Biofilm formation and biofilm dispersal with Streptococcus pneumoniae

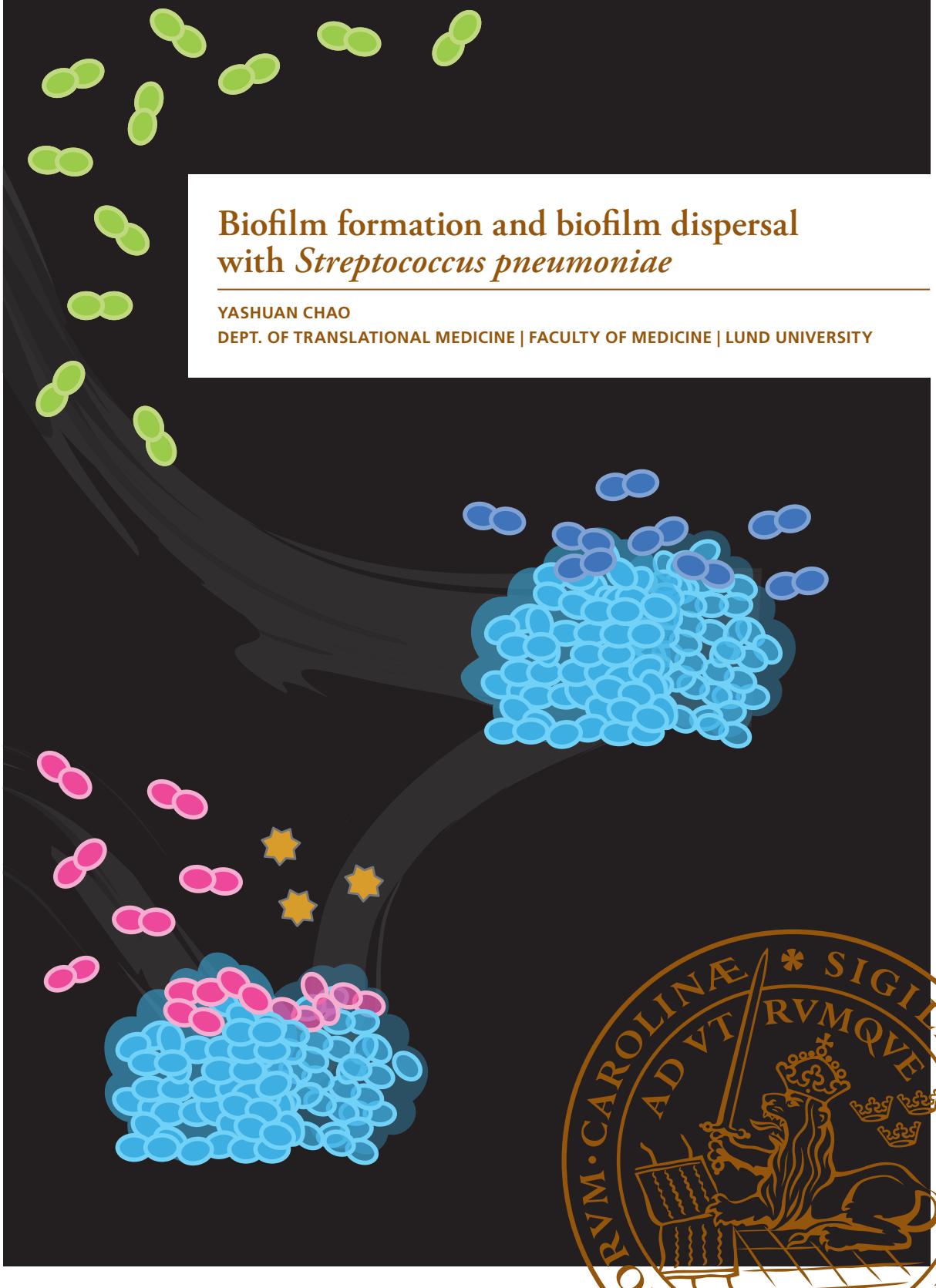
Chao, Yashuan

2019

Document Version:
Publisher's PDF, also known as Version of record

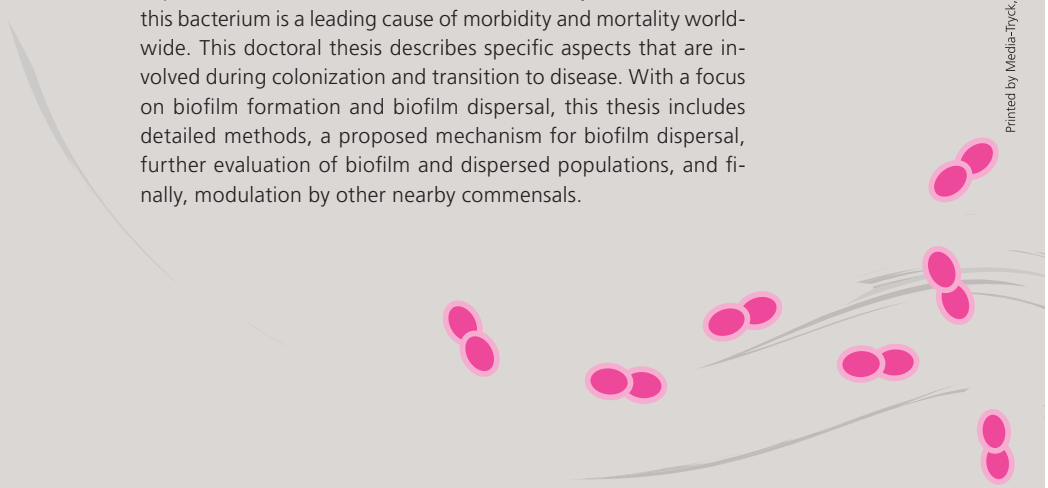
Biofilm formation and biofilm dispersal with *Streptococcus pneumoniae*

YASHUAN CHAO
DEPT. OF TRANSLATIONAL MEDICINE | FACULTY OF MEDICINE | LUND UNIVERSITY



At any given moment, about 25-80% of the world's population carry the bacterium *Streptococcus pneumoniae*. With a population of just over 7 billion, this means up to 5.6 billion of us carry this bacterium right now. How come we're not all feeling sick? It's because this bacterium spends the majority of its life as a harmless commensal. Its primary residence is on the surface behind our nose. Here, the bacterium colonizes by forming biofilms—a community of cells that adhere to a surface and are embedded in a protective matrix. However, a disturbance in the surrounding environment can trigger bacterial release from the biofilm (so-called biofilm dispersal). The dispersed bacteria can then travel to other sites in our bodies and give rise to a wide range of infections, such as middle ear infection, pneumonia, and sepsis. Every year, approximately 1-2 million deaths occur as a result.

S. pneumoniae is a common resident in healthy individuals. Yet, this bacterium is a leading cause of morbidity and mortality worldwide. This doctoral thesis describes specific aspects that are involved during colonization and transition to disease. With a focus on biofilm formation and biofilm dispersal, this thesis includes detailed methods, a proposed mechanism for biofilm dispersal, further evaluation of biofilm and dispersed populations, and finally, modulation by other nearby commensals.



Printed by Media-Tryck, Lund 2019 NORDIC SWAN ECOLABEL 3041 0903

LUND UNIVERSITY

FACULTY OF MEDICINE

Department of Translational Medicine

Lund University, Faculty of Medicine
Doctoral Dissertation Series 2019:84
ISBN 978-91-7619-813-1
ISSN 1652-8220

Biofilm formation and biofilm dispersal
with *Streptococcus pneumoniae*

Biofilm formation and biofilm dispersal with *Streptococcus pneumoniae*

Yashuan Chao

LUND UNIVERSITY

DOCTORAL DISSERTATION

by due permission of the Faculty of Medicine, Lund University, Sweden.
To be defended:
9:00 am on September 19, 2019
Pathology building lecture hall
Jan Waldenströms gata 59, Malmö, Sweden

Faculty opponent
Professor Sven Hammerschmidt
University of Greifswald
Greifswald, Germany

Organization Lund University Faculty of Medicine	**Document name** Doctoral Dissertation
Department off Translational Medicine	**Date of issue** September 19, 2019
Author Yashuan Chao	**Sponsoring organization**

Title Biofilm formation and biofilm dispersal with *Streptococcus pneumoniae*

Abstract

Streptococcus pneumoniae (the pneumococcus) asymptomatically colonizes the human nasopharynx by forming biofilms. Upon exposure to disease triggers, such as fever induced by respiratory virus infection, bacteria are released from the biofilm and can disseminate to other sites and cause infection. As a result, approximately 1-2 million deaths occur every year. These dispersed bacteria have distinct transcriptional and phenotypic profiles as compared with biofilm bacteria and broth-grown, planktonic bacteria. However, the specific mechanisms involved in triggered biofilm dispersal are still under investigation..

The aim of this doctoral thesis was to identify mechanisms that are involved during pneumococcal biofilm formation and biofilm dispersal. We first developed and described methods to study these processes that are associated with colonization and transition to disease. Using these methods, we indicated a role for proteases in biofilm dispersal and proposed a role for serine protease HtrA in heat-induced biofilm dispersal. We also used the methods to derive pneumococcal populations associated with colonization (biofilm bacteria), disease (dispersed bacteria), and conventional broth-grown culture (planktonic bacteria). Proteomic analysis indicated differences between pneumococcal populations, especially regarding metabolic pathways. Most differences were seen between planktonic bacteria and biofilm-derived bacteria. Finally, we used an adapted version of the models and showed that respiratory commensal *Corynebacterium* spp. (corynebacteria) can form biofilms and that subsequent acquisition of the pneumococcus results in dual-species biofilms without affecting the corynebacteria biofilm biomass and function, although with potential protective effects on the pneumococcus. Altogether, this thesis addresses aspects that contribute to the different lifestyles of the pneumococcus.

Key words Biofilm, Biofilm dispersal, *Streptococcus pneumoniae*, Colonization, Proteome, Bacterial phenotype, *Corynebacterium*

Classification system and/or index terms (if any)

Supplementary bibliographical information		**Language** English
ISSN 978-91-7619-813-1 Doctoral Dissertaion Series 2019:84		**ISBN** 1652-8220
Recipient's notes	**Number of pages 190**	**Price**
	Security classification	

Signature  **Date** 2019-07-29

Biofilm formation and biofilm dispersal with *Streptococcus pneumoniae*

Yashuan Chao

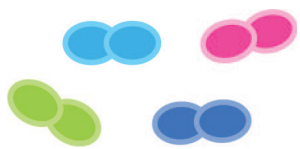
LUND
UNIVERSITY

Cover photo by Yashuan Chao

Faculty of Medicine
Department of Translational Medicine

Doctoral Dissertation Series 2019:84
ISBN 1652-8220
ISSN 978-91-7619-813-1

Printed in Sweden by Media-Tryck, Lund University
Lund 2019



Preface

Thoughts that went into the writing.

The PhD is more than just training to be an independent researcher, it is a personal development program. It is amazing how much I have learned about myself as well as about the world of research over these years. It has been exciting to see this writing come together, from scribbles written on sticky notes to a very long list of thoughts and questions, all sporadically accumulated over the years. Putting everything together has been quite satisfying.

Overall, this thesis consists of six chapters. The first chapter sets the foundation and provides the context for the subject at hand. A focus on specific topics is given in Chapters 2-5. These chapters are intended to highlight and give the framework for the different papers that are appended to this thesis. I have also included a summary in plain English for the papers. Lastly, the final chapter concludes the thesis with a discussion of the papers, central themes, and future perspectives.

My hope is that I have provided enough context at the beginning such that the rest of the chapters fall into place.

July 28, 2019

Acknowledgments

Numerous people have contributed to this thesis, whether it was scientific contribution, administrative assistance, moral support, or just being present. Please know that I am very grateful and that I sincerely appreciate each one of you for the interactions that we have had. This thesis truly would not have come to fruition without the support from all of you. Thank you so much.

I would like to extend an additional thanks to the following:

Department of Translational Medicine, Faculty of Medicine, Lund University, for providing a supportive environment for my PhD studies.

Funders, for contributions without which this thesis could not have been completed, and for experience in research grant writing. Specific funders are acknowledged in the appended papers.

My supervisor, Anders Håkansson

Anders, I will always remember when you asked if I wanted to continue my studies in Sweden. *momentary pause to not sound too impulsive* Yes! The reasons for why it was an easy decision then are the still the same now. You are kind and thoughtful and you truly seek to guide your students. Your vast amount of ideas is inspiring. I appreciate the freedom you give regarding research questions. You have always given me constructive thoughts about my work, while also leaving room for me to develop on my own. You have always extended your support and guidance. Thank you for the opportunity to do research in your group, for trusting me, teaching me, encouraging me, and making sure of my wellbeing.

My co-supervisors, Caroline Bergenfelz and Anna Blom

Caroline, for your contributions in the projects and for being realistic about deadlines, and for always being up for ice cream. **Anna**, for assisting with the admissions process while we were still in the U.S., for continuously being available for advice, and for your inspiring problem-solving skills.

Past and present members of the Division of Experimental Infection Medicine

Fellow PhD students in the lab: **Goutham**, for your deep thinking and your sense of humor (I'll never again be able to patch clones without laughing), and for always cheering me on. Writing is for friends as well! **Feiruz**, for our much-needed café dates and for being on the same wavelength about so many things. To both of you, I am so thankful that we could support each other during the PhD experience.

Anki, for your inspiring creativity, both at work and in home experiments. **Michelle**, for your modesty and for your mentality that we never stop learning. **Sandy**, **Kasper**, **Emily**, **Hanna**, **Jacob**, **Marcus**, and **Selina**, for your curiosity and eagerness to learn.

Collaborators

Johan and **Anahita**, for your enthusiasm and commitment, and for being so easy to work with. **Debby** and **Bas**, for being equally excited and curious about dual-species biofilms. **Melinda**, for your kind and helpful correspondences over the years.

From Lund University

Maria, for delightful conversations and for all of your help with scanning electron microscopy. **Mattias** and **Julia**, for your thoughtful questions and an enjoyable discussion during my half-time seminar. **Anette**, **Cecilia**, **Sandra**, and **Ulrika**, for facilitating an array of administrative tasks that I would otherwise not know how to handle. A special thanks to Eva-Lotta for addressing every one of my questions over the years. **Henric**, **Gerry**, and **Rebecca**, for making things happen effortlessly. **Kristian**, for reviewing my research grant work reports over the years. **Shanice**, for helping me find an apartment when I was first moving to Sweden and for being my first friend in Malmö. **Former and present Wallenberg lab members**, for the welcoming environment and the stimulating discussions.

From University at Buffalo

The short period I spent in Buffalo is not short of great times, and it remains to be one of the highlights of my life. I often reminisce about my times there both in and outside of lab. **Hazeline**, for your kindness and sense of humor and for teaching me lab techniques in Buffalo. I am indebted to your systematic way of teaching and I still use your tips and tricks today. **Ryan**, **Emily, Michelle**, **Alex**, for the summer of 2012 where it all began. A special thanks to **Laura** for teaching me the biofilm ways. **Buffalo family** (you know who you are), for getting through the first year of PPBS together with an

alarming amount of food-related trips and home events and also for attempting to balance that by going to Crunch. **Bralavan**, for our friendship that continues to grow every day.

From University of Colorado Denver

I still can't bring myself to use first names with professors from my university days. **Dr. Roche** and **Matt**, for your enthusiasm and for igniting my interest in microbiology. **Dr. Phiel**, for your dedication to comprehensive learning by employing conceptual thinking and meticulous lab techniques.

