed of cell clusters with a basal diameter of 20-150 µm and surface coverage of 10-20%. CLSM and non-destructive tools, fluorescent hydrophobic and hydrophilic latex beads (diameters from 20 nm to 1 µm) showed that the cell clusters in living biofilms contained no pores or cavities (Figures 2, 3 & 5 in Paper IV). Clusters over 25 µm in height had a core non-penetrable to fluorescent nucleic acid stains within 15 min, indicating limited diffusion

into the clusters (Figures 2 and 3 in Paper IV). EtBr penetrated deeper (17 µm in 15 min) into the clusters than did the less polar SYTO 16 or acridine orange, suggesting that hydrophilic compounds may have better access into these post-ennoblement biofilms (Table 3 in Paper IV).

The cell clusters showed interesting internal structures, as they often contained 1 to 3 spherical internal colonies, packed with bacteria in a density higher than in the surrounding main cluster (Fig 6 in Paper IV). Such heterogenous microbe communities may generate regions with different concentrations of bacterial metabolites (acids, oxidants, reductants etc.). The dense cell clusters only partially covering the steel surface must contain regions with different oxygen contents. These factors may have played a role during the ennoblement, and may promote subsequent corrosion of the steel.

This study demonstrated the poorly permeable nature of biofilms on ennobled stainless steel. Studies of Mattila (2002) then clearly showed the essence of this specific biofilm architecture for the ennoblement. When the water, where the stainless steels were immersed, was enriched with nutrients, the biofilms on the steel grew fluffy and detached easily, and caused no ennoblement of the steel.

4.11. Methods developed in this study

4.11.1. Improved procedure for isolation of primary-biofilm formers

When slimes are sampled from paper machines (Fig 1), a typical sample is composed of coherent sheets or grains of slime (Figs 1a & 2a in the Paper III), adjoined with abundant loose material such as unattached process bacteria, cellulose fibers or papermaking additives. When an untreated sample is transferred on an agar plate and incubated, the abundance of biofilm-associated bacteria may mask the presence of the biofilm-forming bacteria. We observed that the yield of the true primary biofilm-formers was improved by washing the sampled slimes intensively before plating. Paper III presents two examples on the effects of vigorous washing with sterilized water: (1) the white layer (Fig 1a in Paper III) disintegrated, but the orange-pigmented layer detached as cohesive sheets of slime that held together through several washings; and (2) the translucent slime suspended easily contrary to the compact red deposits (Figs 2a & 2b in Paper III).

In the improved isolation procedure the sampled slime is suspended in 5 to 10 volumes of sterilized tap water and is vigorously shaken for 15 s. Compact sheets or pellets of slime are allowed to settle (5-20 s), the supernatant drained, and the procedure repeated 5 to 10 times. The remaining washed pellets of compact slime, freed from the easily suspending bacteria, are likely the causative agents of process disturbances or deterioration of the end-product quality. Bacteria producing these compact slimes are isolated by cultivating the rigorously washed slimes at 45 or 50°C (2-7 d) on R2A or WWA agar plates adjusted to the pH prevailing in the source machine.

4.11.2. The BF-assay

Cowan & Fletcher (1987) introduced the 96-well polystyrene microtiter plates as attachment surfaces for the studies of biofilm formation. Since then the microtiter plate technique has been used as a simple screening tool for adhesion-deficient mutants in studies of biofilm development (O'Toole *et al*. 2000). We modified this technique to serve paper machine

studies. The new variant biofilm formation assay (BF-assay, Fig 9) was used to examine:

- biofilm formation capability of isolates (Papers I & III, and unpublished data)
- species interactions in the biofilm formation (Paper I)
- effects of biocides and of novel alternatives for the biocides on formation of the biofilms (Papers I & III, Table 4.7, Fig 9, and unpublished data)
- impacts of nutrients, pH and liquid flow on the biofilms (4.8)
- cell-to-cell signalling in the paper machine biofilms (4.9)

In addition to the studies with pure bacterial cultures, the BF-assay was used to study process samples. Freshly sampled paper machine slimes were vigorously homogenized and used to seed 6- or 12-well microtiter plates. Table 4.11 shows an example where the BF-assay was successfully applied to assess the effect of a biocide on the propagation of freshly sampled slime.

In this one-day experiment similar biofilms (A_{595} 0.44 ±0.07) grew in all seeded wells with no biocide, proving the presence of primary-biofilm formers in the seed slime. In the presence of 5-10 ppm of the biocide DBNPA more biofilm was produced than in the absence, indicating that the planktic mode of growth was difficult or unfeasible. As much as 40 to 100 ppm of DBNPA was required to block all biofilm formation proving that this particular slime was resistant towards DBNPA. This shows that the efficacy of a biocide towards a problematic slime can be assayed within one day.

Table 4.11. Tolerance of a freshly sampled slime from a fine paper machine towards DBNPA [1]

DBNPA (ppm) in the well	Quantity (A_{595nm} ± SD) of biofilm formed in one day
0	0.44 ± 0.07
5	0.58 ± 0.09
10	0.80 ± 0.22
40	0.22 ± 0.22
100	0.00 ± 0.00

[1]The sampled slime was vigorously homogenized in R2 broth in final concentration of ~5%. Each well of a 12-well microtiter plate was filled with 1.8 ml of this mixture and DBNPA added. The plates were incubated (45°C, 160 rpm), and the biofilm yield quantified as described in Papers I, II & III. Values show average A_{595} ± SD of two to four parallel wells.

4.11.3. FISH protocol for *D. geothermalis*

A FISH protocol was developed for *D. geothermalis* to detect its presence in paper machine biofilms with no need for cultivation methods (Fig 19, Table 4.4). The sequence of DgeoF-probe was compared to the prokaryotic 16S rRNA gene sequences in the RDP database at http://rdp.cme.msu.edu/html/ (reviewed by Maidak *et al*. 2001). Only four strains with completely matching target 16S rRNA sequences were found, covering all strains representing the species *D. geothermalis* in the database: strains AG-3a^T, AG-5a and RSPS-2a from hot springs, and E50053 from paper machine. The probe was separated from the next nearest hit in the RDP database (*Brochothrix thermosphacta* ATCC11509^T) by three mismatching bases indicating that it was specific for *D. geothermalis*.

Air-drying and dehydration of the specimens with ethanol were found sufficient as pretreatments for a good access of the DgeoF-probe into the *D. geothermalis* cells, further permeabilization with lysozyme (5 mg ml^{-1}, 10 min, +20°C) or protease (5 mg ml^{-1}, 10-60 min, 37°C) gave no benefits.

