

Chapter 8

The Importance of Biofilms in Chronic Rhinosinusitis

Jeff G. Leid, Emily K. Cope, Stacy Parmenter, Mark E. Shirtliff, Scot Dowd, Randall Wolcott, Randall Basaraba DVM, Darrell Hunsaker, James Palmer, and Noam Cohen

8.1 Introduction

There is mounting evidence that bacterial and possibly fungal biofilms play an important role in the etiology and persistence of Chronic Rhinosinusitis (CRS). CRS affects nearly 16–25% of the US population each year, with billions of dollars of annual healthcare expenditures dedicated to its treatment (Gliklich and Metson 1995). Unfortunately, the recalcitrant nature of the disease, which often exhibits a chronic relapsing course, significantly contributes to these healthcare costs. The reasons for the persistent nature of the disease are likely secondary to a number of underlying pathophysiologic mechanisms. Asthma, allergic rhinitis, Gram-positive and Gram-negative infections, aspirin-sensitive asthma, fungus, osteitis, nasal polyposis, superantigens, and other factors have been implicated as etiologies contributing to the development of CRS. The chronic inflammation that develops as a fundamental hallmark of the disease can both cause and be a consequence of dysfunctional mucociliary clearance. Ultimately, stasis of sinonasal secretions will lead to subsequent infection and/or persistent inflammation. In some cases, persistent and recurrent infections occur despite multiple therapeutic interventions for CRS. These chronic infections often involve a particularly resistant form of microbial growth that is manifested by communities of bacteria called biofilm.

8.1.1 Background

Medical scientists have worked under the premise espoused more than 160 years ago by Robert Koch that bacterial infections were caused by individual bacteria floating in purulent fluid or invading animal tissues. Methods of culture were developed to grow these bacteria from swabs or needles which withdrew the fluid containing free floating (planktonic) bacteria. Regrowth of these bacteria on media with

J.G. Leid (✉)
Center for Microbial Genetics and Genomics, Northern Arizona University, Flagstaff,
AZ 86011, USA
e-mail: jeff.leid@nau.edu

discs imbedded with antibiotics identified drugs to use to eliminate the infections. Unfortunately, some infections did not respond to the identified antibiotics. These “chronic” infections included osteomyelitis, cystitis, cardiac valvulitis, prostatitis, mastoiditis, dental plaque, sinusitis, etc. Based on over 30 years of research by environmental, industrial, and medical scientists, a massive bank of knowledge is now available to medical researchers highlighting the importance of biofilms in the treatment of chronic infectious diseases in humans.

This includes the upper respiratory tract where the presence of biofilms has been demonstrated in chronic otitis media, cholesteatoma, and chronic adenoiditis among other important diseases (Post, Stoodley et al. 2004). Colonization and infection with *Pseudomonas aeruginosa*, a common microorganism isolated from the sinus mucosa, has been linked to CRS as these bacteria are particularly resistant to antibiotic therapy and have the potential to drive chronic disease. It is well accepted that *P. aeruginosa*, in a biofilm state, plays important roles in bacterial persistence and antibiotic resistance in chronic infections, such as otitis media (see also Chapter 3) and cystic fibrosis lung disease (see also Chapter 10) (Hoiby et al. 2001, Ha et al. 2007). If CRS develops from acute bacterial sinusitis, then this progression into a chronic disease parallels other biofilm-related diseases. However, since the etiology of CRS remains to be defined, it is unclear whether *P. aeruginosa* is a frank pathogen of CRS or whether other communities of bacteria are responsible for antibiotic resistance and chronic inflammation. Clearly the etiology of CRS must be defined so that therapeutic efforts can be targeted to the organisms responsible for the disease.

8.1.2 Biofilms Defined

A biofilm is an assemblage of microbial cells that is irreversibly associated with a surface, although free floating cell clusters can occur, and these microbial communities are often enclosed in a matrix of primary polysaccharide material (Costerton et al. 1999, Webb et al. 2003). Biofilm-associated organisms differ from their planktonic counterparts in the genes that are transcribed, resulting in an altered resistance to antibiotics and the human immune system (Donlan 2000, Becker et al. 2001, Bollinger et al. 2001, Stewart and Costerton 2001, Vallet et al. 2001, Whiteley et al. 2001, Donlan 2002, Donlan and Costerton 2002, Parsek and Singh 2003, Smith and Iglewski 2003, Head and Yu 2004, Ren et al. 2004, Leid et al. 2002, 2005, 2009). These communities form on a wide variety of surfaces including living tissues, indwelling medical devices, industrial or potable water systems, and natural aquatic systems. The interface between a surface and an aqueous medium (e.g., water and blood) provides an ideal environment for the attachment and growth of microorganisms. The nature of the substratum, conditioning films that form on that substratum, hydrodynamics of the aqueous medium, characteristics of that medium, and various properties of the cell surface all play a role in determining the rate and extent of microbial attachment and biofilm formation (Donlan 2000, Becker et al. 2001, Bollinger et al. 2001, Stewart and Costerton 2001, Vallet et al. 2001, Whiteley

et al. 2001, Donlan 2002, Donlan and Costerton 2002, Parsek and Singh 2003, Smith and Iglewski 2003, Head and Yu 2004, Ren et al. 2004).

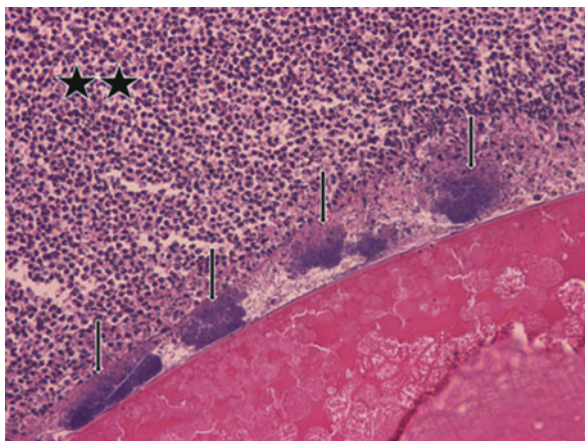
Though every microbial biofilm is unique, some structural attributes are considered universal. Biofilms contain microcolonies of bacterial cells that are separated from other microcolonies by interstitial voids. Easily observed in vitro, liquid flow occurs in these voids (also called water channels) allowing diffusion of nutrients, gasses, and antimicrobial agents. Biofilm architecture is heterogeneous in space and time, constantly changing as a result of internal and external processes. Proximity of cells within or between microcolonies provides an ideal niche for the exchange of extrachromosomal plasmids encoding resistance to multiple antibiotics, exchange of nutrients through gradients, and communication by quorum sensing molecules (Costerton et al. 1999, Donlan 2000, O'Toole et al. 2000, Hoiby et al. 2001, Mah and O'Toole 2001, Leid et al. 2002b, Mah et al. 2003, Parsek and Singh 2003, Webb et al. 2003, Leid et al. 2005). Biofilm-associated organisms have dramatically reduced susceptibility to various types of antimicrobial agents, either because the biofilm structure impedes transport of the agent to the cell surface or because the cells within the biofilm exhibit an altered physiology (Donlan 2002). The clinical relevance of these biofilm communities is the existence of a group or groups of organisms that completely tolerate antibiotic challenge and resist host immunity. These microbes may then serve as a reservoir for further development of antibiotic resistance in the population. A paper in the journal *Nature* correlated the importance of biofilm-specific gene expression and resistance to antibiotics (Mah et al. 2003). For the first time, it was clear that a genetic program specific to the biofilm mode of growth was essential for broad spectrum antibiotic resistance. We have followed up on these studies and demonstrated that a biofilm-specific program also exists that dictates susceptibility to challenge from the host's defenses (Leid et al. 2005, Leid et al. 2009).

8.1.3 Biofilms and Disease

Biofilms have now been implicated in many infectious diseases, including dental caries, periodontitis, otitis media, musculoskeletal infections, necrotizing fasciitis, biliary tract infection, osteomyelitis, bacterial prostatitis, native and prosthetic valve endocarditis, chronic urinary tract infections, cystic fibrosis pneumonia, and now chronic rhinosinusitis (see also the other clinical chapters of this book). Furthermore, nosocomial-type infections are caused by bacterial biofilms. These include ICU pneumonia, sutures, AV shunts, scleral buckles, contact lenses, urinary catheter cystitis, endotracheal tubes, Hickman catheters, central venous catheters, and pressure equalization tubes (Mah and O'Toole 2001, Ehrlich et al. 2002, Post et al. 2004). Although the idea of biofilm communities as the cause of diseases, especially chronic diseases, is still not widely accepted in the practice of medicine, the Centers for Disease Control and Prevention estimates that ~60–70% of all infections are biofilm related. As more focus is directed to microbes growing in the biofilm lifestyle, it is likely that other diseases will be indentified that clearly have biofilm links.

