MINIREVIEW

Biofilms in chronic infections – a matter of opportunity – monospecies biofilms in multispecies infections

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Abstract

It has become evident that aggregation or biofilm formation is an important survival mechanism for bacteria in almost any environment. In this review, we summarize recent visualizations of bacterial aggregates in several chronic infections (chronic otitis media, cystic fibrosis, infection due to permanent tissue fillers and chronic wounds) both as to distribution (such as where in the wound bed) and organization (monospecies or multispecies microcolonies). We correlate these biofilm observations to observations of commensal biofilms (dental and intestine) and biofilms in natural ecosystems (soil). The observations of the chronic biofilm infections point toward a trend of low bacterial diversity and sovereign monospecies biofilm aggregates even though the infection in which they reside are multispecies. In contrast to this, commensal and natural biofilm aggregates contain multiple species that are believed to coexist, interact and form biofilms with high bacterial and niche diversity. We discuss these differences from both the diagnostic and the scientific point of view.

Introduction

It has been clear for decades that some chronic bacterial infections are caused by the ability of bacteria to organize themselves into matrix-enclosed aggregates or microcolonies, also termed biofilms (Donlan & Costerton, 2002; Hall-Stoodley *et al.*, 2006; Bjarnsholt *et al.*, 2008, 2009a, b; James *et al.*, 2008; Kirketerp-Moller *et al.*, 2008; Homoe *et al.*, 2009). Biofilms can be defined as *A coherent cluster of bacterial cells imbedded in a biopolymer matrix, which, compared with planktonic cells, shows increased tolerance to antimicrobials and resists the antimicrobial properties of the host defense*. As stated in this definition, bacteria living in biofilms are very well protected against antibiotics and other antimicrobial agents as well as the host defense, and they thereby become extremely difficult or impossible to eradicate (Fux *et al.*, 2003; Bjarnsholt *et al.*, 2005; Alhede *et al.*, 2009; van Gennip *et al.*, 2009). The classic example of biofilm involvement in chronic infections is *Pseudomonas aeruginosa* in the lungs of patients suffering from cystic fibrosis (CF) (Bjarnsholt *et al.*, 2009a). Because of the bacterial organization in biofilm, this chronic infection is noncurable and eventually results in the death of CF patients (Koch & Høiby, 1993). The severe consequences of biofilm involvement in infections have resulted in a focused effort aimed at identifying whether biofilms are involved in other conditions, some of which have not even been associated previously with bacterial infection. Additionally, it has not been clear which bacterial species were causing the infection, as scrapes or swabs are often culture-negative, possibly due to the strong association of bacteria with each other or to their unculturability. In contrast to these newly discovered infection-

associated biofilms are the endogenous or commensal biofilms in the human body (e.g. the intestine and the oral cavity), whose bacterial diversity and function have been intensely studied for decades. These biofilms are complex due to their high bacterial diversity, but nevertheless, many of the bacteria present have been identified and the succession pattern has been elucidated. Likewise, the role and function of bacterial consortia in natural ecosystems have long been recognized and they may, in many cases, resemble the commensal biofilms in the human body with respect to complexity.

Here, we summarize the recent advances in biofilm research in chronic infections, commensal biofilms and in natural ecosystems. We provide examples of chronic infections where the involvement of bacterial biofilms was just recognized recently and also of well-known biofilm infections where the newest *in situ* detection and identification techniques point toward a trend of low bacterial diversity and sovereign monospecies biofilms, although the infections are multispecies. These observations are in striking contrast to those of commensal and natural biofilms, where multiple species are believed to coexist, in the same biofilm aggregate, interact and form biofilms with high bacterial and niche diversity. We discuss these differences and raise the questions that need to be answered in order to better understand the bacterial organization in various types of biofilms – answers that we believe are important for approaching a new level in biofilm research.

Opportunistic biofilm infections

Here, we define opportunistic biofilm infections as chronic infections in otherwise sterile locations of the human body. As it will become evident later in this review, opportunistic biofilm infections may also occur if the commensal flora is challenged by intruding bacteria disrupting the beneficial ecosystem.

Chronic otitis media

Chronic suppurative otitis media (CSOM) (middle ear infection) without tympanostomy tube insertion is characterized by recurrent chronic suppuration succeeded by varying lengths of silent dry periods. CSOM occurs in patients with chronic otitis media with dry perforations (COM) or following episodes of acute otitis media (AOM) where treatment fails or is not instigated. Once CSOM is established, the disease can often be recalcitrant and difficult to treat. CSOM is often caused by polymicrobial aerobic and anaerobic bacteria.

Common aerobic bacteria found in CSOM, such as *P. aeruginosa*, *Escherichia coli* and *Staphylococcus aureus* and also other pathogenic bacteria including for example pneumococci and *Haemophilus influenzae*, are all known to be potential biofilm producers. During the last decade, biofilm has been morphologically demonstrated experimentally and clinically in different chronic middle ear infectious diseases. The first demonstration was carried out experimentally in the middle ear of chinchillas with chronic otitis media with effusion (COME) (Swords *et al.*, 2004; Jurcisek *et al.*, 2005; Reid *et al.*, 2009) and later directly in human clinical samples of mucosal surface lining from children with COME and with recurrent acute otitis media (rAOM) (Rayner *et al.*, 1998; Post, 2001; Hall-Stoodley *et al.*, 2006). COME is a middle ear disease often seen in small children. Also, biofilm has been found in human cholesteatoma, which is another chronic middle ear disease, as well as in experimentally induced cholesteatomas in gerbils (Chole & Faddis, 2002). Biofilm is frequently found on implanted medical devices and prostheses and has been found on postotorrhea tympanostomy tubes and most recently on a human cochlear implant (Saidi *et al.*, 1999; Bothwell *et al.*, 2003; Pawlowski *et al.*, 2005). Recently, we investigated whether biofilm formation is morphologically present in a high-risk CSOM population from Greenland (Homoe *et al.*, 2009). Using Gram-staining, peptide nuclear acid (PNA) FISH analyses and microscopy, we found morphological evidence of biofilm in otorrhea in five of six (83%) children with CSOM and morphological evidence of biofilm in the mucosa of biopsies from the middle ear in eight of 10 (80%) adults operated for CSOM (Homoe *et al.*, 2009) (see Fig. 1a). These findings have now been confirmed in a study of humans with CSOM in the United States (Lee *et al.*, 2009).

