APMIS ACTA PATHOLOGICA, MICROBIOLOGICA ET IMMUNOLOGICA SCANDINAVICA

ACTA PATHOLOGICA, MICROBIOLOGICA ET IMMUNOLOGICA SCANDINAVICA

The Role of Bacterial Biofilms in Chronic Infections

T. Bjarnsholt

WILEY
Blackwell

Denne afhandling er af Det Sundhedsvidenskabelige Fakultet ved Københavns Universitet antaget til offentligt at forsvares for den medicinske doktorgrad. København, den 31 maj kl 13:00, Haderup Auditoriet, Panum, Blegdamsvej 3B, 2200 København N.

Dekan Ulla Wewer

*Absolutely no benefit can be derived from involving oneself with the natural sciences. One stands there defenseless, with no control over anything. The researcher immediately begins to distract one with his details: now one is to go to Australia, now to the moon; now into an underground cave; now, by Satan, up the arse—to look for an intestinal worm; now the telescope must be used; now the microscope: who the devil can endure it?.**

Med Naturvidenskaberne kan det slet ikke hjælpe at indlade [sig]. Man staaer der værgeløs og kan aldeles ikke controlere. Forskeren begynder strax at adsprede med sine Enkeltheder, nu skal man til Australien nu til Maanen, nu ned i en Hule under Jorden, nuFanden i Vold i Røven – efter en Indvoldsorm; nu skal Teleskopet bruges, nu Mikroskopet: hvo Satan kan holde det ud.

Søren Kierkegaard 1846

*Søren Kierkegaard: A Biography, by Joachim Garff, translated by Bruce H. Kirmmse. 867 pages, Princeton University Press, ISBN: 978069112788

Contents

Cover image: Taking out the defender. The *in vivo* interaction between a *Pseudomonas aeruginosa* biofilm, on a silicone implant, and the responding polymorphonuclear leukocytes.

SEM imaging depicts the interaction at day 1 post insertion of the implant in the peritoneal cavity of a mouse. The leukocytes (yellow) are damaged with obvious cavities in the cell membrane and killed by the bacteria (cyan) following contact with the biofilm.

PREFACE

The work behind the present thesis was initiated during my PhD studies on bacterial communication in biofilms and chronic infections. The study of laboratory biofilms and my subsequent inquiries into their impact in cystic fibrosis sparked my curiosity about how bacteria succeed in biofilms and the role they play in chronic infections. My first grant application for the project 'The role of biofilm in chronic infections' was supported by the Lundbeck Foundation and the Carlsberg Foundation, which meant I was able to proceed and study this phenomenon thanks to both foundations. Throughout this process, I received help from and collaborated with many people to whom I am most grateful. First of all, I would like to thank Niels Høiby because I am so grateful for all your help, support, coaching, constant encouragement, and for inspiring me to write this thesis. I also want to thank Michael Givskov for accepting me as a PhD student, your support and for continuously sharing your laboratory facilities and the help of your staff. I would also like to thank my good friend and colleague Peter Østrup Jensen, because we complement each other fantastically, and without you I would not be where I am today.

Another person who has helped me for many years is Anne Kirstine Nielsen and I am so grateful for all your help in the laboratory. I would like to thank my close colleague and friend Morten Alhede for all his scientific help, inspiration, collaboration, and help with the present thesis. For help with the present thesis, I would also like to thank Oana Ciofu, as well as for our fruitful collaborations. I would like to thank Louise Dahl Christensen and Maria van Gennip (Alhede) for all their hard work with the implant model and experiments in general. For support and inspiration, I would like to thank many good friends and colleagues: Bill Costerton, Søren Molin, Mette Burmølle, Søren J. Sørensen, Claus Moser, Tim Holm Jakobsen, Claus Bøgelund Andersen, Klaus Qvortup, Klaus Kirketerp-Møller, Tanja Pressler, Christine Rønne Hansen, Trine Rolighed Thomsen, Per Halkjær, Vibeke Rudkjøbing, Lise H Christensen, Steen Seier Poulsen, Nils Erik Samdal, Michael Tvede. Hans Petter Hougen, Henrik Calum, Matt Parsek, Mark Shirtliff, Marie Allesen-Holm, Claus Sternberg, Michael Kühl, Preben Homøe, Helle Krogh Johansen, Tim Tolker-Nielsen, and Gerd Döring. In particular, I would like to thank my newly started research group of Kasper Nørskov Kragh, Özge Er, Majken Sønderholm, Stephanie Geisler Crone, Steffen Robert Eickhardt-Sørensen, Morten Alhede, and Anne Kirstine Nielsen. We will rock and roll the future. Thank you guys.

I would also like to thank Lars Christophersen, Ulla R Johansen, Lena Noerregaard, Katja Bloksted, Tina Rathmann, Jette Pedersen, Margit Bæksted, and Tina Wassermann for their help every day. I would like to thank Peter Kindt Fridorff-Jens for his friendship and help in creating our online biofilm course (www.biofilmcourse.ku.dk). The world of science is not always easy to navigate and I would like to thank Charlotte Lex for her mentorship.

I would like to thank the company AdvanDx for their support and constant supply of PNA FISH probes. In addition, I would like to thank all the co-authors of the manuscripts included in this thesis.

I would like to thank Museum of Copenhagen and Marianne Bisballe, for giving me permission to use their illustration of the 'Drop of water' by H.C. Andersen (Figure 23), Grazyna Hahn Poulsen for the illustration of my river analogy (Figure 21) and Claus Lex for help understanding Søren Kierkegaard, and life in general.

Finally, I would like to express my uttermost gratitude and love to my wonderful and beautiful wife Marianne for all her support and help, my son Rasmus, my daugther Milla, my mother, father, Mette, Annie, Niels, and the rest of my fantastic family, because without you I am nothing.

BASIS OF THE THESIS

The main part of the thesis is based on the following papers.
(They are marked as **bold** in the text.)

I. Jensen PØ, **Bjarnsholt T**, Phipps R, Rasmussen TB, Calum H, Christoffersen L, Moser C, Williams P, Pressler T, Givskov M, Høiby N. Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. Microbiology 2007 May;153(Pt 5):1329-38.

II. Christensen LD, Moser C, Jensen PØ, Rasmussen TB, Christophersen L, Kjelleberg S, Kumar N, Høiby N, Givskov M, **Bjarnsholt T.** Impact of *Pseudomonas aeruginosa* quorum sensing on biofilm persistence in an *in vivo* intraperitoneal foreign-body infection model. Microbiology 2007 Jul;153:2312-20.

III. **Bjarnsholt T**, Kirketerp-Møller K, Kristiansen S, Phipps R, Nielsen AK, Jensen PØ, Høiby N, Givskov M. Silver against *Pseudomonas aeruginosa* biofilms. APMIS 2007;115:921–8.

IV. **Bjarnsholt T**, Kirketerp-Møller K, Jensen PØ, Madsen KG, Phipps R, Krogfelt K, Høiby N, Givskov M. Why chronic wounds won't heal: a novel hypothesis. Wound Repair and Regeneration 2008 Jan–Feb;16(1):2-10.

V. Kirketerp-Møller K; Jensen PØ, Fazli M, Madsen KG, Pedersen J, Moser C, Tolker-Nielsen T, Høiby N, Givskov M, **Bjarnsholt T**. Distribution, organization and ecology of bacteria in chronic wounds. J Clin Microbiol 2008 Aug;46(8):2717-22.

VI. **Bjarnsholt T**, Jensen PØ, Fiandaca MJ, Pedersen J, Hansen CR, Andersen CB, Pressler T, Givskov M, Høiby N. *Pseudomonas aeruginosa* biofilms in the respiratory tract of cystic fibrosis patients. Pediatr Pulmonol 2009 Jun;44(6):547-58.

VII. **Bjarnsholt T**, Tolker-Nielsen T, Givskov M, Janssen M, Christensen LH. Detection of bacteria by fluorescence in situ hybridization in culture-negative soft tissue filler lesions. Dermatol Surg 2009;35:1620–1624.

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IX. Van Gennip M, Christensen LD, Alhede M, Phipps R, Jensen PØ, Christophersen L, Pamp SJ, Moser C, Mikkelsen PJ, Koh AY, Tolker-Nielsen T, Pier GB, Høiby N, Givskov M, **Bjarnsholt T.** Inactivation of the rhlA gene in *Pseudomonas aeruginosa* prevents rhamnolipid production, disabling the protection against polymorphonuclear leukocytes**.** APMIS 2009 Jul;117(7):537-46.

X. Werthén M, Henriksson L, Jensen PØ, Sternberg C, Givskov M, **Bjarnsholt T**. An *in vitro* model of bacterial infections in wounds and other soft tissues**.** APMIS 2010 Feb;118 (2):156-64.

XI. **Bjarnsholt T**, Jensen PØ, Jakobsen TH, Phipps R, Nielsen AK, Rybtke MT, Tolker-Nielsen T, Givskov M, Høiby N, Ciofu O. Scandinavian cystic fibrosis study consortium. Quorum sensing and virulence of *Pseudomonas aeruginosa* during lung infection of cystic fibrosis patients. PLoS One 2010 Apr 12;5(4):e10115.

XII. Alhede M, Kragh K, Qvortrup K, Allesen-Holm M, van Gennip M, Christensen LD, Jensen PØ, Nielsen AK, Parsek M, Wozniak D, Molin S, Tolker-Nielsen T, Høiby N, Givskov M, **Bjarnsholt T**. Phenotypes of non-attached *Pseudomonas aeruginosa* aggregates resemble surface attached biofilm. PLoS One 2011;6(11):e27943.

XIII. van Gennip M, Christensen LD, Qvortrup K, Alhede M, Jensen PØ, Høiby N, Givskov M, **Bjarnsholt T.** Interactions between polymorphonuclear leucocytes and *Pseudomonas aeruginosa* biofilms on silicone implants in vivo. Infect Immun. 2012 Aug;80(8):2601-7

IVX. Rudkjøbing VB, Thomsen TR, Alhede M, Kragh KN, Nielsen PH, Johansen UR, Givskov M, Høiby N, **Bjarnsholt T.** True microbiota involved in chronic lung infection of cystic

fibrosis patients found by culturing and 16S rRNA gene analysis. J Clin Microbiol 2011 Dec;49(12):4352-5.

XV. Rudkjøbing VB, Thomsen TR, Alhede M, Kragh KN, Nielsen PH, Johansen UR, Poulsen SS, Givskov M, Høiby N, **Bjarnsholt T.** The microorganisms in chronically infected end-stage and non-end-stage cystic fibrosis patients. FEMS Immunol Med Microbiol. 2012 Jul;65(2):236-44

APMIS 121 (Suppl. 136): 1–51

DOI 10.1111/apm.12099

The role of bacterial biofilms in chronic infections

THOMAS BJARNSHOLT

Københavns Universitet, København N, Denmark

Bjarnsholt T. The role of bacterial biofilms in chronic infections. APMIS 2013; 121 (Suppl. 136): 1–54.

Acute infections caused by pathogenic bacteria have been studied extensively for well over 100 years. These infections killed millions of people in previous centuries, but they have been combated effectively by the development of modern vaccines, antibiotics and infection control measures. Most research into bacterial pathogenesis has focused on acute infections, but these diseases have now been supplemented by a new category of chronic infections caused by bacteria growing in slime-enclosed aggregates known as biofilms. Biofilm infections, such as pneumonia in cystic fibrosis patients, chronic wounds, chronic otitis media and implant- and catheter-associated infections, affect millions of people in the developed world each year and many deaths occur as a consequence. In general, bacteria have two life forms during growth and proliferation. In one form, the bacteria exist as single, independent cells (planktonic) whereas in the other form, bacteria are organized into sessile aggregates. The latter form is commonly referred to as the biofilm growth phenotype. Acute infections are assumed to involve planktonic bacteria, which are generally treatable with antibiotics, although successful treatment depends on accurate and fast diagnosis. However, in cases where the bacteria succeed in forming a biofilm within the human host, the infection often turns out to be untreatable and will develop into a chronic state. The important hallmarks of chronic biofilm-based infections are extreme resistance to antibiotics and many other conventional antimicrobial agents, and an extreme capacity for evading the host defences. In this thesis, I will assemble the current knowledge on biofilms with an emphasis on chronic infections, guidelines for diagnosis and treatment of these infections, before relating this to my previous research into the area of biofilms. I will present evidence to support a view that the biofilm lifestyle dominates chronic bacterial infections, where bacterial aggregation is the default mode, and that subsequent biofilm development progresses by adaptation to nutritional and environmental conditions. I will make a series of correlations to highlight the most important aspects of biofilms from my perspective, and to determine what can be deduced from the past decades of biofilm research. I will try to bridge *in vitro* and *in vivo* research and propose methods for studying biofilms based on this knowledge. I will compare how bacterial biofilms exist in stable ecological habitats and opportunistically in unstable ecological habitats, such as infections. Bacteria have a similar lifestyle (the biofilm) in both habitats, but the fight for survival and supremacy is different. On the basis of this comparison, I will hypothesize how chronic biofilm infections are initiated and how bacteria live together in these infections. Finally, I will discuss different aspects of biofilm infection diagnosis. Hopefully, this survey of current knowledge and my proposed guidelines will provide the basis and inspiration for more research, improved diagnostics, and treatments for well-known biofilm infections and any that may be identified in the future.

Key words: Biofilms; chronic infections

Thomas Bjarnsholt, Københavns Universitet, SUND, Blegdamsvej 3b, 24.1, 2200 København N, Denmark. e-mail: tbjarnsholt@sund.ku.dk

1. INTRODUCTION

Bacterial growth is characterized by two phenotypes, single cells (planktonic) or sessile aggregates. The later is commonly referred to as the biofilm mode of growth. Many divergent definitions of bacterial biofilms exist, but all agree that biofilms are composed of multiple bacteria that form a consortium. The definitions found in the literature differ mainly in

terms of whether cells have to be attached to a surface and whether bacteria form a structured community. The topic of this thesis is medical microbiology and biofilm infections, so I have defined a biofilm as follows:

> *A coherent cluster of bacterial cells embedded in a matrix, which is more tolerant of most antimicrobials and host defences compared with planktonic bacterial cells (1).*

1.1 The development of the biofilm era

Conventional microbiology from 1880 until the middle of the twentieth century is popularly referred to as 'the pure culture period' (2). During this period, bacteria were viewed simply as free-floating single cells, which are also referred to as planktonic. Most studies of bacterial characterization involved the propagation of bacteria in liquid media in test tubes or on agar plates. This seems very peculiar given our current knowledge, because it is estimated that <0.1% of the total microbial biomass is present as a planktonic phenotype (3, 4).

The first observation of surface-associated aggregated bacteria was made by Antonie van Leeuwenhoek (5) in 1684 when he described the 'animals' present in the plaque on teeth. Photomicrographs of aggregating bacteria were produced in 1933 by Henrici (6) and he observed that 'It is quite evident that for the most part water bacteria are not free floating organisms, but grow upon submerged surfaces'. For the purpose of this thesis, microbiology may be divided into two fields: environmental and medical. Environmental microbiology acknowledged the aggregation of bacteria almost 20 years before it was even considered medical microbiology, with the exception of the field of odontology. Aggregates, or flocs, of bacteria have long been used in wastewater treatment plants and the first article to use the term biofilm was published by Rogovska et al. in Microbiology-USSR (MIKROBIOLOGIYA) during 1961 (7).

Publications in the medical field began to acknowledge clumps or heaps of bacteria in 1977 (8), when Høiby described aggregates (heaps) of *Pseudomonas aeruginosa* in the lungs of chronically infected cystic fibrosis (CF) patients. In 1978, Costerton et al. (9) described the presence of surface adhering bacteria embedded in a 'glycocalyx' (matrix), and in 1981 he used the term biofilm for the first time, to describe this phenomenon (10). The phenomenon was reviewed and re-described in 1987 by Costerton et al. (11) as a matrix-enclosed mode of growth.

In 1993, the American Society for Microbiology recognized that the biofilm growth phenotype was relevant to microbiology (12). As a result, the biofilm phenotype became increasingly accepted as an important bacterial trait. In 1999, Costerton et al. (13) defined a biofilm as 'a structured community of bacterial cells enclosed in a self produced polymeric matrix, adherent to a surface'. During the last 15 years, the literature on biofilms has increased dramatically in terms of publications (see Fig. 1) and there are also numerous books on the subject.

1.2 What is a biofilm?

Biofilms have probably been present on the Earth since the first bacteria evolved. In medical microbiology, biofilms are typically involved in chronic persistent infections. Common bacterial infections were very serious before the antibiotic era, when many people died of pneumonia and other acute infections that are now easily cured using antibiotics. Since the development of antibiotics, the world

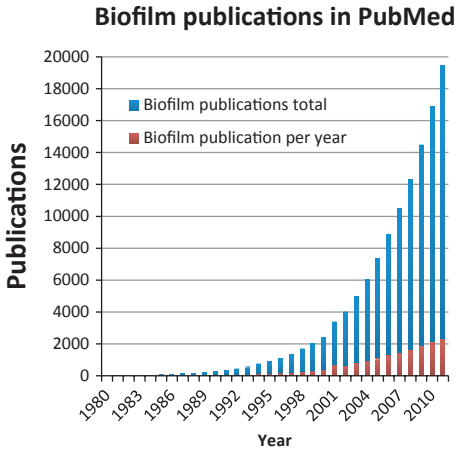

Fig. 1. Accumulated publications on biofilms and per year, derived from the search engine PubMed (http://www.ncbi.nlm.nih.gov/pubmed/).

has experienced an increase in slow-progressing infections with 'lowgrade' pathogenesis compared with acute infections. These slow-progressing infections occur in all age groups where patients experience discomfort, fever, and other clinical signs of infection. However, bacteria were often not detected and the effects of antibiotics were either very disappointing or absent, resulting in persistent infections. The breakthrough in identifying the sources of these persistent infections was a series of *in vitro* and *in vivo* observations made in the 1980s (14). These observations showed that aggregation of bacteria were the cause of slow-progressing infections and Costerton referred to this phenomenon as bacterial biofilms (9). However, biofilms and their extreme tolerance of antimicrobial agents had been discovered 300 years earlier by van Leeuwenhoek (1684) (5). Van Leeuwenhoek observed that animals (bacteria) within the scurf (plaque) on teeth were more resistant to vinegar than animals found outside the plaque, which were killed. This is now known to be one of the major hallmarks of biofilms, i.e., an extreme tolerance of antimicrobial agents. It must to be noted that biofilm antibiotic tolerance should not be confused with antibiotic resistance because, although bacteria within a biofilm tend to survive antibiotic treatment, they become susceptible to the treatment when the biofilm is disrupted (15) (**XII**) (see section 4.1).