Successful *in situ* -hybridization requires stringent conditions that allow binding of the labeled DNA-probe with completely matching target rRNA sequences only (Amann & Schleifer 2001). Sufficient stringency was acquired by adjusting the concentration of formamide in the hybridization buffer to 40% (v/v), and the NaCl concentration in the washing buffer to 80 mM (Fig 19). Under these conditions *D. geothermalis* cells were intensively labeled and the 14 reference strains (chapter 3.2.3) showed no binding of the DgeoF-probe, except for *B. coagulans* E50L1 and *B. licheniformis* D50141 showing weak binding that was easily differentiated from the intensive fluorescence signal originating from *D. geothermalis* cells. Even in more stringent conditions (NaCl 20 mM) we observed weak spot-wise binding of the DgeoF-probe to the *Bacillus* cells, even though several mismatches discriminates DgeoF-probe from these *Bacillus*-species. As the studied *Bacillus*-species produced copious EPS, their weak labelling may have been due to unspecific trapping of the DgeoF-probe by the EPS. The labelling of *Bacillus*-cells diminished when pre-treated with unlabeled unspecific oligonucleotides prior to hybridization, to block any unspecific binding sites. This supports the hypothesis that there was unspecific binding of DgeoF-probe to the EPS of the *Bacillus* cells.

Active cells contain plenty of rRNA molecules that bind DNA-probes in the hybridization, and the active cells will thus exhibit stronger fluorescence than the resting cells. To improve the detection of *D. geothermalis* cells in sampled slimes, we incubated a part of the slimes in R2 or in yeast extract broth (2 g l^{-1}) for 1 h at 50°C to boost the rRNA content of the cells. In subsequent hybridization we observed no benefit from this pre-activation. Slimes that were simply air-dried and dehydrated within six hours from sampling yielded equal fluorescence signal. Kalmbach *et al*. (1997) incubated drinking water biofilms with a mixture of carbon sources prior hybridization, and observed that the percentage of hybridized cells increased from 50% to 80% of the total cell counts. That boosting approach thus clearly improved FISH detection of biofilm cells growing in drinking water, but did not apply for biofilms growing in paper machine water.

4.11.4. Fluorescent stains for microscopy of biofilms

In addition to the microbes, paper machine slimes often contain cellulose fibers and many kinds of papermaking additives that have merged into the growing slimes. Especially filling and coating agents are often autofluorescent, and their abundant presence can disturb or prevent fluorescent microscopic inspection of the slime specimen. We found that the use of fluorescent dyes such as EtBr with at a long Stokes-shift, i.e., large difference between excitation and emission maxima of the dye molecule, eased differentiating the stained bacterial cells from particles of similar size and shape that emit autofluorescence.

Fluorescent dextrans with defined molecular sizes (Lawrence *et al*. 1994; Møller *et al*. 1997) and latex beads of diameter 1 µm (Okabe *et al*. 1997; DeBeer *et al*. 1994) have been used to study the porosity of biofilms. We used fluorescent latex beads of near-to-colloid-sizes, 20 and 100 nm in diameter, in conjunction with CLSM as a new

Table 4.12. Paper machine bacteria deposited in the HAMBI culture collection.

Strain	HAMBI code	Specific features of the strain	Other information
Burkholderia cepacia F28051	2487	Primary-biofilm former	Väisänen *et al*. 1998
B. kururiensis B2842	2494	Degrading paper mill water components	Lindberg *et al*. 2001b
B. multivorans F28L1	2476	Primary-biofilm former	Väisänen *et al*. 1998
B. multivorans F45L5	2491	Primary-biofilm former	Lindberg *et al*. 2001a
B. multivorans F453dL1	2492	Primary-biofilm former	Lindberg *et al*. 2001a
B. multivorans E28L3	2493	Primary-biofilm former	Väisänen *et al*. 1998
Deinococcus geothermalis E50051	2411	Pink-pigmented, primary-biofilm former	Väisänen *et al*. 1998
D. geothermalis C-wire-CTYE-3	2480	Pink-pigmented, primary-biofilm former	Paper III
D. geothermalis C-CTYE-9a	2481	Pink-pigmented, primary-biofilm former	Paper III
Meiothermus silvanus B-R2A5-50-4	2477	Red-pigmented, primary-biofilm former	Paper III
M. silvanus C-wire-CTYE-1	2478	Red-pigmented, primary-biofilm former	Paper III
α-*Proteobacteria* A-col-CFA-6 "*Porphyrobacter*-like"	2479	Orange-pigmented, primary-biofilm former, likely a novel species	EMBL accession no. AJ505839
α-*Proteobacteria* A-col-R2A-4 "*Rhodobacter*-like"	2468	Red-pigmented, primary-biofilm former, likely a novel genus	EMBL accession no. AJ505840
"*Rhodobacter*-like" C-lvk-R2A-1	2469	as above	Paper III
"*Rhodobacter*-like" C-lvk-R2A-2	2421	as above	Paper III
"*Rhodobacter*-like" C-R2A-5d	2512	as above	Paper III
"*Rhodobacter*-like" C-R2A-52d	2511	as above	Paper III
"*Rhodobacter*-like" E-R2A-8a	2509	as above	Paper III
α-*Proteobacteria* A-cf-CFA-6 "*Roseomonas*-like"		Pink-pigmented, frequent inhabitant of paper machines, likely a novel genus	EMBL accession no. AJ512828
Thermomonas haemolytica A50-7-3^T	2467	*Thermomonas* spp. are common in paper machines, type strain (from kaolin)	Busse *et al*. 2002; = DSM 13605^T
T. hydrothermalis SGM-6^T	2518	*Thermomonas* spp. are common in paper machines, type strain (from hot spring)	Alves *et al*. 2003

non-destructive tool (Table 2 in Paper IV). These beads were first used to probe the permeability of living biofilms grown on ennobled stainless steel (Figs 2, 3 & 5 in Paper IV). The study showed that the cell clusters were impermeable even for the smallest latex beads of only 20 nm in diameter indicating the absence of pores or cavities.

Encouraged by this, we used the beads as one of the basic staining methods when examining slimes sampled from paper mills. The fluorescent beads were applied to show the contours of the slime and a nucleic acid stain was applied to show the location of the bacterial cells (Figs 1 & 2 in Paper III). This allows estimation of the compactness and extent of the biofilm matrix, leaving no need for specific staining of the EPS.

4.12. Deposited sequences and bacterial cultures

This study yielded new understanding on the important species in paper machine biofilms. To support research in the field of paper industry microbiology, we deposited several paper machine bacteria in the HAMBI Collection (The Culture Collection of the Faculty of Agriculture and Forestry, University of Helsinki) and three 16S rRNA gene sequences in the EMBL database (reviewed by Stoesser *et al.* 2002), listed in Table 4.12.