Thus, biofilm has been found in several chronic infectious middle ear diseases (Bakaletz, 2007). However, the potential pathogenic role of biofilm in these diseases and the cause and relationship still remain to be elucidated.

Tissue fillers

The use of tissue fillers for esthetic purposes has become increasingly common, and complications following these have accordingly been reported with increasing frequency. Up to a few years ago, it was assumed that these reactions were mainly caused by an autoimmune or an allergic reaction to the injected gel filler, but recent studies have revealed that adverse reactions following the injection with polyacrylamide gel are caused by bacterial infection (Christensen *et al.*, 2005; Christensen *et al.*, 2006; Bjarnsholt *et al.*, 2009b; Christensen, 2009). This consensus, however, was reached with difficulty. Clinical symptoms were unconvincing, cultures taken from the affected site were in general negative and antibiotics such as penicillins were insufficient in curing the reactions (Christensen *et al.*, 2006). It was only when the lesions were treated with steroids or large doses of nonsteroid anti-inflammatory drugs to quench the presumed autoimmunity that abscesses and fistulas appeared,

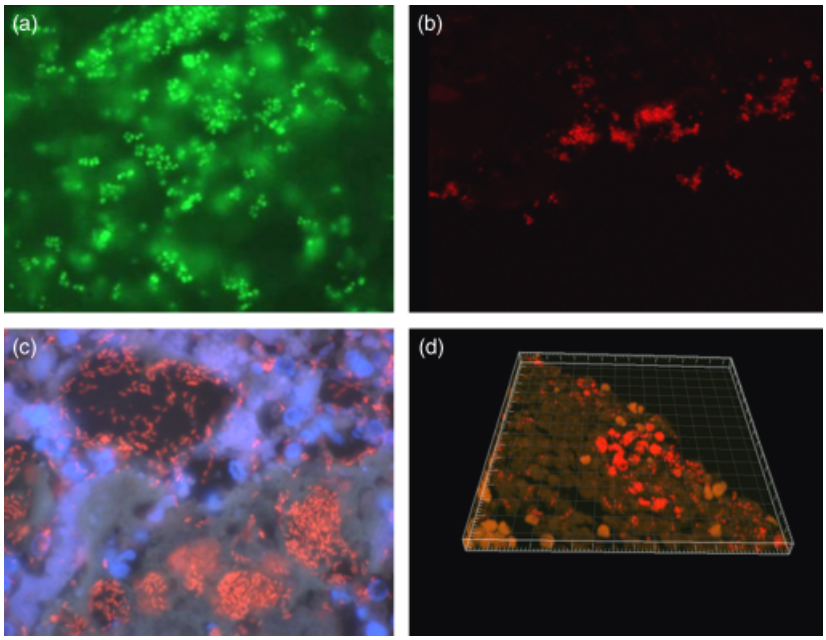


Fig. 1. PNA FISH visualization of bacterial biofilms in different chronic infections. (a) *Staphylococcus aureus* biofilms in otorrhea from a patient with CSOM (Homoe *et al.*, 2009). (b) Unspecific biofilm bacteria in the facial region from a patient injected with a permanent tissue filler (Bjarnsholt *et al.*, 2009b). (c) *Pseudomonas aeruginosa* biofilms in the lung from a patients with cystic fibrosis (Bjarnsholt *et al.*, 2009a). (d) *Pseudomonas aeruginosa* biofilm in a wound from a patient with a chronic leg ulcer (Kirketerp-Moller *et al.*, 2008). All pictures are reprinted with permission from the respective publishers.

and infection was suspected in spite of the above-mentioned negative results (Christensen *et al.*, 2005, 2006). In these cases, ordinary light microscopy showed an amplified foreign-body response, but it also showed the presence of a few polymorphonuclear leukocytes (PMNs) and scattered Gram-positive microorganisms (mainly cocci) (Christensen *et al.*, 2005, 2006), the nature of which was further established by PCR analysis on tissue sections and homogenates (Christensen *et al.*, 2005, 2006). For identification of the bacteria and their positioning within the tissue biopsies, a combination of Gram stain and PNA FISH was performed. Bacteria were detected in biopsies from seven out of eight patients (88%). They inhabited gel and intervening tissue and tended to lie in aggregates, suggesting a biofilm environment (Bjarnsholt, 2009b) (see Fig. 1b). This explains their resistance to antibiotic treatment and supports the assumption that infection with bacteria in aggregates is the cause of culture-negative, steroid-amplified late adverse reactions to polyacrylamide gel.

Cystic fibrosis

Since 1976, considerable success in treating CF patients, suffering from chronic *P. aeruginosa* lung infection, has been gained by intensive treatment using high concentrations of antibiotics at the Copenhagen CF Centre. This was initially done by routine intravenous anti-*P. aeruginosa* treatment for 14 days, every 3rd month. Since 1987, daily inhalation of antibiotics has been added. Before 1976, only 50% of the CF patients would survive 5 years of chronic *P. aeruginosa* lung infection. Today, most CF patients survive for decades with chronic *P. aeruginosa* infection.