The widely accepted conceptual paradigm of a biofilm comes from a multitude of studies that have either looked at bacterial attachment to plastic or glass, under static or shear settings. In many instances, these communities have been described as having large towers or mushrooms extending away from the substratum with community populations ranging between 10^9 and 10^{12} organisms. However, documentation of such extensive communities in vivo from examination of infected tissue is less pictorially and numerically dramatic. Indeed, in the original description of *P. aeruginosa* biofilms in CF sputum by Costerton, the electron micrographs demonstrated clumps of *P. aeruginosa* bacteria containing between 20 and 50 organisms, not extensive communities that are often described from in vitro assays (Costerton et al. 1999, Klausen et al. 2006). When more physiological substrates are employed for in vitro characterization of biofilms, the communities are often much less extensive and mimic the clumps originally described by Costerton (Landry et al. 2006). Parsek's group demonstrated that *P. aeruginosa* growing on either bovine or human mucin under shear forces formed antibiotic resistant communities that phenotypically represented small clusters of organisms. We have demonstrated similar in vivo findings in a mouse model of endophthalmitis (Fig. 8.1, Leid et al. 2002a). These more physiological biofilms were quite different from the extensive phenotypic data of *P. aeruginosa* biofilms that collectively created the original conceptual paradigm of biofilm communities. Nonetheless, these extensive in vitro studies have pushed biofilm research to the forefront of medicine and have defined many important characteristics that are associated with microorganisms growing as heterogeneous communities.

Fig. 8.1 Histogram of biofilm-mediated endophthalmitis. The arrows point to clusters of *Staphylococcus aureus* that are attached to the lens of the eye. The stars represent the inflammatory response to presence of the biofilm organisms



8.1.4 Head and Neck Biofilm-Related Diseases

A comprehensive introduction to biofilms in the Head and Neck was most recently reviewed in Current Opinion in Otolaryngology (Post et al. 2004). Because of

that, we will not detail all of the biofilm diseases here but will highlight a few head and neck diseases that either exhibit a polymicrobial nature or inherent antimicrobial tolerance. Otitis media with effusion has been definitively shown to be associated with mucosal biofilms of known middle ear pathogens, such as *Haemophilus influenzae*, *Streptococcus pneumoniae*, and *Moraxella catarrhalis* (Hall-Stoodley et al. 2006) (see also Chapter 3). Polymicrobial adenoid biofilms have been identified and associated sinusitis has been shown to be reduced in patients after undergoing adenoidectomy (Zuliani et al. 2006). Tonsillitis may at times be a biofilm process, mediated by the presence of multiple organisms, as are all infections involving tracheotomy and tympanostomy tubes and other implanted materials (Vlastarakos et al. 2007). Antibiotic otic drops, in an in vitro study of tympanostomy tubes containing *P. aeruginosa* biofilms, reduced colony forming units by day 5 through day 21. However the antibiotics did not halt progression of the biofilms (Oxley et al. 2007). All of these are examples of biofilm infections that are common to the head and neck, yet are extremely difficult to treat because of the inherent resistance that is associated with biofilm communities.

8.1.5 Factors Contributing to Biofilm Antibiotic Resistance

The inherent resistance of these communities to antibiotics and the host immune system is still being elucidated, although much more is known about antimicrobial resistance mechanisms (Mah and O'Toole 2001, Gilbert et al. 2002, Mah et al. 2003, Parsek and Singh 2003, Bagge et al. 2004, Leid et al. 2005, Ooi et al. 2008) (see also Chapter 13). As mentioned, biofilm-associated organisms have dramatically reduced susceptibility to various types of antimicrobial agents, either because the biofilm structure impedes transport of the agent to the cell surface or because the cells within the biofilm exhibit an altered physiology. A paper in the journal *Nature* correlated the importance of biofilm-specific gene expression and resistance to antibiotics (Mah et al. 2003). This paper was important because it was the first description of biofilm-specific genetic resistance mechanisms against a fairly broad antimicrobial spectrum. Our group has followed up on this study and demonstrated that biofilm-specific genes are also responsible for resistance to the host's immune defenses (Leid et al. 2005). Additionally, communication between the bacteria within the community dictate resistance to the host, and intricate studies have demonstrated that communication of the biofilm organisms can lead directly to leukocidal activity and bacterial persistence (Christensen et al. 2007, Jensen et al. 2007).

8.1.5.1 Antibiotic Resistance

Biofilms evade host defenses and demonstrate decreased susceptibility to systemic and local antibiotic therapy (Davies et al. 1998, Mah and O'Toole 2001). The exopolysaccharide alginate in *P. aeruginosa* could lead to decreased penetration of antibiotics into the biofilm. However, studies showing that antibiotics can diffuse efficiently into biofilms contradict this theory (Mah and O'Toole 2001). Because

water comprises a large portion of the biofilm mass, this allows for diffusion of antibiotics down water channels into the core regions of the biofilm. Resistance could then be conferred by deactivating or neutralizing positively charged antibiotics interacting with the negatively charged polymers of the biofilm matrix. A third theory suggests that bacteria could lie in a non-growing state of suspended animation in the basal layers of the biofilm due to the accrual of waste products and depletion of needed substrates. This could confer relative resistance to antibiotics as most antibiotics work only on dividing bacteria. Finally, decreased diffusion of antibiotics into the bacterial cytoplasm due to fewer porins in the bacterial cell wall is another possible method of resistance. Fewer porins could develop as a stress response due to osmotic forces changing nutrient gradients. In reality, antibiotic resistance is probably a result of a combination of these mechanisms.

Aminoglycoside antibiotics may potentially induce biofilm formation in some bacteria at subtherapeutic doses. Hoffman and colleagues demonstrated the induction of biofilm formation in *P. aeruginosa* and *Escherichia coli* when exposed to subtherapeutic concentrations of these antibiotics (Hoffman et al. 2005). Certain Pseudomonads have a gene named the aminoglycoside response regulator (arr) that confers this biofilm-specific aminoglycoside resistance. The ubiquitous nature of this response in Pseudomonads as well as other bacteria is currently being studied (Hoffman et al. 2005, da Fonseca et al. 2008). As topical sinus irrigations with gentamicin or tobramycin are often prescribed in patients with CRS, this could be a potential source of bacterial biofilm development for *P. aeruginosa* and other microorganisms, especially at subtherapeutic concentrations. A recent paper by O'Toole and colleagues demonstrated that tobramycin, even at high doses, enhanced *P. aeruginosa* biofilm formation on cultured human cells (Anderson et al. 2008). Along these lines, Walker and colleagues demonstrated that live human neutrophils could enhance biofilm formation by serving as necrotic debris that *P. aeruginosa* utilized as scaffolding for biofilm formation (Walker et al. 2005, Parks et al. 2009). This idea of necrotic debris from the host response serving as a microniche for biofilm formation, combined with the antibiotic resistance seen in these communities, could explain the initial pathogenesis of many biofilm diseases, including chronic rhinosinusitis.

Biofilm development is a cyclical process involving initial attachment and mature biofilm formation followed by detachment and potential reseeded of other parts of an implanted medical device or perhaps other organs and tissues in the human body (Costerton et al. 1999). As bacteria transition from planktonic organisms to attached, multicellular communities, specific genes are differentially expressed that aid in biofilm formation and establishment of a community of microorganisms resistant to antibiotics and the human immune system (Sauer et al. 2002, Leid et al. 2002, 2005, 2009). The first step in development is initial, reversible attachment. This step is typically augmented by some conditioning of the attachment surface. During initial attachment, which occurs over seconds and minutes, the bacteria transition from reversible attachment to irreversible attachment and many biofilm specific genes and gene products are up-regulated within 6 h post attachment. Quorum sensing genes are turned on and result in further maturation of the biofilm under specific growth

conditions or secretion of virulence factors that aid in developing the infectious nidus (Parsek and Singh 2003). As the biofilm transitions into a mature community with extensive three-dimensional architecture, exopolymeric substances are (matrix) produced (Hentzer et al. 2001, Nivens et al. 2001, Shirtliff et al. 2002). All of these steps, from initial attachment and biofilm maturation to detachment and reinfection, serve as distinct mechanisms against both antibiotic and human leukocyte killing. Studies of 14 strains of *S. pneumoniae*, a CRS-relevant pathogen like *P. aeruginosa*, clearly demonstrated the complexity of the mature biofilm structure. These strains were shown to differ in architecture, protein mass, and cell counts (Allegrucci et al. 2006), and document the important difference between clinical strains that cause disease and many of the laboratory strains that have lost both genetic diversity and pathogenic potential through serial passage (Jelsbak et al. 2007).