Numerous *in vitro* and *in vivo* biofilm observations show that the causes of most persistent infections are bacterial aggregates or biofilms. The bacteria in these aggregates are physically joined together and they produce an extracellular matrix that contains many different types of extracellular polymeric substances (EPS) including proteins, DNA and polysaccharides. These aggregates can withstand very high doses of antibiotics that would kill planktonic cells. Their tolerance of host defences is also dramatically increased. These characteristics are contained in the biofilm definition presented above. This definition differs in one respect from most other biofilm definitions because it no longer requires that a biotic or abiotic surface is a hallmark. Many chronic infections involve surfaces such as infections on implants, catheters, artificial heart valves, teeth and contact lenses. However, many observations of non-surface-related infections, such as CF, otitis media, chronic wounds and chronic osteomyelitis, have found the same patterns without a surface.

In 2009, Høiby included 'persisting pathology' (16) in the definition of chronic infections.

1.3. Chronic infections

Chronic infections have a slower progression than acute infections and their symptoms are often vague (14) (often referred to as low grade). They are very difficult, if not impossible, to cure with antibiotics (see section 4.1). Chronic inflammation is usually characterized by an adaptive inflammatory response, which is dominated by mononuclear leucocytes and IgG antibodies. In some chronic infections, the inflammatory response is characterized by a chronic inflammatory response and continuous recruitment of polymorphonuclear leucocytes (PMNs). The classic chronic infections before the antibiotic era included tuberculosis and leprosy, which slowly degrade the tissue and affected organs (e.g., lungs) of patients, eventually leading to death.

Chronic infections can develop in patients who suffer from diseases or conditions that cause deficiencies in the primary defensive barriers (innate immunity). This includes disruption of the anatomical (e.g., skin, mucous membranes and cilia) and physiological (e.g., temperature and low pH) inflammatory barriers, as well as phagocytic defects (e.g., PMNs and macrophages). These deficiencies can be divided into congenital abnormalities, the presence of foreign bodies and acquired chronic diseases. The classic example is the chronic lung infection found in patients suffering from the genetic disorder CF (see section 6.3.2). These patients have a reduced volume of periciliary fluid in the airways, which impairs the normal mucociliary clearing of the paranasal sinuses and the lungs, facilitating persistent bacterial infections. The presence of foreign bodies can include artificial limbs and other body parts, and indwelling catheters (11, 13, 17–19), while injected tissue fillers are also now being reported as a site of chronic infections (**VII**). Acquired chronic diseases include diabetes mellitus, arteriosclerosis of the arteries of legs and smoking-induced chronic obstruc-

tive pulmonary disease (COPD). These patients may be prone to the development of a non-healing wound that is chronically infected with bacteria, or the chronic lung infections seen in patients suffering from COPD (20, 21).

2. PURPOSE OF THE STUDIES

Since my PhD studies, my scientific research focus has been the investigation of the occurrence and persistence of biofilms in chronic infections. One of the major aims of this thesis was to compare the structural and physiological characteristics of the two major forms of biofilm infections: surface (biotic and abiotic)-related and non-surface-related biofilms. Another major question was to elucidate the basis of how biofilms tolerate the immune system attacks, i.e., the so-called 'frustrated phagocytosis'. The third major purpose was to improve the diagnosis of biofilm-related infections, where the classical microbiological culture methods are inappropriate, so molecular probes and microscopy were applied to a wide variety of samples from chronic human infections. I investigated biofilm formation, structure and biofilm responses to the immune system and antibacterial agents using *in vitro* methods (**I, III, IX, X, XI, XII**), which were confirmed with *in vivo* animal models (**I, II, IX, XIII**). My questions could not be answered entirely by using available models, so I developed new *in vitro* (**X**) and *in vivo* (**II, XIII**) model systems. I have used molecular probes and antibodies to analyse the presence, organization and distribution of bacteria and biofilms in chronic infections, based on the identification of bacterial aggregates and their matrices. I have applied these methods to a large variety of chronic human infections including chronic *P. aeruginosa* infections in CF patients (**VI, XIV, XV**), soft tissue fillers (**VII**), chronic otitis media infections (**VIII**) and chronic wounds (**IV, V).**

3. HOW BIOFILMS ARE FORMED

3.1. *Pseudomonas aeruginosa* biofilms as a biofilm model

Biofilm developmental processes have been thoroughly studied using surface-based *in vitro* systems (22–28). The most commonly studied bacterium in this context is *P. aeruginosa*. *Pseudomonas aeruginosa* is a Gram-negative rod-shaped bacterium with virtually no specific growth requirements. It is a non-fermentative organism that is capable of growing with or without oxygen. The sequenced genome of *P. aeruginosa* contains 5770 open reading frames and ~10% of its genes encode proteins involved in regulatory processes, which makes it a very diverse and adaptable organism (29).

The ability of *P. aeruginosa* to form biofilms is thought to be one of its main survival strategies when infecting a host, and it is considered to be an important pathogenicity trait (13, 30).

The *P. aeruginosa in vitro* biofilm consists of microcolonies encapsulated by EPS produced by the bacteria, although most of the biofilm is comprised of water channels that are thought to function as a distribution system for nutrients and oxygen (3, 31). An oxygen gradient descends from the surface to the substratum (32–34). *Pseudomonas aeruginosa* can form biofilms on virtually any surface and in any nutritional or environmental conditions.

Classically, the *in vitro*, surface-based biofilm developmental process can be divided into the following different stages: (i) attachment, (ii) maturation and (iii) dispersion, as suggested by Sauer et al. (24) and Klausen et al. (22). The model of *in vitro* surface biofilm development has been subject to changes over the years. An experimental-based model of the formation of *in vitro* biofilms is shown in Fig. 2 (22).

As shown in Fig. 2, cells attach to the surface and form the microcolonies of the biofilm.

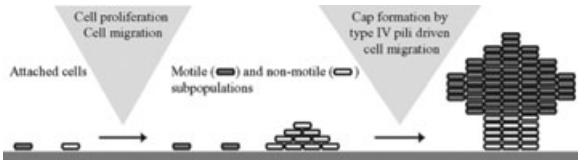

Fig. 2. Formation of an *in vitro* surface-attached *Pseudomonas aeruginosa* biofilm. Initial attachment is followed by clonal growth where one subpopulation of irreversibly attached bacteria forms the base of microcolonies and another subpopulation of non-attached bacteria move on their surface. These non-attached bacteria may eventually climb from the base to form the caps of mushroom structures [adapted from (22) with permission from the publisher].

The surface is covered by motile bacteria, which move by means of twitching motility. Twitching motility is used by *P. aeruginosa* and many other bacteria to move over moist surfaces (35). Mushroom-shaped structures are formed by motile bacteria as they climb up on the stalks using type IV pili, which over time form the caps of the mushrooms. Over the years, a major emphasis has been placed on the development of these biofilm structures. Klausen et al. (23) showed that the formation of these structures is influenced by nutritional and environmental conditions.

Nutrient availability appears to be one of the most important factors affecting *in vitro* microcolony formation in *P. aeruginosa* biofilms. Klausen et al. (23) showed that *P. aeruginosa* formed a highly differentiated biofilm composed of mushroom structures when glucose is used as the carbon source, whereas the same strain formed a flat and undifferentiated biofilm with citrate as a carbon source. It was shown that *P. aeruginosa* cells are less motile when grown on glucose compared with citrate. The non-motile subpopulation that formed the stalks of mushrooms with glucose was not present with citrate.

Klausen et al. also showed that the initial biofilm development with citrate was the same as that with glucose. Microcolony formation occurred by clonal growth, before the bacteria spread on the surface by means of twitching motility to form a flat biofilm with no towers or mushrooms. This surface spreading resulted in clonal mixing that was also observed during the development of non-surface-attached biofilms (**XII**), which was independent of the carbon source, as shown in Fig. 3.

Occasionally, it is stated that biofilm formation, from planktonic to the sessile growth phenotype, is a complex and highly regulated process (36). It has also been suggested that biofilm formation is dependent on the expression of a specific biofilm program (36, 37). However, all the *in vitro* observations available today suggest that it is more likely that biofilm formation proceeds through a series of temporal events that reflect adaptation to nutritional and environmental conditions (38–40). This lack of a biofilm-specific program is supported by an analysis of transcriptomic data by Folsom et al. (41), in which they compared a series of novel and published data sets. They concluded that *P. aeruginosa* biofilms appeared to be iron-depleted, oxygen-limited, with the characteristics of stationary phase growth, and the expression of type IV pili-related genes.

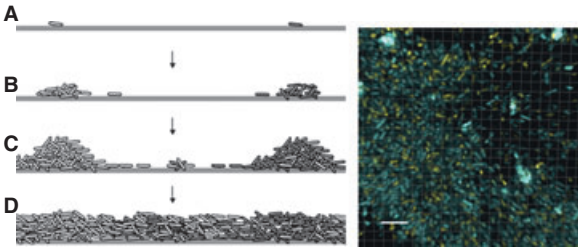

Fig. 3. The left panel shows the formation of a flat *Pseudomonas aeruginosa* biofilm. (A) Attachment, (B) microcolony formation by clonal growth, (C) the bacteria spread out on the substratum, (D) the clonal populations mix [adapted from (23) with permission from the publisher]. The right panel shows the clonal mixing of a non-surface-attached biofilm, which is visualized by the mixing of blue and yellow bacteria (adapted from **XII** with permission from the publisher).

This indicates that biofilm formation, and the successive growth and development, is a complex, but somewhat arbitrary process. This is supported by observations of *P. aeruginosa* biofilm formation, which have revealed a slight variation in biofilm structure in the same strain of *P. aeruginosa* (see Fig. 4). It is also evident from the literature that there are many

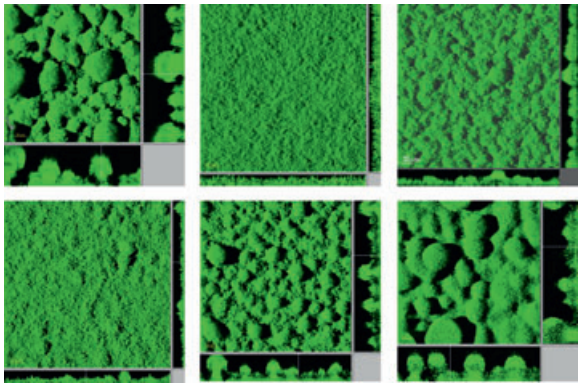

Fig. 4. *Pseudomonas aeruginosa* wild-type grown for 4 days in flow cells using minimal media with glucose as the carbon source. Each frame shows the same wild-type clone on the same day after inoculation, but six different experiments [Jørgensen AA and Molin S, (2003) unpublished results].

discrepancies as to how and when mature biofilm develop during laboratory experiments (24, 40, 42–48).

These arbitrary growth patterns might be caused by the increased mutation rates found in biofilms, as suggested by Conibear et al. (49). This study found that mutation frequencies were elevated in the microcolony structures, which explained the heterogeneity of biofilm development.

A biofilm is thought to maintain equilibrium via growth and dispersal. Dispersal is believed to occur either as single cells or as small microcolonies that are torn from the biofilm, as shown in Fig. 5 (13, 50, 51). The mechanism of dispersion is not fully understood, but Costerton et al. (13) suggested that planktonic dispersion may be a programmed process, whereas clusters are torn away by shear forces (Fig. 5) and/or by prophage-mediated cell death, as proposed by Webb et al. (52).

Dispersal has severe implications for medical biofilms because it provides a mechanism whereby biofilm bacteria can spread throughout an infected organ, or to the whole body. This is the mechanism whereby a chronic infection can cause an acute blood stream infection, which sometimes occurs in patients with infected catheters and implants or those suffering from other biofilm infections such as endocarditis (13, 53–55). Remarkably, it does not occur in patients suffering from CF (see section 6.3.2).

3.2. Why biofilms are formed

What drives bacteria to produce or form a biofilm? Four driving forces are depicted by Jefferson (56) in Fig. 6 and they probably all apply.

Initial aggregation is probably a default mechanism whereby bacteria stick to each other. Further biofilm formation progresses by adaptation to the available nutritional and environmental conditions.

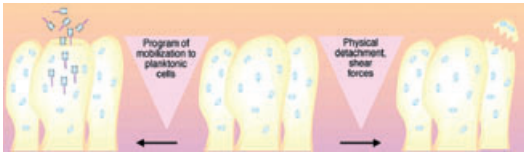

Fig. 5. Dispersal of bacteria from a biofilm. Dispersion can take place as single cells or clusters [adapted from (13) with permission from the author].

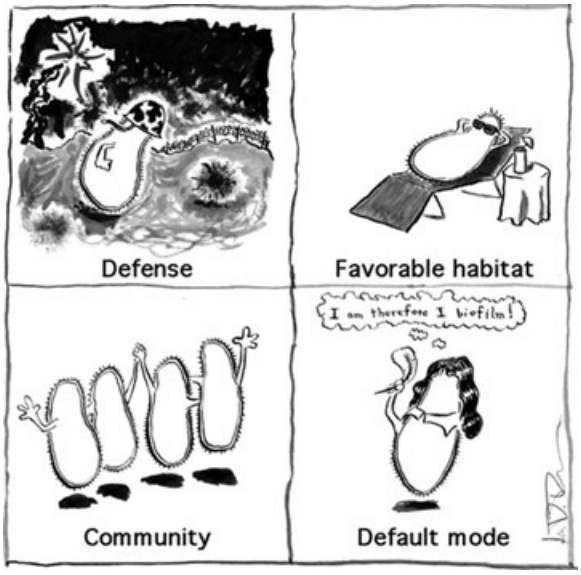

Fig. 6. Cartoon by Jefferson (56) with permission from the publisher, showing the four driving forces underlying biofilm formation.

3.3. The scaffold (united we stand, divided we fall)

Any type of aggregation demands a physical attachment or attractive forces between individual particles within an aggregate, or the aggregate will disintegrate, and bacterial aggregates are no exception. It is generally believed that bacteria are immobilized in aggregates by the matrix or EPS components. Extracellular polymeric substances consist of polysaccharides (57–59), extracellular DNA (60–63) and other macromolecular components such as proteins (64–66), lipids (67), biosurfactants (68, 69), flagella and pili (70–72). Thus, the matrix has been referred to as the 'house of biofilm cells' (73). The initial interaction among bacteria, or between bacteria and a surface, is most often mediated via flagella and/or pili. Bacteria in biofilms are then encapsulated in the EPS, which is either produced by the bacteria or sometimes additionally adapted from the host. Extracellular polymeric substances seems to constitute the scaffolding component for bacteria aggregating in the biofilm (44, 60, 74) and it acts as a scavenger of

free oxygen radicals (75), as well as binding many classes of antibiotics, such as aminoglycosides (76). Apart from this, very little is known about the biofilm matrix, and no complete biochemical profiles exist because different bacteria seem to produce different matrix components.

In most biofilm research, *P. aeruginosa* is the main model organism. The main matrix component that has been investigated in *P. aeruginosa* is undoubtedly polyanion polysaccharide alginate (77, 78). *Pseudomonas aeruginosa* can produce vast amounts of alginate and its mucoid phenotype is most often associated with CF (see section 6.3.2) (79, 80). The development of *in vitro* biofilms by wild-type *P. aeruginosa* does not seem to involve the production or a dependence on alginate as a matrix component (81). This is probably an artefact of *in vitro* systems, because wild-type, non-mucoid *P. aeruginosa* produce small amounts of alginate when infecting experimental animals (82).

Two other very important matrix components produced by *P. aeruginosa* biofilms are the polysaccharides Pel and Psl. Psl consists of repeating D-mannose, D-glucose and L-rhamnose units, while Pel is rich in glucose (83, 84). Psl seems to be involved in all stages of biofilm development and it is a key scaffolding component in mature biofilms (59). Interestingly, Psl is not present in the centres of microcolonies, which is the location of dispersing planktonic bacteria. This suggests that dispersal, at least *in vitro*, is a controlled and not a stochastic event (59).

The matrix can be considered the most important property of a bacterial biofilm, because without it a biofilm would not exist. There is no consensus on the constitution or the appearance of the matrix, but it is evident that its main role is to maintain bacterial assemblages. This was supported by a recent study, in which advanced electron microscopy was used to elucidate the appearance of the matrix (85). Figure 7 shows that the matrix

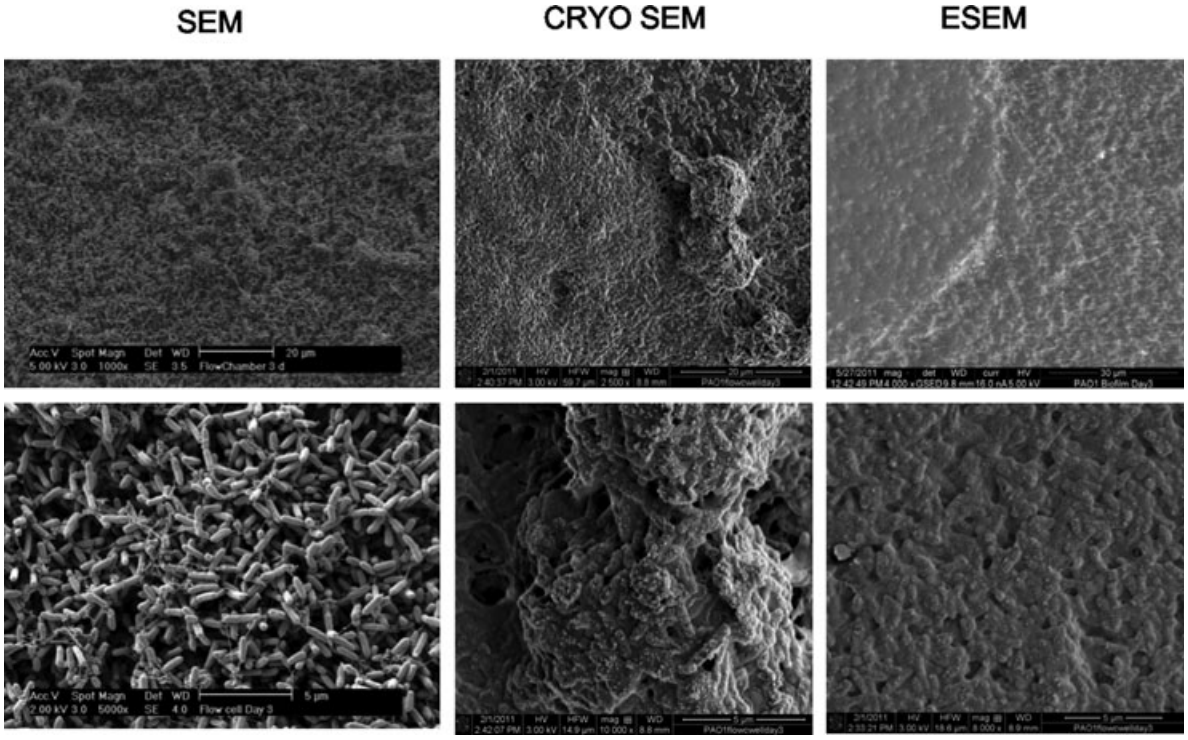

Fig. 7. Comparison of biofilm matrices obtained by standard scanning electron microscopy (SEM), Cryo-SEM and ESEM. The last two methods required no fixation of the sample so they provide a realistic view of the hydrated matrix [adapted from (85) with permission from the publisher].

connecting all the bacteria may be much hydrated.