Despite intensive treatment of chronic *P. aeruginosa* infections, the bacteria persist. The intensive treatment postpones and reduces the damage caused by the chronic infection, but cannot eradicate it. During chronic *P. aeruginosa* infection, CF patients experience a continuous degradation of lung tissue. This is caused in part by the infection and in part by the inflammatory processes. The consequence is a decline in the lung function, which is the primary cause of death in CF patients.

We have recently evaluated the orientation and distribution of *P. aeruginosa* in the conductive and respiratory zones of the lungs of chronic *P. aeruginosa*-infected CF patients using PNA FISH (Bjarnsholt *et al.*, 2009a). Despite the use of a universal eubacterial PNA FISH probe (designed to detect all bacteria) in combination with a specific *P. aeruginosa* PNA FISH probe, *P. aeruginosa* was the only detected bacterium in this study.

In explanted lungs from aggressively treated CF patients, the bacteria were mainly localized in the conductive zone (the upper part of the lung with large and small bronchi) (Fig. 1c). All of the bacteria were imbedded in mucus plugs. The mucus plugs containing bacteria varied considerably in size and spatial orientation. The bacteria within the mucus were dominated by aggregates and only a few planktonic bacteria were detected. A vast number of PMNs surrounded the aggregates. The bacteria were not found adhering to the epithelial wall, demonstrating that the bacteria grew within the mucus and not in or on the lung tissue. Using specific antibodies against alginate, we observed a majority of

mucoid *P. aeruginosa* aggregates intraluminally in the conductive zone, together with a few possible nonmucoid planktonic bacteria.

In the respiratory part of the lungs (the lower part – the alveoli), we observed both dead alveoli and air-filled healthy alveoli. Relatively few bacteria were detected in the respiratory part of the lungs as compared with the conductive part. We found both a few single planktonic cells and small aggregates in the respiratory zone; however, the planktonic bacteria always appeared phagocytosed by PMNs and the aggregated bacteria were closely surrounded by PMNs.

When examining lungs from the nonintensively treated chronically infected CF patients (autopsies from before the aggressive treatment was initiated in Denmark), we observed a different distribution of bacteria. Here, the bacteria in the conductive zone were also found inside the mucus surrounded by an abundance of PMNs, with no bacteria adhering to the airway epithelia. In striking contrast to the explanted lungs, the alveoli of the respiratory zone were filled with aggregating bacteria and PMNs. Most of the lung tissue was extensively destroyed probably caused by the heavy infection and inflammation. Using the specific stain and antibodies against alginate, we detected an abundance of mucoid *P. aeruginosa* aggregates in these nonintensively treated chronically infected patients.

We believe that the present intensive antibiotic therapy of chronic *P. aeruginosa* infections, at the Copenhagen CF Centre, restrains bacteria to the conductive zone, but they are not eradicated. The remaining healthy respiratory zone appears to be protected from massive biofilm infection for a long period. This strongly suggests that the conductive zone serves as a bacterial reservoir where the bacteria are organized in mucoid biofilms within the mucus, protected against antibiotics and host defenses.

Chronic wounds

The increase in obesity worldwide has been followed by a similar increase in diabetes and cardiovascular diseases. Such patients are particularly prone to the development of chronic wounds, which are subject to colonization by a number of bacterial species. In Denmark and in the United States, it has been estimated that 1–2% of the populations, respectively, have a nonhealing wound (Gottrup, 2004). Therefore, chronic wounds have become a burden to the health care systems and the patients undergo suffering, loss of employment and reduced quality of life.

At present, there exists some controversy as to whether opportunistic pathogens play a role in the delayed healing of chronic wounds (Bjarnsholt *et al.*, 2008; Gottrup *et al.*, 2009). This is despite the fact that all studies find the deep dermal tissues of all chronic wounds to harbor multiple bacterial species (Gjodsbol *et al.*, 2006; Dowd *et al.*, 2008; James *et al.*, 2008) (see the next section). Gjodsbol *et al.* (2006) found that more than half of the chronic wounds investigated in their study were colonized with *P. aeruginosa*. Furthermore, the *P. aeruginosa* infected wounds appeared to be significantly larger in terms of area than the wounds that did not contain *P. aeruginosa*. The presence of *P. aeruginosa* also seems to delay or even prevent the healing process (Halbert *et al.*, 1992; Madsen *et al.*, 1996; Gjodsbol *et al.*, 2006).

We have analyzed sections from chronic wounds by PNA FISH and found distinct biofilms (Bjarnsholt *et al.*, 2008; James *et al.*, 2008; Kirketerp-Moller *et al.*, 2008; Fazli *et al.*, 2009) (see Fig. 1d). We have also recently shown that *in vitro* and *in vivo* biofilms of *P. aeruginosa* produce a shielding of excreted rhamnolipids, which offers protection from the bactericidal activity of PMNs (Bjarnsholt *et al.*, 2005; Jensen *et al.*, 2007; Alhede *et al.*, 2009; van Gennip *et al.*, 2009). We propose that this shielding arrests the wound in a chronic inflammatory state, which corresponds to the findings in CF: persistent influx of PMNs, elevated matrix metalloproteases (MMPs) and imbalance of several cytokines (Bjarnsholt *et al.*, 2008). Additionally, it appears that *P. aeruginosa* have accumulated in biofilms at certain locations in wounds. Such biofilms are capable of producing the PMN-eliminating rhamnolipid, which in turn would reduce the number of functional PMNs at the present locations. This would explain the previously reported impairment of the host cells in chronic infections, which might tip the balance even further away from healing, and in a negative feedback loop, causes a further increase in the production of MMPs from the incoming PMNs.