Dance family, for all of the positive energy and the purest form of human connection. **Malmö friends**, for sharing the same appetite for food, beer, and fun. Another tasting event coming soon! **Boulder friends**, for our friendships that stayed the same when everything else changed.

My family, both near and far, for always being supportive of my educational endeavors. 謝謝你為我所做的一切. **Tobi goby**, for keeping me company on those late nights and making sure that I was hydrated, for those dark nights when I had to take care of my biofilms and you would walk with me to lab to make sure I was safe, for your endless care, support, and encouragement, and for always believing in me.

Thank you!

Table of Contents

List of Papers

Paper 1

Yashuan Chao, Caroline Bergenfelz, and Anders P. Håkansson. *In vitro* and *in vivo* biofilm formation by pathogenic Streptococci. Methods in Molecular Biology: Volume 1535. Dec 2016.

Paper 2

Yashuan Chao, Caroline Bergenfelz, and Anders P. Håkansson. Growing and characterizing biofilms formed by *Streptococcus pneumoniae*. Methods in Molecular Biology: Volume 1968. March 2019

Paper 3

Yashuan Chao, Caroline Bergenfelz, and Anders P. Håkansson. Involvement of the serine protease HtrA in heat-induced dispersal of pneumococcal biofilms. Submitted and under review.

Paper 4

Yashuan Chao*, Anahita Bakochi*, Caroline Bergenfelz, Johan Malmström, and Anders P. Håkansson. Proteome profiles of pneumococcal populations associated with colonization and disease. In manuscript. *authors contributed equally

Paper 5

Caroline Bergenfelz, Yashuan Chao, and Anders P. Håkansson. Biofilm formation and inflammatory responses by respiratory tract commensal corynebacteria. In manuscript.

Published work completed during the PhD studies, but that are not included in this thesis:

Yashuan Chao, Laura R. Marks, Melinda M. Pettigrew, and Anders P. Håkansson. *Streptococcus pneumoniae* biofilm formation and dispersion during colonization and disease. Frontiers in Cellular and Infection Microbiology. January 2015.

Popular science summary

At any given moment, about 25-80% of the world's population carry the bacterium *Streptococcus pneumoniae*. With a population of just over 7 billion, this means up to 5.6 billion of us carry this bacterium right now. How come we're not all feeling sick? It's because this bacterium spends the majority of its life as a harmless commensal. Its primary residence is on the surface behind our nose. Here, the bacterium colonizes by forming biofilms—a community of cells that adhere to a surface and are embedded in a protective matrix. However, a disturbance in the surrounding environment can trigger bacterial release from the biofilm (so-called biofilm dispersal). The dispersed bacteria can then travel to other sites in our bodies and give rise to a wide range of infections, such as middle ear infection, pneumonia, and sepsis. Every year, approximately 1-2 million deaths occur as a result.

S. pneumoniae is a common resident in healthy individuals. Yet, this bacterium is a leading cause of morbidity and mortality worldwide. The aim of this doctoral thesis was to identify specific mechanisms that are involved during biofilm formation and biofilm dispersal with *S. pneumoniae*. We first developed and described, in detail, the methods to study these processes. Our methods attempt to mimic the environment where the bacteria normally reside in the body.

Our model systems were first used for studying how bacteria are released from biofilms upon heat exposure, which mimics fever. The biofilm is made up of components like sugars, proteins, and fats. Therefore, we expected that degrading these components would release bacteria from the biofilm. We showed that enzymes that cleave proteins (i.e., proteases) could disperse the biofilms. Similarly, when we blocked the activity of proteases, we saw an inhibition of heat-induced dispersal. We then formed biofilms with a strain that lacks the protease HtrA. These bacteria formed normal biofilms, but did not disperse upon heat exposure as well as strains that expressed the protease HtrA. This suggested to us that protease HtrA is not involved during biofilm formation (associated with colonization), but plays a role during dispersal of biofilms by heat (associated with disease).

We next used the same methods to obtain bacterial populations associated with colonization (biofilm bacteria) or disease (dispersed bacteria) to better understand how they differ. We also included bacteria that were grown in broth (planktonic bacteria) that are commonly used in research studies. We evaluated the abundance of different

proteins in the various bacterial populations to determine each population's traits. We found that the majority of differences between populations were associated with proteins involved in metabolic pathways. Most differences were seen between planktonic bacteria (broth bacteria) and the biofilm-derived bacteria.

Finally, we adapted the biofilm formation methods and used them with commensal *Corynebacterium* species. The presence of *Corynebacterium* in the environment of the nose has been shown to be protective in respiratory health. We found that these bacteria can also form biofilms and with minimal toxicity to respiratory epithelial cells. The bacteria were also able to induce a transient inflammatory response in the epithelial cells. When we added *S. pneumoniae* to the *Corynebacterium* biofilms, they were able to form dual-species biofilms. The presence of *S. pneumoniae* did not seem to impact the *Corynebacterium* biofilm, and there appeared to be a protective effect for *S. pneumoniae* by *Corynebacterium*.

Understanding biofilm formation and biofilm dispersal are aspects that may also provide information for designing new therapeutics. Teasing out the specific mechanisms of how biofilm dispersal occurs as a response to environment signals like fever may provide new targets. These targets would prevent transition to disease while allowing for symptomless colonization to persist. Biofilm and dispersed populations expressed different proteins. The different proteins are potential targets that would be specific for the respective population. For example, a protein that is abundant only in dispersed bacteria would be a specific therapeutic target for dispersed bacteria (disease), but not biofilm bacteria (colonization). Better understanding how commensal bacteria are protective during colonization may lead to the development of probiotics. In conclusion, understanding biofilm formation and biofilm dispersal with *S. pneumoniae* are important aspects for subsequently understanding colonization and disease.

Introduction

The concept of identifying a causative relationship between microbe and disease was introduced in Koch's postulates in 1884[1,2]. As summarized, the following criteria would identify a pathogen and prove the causative relationship between a microbe and its proposed disease:

1. *the microbe must be found in all cases of disease;*
2. *the microbe must be isolated from the diseased host and grown in pure culture in the laboratory;*
3. *introduction of the microbe to a new host must cause the same disease; and,*
4. *the microbe must be re-isolated from the newly diseased host.*

Not too long after, scientists including Koch himself, realized that these postulates had limitations. Still, Koch's postulates have been an important guideline for research in microbiology. Even modified versions exist, such as the relationship between microbial factors and disease[3] as well as between microbes found in the host and health or disease states[4,5]. Overall, there has been a growing understanding of the complex interactions during infectious disease development[6].

One microbe that does not fulfill Koch's postulates is the bacterium *Streptococcus pneumoniae* (the pneumococcus). This bacterium primarily resides in the human upper respiratory tract without causing clinical symptoms. From there, under certain circumstances, the bacterium can migrate to and infect otherwise non-infected host sites, which can then lead to diseases with high morbidity and mortality. Included in this thesis are methods to study distinct life stages of the pneumococcus, such as asymptomatic carriage and transition to disease, as well as a proposed mechanism of how the latter occurs. Bacterial populations associated with these life stages are given a closer look, and finally, the modulation by beneficial microbes is also addressed. Altogether, this thesis attempts to identify what contributes to the different lifestyles of the pneumococcus.

Chapter 1: Life in the human host

Pneumococcus is an altogether amazing cell.

Tiny in size, simple in structure, frail in make-up, it possesses physiological functions of great variety, performs feats of extraordinary intricacy and, attacking man, sets up a stormy disease so often fatal that it must be reckoned as one of the foremost causes of human death.

Benjamin White, 1938

This chapter will provide a brief overview of the different life stages of *Streptococcus pneumoniae*. First, is an introduction to the bacterium and the diseases it causes as well as the current state of therapeutic intervention. The remainder of the chapter focuses on host-microbe interactions and the biological facets that contribute to these interactions from acquisition to disease.

Streptococcus pneumoniae

Since its isolation in 1881[7,8], *Streptococcus pneumoniae* has been an important organism in developing basic principles of biology. In 1928, Griffith used the pneumococcus to demonstrate that bacterial traits could be naturally heritable, termed the transformation principle[9], and the genetic material was later identified to be DNA by Avery, Macleod, and McCarty in 1944[10]. Other significant breakthroughs include the development of Gram's stain for bacterial identification, the first non-protein vaccine, and the concept of antimicrobial resistance[11,12]. These were all discovered while studying the pneumococcus and pneumococcal disease.

Burden of pneumococcal disease

Despite continuous research on the pneumococcus over the last 100+ years, pneumococcal disease remains a major influence on human health. The pneumococcus is a common colonizer of the human nasopharynx and exists predominantly as a commensal, causing no harm to the host. Colonization is the presence and proliferation of bacteria on or in the host without causing disease. Already during childhood, colonization with the pneumococcus occurs in healthy individuals at approximate rates

of 20-60%, which decreases into adulthood to around 2-20%, whereas rates can be up to around 90% of individuals in resource-poor settings [13-18]. However, the bacterium can disseminate from the nasopharynx and infect otherwise non-infected sites, which can lead to a number of diseases (**Figure 1**). Pneumococcal diseases account for a high burden in medical and economic costs[19].

The pneumococcus colonizes the mucosal surface of the nasopharynx. From there, the bacterium can spread to and infect other sites, such as the middle ear (otitis media), sinuses (sinusitis), or the lungs (pneumonia). Invasive diseases occur when the bacterium crosses the mucosal barrier into the bloodstream, which can escalate to sepsis. The pneumococcus may also cross the blood-brain barrier and infect the meninges (meningitis).

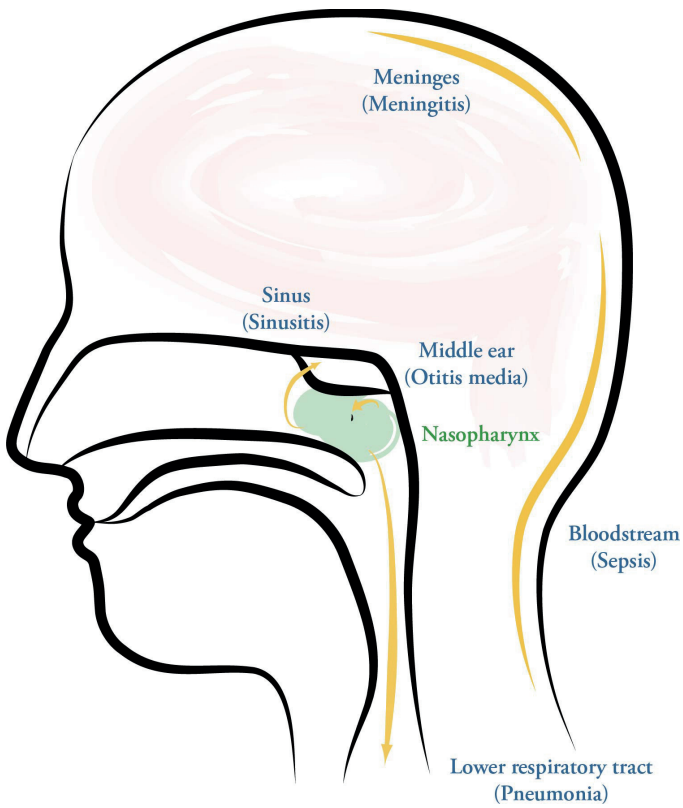


Figure 1. Pneumococcal diseases.
The pneumococcus colonizes the mucosal surface of the nasopharynx. From there, the pneumococcus can spread to and infect other sites, such as the middle ear (otitis media), sinuses (sinusitis), or the lungs (pneumonia). Invasive diseases occur when the bacterium crosses the mucosal barrier into the bloodstream, which can escalate to sepsis. The pneumococcus may also cross the blood-brain barrier and infect the meninges (meningitis).

In 2016, lower respiratory tract infections were estimated to cause nearly 2.4 million deaths worldwide[20]. The pneumococcus was the main etiologic agent and surpassed other etiologies combined, including respiratory syncytial virus, *Haemophilus influenzae* type b, and influenza. The pneumococcus was responsible for over 1.1 million deaths (almost half of the deaths) and 197 million episodes of lower respiratory tract infections, and is considered one of the leading infectious causes of morbidity and mortality[20].

Children and the elderly are the most susceptible populations to pneumococcal disease[21]. Immunocompromised individuals are also susceptible and have higher rates of invasive pneumococcal disease than healthy individuals[22]. In 2016, lower respiratory tract infections caused approximately 650,000 deaths in children under the age of five and nearly 1.1 million deaths in adults older than 70 years[20]. In children under the age of five, the mortality of lower respiratory tract infection increases with lower sociodemographic development[20], where access to healthcare, nutrition, and general hygiene are contributing factors. Interestingly, in the elderly, the mortality rates by location generally do not seem to change[23].

Preventative and treatment approaches

Current therapeutics

Before the introduction of antibiotics for treatment of bacterial infections, most bacterial infectious diseases were deadly. At least 50% and upwards of 90% of patients with pneumococcal bacteremic pneumonia died in the hospital[24]. Treatment with antibiotics has substantially reduced the fatality of disease, and has revolutionized the treatment of infectious diseases to this day. However, reduced susceptibility to antibiotics is increasingly detected in all regions of the world. Antibiotic resistance is considered a global threat to human health, particularly multi-drug resistance (resistance to more than three classes of antibiotics). In 2017, the World Health Organization deemed the pneumococcus as a 'priority pathogen' for which there is an urgent need for new antibiotics[25].

Prevention of pneumococcal disease by vaccines was first introduced in the 1980s. These vaccines target the polysaccharide structure on the surface of the pneumococcus, deemed the capsular polysaccharide or capsule. Vaccines were specifically developed to protect against invasive disease, which is only caused by a subset of the 98 known serotypes[26]—pneumococcal strains that produce a polysaccharide with unique chemical and immunologic (serologic) properties[27]. There are two types of pneumococcal vaccines: polysaccharide vaccine and conjugate vaccine. The 23-valent pneumococcal polysaccharide vaccine was developed in 1983 and contains 23 serotypes, which covered 80-90% of the serotypes causing disease[28]. Children younger than two years of age did not elicit a protective immune response to polysaccharide antigens alone[29].

Therefore, a conjugate of polysaccharide to a non-toxic protein was used in later vaccines, starting with the 7-valent conjugated vaccine. This vaccine contains seven serotypes from the 23-valent vaccine that were common in pediatric invasive disease. Over time, additional serotypes have been added to produce 10-valent and 13-valent conjugate vaccines, with a 15-valent conjugate vaccine in the pipeline[30]

Vaccine implementation has substantially reduced the incidence of invasive pneumococcal disease by serotypes covered in the vaccines[31-34], also known as vaccine types. However, vaccination has also led to serotype replacement, whereby non-vaccine types have emerged in the population in place of vaccine types. Not only have less common serotypes emerged, but prominent vaccine types have also altered their capsule to appear as another serotype, known as serotype (capsular) switching, which allows for the possibility of vaccine escape (**Figure 2**). These phenomena have been well-documented in the post-vaccine era[35-42]. Serotype distribution varies across geographic regions[43,44] and continued surveillance is important for guiding future vaccine development.

