UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

NEW INSIGHTS INTO THE SOCIOMICROBIOLOGY OF *Streptococccus pneumoniae*: EXPLORING BIOFILM FORMATION

HELENA MARGARIDA DA SILVA MORAIS CARROLO

DOUTORAMENTO EM CIÊNCIAS E TECNOLOGIAS DA SAÚDE

ESPECIALIDADE EM MICROBIOLOGIA

2011

UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

NEW INSIGHTS INTO THE SOCIOMICROBIOLOGY OF *Streptococccus pneumoniae*: EXPLORING BIOFILM FORMATION

HELENA MARGARIDA DA SILVA MORAIS CARROLO

DOUTORAMENTO EM CIÊNCIAS E TECNOLOGIAS DA SAÚDE,

ESPECIALIDADE EM MICROBIOLOGIA

2011

UNIVERSIDADE DE LISBOA

FACULDADE DE MEDICINA DE LISBOA

NEW INSIGHTS INTO THE SOCIOMICROBIOLOGY OF *Streptococccus pneumoniae*: EXPLORING BIOFILM FORMATION

HELENA MARGARIDA DA SILVA MORAIS CARROLO

DOUTORAMENTO EM CIÊNCIAS E TECNOLOGIAS DA SAÚDE,

ESPECIALIDADE EM MICROBIOLOGIA

TESE ORIENTADA PELO

PROFESSOR DOUTOR MÁRIO NUNO RAMOS DE ALMEIDA RAMIREZ

To Sternberg and Pasteur who first unveiled the pneumococcus

Resumo

Palavras-chave: *Streptococcus pneumoniae*, competência, lise fágica, formação de biofilmes

A última década assistiu a um aumento das evidências reunidas sobre a elevada diversidade genética existente na população de *Streptococcus pneumoniae.* A competência associada à transformação natural e a transdução fágica são dois recursos biológicos importantes para a plasticidade genómica do pneumococo. Ambos contribuem de forma signficativa para a modificação genómica e têm um impacto visível na capacidade de adaptação e sobrevivência desta bactéria.

Outra estratégia de sobrevivência do pneumococo é a sua capacidade de formação de biofilmes. Estas comunidades bacterianas constituem mecanismos de protecção face a ameaças ambientais tais como os mecanismos de defesa do sistema imunitário do hospedeiro e os antibióticos. Além disso, devido ao facto das bactérias existirem maioritamente sob a forma de biofilmes e de existir uma maior proximidade física entre as bactérias nestas estruturas, os biofilmes são provavelmente o palco principal para a ocorrência das trocas genéticas.

Esta tese contribui para um maior conhecimento da relação entre mecanismos geradores de diversidade genética e a capacidade de formação de biofilmes em *Streptococcus pneumoniae*. Com esse intuito foram seleccionadas duas características biológicas: a) a presença de fagos lisogénicos no genoma bacteriano e b) a diversidade ferotípica, resultando em três estudos independentes: i) o impacto da activação espontânea de fagos lisogénicos na formação de biofilmes pneumocócicos, ii) o estudo de genética de populações sobre a distribuição dos ferótipos e a sua contribuição para a diferenção genética no pneumococo, iii) o estudo da influência dos ferótipos na formação de biofilmes pneumocócicos e na respectiva eficiência de recombinação.

O estudo do efeito da presença de fagos lisogénicos no genoma bacteriano e da sua lise espontânea nos biofilmes pneumocócicos mostrou que, apesar da

indução fágica resultar na morte das respectivas bactérias hospedeiras, a população bacteriana como um todo beneficia da presença dos fagos lisogénicos observando-se um aumento na capacidade de formação de biofilmes. Este estudo permitiu ainda mostrar a existência de uma ligação entre a libertação de DNA e o aumento dos biofilmes pneumocócicos.

O estudo de genética de populações permitiu mostrar que, de entre os dois ferótipos dominantes no pneumococo (CSP1 e CSP2), a maioria das estirpes invasivas caracterizadas apresenta o ferótipo CSP1. Várias associações entre os ferótipos e outros marcadores biológicos foram identificadas indicando que os ferótipos não estão aleatoriamente distribuídos na população pneumocócica. Foram detectadas associações com o serótipo, a resistência antimicrobiana e a linhagem genética; os dados obtidos sugerem igualmente que a transdução fágica pode ser indirectamente arbitrada pelo ferótipo, implicando uma distribuição heterogénea de elementos genéticos de maior dimensão, como é o caso de alguns determinantes genéticos de resistência antimicrobiana. O estudo permitiu ainda mostrar que estirpes filogeneticamente mais próximas têm uma maior probabilidade de partilhar o mesmo ferótipo. Limitações severas à comunicação entre ferótipos parecem estar a conduzir a população pneumocócica para uma situação de afastamento genético continuado, explicando deste modo as duas populações geneticamente distintas que foram detectadas ao longo deste estudo.

No âmbito desta tese foi ainda explorado o impacto dos dois ferótipos dominantes na capacidade de formação de biofilmes e na respectiva eficiência de transformação. As estirpes com CSP1 apresentam biofilmes mais densos e com mais biomassa. Além disso, a adição de CSP sintético respectivo amplifica as diferenças observadas no crescimento dos biofilmes entre ferótipos. Este estudo mostra também que estirpes CSP1 são mais eficientes no processo de transformação em meio líquido e em biofilme.

Em conjunto, os resultados obtidos nesta tese mostram que as trocas genéticas entre estirpes pneumocócicas estão a ocorrer preferencialmente entre estirpes que partilham o mesmo ferótipo e que o aumento na formação de biofilme detectado quer como consequência da presença do fago lisógenico quer

pela via de sinalização do CSP, têm em comum o impacto positivo da libertação de DNA para o meio extracelular resultante da lise de uma fracção de bactérias dentro do biofilme.

Abstract

Keywords: *Streptococcus pneumoniae*, competence, prophage-mediated lysis, biofilm formation

During the last decade it has become more evident that *Streptococcus pneumoniae* (pneumococcus) holds a high genetic diversity throughout its population. Two important biological resources for achieving pneumococcal genetic plasticity are competence/natural transformation and phage transduction. Both factors strongly contribute to genome modification and have a real impact in the capacity of survival and adaptation of this bacterium.

Another important survival skill of the pneumococcus is its ability to form matrix-enclosed biofilms. These microbial communities guarantee protection from environmental threats such as host immune defenses and antibiotics. Moreover, due to the fact that bacteria are believed to spend most of their lifetime in biofilms and that there is a higher physical proximity of bacteria within these structures, biofilms are probably the prefered stage for the occurrence of genetic exchange between pneumococci.

This thesis contributes to a better understanding of the mechanisms that generate pneumococcal genetic diversity and the important bacterial ability to form biofilms. In the pursuit of this goal, two biological conditions were selected: a) prophage carriage and b) pherotype characterization, resulting in three independent studies: i) the study of the impact of prophage spontaneous activation on pneumococcal biofilm formation, ii) a molecular epidemiology study of the distribution of pherotypes and its contribution to pneumococcal genetic differentiation and iii) the study of the influence of pherotypes on pneumococcal biofilm growth and recombination efficiency.

The study of the effect of prophage carriage and its spontaneously induced host lysis on pneumococcal biofilms reveal that, although limited phage induction results in the death of their bacterial hosts, the bacterial population as a whole benefits from prophage carriage and an enhancement in biofilm

formation is observed. Moreover, this study shows a link between the external DNA (eDNA) that is released to the medium due to bacterial lysis and the growth of pneumococcal biofilms.

The molecular epidemiology study performed showed that of the two dominants pherotypes (CSP1 and CSP2) the majority of the invasive isolates screened presented the CSP1 pherotype. Several associations with the pherotypes and other biological markers were observed indicating that pherotypes are not randomly distributed within the pneumococcal population. Associations with serotype, antimicrobial resistance and genetic lineage were unveiled; it was also detected that phage transduction may be indirectly arbitrated by pherotypes, implicating an uneven distribution of large genetic elements such as some genetic determinants of antibiotic resistance. The study also showed that strains that are phylogenetically closer have a higher likelihood of sharing the same pherotype. Severe limitations to inter-pherotype communication may be leading towards an ongoing genetic drift, explaining the two genetically distinct subpopulations that were detected.

Moving forward, we decided to explore the impact of the two major pherotypes on both the capacity to form biofilms and on recombination efficiency. Biofilms of strains presenting CSP1 had increased biofilm mass and were more densely packed. Also, the addition of synthetic cognate CSP amplifies the observed differences in biofilm growth between the pherotypes. The study also revealed that CSP1 strains transform more efficient both in the liquid medium and within the biofilm structure.

Taken together this thesis work has shown that genetic exchanges between pneumococcal strains are occurring preferentially between strains sharing the same pherotypes and that the enhancement of biofilm formation detected both by prophage carriage and by CSP signaling have in common the positive impact of DNA release to the extracellular medium resulting from the lysis of a fraction of the bacteria inside the biofilm.

Acknowledgements

Looking back to these almost 4 years and realizing that, among all the ups and downs that Science will always have, I was able to grab a project and bring it to life, is a true joy.

So, for those who stood with me during this PhD journey here is my "Thank you".

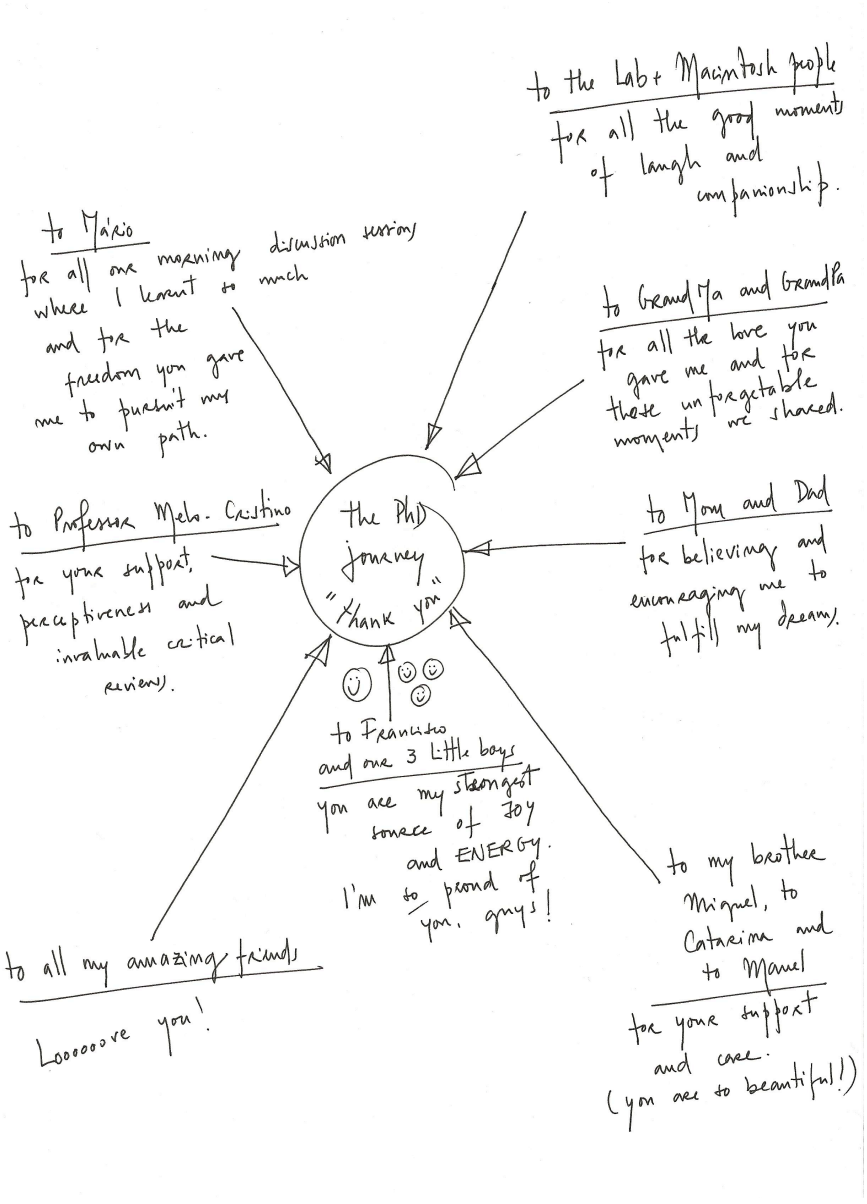

Table of Contents

List of Figures

List of Tables

List of Abbreviations

ABC ATP-binding cassete

ANOVA analysis of variance

ATP adenosine triphosphate

C+Y medium casein-based semi synthetic medium

CFU colony forming unit

CI confidence interval

CLSM confocal laser scanning microscopy

CSP competence-stimulating peptide

DNA deoxyribonucleic acid

DNase deoxyribonuclease

dNTPs deoxynucleotide triphosphates

eDNA external DNA

EPS extracellular polymeric substances

IgA1 immunoglobulin A1

MLST multilocus sequence typing

NovoR novobiocin resistant

OD optical density

OR odds ratio

PCR polymerase chain reaction

PCV7 7-valent pneumococcal conjugate vaccine

PCV13 13-valent pneumococcal conjugate vaccine

PFGE pulsed-field gel electrophoresis

PFU plaque forming unit

PPS pneumococcal conjugate vaccine

PST polystyrene

QS quorum sensing

R/M restriction/modification

SLV single-locus variant

Ssb ssDNA binding protein

ssDNA single-stranded DNA

ST sequence type

StrepR streptomycin resistant

TSA tryptic soy agar

W Wallace coefficient

Chapter 1

Introduction

1. What is Pneumococcus?

1.1. From discovery to vaccines

Streptococcus pneumoniae (pneumococcus) came to light in the golden era of microbiology [1] and since then it has been an object of scientific scrutiny for more than a century.

Two scientists working on opposite sides of the Atlantic Ocean were able to grow pneumococcus *in vitro* and isolated it in culture. Louis Pasteur in France was the first to publish his report, in January 1881, after finding the bacteria in the saliva of a youngster that died with rabies. George Miller Sternberg in the United States sampled his own saliva and successfully grew the bacteria in September 1880, but did not publish his report until April 1881. Their descriptions of the morphology of the isolates were similar each recognizing what is now designated by the diplococcal form of the bacterium and its capsule [1].

The 10-year period following the initial isolation saw rapid extension of the association of the pneumococcus with foci of infection and human disease. During this period pneumococci were isolated from blood, from the lung by direct puncture, from pleural, cerebrospinal and joint fluid [2]. In the same decade, pneumococci was firstly described as an etiological agent of pneumonia, endocarditis, otitis media, arthritis and meningitis [1, 3].

Nevertheless, knowledge of the host's defenses against pneumococcal infections evolved slowly over the final 15 years of the 19th century. The importance of the leukocytes was suggested during this period [4] but the rational use of serotherapy, however, had to wait the recognition of the diversity of pneumococcal capsular types, which did not begin clearly until 1910 [3].

In the beginning of the 20th century, the fortuitous finding of pneumococcus solubility in bile by Neufeld was a major contribution to the identification of this

pathogen [2]. Also in this period, the Quellung reaction [5] was described despite the fact that its first use in the identification of pneumococcal capsular types was not made until 1931 [3].

Study of the pneumococcal capsule has played a major role in the development of immunology. Prior to the second decade of the 20th century, it was widely believed that all antigens were proteins. In 1925, as a result of several years performing chemical studies, it was finally reported by Heidelberger and Avery that "the so-called specific soluble substance of Pneumococcus of the three fixed types is in each instance a polysaccharide" [6]. Subsequent studies marked the foundations of quantitative immunology [7].

The polysaccharide capsule of *Streptococcus pneumoniae* is an important player in the virulence of this organism [3]. Ninety-four serologically distinct capsules have already been recognized [8, 9]. The polysaccharides differ with respect to sugar composition, chemical bonds and presence or absence of side chains. Despite their diversity, all perform the same primary function of providing protection against complement-mediated opsonophagocytosis by blocking the access to the pneumococcus cell wall and associated proteins.

Overall, serotype diversity has been a challenge to effective vaccination. Attempts to prevent pneumococcal pneumonia by vaccination were initiated in 1910s, although failing to establish efficacy [2, 3]. Only in 1945 came out the initial report that type-specific pneumococcal infection in man could be prevented by a tetravalent vaccine of capsular polysaccharides [10].

At the end of the second decade of the 20th century, while trying to produce an effective vaccine against pneumococcal pneumonia, Griffith found that bacteria are capable of transferring genetic information through a process designated by transformation [11]. These observations were the root for the work developed by Avery, MacLeod and McCarthy [10] that culminated on the first report of a biological activity of a nucleic acid and became the cornerstone of molecular genetics. It was the capacity of pneumococci to undergo genetic transformation that lead to the discovery that competence, the process by which cells can bind and uptake exogenous DNA, presented the intriguing property of being tightly controlled at the

population level [12-14]. Competence was thus one of the first examples of a multicellular bacterial response coordinated by a diffusible signal. These processes were later termed quorum-sensing and found to be used by bacteria to sincronize the switch of genetic programs simultaneously at the population level in order to achieve goals that are unattainable by single cells [15].