8.2 Biofilms and Chronic Rhinosinusitis: What Is the Evidence?

Based on the problems arising from recalcitrant infections in chronic rhinosinusitis and emerging research on biofilms in other chronic disease, multiple studies were undertaken to examine the role that biofilms might play in chronic rhinosinusitis (Post et al. 1996). In the first study of biofilms and CRS, Perloff and Palmer examined frontal sinus stents, placed during surgery, by scanning electron microscopy to demonstrate biofilms on the stents of six patients. Morphologic structures characteristic of biofilm growth including water channels, glycocalyx coatings, and a three-dimensional microcolonies on all six of the stents were evident by SEM, and all sinus cultures were positive for *P. aeruginosa*. Additionally, they examined sterile stents in vitro placed in inoculated media for 48 h, which showed the presence of biofilms (Perloff and Palmer 2004). Following this, cultures were taken from 16 consecutive patients and were assessed for biofilm presence on excised mucosa using SEM. All 16 demonstrated signs of infection including cilia loss, and 25% (4/16) demonstrated near total coverage of the ciliary surface and morphologic appearance of a biofilm (Cryer et al. 2004). These studies, including the ability of *P. aeruginosa* to form biofilms on living tissue, were confirmed in an animal model of infection using New Zealand White Rabbits (Perloff and Palmer 2005).

Additional studies have documented biofilm formation in samples taken from patients after functional endoscopic sinus surgery (FESS) by SEM. Ramadan et al. obtained intra-operative samples from the ethmoid bullae from five patients undergoing FESS, all of which demonstrated morphologic criteria of biofilms on SEM (Ramadan et al. 2005). A follow-up study by Sanclement et al. observed biofilms in 80% (24/30) of patients compared to 0/4 controls (Sanclement et al. 2005). In addition, six patient samples were examined by transmission electron microscopy and demonstrated bacterial structures on the mucosal surfaces, which correlated with biofilm structures on SEM in these patients. The limit of all of these studies was the lack of identification of the organisms that were seen as biofilms on the human sinus tissue.

Sanderson et al. used confocal laser scanning microscopy (CLSM) in combination with Fluorescence In Situ Hybridization (FISH) analysis to examine intra-operative samples taken from 18 patients with CRS and five controls undergoing septoplasty (Sanderson et al. 2006). The analysis found 78% (14/18) of patients with detectable bacteria in a biofilm matrix contained *H. influenzae*. They also found *S. pneumoniae*, and *S. aureus*, whereas *P. aeruginosa* was notably absent. Ferguson and Stolz utilized TEM in conjunction with bacterial cultures to demonstrate biofilms on 50% (2/4) of patient samples taken intra-operatively in presumed CRS, both of which grew out *P. aeruginosa*. The other two patients were discovered to have a non-bacterial etiology to the CRS (Ferguson and Stolz 2005). However, the limited patient sample size for this study makes it hard to determine the significance of these findings. Healy and colleagues followed up the initial FISH studies on CRS samples by demonstrating that fungal elements intermixed with *H. influenzae* biofilm communities in patients with allergic rhinosinusitis (Healy et al. 2008). Finally, Psaltis et al. used CLSM to demonstrate biofilms on mucosal biopsies from the middle meatus/ethmoid area in 45% (17/38) patients undergoing FESS for CRS, compared with 0% (0/9) controls undergoing endoscopic transsphenoidal hypophysectomy (Psaltis et al. 2007).

In the largest patient sample study to date, Prince et al. demonstrated that clinical isolates from CRS patients grew as biofilms in vitro using the Calgary Biofilm Assay. Of 157 samples, they noted a biofilm formation rate of 28.6% (Prince et al. 2008). However, it is unclear whether the in vitro CBA approach is an appropriate mimic for the in vivo environment that likely enhances biofilm formation for bacterial species. Speciation of the cultures demonstrated that *S. aureus* was the most commonly isolated organism (33%), but that 20% of patients had either *Pseudomonas* infection or polymicrobial infections containing *P. aeruginosa*. Additionally, this study linked the number of prior surgeries to an increased likelihood of harboring biofilm-forming strains of bacteria (Prince et al. 2008). Collectively, these studies outlined the role of biofilms in CRS and have led to many groups studying a specific organism in the context of CRS pathogenesis.

There is a complete lack of concordance between the studies above, which may be explained by a number of factors in this emerging field. These include differences in sites considered as specimen versus control, lack of universal definition of biofilm from mucosal tissue biopsies, inconsistencies in individual hospital microbiologic laboratory protocols, differences in technique for assessing biofilms (SEM vs. CSLM), and perhaps epidemiologic differences in the endemic microbial flora between regions. Nonetheless, the common theme is that biofilms are present, likely even in “healthy” sinus tissue, and that these biofilm communities are composed of different groups of microorganisms that either promote health or disease. Many examples exist in the literature demonstrating that biofilms of specific microorganisms have the ability to modulate the host immune response, including inflammation. It is quite possible that this situation exists in CRS and results in chronic inflammation. The importance of the polymicrobial nature of the disease is discussed below.

In an examination of the role of biofilms in disease progression, Coticchia and colleagues compared the mucosal surface area in pediatric patients undergoing

adenoidectomy for chronic rhinosinusitis versus those undergoing surgery for obstructive sleep apnea (OSA). In the seven patients with CRS, SEM demonstrated biofilm covering greater than 90% of the adenoid mucosa, as opposed to a mean coverage of 1.9% in those children with OSA (Coticchia et al. 2007). Additional studies have shown that the benefit of adenoidectomy appears unrelated to adenoid size (Maw 1985, Gates 1994), but instead are derived from removal of the biofilm as a source of potential re-infection of the sinus mucosa. This fits with the model of biofilm-mediated chronic infection as described earlier.

With these finding in mind, additional reports have examined the post-operative outcomes for patients with biofilms and continue to suggest clinical relevance for biofilms in disease pathogenesis and persistence. Bendouah et al. followed 19 patients for 1 year post-FESS and patients were assessed for evolution of disease by presence or absence of symptoms, as well as endoscopic examination of the nasal cavities (Bendouah et al. 2006). Only 5/19 patients were deemed to have favorable evolution of symptoms following surgery. None of these patients had *P. aeruginosa* or *S. aureus* isolates that formed biofilms by in vitro culture assays, although 3/5 had biofilm-forming isolates of coagulase-negative staphylococci. Poor post-operative evolution was associated with *S. aureus* or *P. aeruginosa* biofilms; 13 of 14 patients with poor outcomes demonstrated either *S. aureus* or *P. aeruginosa* biofilms, or both, and the authors suggested that biofilms may play a role in the chronicity of the disease. However, no mucosal samples were taken to demonstrate the presence or absence of biofilms in situ in these patients.

In a pre-operative study by Psaltis et al., 40 patients undergoing FESS for CRS had mucosal samples taken intra-operatively. These samples were examined using confocal laser scanning microscopy and 50% were found to have biofilms, but the authors were unable to speciate these biofilms to determine which organisms were present. Although there were no objective differences in pre-operative symptom scores, patients with biofilms had significantly higher Lund–McKay scores than those without. However, after 8 months of follow-up, the biofilm-positive patients were more likely to have ongoing post-operative symptoms and endoscopic findings of continuing mucosal inflammation when compared to the patients in whom no biofilms were noted (Psaltis et al. 2008). These studies provide evidence that biofilms indeed play an active role in perpetuating inflammation in CRS patients and may explain the recurrent and resistant nature of this disease. Although further investigations are needed, persistent infectious biofilms are a likely contributing factor to medically recalcitrant CRS. A greater understanding of biofilm-associated CRS is required to develop novel therapies directed at prevention and eradication.