Considerable evidence also suggests that the matrix has many subsequent roles such as shielding against predators, phagocytes (65, 86), (**I, IX, VI, XII, XIII**) and antibiotics (87, 88), and in the formation of signalling networks (89–91).

4. BIOFILM-RELATED PHENOTYPES

Although specialized organization and programmed aggregation are debatable in the origin of biofilms, bacterial aggregates have an altered phenotype compared with their planktonic counterparts.

4.1. Decreased antimicrobial susceptibility

The most important, and only truly consensual characteristic of bacterial biofilms (apart from the aggregation and matrix), is a decreased susceptibility to antimicrobial agents (44, 92–94) (**III, XII**). This decreased susceptibility has two aspects, tolerance and resistance. Tolerance means that bacteria are not killed, although they are unable to grow in the presence of the drug, whereas resistance allows bacteria to grow in the presence of antibiotics. How is tolerance facilitated by the aggregation of bacteria in biofilms and the biofilm matrix, and what is the source of the conventional resistance found in bacteria in biofilms?

I believe it is very important to distinguish between these two phenomena because all bacteria can become resistant, irrespective of their growth phenotypes, whereas only bacterial aggregates adapt by exhibiting biofilm tolerance (**XII**). Both phenomena are equally important and they may occur simultaneously, although the time perspective is different. Tolerance may arise once a threshold number or density of bacteria has aggregated, whereas resistance will develop over time due to intrinsic and external factors such as mutations. Most chronic infections imply countless bacterial divisions so the accumulation of resistance is a serious problem, which is why tolerance and resistance are equally important for chronic infections in terms of treatment (16). Many bacteria are also naturally resistant to a variety of antibiotics because of penetration barriers, efflux pumps or degrading enzymes. For example, *P. aeruginosa* produces β-lactamase, which inactivates many β-lactam antibiotics by cleavage (95). Several bacterial species are resistant to polymyxins because they have a modified LPS molecule (96). Other active processes such as efflux pump systems have a wide spectrum of activity against substrates including quinolones, tetracycline, chloramphenicol, trimethoprim, β-lactam antibiotics, β-lactamase inhibitors, detergents and solvents (97, 98). Like any bacteria, *P. aeruginosa* can acquire resistance from other bacteria via horizontal gene transfer or uptake (99, 100). However, biofilm tolerance will be emphasized in this thesis to facilitate the understanding of the special biofilm phenotype. 'Normal' resistance has been described in detail in numerous original research and review articles (16, 101–103).

Tolerance is caused by the following factors: (i) the three-dimensional architecture, i.e., the presence of several layers of bacteria promotes the development of nutrient and oxygen gradients (34) and slows down growth in the core of the aggregate; and (ii) the matrix components can bind and/or neutralize antimicrobial agents (104).

Differentiated growth within a biofilm aggregate is documented in several publications (34, 41, 105–107). These investigations demonstrate the presence of areas within a biofilm that are inhabited by stationary phase or even dormant bacteria. This minimizes the effect of most antibiotics because they target active biological processes (92, 108–111). Slow growth is likely to be caused by nutrient- (112) and oxygen- (113) limiting gradients in the biofilm. Studies of entire *in vitro* biofilm transcriptomes reveal that the bulk of bacteria resemble a stationary phase planktonic culture (40, 41). Anaerobic growth also seems to be favoured (40, 41). However, antimicrobial agents that target the membrane, such as colistin, heavy metals and chlorine, appear to have the opposite effect on *in vitro* biofilms, where growing bacteria are more tolerant (111, 114–116). This agrees with the discovery that *in vitro P. aeruginosa* biofilms contain subpopulations with differentiated growth. The outer layer is most similar to exponentially growing cells and is killed by conventional antibiotics, whereas cells in the more central regions

can tolerate these antibiotics because of their halted growth. Efflux pumps might actively pump antibiotic agents out of growing cells in the outer subpopulation.

Independent of bacterial growth, biofilm microenvironments caused by differences in the pH, pCO_2 or pO_2, might also affect the efficacy of antimicrobial compounds. For example, the activity of macrolides and tetracyclines is compromised at low pH while aminoglycosides depend on the availability of oxygen (34).

Extracellular DNA (eDNA) in the matrix has been reported to neutralize the activity of antimicrobials such as tobramycin via its cation-chelating properties (104). The biofilm matrix has also been shown to make biofilm-embedded cells more tolerant to heavy metals such as zinc, copper and lead (114).

4.2. Predator and phagocyte tolerance

A second important, but poorly described, characteristic of bacterial biofilms is the predator and phagocyte tolerance or protection. For a bacterial biofilm to persist in a hostile environment, such as an infection or even in a creek, it needs protection from predators and phagocytes. These predators can include phagotrophic protists, protozoa and phagocytes such as human PMNs, which engulf bacteria to use them as a food source or as a defence mechanism. Protozoa such as free-living amoeba can graze and prey on bacterial biofilms in a wide range of habitats including rivers, activated sludge and water pipes (117, 118). During an infection, the first responses to invading bacteria are via the cellular components of the innate host defence, especially the PMNs (119). Planktonic bacteria are easily engulfed by these professional phagocytes, as shown in *in vitro* experiments (120, 121), whereas mature biofilms seem to be protected (**XII**) (44, 120, 122, 123). This protection seems to be very important in chronic infections because all reports show that the presence of bacterial biofilms promotes the ongoing recruitment of PMNs, etc. (**I, VI, IV, V, XIII)** (124, 125). The exact mechanism has not been fully elucidated, but has been explained as the physical hindrance of the biofilm matrix, and a disabled ability of the phagocytes in killing the biofilm bacteria, a phenomenon termed 'frustrated phagocytosis' (126, 127). Recent research adds further aspects to this phenomenon because phagocytes do come in contact with the bacteria in biofilms and they can even penetrate biofilms (44, 120, 128). However, the bacteria in the biofilms are not killed, which was also evident in my personal *ex vivo* observations (**IV, V, VI**).The defence mechanism appears to be chemical, because the bacteria in biofilms can produce compounds that disable or even kill eukaryotes such as PMNs (**I, IX, XII, XIII**) (121, 129, 130). The production of rhamnolipid in *P. aeruginosa* biofilms appeared to be protective in biofilms in both *in vitro* experiments and in experimental animals (**I, IX, XIII**) (125). *In vitro* experiments showed that a *P. aeruginosa* strain that was unable to produce rhamnolipids produced a thick and mature biofilm, but it was more easily grazed and eradicated when exposed to freshly isolated human PMNs compared with the wild-type strain. However, an unknown secondary defence mechanism must exist, because during the chronic lung infection of CF patients (even though late), *P. aeruginosa* lose its ability to produce rhamnolipid and still persist (**XI**). It is possible that the adapted slow growth (131) combined with persistent alginate production is sufficient to protect bacteria from antibiotics and the host defences.

4.3. Quorum sensing

Another density-dependent trait is the bacterial cell-to-cell communication, known as quorum sensing (QS) (132), where the 'quorum' refers to the minimum number of bacteria aggregated within a specific volume that is required to make a 'decision' to switch on the gene expression of QS-controlled genes (see Fig. 8).

This mechanism is connected with high cell densities, which are clearly found in biofilms, although not exclusively. Cells are not physically aware of the presence or density of other bacteria (133), but they can sense the concentration of signal molecules that indicate the cell density. Thus, bacteria sense signal molecules that are proportional to the cell density. Furthermore, quorate control of gene expression is believed to be an ancient trait in many

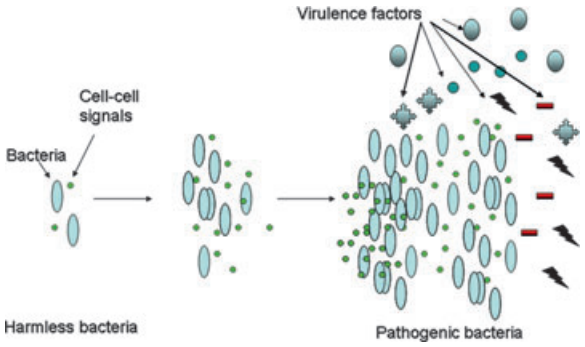

Fig. 8. The principle of quorum sensing (QS). Harmless bacteria do not express virulence factors. As the concentration of QS signal molecules increases with the bacterial density, the expression of QS-regulated genes is initiated and virulence factors are produced, which are excreted into the environment [adapted from (109) with permission from the publisher].

species, which was well established at an early stage in the evolution of bacteria (134).

The principle of signal-mediated gene expression is common in Gram-positive and Gram-negative bacteria, although the molecular mechanisms and signal molecules differ. The first evidence of co-operative behaviour among bacteria was described during the late 1960s and early 1970s by Tomasz (135) and Nealson et al. (136). Nealson et al. (136) studied the biology of light-producing organelles in deep sea fish, where the light was produced by the bacterium *Vibrio fischeri* in a cell density-dependent reaction. The bioluminescence of *V. fisheri* originates from the expression of two luciferase also known as the *lux* genes. The gene products of these two genes increase rapidly when the growth of the bacteria enters the late exponential phase and the early stationary phase of growth.

The first reports of QS as a controller of virulence appeared in the mid-1990s (137). Later analyses of bacterial transcriptomes (138–140) and plant (141), nematode (142), and animal infection studies (**I, II**) (44, 128, 138, 143, 144) have substantiated these reports. In particular, the regulation of rhamnolipid production by *P. aeruginosa* is well known to be regulated by QS (145, 146).

Antibiotic tolerance also appears to be regulated by QS, at least in part, although the complete mechanism has not been fully elucidated (44, 138, 147–149). One link between antibiotic tolerance and QS is QS-regulated eDNA release (60), because DNA is a chelator of aminoglycosides (see section 4.1). This might explain why tobramycin tolerance is partly QS-dependent and why treatment with QS inhibitors has a synergistic action with tobramycin (44, 150).

N-acyl-L-homoserine lactone (AHL) controlled systems in Gram-negative bacteria are the best-studied examples of QS. These QS systems control a wide range of functions in Gram-negative bacteria (151, 152), such as plasmid conjugation in *Agrobacterium tumefaciens* (153), virulence gene expression in *Vibrio cholerae, Burkholderia cepacia* and *P. aeruginosa* (**I, II, IX, XI, XIII**) (138, 154–156), antibiotic production in *Erwinia carotovora* (152), and surface motility by means of swarming in *Serratia liquefaciens*, *P. aeruginosa,* and *B. cepacia* (151, 157–160). AHL signal molecules vary among bacteria and some bacteria produce more than one type of AHL molecule. They all exhibit the same basic structure, i.e., an acyl chain of variable length typically with 4–16 carbons, which in most cases are even numbered (C4, C6, C8, etc.) (161).

The general AHL QS controller is comprised of an *I* gene encoding the AHL synthetase and an *R* gene encoding the receptor. During bacterial growth, the signal molecule is produced by AHL synthetase. The signal molecules form an activated complex with the R receptor protein, which in turn binds to specific regulator sites upstream of the promoter. This binding either facilitates positive or negative regulation of target gene transcription. However, this simple scenario applies to only a limited number of Gram-negative bacteria. For example, QS in *P. aeruginosa* is composed of two AHL systems encoded by *lasR*/*lasI* and *rhlR*/*rhlI,* and a quinolone signal pathway encoded by the *pqs* genes and the PQS signal (162, 163). The entire hierarchy has additional regulatory layers (162, 164). QS systems have also been identified in Gram-positive bacteria where, instead of AHL molecules, small peptides act as signalling molecules, which usually measure 5–17 amino acids in length (165). The signalling peptides are products of oligopeptides that are cleaved and processed within cells. After processing, the signalling peptides are exported

out of cells by active transportation. The secreted peptides then interact with transmembrane receptors in two-component regulatory systems, activating an intercellular response. The basis of this regulation is similar to AHL regulation, i.e., it depends on an increase in bacterial density that leads to an increase in the peptide signal's concentration. Examples of Gram-positive QS-controlled behaviour include: development of genetic competence and sporulation in *Bacillus subtilis* (161) and virulence expression in *Enterococcus faecalis* (166) and *Staphylococcus aureus* (167).

5. ENVIRONMENTAL BIOFILMS

In this thesis, I define environmental biofilms as general ecosystems of aggregating bacteria that are present in their natural habitat with an essential function. These can include bacterial aggregates that live as commensalism with the human body without causing disease or those found in environments such as the soil, submerged surfaces, flocs in wastewater treatment plants and plant rhizospheres.

5.1. Submerged or flooded surfaces

Bacterial biofilms may be found on all abiotic surfaces that are submerged in non-sterile water. If a sterile surface is submerged into water, such as seawater or fresh water, a bacterial biofilm will form almost immediately (168, 169). One of the first intensive studies of bacterial aggregation was performed in an alpine stream (170). The authors identified the sessile bacteria and noted they were surrounded by a slimy substance, which the authors hypothesized to be self-produced and important for the persistence of aggregates on the surface. Complex communities are often observed in fresh and marine waters. Different bacterial species can interact within them, often in symbiosis, and these ecosystems are driven by the capacity to use the available nutrients and sunlight, or a lack of it (171). Thus, these complex biofilms are mixed species biofilms (172). Bacterial colonization of surfaces that are exposed to non-sterile liquids is a substantial problem affecting ship hulls, and industrial pipelines such as oil pipelines and fresh drinking water supplies. Freshwater pipelines are a particular problem because pathogens such as *P. aeruginosa* and *Legionella pneumophila* grow readily within them and this can be an infection route in humans (173–175).

5.2. Activated sludge

Wastewater treatment often depends on bacteria that breakdown organic matter such as nitric oxides, phosphorus and other compounds, into a non-toxic biomass (173, 176). Wastewater treatment is a continuous process where the converted biomass, i.e., the sludge, is introduced into new batches of waste to breakdown. Flocs or aggregates are formed in the sludge, which consist of bacteria and organic and inorganic compounds (177–179). The bacterial composition of flocs depends on the nature of the wastewater. The bacterial aggregates are embedded in EPS, which keeps the aggregate together and provides a stabile and protective environment (see section 3.3). Wastewater is very complex and many different symbiotic bacteria need to be present to breakdown the individual components, i.e., multispecies biofilms (177, 179, 180), as shown in Fig. 9.

5.3. Rhizosphere

Another environmental habitat for bacteria is the thin soil layer adhering to plant roots that remains when the loose soil is shaken away. This layer is known as the rhizosphere and bacteria can grow there in symbiosis with a plant (181). Beneficial microbiota in the rhizosphere promotes plant growth and it functions as biocontrol to protect the plant from soilborne pathogens (182, 183). If this microbiota is compromised, the protection of the plant is lost and a harmful biofilm may form, leading to pathogenesis in the plant (184). The synergy between the plant and the microbiota in the rhizosphere is based on mutual modification of the soil environment by water uptake, and the release of organic materials and growth factors. Different processes affect the nutrient and gas availability, so the bacterial composition of rhizosphere biofilms is multispecies and highly diverse (181).

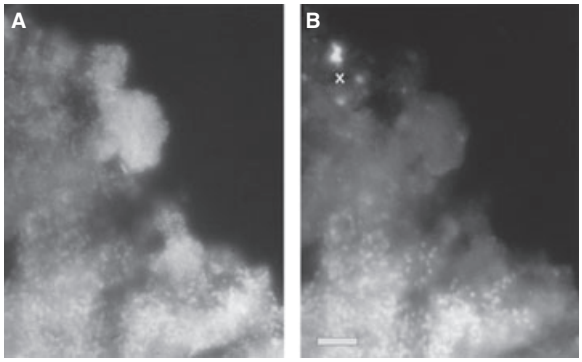

Fig. 9. *In situ* hybridization of sections of sucrose-fed anaerobic granular sludge. The sections were simultaneously hybridized with a fluorescein-labelled oligonucleotide probe that was universal for bacteria, EUB338, and a rhodamine-labelled specific probe for the strain MPOB1, before being viewed by epifluorescence microscopy with a fluorescein-specific (A) and rhodamine-specific (B) filter set. The photomicrographs show the outer layers of a granule. Various morphotypes of rods and cocci hybridized with the bacterial probe, although only the short rods present in the microcolony in the top left corner of the micrographs were visualized by the MPOB1 probe. Bar = 10 mm [adapted from Harmsen et al. (179) with permission from the publisher].

5.4. Oral biofilms

Dental plaque is a classic example of synergism between mammals and bacteria. Dental plaque was the first location in the human body were biofilms were described (185, 186). Forty years ago, researchers exploited electron microscopy to study the development and structure of multispecies dental biofilms (187–189). Later, immunofluorescence and fluorescence *in situ* hybridization (FISH) were combined with confocal laser scanning microscopy (CLSM) and used to explore the spatial distribution and population dynamics of the different bacteria found in the multispecies dental microbiota (190). The teeth (natural and artificial) are easily accessible, so the colonization pattern has been thoroughly investigated (for a review, see (191, 192)).Oral bacteria colonize the pellicle-coated tooth surfaces as single cells and pairs. The initial stages are dominated by bacteria in various stages of cell division and microcolonies are formed as monolayers. Continued cell division in these microcolonies results in the formation of multilayered biofilms. The early colonizers are dominated by streptococci that may comprise up to 60–90% of the initial flora (193). The remaining bacteria are mainly made up of Gram-positive rods, predominantly *Actinomyces* (194). The complexity of the microbiota increases during the next 48 h, as indicated by a high morphological diversity (195) (see Fig. 10). During healthy conditions, the microbiota of the oral cavity provides a beneficial environment, but ecological shifts may occur within the microbial community that result in the two major oral diseases; dental caries and periodontal disease (196, 197).