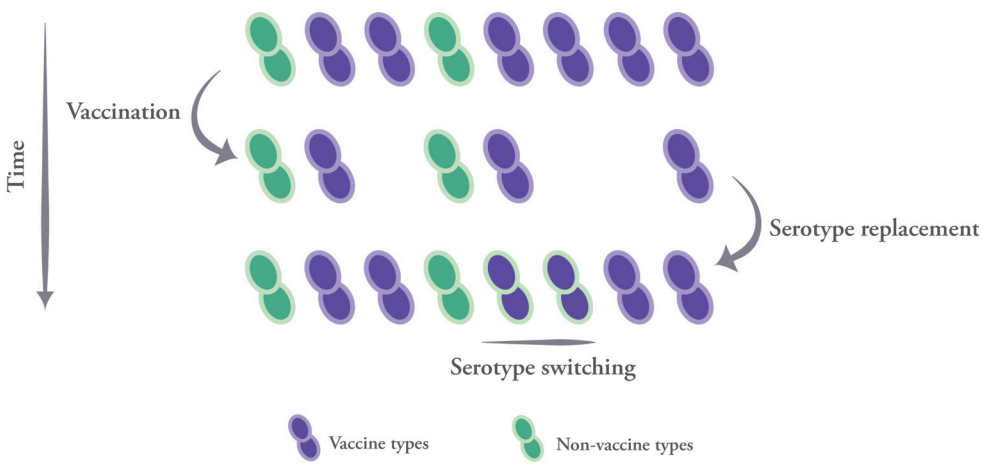


Figure 2. Effects of current vaccines

It has been proposed that targeting a subset of known serotypes in current vaccines was the beginning of an ecological experiment, and that adding new serotypes to the existing vaccines is not a long-term solution as it addresses an immediate problem with another ecological experiment[45]. Given the number of known pneumococcal serotypes, adding more serotypes to the current vaccines is unlikely to cover the majority of serotypes[46]. Despite the shortcomings of current therapeutics, it is clear that usage of

antibiotics and vaccines has decreased the burden of pneumococcal disease. However, in the race against antibiotic resistance and serotype replacement, new strategies are necessary to further protect against pneumococcal infections.

New strategies

There are several pneumococcal protein-based candidates, including whole cell, multi-component (mixture of targets), and chimeric (fusion of targets), many of which are already in clinical trials[28,47,48]. These candidates target surface proteins, which are accessible during nasopharyngeal colonization and would, therefore, provide protection against colonization[48]. This is in contrast to current vaccines that target capsule and primarily protect against invasive disease.

Another preventative approach includes employing the local microbiota. Probiotics are live microorganisms that are beneficial for the host. In clinical trials, probiotics have shown positive effects in upper respiratory tract infections[49,50], and may maintain a healthy upper respiratory tract by competing with pathogens or stimulating the host immune response [50].

Although not experimentally tested, there have been proposals to focus on the host rather than the bacterium. One suggestion is to limit the host immune response, for example, during pneumonia, which may minimize consequences due to excessive inflammation[51]. Another suggestion is to block host receptors, such as endothelium receptors, which may prevent adherence of the pneumococcus to the blood-brain barrier and invasion into the brain from the bloodstream[52]. These suggestions aim to prevent disease progression alongside current therapeutics.

Given that pneumococcal colonization is frequent and is most often harmless, disturbing this primarily commensal bacterium may have unintended effects. Therefore, there may well be an advantage to focusing on disease-specific targets rather than eradication of the bacterium. Such approaches focus on a specific state in commensal disease progression and seek to balance pneumococcal commensalism and protection of the host from pneumococcal disease[53-55]. The work in this thesis attempts to contribute to these approaches as well.

New strategies are serotype-independent and approach prevention in a different manner than current vaccines. These strategies focus on broader coverage and include other key players involved in pneumococcal disease. Progress is being made, and these advances are a result of ongoing research on the pneumococcus and pneumococcal disease.

Host-microbe interactions

A microbe's ability to cause disease—its pathogenicity—is often determined by its virulence factors. These are defined as factors that impair virulence (or harmfulness to the host) when lost. However, in the context of a complex environment within the host, the virulence of a microbe is also influenced by its surroundings. Such factors include the local microbiota and the host's defense mechanisms. It is not the microbe alone, but rather the interactions between the microbe, the microbial community, and other host factors that together contribute to disease development (**Figure 3**).

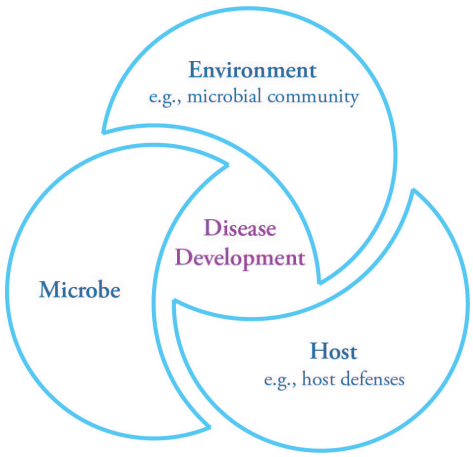


FIGURE 3. Host-microbe interactions contributing to disease development.

Moreover, the interpretation of the basic definition of microbial infection has changed. Many consider colonization as the first stage while others consider colonization as a different process[56]. For the pneumococcus, colonization and infection appear to be two distinct states. Asymptomatic carriage is regarded as a risk factor for the development of pneumococcal disease[57] because while colonization is a necessary step for subsequent development of infection[58], not all colonization events result in infection.

The pneumococcus is an opportunistic pathogen—an organism that causes disease following perturbation of the host. Although pneumococcal diseases occur, the pneumococcus more commonly colonizes without harming the host, i.e., as a commensal. In fact, further classification as a 'commensal opportunist'—a human-specialized, non-obligatory pathogen—has been proposed[59]. For opportunistic pathogens, virulence factors give an advantage in non-infection contexts rather than solely in development of infection[59]. The pneumococcus has traits that allow for its

commensal lifestyle and pathogenicity[60], which will be discussed throughout this thesis. This arsenal of virulence factors contributes to survival by allowing the pneumococcus to colonize and contend alongside nearby microbiota and host defenses as well as, under certain circumstances, invade the host tissue and disseminate to otherwise uninfected sites.

Colonization of the nasopharynx

Following acquisition, the pneumococcus colonizes the mucosal surface of the human nasopharynx. This is its main ecological niche[61], although carriage and spontaneous outbreaks in animals have been documented in the past[62]. Colonization can occur consecutively or with multiple strains as once, and can last for weeks to months[63], with the highest rates of colonization in children under the age of two[64]. It is from this commensal colonization state that transmission from person to person[61] occurs either through aerosolized droplets or direct contact. Transmission is also possible from fomites[65,66]. These states are in contrast to infection and disease, which are dead ends for the bacterium as they are not considered contagious conditions.

Biofilms

Growing literature over the last two decades indicates that the pneumococcus colonizes the human nasopharynx by forming biofilms. The biofilm mode of growth is considered predominant in natural bacterial habitats[67]. The definition of biofilms has evolved since its first mention in the 1970s. It is defined as complex communities of microbes that are attached to a surface and encased within a self-produced matrix, and exhibit an altered phenotype than singly growing planktonic cells[68]. The matrix provides the structural stability and protection to the biofilm. There would be no biofilm without matrix[69], as the matrix is what establishes much of the known characteristic features of biofilms[70].

Biofilms are tolerant to environmental stresses, such as antimicrobial treatment and host defense mechanisms. The matrix functions as a protective barrier and has been shown to limit the penetration of antibiotics in a charge-dependent manner[71]. Biofilm bacteria are less susceptible to antibiotics than planktonic bacteria[72] and often exhibit an altered phenotype with respect to growth and gene expression. A subpopulation of biofilm bacteria may contain dormant, non-dividing cells (or persister cells), which could explain elevated levels of survival following antimicrobial treatment[73]. Finally, chemical gradients within the biofilm may reduce antimicrobial activity and allow for survival of a portion of the biofilm bacteria[74]. This tolerant or transient, non-heritable phenotype[75] is different from heritable antibiotic resistance that result from mutations or acquired genes. In addition to a physical protective barrier, biofilms incorporate host structures, which may contribute to structural stability as well as allow biofilms to masquerade as 'self' structures to hide from the host immune response[76]. Besides its

protective properties, the matrix keeps the bacteria in close proximity to each other and to other resources. The matrix is considered as a 'communal external digestion system', as enzymes are secreted by cells within the biofilm and accumulate in the matrix[69]. The matrix also sequesters nutrients and, in the case of lysed cells, keeps debris to be 'cannibalized' by surviving cells[70]. Altogether, biofilms are protected from the surrounding environment and allow for persistence of the bacteria within them.

Evidence hinting at biofilm formation in the pneumococcus occurred as early as 1992, when the formation of a 'thickened gelatinous layer' was seen when the bacteria were grown on the epithelial surface of human nasal turbinate tissue *ex vivo*[77]. In the presence of the pneumococcus, the epithelium did not show severe damage, although the ciliary beating slowed. Most of the bacterial were well above contact with the tips of the cilia. At the time, this layer was hypothesized to be a mixture of host mucus and bacterial capsular material. The authors concluded that these observations "may be a mechanism of bacterial colonization of the respiratory tract"[77].

Biofilms are considered to constitute the main life form of the pneumococcus during colonization of the human nasopharynx[78-82]. This is in agreement with clinical observations where eradication of pneumococcal colonization is more difficult than eradication of infection[83,84]. In addition, studies have shown that pneumococcal biofilms are less susceptible to antibiotics than broth-grown, planktonic counterparts[82,85-87], and that biofilm bacteria have an altered phenotype compared with planktonic bacteria. For pneumococcal biofilms, extracellular DNA in the matrix is used as a substrate for genetic transformation and spread of acquired antibiotic resistance[88,89]). Different phenotypes between bacterial populations will be addressed more closely in Chapters 2-4 and the composition of biofilms will be discussed in Chapter 3.

The biofilm lifestyle is a way for the pneumococcus to remain in its ecological niche, the nasopharynx, as a commensal. It has been proposed that biofilms are a 'virulence factor' for the pneumococcus[90]. The pneumococcus would likely be eliminated without this form of bacterial life that functions as a reservoir for transmission between hosts. An altered phenotype of the biofilm bacteria and the protective nature of the biofilm structure itself contributes to sustained colonization. Moreover, the proximity of bacterial cells and resources, such as DNA, allow for horizontal gene transfer and acquisition of fitness traits. The biofilm mode of life is a survival advantage for the pneumococcus.

Encounters in the host

Colonization is a dynamic event[91]. Physiological gradients exist along the respiratory tract, which determine niche-specific selection that shapes the distribution of microbial communities[92]. The importance of nasopharyngeal conditions in pneumococcal biofilm formation will be discussed in Chapter 2. In addition to the physiological

conditions, the nasopharyngeal environment also includes the presence of microbes and other host defenses. The pneumococcus must reconcile with these encounters to maintain colonization and persist in the host.

Although pneumococcal colonization is asymptomatic, an initial degree of tissue interaction and penetration likely occurs as colonization is an immunizing event[93-98]. Pneumococcal colonization may act as a natural boosting mechanism of existing immunity[97]. This has been proposed to contribute to pneumococcal disease susceptibility in the elderly[97] where carriage rates are low and would lack the natural boosting mechanism[99].

The nasopharynx is an ecological niche for other microbes as well. Investigation of the microbiota in human health and disease has gained traction since the launch of the Human Microbiome Project in 2007[100]. In the gut, the local microbiota and the immune system have a two-way communication, with the microbiota priming and regulating mucosal and systemic immunity and the host immune system controlling the microbiota composition[101]. Similar, although not as well-studied, host-commensal interactions occur in the respiratory tract that are important for shaping the immune system and maintaining respiratory health[92].

The microbiota in the upper respiratory tract is considered as the gatekeeper to respiratory health and provides colonization resistance against pathogenic microorganisms[92]. Colonization resistance can be by direct commensal-pathogen interactions or by indirect mechanisms, for example, via activation of host immunity by commensals[102]. The microbiota is influenced by several factors already from the mode of delivery, including feeding type, environment conditions, and exposure to therapeutics[92,103-106]. It is a dynamic and diverse reservoir of many commensals and potential pathogens. Distinct microbial profiles are identified in early life and are linked to microbial stability and respiratory health[106]. Typically, a balanced microbiota leads to a stable community that is resilient to infection. In contrast, imbalance leads to a less stable microbial community and more susceptibility to infection for the host. The role of specific commensal species on respiratory health is the topic of Chapter 5.

Transition to disease

A wide range of human infections are associated with microbial biofilms[107]. Biofilms may act directly in disease, such as in dental caries, or indirectly, as on surfaces in the hospital setting[108,109], where the biofilm is a reservoir of potential pathogens. While the pneumococcus forms biofilms during asymptomatic colonization of the nasopharynx, biofilms have been detected during disease *in vivo*, such as otitis media[110-113], chronic sinusitis[114], pneumonia[115], and cardiac microlesions[116]. However, the role of pneumococcal biofilms at disease sites is unclear.

Pneumococcal infection is often associated with concurrent virus infection[117,118], which is involved in the transmission of the pneumococcus from colonized states *in vivo*[119,120]

as well as dissemination to and infection of otherwise non-infected sites[121]. Virus infection and virus-induced host responses, such as fever, are signals recognized by the pneumococcus[121], but the specific mechanisms involved in subsequent release of bacteria from the biofilm (i.e., biofilm dispersal) are less understood. Although, biofilm-dispersed bacteria are distinct from biofilm bacteria[121,122] and these differences help explain the colonization and disease lifestyles of the pneumococcus. Further characterization of these populations will be discussed in Chapter 4.

The pneumococcus is a common resident in healthy individuals. Yet, pneumococcal disease is a prominent cause of serious bacterial infection worldwide. The specific mechanisms involved in the transition from asymptomatic colonization to disease are still under study. The remainder of this thesis describes aspects involved in the transition from asymptomatic colonization to disease with the pneumococcus. With a focus on biofilm formation and biofilm dispersal, this thesis includes detailed methods, a proposed mechanism for biofilm dispersal, further evaluation of associated populations, and finally, modulation by other upper respiratory tract commensals.

Chapter 2: Modeling colonization and transition to disease

Host-microbe interactions are complex and can be difficult to study. To better understand specific aspects, models are valuable tools for unraveling these intricate interactions. These well-studied parts can then be pieced back together to solve a formerly puzzling system. This chapter discusses the *in vitro* models for pneumococcal colonization and transition to disease that are used in this thesis.

Studying biofilms

Biofilms are the predominant microbial lifestyle in nature[67]) and estimated to contribute to 65-80% of infections[123]. However, broth-grown, planktonic cultures are often still used in studies today. This is a problem when studying biofilm-related systems because the broth-grown, planktonic bacteria paradigm may not accurately represent biofilm populations and their function in various niches. To more closely simulate biofilm populations and interactions in the host, it is essential to take in consideration the physiological conditions as well as relevant evaluation methods.

Biofilm formation

Pneumococcal biofilms were first detected during disease states *in vivo*, such as otitis media and chronic sinusitis[111,114]. Biofilm formation during colonization in the mouse nasopharynx was later shown[82,124] and could be recapitulated *in vitro*[82], the latter methods of which have been further developed and are described in Paper 1 and Paper 2 and are also employed in this thesis.

A vast number of *in vitro* studies have contributed to the understanding of pneumococcal biofilm formation (as reviewed[81]). However, many of the studies utilized conventional bacterial culture conditions, such as abiotic surfaces (glass or plastic), a temperature of 37°C, and nutrient-rich media, which are conditions that are not representative of the nasopharyngeal environment where the pneumococcus resides. These conventional conditions were not as supportive for biofilm formation when

compared with conditions that more closely mimic the nasopharyngeal environment, such as the presence of a respiratory epithelial substratum, a temperature of approximately 34°C, and nutrient-limited media mimicking the nutritional conditions of this niche[82]. The conditions mimicking the nasopharynx were more optimal for biofilm formation over the same time period, as visualized by scanning electron microscopy and as measured by antimicrobial susceptibility[82] and transformation efficiency[88]. Together, this suggests that the specific conditions in the nasopharynx are more conducive for pneumococcal biofilm formation.