Although the development of antimicrobial drugs has revolutionized the treatment of bacterial infections, the availability and use of these therapeutic agents have evidenced the adaptability of living organisms. The pneumococcus was among the first bacterial species recognized as being capable of developing resistance to an antimicrobial agent. In 1912 a report was published disclosing the first observations on the development of *in vivo* resistance to an antibacterial drug (optochin: ethyl hydrocuprein) in mice infected with pneumococci [1]. This finding was made a long time before antibioteraphy was considered safe and effective for the treatment of humans and so this phenomenum was rediscovered many years after. In fact, pneumococcal resistant isolates have been described since the introduction of antimicrobial agents. Examples include resistance to sulfonamides reported in the United States in 1943 [16, 17], tetracycline resistance in the United Kingdom in 1963 [18], macrolide resistance in Canada and United States in 1967 [19, 20], penicillin also in 1967 in Australia in 1967 [21], chloramphenicol resistance in Poland in 1970 [22] and sulfamethoxazole-trimethoprim in the United Kingdom in 1972 [23]. Multiply resistant pneumococcal strains, defined as resistant to three or more classes of antibiotics, were first described in 1978 in South Africa [24].

Given the widespread antibiotic resistance to *Streptococcus pneumoniae*, a renewed interest in vaccines for the prevention of pneumococcal infections arose. During the 1970s, the first 23-valent polysaccharide pneumococcal vaccine appeared, being adopted in a very restricted way both geographically and in the population being targeted [1]. The major drawback of this vaccine was the lack of efficacy in infants, calling for further improvement. The result was the heptavalent pneumococcal conjugate vaccine (PCV7), containing the 7 most common pneumococcal serotypes causing invasive infections in children in North America licensed in the US and recommended for routine use in infants in 2000. However, even though the PCV7 vaccine has been reported to reduce pneumococcal disease

[25-27], there are other nonvaccine serotypes arising due to the vaccine selective pressure on the pneumococcal population. To respond to this situation, in 2010 a new 13-valent pneumococcal conjugate vaccine (PCV13) was licensed and its usage is now widely recommended. Nevertheless, pneumococcal conjugated vaccines may not be the ultimate answer for the control of pneumococcal disease. The current unit cost of PCV13 is likely to be unaffordable to many countries where the burden of disease is high [28]. Furthermore, vaccines are limited by the number of serotypes that can be included due to manufacturing considerations and cost of production. In addition, although colonization of vaccine serotypes have been reduced, non-vaccine serotypes have been occupying the vacanted niche, in a process referred to as serotype replacement. This process of replacement has had an impact both on nasopharyngeal colonization and in pneumococcal disease [25].

The ability of pneumococcus to evolve and adapt to new conditions warns against complacency and calls for further action. The lessons learned during the last 130 years will certainly be instructive for the refinement of the current knowledge and for strengthening the future research challenges.

1.2. Biological background and epidemiology

Streptococcus pneumoniae is taxonomically recognized as a group within the pneumoniae-mitis-pseudopneumoniae cluster of the *Streptococcus* genus [29]. It is strikingly similar to four other species *Streptococcus mitis*, *Streptococcus oralis*, *Streptococcus infantis*, and *Streptococcus pseudopneumoniae* [30-33], often leading to problems of identification in microbiology laboratories [29].

The pneumococcus is a Gram-positive pathogenic bacterium, lancet-shaped, which grows in pairs or short chains. Identification of pneumococci has relied heavily upon colony morphology and hemolytic activity on blood agar plates (α hemolysis) [3, 34]. Additional tests include susceptibility to optochin and solubility in bile salts, catalase negativity and agglutination with antipneumococcal polysaccharide sera [5, 35].

The pneumococcus is a common component of the nasopharyngeal mucous membrane microflora [3]; it is believed that most episodes of nasopharyngeal colonization cause no symptoms or disease, although recent data indicate that mild respiratory tract symptoms may occur [36]. There is serial and simultaneous colonization by multiple strains [37-39] with an individual strain generally being carried transiently for weeks to months before it is cleared [40, 41]. Transmission of *Streptococcus pneumoniae* infection occurs *via* respiratory droplets from persons with pneumococcal disease or, more commonly, persons without disease who carry the bacteria in the nasopharynx [42]. When pneumococcal disease occurs, it is the host's own colonizing pneumococci that are the source of the disease-causing strain [1].

The pneumococcus is one of the most common etiologies of respiratory tract infections like sinusitis, otitis media and pneumonia, as well as nonrespiratory infections like meningitis, sepsis and bacteremia [43]. Properties that explain the pathogenic potential of pneumococcus include the presence of a polysaccharide capsule, IgA1 protease, pneumolysin, autolysin, and several surface-exposed proteins that mediate contact with components of the host's tissues and secretions [3, 29, 44].

The vast majority of pneumococcal disease is caused by a relatively small number of serotypes [3]. This could explain the fact that the number of pneumococcal serotypes found colonizing persons in a given community is generally greater than the number causing invasive infection. In what concerns serotype invasiveness-capacity, it has long been observed that certain serotypes appear to be more invasive than other serotypes as they are seldom isolated from asymptomatic carriers but cause a significant fraction of invasive disease [3]. The distribution of serotypes among disease-causing pneumococci is influenced by factors such as the age, the socioeconomic and immune status of the host, but also by the type of disease and geographical region [3]. Reports from population based surveillance of invasive pneumococcal disease show that the incidence is greatest among the very young and the elderly, with males being overrepresented [45, 46]. Pneumococcal infection disproportionately affects the poor [47] and those living in institutional settings [48, 49]. Exposure to children appears to increase the risk of pneumococcal disease in

adults [3]. Children are more commonly colonized with pneumococci than adults, and adults with preschool-aged children in the household have been found to be more likely to be colonized comparing to the rest of the adult population [3, 50].

The rather paradoxical nature of this organism, being a frequent member of the microflora of the human respiratory tract and a major pathogen represents both a vast burden of human disease but also an enormous opportunity for medical and basic science discovery.

2. Strategies for genetic modification in *Streptococcus pneumoniae*

Full genome sequences and extensive surveys of bacterial populations using multilocus sequence typing (MLST) have established the essential role of gene transfer and recombination in bacterial evolution, revealing the high frequency of these events of genetic recombination [51, 52]. Through gene transfer events bacteria can obtain chromosomal genes and genes clusters (islands) as well as plasmids, transposons and prophage bearing genes that have successfully crossed the barriers of natural selection. In this way, bacteria can expand their ecological niches; colonize new habitats and hosts, metabolize new energy sources, synthesize essential nutrients, survive to toxic agents like antibiotics and increase their virulence towards the human and other hosts [53]. While the core genome of the pneumococcus is conserved with little genetic diversity [3, 32], the accessory genome holds a high diversity, both in the presence or absence of genes and in differences within genetic sequences [54, 55].

In order to achieve this genetic diversity, *Streptococcus pneumoniae* can use several strategies such as natural transformation [13, 14], and phage transduction [56].

2.1. Natural transformation

Streptococcus pneumoniae is widely known for its ability to undergo natural transformation - the stable uptake, integration and functional expression of extracellular DNA [3].

Transformable bacteria use related proteins to transport DNA [57, 58], which suggests evolution of this pathway from a common ancestor. As to selective forces

that have shaped these DNA uptake systems, three major hypotheses have been considered: DNA as a nutrient, DNA for repair, DNA for genetic diversity [59].

The hypothesis that pneumococcal transformation occurs for nutrient acquisition was supported by the finding that *Streptococcus pneumoniae* transport system does not require a specific uptake sequence and allows the capture of any double stranded DNA molecule released in the environment [59]. However, this transport system appears to be a wasteful and inefficient food-gathering mechanism since only one of the strands enters the cell, while the complementary strand is degraded in the outside. Moreover, this hypothesis is fragilized by the fact that, instead of using a complex transport apparatus requiring 11-13 proteins [57, 58] it would be more economical to use a released nuclease together with an uptake system for obtaining nucleolytic products [59, 60]. In addition, the finding that internalized ssDNA is actively protected from degradation due to the presence of three competence-induced proteins SsbB, DprA and RecA [61-63] suggests rather the existence of a need to maintain internalized DNA intact.

The hypothesis that the primary function of bacterial transformation is DNA repair was tested in the naturally transforming *Bacillus subtilis* and *Haemophilus influenzae* by determining whether competence for transformation is regulated by DNA damage. The hypothesis was based on the proposal that internalized DNA provided a template for the recovery of damaged regions of the chromosome [64, 65]. Experiments carried out in both microorganisms showed that no induction or enhancement of competence by DNA-damaging agents was observed. [66]. Although it is likely that other explanations rather than DNA repair would fit better the evolutionary function of natural transformation, it has been proposed that degradation of ssDNA could actually play an important role for repair, via temporary replenishment of nucleotide pools [60].

The DNA-for-genetic-diversity hypothesis postulates that the purpose of natural transformation is to capture genetic material from other cells in order to generate genetic diversity and acquire novel traits. This suggestion is supported by the fact that the existing DNA protection from degradation keeps DNA in a form readily available for recombination with the recipient's genome [60]. Progress in the

knowledge of the transformation will probably bring additional arguments to fuel the debate on the biological role of transformation.

2.2. Competence and its regulation

For natural transformation to occur, bacterial cells must first develop a regulated state of competence. This phenotype allow cells to actively lyse and take up DNA from noncompetent pneumococci, thus providing competent bacteria with an efficient predatory mechanism for acquisition of genetic material [67-69].

Pneumococcal competence is subject to a remarkable form of regulation, in which the capacity for highly efficient DNA processing first develops quite suddenly and, simultaneously, in all cells of an exponentially growing culture, is maintained for about 10-15 minutes and then decays nearly as rapidly as it arose [14, 70]. Subsequent cycles of induction and decay are possible, but they will affect only smaller subpopulations within the culture [70].

This remarkable regulation of competence at the population level depends on a developmental switch that is coordinated by quorum sensing *via* a peptide pheromone signal CSP (competence stimulating peptide) that is one of the products of the comCDE operon [71]. The pheromone is ribosomally synthesized as a pro-peptide, product of the *com*C gene, containing 41 amino acids residues and a double glycine motif where cleavage occurs to give a carboxyl-terminal 17-mer oligopeptide (Figure 2.1).

The products of another operon, ComA and ComB, which are, respectively, a transmembrane protein and an ATP-binding cytosolic protein of the ABC transporter family, are responsible for processing and excreting the 17-mer to the outside of the cell. When CSP reaches an extracellular concentration of 1-10ng/ml corresponding to a cell density of about 10^7 cells/ml [72], it binds to the membrane-located histidine kinase receptor ComD, causing its autophosphorilation. Phosphorylated ComD transfers the phosphate group to the intracellular response regulator ComE, enhancing its affinity [73] for its DNA binding sites and leading to an increase in the expression of the comAB and comCDE operons. Activated ComE also drives the

expression of ComW and ComX [74]. ComX is an alternative sigma factor specific for competence, activates transcription of the late *com* genes, required for the full development of competence. *In vivo*, ComW stabilizes ComX against proteolysis and stimulates its activity. [60].

Transcriptome studies using microarrays showed that the whole competence regulon is made of two classes of *com* genes: the early-induced *com* genes (those involved in deciding when conditions are right for development of the competent state) and the late-induced *com* genes (those required for DNA binding, import and recombination) [61].

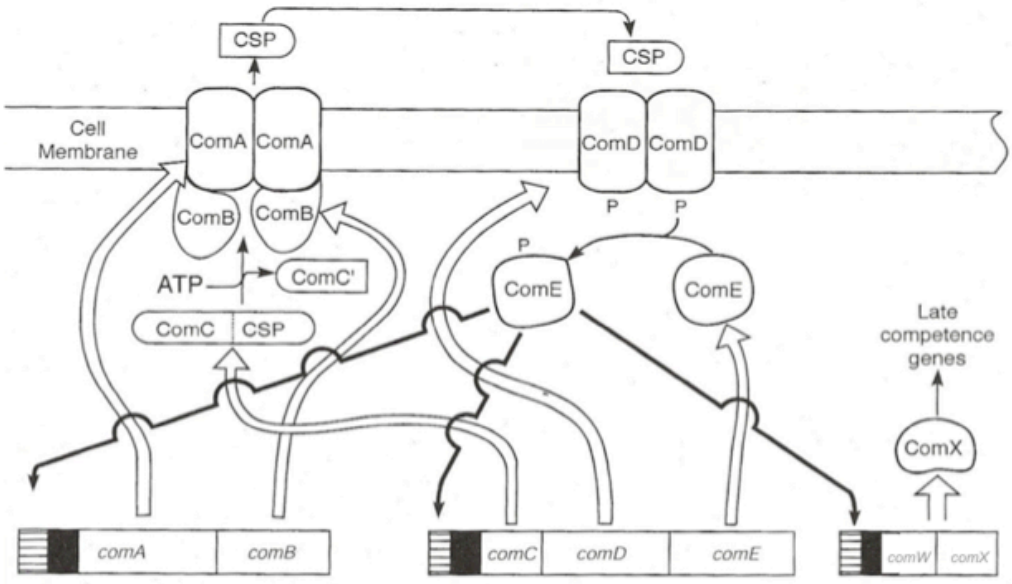

Figure 2.1: **Model of pneumococcal competence regulation.** Accumulated extracelullar CSP signals ComD to phosphorylate ComE, which then enhances synthesis of the early-competence genes of the operons ComAB, ComCDE and ComWX. ComX will then activate the synthesis of late-competence genes that include the genes responsible for transformation. (image adapted from [3]).

Sequencing studies have identified two dominant variants of *Streptococcus pneumoniae* CSP: CSP1 and CSP2 [75]. The large majority of pneumococcal strains isolated from patients encode the CSP1 sequence [75-77] with nearly all the rest of

strains expressing CSP2. In their mature and functional forms of 17-mer oligopeptides, CSP1 and CSP2 differ in eight amino acid residues (Figure 2.2.).

A.

```
CSP1 MKNTVKLEQFVALKEKDLQKIKGG_EMRLSKFFRDFILQRKK

CSP2 MKNTVKLEQFVALKEKDLQKIKGG_EMRISRIILDFLFLRKK

              LEADER_MATURE PEPTIDE
```

Figure 2.2: **The two most common allelic forms of the pneumococcal competence pheromone.** The genes *comC1* and *comC2* encode pro-peptides with identical N-terminal leaders, which are proteolytically processed yielding mature secreted peptide signals. (Image from [60]).

It is known that strains genetically carrying one of the variants are mostly unable to respond to the presence of the other signaling peptide, possibly due to specificity of the ComD receptors for their cognate CSP alleles [75, 76]. It has been suggested that this happens due to the fact that, in these two alternative forms of CSP, the ComD receptors differ in 13 of the first hundred N-terminal residues in the two classes [78].

2.3. Phages

Phages have been recognized as important vectors in the evolution and transfer of genes advantageous to the host bacteria [79], such as the dissemination of virulence factors, that have been found in different species associated with phages or phage-like structures [3, 80, 81]. Nevertheless, phages can also transfer bacterial DNA between cells in the process of transduction [82]. There is increasing evidence from bacterial pathogens that prophages contribute a substantial share of the mobile DNA of their bacterial hosts and seem to be a motor of short-term bacterial evolution [83].

Streptococcus pneumoniae has a high prevalence of lysogenic phages in isolates associated with infection [84, 85]. During lysogeny, the prophage is

integrated in the bacterial chromosome being replicated as part of the host genome. Upon induction, the repressed lysogenic state shifts to lytic growth with the production of viral particles and subsequent phage mediated host lysis to release the phage progeny [79, 84]. In order to exit the bacterium and disseminate its progeny, phages count on lytic enzymes that target the integrity of the cell wall, by disrupting bonds between peptidoglycan monomers, thereby causing cell lysis and the release of phage progeny [86].

It was recognized early that free phages can be found in cultures of lysogenic bacteria, indicating that prophages can spontaneously enter the lytic cycle [87]. In fact, spontaneous phage induction seems to be a common feature of lysogeny, being non-specific of the phage or the bacterial host. The factors that promote this spontaneous induction, both *in vitro* and *in vivo*, are still poorly understood. Recent studies showed that this natural phenomenon may contribute to the pathogenicity observed in *Salmonella typhimurium* [88], increasing the awareness of the potential importance of lysogeny in the context of infection. This spontaneous phage release occurs obviously at low levels, and the phage titer observed is orders of magnitude less than the one produced when the same bacteria are treated with an inducing agent [79, 89, 90].

Inevitably, prophage activation whether spontaneous or induced results in bacterial lysis with the concomitant release of the cellular components to the extracellular medium. Within the release, DNA becomes present in the extracellular environment and becomes available to be rescued in transformation events or to participate in the formation of complex bacterial structures designated by biofilms.