5.5. Intestinal biofilms

The human gut is the next environment after the oral cavity, where commensal multispecies biofilms form. A wide range of bacterial species exists in the human intestine, which interact symbiotically with the host. The number of bacterial species in the intestine has been estimated as ranging from 500 to 1000 (198), although only 20% of this number of species has been cultured. The microbiota of the intestines has numerous functional and beneficial

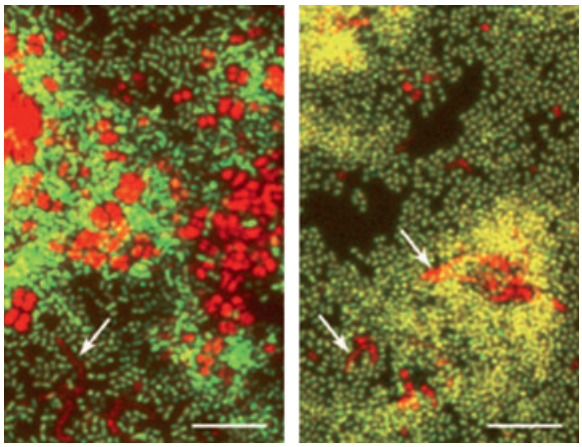

Fig. 10. Examples of multispecies oral biofilms. Confocal laser scanning microscopy images of 24- to 48-hours-old *in situ* biofilms. Biofilms were visualized by fluorescence *in situ* hybridization using an all-bacterium-specific EUB338 probe and a *Streptococcus*-specific STR405 probe simultaneously. Yellow–green represents streptococci and red represents non-streptococci. The image on the left shows filamentous non-streptococci (arrow), while that on the right shows their partial concealment by streptococci (arrows). Scale bar for all images 10 μm [adapted from (195) with permission from the publisher].

roles within the host. Indigestible dietary fibre is metabolized to short-chain fatty acids that nourish the epithelium, promoting the absorption of glucose by inducing the expression of sodium/glucose transporters in the epithelium, thereby enhancing the storage of fat and the synthesis of essential vitamins. The host immune system is stimulated by the microbiota and the binding of pathogenic bacteria to the epithelium is competitively inhibited.

Thus, the intestinal microbiota is a major factor in human health and disease.

If this healthy microbiota is disrupted by antibiotics, chemotherapy or a change in the diet, intestinal colonization by pathogenic bacteria or viruses may occur, leading to disease (199).

This is supported by the use of probiotics because it has been shown that when beneficial microbiota are disturbed as in acute diarrhoea, the balance can be restored if the patient ingests beneficial bacteria (200). Another example is relapsing *Clostridium difficile* antibiotic-associated diarrhoea. Here, *C. difficile* has become very difficult to eradicate. However, it remains unknown whether this is due to *C. difficile* disrupting the normal microbiota biofilm, biofilm production by *C. difficile* itself, or its ability to survive by producing endospores.

It is clear that most beneficial enteric bacteria have the ability to produce biofilm, as shown by Zoetendal et al., (200) who visualized even unculturable bacteria using FISH (see Fig. 11).

It remains unknown whether the biofilms in the intestines are important for stabilizing the homoeostasis of the intestinal microbiota, for inhibiting the virulence traits of pathogenic bacteria, or a combination of both.

5.6. Other environmental biofilm habitats

Of less investigated human habitats with a natural biofilm microbiota in healthy individuals include the female reproductive tract (201), the outer part of the urinary system (202), the outer part of the biliary system (203) and even our skin is a potential biofilm habitat. It is known that bacteria are present in all these non-sterile parts of body (204), although it is still debatable whether they occur as commensals on the bodies of healthy humans or as pathogens in unidentified medical conditions.

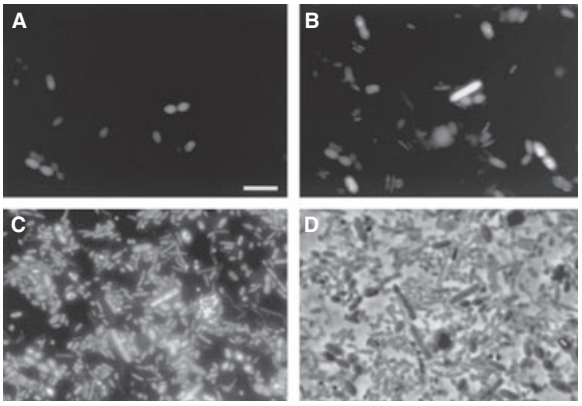

Fig. 11. Intestinal bacteria cells in a microscopic field hybridized with different probes: Cy3-labelled Urobe63 probe (A), FITC-labelled Erec482 probe (B), and DAPI (C). (D) Phase-contrast photomicrograph. Bar = 5 μm. [Adapted from (200) with permission from the publisher, also refer to the reference for probe specifications.]

However, bacteria are present on our skin where they grow and persist as small and large microcolonies on epidermis cells, in hair follicles and in sweat ducts (205, 206). It is commonly assumed that biofilms of differentiated microbiota may have beneficial properties in all the non-sterile regions of the human body.

6. MEDICAL BIOFILMS

6.1. Infections

Our body is covered by skin on the outer surface while mucosal membranes cover the inner surfaces of the body. Microorganisms can cause infection and inflammation (the body's response) by damaging the skin/mucosal membranes or by penetrating the skin/mucosal membranes (207), which leads to damage/inflammation within the body. On the skin, and many mucosal membranes (i.e., mouth, most of the intestinal tract, vagina and distal urethra), there is a normal flora (the human microbiota) that amounts to ten times more bacterial cells than human cells (208) (see sections 5.4–5.6). The interior of the human body is generally sterile, i.e., the blood, brain, muscles, bones, etc. parts of the mucosal membranes (e.g., middle ear, paranasal sinuses, the conductive and respiratory zones of the lungs, the uterus, and upper part of urethra/bladder)

do not harbour a permanent flora, but they are sometimes contaminated with a few bacteria, for example, by aspiration. However, the defence mechanisms of the mucosal membranes rapidly remove these contaminants in healthy humans without causing signs of inflammation or infection. If infection occurs without causing clinical symptoms (subclinical or silent infection), an immune response may be detected later. If an infection causes clinical symptoms, for example, inflammation and fever, it may be cured spontaneously by innate or adaptive immune response and/or by antibiotics, although sometimes it may be lethal. Such infections are known as acute and they can be detected 1–2 weeks after the onset of an antibody response. If the infection persists, despite the immune response and antibiotic therapy, it is known as a chronic/persistent infection.

6.2. Acute infection

Acute infections have a rapid progression, but are normally relatively easy to treat with antibiotics. This may be because the bacteria in acute infections are caused by planktonic bacterial cells (single, small clusters or chains) (see section 4.1). Acute infections are characterized by an innate inflammatory response, which is dominated by PMNs. The vast majority of human infections are acute and can be treated easily by general practitioners, for example, upper airway infections, skin and wound infections, urinary tract infections, enteric infections and pneumonia. More severe and life-threatening infections, such as sepsis, meningitis, and severe cases of pneumonia, are still fairly easily treated at hospitals, if the infection is diagnosed and treated in time.

6.3. Biofilm-related infections

Increasing evidence suggests that the chronicity of persistent bacterial infections is due to bacterial biofilm formation, which contrasts with the planktonic bacteria found in acute infections (**IV, V, VI, VII, VIII, XIII, XV**) (11, 13, 14, 209) as shown in Table 1. The longest recognized biofilm infections are dental infections, such as caries and parodontitis (191, 210, 211) (see Table 1), although these are outside the scope of this thesis.

6.3.1. Chronic wounds – The global increase in obesity has been accompanied by a similar increase in diabetes and cardiovascular diseases. These patients are particularly prone to the development of chronic wounds, which may be colonized by a number of bacterial species (217, 238–240). My research has shown that bacterial biofilms are present in chronic non-healing wounds (**IV, V**) (217, 241) Figure 12, although controversy persists as to whether biofilms have a role in the delayed healing of chronic wounds (242). Most studies show that the deep dermal tissues of all chronic wounds harbour multiple bacterial species (239, 240, 243–245). The most common bacterium found in wounds is *S. aureus*, although *P. aeruginosa* was observed in more than half of the chronic wounds investigated (**V**) (239, 245). Furthermore, *P. aeruginosa*-infected wounds were significantly larger in area than wounds without *P. aeruginosa*, while the presence of *P. aeruginosa* also seemed to delay or even prevent the healing process (246–248).

Many chronic wounds will not heal, despite aggressive treatment, and it was hypothesized that this was due to the presence of bacteria

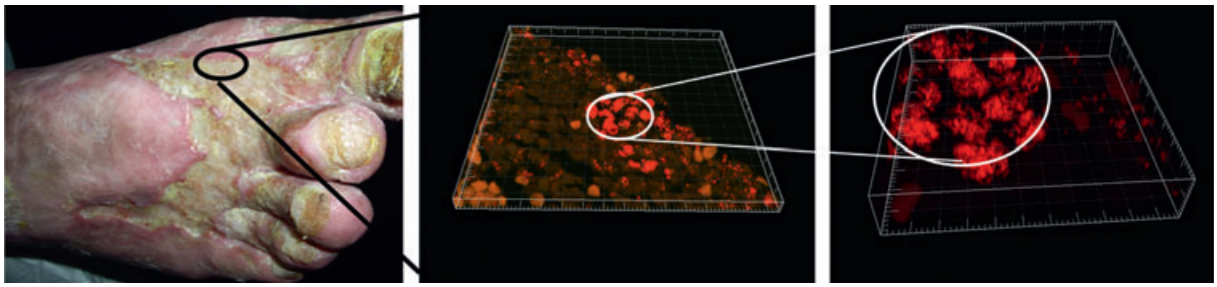

Fig. 12. Biofilms of *Pseudomonas aeruginosa* in a chronic wound visualized using a specific peptide nucleic acid-fluorescence *in situ* hybridization probe (red) with confocal laser scanning microscopy. The right image shows an enlargement of the middle image (adapted from **V** with permission from the publisher).

with a biofilm growth phenotype (249–252). The first direct microscopic evidence of bacterial biofilm involvement in chronic wounds was based on the direct microscopic identification of bacterial aggregates (**IV**) (217, 241). Three publications were published back-to-back in the same issue of the journal 'Wound Repair and Regeneration' during 2008. In my study (**IV**), I detected the presence of bacterial aggregates of *S. aureus* and *P. aeruginosa* using specific peptide nucleic acid (PNA) FISH probes, in combination with CLSM. Peptide nucleic acid FISH allows the direct illuminating of specific target cells within a sample. The presence of biofilms in chronic infected wounds was highly debatable, so the observation of aggregated bacteria was considered insufficient evidence. To elucidate the biofilm growth phenotype that was present, I detected the EPS matrix by illuminating the alginate surrounding *P. aeruginosa*. We also hypothesized that the presence of *P. aeruginosa* maintained the wound in a chronic state, due to the cytolytic effects of the rhamnolipids produced by *P. aeruginosa* (see section 4.2). James et al. (217) demonstrated the elevated presence of microbial aggregates in chronic wounds compared with acute wounds using scanning electron microscopy (SEM).

In a subsequent study, we collected and examined chronic wound samples from 22 different patients, all of whom were suspected to

Table 1. Visual identification of biofilms in chronic infections [modified and expanded from (238)]

Biofilm site	Visualization method	Reference
Dental plaque	Light microscopy	Hodson (211)
	Electron microscopy	Boyde and Lester (212)
	Electron microscopy	Theilade and Theilade (185)
	Light and Electron microscopy	Listgarten (186)
	FISH	Dige et al. (195)
Periodontitis	Electron microscopy	Theilade (213)
	Light microscopy	Berthold and Listgarten (214)
	FISH	Zijnge et al. (215)
Cystic fibrosis lung infections	Light microscopy	Høiby (8),
	Electron microscopy	Lam et al. (216),
	FISH	**VI**
Chronic wounds	FISH	**IV**
	Light and electron microscopy	James et al. (217)
Soft tissue fillers	FISH	**VII**
Otitis media	FISH	Hall-Stoodley et al. (218)
	FISH	**VIII**
Implant associated	Electron microscopy	Marrie et al. (219),
	FISH	Waar et al. (220)
Catheter and shunt associated	Electron microscopy	Marrie et al. (221)
	Electron microscopy	Marrie and Costerton (222)
	Fluorescence microscopy	Stoodley et al. (223)
	FISH and electron microscopy	Parsa et al. (224)
Chronic osteomyelitis	Electron microscopy	Gristina et al. (225),
	Electron microscopy	Marrie and Costerton (226)
	Light and electron microscopy	Sedghizadeh et al. (90)
Chronic rhinosinusitis	Electron microscopy	Cryer et al. (227)
	Electron microscopy	Sanclement et al. (228)
	FISH	Sanderson et al. (229)
	Fluorescence microscopy	Li et al. (230)
Endocarditis	Echocardiography	Stewart et al. (231)
	Electron microscopy	Poyart et al. (232)
UTI	Electron microscopy	Nickel and Costerton (233)
	Light and electron microscopy	Nickel and Costerton (234)
	Light microscopy	Reid et al. (235)
Contact lenses	Electron microscopy	Stapleton and Dart (236)
Human gastrointestinal tract	FISH	Macfarlane and Dillon (237)

be infected by *P. aeruginosa* (**V**). The focus on *P. aeruginosa* was based on our hypothesis that *P. aeruginosa* has a major role in chronic wounds (**IV**).

The wound samples of the 22 different patients were investigated using standard culturing methods and PNA FISH for the direct identification of bacteria. Using standard culturing methods, *S. aureus* was detected in the majority of the wounds, whereas *P. aeruginosa* was observed less frequently. By contrast, PNA FISH showed that a large fraction of the wounds harboured biofilms of *P. aeruginosa* that were embedded in the matrix alginate component. These microcolonies were detected within the wound bed, whereas *S. aureus* was detected on the surface of the wounds, if present. This was supported by our subsequent observations (253) and a study by Davis et al. (241), who demonstrated that *S. aureus* forms microcolonies on the surface of the wound bed that were encased with extracellular matrix. I also participated in a study that showed that bacteria were highly heterogeneously distributed in these chronic infected wounds (245).

6.3.2. Cystic fibrosis – Cystic fibrosis is the most common lethal inherited disease in Caucasians (254). It is a monogenic, autosomal recessive multi-organ disease with a worldwide incidence of gene defects in the range of 1:32 000 to 1:2000 live births (255). The genetic cause of CF was identified in 1989 as a defect in the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) gene, which is located on chromosome 7 (256–258). The CFTR defect causes a decrease in epithelial chloride secretion and an increase in sodium absorption. In the CF lung, this results in dehydrated viscous mucus that is very difficult to clear mechanically, i.e., by coughing. The abnormal mucus viscosity is due to the chronic depletion of water in the periciliary liquid layer and mucus (259, 260). The non-inflammatory defence mechanism, i.e., mucociliary clearance, is impaired so inflammatory defence mechanisms are recruited (PMS, macrophages, IgG, etc.) giving rise to clinical symptoms, i.e., recurrent or chronic bacterial lung infections (255, 261) (see Fig. 13).

Since 1976, CF patients suffering from chronic *P. aeruginosa* lung infection have successfully received intensive treatment with high concentrations of antibiotics at the Copenhagen CF Centre (i.e., maintenance treatment and chronic suppressive treatment) (262, 263). Before 1976, only 50% of CF patients would survive 5 years of chronic *P. aeruginosa* lung

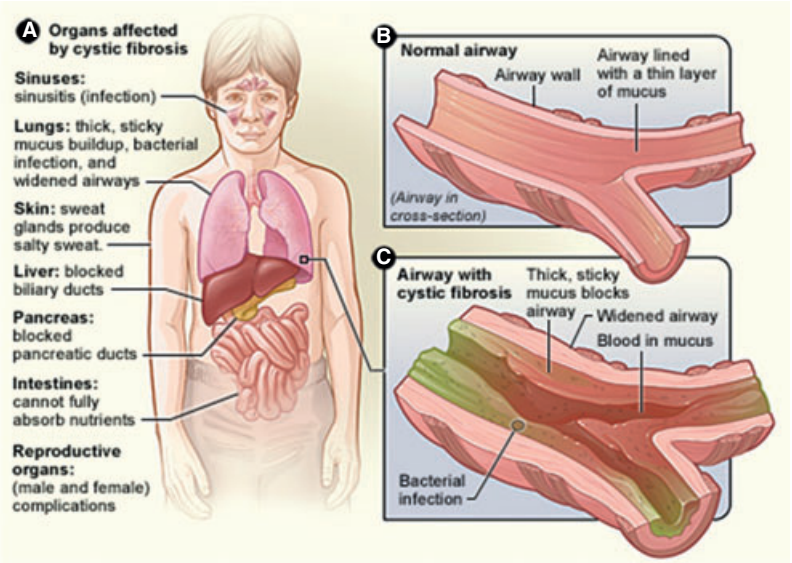

Fig. 13. Schematic introduction to cystic fibrosis (CF). Frame C shows the thick dehydrated mucus that allows bacteria to settle and form a chronic biofilm infection in the CF lung (adapted from: http://www.medicinenet.com/cystic_fibrosis/page3.htm).

infection (**VI**). Most CF patients now survive for decades with chronic *P. aeruginosa* infections (264).

Despite the aggressive and intensive treatment of chronic *P. aeruginosa* infections, the bacteria still persist in the CF lung. The intensive treatment delays and reduces the damage caused by the chronic infection, but it cannot be eradicated. CF patients experience a continuous degradation of lung tissue during chronic *P. aeruginosa* infections. This is caused by infection and inflammatory processes where a pronounced immune response leads to immune complex-mediated tissue destruction (**VI**). There is a consequent decline in lung function, which is the primary cause of death in CF patients (265). Like chronic wounds, CF was believed to be a biofilm disease (32, 79, 216). To investigate the true growth phenotype of bacteria in the CF lung, I evaluated the distribution pattern of *P. aeruginosa* in the conductive and respiratory zones of explanted lungs from chronic *P. aeruginosa*-infected CF patients using PNA FISH (**VI**), just as I did with the chronic wounds (**IV**). I also compared intensively treated explanted lungs with autopsies of non-intensively treated CF patients. As expected, the bacteria were mainly found in aggregates and only a few planktonic bacteria were observed (see Fig. 14). It was evident from my visual observations that bacteria were mainly localized in the conductive zone (the upper part of the lung with large and small bronchi) if the patient was treated intensively with very few in the respiratory zone (the lower part, i.e., the alveoli). All of the aggregates were embedded in mucus plugs, although not all of the mucus plugs contained bacteria. The mucus plugs containing bacteria varied greatly in size and spatial orientation. By contrast, bacteria were distributed throughout the entire lung in both the conductive and respiratory zones in patients that had not been treated intensively. In general, the inflammatory response in the chronic *P. aeruginosa*-infected CF lung was dominated by PMNs, and I observed a vast amount of PMNs surrounding the aggregates. Bacteria did not adhere to the epithelial wall, demonstrating that the bacteria had grown within the mucus rather than adhering to the bronchial inner surface, as previously suggested (32).