5. MAIN CONCLUSIONS

This research resulted in the following novel findings:

We showed that only a fraction of the bacteria found in paper machines is capable of initiating biofilm formation on a clean surface. These bacteria were named as primary-biofilm formers to distinguish them from the secondary biofilm bacteria, i.e. bacteria present in the biofilms but incapable of independently forming biofilm on a clean surface. We hypothesize that targeted prevention of the primary-biofilm formers could block the subsequent stages of biofilm growth, and eradication of the non-biofilm forming species from the circulating waters would not be required.

We showed that the primary-biofilm former *Deinococcus geothermalis* was frequent in paper-machine slimes. This was shown both by cultivation-dependent approach and by a cultivation-independent *in situ* -hybridization protocol developed for the *D. geothermalis*.

D. geothermalis produces strong biofilms that are not removed by one-hour wash with 0.2% NaOH, with 1 M HCl, or with 0.5% SDS. Interestingly, the firm attachment of *D. geothermalis* was laterally slippery. This allowed the attached cells to escape external pushing forces by sliding along the surface. Until our observations, non-motile bacteria were thought to anchor themselves irreversibly in one position on a surface. We named this novel behavior on surfaces “slippery attachment”.

The cell-to-cell and cell-to-surface attachments of *D. geothermalis* are mediated by a small amount of extracellular adhesion threads. The chemistry of cell-to-cell attachment seems to involve uronic acid containing polysaccharides as well as cell surface proteins. Analysis by AFM revealed that the attached cells had a patchy surface with materially different regions exposed. This may explain why *D. geothermalis* attached and grew as biofilms on the various tested non-living substrates with different surface properties.

The availability of nitrogen compounds played a major role in the selection between the planktonic and biofilm modes of growth, whereas availability of organic carbon showed no effect on the growth mode preferred by *D. geothermalis*. Low concentration of nitrogen-containing compounds or phosphate, low ionic strength, and flowing conditions were factors favoring the biofilm mode of growth.

We recognized five taxonomic groups of primary-biofilm formers common to the studied paper machines: pink-pigmented *D. geothermalis*, red-pigmented *Meiothermus silvanus*, a novel genus of “*Rhodobacter*-like” red-pigmented α–*Proteobacteria*, *Thermomonas* spp. and the *Burkholderia cepacia* complex. We also observed that several of the paper machine bacteria were species previously known from microbial mats growing in run-offs (50-60°C) from hot springs in geothermal areas.

We showed that *Bacillus* species were not primary-biofilm formers even if frequently found in paper machine biofilms. They required primary-biofilm forming species to initiate the biofilm build-up. We demonstrated interspecies co-operation and antagonism in growing biofilms. To our knowledge the Paper I is the first publication on species interactions in paper machine biofilms.

We found several new moderately thermophilic bacteria: likely two new genera and several new species of α*–Proteobacteria*.

Cell-to-cell communication of gram-negative bacteria in paper machine biofilms was examined by detecting production of AHL signalling molecules with the aid of biosensor strains, and by experimenting the effects of synthetic signalling molecules to the biofilms. Our results showed no role for the currently recognized AHL signalling molecules in paper machine biofilms.

We showed the value of fluorescent latex beads of near-to-colloid-size combined with CLSM in the analysis of biofilm architecture. Using the latex beads and nucleic acid stains we demonstrated the poorly permeable nature of biofilm microcolonies as an essential characteristic of biofilms grown on stainless steel during ennoblement in Baltic Sea water.

We developed methods: i) an improved procedure for isolation of primary-biofilm forming bacteria; ii) a new variant of the microtiter plate assay for biofilm formation (BF-assay) tuned to examine biofilm formation capability of paper machine isolates, and iii) to examine effects of biocides, nutrients, signalling molecules, pH and liquid flow on biofilm formation. The BF-assay proved suitable also for fast analysis of biocide susceptibility of sampled process slimes.

We showed that many paper machine bacteria were capable of forming biofilms in the presence of either of the two common biocides tested. We found over 40 bacterial strains that increased biofilm formation in response to biocide concentrations inhibiting the free-swimming but not the surface-attached cells. This suggests that biocide stress activated transition from the planktonic growth mode to the biofilm mode of growth. Our results imply that sub-lethal dose of biocide when used in paper machines may cause the bacteria to seek shelter in biofilms thus increasing instead of limiting biofouling in the process. This addresses the importance of testing the efficacy of the intended antimicrobial agents towards true biofilms. Performance of the biocide against biofilms cannot be predicted based on sensitivity data obtained with planktonic bacteria solely.

6. ABSTRACT

Bacterial biofilms impair operation of many industrial processes. In paper machines biofilms detaching from surfaces cause holes or spots in the paper products, or can break the paper web. This thesis showed that only a small proportion of the diverse microflora living in the warm paper-machine waters is capable of initiating the biofilm formation on clean surfaces. We recognized five taxonomic groups of such primary-biofilm formers: the species *Deinococcus geothermalis* and *Meiothermus silvanus*, and members of a novel genus of "*Rhodobacter*-like" α–*Proteobacteria*, the genus *Thermomonas*, and the *Burkholderia cepacia* complex. Based on the number of cultivated isolates and on the *in situ* -hybridization data, the pink-pigmented species *D. geothermalis* was the most frequent. Extracellular adhesion threads, probably including proteins and uronic-acid-containing polysaccharides, mediated its cell-to-cell and cell-to-surface attachment. *D. geothermalis* formed wash-resistant biofilms on different non-living surfaces. We discovered that the firmly adhering cells possessed a laterally slippery attachment mechanism that allowed the cells to slide along the surface without detaching from the surface. This firm, but laterally slippery attachment enabled the cells to escape external pushing forces. This kind of attachment has not been reported earlier for a non-motile (non-flagellated) species.

We showed interspecies interactions in the paper-machine biofilms. *Bacillus* species were frequently found in biofilms, but they were unable to form biofilm on clean surfaces as pure cultures. Their integration into biofilms depended on primary-biofilm formers, other species that initiated the fouling of surfaces. Improved methods were developed to isolate the primary-biofilm formers and to examine the effects of biocides, nutrients, and signalling molecules on the biofilm mode of growth of the bacteria. Low ionic strength and low concentrations of organic nitrogen compounds favored the biofilm mode of growth of *D. geothermalis*. Many of the bacteria isolated from paper machines were able to form biofilms in the presence of biocide concentrations that were inhibitory to free-swimming cells. Several of the biofilm isolates responded to the biocide by increased transition to the biofilm mode of growth. The results suggest that if the biocide will be dosed too low to prevent the surface-attached growth this may actually cause the planktonic bacteria to seek protection by shifting to growth in biofilms and thus increase biofouling of the process. Finally, several novel species of moderately thermophilic bacteria were isolated from paper machines.