8.3 Etiology of CRS

CRS is most likely the manifestation of the interaction of multiple host and environmental factors suggesting that there may be genetic or epigenetic influences that predispose to disease. Environmental factors that have been proposed include viral, bacterial, and/or fungal colonization as well as exposure to inhaled substances, such as cigarette smoke or allergens. The most reported CRS-associated bacteria

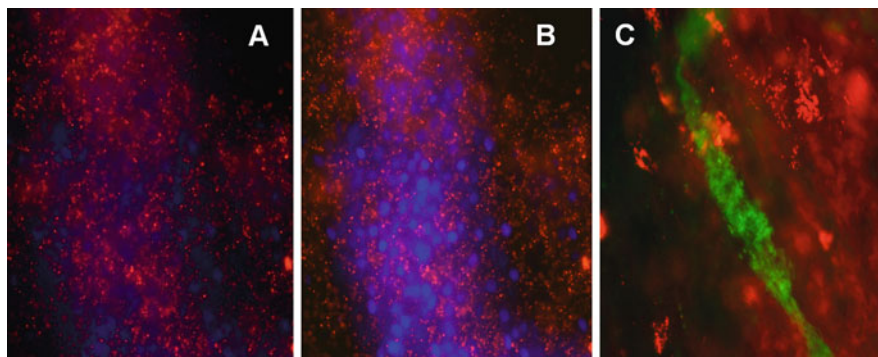


Fig. 8.2 Fluorescent micrographs of explanted sinonasal tissue from human chronic rhinosinusitis patients undergoing functional endoscopic sinus surgery. The *red spheres* are *Haemophilus influenzae* (FISH stain), the *blue* are eukaryotic nuclei (DAPI), and the *green* is a pan-fungal stain (C)

in the literature are *S. aureus*, *P. aeruginosa*, coagulase-negative Staphylococci, *S. pneumoniae*, and *Moraxella catarrhalis*. Using species-specific DNA probes, we recently reported the presence of *H. influenzae* in ~80% of CRS patient lesions (Sanderson et al. 2006, Fig. 8.2a,b). This report was the first to demonstrate the prevalence of *H. influenzae* in CRS-associated nasal tissues. Within the last half decade, much attention has also been directed toward the contribution of fungi (Hamilos and Lund 2004, Gosepath and Mann 2005), specifically *Alternaria* (Kennedy 2004) and *Bipolaris* (Buzina et al. 2003) or toxigenic *S. aureus* (Bernstein et al. 2003) in the sinonasal mucosa during development of polypoid disease. Subsequently, we have demonstrated the presence of fungi and *H. influenzae* in CRS lesions associated with allergic rhinitis (Healy et al. 2008, Fig. 8.2c). These initial studies began a paradigm shift in CRS.

8.4 Evidence that Chronic Rhinosinusitis is a Polymicrobial Disease

As medical technology has advanced, the idea of a single organism causing a disease has become outdated. Sophisticated techniques have identified new groups of microorganisms in every tissue of the human body. The same is certainly true for the human sinus cavity. The use of molecular tools has clearly shown that standard microbial culture techniques only identify 10–40% of the microorganisms present in many diseases. This is confounded by the inherent attachment of these communities making them hard targets for standard clinical diagnostics (Veeh et al. 2003). Although not all of these microbes are likely responsible for the disease state, it is reasonable to assume that combinations of these microorganisms are. Yet, besides studies of dental microorganisms or the organisms in chronic wounds, very little

knowledge of community-directed disease has evolved. Chronic Rhinosinusitis is the perfect opportunity to innovate the world of medicine by introducing, demonstrating, and resolving the importance of microbial communities in a disease that reduces the quality of life in a quarter of the human population. By understanding the importance of the community composition, and not just the presence of frank pathogens (e.g. *S. aureus*, *S. pneumonia*, and *H. influenzae*), we may be able to innovate the treatment strategies for disease by targeting specific pathogens for elimination while promoting the growth of others. We may even be able to turn the microbes against one another by modulating their communication systems. However, we must first identify the pathogens responsible for CRS. At its worst, this line of research will positively benefit the quality of life of millions of patients by resolving the etiology of CRS. At its best, it will revolutionize the practice of medicine by laying the groundwork for disease-specific, patient-specific treatment depending upon the microbes that are present and causing disease.

8.5 Biofilms and CRS – Making the Case for a Paradigm Shift

Biofilms have now been implicated in many infectious diseases, as outlined above (see the other clinical chapters of this book). Many of these diseases are polymicrobial in nature. As mentioned earlier, studies have identified biofilms in the sinus mucosa of patients with CRS (Cryer et al. 2004, Sanderson et al. 2006, Healy et al. 2008). Additionally, CRS has recently been classified into CRS with nasal polyps (CRSwNP) and CRSsNP (without). One possible explanation for the different clinical presentations of the disease may be found in the microbiological members of the offending biofilm community; i.e., heterogeneous biofilms containing fungal elements and/or multiple species could produce a polypoid inflammatory response (Fig. 8.2c above), while biofilms devoid of these microbes produce a non-polypoid mucosal inflammation.

Because medicine has relied on standard clinical microbiological techniques such as bacterial culture, there are currently only a handful of pathogens that have been characterized in CRS. However, many reports have demonstrated that biofilms, simply by their attached nature, are recalcitrant to standard, clinical diagnostic techniques (Veeh et al. 2003). Thus, patients with CRS offer a unique opportunity to investigate the relationship between microbial communities, the *in vivo* ecology, and the resultant disease state(s).

Although we, and others, have demonstrated the importance of biofilms in CRS, the collective scientific literature would also suggest that communities of microorganisms exist in healthy tissue throughout the human body. Therefore, the presence of a biofilm is likely not unique to any polymicrobial disease state. What *is* unique to the development of pathology is the *composition* of the microbial community, interacting through communication systems such as quorum sensing, that *collectively* cause disease. Communication between two common CRS pathogens has already been shown to enhance the virulence of one organism and alter the host response (Ratner et al. 2005). One of the best arguments for this community theory of disease

in CRS, besides the preliminary data presented here, comes from recent publications in the Otorhinolaryngology literature. In the 2008 Jan–Feb issue of *American Journal of Rhinology*, as well as in recent issues of *Laryngoscope*, many authors suggested the importance of single, distinct microorganisms in CRS. Collectively, however, these reports challenge the current CRS paradigm and support the community theory we are proposing here. Most importantly, data from our group support this paradigm shift in the study of CRS as we clearly show distinct microbial communities in CRS vs. non-CRS tissue. Confirmation of these data will elucidate the critical targets for novel therapeutic strategies in the management of this common, costly, and debilitating disease. It is clear to our group that a major point being missed in current CRS studies is the critical polymicrobial combination(s) driving persistent mucosal inflammation leading to the initiation and progression to CRS.

In support of our paradigm-shifting hypothesis, we have utilized diverse molecular approaches to resolve the polymicrobial nature of CRS. The first technique we employed was Terminal Restriction Fragment Length Polymorphism (TRFLP). This molecular protocol has been utilized for some time in environmental microbiology, and we have successfully adapted this technique to CRS. For these studies, total DNA was isolated from the samples, 16S rDNA was amplified by universal primers, and the amplicons run on an Applied Biosystems ABI sequencer. The resultant TRFLP patterns were compared using relative fragment peak heights. NMDS analysis of TRFLP patterns was performed using PC-ORD4 software (MjM Software, Gleneden Beach OR) and cluster analysis was conducted with the same software using the Jaccard distance measure and Ward's method for linking groups. When CRS samples were compared to non-CRS samples by cluster analysis, the CRS microbial communities clustered together (Fig. 8.3, arrow) whereas the control tissue clustered in distinct clades (Fig. 8.3).