6.3.3. Chronic otitis media – The upper respiratory tract consists of the, nose, paranasal sinuses, middle ear and throat, and it is frequently infected in children (266). Children are more susceptible to infection of the middle ear because their Eustachian tube is shorter and less functional compared with the adult ear (267). Infection is most often initiated as an acute viral respiratory infection followed by complicating bacterial infections that may develop into otitis media with effusion, a chronic suppurative bacterial infection with mastoiditis, and even cholesteatomas, even if appropriate antibiotic treatment has been initiated (268).

In the past, a bacterial cause of chronic infections in the middle ear was difficult to confirm because of culture-negative sampling. Recurrences or exacerbations were intriguing and difficult to explain, because bacteria were not isolated. It was suggested that the infection was a local inflammatory reaction without bacteria. However, experiments using animal models demonstrated that bacterial biofilms could cause these infections (269–271). Later, it was shown directly that treatment failure, culture-negative results and recurrent exacerbations were due to bacteria that firmly resided in biofilms (218, 272, 273).

Recently, I was part of a morphological investigation of bacterial biofilms in a high-risk population in Greenland (**VIII**). As with my other biofilm studies, I used PNA FISH to elucidate whether bacteria were present in the aggregates. We observed aggregates that were present in pus discharged from the ears of five of the six (83%) children with chronic suppurative otitis media (CSOM) and we found evidence of biofilms in biopsies from the middle ear in eight of the ten (80%) adults treated for CSOM (see Fig. 15) (**VIII**). These findings were later confirmed in a controlled study of humans with CSOM in the USA (274).

It is now widely accepted that bacterial biofilms have a role in several chronic infectious middle ear diseases (1, 218, 274–278).

6.3.4. Tissue fillers – An emerging problem is the injection of foreign bodies in the form of polyacrylamide gel (soft tissue fillers) under the skin for aesthetic purposes and remodelling after trauma. Many different types of fillers

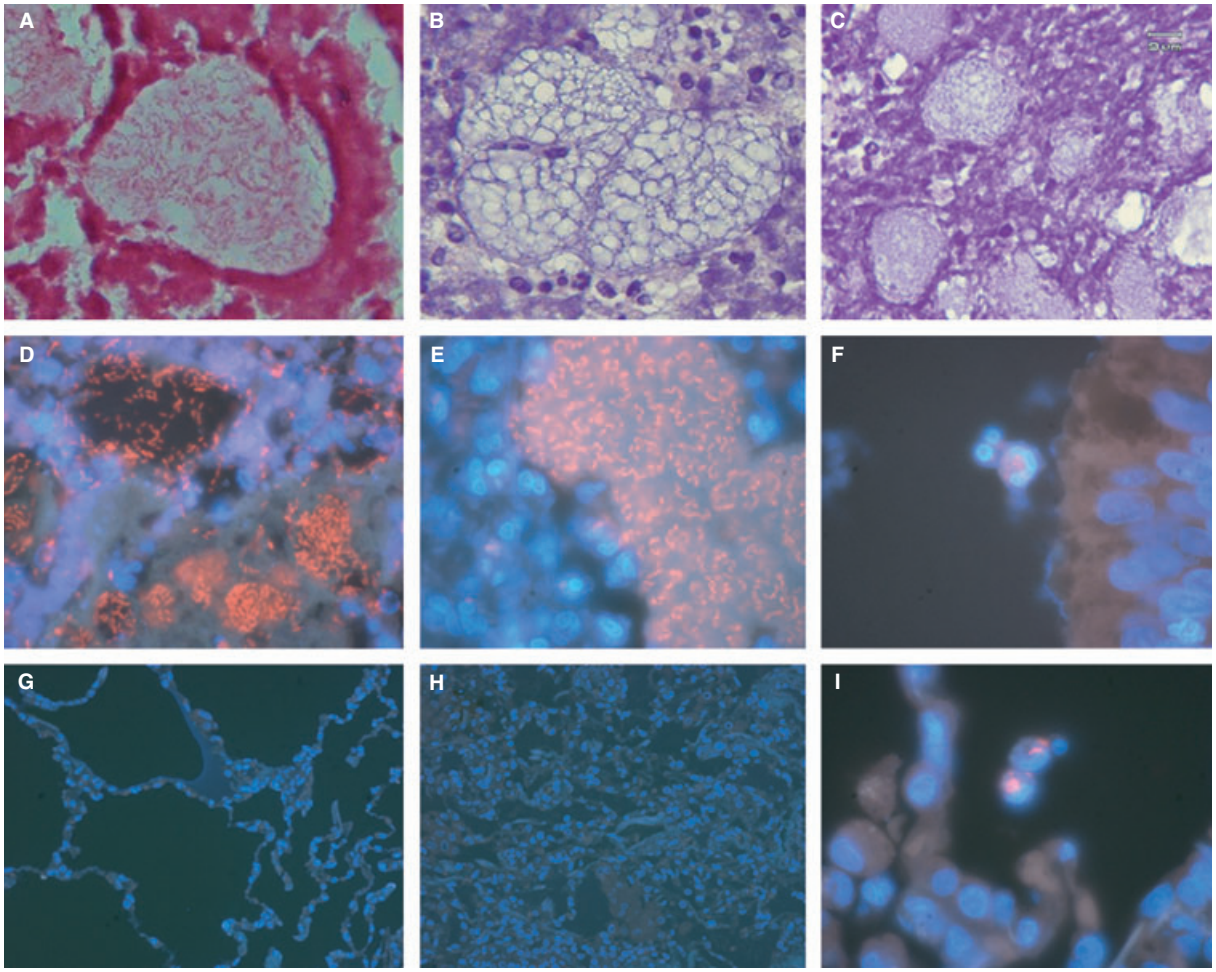

Fig. 14. *Visualization of Pseudomonas aeruginosa* biofilms in the cystic fibrosis (CF) lung. *Pseudomonas aeruginosa* was found in the conductive zone, whereas very few bacteria were detected in the respiratory zone where they were phagocytosed. (A) Bacteria in biofilm within a bronchus visualized using Gram stain (CF male, 41 years of age, chronic *P. aeruginosa* mucoid and non-mucoid infection for 28 years, 46 precipitating antibodies, 114 two-week anti *P. aeruginosa* treatment courses). (B and C) HE staining of bacteria-filled bronchiole. (D and E) Intraluminal *P. aeruginosa* biofilms surrounded by polymorphonuclear leucocytes visualized using peptide nucleic acid-fluorescence *in situ* hybridization, and DAPI. (F) Intact bronchi wall. (G and H) Increasing consolidation of the alveoli, and (I) single phagocytosed *P. aeruginosa* in the respiratory zone (adapted from **VI** with permission from the publisher).

are available ranging from polymers to microparticles, of which some are permanent and other are semi-permanent (279). Fillers are supposedly cleared for toxicity and antigenicity, before they are permitted for human use (279). Fillers are injected subdermally and, as with most foreign materials apart from noble metals, they evoke an inflammatory response where the intensity varies with the type of filler. This inflammatory response is supposed to be short term without complications, although some fillers rely on inflammation to produce the filling effect (280). Most injections have no further complication, but an increasing number of patients develop adverse events such as inflammatory swellings or nodules. If these are left untreated, they often result in fistula formation and the discharge of pus and filler.

Only a few years ago, it was assumed that these reactions were caused by a foreign body reaction towards the injected filler. This was despite suspicions that the adverse reactions

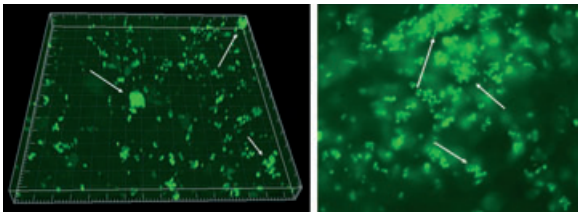

Fig. 15. *Staphylococcus aureus* biofilms (arrows) in otorrhoea from a patient with chronic suppurative otitis media as determined by species-specific peptide nucleic acid-fluorescence *in situ* hybridization. The left frame shows a 3D confocal laser scanning microscopy projection (adapted from **VIII** with permission from the publisher).

after injection of fillers were caused by bacterial infection (281–283). As with the chronic middle ear infections, the samples from the nodules were culture- and often PCR-negative. In addition, as with all other chronic infections, antibiotics had little or no effect on the apparent inflammation (282). It was considered that the adverse events were autoimmune reactions, so patients were treated with steroids or large doses of non-steroidal anti-inflammatory drugs to suppress the inflammatory process. The results were the complete opposite of what was expected, because the treatment usually resulted in full-blown abscess and fistula formation (Fig. 16, frame A). This inflammatory burst fuelled the hypothesis that bacteria were the cause (281, 282).

As with the chronic wound, CF and middle ear observations, I performed PNA FISH on biopsies from patients with adverse reactions due to tissue fillers (**VII**). My observations were the first direct visualizations of bacteria and their locations within the tissues after filler injections. As shown in Fig. 16 frame C, the bacteria detected were organized in aggregates similar to those found in chronic infections of wounds, CF and the middle ear. We believe that the presence of bacteria in biofilms explains the failure of antibiotic treatments and the strong adverse reaction when treated with steroids.

6.3.5. Additional chronic biofilm infections – Other chronic infections that have been linked to the biofilm growth phenotype by other researchers include: chronic osteomyelitis (284), rhinosinusitis (229, 285–287), urinary tract infections (288), tuberculosis (289) and many types of infections associated with foreign bodies inserted in the human body (249, 290).

7. CLINICAL MICROBIOLOGY: THE NEED FOR DIAGNOSTICS AND TREATMENTS FOR BIOFILM INFECTIONS

In clinical microbiology, the main goal is to isolate and identify microorganisms that cause infections in humans, before determining the antibiotic susceptibility of the microorganism identified and giving advice on the prevention and treatment of infections. The major clinical microbiology problems related to harmful bacterial biofilm infections within the human body are the diagnosis of the biofilm and its treatment.

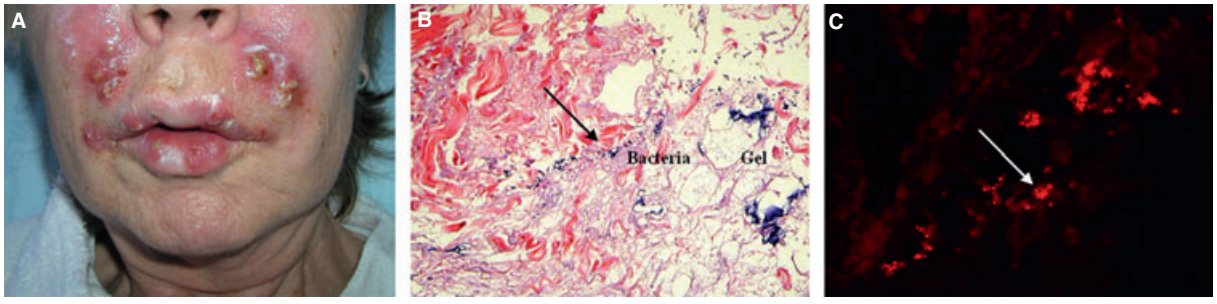

Fig. 16. Adverse reactions to polyacrylamide gel can be seen as swellings or nodules (A). Controversy exists as to whether these are due to bacterial infection or an autoimmune reaction to the filler. However, B and C show that bacteria were definitely present. B shows a histological preparation of tissue from one of these adverse events, while C shows the peptide nucleic acid-fluorescence *in situ* hybridization visualization of bacterial biofilms. The arrows point to aggregates of bacteria [Frame A adopted from Lise H Christensen, Frame B and C adopted from (**VII**) with permission from the publisher].

7.1. Diagnostics

The initial challenge with all infections is to identify the infecting organisms and the focus of the infection. This is usually not a problem for acute infections because specialists in clinical microbiological departments benefit from over 150 years of experience with microscopic techniques, as well as a wide variety of growth media and cultivation conditions. The first task is to obtain a sample for investigation, which is typically identified by the attending medical doctor. Depending on the sample, laboratory technicians make smears for Gram staining and microscopy before selecting different growth media to use and whether the sample should be propagated in solid or liquid media, incubated anaerobically or aerobically, the specific temperatures and duration. PCR is used routinely for some slow-growing or difficult to grow bacteria (e.g., *Mycobacterium tuberculosis, Chlamydia species,* and *Legionella* spp.). After obtaining a pure bacterial culture, species are identified using different biochemical and serological tests, or molecular-based methods such as PNA FISH, Matrix-assisted laser desorption/ionization-Time of Flight (MALDI-TOF), or PCR amplification and 16S rDNA sequencing.

These methods are sometimes more problematic with chronic infections (see Table 1). An exception is CF (see section 6.3.2), where the easily accessible purulent sputum or endolaryngeal secretions can be obtained from patients on a regular basis and they will harbour bacteria if present. However, infections of the sinuses are difficult to access and CF patients are believed to experience these before lung infections (291). Other chronic infections, such as the non-healing wounds and orthopaedic infections, are even more problematic. Clearly, the wound bed is attached to the patient and it cannot readily be removed or homogenized for routine diagnostics. The routine sampling has typically used a swab or a biopsy, although both approached might fail to sample the bacteria because of their heterogeneous distribution within the wound bed, as we have previously shown (**V**) (245, 253). A swab can only collect bacteria found on the surface and not the bacteria embedded in the wound bed (**V**) (253). Given the heterogeneous distribution of bacteria, there is a risk that a 'blind' biopsy will fail to contain any bacteria (245).

7.2. Treatment

After the infecting organism or organisms have been identified, the next task is to treat the infection. Importantly, biofilm infections cannot be treated in the same way as acute infections, as described previously (see section 4.1). The most efficient treatment for a biofilm infection is to mechanically remove the infected area or body part, e.g., dental infections. This is sometimes possible if the focus is a catheter, an implant or an infected organ that is eligible for transplantation. However, it is not always possible or without risk of complications for patients. The two main strategies for preventing or suppressing bacterial biofilm infections are: (i) early aggressive antibiotic treatment before the biofilm is formed; (ii) chronic suppressive antibiotic treatment when the biofilm is established, if it cannot be removed physically (16). As stated in the previous sections, the administration of antibiotics to treat bacterial biofilms demands combinations of several different antibiotics (different targets, subpopulations and penetration) in high doses and for an extended period of time (16) because conventional resistance mechanisms will contribute to the intrinsic biofilm resistance mechanisms.

Due to the risk of inducing traditional resistance mechansims, it is advisable to avoid treating the biofilm infection with an acute infection regime of single drugs in minimal doses for short periods. This may induce further resistance and tolerance, and eventually create opportunities for new bacteria to chronically infect the focus (16, 292).

7.3. Prevention

The most efficient means of combating biofilm infections is to prevent the infection in the first place. Normally, human skin acts as a barrier to prevent bacterial invasion of the body. This natural barrier is compromised by surgery and the insertion of implants. Thus, surgeons have to exercise extreme caution when inserting implants or injecting dermal fillers to avoid the introduction of bacteria. Ultra-clean operating

theatres, surgical instruments, surgical garments and implants are necessary for avoiding direct contamination of the surgical site (293, 294). These precautions are often combined with the prophylactic administration of antibiotics (16). In the future, we might also use drugs that inhibit virulence traits, such as QS inhibitors, to prevent chronic infections (30, 109, 128, 142, 143, 295–297), while antibacterial coatings could be applied to inserted surfaces (298). Early aggressive eradication of intermittent colonization of the lungs has postponed chronic lung infections in CF patients by 10–20 years (254, 263, 299–304).

The best-known example of prevention is our daily treatment to combat dental biofilm formation (see section 5.4). Caries are preventable if the teeth are cleaned mechanically to remove any biofilm using toothbrush and flush (305).

8. SCIENTIFIC EXTRAPOLATIONS AMONG ENVIRONMENTS

As in most other fields of medicine and biology, biofilms research is not an exact science. The results of *in vitro* laboratory work and animal experiments are extrapolated to explain or predict *in vivo* observations in patients and vice versa. Sometimes these extrapolations and correlations are beneficial or true, but not always. It is known that experimental observations should be confirmed by randomized, controlled experiments conducted with groups of patients, for the observations to become facts. In this part of my thesis, I will discuss what I consider to be the most important extrapolations and correlations in the biofilms field.

8.1. In vitro and *in vivo* biofilms

Most of our knowledge and current hypotheses regarding bacterial biofilms originate from studies of biofilm formation conducted using *in vitro* model systems, such as the flow cell (26), where bacteria are grown on a glass surface with a continuously supply of nutrients.

8.1.1. Mushroom-like structures – Bacteria grown in flow cells can produce beautiful structures resembling mushrooms, as described in section 3.1 and shown in Fig. 4.

Throughout the years, a great emphasis has been placed on the development of these three-dimensional structures, i.e., wild-type behaviour (44), and the absence of mushrooms or structure has been correlated with poor biofilm formation (42).

To the best of my knowledge, three-dimensional mushroom-like structures or other highly structured communities have never been identified in experimental animals or chronic human infections.

Given this general focus on three-dimensional structured biofilms and surface-associated biofilms in general, particularly in the area of foreign body-related infections, we developed an experimental animal implant model (**II, XIII**). In this model, we inserted sil-

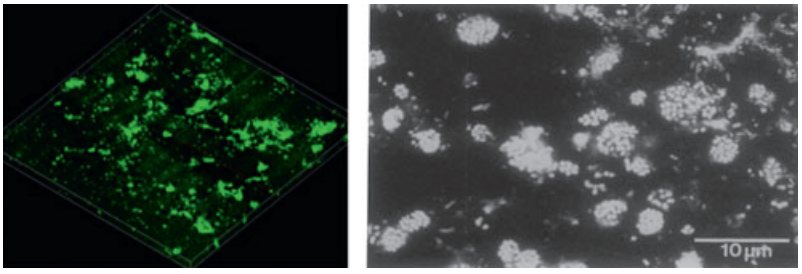

Fig. 17. The left frame shows a biofilm formed on a silicone implant in a mouse model of implant-related infection. The biofilm is 4 days old and it was formed by GFP-tagged *Pseudomonas aeruginosa*. The visualization was performed using confocal laser scanning microscopy (CLSM) immediately after the mouse was euthanized [adapted from (**II**) with permission from the publisher]. The right frame shows a CLSM visualization of an environmental biofilm from the Bow River, Alberta, Canada [adapted from (12) with permission from the publisher].

icone implants pre-coated with *P. aeruginosa* into the peritoneal cavity of mice. The bacteria on the implants formed a biofilm almost immediately, which the mice were unable to clear. *Ex vivo* examination of these implants detected bacterial microcolonies on the silicone implants that resembled the microcolonies found in rivers (12), as shown in Fig. 17, as well as the biofilms observed in chronic wounds (see Fig. 12) (**IV, V**) and CF (see Fig. 14) (**VI**), whereas no three-dimensional structures similar to *in vitro* mushroom structures were observed.