It is well known that bacteria such as *P. aeruginosa* are almost impossible to eradicate from chronic wounds by the use of antibiotics. In addition to this, we recently tested different wound dressings containing silver salts as an antimicrobial agent, which can eradicate planktonic bacteria, but interestingly, none of these contained enough silver to eradicate biofilm bacteria *in vitro* (Bjarnsholt *et al.*, 2007).

We hypothesize that the presence of *P. aeruginosa* in biofilms and the lack of concomitant elimination by attended PMNs are the main causes of inefficient eradication of bacteria by antibiotic treatment and antimicrobial activity of the innate immune system, respectively.

Additional chronic biofilm infections

Other chronic infections that have been linked to the biofilm phenotype, but not described in detail here are osteomyelitis (Brady *et al.*, 2008), rhinosinusitis (Perloff & Palmer, 2004), urinary tract infections (Connell *et al.*, 1997) and all types of infections associated with foreign bodies inserted into the human body (Trampuz & Zimmerli, 2008).

Biofilms in wounds – representative for other chronic infections

Diagnostics

The bacteria associated with chronic wounds have usually been investigated using culture-dependent methods by taking a swab or a biopsy from the wound and using it as an inoculate for various bacterial cultures. The emergence of molecular biology methods and improved sampling techniques has illustrated that the traditional culture-dependent methods often underestimate the bacteria present, and especially in wounds with slow, fastidious or anaerobic biofilm-growing bacteria (Hill *et al*., 2003; Davies *et al*., 2004; Andersen *et al*., 2007; James *et al*., 2008).

The predominant microorganisms in chronic wounds include various anaerobes, *Staphylococcus*, *Corynebacterium*, *Pseudomonas*, *Serratia* and a previously uncharacterized *Bacteroidales* (Wolcott *et al*., 2009; Thomsen *et al*., 2010). In one study, an average of 5.4 species was found in each wound by a combination of molecular methods and cultivation (Thomsen *et al*., 2010).

Different molecular techniques have been used for the identification of bacteria in chronic wounds: fingerprinting techniques (Davies *et al*., 2004; Andersen *et al*., 2007; Dowd *et al*., 2008; Thomsen *et al*., 2010), the 16S rRNA gene cycle (Hill *et al*., 2003; Thomsen *et al*., 2010), FISH (Kirketerp-Moller *et al*., 2008; Fazli *et al*., 2009) (Thomsen *et al*., 2010), pyrosequencing and metagenomics (Dowd *et al*., 2008; Leake *et al*., 2009; Price *et al*., 2009; Wolcott *et al*., 2009) and quantitative PCR (Q-PCR) (Wolcott & Dowd, 2008; Frankel *et al*., 2009; Leake *et al*., 2009; Thomsen *et al*., 2010).

Sequencing of 16S rRNA genes selected on the basis of denaturant gradient gel electrophoresis profiling by Davies *et al*. (2004) allowed the identification of strains that are not detected by cultural means. Interestingly, 40% of the sequences represented microorganisms that could not be cultured from the wound from which they were amplified.

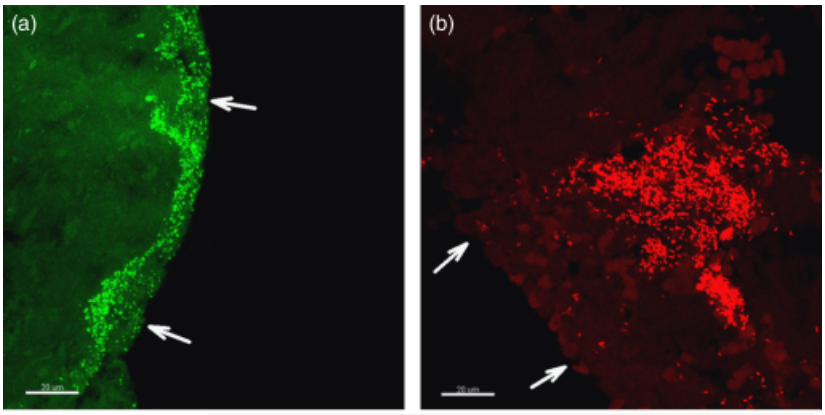
Q-PCR is a promising method for fast characterization of the bacteria present in chronic wounds and was used to establish that the numbers of *S. aureus* and *P. aeruginosa* varied considerably between samples taken at different locations in the same wound (Thomsen *et al*., 2010) (see Fig. 2c). Differences in bacterial populations across the surface of the wounds were also found in several studies (Fazli *et al*., 2009; Wolcott *et al*., 2009) and highlight the importance of sampling techniques during diagnostics.

The Q-PCR methods used for wounds in Leake *et al*. (2009) are rapid and can be run within a few hours, but are limited to major wound-associated bacteria and yeasts. The pyrosequencing methods take 24 h to return results, but are able to detect the relative contribution of any bacteria or yeast in a chronic wound diagnostic sample (Leake *et al*., 2009). The 16S rRNA gene-based PCR methods (Q-PCR and pyrosequencing) provide information on the presence, prevalence and the type of bacterial species in chronic wounds. However, a bias could be the possible detection of DNA from dead (nonviable) bacteria and the techniques fail to yield data on the structural organization and spatial distribution of these bacteria.