Biofilm dispersal

There are different methods to monitor, harvest, and analyze dispersed populations[125]. However, the general trend is that upon an environmental cue, there is a release of bacteria from the biofilm and these dispersed bacteria have unique properties different from the biofilms they are derived from. With pneumococcal biofilms, exposure to virus infection or virus-induced host responses, including elevated temperature (mimicking fever), results in a release of bacteria from the biofilm[121]. The dispersed bacteria and biofilm bacteria are distinct in the genes they express as well as their ability to disseminate and cause infection *in vivo*[121,122]. These studies contribute to the understanding of microbes that have a commensal phenotype and an invasive phenotype. Methods for biofilm dispersal are described in Paper 2, which are employed for studying specific mechanisms for bacterial release in Paper 3 and for further analysis of bacterial populations in Paper 4.

Evaluation of phenotype

Verification is an important aspect of utilizing model systems. For biofilm models, this can be done by comparisons with known biofilm characteristics, such as reduced susceptibility to antimicrobials, altered gene expression, and biofilm matrix formation.

Biomass and antimicrobial susceptibility

Biomass quantification provides a measure of how much material is present, but does not consider the functionality of the biofilm. Therefore, functional assays are also needed. Biofilm biomass is often quantified by staining or viable cell counts. Crystal violet staining is common, which binds negatively charged molecules and does not differentiate between live or dead cells. In that respect, viable cell counts are more specific since only live cells are quantified. In the case of assessing biofilm bacteria as compared with broth-grown, planktonic bacteria of the same strain, functional assays can capitalize on the intrinsic antimicrobial tolerance of biofilms. Generally, the same concentration of an antimicrobial that results in a detectable amount of bacterial cell death of planktonic bacteria will show a reduced amount of death of biofilm bacteria.

This is most easily quantified by viable cell counts. Antibiotics gentamicin (targets protein synthesis) and penicillin G (targets cell wall synthesis)[82] as well as others[86] have been used successfully for this purpose.

Antibiotics can also be employed to determine horizontal genetic exchange of resistance markers and the efficiency of transformation—the uptake of DNA from the environment. Transformation efficiency is markedly higher during biofilm growth both *in vivo* and *in vitro* than during planktonic growth[88]. Of note, in this context, antibiotic resistance results from acquisition of antibiotic resistance genes.

Gene expression analysis

Pneumococcal biofilm, dispersed, and broth-grown planktonic bacteria have been shown to have distinct transcriptional profiles[122]. Biofilm and planktonic bacteria differentially regulate a number of genes, which are also distinguishable between biofilm and dispersed bacteria, namely competence (*comD*), capsule production (*cps2* in strain D39, serotype 2), and pneumolysin (*ply*). Differential regulation of these genes as compared with biofilm bacteria was used for verification of different bacterial populations in this thesis.

Competence is involved in DNA uptake and genetic recombination, and is regulated by the competence stimulating peptide pheromone[126]. The *comD* gene encodes the receptor for the pheromone[127] and is needed for induction of competence and the ability to respond to the pheromone[128]. Therefore, the comD gene can be used to monitor competence. Competent pneumococcal cells are able to kill non-competent pneumococcal cells to acquire DNA in a process called fratricide[129]. Interestingly, the release of DNA during fratricide also involves aggregation of the pneumococcal cells[129]. As pneumococcal biofilms upregulate competence genes[121,122,130], and have also been shown to be primarily composed of dead cells[86], this mechanism may be relevant for aggregation and acquiring DNA from other bacteria during biofilm formation. Indeed, fratricide has been shown to be important for gene transfer between pneumococcal cells in biofilms[89]. Similarly, competence-induced toxin production for acquisition of DNA from other species in a multi-species biofilm has been proposed in the oral bacterium *Streptococcus mutans*[131].Together, this explains, in part, the increase of antibiotic-resistant pneumococcal strains in nasopharyngeal colonization after antibiotic treatment[83,84].

The polysaccharide capsule is an important virulent determinant that shields the pneumococcus from the host immune system. Capsule genes have been found to be downregulated in biofilms as compared with planktonic bacteria[85,86,132]. In one of these studies, there was an apparent reduction in capsule amount in biofilm bacteria nearest the substratum surface[85]. Similarly, reduced amounts of capsule have been identified in bacteria that are in closest contact with epithelial cells during adherence[132]. The reduction of capsule may enhance adhesion and biofilm formation, which is also

supported by non-encapsulated strains forming better biofilms *in vitro* than encapsulated transformants with different serotypes[133]. There is at least one contradicting study where biofilms upregulated capsule gene expression as compared with planktonic bacteria, although the biofilm bacteria were also more effective in pneumonia[130]. This is in contrast to what has been seen using the methods presented in this thesis, where biofilm bacteria downregulated capsule expression and were found in the lungs with no inflammation albeit with similar bacterial loads as compared with planktonic bacteria[121]. Although regulation of capsule expression was opposite in these studies, the population with upregulation of capsule was more virulent in pneumonia models, further supporting the role of capsule in virulence. It is unclear what the reasons are for this discrepancy, but they may arise from different biofilm models.

Pneumolysin is a pore-forming toxin and is well-characterized for its cytotoxicity[134]. Pneumolysin has been shown to be expressed similarly between planktonic bacteria and early biofilm phases, peaking at 6-8 hours and decreasing after 14 hours[135]. The same study showed that pneumolysin-deficient bacteria formed biofilms with less biomass over the same time period, and together suggested a role for pneumolysin during early biofilm assembly. In biofilms formed over longer periods, pneumolysin gene expression has been found to be downregulated[86,121,122,130] and reduced amounts of pneumolysin were detected[86] as compared with planktonic bacteria. The reduction in the amount of pneumolysin toxin during biofilm formation may be an important aspect of colonization.

Visualization

A grain of salt is approximately 100 micrometers in diameter, while the pneumococcus is approximately 1 micrometer in length. Visualization of this bacterium requires the help of microscopes that have powerful magnification, such as a scanning electron microscope. However, it is important to note that sample preparation steps may affect the original samples. Biofilms in this thesis were verified by the naked eye as well as scanning electron microscopy, which employed a sample fixation method that has been shown to preserve the capsule structure[132,136]. Other visualization techniques exist, but require stains or probes for specific structures.

Models are an important aspect of scientific research, especially for studying complex interactions. The methods presented in Paper 1 and Paper 2 are useful for studying specific aspects of biofilm colonization as well as mechanisms of transition from colonization to disease. Methods for biofilm formation and biofilm dispersal are employed in subsequent papers included in this thesis.

Summary: Paper 1 and Paper 2

in plain English

In Paper 1 and Paper 2, we described detailed methods to study how *Streptococcus pneumoniae* biofilms form and how they disperse during colonization and transition to disease. Our models attempt to mimic the nasopharyngeal environment where the bacterium normally resides. These conditions include a temperature of 34°C, low nutrient availability, and an epithelial surface. We used elevated temperature (mimicking fever) to disperse the biofilms. We also described ways to evaluate the biofilms once they are formed. Typically, biofilms are more tolerant to antibiotics and exchange genetic material at high levels as compared with bacteria that are grown in broth. In addition, the biofilm bacteria, dispersed bacteria, and broth bacteria have distinct traits. We used these characteristics in our assessments of the different populations. Imaging by microscopy was another way to evaluate the biofilms. The methods described in Paper 1 and Paper 2 were employed for Paper 3, Paper 4, and Paper 5.

Chapter 3: Dispersal mechanisms – a focus on proteases

Studies regarding biofilm dispersal have done so mostly in the context of developing novel anti-biofilm therapeutics. Fewer studies have done so in the context of disease progression; a critical step in the transition from colonization to local or invasive disease. Biofilm dispersal can be passive or active. Biofilm bacteria released as part of passive processes, such as sloughing due to external force, are expected to retain a biofilm phenotype. Actively dispersed bacteria, where dispersal is triggered as a response to environmental changes, are phenotypically different than the biofilms from which they originate[121,122,137]. An altered phenotype contributes to the understanding of how asymptomatic colonization may transit to dissemination and disease for opportunistic pathogens like the pneumococcus. However, the specific mechanisms of how the pneumococcus senses these signals and, subsequently, exits the biofilm are still under study. This chapter focuses on the latter mechanisms of how bacteria may be released from pneumococcal biofilms.

Biofilm composition

In general, bacterial biofilm matrix is an important structural aspect of the biofilm and is primarily composed of polysaccharides, proteins, lipids, and nucleic acids [138]. Pneumococcal biofilms have been shown to be composed of dead cells[86] and cell lysis is important for matrix formation during biofilm formation[82]. As bacterial biofilm bacteria are encased within the biofilm matrix, which generally comprises more than half of the biofilm biomass[138], enzymes that degrade these matrix components are likely candidates in biofilm dispersal.

Pneumococcal biofilms have been shown to have a matrix composed of carbohydrates and DNA, as indicated by imaging with probes that bind to these components[85]. Treatment with DNases are also able to reduce pneumococcal biofilm biomass[85,133,139]. Extracellular DNA and DNA-binding proteins may serve as a scaffold for bacterial biofilm stability in general[140]. Moreover, the pneumococcus has an enzyme that can degrade the DNA scaffold of neutrophil extracellular traps[141]. Enzymes that degrade

polysaccharides and extracellular DNA may also be involved in triggered biofilm dispersal, although these are not a focus of this thesis.

Proteins can account for up to 60% of the bacterial biofilm matrix[138]. In pneumococcal biofilms, the amount of protein in biofilms has been shown to increase over the duration of biofilm formation and were differentially produced between biofilm and planktonic bacteria[142]. Proteases have been shown to inhibit pneumococcal biofilm formation as well as reduce the amount of existing biofilms[133,139]and is one focus of this thesis.

In general, exogenous treatment of bacterial biofilms with enzymes targeting polysaccharides, proteins, or nucleic acids results in a reduction of biofilm biomass[85,133,139,143-146], which suggests that enzymes targeting the matrix components could have a role in active biofilm dispersal.

Active biofilm dispersal

For the pneumococcus, colonization always precedes pneumococcal disease[58], and changes in the nasopharyngeal environment associated with respiratory virus infection and virus-induced host responses, such as elevated temperature (mimicking fever), can induce the release of bacteria from the biofilm[121]. These dispersed bacteria have distinct transcriptional profiles and phenotypic properties as compared with biofilm bacteria[122], which suggests that the shift from colonization to disease is due to both bacterial release and enhanced virulence of the released bacteria. Elevated temperature has also been shown to disperse biofilms of *Neisseria subflava*[147], a commensal of the oral and upper respiratory tract that is rarely found in invasive disease[148] as well as the opportunistic pathogen *Staphylococcus aureus*[149]. However, the mechanisms involved in temperature-induced dispersal are not well understood. Elucidating the mechanisms involved in biofilm dispersal during the initial transition from nasopharyngeal colonization to infection has prospects for identifying specific therapeutics that target disease progression rather than commensal colonization. Paper 3 addresses a role for proteases in pneumococcal biofilm dispersal upon exposure to febrile-range temperature.

Summary: Paper 3

in plain English

In Paper 3, we used the methods in Paper 1 and Paper 2 to study how bacteria disperse from a biofilm upon heat exposure (mimicking fever). We showed that enzymes that chew up proteins (i.e., proteases) can release bacteria from biofilms. We also showed that if we inhibited protease activity, then bacterial release from biofilms in response to heat was hindered. When we formed biofilms with a strain that had a deletion of a specific protease called HtrA, biofilms formed normally. However, these biofilms did not disperse when exposed to heat. We proposed that protease HtrA is involved in dispersal of biofilms by heat (associated with disease), but not in biofilm formation (associated with colonization). Mechanisms like this one may lead to therapeutic targets for preventing transition to disease while allowing for colonization.

Chapter 4: Further insights into different populations

There are several signals and respective mechanisms involved in bacterial biofilm dispersal, but no universal pattern has clearly emerged[125]. Moreover, biofilm dispersal in the context of disease progression is less defined. The high rates of asymptomatic carriage with the pneumococcus indicate that this bacterium is adapted to the nasopharyngeal niche. Combined with its ability to also cause invasive disease, it is evident that the pneumococcus has the capacity to adapt to its environment and persist in the host. Transcriptome and proteome studies in pneumococcal populations have investigated differences with or without stimuli within broth-grown, planktonic bacteria [150,151] or biofilm bacteria[87,142], and have also compared planktonic with biofilm bacteria[86,137,142,152], but have focused less on the bacteria that are released from the biofilms upon stimulation. This chapter focuses on differences between planktonic, biofilm, and dispersed populations of bacteria.

Planktonic and biofilm bacteria

Transcriptomes and proteomes have been shown to be different between planktonic and biofilm bacteria, as well as during biofilm development[142,153]. Compared with biofilm bacteria, planktonic bacteria upregulated genes associated with energy metabolism, motility and chemotaxis, translation, quorum sensing, with virulence[86,137]. On a protein level, increased protein abundances in planktonic bacteria were associated with energy metabolism, the glycolytic pathway, lipid metabolism, nucleotide metabolism, translation, transcription, and virulence factors[137,152]. Biofilms upregulated genes associated with matrix protein synthesis and, on a protein level, amino acid metabolism, carbohydrate metabolism, and non-glycolytic carbohydrate metabolism[137,152]. These findings are in Table 1.

Table 1. Expression in biofilm bacteria compared with planktonic bacteria in *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*.

mRNA level	
Up in biofilm	**Up in planktonic**
Matrix protein synthesis	Energy metabolism, motility and chemotaxis, translation, quorum sensing, and virulence
Protein level	
Up in biofilm	**Up in planktonic**
Amino acid metabolism, carbohydrate metabolism, and non-glycolytic carbohydrate metabolism	Energy metabolism, glycolytic pathway, lipid metabolism, nucleotide metabolism, translation, transcription, and virulence factors

Biofilm bacteria were more adhesive, less invasive, and elicited a weaker immune response to epithelial cells *in vitro*[121,124]. In animal models, biofilm bacteria were attenuated for invasive disease[86,121] as compared with planktonic bacteria. There is one study where biofilms were found to be more virulent in pneumonia and meningitis models than planktonic bacteria, although these biofilms also upregulated capsule expression[130], which is also in contrast to the other mentioned studies.

Biofilm and dispersed bacteria

Biofilm dispersal as a response to nitric oxide or increased nutrient availability has been investigated in the opportunistic pathogen *Pseudomonas aeruginosa*. Upon exposure to nitric oxide, dispersed bacteria upregulated genes associated with virulence whereas genes associated with iron uptake was observed in biofilm bacteria[137]. Bacteria dispersed by nitric oxide were more evasive and cytotoxic *in vitro* than planktonic bacteria[137]). In another study, upon increased nutrient availability, dispersed bacteria upregulated

genes associated with translation and transport while genes associated with adaption or protection and energy metabolism were observed in biofilm bacteria[154]. Interestingly, there were many hypothetical proteins found to be differentially regulated in dispersed or biofilm bacteria[154].

In the transcriptional analysis of pneumococcal populations, bacteria dispersed following exposure to virus infection or virus-induced signals upregulated carbohydrate metabolism, bacteriocins, and virulence factors[122]. Biofilms upregulated genes associated with competence, amino acid metabolism, nucleotide metabolism, and translation. As compared with biofilm bacteria, dispersed bacteria were more virulent[121,122]. Dispersed bacteria were less adherent, more invasive, and more toxic to epithelial cells *in vitro* compared with biofilm bacteria[121]. In animal models, dispersed bacteria had an increased ability to disseminate and infect otherwise non-infected sites[121]. These findings are in Table 2.