This discrepancy is probably attributable to the design of the flow cell, because they were designed to allow continuous, non-invasive and reproducible monitoring of controlled biofilm development. No other experimental or clinical infection settings would allow such conditions. Grazers and phagocytes are present (see section 4.2) in most environmental and medical biofilm habitats and there is a varying flow (if any flow at all), as well as the presence of pus, other excreted fluids, and many other factors. All of these dynamic and variable factors combined make *in vivo* biofilms very different from flow cell biofilms. However, flow cell biofilms have provided important clinical information on biofilm physiology, although they cannot be correlated directly with *in vivo* biofilms without further observational or experimental evidence.

In support of the flow cell, numerous studies and observations using this method have been extremely fruitful. Almost all hypotheses related to biofilms have originated from flow cell experiments, including antibiotic tolerance, predator and phagocyte tolerance, biofilm formation and biofilm dispersal.

8.2. To surface or not to surface

In the first definition provided by Costerton et al. (13), i.e., 'a structured community of bacterial cells enclosed in a self produced polymeric matrix, adherent to a surface', the authors indicated the necessity of a surface. This was based on the slimy film layer composed of biomaterials found in alpine streams (170) and on submerged ship hulls (306, 307), i.e., biofilms. A surface is present in many of the infections that are now known to involve biofilms (Table 1). However, bacterial aggregates also have been observed in other persistent infections such as chronic wounds and CF, which completely lack a surface (see sections 6.3.1–6.3.4) (216–218) (**IV, V, VI, VII, VIII, XV**).

Why should these aggregates, clumps or heaps (8), be referred to as biofilms? As explained in the introduction, the most important hallmarks of bacterial biofilms are the aggregation of bacteria within a matrix (see section 3.3) and the tolerance of antimicrobial agents (see section 4.1). It is evident from all observations of bacteria in chronic infections that bacteria are present in aggregates and they persist despite antimicrobial chemotherapy. The only difference, and this is open to debate, is the apparent surface. I have never been in any doubt about the lesser importance of the surface, and that aggregation and tolerance are the most crucial factors, because the major differences between planktonic growth and biofilm growth are the biofilm matrix and the gradients (e.g., oxygen, nutrient, generation time, metabolic activity and antibiotic activity) from the surface to the bottom/centre of the biofilm, whereas the surface at the bottom of the biofilm may simply be a surrogate for the gradients.

To elucidate whether aggregated non-surface-attached bacteria displayed the same phenotypes as surface-attached bacteria, we compared the matrix and antibiotic/phagocyte tolerance in these two scenarios (**XII**). All of the features of the aggregated bacteria were similar to the flow cell biofilm, apart from the first layer of bacteria that was attached to the glass surface in the flow cells. All other characteristics were similar in both scenarios, including the aggregation, matrix and protection against antibiotics and phagocytes, even among different subpopulations (**XII**) (Fig. 18).

The most important feature is the build-up of bacterial layers, which leads to QS initiation and nutritional and gaseous gradients within the biofilm. This initiates virulence factor production, such as rhamnolipid production (see section 4.3), the emergence of subpopulations and dormant bacteria.

If the surface had an important role, the standard biofilm phenotypes should be gener-

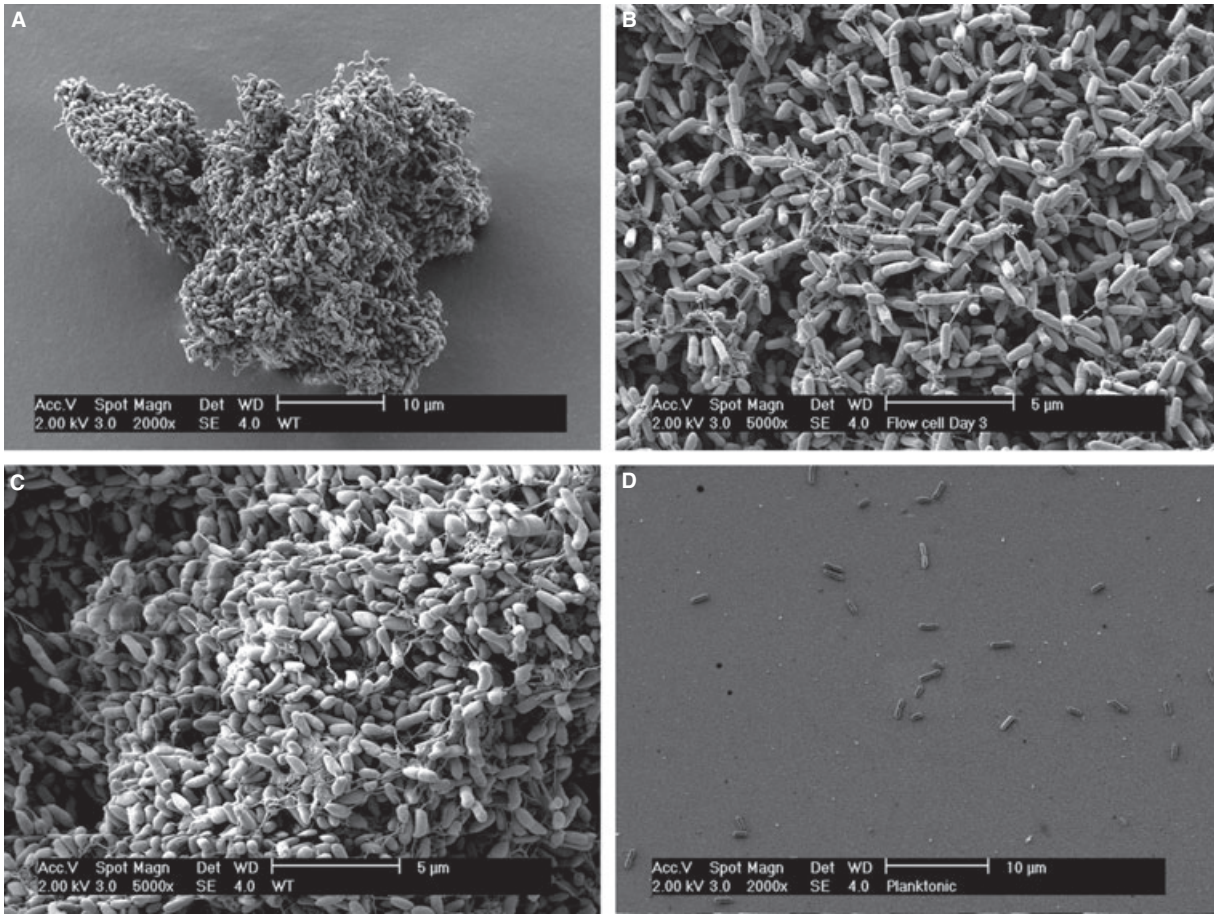

Fig. 18. Scanning electron microscopy of surface- vs non-surface-attached *Pseudomonas aeruginosa* biofilms. (A) Aggregate harvested from a 48-hours-old stationary culture. (B) Details of a 3-day-old biofilm grown in a flow cell. (C) Details of 48-hours-old stationary aggregate. (D) Planktonic cells (OD_{600} = 0.5; adapted from **XII** with permission from the publisher).

ated immediately. Interestingly, during the investigation of biofilm-enabled protection against phagocytes, we observed that immature wild-type *P. aeruginosa* biofilms were easily eradicated when exposed to PMNs (**XIII**) (44). The same phenomenon was observed *ex vivo* on hollow tubes that had been inserted into the peritoneal cavity of mice (**XIII**). SEM showed that single bacterial cells or small groups of surface-attached bacteria were hunted and phagocytosed by PMNs initially. A persistent biofilm did not develop until after day 1, when the PMNs could no longer eradicate the bacteria and they were themselves killed (see Fig. 19).

Single layers of surface-attached bacteria are easily phagocytosed, so it is difficult to imagine that the surface has a role in biofilm-related phenotypes. The only situation where a surface might play a role would be those that experience shear forces or high flow rates, where the stochastic anchoring of a single bacterium could seed a population of bacteria that would develop into a biofilm.

8.3. How should biofilms be studied?

We do not need to define a true biofilm, because all the bacterial aggregates with the distinct phenotypes discussed here should be considered as biofilms. However, we do need to consider which biofilm setup should be used to study a given hypothesis or direct problem, based on the origin of this hypothesis or problem. The goals should also be clear in biofilm research; for example, whether the aim is to test a hypoth-

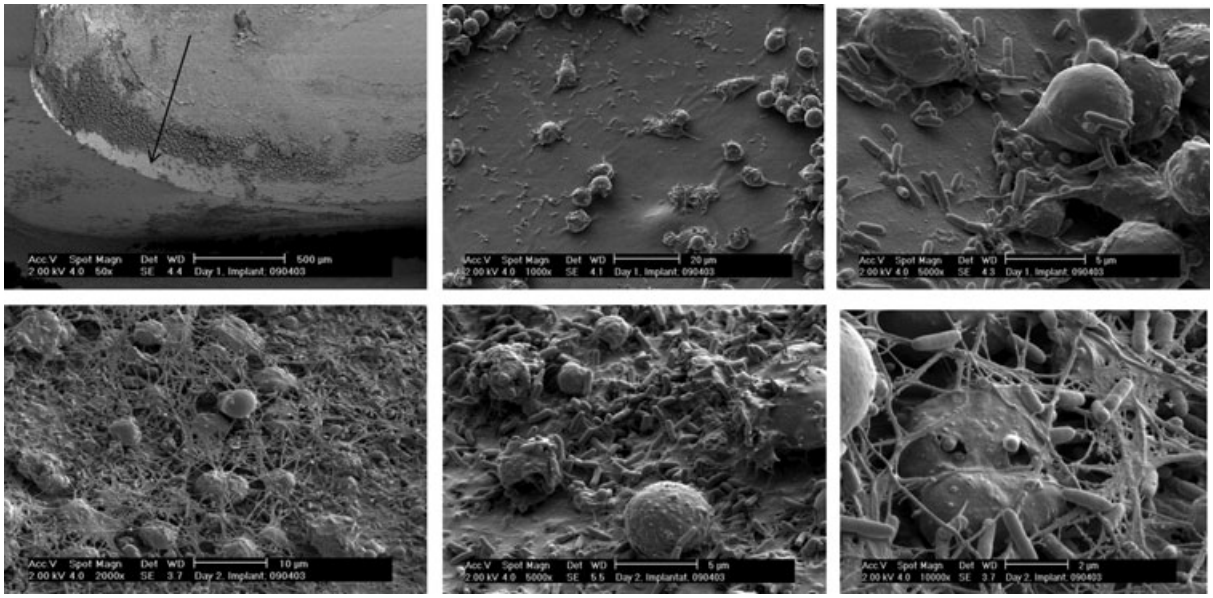

Fig. 19. Scanning electron microscopy images of *ex vivo* interactions between the *Pseudomonas aeruginosa* biofilm and the host defences. The top row was on day 1, where the left picture shows the tube was cleaned of bacteria from the end, and the middle and right picture shows the phagocytosing polymorphonuclear leucocytes (PMNs). The bottom row shows the interactions on day 2, where the bacteria succeeded in forming a protective biofilm that killed the PMNs (adapted from **XIII** with permission from the publisher).

esis, such as attachment, or a specific application such as validating a drug against biofilms. One of the simplest methods for studying surface-attached biofilms is the microtitre plate assay (308), where bacterial attachment to the surfaces of 96 wells is easily monitored by crystal violet staining of the entire biomass. This is a crude and not very reproducible assay, and it does not discriminate between live and dead cells, as well as aggregates that do not adhere to the surface after washing (**XII**). Another surface-based method is the flow cell system. This method is more reproducible and also facilitates discrimination between live and dead bacteria. As with the microtitre plate assay, the weakness with this system is that host materials are missing such as inflammatory components, pus and mucus. Examples may include biofilms in chronic wounds or the CF lung where no hard surface is present, but there is a lot of pus, necrotic tissue, inflammatory cells, etc. Flow cells or microtitre plates could be used to test preliminary questions such as whether a given drug can kill the dormant subpopulation of the wound biofilm or whether the biofilm produces density-specific compounds. Flow cells not microtitre plates should be used if the specific killing efficacy or dispersal is being monitored during the treatment, because the microtitre assay does not allow multiple time-point observations. Observations using a flow cell setup to study specific biofilm behaviour may not be readily extrapolated to an environment lacking a hard surface, such as wounds or the CF lung. Careful consideration should be required when selecting the optimal experimental setup to avoid false negative and false positive results. A classical example is when the microtitre plate assay is used to screen for mutations that prevent bacteria from forming biofilms. We found that an equal amount of biomass was present as suspended aggregates in the wells as was found on the inner surface of the microtitre plates, while bacteria that were unable to attach to the inner surfaces also formed aggregates (**XII**). It is paradoxical that if CF isolates were tested, all the non-adhering cells would be classified as non-biofilm formers, although no surface is available for adhesion in the CF lung. The non-biofilm formers can also form aggregates *in vivo* with the same virulence as their attachable counterparts.

The study of biofilms in these settings requires a model that mimics the pus or mucus

where the bacteria grow. We developed a model of bacterial biofilm growth in a semi-solid matrix of collagen (**X**), similar to that found in a wound bed or the mucopurulent pus of the CF lung. Using this model system, *S. aureus* and *P. aeruginosa* formed aggregates without adhering to a surface. The size and the shape of the aggregates resemble those observed in wound beds. These aggregates displayed the same specialized phenotypes as the flow cell biofilms, i.e., matrix production and high tolerance of antibiotics. Liquid-suspended aggregates were similar to surface-attached biofilms in terms of all their biofilm properties (**XII**). It is possible to add different inflammatory components to both these non-surface models, such as PMNs and antibodies.

However, these models cannot mimic the complex interplay that exists with the actual host defences, which is why animal models also should be used when biofilm interactions with the host are investigated. Many chronic diseases have their own animal models, such as CF (309–311), otitis media (312), chronic wounds (313) and our models of implant-related infections (**II, XIII**) [for a review of *in vivo* biofilm models see (314)].

Modelling all aspects of chronic human infections may not be possible with a single experimental method. The goal must be to identify and ask the right questions using the appropriate available models, or alternatively developing new ones.

8.4. The opportunity of sociomicrobiology

Bacteria form biofilms everywhere and they opportunistically exploit any available carbon, nutrient or energy resources for their metabolic processes. The literature shows that bacteria can thrive almost anywhere and that the biofilm growth phenotype is dominant (3, 4). As described in section 5, the intermixing of different bacterial species is natural where commensalism and synergies exist among species. Different species are mutually dependent in many environmental consortia because of anabolic and catabolic specializations and the availability of nutrients, oxygen, electron acceptors, etc. Most *in vitro* biofilm research has been performed using a single species, although investigations using multispecies and complex consortia have increased because such conditions are prevalent in systems such as dental biofilms (1, 195, 315).

In human infections, especially chronic infections, it is often possible to isolate several bacterial species from the same infection. Wounds, in particular, appear to contain an extreme range of species (217, 238–240). The chronic lung infection of CF patients has also been suggested to be multispecies (316, 317). This infection diversity has been elucidated using traditional identification techniques and molecular methods such as PCR and the new IBIS technology (318). IBIS is a combination of PCR and MALDI-TOF mass spectrometry. This allows the identification of bacterial species and an estimation of the relative abundance of the different bacterial species present. These methods can be used to identify the species present in a sample, but they cannot identify the orientations of each species or determine the species that contribute to pathogenesis. Direct microscopy can reveal the orientation and distribution, but it also fails to attribute pathogenesis.

As with environmental biofilm consortia, it is possible that the presence of many different species is due to their synergistic survival within a host. If this is the case, all the different species present, such as those found in a chronic wound, should occur in close proximity, or in co-aggregates, and they may possibly be engaged in symbiotic relationships.

Our approach to investigating chronic infections for the potential presence of biofilms has applied traditional visualization techniques and novel methods such as PNA FISH and CLSM. The microscopic approach has allowed us to visualize several different bacteria within one tissue or sputum sample. To our surprise, our observations have shown that although different bacterial species were present in the same sample, the majority of the aggregate was formed of a single species (see Figs 12, 14, 15) (**IV, V, VI, VIII, XIV, XV**) (1).

Along these lines, only in extremely rare cases have we observed mixed biofilms, or adjacent biofilms formed of different species in chronic infections. This is in spite of the use of a universal eubacterial PNA FISH probe (designed to detect all bacteria) in combination

with specific PNA FISH probes for bacteria such as *P. aeruginosa* or *S. aureus*.

In our study of the putative CF microbiota, only single or dual species were detected using microscopy and molecular methods (**VI, XIV, XV**), while few species other than *P. aeruginosa* and *S. aureus* were detected in wounds (**IV, V**).

The CF lung is end-stage when eligible for transplantation, so we further investigated the segregation of species in sputum from non-end-stage CF patients that were known to be infected with one to three bacterial or fungal species (**XV**). The microscopic results showed almost complete segregation of the different bacterial species within the sputum as seen in Fig. 20.

8.4.1. Succession or opportunity – It is apparent that chronic infections are often multispecies, but the majority of each aggregate predominantly consists of only one bacterial species per aggregate in multispecies infections. Different bacterial species can form sovereign aggregates in the same infection, but in different locations or niches. Thus, although environmental biofilms are referred to as multispecies, this does not apply to the biofilms found in chronic infections, with the exception of dental infections and foreign body infections that can communicate with a body surface that has a normal flora, for example, catheters or stents.