Visualization, organization and distribution

To better understand the relationship between the presence of particular bacteria in chronic wounds and the disease pathogenesis, it is necessary to not only identify the present bacteria but also to visually characterize the bacterial communities that exist in these wounds. FISH performed with a species-specific PNA probe in combination with a PNA probe detecting all eubacterial species represents a powerful technique for the rapid detection and identification of bacteria, and for the determination of their distribution and structural organization in clinical samples. Because of their uncharged chemical backbone, PNA probes possess unique hybridization characteristics, including rapid and stronger binding to complementary targets compared with traditional DNA probes (Egholm *et al*., 1993). We used PNA-FISH in conjunction with confocal laser scanning microscopy (CLSM) to investigate the structural organization and spatial distribution of bacteria in chronic wounds (Kirketerp-Moller *et al*., 2008; Fazli *et al*., 2009). We found that the bacteria existed in aggregates enclosed in a self-produced extracellular polymeric matrix within the wounds, and the depth of the location of these aggregates was correlated to the depth of the wound bed. The aggregates were mainly composed of single-species bacteria, either *P. aeruginosa* or *S. aureus*. Although the microbial communities of chronic wounds was shown to be polymicrobial (see the previous section), in our investigations, the observation of sovereign multiple-species biofilm aggregates in the wounds was rare, even though several bacterial species were present in the same wound (Kirketerp-Moller *et al*., 2008). In a following study, the analysis of images obtained from a large collection of wound samples by PNA FISH and CLSM revealed that a nonrandom distribution pattern of bacteria existed in the wounds, where *S. aureus* was primarily located close to the wound surface and *P. aeruginosa* was primarily located deeper in the wound bed (see Fig. 2a and b). A detailed quantitative analysis of the distance of bacterial aggregates to the wound surface showed that the aggregates of *P. aeruginosa* were located significantly deeper in the wound bed than those of *S. aureus* (Fazli *et al*., 2009).

This particular distribution of *P. aeruginosa* and *S. aureus* may explain the underrepresentation of *P. aeruginosa* and overrepresentation of *S. aureus* in chronic wounds by



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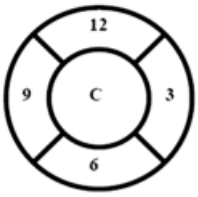

	Method	*S. aureus* Wound E	*S. aureus* Wound F	*P. aeruginosa* Wound E	*P. aeruginosa* Wound F
C	q-PCR	89±11%	200±13%	510±18%	920±9%
	Cultivation			+	
	DGGE	+	+		
3	q-PCR	No sample	86±8%	No sample	300±13%
	Cultivation				
	DGGE		+		
6	q-PCR	240±10%	290±8%	760±7%	8200±8%
	Cultivation			+	
	DGGE	+	+		
9	q-PCR	310±13%	80±5%	47±9%	800±10%
	Cultivation			+	
	DGGE	+	+		
12	q-PCR	180±8%	93±12%	280±3%	15±5%
	Cultivation			+	
	DGGE	+	+		

Fig. 2. (a and b) show the PNA FISH visualization of the different distribution patterns observed for (a) *Staphylococcus aureus* and (b) *Pseudomonas aeruginosa* (Fazli *et al.*, 2009). Schemes below (a and b) show the heterogenous distribution of bacteria in chronic wounds measured by qPCR. (c) The circle illustrates how the sample was divided. Wounds E and F indicate the two different wounds analyzed. Arrows point to the wound surface. Scale bars, 20 μm. Reprinted with permission from the publisher.

conventional culturing of wound swab samples (Kirketerp-Moller *et al.*, 2008), which detects the bacteria that are associated with the wound surface, but may not detect the bacteria that are located inside the wound bed. Conventional cultivation of wound swab as well as biopsy sample in combination with molecular techniques may provide a broader picture of bacterial species that reside in chronic wounds.

Many possible candidates for the important pathogens in chronic wounds have now been identified; the next step is to categorize the true pathogens and develop optimal sampling, identification and treatment regimes.

Natural biofilms

In this review, we define natural biofilms as ecosystems of aggregating bacteria present in their natural habitat having an essential function. This can be aggregating bacteria in commensalism with the human body without causing disease or in nature like the soil. This is in contrast to the above-described biofilms present in otherwise sterile places within the human body.

Dental biofilms

Dental plaque is an archetypical example of a multispecies biofilm. In dental sciences, biofilms are a major research focus because of their etiological role in oral diseases. Normally, bacteria in dental biofilms live in harmony with the host. However, ecological shifts may occur within the microbial community and result in the two major oral diseases: dental caries and periodontal diseases (Marsh, 1994). For example, there is evidence for dental caries as an endogenous disease resulting from a shift in the supragingival flora toward the dominance of acidogenic and acid-tolerant microorganisms (for a review, see Takahashi & Nyvad, 2008). Likewise, a shift in the subgingival microbiota may increase the levels of anaerobic Gram-negative organisms and lead to the development of gingivitis and periodontal diseases (Socransky & Haffajee, 2005).

Dental biofilms are among the best-characterized biofilms in the human body because of their ease of experimentation, *in vivo* as well as *in situ* (see Fig. 3a and b). Teeth (natural and artificial) provide easily accessible nonshedding surfaces that facilitate undisturbed biofilm formation in a natural environment of proper temperature, humidity and nutritional requirements. Already 40 years ago, researchers made use of scanning/transmission electron microscopy to study the development and structure of natural multispecies dental biofilms (Theilade & Theilade, 1970; Listgarten *et al.*, 1975). Since then, numerous studies have provided additional information about these processes from comparative scanning electron microscopic and classical microbiological