To further characterize the microbial communities in CRS, we employed Denaturing High Pressure Liquid Chromatography (DHPLC). In principle, the DHPLC technique provides a “signature” or fingerprint consisting of dozens of absorbance peaks, each of which correspond to a single bacterial species. The area under each peak corresponds to the amount of DNA from that species that is present in the population. By separating and quantifying these mixed samples, as well as through downstream collection and DNA sequencing, it is possible to quickly identify the bacteria in a given population as well as semi-quantitatively provide their relative abundance by peak height. For these studies, 16S rDNA was amplified from total DNA as above and the fragments from the heterogeneous population were injected into a heated HPLC column. These fragments were partially heat denatured. While the double stranded sections of the partially denatured PCR fragments stick tightly to the column, the single strands of the fragments reduce this binding strength. Elution buffer was then passed through the column at increasing concentrations, eluting the 16S rDNA amplicons off of the column depending not only upon the percent GC but also the relative nucleotide sequence order. This allowed the 16S fragments from each species to elute at distinct times and peaks. Once eluted off of the column, the species were detected by spectrometry and peaks were collected for immediate sequencing (see Fig. 8.4). Strikingly, the two

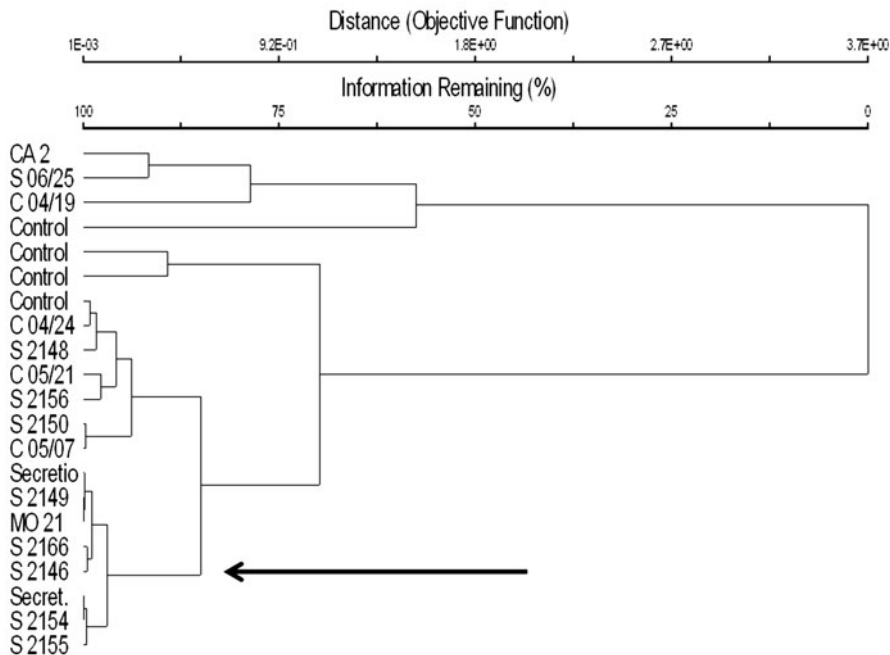


Fig. 8.3 TRFLP data demonstrating the clustering of control or CRS (*arrow*) microbial populations as characterized from explanted human sinonasal tissue

control patients shown in Fig. 8.4 had almost identical microbial profiles. In contrast, the microbial diversity in the CRS patients was large. Upon sequencing, we have identified microbes never before reported in CRS, including two species of *Bordetella*. These data confirm the TRFLP results and, along with 454 sequencing of the microbes in the CRS tissue which is ongoing in our group, will definitively resolve the microbial populations in CRS for the first time. Moreover, these data provide strong evidence for a new polymicrobial community driven paradigm for CRS with resultant and appropriate changes in patient care.

Many studies have shown multiple bacterial species on culture of CRS patients. In a recent Current Opinion in Microbiology, Wargo discusses the evidence for and the implications of the polymicrobial nature of these infections (Wargo and Hogan 2006). Using Fluorescent In Situ Hybridization (FISH), Confocal Scanning Laser Microscopy (CSLM), and explanted human tissues to examine sinus mucosa in patients undergoing Endoscopic Sinus Surgery (ESS) for CRS, the authors found no correlation between the bacteria in the biofilms in the tissue and the bacteria isolated on culture (Leid et al., unpublished). These same patterns have held no matter what molecular approaches we have utilized, including 454 pyrosequencing of human sinus tissue; that is, the molecular data very rarely match the clinical microbiology data obtained from culture. We are presently analyzing the microbial communities within healthy and CRS-associated sinus mucosa through a variety of molecular techniques. The results not only demonstrate a difference in microbial community

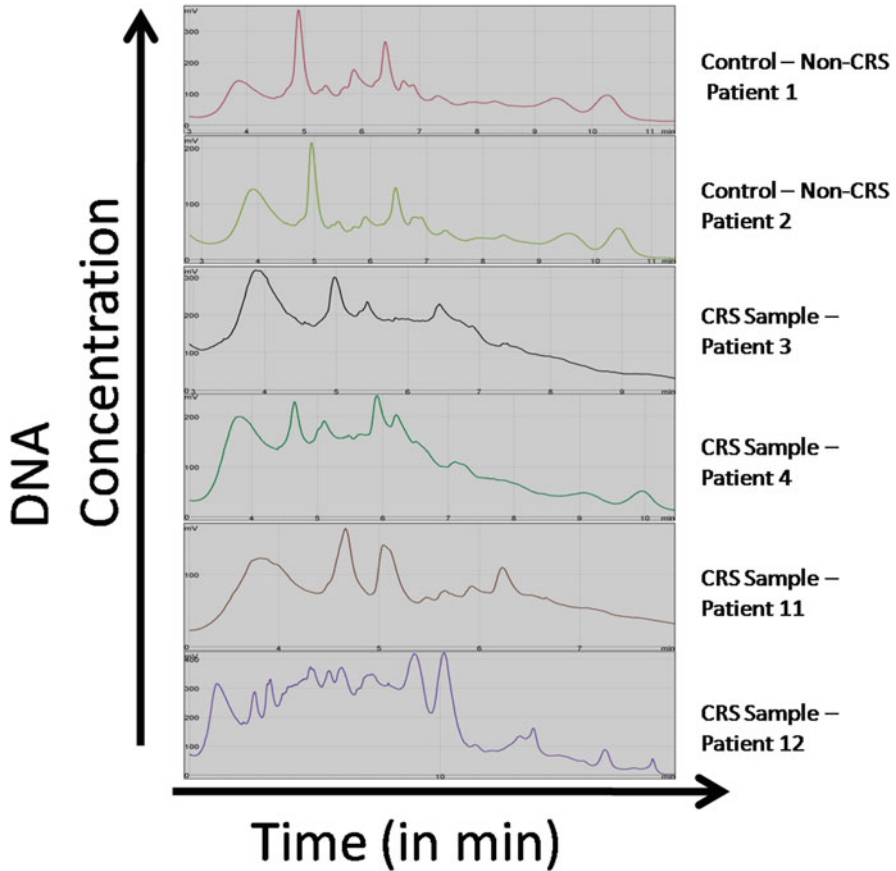


Fig. 8.4 DHPLC profiles of control and CRS patients. Each peak represents a unique bacterial species. Note that the bacterial species are fairly consistent among the control tissues, whereas the CRS patient microbial profiles are diverse

structure, but also solidify the growing evidence of the limitations of data that culture provides because it rarely correlates with the true microbial population in this disease (Psaltis et al. 2007)

8.6 Other Factors Contributing to Development of Biofilm on Rhinosinus Mucosa

It is well recognized that damaged mucosa, dry tissue, and foreign bodies provide excellent substrates for bacterial attachment, leading to biofilm formation. Recent studies by our group have demonstrated biofilm formation on what has been considered to be normal sinonasal mucosa. Environmental factors contributing to CRS were discussed above. Host factors implicated in the development of CRS include ciliary impairment (primary ciliary dyskinesia), mucus homeostasis dysfunction

(cystic fibrosis), atopy, asthma, immuno compromised states (immunoglobulin deficiency or HIV), and paranasal sinus anatomic variations (Cohen and Kennedy 2005). The combination of environmental (microorganisms) and host factors results in the common histopathology finding of mucosal inflammation with the physiologic consequence of marked decrease in sinonasal mucociliary clearance. It is interesting to consider that the majority of these factors also contribute to ciliary damage or dysfunction. Is this relationship due to the contribution of these factors to adherence of bacteria (biofilm communities) to the sinus respiratory epithelium, thereby facilitating biofilm formation? There is still much to be learned from the study of explanted CRS tissue from humans.

8.7 Pathophysiology of the Biofilm Communities

Biofilms have a heterogeneous morphology, because the biofilm phenotype is highly dependent on the surrounding environment. An example of this heterogeneity is demonstrated with bacterial biofilms that form on mucosal surfaces, often referred to as a *mucosal biofilm* (Post et al. 2004). These bacterial biofilms exhibit unique cascades of gene expression when compared to biofilms that form on inert surfaces. Since they form in the special environment of ciliated mucosa, which has known antimicrobial characteristics, these results are interesting because ciliated mucosa is expected to have some protection from biofilm formation. Mucosal biofilms are modified by the host inflammatory response and may incorporate some of the host proteins, waste products, and cellular debris in their composition.