7. ACKNOWLEDGEMENTS

This work was carried out at the Division of Microbiology, Department of Applied Chemistry and Microbiology, University of Helsinki under the supervision of professor Mirja Salkinoja-Salonen. I want to thank Mirja for the great help in interpreting my results and for her boundless, very infectious, enthusiasm for applied microbiology. I admire her knowledge of microbiology and chemistry, and I am grateful for those numerous lessons that she had time to give in the past years.

This work was supported by the Viikki Graduate School in Biosciences of Helsinki University, Kemira Chemicals Oy, the National Technology Agency TEKES (Bimu, Luomuke and ShinePro-projects), Finnish paper industry, the Helsinki University Fund for Center of Excellence, and the Academy of Finland Fund for Center of Excellence.

I owe my gratitude to all the following:

Professor Peter Neubauer and Dr. Kaarina Lähteenmäki, who reviewed this thesis thoroughly and gave valuable comments.

My co-authors Jaro Nuutinen, Katri Mattila, prof. Frederic Rainey, Raimo Mikkola, Ute Schmidt and Esa Kuismanen for sharing their expertise. Special thanks go to Katri for the post-conference adventures in the back country of Yellowstone Natural Park.

My international scientific collaborators: prof. Anatol Eberhard for providing me the synthetic AHLs and performing the electro-spray mass spectrometry; Katrin Bartscht for the immunolabeling of *D. geothermalis* biofilms; Patrick Markus for expertise in the TappingMode AFM; prof. Stephen Farrand and Otto Geisenberger for the biosensor strains; and prof. Edward Moore for the 16S rRNA gene sequence determinations.

The numerous industrial collaborators during the years, especially Pentti Väätänen, Juha Mentu, Päivi Korhonen and Assi Weber.

Outi Väisänen, Irina Tsitko, Tuija Pirttijärvi, Jyrki Juhanoja, Aimo Saano, Douwe Hoornstra, German Jürgens, Maria Andersson, prof. Max Häggblom, Riitta Saastamoinen and Riitta Boeck for their kind co-operation. Special thanks to Helena Vihinen for advices in CLSM.

Justus Reunanen, Inka Harju and Eriikka Heikkinen for assistance in the laboratory.

Viikki Graduate School in Biosciences for appointing my follow-up group. In the beginning I was skeptic, doubting whether this really was needed, but quite soon I changed my opinion completely. It felt good to have a group of experienced scientist that followed my progress and gave advices, so many thanks to prof. Carl Gahmberg, Benita Westerlund, Kaarina Lähteenmäki and Michael Hess.

Emeritus professor Seppo Niemelä, who encouraged me to go into the fascinating world of microscopy.

Anssi and Rane, my long time colleagues, for their friendship and for those numerous coffee breaks that we shared.

All my former and current colleagues (Joanna, Chris, Pauliina, Pekka, Kimmo, Irmgard, Jukka, Tuomas, Päivi, Jouni, Hanna L., Mari R., Camelia, Mika, Hanna S., Elina, Jaakko, Ranad, Kari, Jussi, Timo T., Timo N., Shea, Zewdu, Leone, and all the others not mentioned here) for the nice working atmosphere, for their friendship, and for all the discussions about work and leisure.

Leena Steininger, Hannele Tukiainen and Tuula Suortti for the secretarial help, and all the other persons at Division of Microbiology for assisting in many practical tasks.

Instrument Center of Faculty of Agriculture and Forestry for their services.

I would also like to thank all my relatives and friends, including the floorbandy teams BI Knockouts and LaKu, who may not have noticed it, but had an important role in the maturation of this thesis, by sustaining fresh connections to the real life outside the laboratory.

I am indebted to my dear parents Sakari and Maija-Liisa for their encouragement through the years. I am grateful to them, as well as to Marja and Olli, for all the moments that they were playing with their first grand child Onni, thus giving me a change to finalize this work.

Finally, I wish to thank so much my dear wife Satu and my son Onni, who are the biggest joy in my life. They have succeeded to balance my life so that it has not been overwhelmed by science. I feel fortunate to have such a great family. Special thanks for the patience during the last squeeze of this thesis.



Helsinki, April 2003

8. REFERENCES

Alves MP, FA Rainey, MF Nobre and MS DaCosta. 2003. *Thermomonas hydrothermalis* sp. nov., a new slightly thermophilic γ–*Proteobacterium* isolated from a hot spring in central Portugal. Syst Appl Microbiol 26: 70-75.

Amann RI, W Ludwig and K-H Schleifer. 1995. Review. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. Microbiol Rev 59: 143-169.

Amann RI. 1995. In situ identification of microorganisms by whole cell hybridization with rRNA-targeted nucleic acid probes, 3.3.6: 1-15. In: Akkermans ADL, JD van Elsas and FJ de Bruijn (Eds), Molecular Microbial Ecology Manual, Kluwer Academic Publishers, Dordrecht, The Netherlands.

Amann R. 2000. Review. Who is out there? Microbial aspects of biodiversity. System Appl Microbiol 23: 1-8.

Amann R, BM Fuchs and S Behrens. 2001. The identification of microorganisms by fluorescence *in situ* hybridization. Curr Opin Biotechnol 12: 231-236.

Amann R and K-H Schleifer. 2001. Nucleic acid probes and their application in environmental microbiology, p. 67-82. In: Boone DR, RW Castenholz and GM Garrity (Eds), Bergey's Manual of Systematic Bacteriology, Second edition, Bergey's Manual Trust, Springer-Verlag, New York, USA.

Auerbach ID, C Sorensen, HG Hansma and PA Holden. 2000. Physical morphology and surface properties of unsaturated *Pseudomonas putida* biofilms. J Bacteriol 182: 3809-3815.

Bachofen R and A Schenk. 1998. Quorum sensing autoinducers: Do they play a role in natural microbial habitats? Microbiol Res 153: 61-63.

Battista JR and FA Rainey. 2001. Genus *Deinococcus*, p. 396-403. In: Boone DR, RW Castenholz and GM Garrity (Eds), Bergey's Manual of Systematic Bacteriology, Second edition, Springer-Verlag, New York, USA.

Beech IB. 1996a. The potential use of atomic force microscopy for studying corrosion of metals in the presence of bacterial biofilms. Int Biodeterior Biodegrad 37: 141-149.

Beech IB, CWS Cheung, DB Johnson and JR Smith. 1996b. Comparative studies of bacterial biofilms on steel surfaces using atomic force microscopy and environmental scanning electron microscopy. Biofouling 10: 65-77.

Blanco MA, C Negro, I Gaspar and J Tijero. 1996. Mini-review. Slime problems in the paper and board industry. Appl Microbiol Biotechnol 46: 203-208.

Borenstein SW. 1994. Microbiologically influenced corrosion handbook. Woodhead Publishing, Cambridge, UK, 288 pp.

Bremer PJ, GG Geesey and B Drake. 1992. Atomic force microscopy examination of the topography of a hydrated bacterial biofilm on a copper surface. Curr Microbiol 24: 223-230.