This difference in the bacterial organization of environmental biofilms and infection bio-

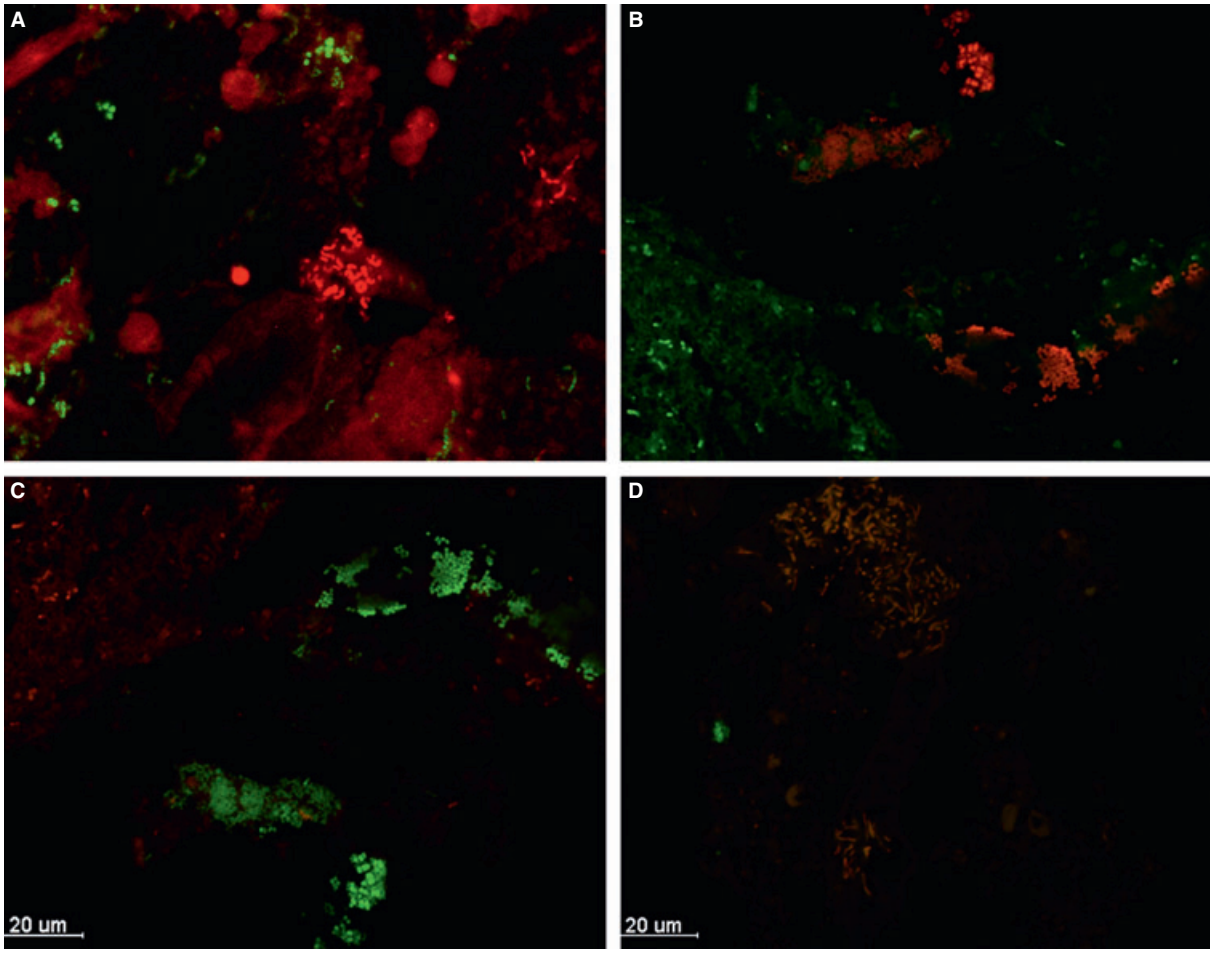

Fig. 20. Peptide nucleic acid-fluorescence *in situ* hybridization visualization of bacterial aggregates/biofilms in expectorated sputum from non-end-stage cystic fibrosis patients. Frames (A, C and D) show *Pseudomonas aeruginosa* (red) and other bacterial species (green) while frame (B) shows *Staphylococcus aureus* (red) and other bacteria (green). The images show that the biofilms of different species appear to be segregated from each other (adapted from **XV** with permission from the publisher).

films might be attributable to selection pressure from the body's defence mechanisms, which can eliminate many non-pathogenic bacteria, antibiotic therapy and the availability of nutrients and/or commensalism. The co-aggregations found in environmental locations could be explained by the sharing of beneficial metabolic intermediate compounds among different bacteria. Bacteria found in environmental consortia may have survived selection via synergistic exploitation of, and adaptation to, diverse niches, and the limited availability and/or capacity to utilize the available nutrients (319). This may have caused the high bacterial diversity and multispecies biofilms found in these environments (see section 5). This includes habitats that are directly created by certain bacteria and the species heterogeneity that is determined by the presence of the originating microorganisms. Thus, a complex succession takes place during the formation of these biofilms (320), including dental biofilms, which includes random bacterial settlement. This succession depends on early colonizers, increased competition among the species present and niche differentiation. This results in highly diverse and heterogeneous biofilms at the structural, resource, functional and taxonomic levels.

In infections, the order of appearance is completely different, i.e., the opportunity of being present. During infection processes, bacteria damage the skin/mucosa or penetrate a surface to invade a sterile body area where they induce damage/inflammation of the invaded tissue, i.e., disease. For these intruding or infecting, i.e., pathogenic or facultative pathogenic (opportunist) bacteria, the key challenge is to survive their encounter with the host defence system. The host defences are very efficient and not all bacteria can persist, especially the planktonic growth phenotype, unless they can protect themselves with capsules or toxins. This makes the encounter a very effective constraint on the bacterial diversity found in chronic infections. The initial phase of biofilm formation may be the crucial point in infections. Opportunistic pathogenic bacteria need to be in the right place at the right time to establish an infection by evading the host defence system. It is highly unlikely that another species will encounter a specific chronic biofilm infection, endure the already recruited host defence, and last but not least, merge with the aggregates of the already present bacteria. The initial opportunist would not persist in isolation if they required another species to do so. It seems unlikely that specialized consortia of bacteria will exploit an opportunity, except in areas such as the teeth, as mentioned earlier. It may be possible that dominant aggregates of different species occasionally interact with each other by signalling or other chemical interaction, but this needs to be investigated.

One may speculate that succession of biofilms in chronic infections is maintained at an evolutionarily early stage, which is equivalent to the initial stages of environmental biofilm consortia, rather than evolving from homogeneity to highly differentiated heterogeneity and niche differentiation.

Additional conditions may favour the growth of single species aggregates in chronic infections. Dead cells (both eukaryotic and prokaryotic) and a constant blood supply probably contribute to nutrient availability. This may impose a low selection pressure for coexistence and niche differentiation, i.e., a symbiotic relationship among different bacteria is not crucial for the growth and proliferation of a single bacterial species. Bacterial pathogenic colonization or infection of a host can result in host death or eradication, which may have prevented a specific bacterial chronic infection population or infectious microbiota from evolving in human beings, with the exception of the dental area.

Evolutionary studies show that the microbiota of the CF lung does not diversify over time, but instead the bacteria themselves become specialized within a species (321, 322). Evolutionary observations also suggest that bacteria such as *P. aeruginosa* do not make a transition from opportunists to primary pathogens in CF patients (i.e., the chronic phenotype) by means of toxin production, and instead they achieve a persistent state by evading the host defence mechanisms by alginate production. The body subsequently induces a pronounced immune response that leads to immune complex-mediated inflammation and tissue damage, i.e., indirect pathogenicity (**VI**).

However, visualization methods such as PNA FISH have limitations like other molecular methods – their relatively low sensitivity. Thus, it cannot be excluded that more than

one species may be present in an apparently monospecies aggregate, although they must be present in very low numbers or they must possess an activity level (and thereby rRNA content) lower than the detection limit of FISH-based detection. Thus, the possible low-level presence of another species of bacteria that produces very little rRNA suggests that they either have an insignificant role or no role.

8.4.2. Low diversity and high pathogenicity – Another interesting aspect of the single species phenomenon is that there appears to be an association between low diversity and high pathogenicity. In environmental biofilms, external factors may change that could possibly inhibit the growth of certain bacteria within the consortium, leading to a shift towards lower diversity. This might be a pH shift or the iron level in the soil, which could create a new niche allowing a plant pathogen to overtake the rhizosphere of a plant (323–325). In the colonizing biofilms found in humans, such as dental and intestinal biofilms, a shift in pH or nutritional state may produce new niches that allow potential pathogens to succeed and lead to pathogenicity. The development of dental caries is such an event, which is caused by a lowering of the pH in the oral cavity. Dental caries are probably a result of increased biofilm consumption of fermentable carbohydrates such as sucrose (193, 326). The healthy oral cavity usually has low numbers of *Streptococcus mutans* in the beneficial plaque, low numbers of lactobacilli and high numbers of *Streptococcus sanguinis* (327), as well as many other bacteria (193). In conditions of low pH, acid-sensitive bacteria such as *S. sanguinis* decrease whereas acidogenic and aciduric bacteria (*S. mutans* and lactobacilli) increase and competitive succeed in the microbiota. This shift also leads to increased acid production, which exacerbates the demineralization of teeth enamel, leading to caries. This succession of pathogens is known as the ecological plaque hypothesis, or a ecological catastrophe (191, 210, 328–330).

8.5. Diagnosing biofilms in infections

The aetiology of many chronic diseases has been difficult to elucidate, because the isolation of bacteria using standard culture method has often failed (**V, VII**) (224, 331, 332). It was proposed that bacteria adhering to surfaces, and those in biofilm in general, were unculturable because of the biofilm growth phenotype (224, 331–333). However, bacteria were readily detected by FISH and microscopy, and by PCR (273, 275). As biofilm research has developed, it has been realized that bacteria can attach to most available surfaces, such as foreign bodies, host tissues or other bacteria. A special feature of the biofilm growth phenotype that makes the bacteria in the biofilm unculturable is unlikely. First, any invading or 'displaced' bacteria must be viable, otherwise they would not multiply, spread and evoke an immune response, because if they did not, they would probably disappear and there would be no inflammation or tissue damage to indicate their presence. Second, Whiteley et al. showed that the transcriptomes of biofilm-producing bacteria are similar to those of planktonic growing bacteria, and any differences were mainly related to antibiotic tolerance (334). Furthermore, no genes or gene products related to biofilm growth have yet been identified that could lead to unculturability (41). This indicates that the problem of culturing is not that the bacteria found in biofilms are unculturable. The problem is that they have to be released from the surface, tissue or aggregate. The solution to this problem is simple. A biofilm-infected sample or object has to be treated appropriately using ultrasound (sonication) to release the bacteria, as vigorous vortexing or mechanical scraping have been shown to be insufficient for this purpose (335–339). Another potential difficulty is that most bacteria are unculturable due to growth media limitations or if the patient was treated with antibiotics before sampling, although most recognized pathogenic bacteria (with some exceptions, e.g., *Treponema pallidum*) do grow on standard growth media or in cell culture, as well as in the human body. It has been suggested that the few pathogenic bacteria that are unculturable on growth media are activated to grow when they grow together with eukaryotic cells (340). This was also emphasized by Sampedro et al. in an investigation of spinal implants (341). They showed that PCR and culturing was suitable for the detection of

bacteria on spinal implants, if they were present in the host environment in sufficient numbers to establish an infection (342).

The problems of diagnosing bacteria in chronic infections are far from being solved by sonication prior to culturing and PCR. As shown in Table 2, each of these methods has its advantages and limitations.

In all techniques, i.e., culturing, PCR, IBIS or microscopy, the major problem is the collection of the bacteria because of their heterogeneous distribution (**V**) (245, 253) and strong attachment to the surfaces of catheters and implants. One advantage of direct growth, and to some extent IBIS plexID (318), is that bacterial susceptibility to antibiotics can be investigated if they grow or are identified. However, a pitfall is that antibiotic susceptibility is performed on bacteria in their planktonic form and such tests can be irrelevant because of biofilm-enabled tolerance (16) (see section 4.1).

The advantages of PCR are that this method will detect bacteria independent of their growth. The major disadvantage is that PCR does not discriminate between live and dead cells. Furthermore, the detection of a bacterium by PCR does not necessarily indicate that it contributes to the pathogenesis of the infection because it may be a contaminant. Microscopy facilitates the direct visualization of the infecting bacteria, the surrounding tissue and inflammatory cells (**IV, V, VI, VII, VIII, XIII, XV**). A disadvantage of microscopy is that observation might prove difficult if only a few bacteria are present.

Specific criteria to aid the diagnosis of biofilm infections have been published in recent years. The first suggested criteria included: (i) bacteria adherent to a surface; (ii) direct observation of a bacterial cell cluster in a matrix; (iii) confined to a particular location; and (iv) failure of antibiotics to clear the infection, despite using concentrations that would kill bacteria in their planktonic state (343). These criteria were then re-evaluated to include: (i) only associated with a surface, but not firmly attached; (ii) a culture-negative result despite a clinically documented high suspicion of infection; and (iii) direct observation of protection against the host defences (344). This was further expanded by Høiby to include a distinction between acute and chronic infections (see Table 3) (16).

Only a few universal diagnostic molecular biofilm markers have been identified (345). Great efforts have been made, but a common difficulty is the heterogeneity of bacterial biofilms. This has also been empathized when designing vaccines against biofilms (345, 346).

A further problem associated with the difficulties of identifying bacteria comes with understanding the significance of the many microorganisms identified by increasingly sensitive molecular techniques.

The problem of bacterial detection using methods based on DNA/RNA, and even cul-

Table 2. Advantages and disadvantages of different methods used for identifying infectious bacteria in humans

Method	Advantages	Pitfalls and difficulties
Culturing	Bacterial presence is confirmed Antibiotic susceptibility Direct quantification	Heterogeneous distribution Finding the focus Pathogens vs contamination Biofilms or planktonic samples can be culture-negative
PCR and IBIS	Fast results even when culture is negative Low cut-off	Heterogeneous distribution Finding the focus Pathogens vs contamination Biofilm or planktonic
Microscopy	Biofilms are confirmed Interactions with tissues Inflammatory cells Results even when culture-negative	Heterogeneous distribution Finding the focus

Table 3. Some general features of biofilm infections in humans compared with acute planktonic infections and superficial colonization/normal flora found on the skin and mucosal membranes. Bold indicates biofilm-specific features.

Features of biofilm infections	Necessary condition for biofilm infection	Also found in acute planktonic infection	Also found in colonization/normal flora on skin and mucosal membranes
Aggregates of bacteria embedded in a self-produced polymer matrix	Yes	No	No/Yes
Tolerant of clinically relevant PK/PD* doses of antibiotics, despite the susceptibility of planktonic cells	Yes	No	No/Yes
Tolerant of innate and adaptive immune responses	Yes	No	No/Yes—unknown
Inflammation	Yes	Yes	No
Chronic infections	Yes	No	No
Foreign body-associated infections	No	Initial	No
Located on surfaces	No	Yes	Yes
Localized infection	Yes	Yes	Yes
Focus of spreading or local exacerbation	Yes	Yes	Yes

*PK/PD, pharmacokinetic/pharmacodynamic [adapted from (16)]

ture, is that a positive test result does not provide any information on pathogenicity or its absence. This makes it very difficult to discriminate between pathogens, passive bystanders and contamination. Contamination is a particular problem when using increasingly sensitive methods. This has been evident when mapping the CF microbiota, because the samples have to pass through the upper respiratory tract which is colonized with many different bacteria (191, 192, 347). We aimed to overcome this by investigating explanted lungs (**VI, XIV**), although this had the disadvantage that only end-stage microbiota could be investigated (see section 8.4).

In 1884, during his investigation of the aetiology of tuberculosis, Robert Koch proposed that:

> *...it is necessary to obtain a perfect proof to satisfy oneself that the parasite and the disease are not only correlated, but actually casually related, and the parasite is the actual direct cause of the disease. This can only be done by completely separating the parasite from the diseased organism, and from all of the products of the disease which could be subscribed to a disease-inducing influence, and then introducing the isolated parasite into healthy organisms and induce the disease a new with all its characteristic symptoms and properties.*

The dilemma of biofilm-related infections is that bacteria can be difficult to isolate and/or that numerous species are identified. This direct evidence has also been a problem with viral infections. In 1936, Rivers proposed that: (i) a specific virus must be found associated with a disease with a degree of regularity; and (ii) the virus must be shown to occur in the sick individual, not as an incidental or accidental finding, but as the cause of the disease under investigation (348). In the search for the causative agent of infectious mononucleosis, the Epstein–Barr virus was identified on immunological grounds alone, which showed beyond reasonable doubt that it was the agent (349–351). This led to the production of a set of factors to support immunological proof that an agent caused a specific disease (351).

The use of specific immune responses has also been used in the diagnostics of chronic infections of the CF lung (352, 353) and endocarditis (354, 355). In 1965, Hill proposed nine factors (see Table 4) for determining a possible causal relationship between an infectious organism and a specific disease.

These modified Koch's criteria were developed further for chronic infections in CF patients by Høiby (356).

Using these tools and guidelines in combination with relevant animal models, we may be able to distinguish between the causative agents (should be treated) of specific chronic

infections and irrelevant commensals (should not be treated). It is crucial to make this distinction to optimize the validity of new diagnostic methods such as IBIS (318), or we will accumulate an abundance of useless and misleading information.

9. DISCUSSION

The philosopher Søren Kirkegaard (see page 3) was disdainful of scientists who crave to dissect nature and explore our world and its surroundings to find true meaning, which is certainly thought-provoking. Is it necessary to explore everything? Is it necessary to distinguish between surface and non-surface-attached bacteria, and single and multispecies biofilms? The great explorers of our world several hundred years ago were curious, but they were also driven by the great rewards and fame that new explorations would provide. Today, as always, science is driven by the passion for fame and fortune, but also for new insights and progress that makes a difference, e.g., to patients. In the science of biofilms, it has been imperative for me to understand how to study biofilms, how they are formed, how they are detected and identified, and how they are treated when they cause an infection. To do this, I knew initially that I had to use a microscope to study the 'small animals' (bacteria) (5), pathogenic or commensal, and their detailed interactions, before applying other methods based on these microscopic observations. The classic example is Louis Pasteur (1822–1895) who observed and sketched the aggregations of bacteria that caused the acidification of wine, which led to his famous discovery of pasteurization.