S. aureus and *P. aeruginosa* are notorious pathogens in both lower and upper airway disease. Both organisms are able to produce biofilms. *S. aureus* can produce exotoxins that are active as superantigens to specific immunity. Some believe the superantigens play a role in the development of CRS in certain individuals (Bachert et al. 2002, Tripathi et al. 2004). On the other hand, *P. aeruginosa* is a Gram-negative bacterium that is frequently associated with long-term respiratory diseases. Gram-negative bacterial CRS is particularly recalcitrant in nature. Gram-negative sinusitis, specifically *Pseudomonas*, has been studied extensively in the past and has been noted to cause an intense transmucosal injury far greater than experimental sinusitis using other bacteria associated with sinusitis, such as *S. pneumoniae* (Bolger et al. 1997). However, these results may have been more linked to the choice of the animal model than the role of these pathogens in CRS. Persistent CRS disease with either of these infectious organisms that is recalcitrant to antibiotic therapy is nicely explained by the presence of bacterial biofilms.

8.8 Current Paradigms in the Medical and Surgical Management of CRS

The definition of maximal medical therapy for the management of chronic rhinosinusitis has not been standardized. The medications used, timing, and doses vary from physician to physician and across the specialties treating the disease. There

are no published guidelines or FDA approved medications for CRS, most likely a result of the undefined disease etiology. What is uniformly accepted is the general principle of maximal medical therapy to promote ventilation of obstructed sinus and drainage of stagnant secretions, by decreasing mucosal inflammatory responses and eliminating inciting bacterial and/or fungal infection. A common treatment regimen consists of a prolonged course of a broad spectrum antibiotic, oral steroid, nasal saline irrigations, and nasal steroid sprays +/- nasal antihistamine sprays based on evidence of atopy. The choice of antibiotic is ideally guided by endoscopic culture of the middle meatus. When one is not attainable, a broad-spectrum antibiotic that covers Gram-positive, Gram-negative, and anaerobic microorganisms is chosen. The exact duration of therapy varies from physician to physician, but patients are commonly treated with an initial three-week course, augmented by an additional three weeks of therapy for sub-optimal response. The course of antibiotics is accompanied by a three-week tapering course of oral steroids. After the initial three weeks, a repeat CT scan and nasal endoscopy is performed. If there is symptomatic improvement, but still significant findings of rhinosinusitis radiographically or endoscopically, an additional three weeks of antibiotics and/or oral steroids may be prescribed. If there is no symptomatic or clinical improvement, the patient is given the option of a longer course of medical therapy or surgical intervention.

The surgical management of CRS and nasal polyposis has evolved over the years. External facial incisions, extensive nasal packing, and prolonged hospital stays have been replaced by outpatient surgery, endoscopic techniques, minimal packing, and computer aided technology that have stretched the limits of sinus surgery. As a result, functional endoscopic sinus surgery (FESS) has become a popular adjunct to the medical management of chronic rhinosinusitis. Utilizing mucosa sparing techniques, the extirpation of sinus cells and the creation and maintenance of patient sinuses have demonstrated excellent long term results through subjective and objective measures (Senior et al. 1998). By employing these therapeutic approaches, many patients with CRS demonstrate improvement following appropriate medical and surgical therapies. However, a subpopulation of symptomatic patients exists with recalcitrant CRS. With its poorly defined etiology, the current standard of care for CRS is being administered in an imperfect fashion. Resolving the microbial communities in CRS, and linking those communities to patient treatment outcomes, will have a real and dramatic impact on patient health.

8.9 Treatments

A number of techniques have been evaluated for their capability to manage and control biofilms in environmental science. Materials and coatings to help reduce initial cell adhesion to surfaces and a variety of treatments aimed at decreasing or destroying already formed biofilms have been evaluated. These include heat, chemical treatments, antibiotics, sonication, quorum-sensing analogs, cleaning regimens, low-power laser therapy, and lectins (Oulahal-Lagsir et al. 2000, Hammer

and Bassler 2003, Nandakumar et al. 2004, Parkar et al. 2004, Sheehan et al. 2004, Woodworth et al. 2007). Furthermore, new investigations into biological control agents, such as bacteriophages and protozoa, have shown promise although they are still years away from clinical application (Kadouri and O'Toole 2005). Many of these treatments are prohibitive in humans due to detrimental effects on host cells. The pursuit of a reliable method for the elimination of human biofilm infections is ongoing and likely the holy grail of biofilm research (see also Chapter 14).

Although CRS may have many independent inciting factors, including bacterial infection (whether planktonic or biofilm-mediated), genetics, reactive airways, anatomic abnormality, fungal infection, and allergy, the mainstays of therapy remain the same: anti-inflammatory and antimicrobial agents combined with surgical ventilation. Even though FESS has a high success rate, there are large population of patients that progress back to CRS. Other treatments for biofilms include novel methods of antibiotic therapy. Investigators have demonstrated that low dose macrolide therapy at levels far below the established minimal inhibitory concentration for *Pseudomonas* can decrease biofilm formation (Gillis and Iglewski 2004, Wozniak and Keyser 2004). However, the underlying mechanism behind this decrease has yet to be elucidated. Additionally, studies also demonstrate that antibiotic challenge can actually *increase* biofilm formation in *P. aeruginosa*, thus bringing into question the effective use of antibiotics in therapy (Anderson et al. 2008). Indeed, recent publications suggest that antimicrobial therapy of CRS was unwarranted because of the lack of success of treatment.

Topical saline irrigations are often utilized as a mechanical debridement of the mucosal surface following sinus surgery. Chemical surfactants, such as detergents, have anti-microbial activity by disruption of bacterial cell walls. Baby shampoo is an inexpensive, commercially available solution containing multiple chemical surfactants. Our prior studies demonstrated an in vitro anti-biofilm effect on *Pseudomonas* biofilm formation using a 1% solution of baby shampoo (Chiu et al. 2008). We subsequently studied its effects in a prospective study of symptomatic post-functional endoscopic sinus surgery (FESS) patients who irrigated twice a day for 4 weeks. Sixty percent of patients noted improvement in specific symptoms of thickened mucus and postnasal drainage with the formulation. Other surfactant-containing agents are currently under investigation including a combination of citric acid and zwitterionic surfactant. One study demonstrated significant reductions in biofilms on human chronic sinusitis mucosal specimens with hydrodynamic administration of this solution (Desrosiers et al. 2007).

Future directions for biofilm-associated CRS include investigations into the nature of the biofilm at the molecular and cellular levels. Molecular targets of specific aspects of the biofilm lifecycle continue to show promise. Disrupting the type IV *pili* attachment phases of *Pseudomonas* is one potential target of ongoing research (Gallant et al. 2005). Disrupting quorum sensing could be the most specific and unique target for biofilm therapeutics. A variety of novel mechanisms, including the substitution of furanones and the enzymatic cleavage of acyl-homoserine lactones (one of the quorum sensing signals), can interfere with quorum sensing (Koch et al. 2005). Targeting quorum-sensing signals at the molecular level is an area

of continuing research and has potential for biofilm interventions and eradication (Dong et al. 2001). However, multiple lines of investigation should be considered in order to maximize the development of biofilm-specific treatments that show promise in animal models of chronic disease.

8.10 Scientific Challenge/Importance of CRS – Challenge to an Existing Paradigm

While multiple etiologies, including host and environmental factors, contribute to the development of CRS, a universal histopathologic finding is uncharacterized mucosal inflammation. Regardless of the respective causes of CRS, clinicians are left with a significant patient population with persistent and recurrent inflammation and infections that are recalcitrant to antimicrobial or surgical therapy. These patients have chronic sinonasal irritation with a dramatic loss in their quality of life. Many studies have investigated CRS-associated local (sinonasal) and systemic inflammation in the context of specific bacterial and fungal pathogens. The current paradigm of CRS is that the presence of a single pathogen, such as *Staphylococcus aureus*, *P. aeruginosa*, or *Streptococcus pneumoniae*, leads to inflammatory events which over time result in chronic disease. To innovate the approach to clinical treatment of CRS, scientists must begin to focus on the polymicrobial nature of the disease. A unique approach to understand this disease may be based on the microbial ideals of ecology, versus the standard approach of studying virulence factors or single genes of single organisms. This new direction in CRS research, and the role of polymicrobial biofilms, will bring fundamental changes in patient treatment and improved patient health.