Budavari S (Ed). 1996. The Merck Index – An encyclopedia of chemicals, drugs, and biologicals. Twelfth Edition. Merck & Co., Whitehouse Station, NJ, USA.

Busscher HJ, AT Poortinga and R Bos. 1998. Lateral and perpendicular interaction forces involved in mobile and immobile adhesion of microorganisms on model solid surfaces. Curr Microbiol 37: 319-323.

Busse H-J, P Kämpfer, ERB Moore, J Nuutinen, IV Tsitko, EBM Denner, L Vauterin, M Valens, R Rosselló-Mora and MS Salkinoja-Salonen. 2002. *Thermomonas haemolytica* gen. nov., sp. nov., a γ–*Proteobacterium* from kaolin slurry. Int J Syst Evol Microbiol 52: 473-483.

Carpentier B and O Cerf. 1993. Review. Biofilms and their consequences, with particular reference to hygiene in the food industry. J Appl Bacteriol 75: 499-511.

Cha C, P Gao, Y-C Chen, PD Shaw and SK Farrand. 1998. Production of acyl-homoserine lactone quorum-sensing signals by gram-negative plant-associated bacteria. Molec Plant-Microbe Interact 11: 1119-1129.

References

Chaudhary A, LK Gupta, JK Gupta and UC Banerjee. 1997. Studies on slime-forming organisms of a paper mill – slime production and its control. J Industr Microbiol Biotechnol 18: 348-352.

Chen X, S Schauder, N Potier, AV Dorsselaer, I Pelczer, BL Bassler and FM Hughson. 2002. Structural identification of a bacterial quorum-sensing signal containing boron. Nature 415: 545-549.

Claus G and R Müller. 1996. Biofilms in a paper mill process water system, p. 429-437. In: Heitz E, H-C Flemming and W Sand (Eds), Microbially influenced corrosion of materials, Springer-Verlag, Berlin, Germany.

Conway B-A, V Venu and DP Speert. 2002. Biofilm formation and acyl homoserine lactone production in the *Burkholderia cepacia* complex. J Bacteriol 184: 5678-5685.

Costerton JW, Z Lewandowski, D DeBeer, D Caldwell, D Korber and G James. 1994. Mini-review. Biofilms, the customized microniche. J Bacteriol 176: 2137-2142.

Costerton JW and HM Lappin-Scott. 1995. Introduction to Microbial Biofilms, p. 1-11. In: Lappin-Scott HM and JW Costerton (Eds), Microbial biofilms, Plant and microbial biotechnology research series: 5, University Press, Cambridge, UK.

Costerton JW, PS Stewart and EP Greenberg. 1999. Review. Bacterial biofilms: a common cause of persistent infections. Science 284: 1318-1322.

Cowan MM and M Fletcher. 1987. Rapid screening method for detection of bacterial mutants with altered adhesion abilities. J Microbiol Methods 7: 241-249.

Czechowski MH and P Stoodley. 2002. Antimicrobials and biofilms. J Industr Microbiol Biotechnol 29: 325.

Das JR, M Bhakoo, MV Jones and P Gilbert. 1998. Changes in the biocide susceptibility of *Staphylococcus epidermidis* and *Escherichia coli* cells associated with rapid attachment to plastic surfaces. J Appl Microbiol 84: 852-858.

Davies DG, MR Parsek, JP Pearson, BH Iglewski, JW Costerton and EP Greenberg. 1998. The involvement of cell-to-cell signals in the development of bacterial biofilms. Science 280: 295-298.

DeBeer D, P Stoodley, F Roe and Z Lewandowski. 1994. Effects of biofilm structures on oxygen distribution and mass transport. Biotechnol Bioeng 43: 1131-1138.

Dong Y-H, L-H Wang, J-L Xu, H-B Zhang, X-F Zhang and L-H Zhang. 2001. Quenching quorum-sensing-dependent bacterial infection by an *N*-acyl homoserine lactonase. Nature 411: 813-817.

Dong Y-H, AR Gusti, Q Zhang, J-L Xu and L-H Zhang. 2002. Identification of quorum-quenching *N*-acyl homoserine lactonases from *Bacillus* species. Appl Environ Microbiol 68: 1754-1759.

Donlan RM and JW Costerton. 2002. Review. Biofilms: survival mechanisms of clinically relevant microorganisms. Clin Microbiol Rev 15: 167-193.

Dufréne YF. 2002. Mini-review. Atomic force microscopy, a powerful tool in microbiology. J Bacteriol 184: 5205-5213.

Dunne WM Jr. 2002. Review. Bacterial adhesion: Seen any good biofilms lately? Clin Microbiol Rev 15: 155-166.

Elvers KT, K Leeming, CP Moore and HM Lappin-Scott. 1998. Bacterial-fungal biofilms in flowing water photo-processing tanks. J Appl Microbiol 84: 607-618.

Elvers KT, K Leeming and HM Lappin-Scott. 2002. Binary and mixed population biofilms: Time-lapse image analysis and disinfection with biocides. J Industr Microbiol Biotechnol 29: 331-338.

Ferreira AC, MF Nobre, FA Rainey, MT Silva, R Wait, J Burghardt, AP Chung and MS DaCosta. 1997. *Deinococcus geothermalis* sp. nov. and *Deinococcus murrayi* sp.nov., two extremely radiation-resistant and slightly thermophilic species from hot springs. Int J Syst Bacteriol 47: 939-947.

Finnish Environment Institute (SYKE, Helsinki, Finland). 2002. Limantorjuntakemikaalien myynti vuonna 2001 (Total sales of slimicides in Finland in 2001, in Finnish), Ympäristö 8: 19. http://www.ymparisto.fi/ympsuo/kemik/suke_myynti.htm (19.1.2003)

Flemming H-C. 2002. Mini-review. Biofouling in water systems – cases, causes and countermeasures. Appl Microbiol Biotechnol 59: 629-640.

Flint SH, PJ Bremer and JD Brooks. 1997. Biofilms in dairy manufacturing plant – description, current concerns and methods of control. Biofouling 11: 81-97.

Flint S, J Palmer, K Bloemen, J Brooks and R Crawford. 2001. The growth of *Bacillus stearothermophilus* on stainless steel. J Appl Microbiol 90: 151-157.

Fotiadis D, S Scheuring, SA Müller, A Engel and DJ Müller. 2002. Review. Imaging and manipulation of biological structures with the AFM. Micron 33: 385-397.

Fuchs BM, G Wallner, W Beisker, I Schwippl, W Ludwig and R Amann. 1998. Flow cytometric analysis of the *in situ* accessibility of *Escherichia coli* 16S rRNA for fluorescently labelled oligonucleotide probes. Appl Environ Microbiol 64: 4973-4982.