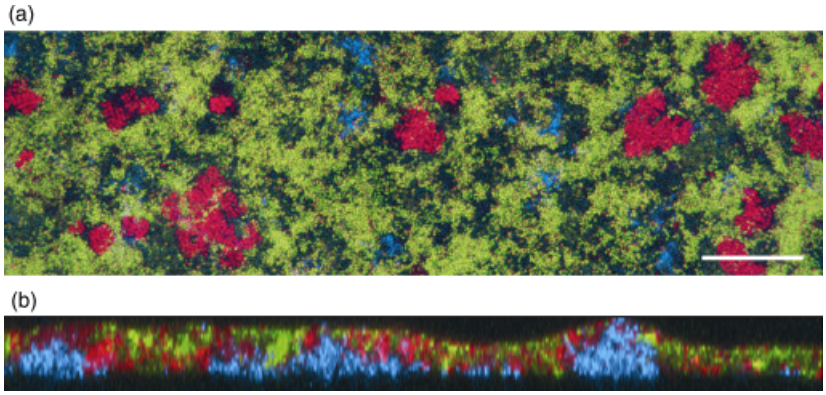

Fig. 3. (a and b) CLSM images of 48-h *in situ* dental biofilms. Biofilms were stained simultaneously with all-bacterium-specific EUB338 probe, *Streptococcus*-specific STR405 probe and *Actinomyces naeslundii*-specific ACT476 probe. Yellow-green, blue and red represent streptococci, *A. naeslundii* and other bacteria, respectively. (a) Maximum projection image of relative thin 48-h biofilm showing complete surface coverage with the dominance of streptococci. Well-defined microcolonies of large coccoid nonstreptococci are observed as well as microcolonies of *A. naeslundii*. Scale bar = 25 μm (Dige, 2008). (b) Sagittal (*x-z*, *y-z*) section of a multilayered dental biofilm. Note that *A. naeslundii* (blue) is predominantly located in the inner part of the biofilms next to the surface (bottom of the images). Some microcolonies of *A. naeslundii* extended almost throughout the entire thickness of the biofilm. The width of (b) is 200 μm (Dige *et al.*, 2009a). Pictures are reprinted with permission from the publisher.

studies (for a review, see Nyvad, 1993). Overall, these studies have described oral bacteria to colonize the surface of pellicle-coated tooth surfaces as single cells and pairs. The initial stages are dominated by bacteria in various stages of cell division, and microcolonies of monolayers are formed. Continued cell division in these microcolonies results in the formation of multilayers. The early colonizers are dominated by streptococci that may comprise up to 60–90% of the initial flora. The remaining bacteria are mostly made up of Gram-positive rods, of which the majority is *Actinomyces*. During the following 48 h, the complexity of the microbiota increases, as indicated by a high morphological diversity.

A major drawback of the above studies was the inabilty to determine the identity and organization of the bacteria in an intact biofilm. The development of molecular methods such as immunofluorescence and FISH combined with CLSM has offered new opportunities to further explore the spatial distribution and population dynamics of the different bacteria of multispecies dental biofilms as demonstrated elegantly *in vitro* by Thurnheer *et al.* (2004). The combined use of these technologies has shown that dental biofilm bacteria *in vivo* engage in interspecies interactions, which may be of importance in the establishment of functioning microbial communities (Palmer *et al.*, 2003; Diaz *et al.*, 2006; Chalmers *et al.*, 2008). So far, only a few studies of dental biofilms have taken advantage of these methods for studying temporal shifts in the bacterial composition in combination with observations of biofilm architecture *in vivo*, and these studies have mainly focused on streptococci (Palmer *et al.*, 2003; Diaz *et al.*, 2006; Al-Ahmad *et al.*, 2007; Dige *et al.*, 2007; Hannig *et al.*, 2007; Dige *et al.*, 2009b). A recent publication highlighted the temporospatial relationship and the population dynamics of *Actinomyces* relative to streptococci in the initial stages of biofilm formation including the presence of prominences (chimneys) of multilayered complex microcolonies (Dige *et al.*, 2009a). A remarkable observation of the study was the preferential colonization of *Actinomyces naeslundii* in the deeper regions of the biofilm, which was proposed to have important ecological consequences in caries. Therefore, when applying such new technologies, studies of the structural composition and metabolism of dental biofilms may not only help to advance our understanding of oral diseases but may also serve to demonstrate concepts of universal interest in microbial ecology.

Intestinal biofilms

The human gut represents a second environment where commensal multispecies biofilm aggregates may form naturally inside humans. In the human intestine, a wide number of bacterial species exist and interact with the normal host in symbiosis. The number of bacterial species in the intestine has been found to vary from 500 to 1000 (Eckburg *et al.*, 2005).

To date, only 20% of the different species have been cultured. The total number of culturable bacteria, colonizing the proximal part of the gastrointestinal tract (stomach and duodenum), is 10^3 CFU g^{-1} material and increases in number to 10^4 CFU g^{-1} in the jejunum, 10^7 CFU g^{-1} in the terminal ileum and finally 10^{12} CFU g^{-1} in the colon (Guarner, 2006). The microbial communities play numerous functional and beneficial roles in the host. Indigestible dietary material is metabolized to short-chain fatty acids that

nourish the epithelium, promote the absorption of glucose by inducing the expression of sodium/glucose transporters in the epithelium, enhance the storage of fat and synthesize essential vitamins. The host immune system is stimulated by the microbial communities and binding of pathogenic bacteria to the epithelium is competitively inhibited.

The intestinal microbial communities are thus a major factor in human health and disease. It is likely that biofilms may be an important factor to establish and preserve a 'normal' microbial community 'feeding' the intestine with planktonic beneficial bacteria maintaining the bacterial homeostasis in the gut.

Once disrupted, either by antibiotics, chemotherapy or a change in the diet, intestinal colonization of pathogenic bacteria or viruses may occur, leading to disease in the host. In one study, high numbers of patients with Crohn's disease were colonized with adherent-invasive *E. coli* (Rolhion & Darfeuille-Michaud, 2007), and the authors reasoned that these pathogenic strains of *E. coli* may be involved in the chronic inflammatory reactions seen in Crohn's disease. Relapsing *Clostridium difficile* antibiotic-associated diarrhea may likewise, among other things, be due to biofilm production caused by *C. difficile* itself, resulting in difficulty in eradicating the organism.