8.11 Conclusions

Since their initial characterization over four decades ago, biofilms have become a major focus of study for many chronic diseases. Because of their inherent resistance, both to antimicrobial agents and to components of the host's immune system, they often initiate inflammatory responses that ultimately destroy healthy tissue. Although these communities have been associated with a number of diseases in humans, only recently this concept has been applied to chronic rhinosinusitis. As with most biofilm-mediated diseases, the original observations were of microbial communities attached to human cells, visualized by electron microscopy. These early studies were paramount to the study of CRS because the biofilm phenotype explained much of the disease pathology. However, the etiology of the disease remains undefined, and because of this, patient treatment regimes are incomplete at best. Functional endoscopic sinus surgery, where biofilm-containing tissue is removed, has provided a great deal of clinical relief to patients, but this therapy is not effective for approximately 30% of CRS patients. In order to improve patient treatment, the microorganisms, living as biofilm communities, must be defined. Utilizing

microbial ecological approaches, as well as state-of-the-art molecular techniques, it is clear that CRS is a polymicrobial disease. Once the offending microbes are elucidated, it may be possible to either drive the selection and expansion of specific microbes through quorum sensing analogs (for example) or develop selective means of bacterial-specific killing (for example, bacteriophage therapy). As the data supporting the contribution of biofilms to the persistence of CRS build, it becomes more evident that novel anti-biofilm therapies must be developed.

References

- Allegrucci M, Hu FZ et al (2006) Phenotypic characterization of *Streptococcus pneumoniae* biofilm development. *J Bacteriol* 188(7):2325–2335
- Anderson GG, Moreau-Marquis S et al (2008) In vitro analysis of tobramycin-treated *Pseudomonas aeruginosa* biofilms on cystic fibrosis-derived airway epithelial cells. *Infect Immun* 76(4):1423–1433
- Bachert C, Gevaert P et al (2002) Staphylococcus aureus superantigens and airway disease. *Curr Allergy Asthma Rep* 2(3):252–258
- Bage N, Hentzer M et al (2004) Dynamics and spatial distribution of beta-lactamase expression in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* 48(4):1168–1174
- Becker P, Hufnagle W et al (2001) Detection of differential gene expression in biofilm-forming versus planktonic populations of *Staphylococcus aureus* using micro-representational-difference analysis. *Appl Environ Microbiol* 67(7):2958–2965
- Bendouah Z, Barbeau J et al (2006) Biofilm formation by *Staphylococcus aureus* and *Pseudomonas aeruginosa* is associated with an unfavorable evolution after surgery for chronic sinusitis and nasal polyposis. *Otolaryngol Head Neck Surg* 134(6):991–996
- Bernstein JM, Ballou M et al (2003) A superantigen hypothesis for the pathogenesis of chronic hyperplastic sinusitis with massive nasal polyposis. *Am J Rhinol* 17(6):321–326
- Bolger WE, Leonard D et al (1997) Gram negative sinusitis: a bacteriologic and histologic study in rabbits. *Am J Rhinol* 11(1):15–25
- Bollinger N, Hassett DJ et al (2001) Gene expression in *Pseudomonas aeruginosa*: evidence of iron override effects on quorum sensing and biofilm-specific gene regulation. *J Bacteriol* 183(6):1990–1996
- Buzina W, Braun H et al (2003) *Bipolaris spicifera* causes fungus balls of the sinuses and triggers polypoid chronic rhinosinusitis in an immunocompetent patient. *J Clin Microbiol* 41(10):4885–4887
- Chiu AG, Palmer JN et al (2008) Baby shampoo nasal irrigations for the symptomatic post-functional endoscopic sinus surgery patient. *Am J Rhinol* 22(1):34–37
- Christensen LD, Moser C et al (2007) Impact of *Pseudomonas aeruginosa* quorum sensing on biofilm persistence in an in vivo intraperitoneal foreign-body infection model. *Microbiology* 153(Pt 7):2312–2320
- Cohen NA, Kennedy DW (2005) Endoscopic sinus surgery: where we are-and where we're going. *Curr Opin Otolaryngol Head Neck Surg* 13(1):32–38
- Costerton JW, Stewart PS et al (1999) Bacterial biofilms: a common cause of persistent infections. *Science* 284(5418):1318–1322
- Coticchia J, Zuliani G et al (2007) Biofilm surface area in the pediatric nasopharynx: Chronic rhinosinusitis vs obstructive sleep apnea. *Arch Otolaryngol Head Neck Surg* 133(2):110–114
- Cryer J, Schipor I et al (2004) Evidence of bacterial biofilms in human chronic sinusitis. *ORL J Otorhinolaryngol Relat Spec* 66(3):155–158

- da Fonseca EL, Freitas Fdos S et al (2008) Detection of new arr-4 and arr-5 gene cassettes in clinical *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* strains from Brazil. *Antimicrob Agents Chemother* 52(5):1865–1867
- Davies DG, Parsek MR et al (1998) The involvement of cell-to-cell signals in the development of a bacterial biofilm. *Science* 280(5361):295–298
- Desrosiers M, Myntti M et al (2007) Methods for removing bacterial biofilms: in vitro study using clinical chronic rhinosinusitis specimens. *Am J Rhinol* 21(5):527–532
- Dong YH, Wang LH et al (2001) Quenching quorum-sensing-dependent bacterial infection by an N-acyl homoserine lactonase. *Nature* 411(6839):813–817
- Donlan RM (2000) Role of biofilms in antimicrobial resistance. *ASAIO J* 46(6):S47–52
- Donlan RM (2002) Biofilms: microbial life on surfaces. *Emerg Infect Dis* 8(9):881–890
- Donlan RM, Costerton JW (2002) Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* 15(2):167–193
- Ehrlich GD, Veeh R et al (2002) Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA* 287(13):1710–1715
- Ferguson BJ, Stolz DB (2005) Demonstration of biofilm in human bacterial chronic rhinosinusitis. *Am J Rhinol* 19(5):452–457
- Gallant CV, Daniels C et al (2005) Common beta-lactamases inhibit bacterial biofilm formation. *Mol Microbiol* 58(4):1012–1024
- Gates GA (1994) Adenoidectomy for otitis media with effusion. *Ann Otol Rhinol Laryngol Suppl* 163:54–58
- Gilbert P, Maira-Litran T et al (2002) The physiology and collective recalcitrance of microbial biofilm communities. *Adv Microb Physiol* 46:202–256
- Gillis RJ, Iglewski BH (2004) Azithromycin retards *Pseudomonas aeruginosa* biofilm formation. *J Clin Microbiol* 42(12):5842–5845
- Gliklich RE, Metson R (1995) The health impact of chronic sinusitis in patients seeking otolaryngologic care. *Otolaryngol Head Neck Surg* 113(1):104–109
- Gosepath J, Mann WJ (2005) Role of fungus in eosinophilic sinusitis. *Curr Opin Otolaryngol Head Neck Surg* 13(1):9–13
- Ha KR, Psaltis AJ et al (2007) A sheep model for the study of biofilms in rhinosinusitis. *Am J Rhinol* 21(3):339–345
- Hall-Stoodley L, Hu FZ et al (2006) Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* 296(2):202–211
- Hamilos DL, Lund VJ (2004) Etiology of chronic rhinosinusitis: the role of fungus. *Ann Otol Rhinol Laryngol Suppl* 193:27–31
- Hammer BK, Bassler BL (2003) Quorum sensing controls biofilm formation in *Vibrio cholerae*. *Mol Microbiol* 50(1):101–104
- Head NE, Yu H (2004) Cross-sectional analysis of clinical and environmental isolates of *Pseudomonas aeruginosa*: biofilm formation, virulence, and genome diversity. *Infect Immun* 72(1):133–144
- Healy DY, Leid JG et al (2008) Biofilms with fungi in chronic rhinosinusitis. *Otolaryngol Head Neck Surg* 138(5):641–647
- Hentzer M, Teitzel GM et al (2001) Alginate overproduction affects *Pseudomonas aeruginosa* biofilm structure and function. *J Bacteriol* 183(18):5395–5401
- Hoffman LR, D'Argenio DA et al (2005) Aminoglycoside antibiotics induce bacterial biofilm formation. *Nature* 436(7054):1171–1175
- Hoiby N, Krogh Johansen H et al (2001) *Pseudomonas aeruginosa* and the in vitro and in vivo biofilm mode of growth. *Microbes Infect* 3(1):23–35
- Jelsbak L, Johansen HK et al (2007) Molecular epidemiology and dynamics of *Pseudomonas aeruginosa* populations in lungs of cystic fibrosis patients. *Infect Immun* 75(5):2214–2224
- Jensen PO, Bjarnsholt T et al (2007) Rapid necrotic killing of polymorphonuclear leukocytes is caused by quorum-sensing-controlled production of rhamnolipid by *Pseudomonas aeruginosa*. *Microbiology* 153(Pt 5):1329–1338