Grantham MC and PM Dove. 1996. Investigation of bacterial-mineral interactions using Fluid Tapping Mode atomic force microscopy. Geochim Cosmochim Acta 60: 2473-2480.

Granum PE and T Lund. 1997. *Bacillus cereus* and its food poisoning toxins. FEMS Microbiol Lett 157: 223-228.

Greenberg EP. 1999. Quorum sensing in Gram-negative bacteria: acylhomoserine lactone signalling and cell-cell communication, p. 71-84. In: England R, G Hobbs, NJ Bainton and D McL Roberts (Eds), Microbial signalling and communication, Symposium 57 of the Society for General Microbiology, Cambridge University Press, Cambridge, UK.

Grobe KJ, J Zahller and PS Stewart. 2002. Role of dose concentration in biocide efficacy against *Pseudomonas aeruginosa* biofilms. J Industr Microbiol Biotechnol 29: 10-15.

Han W, SM Lindsay and T Jing. 1996. A magnetically driven oscillating probe microscope for operation in liquids. Appl Phys Lett 69: 4111-4113.

Harju-Jeanty P and P Väätänen. 1984. Detrimental micro-organisms in paper and cardboard mills. Paperi ja Puu 3: 245-251.

Harkes G, J Dankert and J Feijen. 1992. Bacterial migration along solid surfaces. Appl Environ Microbiol 58: 1500-1505.

Haugland RP. 2001. Handbook of fluorescent probes and research products. Eighth edition, Molecular Probes Europe, PoortGebouw, Leiden, The Netherlands.

Hentzer M, K Riedel, TB Rasmussen, A Heydorn, JB Andersen, MR Parsek, SA Rice, L Eberl, S Molin, N Høiby, S Kjelleberg and M Givskov. 2002. Inhibition of quorum sensing in *Pseudomonas aeruginosa* biofilm bacteria by a halogenated furanone compound. Microbiol 148: 87-102.

Hughes-van Kregten MC. 1988. Slime flora of New Zealand paper mills. Appita 41: 470-474.

Husmark U. 1993. Adhesion mechanisms of bacterial spores to solid surfaces. Ph.D. Thesis. Department of Food Science, Chalmers University of Technology, Göteborg, Sweden. 85 pp.

Hussain M, MH Wilcox and PJ White. 1993. Review. The slime of coagulase-negative staphylococci: Biochemistry and relation to adherence. FEMS Microbiol Rev 104: 191-208.

Johnsrud SC. 1997. Biotechnology for solving slime problems in the pulp and paper industry, p. 311-328. In: Scheper T (Ed), Advances in Biochemical Engineering / Biotechnology, Vol. 57, Springer-Verlag, Berlin, Germany.

Jones MV. 1995. Fungal biofilms; eradication of a common problem, p. 19-23. In: Wimpenny J, P Handley, P Gilbert and HM Lappin-Scott (Eds), The life and death of biofilm, Bioline, Gardiff, UK. Cited from Elvers *et al*. (1998).

Kalmbach S, W Manz and U Szewzyk. 1997. Dynamics of biofilm formation in drinking water: phylogenetic affiliation and metabolic potential of single cells assessed by formazan reduction and *in situ* hybridization. FEMS Microbiol Ecol 22: 265-279.

References

Kalmbach S, W Manz, B Bendinger and U Szewzyk. 2000. *In situ* probing reveals *Aquabacterium commune* as a widespread and highly abundant bacterial species in drinking water biofilms. Wat Res 34: 575-581.

Kemira Chemicals Oy (Vaasa, Finland). Käyttöturvallisuustiedote (Safety data sheet, in Finnish). The newest updates available at 30.1.2003 for all authorized biocide products.

Kolari M, MS Salkinoja-Salonen, H Laatikainen, P Tammela, P Vuorela ja P Väätänen. 2002. Paperi- ja kartonkikoneiden termofiilisten mikrobien biofilminmuodostuksen esto. Patenttihakemus FI20021986 (Patent application, in Finnish).

Kolari M, FA Rainey ja MS Salkinoja-Salonen. 2003. Pikamenetelmä paperi- ja kartonkikoneiden termofiilisten biofilminmuodostajabakteerien osoittamiseen. Patenttihakemus (Patent application in preparation, in Finnish).

Korber DR, JR Lawrence, HM Lappin-Scott and JW Costerton. 1995. Growth of microorganisms on surfaces, p. 15-45. In: Lappin-Scott HM and JW Costerton (Eds), Microbial biofilms, Plant and microbial biotechnology research series: 5, University Press, Cambridge, UK.

Kuchma SL and GA O'Toole. 2000. Review. Surface-induced and biofilm-induced changes in gene expression. Curr Opin Biotechnol 11: 429-433.

Kumamoto CA. 2002. *Candida* biofilms. Curr Opin Microbiol 5: 608-611.

Lawrence JR, DR Korber, BD Hoyle, JW Costerton and DE Caldwell. 1991. Optical sectioning of microbial biofilms. J Bacteriol 173: 6558-6567.

Lawrence JR, GM Wolfaardt and DR Korber. 1994. Determination of diffusion coefficients in biofilms by confocal laser microscopy. Appl Environ Microbiol 60: 1166-1173.

Lawrence JR, DR Korber, GM Wolfaardt, DE Caldwell and TR Neu. 2002a. Analytical imaging and microscopy techniques, p. 39-61. In: Hurst CJ, RL Crawford, MJ McInerney, GR Knudsen and LD Stetzenbach (Eds), Manual of environmental microbiology, Second edition, American Society for Microbiology, ASM Press, Washington, DC, USA.

Lawrence JR, TR Neu and KC Marshall. 2002b. Colonization, adhesion, aggregation, and biofilms, p. 466-477. In: Hurst CJ, RL Crawford, MJ McInerney, GR Knudsen and LD Stetzenbach (Eds), Manual of environmental microbiology, Second edition, American Society for Microbiology, ASM Press, Washington, DC, USA.

Lin Y-H, J-L Xu, J Hu, L-H Wang, SL Ong, JR Leadbetter and L-H Zhang. 2003. Acyl-homoserine lactone acylase from *Ralstonia* strain XJ12B represents a novel and potent class of quorum-quenching enzymes. Mol Microbiol 47: 849-860.

Lindberg LE, BR Holmbom, OM Väisänen, A Weber and MS Salkinoja-Salonen. 2001a. Sugar composition of biofilms produced by paper mill bacteria. Appl Microbiol Biotechnol 55: 638-643.

Lindberg LE, BR Holmbom, OM Väisänen, A Weber and MS Salkinoja-Salonen. 2001b. Degradation of paper mill water components in laboratory tests with pure cultures of bacteria. Biodegradation 12: 141-148.

Lindsay D, VS Brözel, JF Mostert and A von Holy. 2000. Physiology of dairy-associated *Bacillus* spp. over a wide pH range. Int J Food Microbiol 54: 49-62.