Table 2. Expression in biofilm bacteria compared with dispersed bacteria

mRNA level		
Up in biofilm	**Up in dispersed**	**Bacteria, Dispersal agent**
Iron uptake	Virulence	*Pseudomonas aeruginosa,* Nitric oxide
Adaption/protection and energy metabolism,	Translation and transport	*Pseudomonas aeruginosa,* Increase in nutrient availability
Protein level		
Up in biofilm	**Up in dispersed**	**Bacteria, Dispersal agent**
Competence, amino acid metabolism, nucleotide metabolism, and translation	Carbonhydrate metabolism, bacterioincs, and virulence factors	*Streptococcus pneumoniae*, Virus infection

Temperature-induced biofilm dispersal

Fever is often associated with virus infection. Elevated temperature (mimicking fever) is able to disperse pneumococcal biofilms, and these dispersed bacteria have distinct transcriptional profiles and phenotypic properties as compared with biofilm bacteria[121,122]. These distinct populations are associated with colonization (biofilm bacteria) and disease (dispersed bacteria)[121]. With regard to the effect of temperature modulation, there is a study that evaluates the proteome in fish-pathogenic *Streptococcus agalactiae* at 32°C compared with 22°C[155]. Generally, there were not major differences between temperatures, which was suggested to coincide with a pathogen that would be exposed to temperature variations in the water. The differentially regulated proteins that were upregulated in higher temperature were primarily involved in metabolic pathways, such as amino acid metabolism, carbohydrate metabolism, and lipid metabolism. Interestingly, there was a low correlation between the transcriptome and

the proteome. For example, there was one differentially expressed translation gene, but 11 translation proteins with differential abundance. In the case of nucleotide metabolism, regulation was observed in opposite directions on the gene level compared to the protein level. The combination of relatively unchanged expression between temperatures and differential expression of some genes and proteins in *S. agalactiae* provides insight into the host-microbe interactions[155].

Colonization is an important step in the route to invasive disease. Therefore, describing the bacteria that leave the colonizing state is essential for understanding the transition from asymptomatic colonization to disease. Signals from the surrounding environment may be an important step for preparing the dispersed bacteria for an environment other than the nasopharyngeal niche during colonization. Characterizing the bacterial populations associated with colonization (biofilms) and invasive disease (dispersed bacteria) offers opportunities for identifying specific therapeutics that target these populations. To further characterize the previously identified transcriptional differences[122], Paper 4 examines the proteome profiles of pneumococcal populations associated with conventional culture (planktonic bacteria) as well as physiological niches, specifically colonization (biofilms) and invasive disease (dispersed bacteria).

Summary for Paper 4

in plain English

In Paper 4, we used the methods in Paper 1 and Paper 2 to obtain bacterial populations associated with colonization and disease. Biofilm bacteria are associated with colonization. Bacteria that are dispersed from biofilms in response to elevated temperature (mimicking fever) are associated with disease. We also used bacteria that were grown in broth (planktonic bacteria). We evaluated the abundance of different proteins that the bacterial populations had. Most of the identified proteins were associated with metabolism. There were differences between biofilm bacteria and dispersed bacteria. Even more differences were seen between broth bacteria and biofilm bacteria or dispersed bacteria. Generally, biofilm bacteria appeared to be less metabolically active than the other two populations. The differences between populations may be potential targets for specific population. For example, a protein that is abundant only in dispersed bacteria would be a specific therapeutic target for dispersed bacteria (disease), but not biofilm bacteria (colonization).

Chapter 5: In the context of the nasopharyngeal microbiota

Until now, this thesis has primarily focused on the pneumococcus under relatively defined situations. However, this bacterium is not alone in host environments. The nasopharynx is also an ecological niche for other commensals and pathogens. This chapter focuses on the microbiota related to respiratory health.

Other nasopharyngeal commensals

The microbiota of the upper respiratory tract is considered the gatekeeper to respiratory health as it provides colonization resistance against respiratory pathogens and is thought to influence the development of respiratory tract infections[92]. During early life, the absence of beneficial bacteria, the presence and abundance of potential pathogens, and an influx of oral species into the nasopharyngeal niche has been observed prior to and during respiratory tract infections[156]. In adults and elderly, the microbiota was shown to differ in the nostrils as well as the oropharynx, with a loss of microbial topography with age[157]. Interestingly, the microbiota of the nostrils of the elderly resembled the oropharyngeal microbiota of adults, suggesting displacement by oropharyngeal microbiota, which may contribute to the increased risk of respiratory infections in the elderly[157].

Already at 1.5 months of age, distinct microbial profiles can be identified in healthy individuals and are linked with microbial stability and respiratory health[106]. Profiles dominated by *Corynebacterium/Dolosigranulum* or *Moraxella* were more stable over time, whereas microbial instability was associated with *Streptococcus*- or *Haemophilius*-dominated profiles[106]. *Corynebacterium* spp. (corynebacteria) commonly colonize the nasal passage of children and adults[106,158-162]. Corynebacteria are thought to be protective members of the normal microbiota, as presence and abundance of corynebacteria have been shown to be negatively associated with pneumococcal carriage as well as the number of acute respiratory infections[103,106,160,162,163]. However, the specific mechanistic interactions between corynebacteria and the pneumococcus are not well understood.

Colonization resistance by commensals arises from interactions with pathogens[164]. *Corynebacterium accolens* has been shown to inhibit the growth of the pneumococcus *in vitro* on agar by producing fatty acids from human skin surface triacylglycerols[165]. Modification of the environment is one indirect mechanism by which corynebacteria may shape the nasopharyngeal microbiota. As the pneumococcus forms biofilms during colonization of the nasopharynx, it is possible that other colonizing commensals do so as well. *Corynebacterium pseudodiphtheriticum* has been reported to form biofilms on abiotic surfaces[166]. Paper 5 addresses corynebacteria biofilm formation in conditions mimicking the nasopharyngeal environment by utilizing methods adapted from Paper 1 and Paper 2.

Nasopharyngeal colonization is a commonality between the pneumococcus and corynebacteria. While microbiota composition is related to respiratory health, the interplay between the host and corynebacteria and the pneumococcus during colonization are not well understood. Better understanding these host-microbe interactions may reveal therapeutic avenues to improve human health. Paper 5 explores the interactions between commensal corynebacteria, the host epithelial cells, and the pneumococcus, with a focus on biofilm formation and inflammatory responses by corynebacteria.

Summary for Paper 5

in plain English

In Paper 5, we used an adapted version of the biofilm formation methods in Paper 1 and Paper 2 to study biofilm formation with commensal *Corynebacterium* species. The presence of these bacteria has been shown to be protective in respiratory health, with fewer reported cases of respiratory infections. We showed that *Corynebacterium* could form biofilms in our model that mimics the nasopharyngeal environment. We assessed these biofilms for tolerance to antibiotics and visually by microscopy. The *Corynebacterium* formed biofilms with minimal toxicity to the cells and were able to induce a transient inflammatory response in respiratory epithelial cells. We then investigated the acquisition of *Streptococcus pneumoniae* to existing *Corynebacterium* biofilms. We found that the presence of *S. pneumoniae* did not appear to affect the *Corynebacterium* biofilms, and that the *Corynebacterium* may have a protective effect for *S. pneumoniae*. Understanding the role of commensal bacteria interactions may lead to probiotic therapeutics.

Chapter 6: Conclusions

Standing on the shoulders of giants.

A key thing is knowing who's shoulders you are standing on, and help the others who could stand on your shoulders

Bill Buchanan

This concluding chapter focuses on a discussion of the papers included in this thesis, central themes, as well as future perspectives.

Discussion of papers

Aims

The aim of this thesis was to identify specific mechanisms involved during biofilm formation and biofilm dispersal with *Streptococcus pneumoniae*.

- to develop and improve methods to study pneumococcal biofilm formation and biofilm dispersal that are associated with colonization and transition to disease
- to investigate the role of proteases in pneumococcal biofilm dispersal
- to examine the proteome of pneumococcal populations associated with colonization (biofilm bacteria), disease (dispersed bacteria), and conventional broth-grown culture (planktonic bacteria)
- to assess interactions between commensal *Corynebacterium* spp., the pneumococcus, and respiratory epithelial cells, with a focus on biofilm formation

Summary of results

In **Paper 1** and **Paper 2**, we described detailed and improved *in vitro* and *in vivo* methods to model biofilm formation and biofilm dispersal with the pneumococcus. The *in vitro* approach attempts to mimic the nasopharyngeal environment by including features such as a respiratory epithelial substratum, an approximate nasopharyngeal temperature of 34°C, and nutrient-limiting media. We also described methods to evaluate biofilm formation. This included assessments of the functionality of the biofilms via known characteristics of biofilms, such as antibiotic tolerance, high transformation efficiency, and altered gene expression as compared with broth-grown, planktonic bacteria. Methods for visualizing biofilms by scanning electron microscopy were also included. Biofilm dispersal as a model for transition from colonization via response to signals in the environment was described. Gene expression analysis was also used for verifying the phenotype of dispersed bacteria as compared with the biofilms they originated from.

In **Paper 3**, we utilized the methods in Paper 1 and Paper 2 and investigated pneumococcal biofilm dispersal by exposure to elevated temperature (mimicking fever) in lab strain D39 (serotype 2) and clinical isolate EF10175 (serotype 19F). We showed that exogenous addition of serine proteases (trypsin and proteinase K) and cysteine protease (papain) could release bacteria from the biofilm into the supernatant. We further showed a role for serine protease activity in heat-induced biofilm dispersal by inhibition with serine protease inhibitors during heat-induced dispersal. By utilizing strains lacking the surface-exposed serine proteases HtrA or PrtA as compared with wild-type isogenic strains, we identified a role for serine protease HtrA, but not PrtA, in heat-induced biofilm dispersal. By our biofilm assessment methods in Paper 1 and Paper 2, biofilm formation was not affected by the mutations. We proposed a role for serine protease HtrA in heat-induced biofilm dispersal, but not biofilm formation. Understanding how bacterial are released during biofilm dispersal has the potential to interfere with disease-specific targets rather than colonization.

In **Paper 4**, we utilized the methods in Paper 1 and Paper 2 to obtain bacterial populations associated with colonization (biofilm bacteria) and disease (heat-dispersed bacteria), and also used conventional broth-grown culture (planktonic bacteria). We used lab strain D39 (serotype 2) and clinical isolate EF01715 (serotype 19F). The biofilms were validated by biomass and antibiotic tolerance and biofilm dispersal was confirmed by increase of bacterial release upon heat treatment. Proteome cluster analysis indicated differences between biofilm bacteria and dispersed bacteria, and even more so as compared with planktonic bacteria.

When comparing proteome profiles and previous transcriptional profiles of genes upregulated in dispersed bacteria as compared with biofilm bacteria, about half of the genes and respective proteins overlapped in the direction of expression, representing pathways involved in competence, amino acid metabolism, ABC transport systems, and

carbohydrate metabolism. Nucleotide metabolism, vitamins and cofactors, and putative proteins were regulated in different directions. In the proteome analysis, differences between populations were primarily associated with metabolic pathways.

As compared with biofilm bacteria, dispersed bacteria upregulated proteins associated with amino acid metabolism, carbohydrate metabolism, nucleotide metabolism, and translation. Biofilm bacteria upregulated proteins associated with genetic information processing and ABC transport systems. As compared with biofilm bacteria, planktonic bacteria upregulated nucleotide metabolism, translation, and carbohydrate metabolism. Biofilms upregulated replication and repair and transport systems. Together, the proteome profile of biofilm bacteria appeared to be less metabolically active and had increased expression of replication and repair, ABC transport systems, and metabolism of cofactors and vitamins as compared with dispersed bacteria as well as planktonic bacteria, which corresponds with their sessile life. These data further indicate differences between broth-grown planktonic bacteria and biofilm-derived bacteria, and also provides targets for specific populations associated with colonization (biofilms) or disease (dispersed bacteria).

In **Paper 5**, we utilized an adapted version of the biofilm formation methods in Paper 1 and Paper 2 and assessed interactions between *Corynebacterium* spp. (corynebacteria), the pneumococcus, and respiratory tract epithelial cells. While the paper also addressed the inflammatory response by respiratory cells, the focus of this thesis was on the biofilm aspects. We formed biofilms with four clinical isolates of corynebacteria over 72 hours, and generally saw no difference between a substratum of live or pre-fixed epithelial cells. Biomass was generally unchanged over 24-72 hours, but antibiotic susceptibility decreased over time, indicating formation of functional biofilms. A plastic substratum was not conducive for biofilm formation, as most of the strains remained susceptible to antibiotic treatment over time as compared with biofilms formed on epithelial substrata. As the pneumococcus is toxic to epithelial cells over 24 hours, we utilized a pre-fixed epithelial substratum to assess interactions with corynebacteria. Corynebacteria biofilms were formed over 48 hours, then formed in the presence or absence of the pneumococcus for another 24 hours. Generally, the presence of the pneumococcus did not affect corynebacteria biofilms (in biomass or susceptibility to antibiotics). However, the pneumococcus was less susceptible to antibiotic treatment when formed with corynebacteria, suggesting a protective effect of the presence of corynebacteria. A better understanding of microbial interactions offers opportunities for probiotic strategies.

Contributions to the field

Our main contribution has been to further understand the pneumococcus in the context of biofilm formation and biofilm dispersal that are associated with colonization and disease, respectively. The involvement of proteases with pneumococcal biofilm dispersal may lead to identifying other similar mechanisms of triggered dispersal, which could be useful as a target for specific prevention of the initial transition from colonization to infection. The proteome analysis further revealed differences between relevant pneumococcal populations associated with colonization, disease, as well as conventional broth-grown culture. These differences may be useful for understanding the pneumococcus in different niches as well as identifying therapeutic targets directed at colonization or transition to disease. Finally, we have demonstrated that commensal corynebacteria form biofilms and that they may be protective for the pneumococcus in dual-species biofilms. The detailed methods to model and evaluate biofilm formation and biofilm dispersal could be adapted and used for other opportunistic pathogens that have colonization states that may transit to infection states.

Central themes

The role of colonization

The pneumococcus is well-adapted for colonizing the nasopharynx, which is evident by the high rates of carriage. However, the prevalence of non-invasive and invasive diseases indicates that the pneumococcus is also equipped to survive in other host sites. The transcriptome[122] and proteome differences of populations associated with colonization (biofilm bacteria) and disease (dispersed bacteria) indicate that the pneumococcus responds to changes in the environment. These signals may be an important step for preparing the dispersed bacteria for a new environment. As colonization and infection appear to be two different states for the pneumococcus, this may be an important aspect to consider for future therapeutics. Implementation of current vaccines has led to serotype replacement, which may be a caution to targeting colonization that is abundant and typically asymptomatic.

Pneumococcal colonization may act as a natural boosting mechanism of existing immunity[97]. This may explain why the elderly have low carriage rates[99] but high susceptibility to pneumococcal disease[97]. In addition, loss of microbial topography occurs with age, as well as an apparent displacement of nostril microbiota with oropharyngeal microbiota[157]. These are interesting findings in light of studies indicating that stable microbial profiles in the nasopharynx are associated with lower rates of respiratory infection in the first two years of life[106].

Different populations

To focus on elevated temperature as a dispersal signal, we used pre-fixed epithelial cells for biofilm formation and biofilm dispersal. Elevated temperature alone (mimicking fever) is a dispersal signal, but is likely more complicated *in vivo*. The biofilm and heat-dispersed populations were associated with colonization and disease in animal models[121]. Treatment with enzymes has been shown to prevent biofilm formation or reduce already formed biofilms, but have not addressed biofilm dispersal as a mechanism for disease progression. A focus on the specific mechanisms for biofilm dispersal is important for understanding the transition phase from colonization to infection.