- Kadouri D, O'Toole GA (2005) Susceptibility of biofilms to *Bdellovibrio bacteriovorus* attack. *Appl Environ Microbiol* 71(7):4044–4051
- Kennedy DW (2004) Pathogenesis of chronic rhinosinusitis. *Ann Otol Rhinol Laryngol Suppl* 193:6–9
- Klausen M, Gjermansen M et al (2006) Dynamics of development and dispersal in sessile microbial communities: examples from *Pseudomonas aeruginosa* and *Pseudomonas putida* model biofilms. *FEMS Microbiol Lett* 261(1):1–11
- Koch B, Liljefors T et al (2005) The LuxR receptor: the sites of interaction with quorum-sensing signals and inhibitors. *Microbiology* 151(Pt 11):3589–3602
- Landry RM, An D et al (2006) Mucin-*Pseudomonas aeruginosa* interactions promote biofilm formation and antibiotic resistance. *Mol Microbiol* 59(1):142–151
- Leid JG, Costerton JW et al (2002) Immunology of Staphylococcal biofilm infections in the eye: new tools to study biofilm endophthalmitis. *DNA Cell Biol* 21(5–6):405–413
- Leid JG, Shirtliff ME et al (2002) Human leukocytes adhere to, penetrate, and respond to *Staphylococcus aureus* biofilms. *Infect Immun* 70(11):6339–6345
- Leid JG, Willson CJ et al (2005) The exopolysaccharide alginate protects *Pseudomonas aeruginosa* biofilm bacteria from IFN- γ -mediated macrophage killing. *J Immunol* 175(11):7512–7518
- Leid JG, Kerr M et al (2009) Flagellar-mediated biofilm defense mechanisms of *Pseudomonas aeruginosa* against host derived lactoferrin. *Infection and Immunity* 77:4559–4566
- Mah TF, O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. *Trends Microbiol* 9(1):34–39
- Mah TF, Pitts B et al (2003) A genetic basis for *Pseudomonas aeruginosa* biofilm antibiotic resistance. *Nature* 426(6964):306–310
- Maw AR (1985) Age and adenoid size in relation to adenoidectomy in otitis media with effusion. *Am J Otolaryngol* 6(3):245–248
- Nandakumar K, Obika H et al (2004) Recolonization of laser-ablated bacterial biofilm. *Biotechnol Bioeng* 85(2):185–189
- Nivens DE, Ohman DE et al (2001) Role of alginate and its O acetylation in formation of *Pseudomonas aeruginosa* microcolonies and biofilms. *J Bacteriol* 183(3):1047–1057
- O'Toole G, Kaplan HB et al (2000) Biofilm formation as microbial development. *Annu Rev Microbiol* 54:49–79
- Ooi EH, Wormald PJ et al (2008) Innate immunity in the paranasal sinuses: a review of nasal host defenses. *Am J Rhinol* 22(1):13–19
- Oulahal-Lagsir N, Martial-Gros A et al (2000) Ultrasonic methodology coupled to ATP bioluminescence for the non-invasive detection of fouling in food processing equipment—validation and application to a dairy factory. *J Appl Microbiol* 89(3):433–441
- Oxley KS, Thomas JG et al (2007) Effect of ototopical medications on tympanostomy tube biofilms. *Laryngoscope* 117(10):1819–1824
- Parkar SG, Flint SH et al (2004) Evaluation of the effect of cleaning regimes on biofilms of thermophilic bacilli on stainless steel. *J Appl Microbiol* 96(1):110–116
- Parks QM, Young RL et al (2009) Neutrophil enhancement of *Pseudomonas aeruginosa* biofilm development: human F-actin and DNA as targets for therapy. *J Med Microbiol* 58(Pt 4):492–502
- Parsek MR, Singh PK (2003) Bacterial biofilms: an emerging link to disease pathogenesis. *Annu Rev Microbiol* 57:677–701
- Perloff JR, Palmer JN (2004) Evidence of bacterial biofilms on frontal recess stents in patients with chronic rhinosinusitis. *Am J Rhinol* 18(6):377–380
- Perloff JR, Palmer JN (2005) Evidence of bacterial biofilms in a rabbit model of sinusitis. *Am J Rhinol* 19(1):1–6
- Post JC, Aul JJ et al (1996) PCR-based detection of bacterial DNA after antimicrobial treatment is indicative of persistent, viable bacteria in the chinchilla model of otitis media. *Am J Otolaryngol* 17(2):106–111

- Post JC, Stoodley P et al (2004) The role of biofilms in otolaryngologic infections. *Curr Opin Otolaryngol Head Neck Surg* 12(3):185–190
- Prince AA, Steiger JD et al (2008) Prevalence of biofilm-forming bacteria in chronic rhinosinusitis. *Am J Rhinol* 22(3):239–245
- Psaltis AJ, Ha KR et al (2007) Confocal scanning laser microscopy evidence of biofilms in patients with chronic rhinosinusitis. *Laryngoscope* 117(7):1302–1306
- Psaltis AJ, Weitzel EK et al (2008) The effect of bacterial biofilms on post-sinus surgical outcomes. *Am J Rhinol* 22(1):1–6
- Ramadan HH, Sanclement JA et al (2005) Chronic rhinosinusitis and biofilms. *Otolaryngol Head Neck Surg* 132(3):414–417
- Ratner AJ, Lysenko ES et al (2005) Synergistic proinflammatory responses induced by polymicrobial colonization of epithelial surfaces. *Proc Natl Acad Sci USA* 102(9):3429–3434
- Ren D, Bedzyk LA et al (2004) Gene expression in *Escherichia coli* biofilms. *Appl Microbiol Biotechnol* 64(4):515–524
- Sanclement JA, Webster P et al (2005) Bacterial biofilms in surgical specimens of patients with chronic rhinosinusitis. *Laryngoscope* 115(4):578–582
- Sanderson AR, Leid JG et al (2006) Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. *Laryngoscope* 116(7):1121–1126
- Sauer K, Camper AK et al (2002) *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* 184(4):1140–1154
- Senior BA, Kennedy DW et al (1998) Long-term results of functional endoscopic sinus surgery. *Laryngoscope* 108(2):151–157
- Sheehan E, McKenna J et al (2004) Adhesion of *Staphylococcus* to orthopaedic metals, an in vivo study. *J Orthop Res* 22(1):39–43
- Shirliff ME, Mader JT et al (2002) Molecular interactions in biofilms. *Chem Biol* 9(8):859–871
- Smith RS, Iglewski BH (2003) *P. aeruginosa* quorum-sensing systems and virulence. *Curr Opin Microbiol* 6(1):56–60
- Stewart PS, Costerton JW (2001) Antibiotic resistance of bacteria in biofilms. *Lancet* 358(9276):135–138
- Tripathi A, Conley DB et al (2004) Immunoglobulin E to staphylococcal and streptococcal toxins in patients with chronic sinusitis/nasal polyposis. *Laryngoscope* 114(10):1822–1826
- Vallet I, Olson JW et al (2001) The chaperone/usher pathways of *Pseudomonas aeruginosa*: identification of fimbrial gene clusters (cup) and their involvement in biofilm formation. *Proc Natl Acad Sci USA* 98(12):6911–6916
- Veeh RH, Shirliff ME et al (2003) Detection of *Staphylococcus aureus* biofilm on tampons and menses components. *J Infect Dis* 188(4):519–530
- Vlastarakos PV, Nikolopoulos TP et al (2007) Biofilms in ear, nose, and throat infections: how important are they? *Laryngoscope* 117(4):668–673
- Walker TS, Tomlin KL et al (2005) Enhanced *Pseudomonas aeruginosa* biofilm development mediated by human neutrophils. *Infect Immun* 73(6):3693–3701
- Wargo MJ, Hogan DA (2006) Fungal – bacterial interactions: a mixed bag of mingling microbes. *Curr Opin Microbiol* 9(4):359–364
- Webb JS, Givskov M et al (2003) Bacterial biofilms: prokaryotic adventures in multicellularity. *Curr Opin Microbiol* 6(6):578–585
- Whiteley M, Bangera MG et al (2001) Gene expression in *Pseudomonas aeruginosa* biofilms. *Nature* 413(6858):860–864
- Woodworth BA, Antunes MB et al (2007) Murine tracheal and nasal septal epithelium for air-liquid interface cultures: a comparative study. *Am J Rhinol* 21(5):533–537
- Wozniak DJ, Keyser R (2004) Effects of subinhibitory concentrations of macrolide antibiotics on *Pseudomonas aeruginosa*. *Chest* 125(2 Suppl):62S–69S; quiz 69S
- Zuliani G, Carron M et al (2006) Identification of adenoid biofilms in chronic rhinosinusitis. *Int J Pediatr Otorhinolaryngol* 70(9):1613–1617