3. Social interactions of *Streptococcus pneumoniae*

Several biological processes result from social interaction events within populations. Understanding how interaction among cells enables the spread of information and leads to dynamic group-behaviors is the fundamental role of sociomicrobiology [91, 92] .As research in this area moves forward, it is becoming clear that bacteria are far from being the solitary organisms that were once perceived. In fact, they lead highly social lives regulated by the ability to detect the density and diversity of bacteria around them [92-96]. This ability is phylogenetically widespread, which suggests an early origin in bacterial evolution [97]. The bacterial group behaviors include coordinated control of virulence, luminescence, competence and biofilm formation [98].

3.1. Quorum sensing

The bacterial ability of coordinating gene expression in accordance to an environmental stimulus is a process termed quorum sensing (QS). This bacterial cell-to-cell communication mechanism provides significant benefits in hosts colonization, defense against competitors, adaptation to varying physical conditions, cellular differentiation and species evolution [99].

The underlying mechanism of QS is the production of diffusible signal molecules by the bacteria (autoinducers), that accumulate in a cell density-dependent manner, interact with specific receptors on self and on neighboring cells, and then regulate expression of specific target genes. By integrating this with other environmental stimuli, bacteria are capable of exhibiting complex responses and taking part in sophisticated interactions [100]. QS is phylogenetically widespread pointing towards an early origin in bacterial evolution [97].

To date, several types of QS systems are known: one for Gram-negative bacteria mediated by *N*-acyl homoserine lactone [101] derivates [102, 103], another for Gram-positive bacteria relying on polypeptides [15, 72, 104] and a third type of

QS system, AI-2, that has been proposed as a inter-species signaling system common to a widespread range of bacterial species [105, 106].

In Gram-positive bacteria, QS regulates a number of physiological activities, including those involving sporulation in *Bacillus subtilis* [107], antibiotic biosynthesis in *Lactococcus lactis* [108], induction of virulence factors in *Staphylococcus aureus* [109] and competence development in *Streptococcus pneumoniae [72]*. Among streptococci, the best characterized quorum sensing system belongs to *Streptococcus pneumoniae*. Although initially QS was believed to act mainly to acquire and incorporate foreign DNA into the chromosome, studies implicating its involvement in biofilm formation, fratricide as well as virulence suggested a broader and more varied role for this system in *Streptococcus pneumoniae*.

3.2. Fratricide

Streptococcus pneumoniae has long been known to possess a pathway leading to the lytic death of a large proportion of cells within a population. This lytic death and subsequent release of DNA were recently shown to be induced by the competence pathway [67, 68].

Undoubtedly, individuals will sometimes become infected with two or more pneumococcal strains at the same time, resulting in their cohabitation in the nasopharynx milieu [37, 110]. Fratricide is then committed by pneumococci that enter the competence state first and promote the lysis of their noncompetent siblings that share the same niche, through the expression of a putative murein hydrolase CbpD [111], major autolysin lytA and lysozyme lytC, and a two-peptide bacteriocin cibAB and its immunity factor cibC [111]. As the DNA released from the lysed cells can be taken up by the competent “attacker” cells, the rate of gene transfer is greatly increased [112].

One possible explanation for the advantage of competent pneumococci attacking their noncompetent siblings may be that this process contributes to the

virulence of this bacterium by exacerbating an infection, through the release of virulence factors and inflammatory mediators [111, 112]. Another non-mutually exclusive explanation is that fratricide may be a direct consequence of the existence of two predominant pherotypes. Regarding the pherotype impact on genetic exchange, the first model proposes that the lysis of bacteria belonging to the other pherotype is occurring favoring genetic diversity within the pneumococcal population [113, 114]. The second model proposes limitations to pneumococcal genetic exchanges regarding the absence of interpherotype communication [115, 116].

3.3. Biofilms

Bacteria in their natural ecosystems preferentially grow as matrix-enclosed biofilms. This microbial lifestyle appears to be ancient and an integral component of the prokaryotic life cycle [117]. Bacterial biofilms have a structurally complex and dynamic structure that can be broadly divided into three major phases [118-120]. The first phase corresponds to the adhesion of planktonic bacteria to surfaces. This initial attachment is either mediated by electrostatic contacts or relies on the interaction of bacterial surface structures with inert or protein-carbohydrate-coated surfaces [118]. The second phase involves the proliferation of the primary colonizers and the maturation of the biofilm. During this phase, either bacteria multiply without releasing progeny cells from the biofilm, or the primary colonizers recruit and coaggregate planktonic members of the same species or other species [119]. At the same time, bacteria inside the biofilm produce extracellular polymeric substances (EPS), which stabilize the biofilm architecture. In a final third phase, previously sessile members of the mature biofilm detach and act as primary colonizers at different sites [120].

Biofilms results in several benefits, including protection from environmental threats such as host immune defenses and antibiotics. Thus, bacteria in biofilms are characteristically highly resistant to immune-mediated clearance [121-123] and, in some studies, these biological structures have been proposed as the way bacteria organize themselves when colonizing asymptomatically their human host [124-126].

The extracellular matrix is implicated in the bacterial protection within the biofilm. This structure consists of polysaccharides, proteins and nucleic acids [127]. The role of extracellular DNA (eDNA) as a critical element of the matrix is being increasingly recognized, both in providing structural stability as well as protection against antimicrobial agents [128-130].

Many of the developmental, metabolic, genetic, and physical properties of biofilms are regulated by QS systems [131, 132]. The first report to suggest that CSPs were involved in biofilm formation in Gram-positive bacteria was made in *Streptococcus gordinii*. In this organism, a transposon mediated knock-out mutation in the *comD* gene resulted in a phenotype that was defective in biofilm formation [133]. Further evidence came from a report in *Streptococcus mutans* where the inactivation of any component of the *comCDE* operon resulted in a phenotype of defective competence and biofilm formation [134]. An alternative approach showed that the addition of synthetic CSP favored the biofilm mode of growth of *Streptococcus intermedius* without affecting the rate of culture growth [135].

Recent studies demonstrated the potential of *Streptococcus pneumoniae* to produce biofilms *in vivo [136, 137]*. Pneumococcal biofilms were indeed detected on affected tissues in patients with chronic rhinosinusitis [136], children with otitis media [137], as well as in a chinchilla model of otitis [138]. The presence of these bacterial communities at the site of infection implicates them in these disorders, although their significance in the infection process is still a matter of debate. Two recent studies have failed to find an association between the ability to form biofilms and the origin of the isolates, i.e., if the isolates had been recovered from asymptomatic carriers or caused invasive infections [139, 140]. Although these two studies question the role of biofilms in determining the invasive potential of pneumococci, the transcriptional profile of several known virulence-related genes in *Streptococcus pneumoniae* isolated from lungs and brains of infected mice is similar to that found in biofilms formed *in vitro*, suggesting a possible biofilm-like state of *Streptococcus pneumoniae* associated with tissues [141]. In addition, a link was established between pneumococcal biofilm formation and the asymptomatic colonization of the nasopharynx, the most frequent state of pneumococci [142].

4. Thesis outline

This thesis describes the results of a research work developed between 2008 and 2011 in the Molecular Microbiology and Infection Unit, under the supervision of Prof. Dr. Mário Ramirez. The main goal of this work was to delve into the large genetic diversity of *Streptococcus pneumoniae* and to explore biological parameters with a significant impact on the biofilm formation of this species.

The results, final remarks and future perspectives resulting from this work are presented in the following chapters.

Chapter 2 explores the impact of spontaneous prophage activation and the consequent bacterial host lysis on *Streptococcus pneumoniae* biofilm formation. Our data provides evidence that prophage-mediated lysis enhances biofilm formation through the release of eDNA. Overall, the results suggest that in this context the bacterial population as a whole could benefit from limited prophage induction. These results have been published in: Margarida Carrolo, Maria Joao Frias, Francisco R Pinto, Jose Melo-Cristino and Mario Ramirez (2010) Prophage Spontaneous Activation Promotes DNA Release Enhancing Biofilm Formation in *Streptococcus pneumoniae*, PLoS One. 5 (12): e15678. (*Individual contributions*: Margarida Carrolo was responsible for the biofilms experiments, the CLSM results, and participated in data analysis. Maria Joao Frias constructed the mutants with the exception of R36AΔ*lytA*, performed the experiments of purification and quantification of eDNA and participated in data analysis. Francisco Pinto participated in the data analysis. All authors have contributed to the study design and have critically revised the manuscript).

Chapter 3 presents the results from a molecular epidemiological study. A pneumococcal population was scrutinized for the distribution of the two major pherotypes and associations with serotype, antimicrobial resistance and genetic lineage were evaluated. Results show that pherotype is a clonal property of pneumococci. The original work described in this chapter has been integrally published in: Margarida Carrolo, Francisco R Pinto, Jose Melo-Cristino and Mario Ramirez (2009) Pherotypes are driving genetic differentiation within *Streptococcus*

pneumoniae, BMC Microbiology. 9:191. (*Individual contributions*: Margarida Carrolo was responsible for designing and performing all the experimental work, for writing the manuscript and participating in the data analysis. Francisco Pinto was responsible for the infinite allele model of MLST evolution and participated in the data analysis. Mário Ramirez was responsible for the population genetics analysis. All authors have contributed to the study design and have critically revised the manuscript).

Chapter 4 focuses on the influence of pherotypes on biofilm growth and genetic recombination in pneumococci. Here we show that CSP1 strains present both a higher capacity to form biofilms and also a stronger recombination efficiency. These observations may explain the higher prevalence of CSP1 strains in the pneumococcal population. The results presented in this chapter are in the process of submission to PLoS ONE under the title "Pherotype influences biofilm growth and recombination in *Streptococcus pneumoniae*". (*Individual contributions*: Margarida Carrolo was responsible for designing and performing all the experimental work, for writing the manuscript and participated in the data analysis. Francisco Pinto participated in the data analysis. All authors have contributed to the study design and have critically revised the manuscript).

Chapter 5 comprises an integrative discussion of the results and holds future perspectives on the better understanding of the sociomicrobiology of *Streptococcus pneumoniae*.

5. Objectives

Recently, eDNA was found to be a by-product of cell lysis with a critical structural influence on the biofilm matrix. So as the **first objective** of this thesis work we decided to explore the effect of prophage spontaneous activation with the consequence bacterial host lysis on pneumococcal biofilms.

Moving forward, the **second objective** of this thesis work was to study the distribution of the two major pherotypes in the pneumococcal population and their association with serotype, antimicrobial resistance and genetic lineage. Moreover these results would allow the view of the impact of pherotypes on pneumococcal gene flow and to probe the consequences of gene flow limitations on the evolution of pneumococcus.

The previously described link between competence and biofilm formation in other bacteria was the motivation that drove us to the **third objective** of this work which was the study of the influence of the pherotypes on biofilm growth and recombination in *Streptococcus pneumoniae*.

Overall, these studies highlight the importance of studying the sociomicrobiology of *Streptococcus pneumoniae* and the influence of it on the biofilm structures that *per se* may have a strong impact on the response of pneumococci to antibiotics and human immune defenses.

Bibliography

1. *Pneumococcal Vaccines.* Washington, DC: ASM Press; 2008.

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

3. In *The Pneumococcus.* Edited by Toumanen EI, Mitchell TJ, Morrison DA, Spratt BG. Washington, DC: ASM Press; 2004.

4. Gamaléia N: **Sur l'étiologie de la pneumonie fibrineuse chez l'homme.** *Annales de l'Institut Pasteur* 1888, **2:**440-459.

5. Prescott LM, Harley JP, Klein DA: **Medical Immunology.** In *Microbiology.* 6th edition edition. New York: McGraw Hill; 2005.

6. Avery OT, Heidelberger M: **Immunological Relationships of Cell Constituents of Pneumococcus: Second Paper.** *J Exp Med* 1925, **42:**367-376.

7. Heidelberger M, Kendall FE: **A Quantitative Study of the Precipitin Reaction between Type Iii Pneumococcus Polysaccharide and Purified Homologous Antibody.** *J Exp Med* 1929, **50:**809-823.

8. Song JH, Baek JY, Ko KS: **Comparison of capsular genes of *Streptococcus pneumoniae* serotype 6A, 6B, 6C, and 6D isolates.** *J Clin Microbiol* 2011, **49**: 1758-64.

9. Calix JJ, Nahm MH: **A new pneumococcal serotype, 11E, has a variably inactivated *wcjE* gene.** *J Infect Dis* 2010 **202:**29-38.

10. Macleod CM, Hodges RG, Heidelberger M, Bernhard WG: **Prevention of Pneumococcal Pneumonia by Immunization with Specific Capsular Polysaccharides.** *J Exp Med* 1945, **82:**445-465.

11. Griffith F: **The Significance of Pneumococcal Types.** *J Hyg (Lond)* 1928, **27:**113-159.

12. Pakula R, Walczak W: **On the nature of competence of transformable streptococci.** *J Gen Microbiol* 1963, **31:**125-133.

13. Tomasz A, Hotchkiss RD: **Regulation of the Transformability of Pneumococcal Cultures by Macromolecular Cell Products.** *Proc Natl Acad Sci U S A* 1964, **51:**480-487.

14. Tomasz A: **Control of the competent state in Pneumococcus by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria.** *Nature* 1965, **208:**155-159.

15. Waters CM, Bassler BL: **Quorum sensing: cell-to-cell communication in bacteria.** *Annu Rev Cell Dev Biol* 2005, **21:**319-346.

16. Frisch AW, Price AE, Myers GB: **Type VIII Pneumococcus: development of sulfadiazine resistance, transmission by cross-infection, and persistence in carriers.** *Annals Intern Med* 1943, **18:**271-278.

17. Hamburger M, Schmidt LH, Sesler CL, Ruegsegger JM, Grupen ES: **The occurence of sulfonamide-resistant pneumococci in clinical practice.** *J Infect Dis* 1943, **73:**12-30.

18. Evans W, Hansman D: **Tetracycline-resistant pneumococci.** *Lancet* 1963, **1:**451.

19. Dixon JMS: **Pneumococcus resistant to erythromycin and lincomycin.** *Lancet* 1967, **1:**573.

20. Kislak JW: **Type 6 pneumococcus resistant to erythromycin and lincomycin.** *New Engl J Med* 1995, **276:**852.

21. Hansman D, Bullun MM: **A resistant pneumococcus.** *Lancet* 1967, **2:**264-265.

22. Cybulska J, Jeljaszewicz J, Lund E, Munksgaard A: **Prevalence of types of *Diplococcus pneumoniae* and their susceptibility to 30 antibiotics.** *Chemotherapy* 1970, **15:**304-316.

23. Howe JG, Wilson TS: **Co-trimoxazole-resistant pneumococci.** *Lancet* 1972, **2:**184-185.

24. Jacobs MR, Koornhof HJ, Robins-Browne RM, Stevenson CM, Vermaak ZA, Freiman I, Miller GB, Witcomb MA, Isaacson M, Ward JI, Austrian R: **Emergence of multiply resistant pneumococci.** *N Engl J Med* 1978, **299:**735-740.

25. Weinberger DM, Malley R, Lipsitch M: **Serotype replacement in disease after pneumococcal vaccination.** *Lancet* 2011 [epub ahead of print].

26. Casado-Flores J, Rodrigo C, Aristegui J, Martinon JM, Fenoll A, Mendez C: **Decline in pneumococcal meningitis in Spain after introduction of the heptavalent pneumococcal conjugate vaccine.** *Pediatr Infect Dis J* 2008, **27:**1020-1022.

27. Aguiar SI, Brito MJ, Goncalo-Marques J, Melo-Cristino J, Ramirez M: **Serotypes 1, 7F and 19A became the leading causes of pediatric invasive**

pneumococcal infections in Portugal after 7 years of heptavalent conjugate vaccine use. *Vaccine*, **28:**5167-5173.

28. Obaro SK: **Confronting the pneumococcus: a target shift or bullet change?** *Vaccine* 2000, **19:**1211-1217.

29. Kilian M, Poulsen K, Blomqvist T, Havarstein LS, Bek-Thomsen M, Tettelin H, Sorensen UB: **Evolution of *Streptococcus pneumoniae* and its close commensal relatives.** *PLoS One* 2008, **3:**e2683.

30. Arbique JC, Poyart C, Trieu-Cuot P, Quesne G, Carvalho Mda G, Steigerwalt AG, Morey RE, Jackson D, Davidson RJ, Facklam RR: **Accuracy of phenotypic and genotypic testing for identification of *Streptococcus pneumoniae* and description of *Streptococcus pseudopneumoniae* sp. nov.** *J Clin Microbiol* 2004, **42:**4686-4696.

31. Hanage WP, Kaijalainen T, Herva E, Saukkoriipi A, Syrjanen R, Spratt BG: **Using multilocus sequence data to define the pneumococcus.** *J Bacteriol* 2005, **187:**6223-6230.

32. Whatmore AM, Efstratiou A, Pickerill AP, Broughton K, Woodard G, Sturgeon D, George R, Dowson CG: **Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, *Streptococcus oralis*, and *Streptococcus mitis*: characterization of "Atypical" pneumococci and organisms allied to *S. mitis* harboring *S. pneumoniae* virulence factor-encoding genes.** *Infect Immun* 2000, **68:**1374-1382.

33. Carvalho MG, Steigerwalt AG, Thompson T, Jackson D, Facklam RR: **Confirmation of nontypeable *Streptococcus pneumoniae*-like organisms isolated from outbreaks of epidemic conjunctivitis as Streptococcus pneumoniae.** *J Clin Microbiol* 2003, **41:**4415-4417.