In our previous transcriptome analysis, biofilm, dispersed, and broth-grown, planktonic populations were observed to have distinct transcriptional profiles[122]. We, therefore, wanted to address these differences on a protein level. Biofilms dispersed upon exposure to an elevated temperature of 38.5°C, which was sufficient for observing proteomic changes between dispersed and biofilm populations. However, only about half of the identified genes (and the proteins they encode) were regulated in the same direction when comparing transcriptional data to proteomic data. A low correlation between the transcriptome and proteome was seen when comparing fish-pathogenic *Streptococcus agalactiae* at 32°C and 22°C[155]. This may be due to differences in the turnover rate between mRNA transcripts and proteins.

Generally, our proteome analysis revealed that biofilms were more metabolically quiescent as compared with dispersed bacteria or broth-grown, planktonic bacteria, which is similar to what has been observed in other studies[86,122,137,142,152]. The differentially regulated pathways provide targets that are specific to a bacterial population (e.g., biofilm or dispersed) that is associated with colonization and disease, respectively. It is important to note that the observed proteomic changes are involved in an initial response of the pneumococcus to elevated temperature. As the pneumococcus invades other host sites, proteome profiles are likely to change. A study has shown that subsequent planktonic growth of pneumococcal biofilm bacteria partially restored virulence in animal models, suggesting that this population had an intermediate phenotype[86]. This supports the notion that the pneumococcus adapts to its surroundings.

Methodological considerations

Studying pneumococcal biofilms is challenging. There are different biofilms models with varying conditions in regards to media, temperature, the substratum, and the duration of time. These additional variables make it more difficult to compare studies, especially when there are contradicting findings. The variability among results suggests that the model system influences the results, which is important to note. There is more

and more evidence of phenotypic differences between broth-grown, planktonic bacteria and biofilm bacteria. These differences, which may be associated with the niche, need to be taken into consideration when studying these populations.

Another challenge is studying heterogeneity in biofilms. The proteomic analysis of the biofilm population in our studies address the average expression profile. This could also mean that the biofilm proteomic profile is a reflection of the matrix proteome as well. Another potential limitation is studying pneumococcal biofilms in pure-culture systems. Host-microbe interactions are complex and involves the microbe, other microbes in the environment, as well as host defenses. In this thesis, we have primarily studied the pneumococcus in conditions mimicking the nasopharynx but without dynamic input from the host or other microbes. Our work with dual-species biofilms with corynebacteria and the pneumococcus is of continued interest to begin to understand more complex interactions.

A decent part of my PhD studies has been devoted to developing the biofilm methods, the methods of which are an important foundation for the rest of the thesis work. From our experience with developing the *in vitro* biofilm models, the specific parameters have a profound influence. While some outcomes were a result of intentional manipulation, others happened with the best reason being that you blinked at the bacteria the wrong way. Regardless of these variabilities, the use of models attempts to simplify complex interactions to better understand specific questions, but is possibly limited by the simplicity. It is a trade-off. The best one can do is to recognize the limitations and the implications.

Future

Biofilm dispersal mechanisms

We have explored the role of proteases in pneumococcal biofilm dispersal, but other enzymes that target the matrix are of interest, such as DNases. It will be important to verify the role of HtrA in biofilm formation and biofilm dispersal *in vivo*. We hypothesize that the mutant strain lacking HtrA will colonize the nasopharynx of the animals (as biofilm formation *in vitro* was comparable to the wild-type isogenic strain), but will not disseminate to the middle ears, lungs, or bloodstream following exposure to elevated temperature (as biofilm dispersal *in vitro* was impaired). It is of interest to investigate whether immunization with HtrA will specifically target disease-causing (dispersed) population but allow for asymptomatic colonization.

Pneumococcal populations

We intend to investigate the biofilm, dispersed, and planktonic populations in other pneumococcal clinical isolates, which is underway. As there were not as distinct changes in the proteomic analysis between biofilm and dispersed bacteria, we hypothesize that the duration of exposure has an effect. Our planktonic population have only been cultured in broth-grown conditions or kept as frozen glycerol stocks. The biofilm population has been formed over 72 hours, whereas heat dispersal is over 4 hours. Longer duration of exposure is of interest.

As of now, we have not addressed the passively released bacteria from biofilms at 34°C or the remaining biofilm after heat-induced dispersal, but that is underway. We are also interested in investigating the effect of temperature on broth-grown planktonic bacteria. The question is if elevated temperature alone has similar effects on broth-grown planktonic bacteria as seen in heat-induced biofilm dispersal or if egress from a biofilm phase is necessary. For example, will bacteria grown planktonically at 34°C and subsequently exposed to 38.5°C have a similar phenotype as bacteria that are heat-dispersed from biofilms.

Of interest is to compare biofilm formation and biofilm dispersal across non-vaccine serotypes that are currently circulating in the population. Different serotypes exhibit different invasive potentials, and we wonder if there is a link between a strain's ability to form biofilms and its invasive potential.

Transcriptional changes do not necessarily correspond to changes on the protein level. Therefore, it is important to validate using other methods. Since the majority of the proteome analysis revealed regulation of proteins in metabolic pathways, other methods to validate the metabolic differences can be used, such as enzymatic assays or

metabolome analysis. In addition, metabolism may also influence host-microbe interactions via metabolite-responsive regulators[167] and metabolic proteins may also have alternative functions[152].

Microbiota

We are planning to visualize the *Corynebacterium* spp. (corynebacteria) biofilms, in single-species biofilms and in dual-species biofilms with the pneumococcus by scanning electron microscopy.

Investigating dual-species biofilms and the order of acquisition are ongoing. We have focused on corynebacteria in this thesis, but have also explored other commensal species involved in respiratory health, such as *Moraxella*. The presence of corynebacteria and *Moraxella* are associated with lower rates of respiratory infection, but we are also interested in evaluating dual-species biofilms with microbiota species that are associated with increased rates of respiratory infection as well.

We are also investigating if the presence of corynebacteria in a dual-species biofilm will modulate heat-induced biofilm dispersal of the pneumococcus. Furthermore, evaluating dual-species biofilms with gene expression analysis may reveal differentially regulated genes that may be involved in modulating the pneumococcus.

Methods

As I have emphasized the importance of incorporating physiological conditions in model systems, we do have an interest in further developing our current models. Incorporating a nasopharyngeal cell line, nutrient-poor media with other carbon sources (e.g., galactose) as well as mucins are some of our interests. With regards to imaging our biofilms, we would like to incorporate live cell imaging to better understand the dynamics of biofilm formation and biofilm dispersal.

References

1 Koch, R. The etiology of tuberculosis [Koch's postulates]. *The germ theory of disease*, 116-118 (1884).

2 Koch, R. Die Aetiologie der Tuberkulose. *Mittbeihgen aus dem Kaiserlichen Gesundbeitsamte* **2**, 1-88 (1884).

3 Falkow, S. Molecular Koch's postulates applied to bacterial pathogenicity--a personal recollection 15 years later. *Nat Rev Microbiol* **2**, 67-72, doi:10.1038/nrmicro799 (2004).

4 Neville, B. A., Forster, S. C. & Lawley, T. D. Commensal Koch's postulates: establishing causation in human microbiota research. *Curr Opin Microbiol* **42**, 47-52, doi:10.1016/j.mib.2017.10.001 (2018).

5 Vonaesch, P., Anderson, M. & Sansonetti, P. J. Pathogens, microbiome and the host: emergence of the ecological Koch's postulates. *FEMS Microbiol Rev* **42**, 273-292, doi:10.1093/femsre/fuy003 (2018).

6 Casadevall, A. & Pirofski, L. A. Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. *Infect Immun* **68**, 6511-6518, doi:10.1128/iai.68.12.6511-6518.2000 (2000).

7 Sternberg, G. M., Joseph Meredith Toner, C. & National Board of, H. *A fatal form of septicaemia in the rabbit produced by the subcutaneous injection of human saliva : an experimental research*. (Printed by John Murphy & Co., 1881).

8 Pasteur, L. Note sur la maladie nouvelle provoquee par la salive d'un enfant mort de la rage. *Bulletin de l'Academie de Medicine* **10**, 94-103 (1881).

9 Griffith, F. The Significance of Pneumococcal Types. *J Hyg (Lond)* **27**, 113-159, doi:10.1017/s0022172400031879 (1928).

10 Avery, O. T., Macleod, C. M. & McCarty, M. Studies on the Chemical Nature of the Substance Inducing Transformation of Pneumococcal Types : Induction of Transformation by a Desoxyribonucleic Acid Fraction Isolated from Pneumococcus Type Iii. *J Exp Med* **79**, 137-158, doi:10.1084/jem.79.2.137 (1944).

11 Watson, D. A., Musher, D. M., Jacobson, J. W. & Verhoef, J. A brief history of the pneumococcus in biomedical research: a panoply of scientific discovery. *Clin Infect Dis* **17**, 913-924, doi:10.1093/clinids/17.5.913 (1993).

12 Austrian, R. Pneumococcus: the first one hundred years. *Rev Infect Dis* **3**, 183-189 (1981).

13 Adegbola, R. A. *et al.* Carriage of *Streptococcus pneumoniae* and other respiratory bacterial pathogens in low and lower-middle income countries: a systematic review and meta-analysis. *PLoS One* **9**, e103293, doi:10.1371/journal.pone.0103293 (2014).

14 Wang, L., Fu, J., Liang, Z. & Chen, J. Prevalence and serotype distribution of nasopharyngeal carriage of *Streptococcus pneumoniae* in China: a meta-analysis. *BMC Infect Dis* **17**, 765, doi:10.1186/s12879-017-2816-8 (2017).

15 Navne, J. E. *et al.* Nasopharyngeal bacterial carriage in young children in Greenland: a population at high risk of respiratory infections. *Epidemiol Infect* **144**, 3226-3236, doi:10.1017/S0950268816001461 (2016).
16 van Hoek, A. J. *et al.* Pneumococcal carriage in children and adults two years after introduction of the thirteen valent pneumococcal conjugate vaccine in England. *Vaccine* **32**, 4349-4355, doi:10.1016/j.vaccine.2014.03.017 (2014).
17 Bosch, A. *et al.* Nasopharyngeal carriage of *Streptococcus pneumoniae* and other bacteria in the 7th year after implementation of the pneumococcal conjugate vaccine in the Netherlands. *Vaccine* **34**, 531-539, doi:10.1016/j.vaccine.2015.11.060 (2016).
18 Milucky, J. *et al. Streptococcus pneumoniae* colonization after introduction of 13-valent pneumococcal conjugate vaccine for US adults 65 years of age and older, 2015-2016. *Vaccine* **37**, 1094-1100, doi:10.1016/j.vaccine.2018.12.075 (2019).
19 Drijkoningen, J. J. & Rohde, G. G. Pneumococcal infection in adults: burden of disease. *Clin Microbiol Infect* **20 Suppl 5**, 45-51, doi:10.1111/1469-0691.12461 (2014).
20 Collaborators, G. B. D. L. R. I. Estimates of the global, regional, and national morbidity, mortality, and aetiologies of lower respiratory infections in 195 countries, 1990-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet Infect Dis* **18**, 1191-1210, doi:10.1016/S1473-3099(18)30310-4 (2018).
21 O'Brien, K. L. *et al.* Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* **374**, 893-902, doi:10.1016/S0140-6736(09)61204-6 (2009).
22 van Aalst, M. *et al.* Incidence of invasive pneumococcal disease in immunocompromised patients: A systematic review and meta-analysis. *Travel Med Infect Dis* **24**, 89-100, doi:10.1016/j.tmaid.2018.05.016 (2018).
23 Collaborators, G. B. D. M. Global, regional, and national under-5 mortality, adult mortality, age-specific mortality, and life expectancy, 1970-2016: a systematic analysis for the Global Burden of Disease Study 2016. *Lancet* **390**, 1084-1150, doi:10.1016/S0140-6736(17)31833-0 (2017).
24 Austrian, R. & Gold, J. Pneumococcal Bacteremia with Especial Reference to Bacteremic Pneumococcal Pneumonia. *Ann Intern Med* **60**, 759-776, doi:10.7326/0003-4819-60-5-759 (1964).
25 (World Health Organization, 2017).
26 Geno, K. A., Saad, J. S. & Nahm, M. H. Discovery of Novel Pneumococcal Serotype 35D, a Natural WciG-Deficient Variant of Serotype 35B. *J Clin Microbiol* **55**, 1416-1425, doi:10.1128/JCM.00054-17 (2017).
27 Geno, K. A. *et al.* Pneumococcal Capsules and Their Types: Past, Present, and Future. *Clin Microbiol Rev* **28**, 871-899, doi:10.1128/CMR.00024-15 (2015).
28 Daniels, C. C., Rogers, P. D. & Shelton, C. M. A Review of Pneumococcal Vaccines: Current Polysaccharide Vaccine Recommendations and Future Protein Antigens. *J Pediatr Pharmacol Ther* **21**, 27-35, doi:10.5863/1551-6776-21.1.27 (2016).
29 Bridy-Pappas, A. E., Margolis, M. B., Center, K. J. & Isaacman, D. J. *Streptococcus pneumoniae*: description of the pathogen, disease epidemiology, treatment, and prevention. *Pharmacotherapy* **25**, 1193-1212, doi:10.1592/phco.2005.25.9.1193 (2005).
30 Merck Announces Results from Phase 2 Trial of Investigational 15-valent Pneumococcal Conjugate Vaccine (V114) in Infants. (2019).

31 Shapiro, E. D. *et al.* The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. *N Engl J Med* **325**, 1453-1460, doi:10.1056/NEJM199111213252101 (1991).
32 Richter, S. S. *et al.* Pneumococcal serotypes before and after introduction of conjugate vaccines, United States, 1999-2011(1.). *Emerg Infect Dis* **19**, 1074-1083, doi:10.3201/eid1907.121830 (2013).
33 Whitney, C. G. *et al.* Decline in invasive pneumococcal disease after the introduction of protein-polysaccharide conjugate vaccine. *N Engl J Med* **348**, 1737-1746, doi:10.1056/NEJMoa022823 (2003).
34 Hammitt, L. L. *et al.* Indirect effect of conjugate vaccine on adult carriage of *Streptococcus pneumoniae*: an explanation of trends in invasive pneumococcal disease. *J Infect Dis* **193**, 1487-1494, doi:10.1086/503805 (2006).
35 Weil-Olivier, C., van der Linden, M., de Schutter, I., Dagan, R. & Mantovani, L. Prevention of pneumococcal diseases in the post-seven valent vaccine era: a European perspective. *BMC Infect Dis* **12**, 207, doi:10.1186/1471-2334-12-207 (2012).
36 Hicks, L. A. *et al.* Incidence of pneumococcal disease due to non-pneumococcal conjugate vaccine (PCV7) serotypes in the United States during the era of widespread PCV7 vaccination, 1998-2004. *J Infect Dis* **196**, 1346-1354, doi:10.1086/521626 (2007).
37 Hsu, K. K., Shea, K. M., Stevenson, A. E., Pelton, S. I. & Massachusetts Department of Public, H. Changing serotypes causing childhood invasive pneumococcal disease: Massachusetts, 2001-2007. *Pediatr Infect Dis J* **29**, 289-293, doi:10.1097/INF.0b013e3181c15471 (2010).
38 Davis, S. M., Deloria-Knoll, M., Kassa, H. T. & O'Brien, K. L. Impact of pneumococcal conjugate vaccines on nasopharyngeal carriage and invasive disease among unvaccinated people: review of evidence on indirect effects. *Vaccine* **32**, 133-145, doi:10.1016/j.vaccine.2013.05.005 (2013).
39 Ghaffar, F. *et al.* Effect of the 7-valent pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae* in the first 2 years of life. *Clin Infect Dis* **39**, 930-938, doi:10.1086/423379 (2004).
40 Huang, S. S. *et al.* Continued impact of pneumococcal conjugate vaccine on carriage in young children. *Pediatrics* **124**, e1-11, doi:10.1542/peds.2008-3099 (2009).
41 Hanage, W. P. *et al.* Evidence that pneumococcal serotype replacement in Massachusetts following conjugate vaccination is now complete. *Epidemics* **2**, 80-84, doi:10.1016/j.epidem.2010.03.005 (2010).
42 Wyres, K. L. *et al.* Pneumococcal capsular switching: a historical perspective. *J Infect Dis* **207**, 439-449, doi:10.1093/infdis/jis703 (2013).
43 Balsells, E., Guillot, L., Nair, H. & Kyaw, M. H. Serotype distribution of *Streptococcus pneumoniae* causing invasive disease in children in the post-PCV era: A systematic review and meta-analysis. *PLoS One* **12**, e0177113, doi:10.1371/journal.pone.0177113 (2017).
44 Cui, Y. A., Patel, H., O'Neil, W. M., Li, S. & Saddier, P. Pneumococcal serotype distribution: A snapshot of recent data in pediatric and adult populations around the world. *Hum Vaccin Immunother* **13**, 1-13, doi:10.1080/21645515.2016.1277300 (2017).
45 Hanage, W. P. Serotype replacement in invasive pneumococcal disease: where do we go from here? *J Infect Dis* **196**, 1282-1284, doi:10.1086/521630 (2007).
46 Rodgers, G. L. & Klugman, K. P. The future of pneumococcal disease prevention. *Vaccine* **29 Suppl 3**, C43-48, doi:10.1016/j.vaccine.2011.07.047 (2011).