Ludwig W and H-P Klenk. 2001. Overview: a phylogenetic backbone and taxonomic framework for procaryotic systematics, p. 49-65. In: Boone DR, RW Castenholz and GM Garrity (Eds), Bergey's Manual of Systematic Bacteriology, Second edition, Bergey's Manual Trust, Springer-Verlag, New York, USA.

Lynch MJ, S Swift, DF Kirke, CW Keevil, CER Dodd and P Williams. 2002. The regulation of biofilm development by quorum sensing in *Aeromonas hydrophila*. Environ Microbiol 4: 18-28.

Maidak BL, JR Cole, TG Lilburn, CT Jr Parker, PR Saxman, RJ Farris, GM Garrity, GJ Olsen, TM Schmidt and JM Tiedje. 2001. The RDP-II (Ribosomal Database Project). Nucleic Acids Res 29: 173-174. http://rdp.cme.msu.edu/html/

Maillard J-Y. 2002. Bacterial target sites for biocide action. J Appl Microbiol Symposium Supplement, 92: 16S-27S.

Makarova KS, L Aravind, YI Wolf, RL Tatusov, KW Minton, EV Koonin and MJ Daly. 2001. Genome of the extremely radiation-resistant bacterium *Deinococcus radiodurans* viewed from the perspective of comparative genomics. Microbiol Mol Biol Rev 65: 44-79.

Manz W, K Wendt-Potthoff, TR Neu, U Szewzyk and JR Lawrence. 1999. Phylogenetic composition, spatial structure, and dynamics of lotic bacterial biofilms investigated by fluorescent *in situ* hybridization and confocal laser scanning microscopy. Microb Ecol 37: 225-237.

Martinelli D, M Bunk, B Cadalbert and W Kolb. 2002. The language of bacteria: Biofilms in the paper industry. Paper Technol 43: 47-49.

Mattila K, A Weber and MS Salkinoja-Salonen. 2002. Structure and on-site formation of biofilms in paper machine water flow. J Industr Microbiol Biotechnol 28: 268-279.

Mattila K. 2002. Biofilms on stainless steels exposed to process waters. Dissertationes Biocentri Viikki Universitatis Helsingiensis 15/2002, Ph.D. thesis, University of Helsinki, Helsinki, Finland.

McBain AJ, AH Rickard and P Gilbert. 2002. Review. Possible implications of biocide accumulation in the environment on the prevalence of bacterial antibiotic resistance. J Industr Microbiol Biotechnol 29: 326-330.

McClean KH, MK Winson, L Fish, A Taylor, SR Chhabra, M Camara, M Daykin, JH Lamb, S Swift, BW Bycroft, GSAB Stewart and P Williams. 1997. Quorum sensing and *Chromobacterium violaceum*: exploitation of violacein production and inhibition for the detection of *N*-acylhomoserine lactones. Microbiol 143: 3703-3711.

McCoy JW. 1983. The chemical treatment of cooling water. Second edition, Chemical Publishing Co, NY, USA, 301 pp.

McLean RJC, M Whiteley, DJ Stickler and WC Fuqua. 1997. Evidence of autoinducer activity in naturally occurring biofilms. FEMS Microbiol Letters 154: 259-263.

Møller S, AR Pedersen, LK Poulsen, E Arvin and S Molin. 1996. Activity and three-dimensional distribution of toluene-degrading *Pseudomonas putida* in a multispecies biofilm assessed by quantitative in situ hybridization and scanning confocal laser microscopy. Appl Environ Microbiol 62: 4632-4640.

Møller S, DR Korber, G Wolfaardt, S Molin and DE Caldwell. 1997. Impact of nutrient composition on a degradative biofilm community. Appl Environ Microbiol 63: 2432-2438.

Murray RGE, RN Doetsch and CF Robinow. 1994. Determinative and cytological light microscopy, p. 22-40. In: Gerhardt P, RGE Murray, WA Wood and NR Krieg (Eds), Methods for General and Molecular Bacteriology, American Society for Microbiology, Washington DC, USA.

Neu TR, GDW Swerhone and JR Lawrence. 2001. Assessment of lectin-binding analysis for *in situ* detection of glycoconjugates in biofilm systems. Microbiology 147: 299-313.

Okabe S, T Yasuda and Y Watanabe. 1997. Uptake and release of inert fluorescence particles by mixed population biofilms. Biotechnol Bioeng 53: 459-469.

Oosthuizen MC, B Steyn, J Theron, P Cosette, D Lindsay, A von Holy and VS Brözel. 2002. Proteomic analysis reveals differential protein expression by *Bacillus cereus* during biofilm formation. Appl Environ Microbiol 68: 2770-2780.

Oppong D, VM King, X Zhou and JA Bowen. 2000. Cultural and biochemical diversity of pink-pigmented bacteria isolated from paper mill slimes. J Industr Microbiol Biotechnol 25: 74-80.

O'Toole G, HB Kaplan and R Kolter. 2000. Review. Biofilm formation as microbial development. Annu Rev Microbiol 54: 49-79.

Parkar SG, SH Flint, JS Palmer and JD Brooks. 2001. Factors influencing attachment of thermophilic bacilli to stainless steel. J Appl Microbiol 90: 901-908.

References

Paulus W. 1993. Microbicides for the protection of materials – A handbook. Chapman & Hall, London, UK, 496 pp.

Pellegrin V, S Juretschko, M Wagner and G Cottenceau. 1999. Morphological and biochemical properties of a *Sphaerotilus* sp. isolated from paper mill slimes. Appl Environ Microbiol 65: 156-162.

Peng J-S, W-C Tsai and C-C Chou. 2001. Surface characteristics of *Bacillus cereus* and its adhesion to stainless steel. Int J Food Microbiol 65: 105-111.

Pirttijärvi TSM, TH Graeffe and MS Salkinoja-Salonen. 1996. Bacterial contaminants in liquid packaging boards: assessment of potential for food spoilage. J Appl Bacteriol 81: 445-458.

Pirttijärvi TSM, MA Andersson, AC Scoging and MS Salkinoja-Salonen. 1999. Evaluation of methods for recognising strains of *Bacillus cereus* group with food poisoning potential among industrial and environmental contaminants. Syst Appl Microbiol 22: 133-144.

Pirttijärvi TSM. 2000. Contaminant aerobic sporeforming bacteria in the manufacturing processes of food packaging board and food. Dissertationes Biocentri Viikki Universitatis Helsingiensis 14/2000, Ph.D. thesis, University of Helsinki, Helsinki, Finland.

Poortinga AT, R Bos and HJ Busscher. 2000. Controlled electrophoretic deposition of bacteria to surfaces for the design of biofilms. Biotechnol Bioeng 67: 117-120.