34. Chandler LJ, Reisner BS, Woods GL, Jafri AK: **Comparison of four methods for identifying *Streptococcus pneumoniae.*** *Diagn Microbiol Infect Dis* 2000, **37:**285-287.

35. Musher DM: ***Streptococcus pneumoniae*.** In *Mandell, Douglas, and Bennet's Principles and Practice of Infectious Diseases* Edited by Mandell GL, Bennett JE, Dolin R. Philadelphia, PA: Churchill Livingstone; 2000.

36. Sleeman KL, Daniels L, Gardiner M, Griffiths D, Deeks JJ, Dagan R, Gupta S, Moxon ER, Peto TE, Crook DW: **Acquisition of *Streptococcus pneumoniae* and nonspecific morbidity in infants and their families: a cohort study.** *Pediatr Infect Dis J* 2005, **24:**121-127.

37. Brugger SD, Hathaway LJ, Muhlemann K: **Detection of *Streptococcus pneumoniae* strain cocolonization in the nasopharynx.** *J Clin Microbiol* 2009, **47:**1750-1756.

38. Gray BM, Converse GM, 3rd, Dillon HC, Jr.: **Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life.** *J Infect Dis* 1980, **142:**923-933.

39. Frazao N, Sa-Leao R, de Lencastre H: **Impact of a single dose of the 7-valent pneumococcal conjugate vaccine on colonization.** *Vaccine*, **28:**3445-3452.

40. Gray BM, Converse GM, 3rd, Dillon HC, Jr.: **Serotypes of *Streptococcus pneumoniae* causing disease.** *J Infect Dis* 1979, **140:**979-983.

41. Smith T, Lehmann D, Montgomery J, Gratten M, Riley ID, Alpers MP: **Acquisition and invasiveness of different serotypes of *Streptococcus pneumoniae* in young children.** *Epidemiol Infect* 1993, **111:**27-39.

42. Musher DM: **How contagious are common respiratory tract infections?** *N Engl J Med* 2003, **348:**1256-1266.

43. Weiser JN: **The pneumococcus: why a commensal misbehaves.** *J Mol Med*, **88:**97-102.

44. Mitchell TJ: **The pathogenesis of streptococcal infections: from tooth decay to meningitis.** *Nat Rev Microbiol* 2003, **1:**219-230.

45. Klein JO: **The epidemiology of pneumococcal disease in infants and children.** *Rev Infect Dis* 1981, **3:**246-253.

46. Robinson KA, Baughman W, Rothrock G, Barrett NL, Pass M, Lexau C, Damaske B, Stefonek K, Barnes B, Patterson J, et al: **Epidemiology of invasive *Streptococcus pneumoniae* infections in the United States, 1995-1998: Opportunities for prevention in the conjugate vaccine era.** *JAMA* 2001, **285:**1729-1735.

47. Pastor P, Medley F, Murphy TV: **Invasive pneumococcal disease in Dallas County, Texas: results from population-based surveillance in 1995.** *Clin Infect Dis* 1998, **26:**590-595.

48. Takala AK, Jero J, Kela E, Ronnberg PR, Koskenniemi E, Eskola J: **Risk factors for primary invasive pneumococcal disease among children in Finland.** *JAMA* 1995, **273:**859-864.

49. Levine OS, Farley M, Harrison LH, Lefkowitz L, McGeer A, Schwartz B: **Risk factors for invasive pneumococcal disease in children: a population-based case-control study in North America.** *Pediatrics* 1999, **103:**E28.

50. Hendley JO, Sande MA, Stewart PM, Gwaltney JM, Jr.: **Spread of *Streptococcus pneumoniae* in families. I. Carriage rates and distribution of types.** *J Infect Dis* 1975, **132:**55-61.

51. Gogarten JP, Doolittle WF, Lawrence JG: **Prokaryotic evolution in light of gene transfer.** *Mol Biol Evol* 2002, **19:**2226-2238.

52. Feil EJ: **Small change: keeping pace with microevolution.** *Nat Rev Microbiol* 2004, **2:**483-495.

53. Levin BR, Cornejo OE: **The population and evolutionary dynamics of homologous gene recombination in bacterial populations.** *PLoS Genet* 2009, **5:**e1000601.

54. Oggioni MR, Memmi G, Maggi T, Chiavolini D, Iannelli F, Pozzi G: **Pneumococcal zinc metalloproteinase ZmpC cleaves human matrix metalloproteinase 9 and is a virulence factor in experimental pneumonia.** *Mol Microbiol* 2003, **49:**795-805.

55. Oggioni MR, Pozzi G: **Comparative genomics for identification of clone-specific sequence blocks in *Streptococcus pneumoniae*.** *FEMS Microbiol Lett* 2001, **200:**137-143.

56. Bernheimer HP: **Lysogenic pneumococci and their bacteriophages.** *J Bacteriol* 1979, **138:**618-624.

57. Claverys JP, Martin B: **Bacterial "competence" genes: signatures of active transformation, or only remnants?** *Trends Microbiol* 2003, **11:**161-165.

58. Chen I, Dubnau D: **DNA uptake during bacterial transformation.** *Nat Rev Microbiol* 2004, **2:**241-249.

59. Dubnau D: **DNA uptake in bacteria.** *Annu Rev Microbiol* 1999, **53:**217-244.

60. Guiral S, Moscoso M, Dagkessamanskaia A, Claverys JP: **Competence in *Streptococcus pneumoniae*: What is it For?** In *Molecular Biology of Streptococci.* Edited by Hakenbeck R, Chhatwal S. Norfolk: Horizon Bioscience; 2007.

61. Johnsborg O, Eldholm V, Havarstein LS: **Natural genetic transformation: prevalence, mechanisms and function.** *Res Microbiol* 2007, **158:**767-778.

62. Morrison DA, Mannarelli B: **Transformation in pneumococcus: nuclease resistance of deoxyribonucleic acid in the eclipse complex.** *J Bacteriol* 1979, **140:**655-665.

63. Berge M, Mortier-Barriere I, Martin B, Claverys JP: **Transformation of *Streptococcus pneumoniae* relies on DprA- and RecA-dependent protection of incoming DNA single strands.** *Mol Microbiol* 2003, **50:**527-536.

64. Michod RE, Wojciechowski MF, Hoelzer MA: **DNA repair and the evolution of transformation in the bacterium *Bacillus subtilis.*** *Genetics* 1988, **118:**31-39.

65. Wojciechowski MF, Hoelzer MA, Michod RE: **DNA repair and the evolution of transformation in *Bacillus subtilis.* II. Role of inducible repair.** *Genetics* 1989, **121:**411-422.

66. Redfield RJ: **Evolution of natural transformation: testing the DNA repair hypothesis in *Bacillus subtilis* and *Haemophilus influenzae*.** *Genetics* 1993, **133:**755-761.

67. Moscoso M, Claverys JP: **Release of DNA into the medium by competent *Streptococcus pneumoniae:* kinetics, mechanism and stability of the liberated DNA.** *Mol Microbiol* 2004, **54:**783-794.

68. Steinmoen H, Knutsen E, Havarstein LS: **Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population.** *Proc Natl Acad Sci U S A* 2002, **99:**7681-7686.

69. Steinmoen H, Teigen A, Havarstein LS: **Competence-induced cells of *Streptococcus pneumoniae* lyse competence-deficient cells of the same strain during cocultivation.** *J Bacteriol* 2003, **185:**7176-7183.

70. Morrison DA, Lee MS: **Regulation of competence for genetic transformation in *Streptococcus pneumoniae*: a link between quorum sensing and DNA processing genes.** *Res Microbiol* 2000, **151:**445-451.

71. Claverys JP, Havarstein LS: **Extracellular-peptide control of competence for genetic transformation in *Streptococcus pneumoniae.*** *Front Biosci* 2002, **7:**d1798-1814.

72. Havarstein LS, Coomaraswamy G, Morrison DA: **An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae.*** *Proc Natl Acad Sci U S A* 1995, **92:**11140-11144.

73. Ween O, Gaustad P, Havarstein LS: **Identification of DNA binding sites for ComE, a key regulator of natural competence in *Streptococcus pneumoniae*.** *Mol Microbiol* 1999, **33:**817-827.

74. Luo P, Li H, Morrison DA: **Identification of ComW as a new component in the regulation of genetic transformation in *Streptococcus pneumoniae*.** *Mol Microbiol* 2004, **54:**172-183.

75. Pozzi G, Masala L, Iannelli F, Manganelli R, Havarstein LS, Piccoli L, Simon D, Morrison DA: **Competence for genetic transformation in encapsulated**

strains of *Streptococcus pneumoniae*: two allelic variants of the peptide pheromone. *J Bacteriol* 1996, **178:**6087-6090.

76. Ramirez M, Morrison DA, Tomasz A: **Ubiquitous distribution of the competence related genes *comA* and *comC* among isolates of *Streptococcus pneumoniae*.** *Microb Drug Resist* 1997, **3:**39-52.

77. Carrolo M, Pinto FR, Melo-Cristino J, Ramirez M: **Pherotypes are driving genetic differentiation within *Streptococcus pneumoniae*.** *BMC Microbiol* 2009, **9:**191.

78. Whatmore AM, Barcus VA, Dowson CG: **Genetic diversity of the streptococcal competence (com) gene locus.** *J Bacteriol* 1999, **181:**3144-3154.

79. Little JW: **Lysogeny, prophage induction, and lysogenic conversion.** In *Phages, their role in bacterial pathogenesis and biotechnology.* Edited by Waldor MK, Friedman DI, Adhya SL. Washington, DC: ASM Press; 2005.

80. Cheetham BF, Katz ME: **A role for bacteriophages in the evolution and transfer of bacterial virulence determinants.** *Mol Microbiol* 1995, **18:**201-208.

81. Barksdale L, Arden SB: **Persisting bacteriophage infections, lysogeny, and phage conversions.** *Annu Rev Microbiol* 1974, **28:**265-299.

82. Sternberg NL, Maurer R: **Bacteriophage-mediated generalized transduction in *Escherichia coli* and *Salmonella typhimurium.*** *Methods Enzymol* 1991, **204:**18-43.

83. Canchaya C, Fournous G, Chibani-Chennoufi S, Dillmann ML, Brussow H: **Phage as agents of lateral gene transfer.** *Curr Opin Microbiol* 2003, **6:**417-424.

84. Ramirez M, Severina E, Tomasz A: **A high incidence of prophage carriage among natural isolates of *Streptococcus pneumoniae*.** *J Bacteriol* 1999, **181:**3618-3625.

85. Severina E, Ramirez M, Tomasz A: **Prophage carriage as a molecular epidemiological marker in *Streptococcus pneumoniae*.** *J Clin Microbiol* 1999, **37:**3308-3315.

86. Fischetti VA: **Bacteriophage lysins as effective antibacterials.** *Curr Opin Microbiol* 2008, **11:**393-400.

87. Lwoff A: **Lysogeny.** *Bacteriol Rev* 1953, **17:**269-337.

88. Figueroa-Bossi N, Bossi L: **Inducible prophages contribute to Salmonella virulence in mice.** *Mol Microbiol* 1999, **33:**167-176.

89. Bossi L, Fuentes JA, Mora G, Figueroa-Bossi N: **Prophage contribution to bacterial population dynamics.** *J Bacteriol* 2003, **185:**6467-6471.

90. Livny J, Friedman DI: **Characterizing spontaneous induction of Stx encoding phages using a selectable reporter system.** *Mol Microbiol* 2004, **51:**1691-1704.

91. Parsek MR, Greenberg EP: **Sociomicrobiology: the connections between quorum sensing and biofilms.** *Trends Microbiol* 2005, **13:**27-33.

92. Xavier JB: **Social interaction in synthetic and natural microbial communities.** *Mol Syst Biol*, **7:**483.

93. Velicer GJ: **Social strife in the microbial world.** *Trends Microbiol* 2003, **11:**330-337.

94. West SA, Griffin AS, Gardner A, Diggle SP: **Social evolution theory for microorganisms.** *Nat Rev Microbiol* 2006, **4:**597-607.

95. Nadell CD, Xavier JB, Foster KR: **The sociobiology of biofilms.** *FEMS Microbiol Rev* 2009, **33:**206-224.

96. Nadell CD, Xavier JB, Levin SA, Foster KR: **The evolution of quorum sensing in bacterial biofilms.** *PLoS Biol* 2008, **6:**e14.

97. Lerat E, Moran NA: **The evolutionary history of quorum-sensing systems in bacteria.** *Mol Biol Evol* 2004, **21:**903-913.

98. Spoering AL, Gilmore MS: **Quorum sensing and DNA release in bacterial biofilms.** *Curr Opin Microbiol* 2006, **9:**133-137.

99. Shapiro JA: **Thinking about bacterial populations as multicellular organisms.** *Annu Rev Microbiol* 1998, **52:**81-104.

100. Gray KM: **Intercellular communication and group behavior in bacteria.** *Trends Microbiol* 1997, **5:**184-188.

101. Mizrachi Nebenzahl Y, Porat N, Lifshitz S, Novick S, Levi A, Ling E, Liron O, Mordechai S, Sahu RK, Dagan R: **Virulence of *Streptococcus pneumoniae* may be determined independently of capsular polysaccharide.** *FEMS Microbiol Lett* 2004, **233:**147-152.

102. Eberhard A, Burlingame AL, Eberhard C, Kenyon GL, Nealson KH, Oppenheimer NJ: **Structural identification of autoinducer of *Photobacterium fischeri* luciferase.** *Biochemistry* 1981, **20:**2444-2449.

103. Nasser W, Reverchon S: **New insights into the regulatory mechanisms of the LuxR family of quorum sensing regulators.** *Anal Bioanal Chem* 2007, **387:**381-390.

104. Balaban N, Goldkorn T, Nhan RT, Dang LB, Scott S, Ridgley RM, Rasooly A, Wright SC, Larrick JW, Rasooly R, Carlson JR: **Autoinducer of virulence as a target for vaccine and therapy against *Staphylococcus aureus*.** *Science* 1998, **280:**438-440.

105. Winans SC, Bassler BL: **Mob psychology.** *J Bacteriol* 2002, **184:**873-883.

106. Xavier KB, Bassler BL: **Regulation of uptake and processing of the quorum-sensing autoinducer AI-2 in *Escherichia coli.*** *J Bacteriol* 2005, **187:**238-248.

107. Grossman AD: **Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis.*** *Annu Rev Genet* 1995, **29:**477-508.

108. Kuipers OP, Beerthuyzen MM, de Ruyter PG, Luesink EJ, de Vos WM: **Autoregulation of nisin biosynthesis in *Lactococcus lactis* by signal transduction.** *J Biol Chem* 1995, **270:**27299-27304.

109. Ji G, Beavis RC, Novick RP: **Cell density control of staphylococcal virulence mediated by an octapeptide pheromone.** *Proc Natl Acad Sci U S A* 1995, **92:**12055-12059.

110. Claverys JP, Martin B, Havarstein LS: **Competence-induced fratricide in streptococci.** *Mol Microbiol* 2007, **64:**1423-1433.

111. Guiral S, Mitchell TJ, Martin B, Claverys JP: **Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements.** *Proc Natl Acad Sci U S A* 2005, **102:**8710-8715.

112. Claverys JP, Havarstein LS: **Cannibalism and fratricide: mechanisms and *raisons d'etre*.** *Nat Rev Microbiol* 2007, **5:**219-229.

113. Claverys JP, Prudhomme M, Martin B: **Induction of competence regulons as a general response to stress in gram-positive bacteria.** *Annu Rev Microbiol* 2006, **60:**451-475.

114. Cornejo OE, McGee L, Rozen DE: **Polymorphic competence peptides do not restrict recombination in *Streptococcus pneumoniae.*** *Mol Biol Evol*, **27:**694-702.

115. Havarstein LS, Hakenbeck R, Gaustad P: **Natural competence in the genus Streptococcus: evidence that streptococci can change pherotype by interspecies recombinational exchanges.** *J Bacteriol* 1997, **179:**6589-6594.

116. Tortosa P, Dubnau D: **Competence for transformation: a matter of taste.** *Curr Opin Microbiol* 1999, **2:**588-592.

117. Lembke C, Podbielski A, Hidalgo-Grass C, Jonas L, Hanski E, Kreikemeyer B: **Characterization of biofilm formation by clinically relevant serotypes of group A streptococci.** *Appl Environ Microbiol* 2006, **72:**2864-2875.

118. Hall-Stoodley L, Costerton JW, Stoodley P: **Bacterial biofilms: from the natural environment to infectious diseases.** *Nat Rev Microbiol* 2004, **2:**95-108.

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

120. Watnick P, Kolter R: **Biofilm, city of microbes.** *J Bacteriol* 2000, **182:**2675-2679.

121. Anderson GG, O'Toole GA: **Innate and induced resistance mechanisms of bacterial biofilms.** *Curr Top Microbiol Immunol* 2008, **322:**85-105.