47 Lagousi, T., Basdeki, P., Routsias, J. & Spoulou, V. Novel Protein-Based Pneumococcal Vaccines: Assessing the Use of Distinct Protein Fragments Instead of Full-Length Proteins as Vaccine Antigens. *Vaccines (Basel)* **7**, doi:10.3390/vaccines7010009 (2019).
48 Pichichero, M. E. Pneumococcal whole-cell and protein-based vaccines: changing the paradigm. *Expert Rev Vaccines* **16**, 1181-1190, doi:10.1080/14760584.2017.1393335 (2017).
49 Popova, M. *et al.* Beneficial effects of probiotics in upper respiratory tract infections and their mechanical actions to antagonize pathogens. *J Appl Microbiol* **113**, 1305-1318, doi:10.1111/j.1365-2672.2012.05394.x (2012).
50 Coleman, A. & Cervin, A. Probiotics in the treatment of otitis media. The past, the present and the future. *Int J Pediatr Otorhinolaryngol* **116**, 135-140, doi:10.1016/j.ijporl.2018.10.023 (2019).
51 Dockrell, D. H., Whyte, M. K. B. & Mitchell, T. J. Pneumococcal pneumonia: mechanisms of infection and resolution. *Chest* **142**, 482-491, doi:10.1378/chest.12-0210 (2012).
52 Iovino, F., Seinen, J., Henriques-Normark, B. & van Dijl, J. M. How Does *Streptococcus pneumoniae* Invade the Brain? *Trends Microbiol* **24**, 307-315, doi:10.1016/j.tim.2015.12.012 (2016).
53 Greene, C. J. *et al.* Novel Strategy To Protect against Influenza Virus-Induced Pneumococcal Disease without Interfering with Commensal Colonization. *Infect Immun* **84**, 1693-1703, doi:10.1128/IAI.01478-15 (2016).
54 Jones, C. H. *et al.* Comprehensive vaccine design for commensal disease progression. *Sci Adv* **3**, e1701797, doi:10.1126/sciadv.1701797 (2017).
55 Li, Y. *et al.* Directed vaccination against pneumococcal disease. *Proc Natl Acad Sci U S A* **113**, 6898-6903, doi:10.1073/pnas.1603007113 (2016).
56 Dani, A. Colonization and infection. *Cent European J Urol* **67**, 86-87, doi:10.5173/ceju.2014.01.art19 (2014).
57 Darboe, M. K., Fulford, A. J., Secka, O. & Prentice, A. M. The dynamics of nasopharyngeal *Streptococcus pneumoniae* carriage among rural Gambian mother-infant pairs. *BMC Infect Dis* **10**, 195, doi:10.1186/1471-2334-10-195 (2010).
58 Bogaert, D., De Groot, R. & Hermans, P. W. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* **4**, 144-154, doi:10.1016/S1473-3099(04)00938-7 (2004).
59 Brown, S. P., Cornforth, D. M. & Mideo, N. Evolution of virulence in opportunistic pathogens: generalism, plasticity, and control. *Trends Microbiol* **20**, 336-342, doi:10.1016/j.tim.2012.04.005 (2012).
60 Weiser, J. N. The pneumococcus: why a commensal misbehaves. *J Mol Med (Berl)* **88**, 97-102, doi:10.1007/s00109-009-0557-x (2010).
61 Austrian, R. Some aspects of the pneumococcal carrier state. *J Antimicrob Chemother* **18 Suppl A**, 35-45, doi:10.1093/jac/18.supplement_a.35 (1986).
62 Maxwell, F. Recent advances in the epidemiology of pneumococcal infections. *Medicine* **21** (1942).
63 Sleeman, K. L. *et al.* Capsular serotype-specific attack rates and duration of carriage of *Streptococcus pneumoniae* in a population of children. *J Infect Dis* **194**, 682-688, doi:10.1086/505710 (2006).

64 Gray, B. M., Converse, G. M., 3rd & Dillon, H. C., Jr. Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life. *J Infect Dis* **142**, 923-933 (1980).
65 Walsh, R. L. & Camilli, A. *Streptococcus pneumoniae* is desiccation tolerant and infectious upon rehydration. *MBio* **2**, e00092-00011, doi:10.1128/mBio.00092-11 (2011).
66 Marks, L. R., Reddinger, R. M. & Hakansson, A. P. Biofilm formation enhances fomite survival of *Streptococcus pneumoniae* and *Streptococcus pyogenes. Infect Immun* **82**, 1141-1146, doi:10.1128/IAI.01310-13 (2014).
67 Costerton, J. W., Geesey, G. G. & Cheng, K. J. How bacteria stick. *Sci Am* **238**, 86-95 (1978).
68 Donlan, R. M. & Costerton, J. W. Biofilms: survival mechanisms of clinically relevant microorganisms. *Clin Microbiol Rev* **15**, 167-193, doi:10.1128/cmr.15.2.167-193.2002 (2002).
69 Flemming, H. C. & Wingender, J. The biofilm matrix. *Nat Rev Microbiol* **8**, 623-633, doi:10.1038/nrmicro2415 (2010).
70 Flemming, H. C. *et al.* Biofilms: an emergent form of bacterial life. *Nat Rev Microbiol* **14**, 563-575, doi:10.1038/nrmicro.2016.94 (2016).
71 Tseng, B. S. *et al.* The extracellular matrix protects *Pseudomonas aeruginosa* biofilms by limiting the penetration of tobramycin. *Environ Microbiol* **15**, 2865-2878, doi:10.1111/1462-2920.12155 (2013).
72 Brooun, A., Liu, S. & Lewis, K. A dose-response study of antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother* **44**, 640-646, doi:10.1128/aac.44.3.640-646.2000 (2000).
73 Lewis, K. Persister cells, dormancy and infectious disease. *Nat Rev Microbiol* **5**, 48-56, doi:10.1038/nrmicro1557 (2007).
74 Stewart, P. S. & Costerton, J. W. Antibiotic resistance of bacteria in biofilms. *Lancet* **358**, 135-138, doi:10.1016/s0140-6736(01)05321-1 (2001).
75 Trivedi, U. *et al.* Prevalence of Multiple Antibiotic Resistant Infections in Diabetic versus Nondiabetic Wounds. *J Pathog* **2014**, 173053, doi:10.1155/2014/173053 (2014).
76 Blanchette, K. A. & Orihuela, C. J. Future perspective on host-pathogen interactions during bacterial biofilm formation within the nasopharynx. *Future Microbiol* **7**, 227-239, doi:10.2217/fmb.11.160 (2012).
77 Feldman, C. *et al.* The interaction of *Streptococcus pneumoniae* with intact human respiratory mucosa *in vitro*. *Eur Respir J* **5**, 576-583 (1992).
78 Shak, J. R., Vidal, J. E. & Klugman, K. P. Influence of bacterial interactions on pneumococcal colonization of the nasopharynx. *Trends Microbiol* **21**, 129-135, doi:10.1016/j.tim.2012.11.005 (2013).
79 Munoz-Elias, E. J., Marcano, J. & Camilli, A. Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization. *Infect Immun* **76**, 5049-5061, doi:10.1128/IAI.00425-08 (2008).
80 Gilley, R. P. & Orihuela, C. J. Pneumococci in biofilms are non-invasive: implications on nasopharyngeal colonization. *Front Cell Infect Microbiol* **4**, 163, doi:10.3389/fcimb.2014.00163 (2014).

81 Chao, Y., Marks, L. R., Pettigrew, M. M. & Hakansson, A. P. *Streptococcus pneumoniae* biofilm formation and dispersion during colonization and disease. *Front Cell Infect Microbiol* **4**, 194, doi:10.3389/fcimb.2014.00194 (2014).
82 Marks, L. R., Parameswaran, G. I. & Hakansson, A. P. Pneumococcal interactions with epithelial cells are crucial for optimal biofilm formation and colonization *in vitro* and i*n vivo*. *Infect Immun* **80**, 2744-2760, doi:10.1128/IAI.00488-12 (2012).
83 Dabernat, H. *et al.* Effects of cefixime or co-amoxiclav treatment on nasopharyngeal carriage of *Streptococcus pneumoniae* and *Haemophilus influenzae* in children with acute otitis media. *J Antimicrob Chemother* **41**, 253-258, doi:10.1093/jac/41.2.253 (1998).
84 Dagan, R. *et al.* Dynamics of pneumococcal nasopharyngeal colonization during the first days of antibiotic treatment in pediatric patients. *Pediatr Infect Dis J* **17**, 880-885 (1998).
85 Hall-Stoodley, L. *et al.* Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in *Streptococcus pneumoniae* clinical isolates. *BMC Microbiol* **8**, 173, doi:10.1186/1471-2180-8-173 (2008).
86 Sanchez, C. J. *et al. Streptococcus pneumoniae* in biofilms are unable to cause invasive disease due to altered virulence determinant production. *PLoS One* **6**, e28738, doi:10.1371/journal.pone.0028738 (2011).
87 Allan, R. N. *et al.* Low Concentrations of Nitric Oxide Modulate S*treptococcus pneumoniae* Biofilm Metabolism and Antibiotic Tolerance. *Antimicrob Agents Chemother* **60**, 2456-2466, doi:10.1128/AAC.02432-15 (2016).
88 Marks, L. R., Reddinger, R. M. & Hakansson, A. P. High levels of genetic recombination during nasopharyngeal carriage and biofilm formation in *Streptococcus pneumoniae. MBio* **3**, doi:10.1128/mBio.00200-12 (2012).
89 Wei, H. & Havarstein, L. S. Fratricide is essential for efficient gene transfer between pneumococci in biofilms. *Appl Environ Microbiol* **78**, 5897-5905, doi:10.1128/AEM.01343-12 (2012).
90 Brooks, L. R. K. & Mias, G. I. *Streptococcus pneumoniae*'s Virulence and Host Immunity: Aging, Diagnostics, and Prevention. *Front Immunol* **9**, 1366, doi:10.3389/fimmu.2018.01366 (2018).
91 Pelton, S. I. Regulation of bacterial trafficking in the nasopharynx. *Paediatr Respir Rev* **13**, 150-153, doi:10.1016/j.prrv.2012.04.001 (2012).
92 Man, W. H., de Steenhuijsen Piters, W. A. & Bogaert, D. The microbiota of the respiratory tract: gatekeeper to respiratory health. *Nat Rev Microbiol* **15**, 259-270, doi:10.1038/nrmicro.2017.14 (2017).
93 Richards, L., Ferreira, D. M., Miyaji, E. N., Andrew, P. W. & Kadioglu, A. The immunising effect of pneumococcal nasopharyngeal colonisation; protection against future colonisation and fatal invasive disease. *Immunobiology* **215**, 251-263, doi:10.1016/j.imbio.2009.12.004 (2010).
94 Pichichero, M. E. *et al.* Antibody response to *Streptococcus pneumoniae* proteins PhtD, LytB, PcpA, PhtE and Ply after nasopharyngeal colonization and acute otitis media in children. *Hum Vaccin Immunother* **8**, 799-805, doi:10.4161/hv.19820 (2012).
95 Granat, S. M. *et al.* Epidemiological evidence for serotype-independent acquired immunity to pneumococcal carriage. *J Infect Dis* **200**, 99-106, doi:10.1086/599364 (2009).

96 Musher, D. M. *et al.* Emergence of antibody to capsular polysaccharides of *Streptococcus pneumoniae* during outbreaks of pneumonia: association with nasopharyngeal colonization. *Clin Infect Dis* **24**, 441-446, doi:10.1093/clinids/24.3.441 (1997).
97 Ferreira, D. M. *et al.* Controlled human infection and rechallenge with *Streptococcus pneumoniae* reveals the protective efficacy of carriage in healthy adults. *Am J Respir Crit Care Med* **187**, 855-864, doi:10.1164/rccm.201212-2277OC (2013).
98 McCool, T. L., Cate, T. R., Moy, G. & Weiser, J. N. The immune response to pneumococcal proteins during experimental human carriage. *J Exp Med* **195**, 359-365, doi:10.1084/jem.20011576 (2002).
99 Ridda, I. *et al.* Lack of pneumococcal carriage in the hospitalised elderly. *Vaccine* **28**, 3902-3904, doi:10.1016/j.vaccine.2010.03.073 (2010).
100 Turnbaugh, P. J. *et al.* The human microbiome project. *Nature* **449**, 804-810, doi:10.1038/nature06244 (2007).
101 Stecher, B. The Roles of Inflammation, Nutrient Availability and the Commensal Microbiota in Enteric Pathogen Infection. *Microbiol Spectr* **3**, doi:10.1128/microbiolspec.MBP-0008-2014 (2015).
102 Kamada, N., Chen, G. Y., Inohara, N. & Nunez, G. Control of pathogens and pathobionts by the gut microbiota. *Nat Immunol* **14**, 685-690, doi:10.1038/ni.2608 (2013).
103 Bosch, A. *et al.* Maturation of the Infant Respiratory Microbiota, Environmental Drivers, and Health Consequences. A Prospective Cohort Study. *Am J Respir Crit Care Med* **196**, 1582-1590, doi:10.1164/rccm.201703-0554OC (2017).
104 Bosch, A. *et al.* Development of Upper Respiratory Tract Microbiota in Infancy is Affected by Mode of Delivery. *EBioMedicine* **9**, 336-345, doi:10.1016/j.ebiom.2016.05.031 (2016).
105 Biesbroek, G. *et al.* The impact of breastfeeding on nasopharyngeal microbial communities in infants. *Am J Respir Crit Care Med* **190**, 298-308, doi:10.1164/rccm.201401-0073OC (2014).
106 Biesbroek, G. *et al.* Early respiratory microbiota composition determines bacterial succession patterns and respiratory health in children. *Am J Respir Crit Care Med* **190**, 1283-1292, doi:10.1164/rccm.201407-1240OC (2014).
107 Costerton, J. W., Stewart, P. S. & Greenberg, E. P. Bacterial biofilms: a common cause of persistent infections. *Science* **284**, 1318-1322, doi:10.1126/science.284.5418.1318 (1999).
108 Bloomfield, S. *et al.* Lesser-known or hidden reservoirs of infection and implications for adequate prevention strategies: Where to look and what to look for. *GMS Hyg Infect Control* **10**, Doc04, doi:10.3205/dgkh000247 (2015).
109 Kotaskova, I. *et al.* Molecular Techniques Complement Culture-Based Assessment of Bacteria Composition in Mixed Biofilms of Urinary Tract Catheter-Related Samples. *Front Microbiol* **10**, 462, doi:10.3389/fmicb.2019.00462 (2019).
110 Ehrlich, G. D. *et al.* Mucosal biofilm formation on middle-ear mucosa in the chinchilla model of otitis media. *JAMA* **287**, 1710-1715, doi:10.1001/jama.287.13.1710 (2002).
111 Hall-Stoodley, L. *et al.* Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* **296**, 202-211, doi:10.1001/jama.296.2.202 (2006).