The relevance of my biofilm research was stated by the National Institutes of Health's Program on Immunology of Biofilms (PA07-288), as follow:

> *One of the limitations of early approaches used to study infectious organisms in disease is that planktonic (pure freely suspended cultures of) bacteria were employed. Although much information on the immune response came from the study of planktonic bacteria, it is now clear that bacteria in the clinical environment live more often as communities of microorganisms (biofilms) than as single cell suspensions.*

My contribution to the growing field of biofilms may be divided into three categories. The main category is additional evidence that the biofilm growth phenotype is associated with chronic bacterial infections. The biofilm phenotype found in chronic infections seems to protect the bacteria, and biofilm formation could be explained using a 'united we stand – divided we fall' paradigm. The denominator of these chronic infections are an extreme tolerance and resistance to antimicrobial agents and a capacity to evade the host defences (**I, II, IV, V, VI, VII, VIII, IX, XI, XII, XIII**) (65). The second category is the study of biofilm-enabled antimicrobial tolerance (**III, X, XII**) (16, 44, 128, 357). Interestingly, my research has shown that biofilm-enabled tolerance of antibiotics is reversible, at least in model systems (**XII**). In theory, this means

Table 4. Hill's epidemiologic criteria for causal associations [modified from (350)]

Causal criterion	Causal association
Strength of association	What is the relative risk?
Consistency of association	Is there agreement among repeated observations in different places, at different times, using different methodology, by different researchers, under different circumstances?
Specificity of association	Is the outcome unique to the exposure?
Temporality	Does exposure precede the outcome variable?
Biological gradient	Is there evidence of a dose–response relationship?
Plausibility	Does the causal relationship make biological sense?
Consistency	Is the causal association compatible with present knowledge of the disease?
Experimentation	Does controlled manipulation of the exposure variable change the outcome?
Analogy	Does the causal relationship conform to a previously described relationship?

that chronic biofilm infections can be treated or prevented if the biofilm is dissolved or bacteria are actively prevented from aggregating. The third category is the capacity of biofilms to evade phagocytes such as PMNs. No solid barrier appears to protect the bacteria and the protection seems to be derived from excreted chemicals that kill, paralyse or divert phagocytes (**I, II, IX, XIII**) (125).

As with many other biofilm researchers, *P. aeruginosa* has been my preferred model organism for biofilm research. Based on our *ex vivo* observations, this was an appropriate choice for studying biofilm-related infections because it is involved in several chronic infections (**IV, V, VI**) (253), which have much in common. First of all, *in vitro* and *in vivo* experimental *P. aeruginosa* biofilms excrete rhamnolipids that provide protection from phagocytes and grazers (see section 4.2) (**I, IX, XIII**) (44, 125, 358). Plenty of evidence indicates that rhamnolipids also have this protective role in chronic human infections (359, 360). Evidence of this major role came from clinical isolates of *P. aeruginosa* that do not lose their capacity to produce rhamnolipids over decades in chronic infections, although many other toxins are lost (**XI**). PMN debris such as DNA and myeloperoxidase has also been observed surrounding aggregates of *ex vivo* samples of human infections similar to that observed in *in vitro* and *in vivo* model systems (**V, VI, XIII**). Interestingly, *P. aeruginosa* microcolonies are connected to a massive accumulation of PMNs, although the PMNs appear to be prevented from penetrating the microcolonies. This fatal attraction could be caused by the QS signal molecules of *P. aeruginosa*, because our *in vitro* experiments showed that they attract PMNs (**I**). Rhamnolipid production by *P. aeruginosa* is regulated by QS (see section 4.3), while QS regulation depends on the build-up of signal molecules produced by the bacteria. This is a cunning strategy whereby *P. aeruginosa* avoids the cellular components of the host defences by waiting for the build-up of a critical mass before producing its protection, the rhamnolipid (30). *Pseudomonas aeruginosa* then lures the approaching PMNs to their death. This leads to a chronic inflammatory condition, the continuous influx and dominant presence of neutrophils, and the efflux of intracellular degradation enzymes from the dead neutrophils, such as reactive oxygen species and matrix metalloproteases (MMPs) (361–363).

This is also our hypothesis of chronic wounds, where *P. aeruginosa* arrests the wound in a chronic inflammatory state (**IV**) (361–364). Many similarities exist between chronic infections of the CF lung and wounds, i.e., the persistent influx of PMNs, elevated MMPs and an imbalance of several cytokines (254, 299, 300, 365, 366). These observations may explain the previously reported impairment of host cells in chronic infections, and why the balance is tipped even further away from healing. A negative feedback loop might further increase the release of destructive enzymes from incoming PMNs.

Another interesting similarity between chronic wounds and CF is that the chronic *P. aeruginosa* infection is preceded by intermittent colonization/infection with other bacteria, especially *S. aureus* (304, 367, 368). Whether this is a real phenomenon or simply a coincidence is not yet known, but it might be speculated that bacteria such as *S. aureus* induce the host defences to a state that favours *P. aeruginosa* (synergism). Another possible factor affecting this phenomenon is that *S. aureus* does not present a therapeutic problem in CF and chronic wounds. It may be hypothesized that the eradication of *S. aureus* creates a vacuum and an opportunity for bacteria such as *P. aeruginosa*.

In fact, chronic infections appear to depend on a preceding compromising event, because healthy people do not appear to acquire chronic infections. First of all, the primary defences of the human body have to be compromised (see section 5.3). Second, a stochastic event such as a viral infection, stress, intoxication or acute infection is probably required to dampen or occupy the host response, thereby allowing bacteria to settle in protective biofilms. These events might be compared to the obstruction of a river, which at some point will cause flooding of the surrounding dry land, as shown in Fig. 21.

On the basis of my *in vitro* and *in vivo* observations of bacterial aggregates, I believe it is clear that bacteria aggregate as a default and that subsequent biofilm development progresses via adaptations to nutritional and environmental conditions.

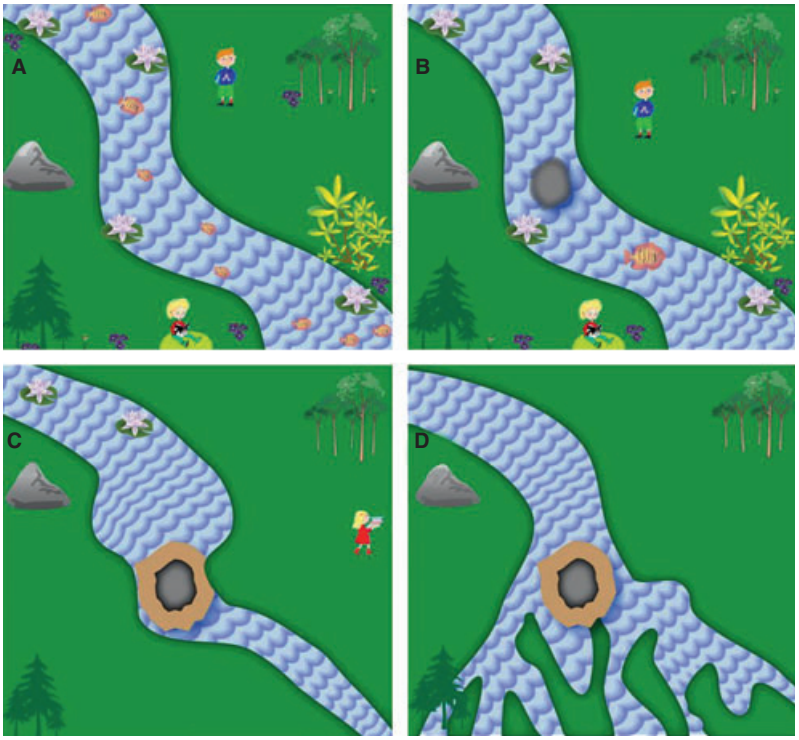

Fig. 21. Frame (A) shows the river in natural balance. Frame (B) is an event that compromises the primary defences (e.g., insertion of implants or catheters, or thickened mucus in the cystic fibrosis airway) as shown by the presence of a rock in the river. In itself, this event does not cause any persistent pathology, but bacteria are allowed to settle and persist on the rock (implant etc.) if natural predators (phagocytes = host defences) are impaired or sluggish as a consequence of pollution (viral infection etc.). A persistent biofilm is formed, Frame (C). This persistent biofilm eventually causes the river to flood (e.g., severe acute infections or impaired lung function), Frame (D). The only solution is to remove the rock, which symbolizes the removal of the catheter, amputation of limbs or the transplantation of organs to prevent the death of the patient.

As stated previously, bacterial biofilms exist in environmental ecological habitats and opportunistic ecological habitats, such as infections. The lifestyle is the same, except that infectious biofilms are formed in places where a preceding microbiota was absent so the fight for survival and supremacy is different. The consequence is that chronic infections are dominated by monospecies biofilms, although the infections are most likely multispecies, whereas environmental biofilms are dominated by multispecies (**V, XIV, XV**) (1, 217, 239, 240, 245, 253).

10. CONCLUSION

My goal many years ago was to elucidate the role of biofilms in chronic infection. I have studied biofilms in infections and in the laboratory, how they are formed, how they persist with antibiotics, how to diagnose them and how they can be treated. I soon discovered that, like all other natural phenomena and processes, biofilms appear very difficult and complex when viewed from a distance. However, my microscopic examinations of biofilms have shown that all the interactions and pathways are simple. The biofilm era has been dominated by a tendency to complicate the processes of biofilm formation, regulation and protection. However, based on my *in vitro* and *in vivo* observations of aggregating bacteria, I believe that biofilm formation and persistence is less complex than postulated in the past, although we still have many unanswered questions. I propose a simplistic view of biofilms based on a 'united we stand – divided we fall'

paradigm, much like the flocking behaviour of starlings when protecting themselves from raptors as seen in Fig. 22.

11. FUTURE PERSPECTIVES

Although I propose that biofilms can be as simple as one on top of another, we have many unanswered question.

Much evidence has been accumulated showing that chronic infections involve the biofilm growth phenotype; whereas it is assumed that acute infections involve the planktonic growth phenotype. It will be important to elucidate the role of the preferred lifestyle that bacteria choose in acute infections compared with chronic infections in specific environments. The switch between planktonic and biofilm lifestyles should be elucidated to map this interplay.

As with my river analogy (Fig. 21), it will be interesting to investigate how bacterial aggregation is initiated, how chronic infections are initiated and how the infection proceeds. To understand these events, we need to estimate the minimum number of bacterial cells present in an aggregate that is required to initiate the development of a localized, chemical and physiological microenvironment, which I assume is present within a biofilm.

Subsequently, I would like to elucidate the social interactions in biofilms. In my work presented in this thesis, I have mainly identified single species biofilms (one bacterial species per aggregate) during infectious processes. This contrasts with the multitude of reports stating that infections are often multispecies, i.e., several bacterial species identified within one infection, such as those seen in dental biofilms, for example, plaques and periodontitis. I have only rarely observed multispecies biofilms (several bacterial species mixed in the same aggregate) (**IV, V, VI, VIII, XV**) (253). It will be interesting to observe whether these few multispecies biofilms really function as a consortium containing several different bacterial species 'working' together, or whether they just 'happen' to be adjacent to each other.

At the micro-scale level, I wonder if this is comparable to a large city, where it appears that all the people live together when viewed from above, but a closer look reveals that they are living separately in small 'boxes'. Are there 'compartments' in which the most closely related individuals form groups, as illustrated in the fairytale 'The drop of water' by Andersen HC (see Fig. 23).

Another area of investigation should be to determine whether single species biofilms collaborate in a multispecies infection.

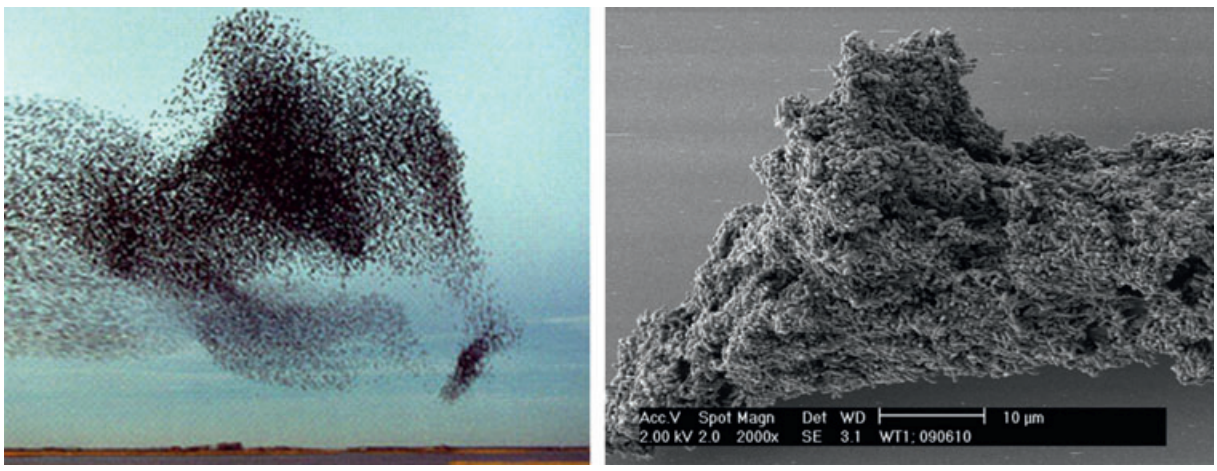

Fig. 22. The left frame shows starlings flocking to protect themselves from raptors, while the right frame shows *Pseudomonas aeruginosa* forming a 'flock' or biofilm to protect themselves from antibiotics, phagocytes and grazers.

These perspectives are more or less basic science. The main challenge will be to identify bacteria that have a role and to exclude those that have none. It will also be an ongoing challenge to develop tools for the rapid and efficient diagnosis of chronic infections. No one has yet identified a molecule or product that is produced by all the bacteria within a biofilm, not to mention by all bacteria. In CF patients with chronic *P. aeruginosa* lung infections, the presence of alginate in the sputum (369) and the growth of the mucoid phenotype indicates biofilm infection (80). Another approach could be to identify a host-produced marker such as antibodies or interleukins. The problem is that most chronic infections evoke a localized inflammatory response that might not be measurable in the circulating blood. Until now, a general antibody response has been very useful for detecting *P. aeruginosa* biofilm infections in the lungs of CF patients (352, 370–373), while specific antibodies to alginate can also be used (78, 374) in endocarditis (354, 355).

Finally, great benefits will be derived from an optimized and efficient treatment regime for biofilm infections. If mechanical disruption of the biofilm at the site of infection was possible, local high concentrations of antibiotics would probably be effective. Thus, matrix-degrading enzymes could prove useful, again in combination with antibiotics, as seen with the DNase treatment of CF patients (375). Another approach may be the use of anti-virulence drugs, such as drugs that inhibit QS regulation, which could inhibit the production of virulence factors, for example, rhamnolipids. This approach is actually used in CF patients where azithromycin fulfils these criteria (376). Directly targeting virulence factors with neu-

Fig. 23. The drop of water magnifies the individuals in the picture of Copenhagen as I have magnified the bacteria in biofilms by microscopy. Picture reproduced with permission from the Museum of Copenhagen and Marianne Bisballe.

tralizing agents such as antibodies is yet another possibility, although this may not be useful because CF patients with chronic *P. aeruginosa* lung infection produce neutralizing antibodies for many toxins produced by these bacteria (374, 377–379).

A completely opposite approach would be to prevent bacteria from settling into biofilms or killing their planktonic phenotype before they switch to the protective biofilm phenotype. This strategy has already proven its worth for many years, because the early aggressive treatment of lung infections in CF patients has postponed the onset of chronic infections and prolonged the life of CF patients by decades (263, 264, 299, 300, 302, 304, 380, 381).

One thing that seems certain is that although we have started to unravel the mechanisms behind the biofilm phenotype, we have just scratched the surface.

CONFLICT OF INTEREST

The author declares no conflicts of interest.

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13. SUMMARY IN DANISH (DANSK RESUMÉ)

Akutte infektioner forårsaget af patogene bakterier har været studeret dybtgående i mere end 100 år. Disse infektioner dræbte millioner af mennesker i de forrige århundreder, men kan nu bekæmpes effektivt med udviklingen af de moderne vacciner, antibiotika og forbedret hygiejne. Mens den meste forskning inden for bakteriel patogenese har fokuseret på de akutte infektioner, er betydningen af disse sygdomme nu suppleret med en ny kategori af kroniske infektioner forårsaget af bakterier som vokser i slim-indkapslede aggregater, også kendt som biofilm. Biofilminfektioner såsom lungebetændelse hos patienter med cystisk fibrose, kroniske sår, kronisk mellemørebetændelse og infektioner på implantater og katetre, påvirker millioner af mennesker i den udviklede verden hvert år, mange med dødsfald til følge. Dybest set, kan bakterier optræde i to livsformer ved vækst og formering. I den ene form, optræder bakterierne som enkelte uafhængige celler (planktoniske), og i den anden form er bakterierne organiseret og fikseret i aggregater. Den sidstnævnte form bliver almindeligvis benævnt som biofilmvækstfænotypen. Akutte infektioner antages at involvere planktoniske bakterier og kan normalt behandles med antibiotika, men en succesfuld behandling afhænger af en præcis og hurtig diagnose. I de tilfælde, hvor det lykkes bakterierne at danne en biofilm inde i et menneske, viser infektionen sig oftest at være uhelbredelig og vil udvikle sig til en kronisk tilstand. De vigtige egenskaber ved kroniske, biofilmbaserede infektioner er en ekstrem resistens over for antibiotika, samt en række andre konventionelle antimikrobielle stoffer og en ekstrem evne til at undgå

værtens immunforsvar. I denne afhandling vil jeg samle den aktuelle viden om biofilm med vægt på kroniske infektioner, samt retningslinjer for diagnose og behandling af disse infektioner, og relatere dette til min seneste forskning inden for biofilmområdet. Jeg vil fremlægge beviser til støtte for det synspunkt, at biofilmfænotypen dominerer kroniske bakterielle infektioner, at bakteriernes sammenklumpning er en naturlig egenskab, og at den efterfølgende udvikling af biofilm sker som en tilpasning til de ernæringsmæssige og miljømæssige forhold. Jeg vil lave en række sammenligninger med det formål at fremhæve, ud fra mit synspunkt, de vigtigste aspekter af biofilm og hvad der skal udledes af de sidste årtiers forskning i biofilm. Jeg vil forsøge at bygge bro mellem in vitro- og in vivo- forskning og foreslå, hvordan man kan studere biofilm med denne viden i tankerne. Jeg vil sammenligne hvordan bakterielle biofilm findes både i økologiske stabile miljøer og opportunistiske økologiske ustabile miljøer, såsom infektioner. Bakterierne deler samme livsstil (biofilm) i begge habitformer, men kampen for overlevelse og for overherredømme er anderledes. Baseret på dette vil jeg også forsøge at forudsige og illustrere, hvordan kroniske biofilminfektioner opstår, og hvordan bakterier lever sammen i infektioner. Endelig vil jeg diskutere forskellige aspekter af diagnosticeringen af biofilminfektioner. Denne gennemgang af viden og de efterfølgende retningslinjer kan forhåbentlig danne grundlag og inspiration for meget mere forskning og for forbedret diagnostik og behandling af de accepterede biofilminfektioner og muligvis også for de infektioner, som vil blive identificeret som biofilminfektioner i fremtiden.