122. Mah TF, O'Toole GA: **Mechanisms of biofilm resistance to antimicrobial agents.** *Trends Microbiol* 2001, **9:**34-39.

123. Xu KD, McFeters GA, Stewart PS: **Biofilm resistance to antimicrobial agents.** *Microbiology* 2000, **146 (Pt 3):**547-549.

124. Johnsborg O, Eldholm V, Bjornstad ML, Havarstein LS: **A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species.** *Mol Microbiol* 2008, **69:**245-253.

125. Neil RB, Shao JQ, Apicella MA: **Biofilm formation on human airway epithelia by encapsulated *Neisseria meningitidis* serogroup B.** *Microbes Infect* 2009, **11:**281-287.

126. Satorres SE, Alcaraz LE, Cargnelutti E, Di Genaro MS: **IFN-gamma plays a detrimental role in murine defense against nasal colonization of *Staphylococcus aureus*.** *Immunol Lett* 2009, **123:**185-188.

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

128. Mulcahy H, Charron-Mazenod L, Lewenza S: **Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms.** *PLoS Pathog* 2008, **4:**e1000213.

129. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS: **Extracellular DNA required for bacterial biofilm formation.** *Science* 2002, **295:**1487.

130. Vilain S, Pretorius JM, Theron J, Brozel VS: **DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms.** *Appl Environ Microbiol* 2009, **75:**2861-2868.

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

132. Federle MJ, Bassler BL: **Interspecies communication in bacteria.** *J Clin Invest* 2003, **112:**1291-1299.

133. Loo CY, Corliss DA, Ganeshkumar N: ***Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes.** *J Bacteriol* 2000, **182:**1374-1382.

134. Li YH, N. , Tang MB, Aspiras PC, Lau JHL, Ellen RP, Cvitkovitch DG: **A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation.** *J Bacteriol* 2002, **184:**2699-2708.

135. Petersen FC, Pecharki D, Scheie AA: **Biofilm mode of growth of *Streptococcus intermedius* favored by a competence-stimulating signaling peptide.** *J Bacteriol* 2004, **186:**6327-6331.

136. Sanderson AR, Leid JG, Hunsaker D: **Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis.** *Laryngoscope* 2006, **116:**1121-1126.

137. Hall-Stoodley L, Hu FZ, Gieseke A, Nistico L, Nguyen D, Hayes J, Forbes M, Greenberg DP, Dice B, Burrows A, et al: **Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media.** *JAMA* 2006, **296:**202-211.

138. Reid SD, Hong W, Dew KE, Winn DR, Pang B, Watt J, Glover DT, Hollingshead SK, Swords WE: **Streptococcus pneumoniae forms surface-attached communities in the middle ear of experimentally infected chinchillas.** *J Infect Dis* 2009, **199:**786-794.

139. Lizcano A, Chin T, Sauer K, Tuomanen EI, Orihuela CJ: **Early biofilm formation on microtiter plates is not correlated with the invasive disease potential of *Streptococcus pneumoniae.*** *Microb Pathog* 2010, **48:**124-130.

140. Tapiainen T, Kujala T, Kaijalainen T, Ikaheimo I, Saukkoriipi A, Renko M, Salo J, Leinonen M, Uhari M: **Biofilm formation by *Streptococcus pneumoniae* isolates from paediatric patients.** *APMIS* 2010, **118:**255-260.

141. Oggioni MR, Trappetti C, Kadioglu A, Cassone M, Iannelli F, Ricci S, Andrew PW, Pozzi G: **Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis.** *Mol Microbiol* 2006, **61:**1196-1210.

142. Munoz-Elias EJ, Marcano J, Camilli A: **Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization.** *Infect Immun* 2008, **76:**5049-5061.

Chapter 2

PLoS one

Prophage Spontaneous Activation Promotes DNA Release Enhancing Biofilm Formation in *Streptococcus pneumoniae*

Margarida Carrolo[1☯], Maria João Frias[1☯], Francisco Rodrigues Pinto[2], José Melo-Cristino[1], Mário Ramirez[1]*

1 Instituto de Microbiologia, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Lisboa, Portugal, **2** Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, Lisboa, Portugal

Abstract

Streptococcus pneumoniae (pneumococcus) is able to form biofilms *in vivo* and previous studies propose that pneumococcal biofilms play a relevant role both in colonization and infection. Additionally, pneumococci recovered from human infections are characterized by a high prevalence of lysogenic bacteriophages (phages) residing quiescently in their host chromosome. We investigated a possible link between lysogeny and biofilm formation. Considering that extracellular DNA (eDNA) is a key factor in the biofilm matrix, we reasoned that prophage spontaneous activation with the consequent bacterial host lysis could provide a source of eDNA, enhancing pneumococcal biofilm development. Monitoring biofilm growth of lysogenic and non-lysogenic pneumococcal strains indicated that phage-infected bacteria are more proficient at forming biofilms, that is their biofilms are characterized by a higher biomass and cell viability. The presence of phage particles throughout the lysogenic strains biofilm development implicated prophage spontaneous induction in this effect. Analysis of lysogens deficient for phage lysin and the bacterial major autolysin revealed that the absence of either lytic activity impaired biofilm development and the addition of DNA restored the ability of mutant strains to form robust biofilms. These findings establish that limited phage-mediated host lysis of a fraction of the bacterial population, due to spontaneous phage induction, constitutes an important source of eDNA for the *S. pneumoniae* biofilm matrix and that this localized release of eDNA favors biofilm formation by the remaining bacterial population.

Citation: Carrolo M, Frias MJ, Pinto FR, Melo-Cristino J, Ramirez M (2010) Prophage Spontaneous Activation Promotes DNA Release Enhancing Biofilm Formation in *Streptococcus pneumoniae*. PLoS ONE 5(12): e15678. doi:10.1371/journal.pone.0015678

Editor: Jean-Pierre Vartanian, Institut Pasteur, France

Received August 13, 2010; **Accepted** November 22, 2010; **Published** December 20, 2010

Funding: M.C. and M.J.F. are supported by research grants from Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/35854/2007 and SFRH/BD/38543/2007 respectively). This work was partly supported by Fundação para a Ciência e Tecnologia, Portugal (PTDC/SAU-ESA/64888/2006 and PIC/IC/83065/2007), Fundação Calouste Gulbenkian and an unrestricted grant from GlaxoSmithKline. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have read the journal's policy and have the following conflicts. J.M.C. has received research grants administered through his university and has consulted and received honoraria for speaking from Pfizer, Bial, GlaxoSmithKline and Novartis. M.R. has consulted for Wyeth Pharmaceuticals. The remaining authors have declared that no competing interests exist. This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

* E-mail: ramirez@fm.ul.pt

☯ These authors contributed equally to this work.

Introduction

Biofilms, the most frequently encountered physiological form adopted by microorganisms, are surface-adapted communities that constitute a protected mode of bacterial growth allowing survival in hostile environments [1–4]. Recent studies demonstrated the potential of *Streptococcus pneumoniae* to produce biofilms *in vivo* [5,6]. Pneumococcal biofilms were indeed detected on affected tissues in patients with chronic rhinosinusitis [5], children with otitis media [6], as well as a chinchilla model of otitis [7]. The presence of these communities at the site of infection implicates them in these disorders, although their significance in the infection process is a matter of debate. Two recent studies have failed to find an association between the ability to form biofilms and whether the isolates had been recovered from asymptomatic carriers or caused invasive infections [8,9]. Moreover, a mouse model of invasive infection failed to show any association between the capacity to cause bacteremia and the ability of the strains to form robust biofilms [8]. Although these two studies question the role of biofilms in determining the invasive potential of pneumococci, the transcriptional profile of several known virulence-related genes in *S. pneumoniae* isolated from lungs and brains of infected mice is similar to that in biofilms formed *in vitro*, suggesting a possible biofilm-like state of *S. pneumoniae* associated with tissues [10]. In addition, a link was established between pneumococcal biofilm formation and the asymptomatic colonization of the nasopharynx [11], the most frequent state of pneumococci. Overall, these studies highlight the importance of studying *S. pneumoniae* biofilms, particularly of identifying the factors that influence the formation of these structures.

Bacterial biofilms are encased within an extracellular matrix consisting of polysaccharides, proteins and nucleic acids [2]. Although polysaccharides and proteins are important components, the role of extracellular DNA (eDNA) as a critical element of the matrix is increasingly recognized, both in providing structural stability as well as protection against antimicrobial agents [12–15].

In Gram-positive bacteria, such as *Enterococcus faecalis* and *Staphylococcus epidermidis*, autolysins (bacterial murein hydrolases) were recently implicated in biofilm formation, apparently by mediating bacterial lysis with the consequent release of eDNA [15–18]. Pneumococcal cells are characterized by the presence of a major autolysin LytA, an N-acetyl-muramyl-L-alanine amidase, which is responsible for the unusual property of massive cellular lysis displayed in the stationary phase of liquid cultures [19]. Furthermore, cell lysis dependent on LytA was also detected upon competence development, which results in DNA release into the medium [20,21]. The observation that *S. pneumoniae* biofilm formation is influenced by the presence of eDNA [22,23] and that LytA mutants have a decreased capacity to form biofilms [23], hints that LytA-induced pneumococcal lysis could be related to biofilm formation through the release of eDNA.

In addition to autolytic events, cell lysis in *S. pneumoniae* can also be mediated by lysogenic phages, which have a high prevalence (76%) in isolates associated with infection [24,25]. During lysogeny, the prophage is integrated in the bacterial chromosome being replicated as part of the hosts genome. Upon induction, the repressed lysogenic state shifts to lytic growth with the production of viral particles and subsequent phage mediated host lysis to release the phage progeny [24,26]. It was recognized early that free phages can be found in cultures of lysogenic bacteria in the absence of a known inducing agent, indicating that some prophages spontaneously enter the lytic cycle [27]. Spontaneous phage induction seems to be a common feature of lysogeny, being non-specific of the phage or the bacterial host, although the factors that promote spontaneous induction, either *in vitro* or *in vivo*, are poorly understood. Recent studies showed that this natural phenomenon may contribute to pathogenicity in *Salmonella* [28], increasing the awareness of the potential importance of lysogeny in the context of infection. This spontaneous phage release occurs obviously at low levels, and the phage titer observed is orders of magnitude less than the one produced when the same bacteria are treated with an inducing agent [26,29,30].

Whether agent-induced or spontaneous, it was believed that phages of *S. pneumoniae* relied exclusively on their own lysins to hydrolyze host cell wall peptidoglycan and release the phage progeny [31]. Recently, it was shown that pneumococcal lysogenic phages achieve an optimal exit strategy by orchestrating the coordinated action of the phage-encoded lysin and the bacterial major autolysin LytA [32].

Inevitably, prophage activation results, through bacterial lysis, in the release of the cellular components to the extracellular medium. Since eDNA is increasingly recognized as a critical element for biofilm formation, we hypothesized that spontaneous induction of lysogenic phages could have a positive effect on pneumococcal biofilms. To test this, we have evaluated biofilm formation and eDNA release of isogenic strains differing in carriage of a prophage and having functional or being deleted in the major phage and bacterial lysins.

Results

Lysogenic phages enhance biofilm development

In order to evaluate the impact of lysogeny in biofilm formation a well established *in vitro* system, based on an abiotic surface as the growth substrate, was used allowing proper investigation of the initial stages of biofilm formation [8–10,22,23]. We started by monitoring biofilm development of the isogenic pair of *S. pneumoniae* strains R36A and R36AP, which differ only in the presence of a prophage (R36AP is a lysogen of phage SV1). Biofilm growth was followed at specific time points between 6 h and 30 h of incubation by biomass quantification and viable cell counts (Fig. 1A and 1B). The evaluation of cell viability by CFUs was consistent with biomass quantification obtained by crystal-violet staining. The biofilm of the lysogenic strain R36AP reaches its maximal development at 24 h and from that time onwards a decrease in biomass occurs. We reasoned that this decrease is inherent to the experimental conditions used, probably due to nutrient depletion, accumulation of toxic substances or intrinsic properties of the biofilm. In contrast, for the wild type non-lysogenic strain R36A the highest biofilm mass values are registered at 26 h, decreasing afterwards in a behavior similar to that of strain R36AP. This observation is consistent with a slower biofilm growth of strain R36A, resulting in delayed development. The lysogenic strain showed improved biofilm growth at all time points and also a higher maximal biofilm mass than its non-lysogenic parent. In agreement with these findings, images of CLSM show denser and thicker biofilms for R36AP (Fig. 2A and 2B). Since the lysogen R36AP is indistinguishable from its parental strain R36A in planktonic growth [32], the differences observed must be attributed to the influence of the lysogenic phage on biofilm formation.

Spontaneous prophage induction enhances biofilm development due to host lysis

It was previously shown that the main pneumococcal autolysin LytA is important in normal biofilm development since its inactivation resulted in diminished biofilm formation, possibly by a mechanism dependent on its regulated lytic activity [23]. Thus, autolytic events may be helpful in the establishment of robust *S. pneumoniae* biofilms. It is well known that spontaneous phage induction results in the lysis of a fraction of the bacterial population [29] and we speculated that such induction could also occur within pneumococcal biofilms. Accordingly, the enhanced biofilm formation of the lysogenic strain R36AP could be explained by limited phage triggered lysis. To test this hypothesis, we compared biofilm development of the lysogenic strain R36AP to that of the derived mutants for phage lysin Svl (strain R36APΔ*svl*), bacterial autolysin LytA (R36APΔ*lytA*) or both lysins (R36APΔ*lytA*Δ*svl*). As shown in Fig. 1A and 1B biofilm growth is significantly impaired in the absence of the phage lysin with a shift in the biofilm biomass peak from 24 h to 26 h, analogous to the growth pattern observed for the non-lysogenic strain (R36A). A similar behavior was observed for the lysogen in the absence of the bacterial autolysin. In fact, the presence of at least one lysin is essential, as the double mutant was largely deficient in biofilm formation. Accordingly, the non-lysonenic R36AΔ*lytA* strain is also severely impaired in biofilm formation, supporting an important role of bacterial lysis in biofilm formation. This inability to form biofilms is not due to a growth defect since all mutants presented identical planktonic growth to the parent lysogen [32]. These results are consistent with the hypothesis that the positive impact of prophages in pneumococcal biofilm development is due to spontaneous induction of the lytic cycle resulting in cell lysis.

In order to confirm if phage induction was indeed occurring in the biofilm, we measured the phage particles released during biofilm development of strain R36AP by determining the number of PFUs throughout biofilm growth (Fig. 1C). We observed the presence of phages in the biofilm at all time points, indicating that spontaneous phage induction is occurring continuously and paralleling the increase in viable cells. A substantial increase in the number of PFUs coincides with the peak of biofilm development (Fig. 1A and 1B), indicating increased phage induction at the later stages of biofilm formation. This higher phage induction is not due to a massive triggering of the phage

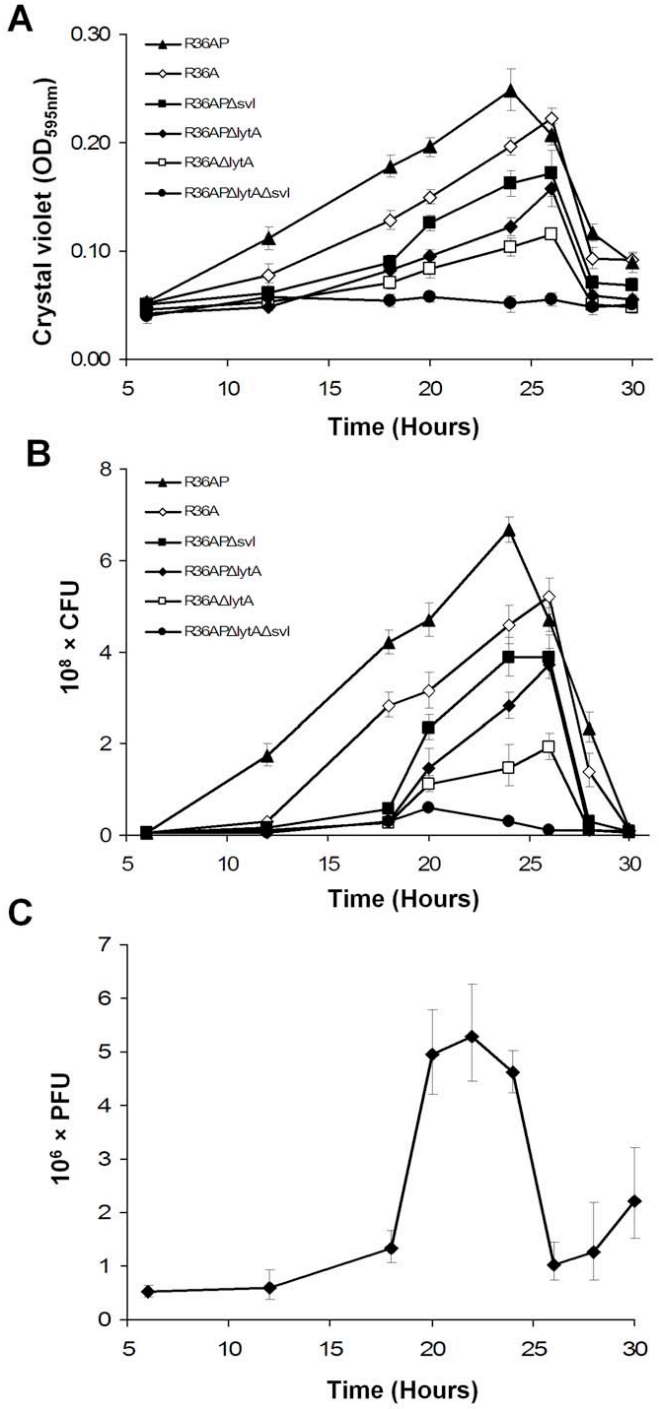

Figure 1. Effect of lysogeny and phage induction in *Streptococcus pneumoniae* biofilm development. A) Biofilm development monitored as biomass from 6 h to 30 h. R36A non-lysogenic strain; R36AP lysogenic derivative of R36A; R36AP*Δsvl*, R36AP*ΔlytA*, R36A*ΔlytA* and R36AP*ΔlytAΔsvl* are mutants in which the phage lysin (Svl), the bacterial autolysin (LytA) or both were deleted. Results are an average of 9 independent replicates. **B**) Biofilm development monitored as CFUs from 6 h to 30 h. The strains are the same indicated in panel A. Results are an average of 6 independent replicates. **C**) The presence of phage in the R36AP biofilm was determined by the production of plaques on R36A. PFUs were determined throughout biofilm development from 6 h to 30 h. Results are an average of 2 to 7 independent replicates for each time point. In all panels error bars represent 95% confidence intervals for the sample mean.
doi:10.1371/journal.pone.0015678.g001

lytic cycle related to this stage of biofilm growth since no increase of PFUs concomitant with biofilm dispersal was observed. Altogether, these results strongly support a role of spontaneous phage induction during biofilm development in the different phenotype shown by lysogenic strains.