Poortinga AT, R Bos and HJ Busscher. 2001. Reversibility of bacterial adhesion at an electrode surface. Langmuir 17: 2851-2856.

Prater CB, PG Maivald, KJ Kjoller and MG Heaton. TappingMode imaging applications and technology. Application Note AN04, Digital Instruments, Santa Barbara, CA, USA. http://www.di.com (1.2.2003)

Raaska L, J Sillanpää, A-M Sjöberg and M-L Suihko. 2002. Potential microbiological hazards in the production of refined paper products for food applications. J Industr Microbiol Biotechnol 28: 225-231.

Rickard AH, P Gilbert, NJ High, PE Kolenbrander and PS Handley. 2003. Review. Bacterial co-aggregation: and integral process in the development of multi-species biofilms. Trends Microbiol 11: 94-100.

Riedel K, M Hentzer, O Geisenberger, B Huber, A Steidle, H Wu, N Høiby, M Givskov, S Molin and L Eberl. 2001. *N*-Acylhomoserine-lactone-mediated communication between *Pseudomonas aeruginosa* and *Burkholderia cepacia* in mixed biofilms. Microbiol 147: 3249-3262.

Rupp, ME, PD Fey, C Heilmann and F Götz. 2001. Characterization of the importance of *Staphylococcus epidermidis* autolysin and polysaccharide intercellular adhesin in the pathogenesis of intravascular catheter-associated infection in a rat model. J Infect Dis 183: 1038-1042.

Salzburger W. 1996. Paper and additives, p. 415-427. In: Heitz E, H-C Flemming and W Sand (Eds), Microbially influenced corrosion of materials, Springer-Verlag, Berlin, Germany.

Savcor Process Oy (Mikkeli, Finland). MAF (Microbiological AntiFouling) system. http://www.savcor.com/divisions/process/case_maf.asp (20.2.2003)

Sharon N. 1975. Complex carbohydrates: their chemistry, biosynthesis, and functions. Addison-Wesley Publishing Company, Reading, MA, USA.

Steele A, DT Goddard and IB Beech. 1994. An atomic force microscopy study of the biodeterioration of stainless steel in the presence of bacterial biofilms. Int Biodeterior Biodegrad 34: 35-46.

Stoesser G, W Baker, A van den Broek, E Camon, M Garcia-Pastor, C Kanz, T Kulikova, R Leinonen, Q Lin, V Lombard, R Lopez, N Redaschi, P Stoehr, M Tuli, K Tzouvara and R Vaughan. 2002. The EMBL Nucleotide Sequence Database. Nucleic Acids Res 30: 21-26. http://www.ebi.ac.uk/embl/

Stoodley P, Z Lewandowski, JD Boyle and HM Lappin-Scott. 1999. The formation of migratory ripples in a mixed species bacterial biofilm growing in turbulent flow. Environ Microbiol 1: 447-455.

Stoodley P, K Sauer, DG Davies and JW Costerton. 2002. Review. Biofilms as complex differentiated communities. Annu Rev Microbiol 56: 187-209.

Suominen I, M-L Suihko and MS Salkinoja-Salonen. 1997. Microscopic study of migration of microbes in food-packaging paper and board. J Industr Microbiol Biotechnol 19: 104-113.

Szewzyk U, R Szewzyk, W Manz and K-H Schleifer. 2000. Review. Microbiological safety of drinking water. Annu Rev Microbiol 54: 81-127.

Telegdi J, Zs Keresztes, G Pálinkás, E Kálmán and W Sand. 1998. Microbially influenced corrosion visualized by atomic force microscopy. Appl Phys A 66: S639-642.

Tolker-Nielsen T, UC Brinch, PC Ragas, JB Andersen, CS Jacobsen and S Molin. 2000. Development and dynamics of *Pseudomonas* sp. biofilms. J Bacteriol 182: 6482-6489.

Vermis K, C Vandekerckhove, HJ Nelis and PAR Vandamme. 2002. Evaluation of restriction fragment length polymorphism analysis of 16S rDNA as a tool for genomovar characterization within the *Burkholderia cepacia* complex. FEMS Microbiol Letters 214: 1-5.

Vigeant MAS and RM Ford. 1997. Interactions between motile *Escherichia coli* and glass in media with various ionic strengths, as observed with a three-dimensional-tracking microscope. Appl Environ Microbiol 63: 3474-3479.

Väisänen O, S Elo, S Marmo and M Salkinoja-Salonen. 1989a. Enzymatic characterization of bacilli from food packaging paper and board machines. J Industr Microbiol 4: 419-428.

Väisänen O and M Salkinoja-Salonen. 1989b. Use of phage typing and fatty acid analysis for the identification of bacilli isolated from food packaging paper and board machines. System Appl Microbiol 12: 103-111.

Väisänen OM, J Mentu and MS Salkinoja-Salonen. 1991a. Bacteria in food packaging paper and board. J Appl Bacteriol 71: 130-133.

Väisänen OM, NJ Mwaisumo and MS Salkinoja-Salonen. 1991b. Differentiation of dairy strains of the *Bacillus cereus* group by phage typing, minimum growth temperature, and fatty acid analysis. J Appl Bacteriol 70: 315-324.

Väisänen OM, E-L Nurmiaho-Lassila, SA Marmo and MS Salkinoja-Salonen. 1994. Structure and composition of biological slimes on paper and board machines. Appl Environ Microbiol 60: 641-653.

Väisänen OM, A Weber, A Bennasar, FA Rainey, H-J Busse and MS Salkinoja-Salonen. 1998. Microbial communities of printing paper machines. J Appl Microbiol 84: 1069-1084.

Watnick P and R Kolter. 2000. Mini-review. Biofilm, city of microbes. J Bacteriol 182: 2675-2679.

Whiteley M, MG Bangera, RE Bumgarner, MR Parsek, GM Teitzel, S Lory and EP Greenberg. 2001. Gene expression in *Pseudomonas aeruginosa* biofilms. Nature 413: 860-864.

Winson MK, S Swift, L Fish, JP Troup, F Jørgensen, SR Chhabra, BW Bycroft, P Williams and GSAB Stewart. 1998. Construction and analysis of *luxCDABE*-based plasmid sensors for investigating *N*-acyl homoserine lactone-mediated quorum sensing. FEMS Microbiol Letters 163: 185-192.

Wolfaardt GM, JR Lawrence, RD Robarts and DE Caldwell. 1998. In situ characterization of biofilm exopolymers involved in the accumulation of chlorinated organics. Microbiol Ecol 35: 213-223.

Zuber P, M Nakano and M Marahiel. 1993. Peptide antibiotics, p. 897-916. In: Sonenshein AL, JA Hoch and R Losick (Eds), *Bacillus subtilis* and other gram-positive bacteria, American Society for Microbiology, Washington DC, USA.