Most of the normally occurring enteric bacteria have the ability to produce biofilm, and future studies might elucidate whether this may be important in maintaining the homeostasis of the intestinal microbial communities, be a virulence factor for pathogenic bacteria or a combination of the two.

Biofilms in soil

Soil contains an astonishingly high bacterial number ($\sim 10^9$ cells g^{-1} soil) and diversity ($\sim 10^6$ species g^{-1} soil) (Torsvik *et al.*, 1990; Gans *et al.*, 2005). This, combined with a relatively large and diverse surface area available for bacterial attachment, indicates an obvious potential for multispecies biofilm formation in this environment. However, little is known about the bacterial organization and diversity of biofilms in soil, which may be due to difficulties of studying this structurally complex environment at the microscopic scale without disturbing the soil and biofilm structures.

Several observations indicate that biofilms are formed in soil and that different bacterial species coexist and interact in these. Additionally, multispecies biofilms may be of major importance for both the soil ecology and the fitness of the bacteria present. Firstly, bacteria are not evenly distributed in the soil environment. The highest bacterial densities are found near nutrient sources i.e. on roots or decaying organic material (Foster, 1988; Pearce *et al.*, 1995; Nunan *et al.*, 2003). In the bulk soil, bacteria are found in patches or microcolonies containing low cell numbers, often composed of different bacterial species (Nunan *et al.*, 2003; Grundmann, 2004). However, when exposed to nutrient sources, these microcommunities have the potential to develop into multispecies biofilms with high bacterial density (Nunan *et al.*, 2003). Secondly, several biological processes depend on the activities of multispecies cooperating consortia, including degradation of decaying organic material (Nannipieri *et al.*, 2002) and removal of xenobiotic compounds in the soil. In several studies, the efficiency of these processes was enhanced when multiple species originally isolated from soil were present in biofilms, including reduction of mercury (von Canstein *et al.*, 2002) and degradation of polycyclic aromatic hydrocarbon (Stach & Burns, 2002). Finally, the bacteria gain many advantages from organizing into soil biofilm communities, including increased protection from the harsh conditions encountered by soil bacteria, for example drought (Roberson & Firestone, 1992) and exposure to antibiotics, heavy metals and other antibacterial compounds, by maintaining themselves in an environment as stable as possible (Jefferson, 2004).

Apart from the functional studies referred to above, which indicated an enhanced efficiency of multispecies biofilm, indications of interspecific bacterial interactions among biofilm-associated soil bacteria have been reported (Burmolle *et al.*, 2007; Hansen *et al.*, 2007). Hansen and colleagues demonstrated how a bacterial strain originally isolated from soil evolved by simple mutations, as a consequence of the association with another soil bacterium in a biofilm, in order to adapt to the conditions of coexistence. Additionally, we have isolated soil bacteria based on their ability of surface/cell–cell attachment, and followed the succession in an early multispecies biofilm. In this setting, we were able to distinguish between early, intermediate and late colonizers in the species present and we also observed several examples of synergistic interactions with respect to biofilm formation when cocultivating biofilm isolates [Burmolle *et al.* (2007) + unpublished data].

These studies and observations indicate that the biofilms formed in soil consist of multiple species cooperating and sheltering themselves from the surrounding environment. Thus, soil biofilms may resemble those formed in the oral cavity and the intestine, and these environments also all have in common the high bacterial number and diversity and various available surfaces. Additionally, bacteria are natural inhabitants in these environments, with essential functions for maintaining the balance, which is in contrast to biofilms in infections.

Discussion and conclusion

Diagnoses of biofilm in chronic infections

The initial problem or 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 the bacteria are readily obtained by swapping or sampling the infected area. For chronic infections, it is usually more problematic. An exception is CF, in which the easily accessible purulent sputum coughed up by the patients on a regular basis harbors the bacteria. For the other chronic infections, routine sampling has been carried out either using a swab, a scrape or a biopsy; however, all might fail to sample the bacteria. In a chronic wound, the swab sampling will only collect the bacteria on the surface and not the bacteria embedded in the wound bed. On the other hand, because the bacteria are very heterogeneously distributed, chances are that a biopsy fails to contain any bacteria present otherwise. Also, for implant- and catheter-related infections, identification of the bacteria has been proven to be difficult. Until recently, bacteria on surfaces and biofilm in general were considered unculturable. The problem might rather be surface adherence: that the bacteria simply attach extremely well to the surface of the foreign bodies. The majority of the cells present may not be unculturable, but they may have to be released from the surface, and for this purpose, vigorous vortexing or even mechanical scraping is usually not enough. The implant or catheter has to be treated with ultrasound (sonication) to release the bacteria (Bjerkan *et al.*, 2009; Rieger *et al.*, 2009; Achermann *et al.*, 2010). In the light of this, it can never be ruled out that some bacteria within the persistent biofilm may be so dormant that they prove impossible to grow on any media. Also, some pathogenic bacteria that are unculturable on growth media are believed by some to be activated when present in the host environment, and they are then able to establish an infection (Brown & Barker, 1999).

The problems of diagnosing the bacteria in these chronic infections are far from solved. Today, bacteria can be detected by culturing, PCR, microscopy or radiography. Each method has its own advantages and limitations. For culturing, the problem is to collect the bacteria, either next to the surface, which is sampled, or from the catheter or implant. On the other hand, if bacteria are cultured, their resistance against antibiotics can be investigated easily.