Released eDNA through phage-mediated lysis is a key factor for biofilm enhancement

Extracellular DNA is an essential matrix component produced by many bacterial species during biofilm development [13,15,16,33,34]. Therefore, we hypothesized that in *S. pneumoniae* phage-mediated lysis of a fraction of the bacterial population within the biofilm could provide an extra source of eDNA for incorporation in the biofilm matrix.

We performed a DNase I susceptibility assay by incubating this enzyme for 24 h with R36A and R36AP in conditions allowing biofilm formation. A gradient of DNase I concentrations, ranging from 0.5 to 50 μg/ml was used. Biofilm biomass quantification indicates that DNase I reduces biofilm formation in a dose dependent way (Fig. 3A). As expected, the biomass reduction is directly related to a decrease of viable cells in the biofilm (data not shown). The effect of DNase I is similar in R36A and R36AP biofilms, however, R36AP biofilms always show a higher biomass than R36A biofilms at all tested DNase I concentrations, suggesting that R36AP biofilms are richer in eDNA. In fact, the biomass of the R36AP biofilm incubated with 0.5 μg/ml of DNase I, is similar to that of the R36A biofilm incubated in the absence of DNase I, indicating that the presence of this enzyme reduced the extra eDNA present in the R36AP matrix, resulting in a biofilm similar to that of R36A. Taken together, the data enable an argument to be made for the beneficial effect of lysogenic phages in biofilm development due to an increased presence of eDNA in the matrix.

To further explore this potential role of eDNA on biofilm development, we decided to determine the effect of the addition of external DNA to the medium since the time of seeding, on biofilm mass measured at 24 h of growth. DNA was extracted from the R36A strain (homologous DNA) and used at a final concentration of 10, 100 and 1000 ng/ml. To rule out any specific effect of pneumococcal DNA, the same experiments were repeated using DNA isolated from salmon sperm (heterologous DNA). As shown in Fig. 3B, incubation with DNA since biofilm seeding enhances biofilm development in a dose dependent manner, with a significant effect detected with as little as 10 ng/ml. This biomass increase parallels the number of viable cells in the biofilm (data not shown). Moreover, this effect is observed with both homologous and heterologous DNA, indicating that this was due to an intrinsic property of the DNA molecule and independent of the exact nucleotide sequence and donor organism.

Microscopy was used to explore the differences between untreated R36AP biofilms and those treated with 50 μg/ml of DNase I and 1000 ng/ml of DNA. In agreement with the results obtained by biomass quantification, treatment with DNase I resulted in sparser and thinner biofilms when compared to control (Fig. 2A and 2D). On the other hand, supplementation of the medium with DNA resulted in a more densely packed and thicker biofilm (Fig. 2A and 2C). These results further support that the limited lysis promoted by lysogenic phages during biofilm development leads to higher eDNA release resulting in stronger biofilm growth.

Due to the different kinetics of biofilm development of the lysogenic and non-lysogenic strains, we wanted to clarify if the role of DNA was only critical in the initial steps of biofilm establishment (initial cell attachment) or if its presence was necessary throughout the subsequent early phases of biofilm

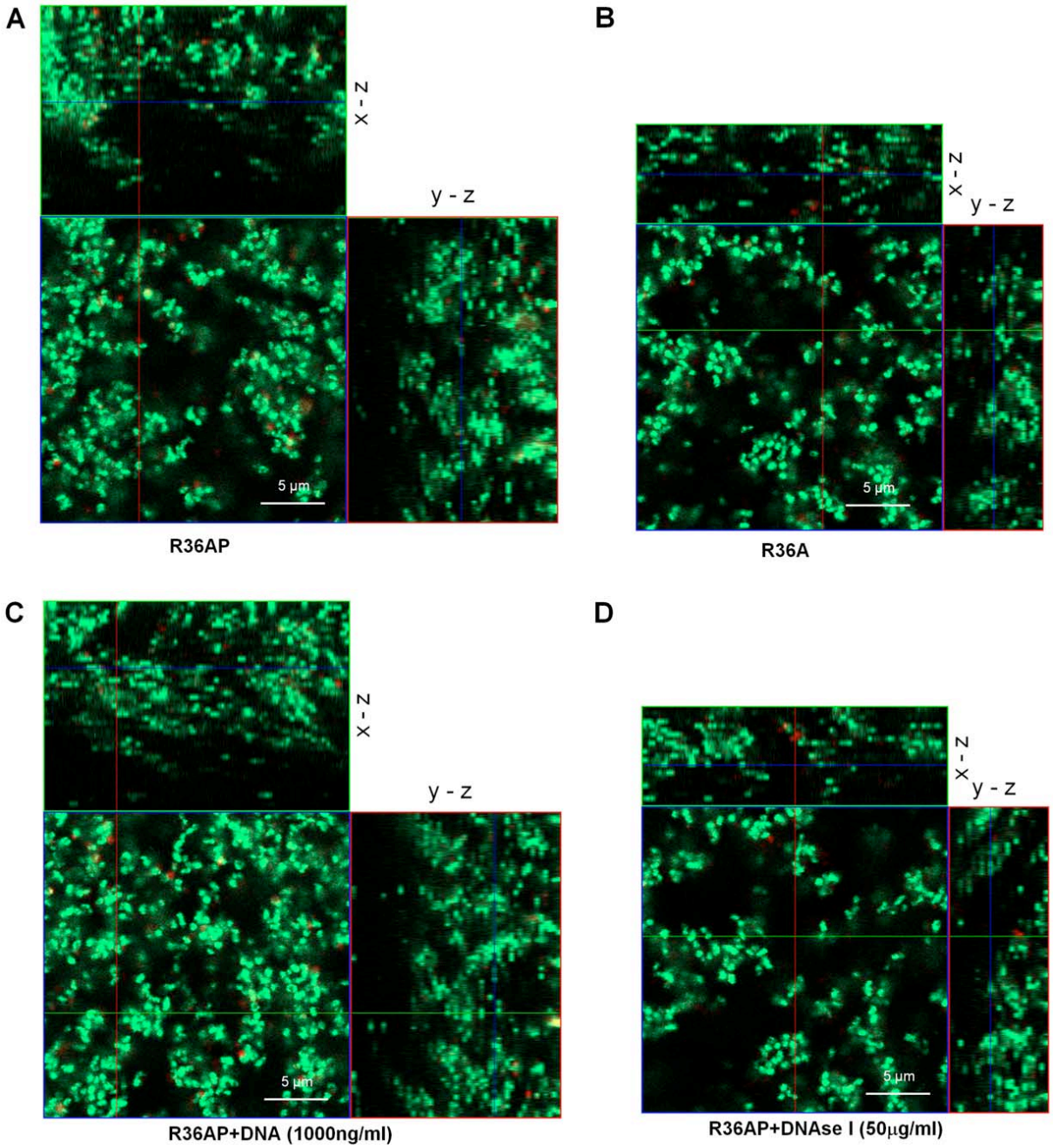

Figure 2. Confocal laser scanning microscopy images of R3A6P and R36A biofilms. Staining was done with Syto 9/PI (Live/Dead BacLight Bacterial Viability kit) and images were acquired at 630×amplification. Only live cells internalize Syto 9 (fluorescing green) whereas dead cells allow the uptake of PI (fluorescing red). The large images are optical sections of top views and the small images to the right and above are optical sections of side views. The depth of the biofilm is indicated by the height of the *z*-stack. The inset scale bar represents 5 µm. **A**) Biofilm formed by the lysogenic strain R36AP. **B**) Biofilm of the non-lysogenic strain R36A. **C**) The biofilm was grown in the presence of salmon sperm DNA at 1000 ng/ml. **D**) The biofilm was grown in medium supplemented with DNase I at 50 µg/ml. In all panels the results are representative images of 3 independent experiments and biofilm growth was evaluated at 24 h.
doi:10.1371/journal.pone.0015678.g002

development. To this end, the wells where the biofilms were grown were pre-coated with DNA followed by incubation of the bacteria in DNA-free medium. After 24 h, biofilm mass was similar to the uncoated control (Fig. 3B), indicating that the observed DNA effect is not related with the initial adherence process.

Furthermore, we also examined whether DNA acts as a structural component of the biofilm or if the availability of extra nutrients, due to the presence of DNA in the medium, could explain the enhanced biofilm development. With that in mind, we grew biofilms in the presence of sonicated DNA and compared them with biofilms formed in the presence of intact DNA, using both homologous and heterologous DNA. In the presence of fragmented DNA, biofilm development assessed at 24 h was similar to that of biofilms grown in the absence of DNA and substantially less to that observed with intact DNA (Fig. 3B). This data revealed that large DNA fragments were essential for the enhancement of biofilm growth and suggested that DNA had an important structural role in biofilm architecture.

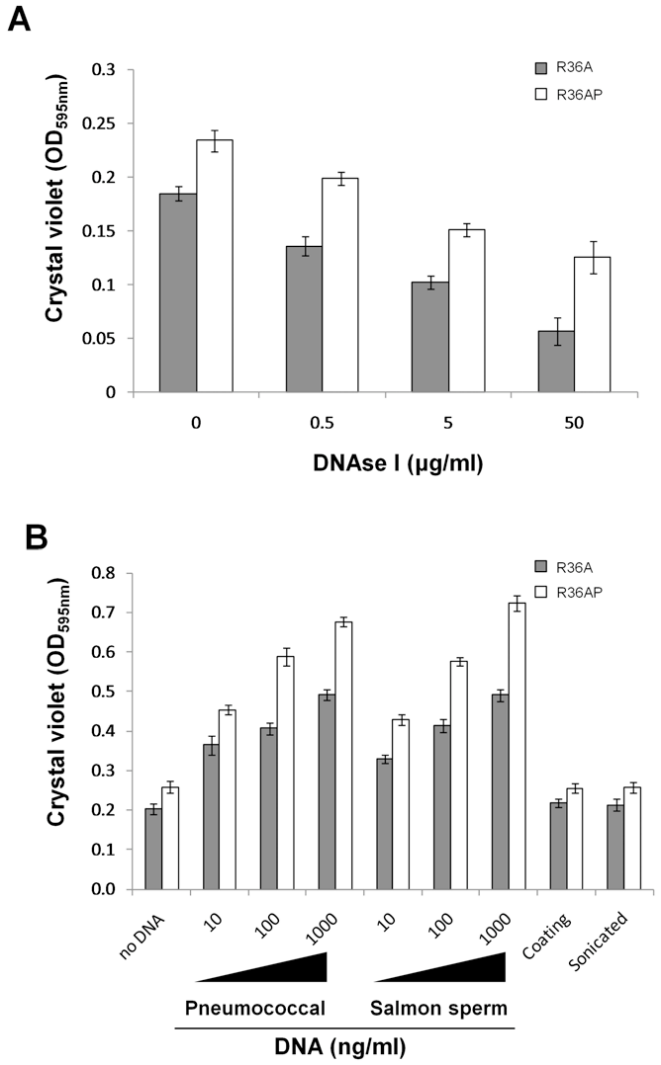

Figure 3. Effect of DNAse I and DNA on biofilm mass. A) The lysogenic strain R36AP and its non-lysogenic parent R36A were exposed from seeding to DNAse I at final concentrations of 0.5, 5 and 50 µg/ml. Biofilm mass was quantified after 24 h of incubation. **B**) R36AP and R36A were exposed from seeding to DNA from R36A or salmon sperm at final concentrations of 10, 100 and 1000 ng/ml. In separate experiments, the effect on biofilm development of coating the wells with 1000 ng/ml of R36A DNA prior to seeding and the addition of sonicated R36A DNA at 1000 ng/ml since the time of seeding was also determined. Biofilm mass was assessed at 24 h of incubation. In all panels, the results are an average of 9 independent replicates and error bars represent 95% confidence intervals for the sample mean.
doi:10.1371/journal.pone.0015678.g003

To confirm if a higher eDNA release due to phage spontaneous induction is related to a strong biofilm development we determined the actual eDNA released into the biofilm of each strain, after 24 h of growth, by quantitative real-time PCR. Strains with a higher capacity to form biofilm (R36AP, R36A, R36APΔ*svl* and R36APΔ*lytA*) contain significantly more eDNA in comparison to the almost undetectable levels present in R36APΔ*lytA*Δ*svl* and R36AΔ*lytA*, two strains with poor biofilm forming capacity (Fig. 4A). The marked difference observed between lytic and non-lytic strains suggest that lytic events resulting in eDNA release have a strong positive impact in biofilm development.

DNA release upon phage induction is dependent on lysis. So, we reasoned that the addition of external DNA to biofilms of the mutant strains R36APΔ*svl*, R36APΔ*lytA* and R36APΔ*lytA*Δ*svl* would allow the development of more robust biofilms. Indeed, when the mutant strains were given exogenous DNA, biofilm development was strongly increased in R36APΔ*svl* and R36APΔ*lytA* (Fig. 4B). The addition of a large excess of DNA overcomes the impairments created by the ablation of either the phage or bacterial lysins, with the formation of more biofilm in the presence of DNA by these mutants than that observed when R36AP, where both lysins are functional, was incubated in the absence of exogenous DNA (Fig. 4B). As pointed out previously, the mutant lacking both lysin activities (R36APΔ*lytA*Δ*svl*) was incompetent to form stable biofilms and, even in the presence of excess DNA, failed to recover to the R36AP level. Thus the addition of DNA does not fully overcome the abolishment of the two major lysins present in R36AP. This is in contrast to R36AΔ*lytA* that responds well to the addition of external DNA. Although both mutants present similar amounts of eDNA (Fig. 4A), the R36AΔ*lytA* strain forms more biofilm biomass than strain R36APΔ*lytA*Δ*svl* (Fig. 1A) and this effect is even more pronounced in the number of viable bacteria in the biofilm (Fig. 1B). It has been previously shown that even when phage and bacterial lysins are deleted, phage induction decreases cell viability as phages express holins that collapse the cell membrane potential resulting in host cell death [32]. Thus, this difference in cell viability between R36AΔ*lytA* and R36APΔ*lytA*Δ*svl* may be sufficient to compromise the enhancement of biofilm development in the presence of added DNA observed in the later strain.

To test if the presence of a capsular polysaccharide could influence our results, we characterized the behavior of strain SVMC28 and its mutants in both phage and bacterial lysins. SVMC28 is an encapsulated strain and the natural host of the SV1 phage. The results obtained were superimposable to those of strain R36AP and its mutants, with the same relative biomass produced by the parental strain and its mutants in the absence of DNA and the same effect seen upon DNA addition (Fig. 4C). This indicates that our observations were reproducible in different genetic backgrounds and, more importantly, that the capsule did not qualitatively alter our conclusions Overall, our results indicate that the release of eDNA through controlled lytic events is a key factor for biofilm formation in *S. pneumoniae* and that lysogenic phages are important adjuvants for its incorporation in the biofilm matrix independently of the presence of a capsular polysaccharide.

Discussion

Prophages are extremely common among *S. pneumoniae* isolates causing infections in humans [24]. The lysogenic lifestyle results in the establishment of the phage genome inside the bacterial host where it can remain in a dormant state replicating together with the bacterial chromosome. An important feature is the possible transition from the repressed lysogenic state to lytic development, that ultimately leads to host cell death and release of the newly produced phage particles. Prophage induction can occur spontaneously in a fraction of the lysogenic bacterial population or massively upon external stimuli [26,27].