112 Reid, S. D. *et al. Streptococcus pneumoniae* forms surface-attached communities in the middle ear of experimentally infected chinchillas. *J Infect Dis* **199**, 786-794, doi:10.1086/597042 (2009).
113 Weimer, K. E. *et al.* Coinfection with Haemophilus influenzae promotes pneumococcal biofilm formation during experimental otitis media and impedes the progression of pneumococcal disease. *J Infect Dis* **202**, 1068-1075, doi:10.1086/656046 (2010).
114 Sanderson, A. R., Leid, J. G. & Hunsaker, D. Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. *Laryngoscope* **116**, 1121-1126, doi:10.1097/01.mlg.0000221954.05467.54 (2006).
115 Sanchez, C. J. *et al.* The pneumococcal serine-rich repeat protein is an intra-species bacterial adhesin that promotes bacterial aggregation i*n vivo* and in biofilms. *PLoS Pathog* **6**, e1001044, doi:10.1371/journal.ppat.1001044 (2010).
116 Shenoy, A. T. *et al. Streptococcus pneumoniae* in the heart subvert the host response through biofilm-mediated resident macrophage killing. *PLoS Pathog* **13**, e1006582, doi:10.1371/journal.ppat.1006582 (2017).
117 Pettigrew, M. M. *et al.* Viral-bacterial interactions and risk of acute otitis media complicating upper respiratory tract infection. *J Clin Microbiol* **49**, 3750-3755, doi:10.1128/JCM.01186-11 (2011).
118 Klein, E. Y. *et al.* The frequency of influenza and bacterial coinfection: a systematic review and meta-analysis. *Influenza Other Respir Viruses* **10**, 394-403, doi:10.1111/irv.12398 (2016).
119 Diavatopoulos, D. A. *et al.* Influenza A virus facilitates *Streptococcus pneumoniae* transmission and disease. *FASEB J* **24**, 1789-1798, doi:10.1096/fj.09-146779 (2010).
120 Ortigoza, M. B., Blaser, S. B., Zafar, M. A., Hammond, A. J. & Weiser, J. N. An Infant Mouse Model of Influenza Virus Transmission Demonstrates the Role of Virus-Specific Shedding, Humoral Immunity, and Sialidase Expression by Colonizing Streptococcus pneumoniae. *MBio* **9**, doi:10.1128/mBio.02359-18 (2018).
121 Marks, L. R., Davidson, B. A., Knight, P. R. & Hakansson, A. P. Interkingdom signaling induces *Streptococcus pneumoniae* biofilm dispersion and transition from asymptomatic colonization to disease. *MBio* **4**, doi:10.1128/mBio.00438-13 (2013).
122 Pettigrew, M. M. *et al.* Dynamic changes in the *Streptococcus pneumoniae* transcriptome during transition from biofilm formation to invasive disease upon influenza A virus infection. *Infect Immun* **82**, 4607-4619, doi:10.1128/IAI.02225-14 (2014).
123 Wolcott, R. D. & Ehrlich, G. D. Biofilms and chronic infections. *JAMA* **299**, 2682-2684, doi:10.1001/jama.299.22.2682 (2008).
124 Blanchette-Cain, K. *et al. Streptococcus pneumoniae* biofilm formation is strain dependent, multifactorial, and associated with reduced invasiveness and immunoreactivity during colonization. *MBio* **4**, e00745-00713, doi:10.1128/mBio.00745-13 (2013).
125 Guilhen, C., Forestier, C. & Balestrino, D. Biofilm dispersal: multiple elaborate strategies for dissemination of bacteria with unique properties. *Mol Microbiol* **105**, 188-210, doi:10.1111/mmi.13698 (2017).
126 Havarstein, L. S., Coomaraswamy, G. & Morrison, D. A. An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae. Proc Natl Acad Sci U S A* **92**, 11140-11144, doi:10.1073/pnas.92.24.11140 (1995).

127 Havarstein, L. S., Gaustad, P., Nes, I. F. & Morrison, D. A. Identification of the streptococcal competence-pheromone receptor. *Mol Microbiol* **21**, 863-869, doi:10.1046/j.1365-2958.1996.521416.x (1996).
128 Pestova, E. V., Havarstein, L. S. & Morrison, D. A. Regulation of competence for genetic transformation in *Streptococcus pneumoniae* by an auto-induced peptide pheromone and a two-component regulatory system. *Mol Microbiol* **21**, 853-862, doi:10.1046/j.1365-2958.1996.501417.x (1996).
129 Havarstein, L. S., Martin, B., Johnsborg, O., Granadel, C. & Claverys, J. P. New insights into the pneumococcal fratricide: relationship to clumping and identification of a novel immunity factor. *Mol Microbiol* **59**, 1297-1307, doi:10.1111/j.1365-2958.2005.05021.x (2006).
130 Oggioni, M. R. *et al.* Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. *Mol Microbiol* **61**, 1196-1210, doi:10.1111/j.1365-2958.2006.05310.x (2006).
131 Kreth, J., Merritt, J., Shi, W. & Qi, F. Co-ordinated bacteriocin production and competence development: a possible mechanism for taking up DNA from neighbouring species. *Mol Microbiol* **57**, 392-404, doi:10.1111/j.1365-2958.2005.04695.x (2005).
132 Hammerschmidt, S. *et al.* Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. *Infect Immun* **73**, 4653-4667, doi:10.1128/IAI.73.8.4653-4667.2005 (2005).
133 Moscoso, M., Garcia, E. & Lopez, R. Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *J Bacteriol* **188**, 7785-7795, doi:10.1128/JB.00673-06 (2006).
134 Los, F. C., Randis, T. M., Aroian, R. V. & Ratner, A. J. Role of pore-forming toxins in bacterial infectious diseases. *Microbiol Mol Biol Rev* **77**, 173-207, doi:10.1128/MMBR.00052-12 (2013).
135 Shak, J. R. *et al.* Novel role for the *Streptococcus pneumoniae* toxin pneumolysin in the assembly of biofilms. *MBio* **4**, e00655-00613, doi:10.1128/mBio.00655-13 (2013).
136 Fassel, T. A., Mozdziak, P. E., Sanger, J. R. & Edmiston, C. E. Paraformaldehyde effect on ruthenium red and lysine preservation and staining of the staphylococcal glycocalyx. *Microsc Res Tech* **36**, 422-427, doi:10.1002/(SICI)1097-0029(19970301)36:5<422::AID-JEMT12>3.0.CO;2-U (1997).
137 Chua, S. L. *et al.* Dispersed cells represent a distinct stage in the transition from bacterial biofilm to planktonic lifestyles. *Nat Commun* **5**, 4462, doi:10.1038/ncomms5462 (2014).
138 Flemming, H. C. & Wingender, J. Relevance of microbial extracellular polymeric substances (EPSs)--Part I: Structural and ecological aspects. *Water Sci Technol* **43**, 1-8 (2001).
139 Domenech, M., Garcia, E. & Moscoso, M. Biofilm formation in *Streptococcus pneumoniae*. *Microb Biotechnol* **5**, 455-465, doi:10.1111/j.1751-7915.2011.00294.x (2012).
140 Novotny, L. A., Jurcisek, J. A., Goodman, S. D. & Bakaletz, L. O. Monoclonal antibodies against DNA-binding tips of DNABII proteins disrupt biofilms *in vitro* and induce bacterial clearance *in vivo*. *EBioMedicine* **10**, 33-44, doi:10.1016/j.ebiom.2016.06.022 (2016).
141 Beiter, K. *et al.* An endonuclease allows *Streptococcus pneumoniae* to escape from neutrophil extracellular traps. *Curr Biol* **16**, 401-407, doi:10.1016/j.cub.2006.01.056 (2006).

142 Allegrucci, M. *et al.* Phenotypic characterization of *Streptococcus pneumoniae* biofilm development. *J Bacteriol* **188**, 2325-2335, doi:10.1128/JB.188.7.2325-2335.2006 (2006).
143 Ma, L. *et al.* Assembly and development of the *Pseudomonas aeruginosa* biofilm matrix. *PLoS Pathog* **5**, e1000354, doi:10.1371/journal.ppat.1000354 (2009).
144 Lauderdale, K. J., Malone, C. L., Boles, B. R., Morcuende, J. & Horswill, A. R. Biofilm dispersal of community-associated methicillin-resistant *Staphylococcus aureus* on orthopedic implant material. *J Orthop Res* **28**, 55-61, doi:10.1002/jor.20943 (2010).
145 Chaignon, P. *et al.* Susceptibility of staphylococcal biofilms to enzymatic treatments depends on their chemical composition. *Appl Microbiol Biotechnol* **75**, 125-132, doi:10.1007/s00253-006-0790-y (2007).
146 Domenech, M., Garcia, E. & Moscoso, M. *In vitro* destruction of *Streptococcus pneumoniae* biofilms with bacterial and phage peptidoglycan hydrolases. *Antimicrob Agents Chemother* **55**, 4144-4148, doi:10.1128/AAC.00492-11 (2011).
147 Kaplan, J. B. & Fine, D. H. Biofilm dispersal of *Neisseria subflava* and other phylogenetically diverse oral bacteria. *Appl Environ Microbiol* **68**, 4943-4950, doi:10.1128/aem.68.10.4943-4950.2002 (2002).
148 Liu, G., Tang, C. M. & Exley, R. M. Non-pathogenic *Neisseria:* members of an abundant, multi-habitat, diverse genus. *Microbiology* **161**, 1297-1312, doi:10.1099/mic.0.000086 (2015).
149 Reddinger, R. M., Luke-Marshall, N. R., Hakansson, A. P. & Campagnari, A. A. Host Physiologic Changes Induced by Influenza A Virus Lead to *Staphylococcus aureus* Biofilm Dispersion and Transition from Asymptomatic Colonization to Invasive Disease. *MBio* 7, doi:10.1128/mBio.01235-16 (2016).
150 Bittaye, M., Cash, P. & Forbes, K. Proteomic variation and diversity in clinical *Streptococcus pneumoniae* isolates from invasive and non-invasive sites. *PLoS One* **12**, e0179075, doi:10.1371/journal.pone.0179075 (2017).
151 Chai, M. H. *et al.* Proteomic comparisons of opaque and transparent variants of *Streptococcus pneumoniae* by two dimensional-differential gel electrophoresis. *Sci Rep* 7, 2453, doi:10.1038/s41598-017-02465-x (2017).
152 Allan, R. N. *et al.* Pronounced metabolic changes in adaptation to biofilm growth by *Streptococcus pneumoniae*. *PLoS One* **9**, e107015, doi:10.1371/journal.pone.0107015 (2014).
153 Sauer, K., Camper, A. K., Ehrlich, G. D., Costerton, J. W. & Davies, D. G. *Pseudomonas aeruginosa* displays multiple phenotypes during development as a biofilm. *J Bacteriol* **184**, 1140-1154, doi:10.1128/jb.184.4.1140-1154.2002 (2002).
154 Sauer, K. *et al.* Characterization of nutrient-induced dispersion in *Pseudomonas aeruginosa* PAO1 biofilm. *J Bacteriol* **186**, 7312-7326, doi:10.1128/JB.186.21.7312-7326.2004 (2004).
155 Tavares, G. C. *et al.* Transcriptome and Proteome of Fish-Pathogenic *Streptococcus agalactiae* Are Modulated by Temperature. *Front Microbiol* **9**, 2639, doi:10.3389/fmicb.2018.02639 (2018).
156 Man, W. H. *et al.* Loss of Microbial Topography between Oral and Nasopharyngeal Microbiota and Development of Respiratory Infections Early in Life. *Am J Respir Crit Care Med*, doi:10.1164/rccm.201810-1993OC (2019).

157 Whelan, F. J. *et al.* The loss of topography in the microbial communities of the upper respiratory tract in the elderly. *Ann Am Thorac Soc* **11**, 513-521, doi:10.1513/AnnalsATS.201310-351OC (2014).
158 Oh, J., Conlan, S., Polley, E. C., Segre, J. A. & Kong, H. H. Shifts in human skin and nares microbiota of healthy children and adults. *Genome Med* **4**, 77, doi:10.1186/gm378 (2012).
159 Kaspar, U. *et al.* The culturome of the human nose habitats reveals individual bacterial fingerprint patterns. *Environ Microbiol* **18**, 2130-2142, doi:10.1111/1462-2920.12891 (2016).
160 Teo, S. M. *et al.* The infant nasopharyngeal microbiome impacts severity of lower respiratory infection and risk of asthma development. *Cell Host Microbe* **17**, 704-715, doi:10.1016/j.chom.2015.03.008 (2015).
161 Pettigrew, M. M. *et al.* Upper respiratory tract microbial communities, acute otitis media pathogens, and antibiotic use in healthy and sick children. *Appl Environ Microbiol* **78**, 6262-6270, doi:10.1128/AEM.01051-12 (2012).
162 Laufer, A. S. *et al.* Microbial communities of the upper respiratory tract and otitis media in children. *MBio* **2**, e00245-00210, doi:10.1128/mBio.00245-10 (2011).
163 Konno, M. *et al.* Study of upper respiratory tract bacterial flora: first report. Variations in upper respiratory tract bacterial flora in patients with acute upper respiratory tract infection and healthy subjects and variations by subject age. *J Infect Chemother* **12**, 83-96, doi:10.1007/s10156-006-0433-3 (2006).
164 Brugger, S. D., Bomar, L. & Lemon, K. P. Commensal-Pathogen Interactions along the Human Nasal Passages. *PLoS Pathog* **12**, e1005633, doi:10.1371/journal.ppat.1005633 (2016).
165 Bomar, L., Brugger, S. D., Yost, B. H., Davies, S. S. & Lemon, K. P. *Corynebacterium accolens* Releases Antipneumococcal Free Fatty Acids from Human Nostril and Skin Surface Triacylglycerols. *MBio* **7**, e01725-01715, doi:10.1128/mBio.01725-15 (2016).
166 Souza, M. C. *et al.* Biofilm formation and fibrinogen and fibronectin binding activities by *Corynebacterium pseudodiphtheriticum* invasive strains. *Antonie Van Leeuwenhoek* **107**, 1387-1399, doi:10.1007/s10482-015-0433-3 (2015).
167 Richardson, A. R., Somerville, G. A. & Sonenshein, A. L. Regulating the Intersection of Metabolism and Pathogenesis in Gram-positive Bacteria. *Microbiol Spectr* **3**, doi:10.1128/microbiolspec.MBP-0004-2014 (2015).