For bacterial detection using methods based on DNA/RNA, the problem is that extraction does not reveal any information on the spatial distribution of the present bacteria. It should also be noticed that just because a bacterium is present does not necessarily indicate that it contributes to the pathogenesis of the infection, and so it may not require treatment.

Microscopy enables the direct visualization of the infecting present bacteria and their organization. Again, the bacteria have to be present in the collected sample, meaning that many biopsies have to be analyzed for the correct diagnostics of for example a wound.

Bacterial organization in chronic infections and in natural biofilms

Based on our own observations and the current literature, we believe it is evident that chronic infections most often are multispecies. This has been determined using various methods. On the other hand, at least based on our own visualizations of the bacterial biofilms in the previously described clinical infections, the sovereign aggregates themselves predominantly consist of only one bacterial species per aggregate, even in multispecies infections. Different bacterial species can form sovereign aggregates in the same infection, but at different locations, and thus these biofilms cannot be referred to as multispecies; only the infection can be characterized as mutispecies. We believe that this is in striking contrast to what we have termed the 'natural biofilms', where several bacterial species can intermix within the same sovereign aggregate such as dental biofilms in which different bacterial species clearly live intermingled. The reasons for this difference in bacterial organization might be the selection pressure, availability of nutrients and commensalism. The coaggregation in natural locations could be explained by the beneficial catabolism and anabolism of compounds shared among the different bacteria. Because of the high bacterial diversity in these environments in combination with an often-limited availability of nutrients and suitable niches, the present bacteria may have survived selection by exploiting and adapting to all sorts of possible niches, including those directly created or affected by the presence of other microorganisms. Thus, a complex succession takes place during the formation of these biofilms (Jackson, 2003), which includes random bacterial settlement (how random is determined by a range of factors) of early colonizers, an increased competition among the present species and a niche differentiation resulting in very diverse and heterogeneous biofilms at the structural, resource, functional and taxonomical levels.

The scenario is very different in infections. Here, the key challenge for the colonizing bacteria is to survive the encounter with the host defense system, which may be a very efficient restricting factor of the bacterial diversity in chronic infections. The initial phase of biofilm formation in infections may be the crucial point; by chance, the right (opportunistic) pathogenic bacteria need to be at the right place at the right time in order to establish an infection in spite of the activities of the host defense system. Because most harmful chronic infections form in locations of the human body where the host response acts to maintain sterility, the bacterial diversity here is different from and much lower than that of the natural systems. Chances of another species then encountering this chronic biofilm infection and subsequently withstanding the already recruited host defense system and (out)competing the established species are low. Thus, it may be that succession

in biofilms in chronic infections is maintained at a successional stage equivalent to the initial one in natural biofilms, where species by chance settle and some early colonizers establish, which then, at a later point, leads to niche differentiation and heterogeneity. In the natural biofilms, however, environmental factors may eventually change, leading to a shift in the balance toward a lower diversity, which is often associated with pathogenicity in the natural human biofilms (see previous sections on dental and intestinal biofilms) and it is in fact intriguing that, at least for the systems described above, there appears to an association between low diversity and high pathogenicity. Such a shift in balance may be compared with environmental perturbations, such as pollution, which also cause decreased bacterial diversity (Torsvik *et al*., 1996).

Some additional conditions may contribute to why multispecies biofilms are not formed in chronic infections. Dead cells (both eukaryotic and prokaryotic) and a constant blood supply result in high nutrient availability in these environments, imposing a lower selection pressure for coexistence and niche differentiation i.e. symbiosis is not a crucial requisite for growth. Also, as stated above, the bacterial diversity is low in locations subjected to chronic infections and the present bacterial populations have not coevolved during the existence of human beings.

The above assumptions are based on observations from various environments, achieved using the different techniques described above, primarily cultivation, PCR and FISH. These techniques all have limitations; cultivation and FISH have a relatively low sensitivity and PCR is subject to false positives and the lack of information concerning the spatial organization. We therefore, of course, cannot rule out the possibility that there may in fact be several species present in the apparently monospecies bacterial aggregates, but at very low relative frequencies or with an activity level (and thereby rRNA content) lower than the detection limits for the FISH-based detection that is applied in most studies. The implications of FISH variants with a higher sensitivity, such as CARD-FISH (Pernthaler & Pernthaler, 2007), may provide further information on this.

Additionally, most studies on chronic infection are based on a limited patient material. Further investigations into this complex topic, reviewed in this article, would benefit from studies on larger patient populations.

In conclusion, we suggest that clinical biofilms consist of single bacterial species (much like an individual colony on a plate), even though the infections can be multispecies. Additionally, just because many bacteria are present, they do not necessarily participate in the infection. And the natural biofilms like in intestinal, dental and soil clearly show different bacterial species living intermingled. Thus, this might not be a generalizable hypothesis, but is related to the clinical site and types of bacteria.

Future aspects

Here, we have presented our interpretation of a series of facts. The final problem, which may be the biggest challenge for research of biofilm infections in the coming years, is to reveal the significance (are they contributing to pathogenesis?) of the numerous identified microorganisms (especially the anaerobes) by molecular techniques. In addition, when it comes to chronic infections claimed to involve biofilms, we have to be able to visualize the presence of the detected bacteria in aggregates and evaluate their significance for the function of the biofilm (e.g. virulence and structural organization).

In spite of the apparently large differences in biofilms in chronic infections and the natural, 'commensal' biofilms, many of the relevant questions are similar: who is there, what is their function and what do they gain from being there? – and if multiple species are in fact present in single – or different, but closely positioned – sovereign aggregates, how do these different species affect each other synergistically and antagonistically to finally determine the overall function of the biofilm? We believe that the broad current knowledge in bacterial biofilms provides a robust foundation for taking biofilm research one step further by addressing the above questions.

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