Here we investigated the impact of lysogeny in *S. pneumoniae* biofilm formation exploring its role in the early development of these structures. Our data provided evidence that prophage carriage had a positive impact on pneumococcal biofilm formation through spontaneous induction of the lytic cycle. Phage induction results in the death of their bacterial hosts, however we showed that this phage-mediated lysis enhances biofilm formation, suggesting that in this context the bacterial population as a whole could benefit from limited prophage induction. Studies on gene expression in biofilms of various species have identified phage genes as overexpressed

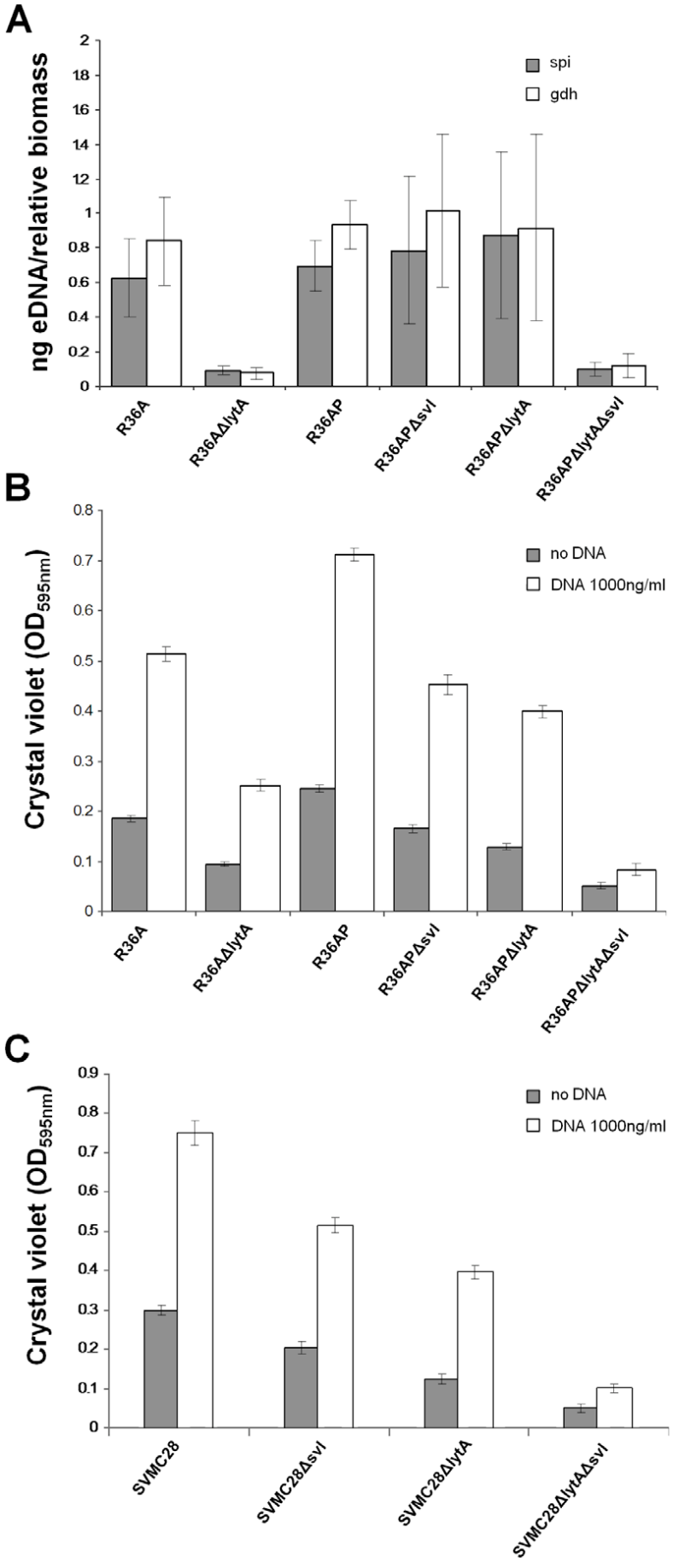
A
ng eDNA/relative biomass
spi
gdh
R36A
R36AΔlytA
R36AP
R36APΔsvl
R36APΔlytA
R36APΔlytAΔsvl
B
Crystal violet (OD595nm)
no DNA
DNA 1000ng/ml
R36A
R36AΔlytA
R36AP
R36APΔsvl
R36APΔlytA
R36APΔlytAΔsvl
C
Crystal violet (OD595nm)
no DNA
DNA 1000ng/ml
SVMC28
SVMC28Δsvl
SVMC28ΔlytA
SVMC28ΔlytAΔsvl

Figure 4. eDNA quantification and DNA impact on biofilm mass. A) Extracellular DNA was isolated from the biofilm matrices of R36A, R36AΔ*lytA*, R36AP, R36APΔ*svl*, R36APΔ*lytA* and R36APΔ*lytA*Δ*svl* and quantitative real-time PCR of two chromosomal genes, *spi* and *gdh*, was done. The relative biomass was quantified at $OD_{595\ nm}$ and the eDNA measurements were normalized to total biofilm mass. **B**) The effect of salmon sperm DNA (1000 ng/ml) added from seeding on biofilm biomass at 24 h was tested. R36APΔ*svl*, R36APΔ*lytA*, R36AΔ*lytA* and R36APΔ*lytA*Δ*svl* are mutants in which the phage lysin (Svl), the bacterial autolysin (LytA) or both were deleted. **C**) The same experiments described in panel B were done with the encapsulated wild type host of phage SV1, strain SVMC28, and its mutants. SVMC28Δ*svl*, SVMC28Δ*lytA* and SVMC28Δ*lytA*Δ*svl* are mutants in which the phage lysin (Svl), the bacterial autolysin (LytA) or both were deleted. In all panels, the results are an average of 9 independent replicates and error bars represent 95% confidence intervals for the sample mean.
doi:10.1371/journal.pone.0015678.g004

relative to planktonic growth while other studies showed the existence of lysis inside biofilms and proposed that it could increase biofilm fitness [35–40]. Our results corroborate this previous proposal in the context of *S. pneumoniae* biofilms, clearly identifying the phage activated lytic machinery as a key player in this effect. Interestingly, phage mediated bacterial lysis within the biofilm has also been described in other bacterial species. However, in contrast to our study, in those cases, phage induction results in the death of a large fraction of the bacterial population and occurs in the later stages of biofilm development [36,38–40].

The impact of lysogenic phages on pneumococcal populations is still an open question since comparative genomic analysis did not reveal any phage-encoded virulence factors, contrary to other related streptococcal pathogens such as *Streptococcus pyogenes* [41,42]. The observed biofilm potentiating role of lysogenic phages and the proposed importance of these structures in colonization [11] could explain in part the high incidence of lysogeny in *S. pneumoniae* natural populations [24,25]. Furthermore, a high frequency of lysogeny is characteristic of many bacterial pathogens [43] as well as of bacterial populations in the environment [44], raising the possibility that the influence of lysogeny on the ability of pneumococci to form biofilms could be paralleled in other bacterial species.

The mechanism by which spontaneous prophage-mediated cell lysis leads to increased biofilm development was also addressed in this study. We gathered evidence that DNA released through this process to the extracellular environment contributes to biofilm formation in *S. pneumoniae*. An approximately six-fold increase in eDNA was detected in strains carrying prophages and functional bacterial or phage lysins. These lysogenic strains were also characterized by forming biofilms with a higher biomass and cell viability. This role of eDNA is consistent with previous findings in this species, although in those studies the source of eDNA was not identified [6,23]. We observed that eDNA is not involved in the initial attachment stage, since pre-treatment of the plastic substrate with DNA did not increase biofilm formation. In agreement, a high concentration of DNAse I added from the onset of biofilm incubation still allowed bacterial surface attachment and biofilm formation, although in these conditions bacteria failed to form the thick and dense structures observed in the absence of DNase I. To our knowledge, this is the first study of the role of eDNA in initial adhesion of pneumococcal cells to a surface. Although in some bacterial species eDNA plays an important role in this initial step [16,45], similar results to ours were already observed with another Gram-positive bacterium [15]. Being such a complex lifestyle, it is plausible that in different microorganisms the importance of the various mechanisms for biofilm establishment is also different. The factors or substances that promote initial attachment remain to be identified in *S. pneumoniae*. However, eDNA played an important role already in the early stages of biofilm development, since spontaneous phage induced lysis is detected in the early hours of biofilm establishment and the R36AP lysogen showed a more robust biofilm development at all time points. Accordingly, a mutant lacking the phage lysin produced less biofilm and in a delayed fashion, a behaviour that was similar to the mutants lacking the major bacterial autolysin LytA. Both observations are consistent with a possibly slower accumulation of eDNA in the matrix and with an important role of eDNA at various stages of biofilm formation.

Our data indicates that eDNA is an important structural component of *S. pneumoniae* biofilms, ensuring stability of the overall architecture of these structures. Although DNAse I treatment resulted in eDNA degradation with the consequent reduction in biofilm formation, the critical result that definitely establishes this structural role of DNA was the observation that addition of fragmented DNA did not affect biofilm development, whereas intact DNA led to increases in both mass and bacterial viability in biofilms, indicating that the long strands of DNA may allow more intercellular cohesion thereby increasing biofilm stability. These results are supported by studies in other species that have proposed DNA as an essential component of the extracellular polymeric substance that constitutes the biofilm matrix [13,15,16,23,46]. Thus, cell lysis mediated by lysogenic phages influences the matrix composition, thereby contributing to the pneumococcal biofilm structural stability. Since spontaneous phage induction occurs in different areas of the biofilm, it is expected to contribute significantly to the abundance and widespread localization of eDNA.

In contrast to limited cell lysis due to spontaneous phage induction, massive phage induction in the presence of an external inducing agent could disrupt biofilms drastically, an hypothesis supported by the use of lytic phages as powerful anti-biofilm agents active against different microorganisms [47,48]. In fact, preliminary results from our group indicate that Mitomycin C phage induction is able to disrupt to a large extent biofilms of lysogenic strains. If the proportion of induced cells is large, more cells lyse than are contributing to the biofilm resulting in an overall loss of biofilm mass. This is in agreement with the natural biofilm demise mediated by substantial phage induction proposed for some *Pseudomonas aeruginosa* strains that facilitate differentiation and dispersal of biofilm associated bacteria [36,39,40]. The beneficial or detrimental effect of prophage induction on biofilm formation seems to be quantitatively regulated by the proportion of lysogenic bacteria undergoing lytic induction.

In conclusion, we showed that limited activation of prophages into the lytic cycle, thereby promoting host lysis and eDNA release, contributes to enhanced pneumococcal biofilm production. This more efficient biofilm development afforded by lysogenic phages may be an important aspect in the biology of the bacteria since lysogeny is highly prevalent in pneumococci. Our data provided new insights into the factors that influence the formation and maintenance of biofilms whose occurrence and importance *in vivo* is increasingly recognized.

Materials and Methods

Bacterial strains, culture conditions and DNA manipulations

Bacterial strains SVMC28 and R36A were obtained from the Rockefeller University collection (A. Tomasz). R36A is a non-

lysogenic, non-encapsulated strain [24]. SVMC28 is an encapsulated (serotype 23F) clinical isolate, lysogenic for phage SV1 encoding the Svl phage lysin [32]. R36AΔ*lytA* was kindly provided by S. Filipe. SVMC28 derived mutants SVMC28Δ*svl*, SVMC28Δ*lytA* and SVMC28Δ*svl*Δ*lytA* belong to the Faculdade de Medicina de Lisboa collection. The SV1-lysogenized strains R36AP, R36APΔ*lytA*, R36APΔ*svl* and R36APΔ*lytA*Δ*svl* are also from the Faculdade de Medicina de Lisboa collection. All strains were described previously [32]. All *S. pneumoniae* strains were grown in a casein-based semi-synthetic medium (C+Y) at 37°C without aeration or in tryptic soy agar (TSA) (Oxoid, Hampshire, England) supplemented with 5% (v/v) sterile sheep blood incubated at 37°C in 5% CO_2. For overnight cultures, pneumococcal mutant strains were grown in the presence of 2 μg/ml erythromycin or 4 μg/ml chloramphenicol (Sigma, Steinheim, Germany) or both, as appropriate. After selective growth, the culture was diluted 1:100 in fresh medium and grown until the appropriate optical density. Chromosomal DNA from *S. pneumoniae* strain R36A was isolated following previously described procedures [49]. Sperm salmon DNA was purchased from Invitrogen Co. (Carlsbad, California, USA).

Biofilm biomass quantification

Biofilm formation was determined by the ability of cells to grow adherent to 96-well flat-bottom polystyrene microtiter plates (Nunc^{TM}, Roskilde, Denmark) in static conditions. Cells were grown in C+Y medium, with selective antibiotic when necessary, to an optical density measured at 600 nm (OD_{600nm}) between 0.5 and 0.6 and then diluted 1:4 in fresh medium to a final volume of 200 μl per well. Microtiter plates were incubated at 37°C and biofilm mass was determined by staining with crystal violet [23] and measuring the OD_{595nm} using a plate reader (Tecan Infinite M200 with i-control^{TM} software V1.40). The incubation times at which the biomass was quantified were selected based on preliminary experiments in order to monitor the dynamics of biofilm growth and dispersal. Shorter time intervals were selected when biofilm mass showed steeper variations. The incubation times at which biomass was quantified were 6 h, 12 h, 18 h, 20 h, 24 h, 26 h, 28 h and 30 h of incubation. A control with only C+Y medium was also done for all time points and the values were subtracted to those measured for all strains.

Quantitative determination of biofilm formation was also evaluated in the presence of desoxiribonuclease I (DNase I) and DNA, incorporated in the medium. DNase I (Sigma, Steinheim, Germany) was used at a final concentration of 0.5, 5 or 50 μg/ml and biofilm mass was measure after 24 h of incubation. DNA from R36A strain or salmon sperm was added at 10, 100 or 1000 ng/ml to the medium and determination of biofilm formation was carried out 24 h post incubation. Values obtained from medium supplemented with DNAse I and DNA were subtracted in all strains. To test if DNA was important in biofilm adherence, the plate wells were incubated with 1000 ng/ml of R36A DNA overnight at 4°C to condition the plastic surface. The solution was then discarded and the biofilm was seeded as described before. Biofilm mass was determined at 24 h post incubation. To determine if the impact of DNA on biofilm formation was due to a structural role, DNA from R36A was broken by sonication for 5 min at 0.63 A and 50–60 Hz in a Transsonic T570 (Elma, Germany), and added to the medium at 1000 ng/ml. DNA fragmentation was confirmed by agarose gel electrophoresis. Biofilm formation was compared to biofilms grown in the presence of 1000 ng/ml of intact DNA.

Biofilm colony forming units (CFU) assays

Biofilms were grown in 96-well plates at 37°C as described for the biofilm biomass quantification assay. CFUs were determined at the selected time points between 6 h and 30 h of incubation. Liquid medium with bacteria was gently removed from the wells, which were washed twice with phosphate buffered saline (PBS) 1×, pH 7.2 (Invitrogen, Grand Island, New York) to eliminate unbound bacteria without disturbing the adherent biofilm. 200 μl of PBS were then added to each well and biofilms were scraped thoroughly, including well edges. The well contents were recovered and the total CFU number was determined by serial dilution and plating on appropriate media.

To test the effect of DNase I and DNA on biofilm development, DNase I was added to the growth medium to a final concentration of 0.5, 5 or 50 μg/ml. After 24 h of incubation at 37°C, CFUs were determined as described above. When using DNA to evaluate its effect on biofilm formation, DNA from salmon sperm was added to the growth medium at a final concentration of 1000 ng/ml and CFUs were determined as described above.

Phage plaque assays

Plaque assays were performed as described elsewhere [32].In detail, basal plates were made by pouring C+Y medium with 170 U catalase per ml and 1% agar into Petri dishes. A lawn culture of R36A strain grown to an OD_{600nm} of 0.2 was mixed with soft agar containing C+Y supplemented with 170 U catalase per ml and 0.35% agar. The entire mixture was spread onto basal plates. After hardening, phage preparations were applied in 10 μL aliquots directly on the soft agar with the R36A indicator strain. Incubation was performed at 30°C for 18 h. To obtain the phage preparation, at the chosen time points after biofilm seeding each well was scraped thoroughly including well edges. The harvested biofilms were filtered through a 0.45 μm-pore-size membrane followed by filtering with a 100 000 MWCO polyethersulfone membrane (Vivaspin concentrator, Sartorius Stedim biotech, Goettingen, Germany), that retains and concentrates the SV1 phage [32]. The phage concentrate was stored at 4°C for a maximum of 24 h until usage. The filtrate containing proteins <100 KDa, that could cause bacterial lysis such as LytA and bacteriocins, was also used to eliminate the possibility that lysis of the indicator strain was caused by bacterial products and not caused by phage infection. Images of the plates were acquired with the high-performance stereo-microscope Leica MZ7.5 (Leica Microsystems, Germany) and the number of plaque forming units (PFUs) was counted manually by visual inspection of the image.

Confocal laser scanning microscopy (CLSM)

Biofilms were stained by using a Live/Dead BacLight bacterial viability kit (Invitrogen, Carlbad, USA) and examined by CLSM. Syto9/PI labeled biofilms allowed for monitoring the viability of bacterial populations as a function of the membrane integrity of the cell. Cells with a compromised membrane (dead cells) will stain red whereas cells with an intact membrane (live cells) will stain green. Whenever DNA and DNase I effects were tested, the medium was supplemented before biofilm seeding (t = 0). In all experiments, biofilms were analyzed after 24 h of incubation. Images were acquired on a Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany) using a PlanApochromat 63×/1.4 objective for cell viability assays and a C-AproChromat 40×/1.2. Syto 9 fluorescence was detected using the 488 nm laser line of an Ar laser (45 mW nominal output) and a BP 505–550 filter. PI fluorescence was detected using a DPSS 561 nm laser (15 mW nominal output) and a LP 575 filter. For imaging, the laser power was attenuated to 1–2% of its maximum value. The pinhole aperture was set to 1 Airy unit.

Purification and quantification of eDNA. Biofilms were grown in 96-well plates at 37°C as reported above. eDNA was

purified from 24 h biofilms exactly as previously described [50]. eDNA was quantified by real-time PCR using the primes gdh-up (5′-ATGGACAAACCAGCNAGYTT) and gdh-dn (5′-GCTTG-AGGTCCCATRCTNCC) and spi-up (5′-TTATTCCTCCTG-ATTCTGTC) and spi-dn (GTGATTGGCCAGAAGCGGAA), amplifying the *gdh* and *spi* genes used for multilocus sequence typing (MLST), respectively. These are housekeeping genes located far apart in the R36A chromosome. PCRs were performed on non-diluted samples with the SYBR Green Jump Start Taq Ready Mix (Sigma, Steinheim, Germany), according to the manufacturers recommendations. Purified R36A genomic DNA at known concentrations was also subjected to quantitative real-time PCR with each primer pair to generate a standard curve used to calculate the concentration of eDNA in the unknown samples. PCR was performed in a 7500 Fast Real-Time PCR System (Applied Biosystems, Life Technologies, Carlsbad, California, USA). To account for potential differences in biomass, the average OD_{595nm} of each biofilm was determined and used to calculate the relative OD_{595nm} of each biofilm with respect to the OD_{595nm} of the wild type R36A biofilm. The nanogram of eDNA per relative biomass of each biofilm was then calculated by dividing its total eDNA (ng) by its relative OD_{595nm}.

Acknowledgments

We thank José Rino and Marco Antunes for their support with the microscopy work.

Author Contributions

Conceived and designed the experiments: MC MJF FRP JM-C MR. Performed the experiments: MC MJF. Analyzed the data: MC MJF FRP MR. Wrote the paper: MC MJF FRP JM-C MR.

References

1. Kolter R, Greenberg EP (2006) Microbial sciences: the superficial life of microbes. Nature 441: 300–302.
2. Costerton JW, Stewart PS, Greenberg EP (1999) Bacterial biofilms: a common cause of persistent infections. Science 284: 1318–1322.
3. Mah TF, O'Toole GA (2001) Mechanisms of biofilm resistance to antimicrobial agents. Trends Microbiol 9: 34–39.
4. Hall-Stoodley L, Stoodley P (2009) Evolving concepts in biofilm infections. Cell Microbiol 11: 1034–1043.
5. Sanderson AR, Leid JG, Hunsaker D (2006) Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. Laryngoscope 116: 1121–1126.
6. Hall-Stoodley L, Hu FZ, Gieseke A, Nistico L, Nguyen D, et al. (2006) Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. Jama 296: 202–211.
7. Reid SD, Hong W, Dew KE, Winn DR, Pang B, et al. (2009) *Streptococcus pneumoniae* forms surface-attached communities in the middle ear of experimentally infected chinchillas. J Infect Dis 199: 786–794.
8. Lizcano A, Chin T, Sauer K, Tuomanen EI, Orihuela CJ (2010) Early biofilm formation on microtiter plates is not correlated with the invasive disease potential of *Streptococcus pneumoniae*. Microb Pathog 48: 124–130.
9. Tapiainen T, Kujala T, Kaijalainen T, Ikaheimo I, Saukkoriipi A, et al. (2010) Biofilm formation by *Streptococcus pneumoniae* isolates from paediatric patients. Apmis 118: 255–260.
10. Oggioni MR, Trappetti C, Kadioglu A, Cassone M, Iannelli F, et al. (2006) Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol Microbiol 61: 1196–1210.
11. Munoz-Elias EJ, Marcano J, Camilli A (2008) Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization. Infect Immun 76: 5049–5061.
12. Mulcahy H, Charron-Mazenod L, Lewenza S (2008) Extracellular DNA chelates cations and induces antibiotic resistance in *Pseudomonas aeruginosa* biofilms. PLoS Pathog 4: e1000213.
13. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS (2002) Extracellular DNA required for bacterial biofilm formation. Science 295: 1487.
14. Vilain S, Pretorius JM, Theron J, Brozel VS (2009) DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms. Appl Environ Microbiol 75: 2861–2868.
15. Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, et al. (2009) Contribution of autolysin and sortase A during *Enterococcus faecalis* DNA-dependent biofilm development. Infect Immun 77: 3626–3638.
16. Qin Z, Ou Y, Yang L, Zhu Y, Tolker-Nielsen T, et al. (2007) Role of autolysin-mediated DNA release in biofilm formation of *Staphylococcus epidermidis*. Microbiology 153: 2083–2092.
17. Thomas VC, Hiromasa Y, Harms N, Thurlow L, Tomich J, et al. (2009) A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*. Mol Microbiol 72: 1022–1036.
18. Thomas VC, Thurlow LR, Boyle D, Hancock LE (2008) Regulation of autolysis-dependent extracellular DNA release by *Enterococcus faecalis* extracellular proteases influences biofilm development. J Bacteriol 190: 5690–5698.
19. Tomasz A, Moreillon P, Pozzi G (1988) Insertional inactivation of the major autolysin gene of *Streptococcus pneumoniae*. J Bacteriol 170: 5931–5934.
20. Steinmoen H, Knutsen E, Havarstein LS (2002) Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population. Proc Natl Acad Sci U S A 99: 7681–7686.
21. Moscoso M, Claverys JP (2004) Release of DNA into the medium by competent *Streptococcus pneumoniae*: kinetics, mechanism and stability of the liberated DNA. Mol Microbiol 54: 783–794.
22. Hall-Stoodley L, Nistico L, Sambanthamoorthy K, Dice B, Nguyen D, et al. (2008) Characterization of biofilm matrix, degradation by DNase treatment and evidence of capsule downregulation in *Streptococcus pneumoniae* clinical isolates. BMC Microbiol 8: 173.
23. Moscoso M, Garcia E, Lopez R (2006) Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. J Bacteriol 188: 7785–7795.
24. Ramirez M, Severina E, Tomasz A (1999) A high incidence of prophage carriage among natural isolates of *Streptococcus pneumoniae*. J Bacteriol 181: 3618–3625.
25. Severina E, Ramirez M, Tomasz A (1999) Prophage carriage as a molecular epidemiological marker in *Streptococcus pneumoniae*. J Clin Microbiol 37: 3308–3315.
26. Little JW (2005) Lysogeny, prophage induction, and lysogenic conversion. In: Waldor MK, Friedman DI, Adhya SL, eds. Phages, their role in bacterial pathogenesis and biotechnology. Washington, DC: ASM Press. pp 37–54.
27. Lwoff A (1953) Lysogeny. Bacteriol Rev 17: 269–337.
28. Figueroa-Bossi N, Bossi L (1999) Inducible prophages contribute to *Salmonella* virulence in mice. Mol Microbiol 33: 167–176.
29. Bossi L, Fuentes JA, Mora G, Figueroa-Bossi N (2003) Prophage contribution to bacterial population dynamics. J Bacteriol 185: 6467–6471.
30. Livny J, Friedman DI (2004) Characterizing spontaneous induction of Stx encoding phages using a selectable reporter system. Mol Microbiol 51: 1691–1704.
31. Lopez R, Garcia E (2004) Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. FEMS Microbiol Rev 28: 553–580.
32. Frias MJ, Melo-Cristino J, Ramirez M (2009) The autolysin LytA contributes to efficient bacteriophage progeny release in *Streptococcus pneumoniae*. J Bacteriol 191: 5428–5440.
33. Allesen-Holm M, Barken KB, Yang L, Klausen M, Webb JS, et al. (2006) A characterization of DNA release in *Pseudomonas aeruginosa* cultures and biofilms. Mol Microbiol 59: 1114–1128.
34. Spoering AL, Gilmore MS (2006) Quorum sensing and DNA release in bacterial biofilms. Curr Opin Microbiol 9: 133–137.
35. Mai-Prochnow A, Evans F, Dalisay-Saludes D, Stelzer S, Egan S, et al. (2004) Biofilm development and cell death in the marine bacterium *Pseudoalteromonas tunicata*. Appl Environ Microbiol 70: 3232–3238.
36. Webb JS, Thompson LS, James S, Charlton T, Tolker-Nielsen T, et al. (2003) Cell death in *Pseudomonas aeruginosa* biofilm development. J Bacteriol 185: 4585–4592.
37. Mai-Prochnow A, Webb JS, Ferrari BC, Kjelleberg S (2006) Ecological advantages of autolysis during the development and dispersal of *Pseudoalteromonas tunicata* biofilms. Appl Environ Microbiol 72: 5414–5420.
38. Webb JS, Lau M, Kjelleberg S (2004) Bacteriophage and phenotypic variation in *Pseudomonas aeruginosa* biofilm development. J Bacteriol 186: 8066–8073.
39. Kirov SM, Webb JS, O'May C Y, Reid DW, Woo JK, et al. (2007) Biofilm differentiation and dispersal in mucoid *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. Microbiology 153: 3264–3274.
40. Rice SA, Tan CH, Mikkelsen PJ, Kung V, Woo J, et al. (2009) The biofilm life cycle and virulence of *Pseudomonas aeruginosa* are dependent on a filamentous prophage. Isme J 3: 271–282.
41. Desiere F, McShan WM, van Sinderen D, Ferretti JJ, Brussow H (2001) Comparative genomics reveals close genetic relationships between phages from dairy bacteria and pathogenic Streptococci: evolutionary implications for prophage-host interactions. Virology 288: 325–341.
42. Banks DJ, Beres SB, Musser JM (2002) The fundamental contribution of phages to GAS evolution, genome diversification and strain emergence. Trends Microbiol 10: 515–521.
43. Brussow H, Canchaya C, Hardt WD (2004) Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. Microbiol Mol Biol Rev 68: 560–602.

44. Williamson SJ, Cary SC, Williamson KE, Helton RR, Bench SR, et al. (2008) Lysogenic virus-host interactions predominate at deep-sea diffuse-flow hydrothermal vents. Isme J 2: 1112–1121.
45. Harmsen M, Lappann M, Knochel S, Molin S (2010) Role of extracellular DNA during biofilm formation by *Listeria monocytogenes*. Appl Environ Microbiol 76: 2271–2279.
46. Moscoso M, Garcia E, Lopez R (2009) Pneumococcal biofilms. Int Microbiol 12: 77–85.
47. Donlan RM (2009) Preventing biofilms of clinically relevant organisms using bacteriophage. Trends Microbiol 17: 66–72.
48. Lu TK, Collins JJ (2007) Dispersing biofilms with engineered enzymatic bacteriophage. Proc Natl Acad Sci U S A 104: 11197–11202.
49. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, et al. (1999) Current protocols in molecular biology. New York, NY: Wiley-Interscience.
50. Mann EE, Rice KC, Boles BR, Endres JL, Ranjit D, et al. (2009) Modulation of eDNA release and degradation affects *Staphylococcus aureus* biofilm maturation. PLoS One 4: e5822.

Chapter 3

BMC Microbiology

BioMed Central

Research article

Open Access

Pherotypes are driving genetic differentiation within *Streptococcus pneumoniae*

Margarida Carrolo†[1], Francisco R Pinto†[2], Jose Melo-Cristino[1] and Mario Ramirez*[1]

Address: [1]Instituto de Microbiologia, Instituto de Medicina Molecular, Faculdade de Medicina, Universidade de Lisboa, Av. Prof. Egas Moniz, 1649-028 Lisboa, Portugal and [2]Centro de Química e Bioquímica, Faculdade de Ciências, Universidade de Lisboa, Campo Grande, 1749-016 Lisboa, Portugal

Email: Margarida Carrolo - mcarrolo@fm.ul.pt; Francisco R Pinto - frpinto@fc.ul.pt; Jose Melo-Cristino - melo_cristino@fm.ul.pt; Mario Ramirez* - ramirez@fm.ul.pt

* Corresponding author †Equal contributors

Published: 7 September 2009

BMC Microbiology 2009, **9**:191 doi:10.1186/1471-2180-9-191

Received: 21 May 2009

Accepted: 7 September 2009

This article is available from: http://www.biomedcentral.com/1471-2180/9/191

Abstract

Background: The boundaries of bacterial species and the mechanisms underlying bacterial speciation are matters of intense debate. Theoretical studies have shown that recombination acts as a strong cohesive force preventing divergence in bacterial populations. *Streptococcus pneumoniae* populations have the telltale signs of high recombination with competence implicated as the major driving force behind gene exchange. Competence in *S. pneumoniae* is triggered by a quorum-sensing mechanism controlled by the competence-stimulating peptide pheromone.

Results: We studied the distribution of the two major pherotypes in the pneumococcal population and their association with serotype, antimicrobial resistance and genetic lineage. Using multilocus sequence data we evaluated pherotype influence on the dynamics of horizontal gene transfer. We show that pherotype is a clonal property of pneumococci. Standard population genetic analysis and multilocus infinite allele model simulations support the hypothesis that two genetically differentiated populations are defined by the major pherotypes.

Conclusion: Severe limitations to gene flow can therefore occur in bacterial species in the absence of geographical barriers and within highly recombinogenic populations. This departure from panmixia can have important consequences for our understanding of the response of pneumococci to human imposed selective pressures such as vaccination and antibiotic use.

Background

Horizontal gene transfer and recombination, although recognized as important mechanisms in the evolution of certain phenotypes such as penicillin resistance in both *Neisseria meningitidis* and *Streptococcus pneumoniae*, were considered to be rare [1,2]. Full genome sequences and extensive surveys of bacterial populations using multilocus sequence typing (MLST) have challenged this view and established the essential role of horizontal gene transfer and recombination in bacterial evolution, revealing the high frequency of these events [3,4].

Streptococcus pneumoniae (pneumococcus) is an important human pathogen, taxonomically recognized as a group within the pneumoniae-mitis-pseudopneumoniae cluster of the *Streptococcus* genus [5]. The capacity of pneumo-

cocci to undergo genetic transformation was recognized early in the study of this bacterium [6] and it was later found that competence presented the intriguing property of being tightly controlled at the population level [7]. Competence was thus one of the first examples of a multicellular bacterial response coordinated by a diffusible signal. These processes were later termed quorum-sensing and found to be used by both Gram positive and Gram negative bacteria to synchronize the switch of genetic programs simultaneously at the population level in order to achieve goals that are unattainable by single cells [8]. Several molecules are used by bacteria to regulate their quorum-sensing mechanisms, with modified or unmodified oligopeptides being used by Gram positive and Gram-negative bacteria [8]. In *S. pneumoniae*, a secreted unmodified 17-aminoacid peptide pheromone, termed the competence-stimulating peptide (CSP), is responsible for quorum-sensing [9]. The product of the *comC* gene is secreted and processed by an ABC transporter (ComAB) resulting in the accumulation of CSP in the medium. A two-component regulatory system consisting of a histidine kinase receptor (ComD) and its cognate response regulator (ComE) are then responsible for sensing the CSP concentration and triggering the competence response.

In pneumococci several distinct mature CSPs have been identified, although the vast majority of strains produce one of two variants: CSP-1 or CSP-2 (also designated CSP-α and CSP-β, respectively) [5,10-12]. Each different CSP variant identifies a pherotype and strains genetically carrying one of the variants are mostly unable to respond to the presence of the other signaling peptide, possibly due to specificity of the ComD receptors for their cognate CSP alleles [10,11].

Competent bacteria will recognize and bind naked double stranded DNA fragments present in their environment, and translocate these fragments in a single stranded form across the membrane and into the cytoplasm. A number of genes facilitating recombination of the incoming DNA with the bacterial chromosome are also upregulated at competence, favoring the integration of the foreign DNA fragment that may permanently change the cell genotype and phenotype [9]. Competent cells are also endowed with the capacity to kill non-competent pneumococci in a mechanism named fratricide [13,14] and this may be a key property for transformation *in vivo* by providing a source of free DNA.

Pneumococcal fratricide is committed by cells that are competent and thus able to lyse non-competent siblings [13,15-17] with the concomitant release of DNA that will become available for transformation. The existence of two predominant pherotypes in *S. pneumoniae* and the documented occurrence of co-colonization [18,19], led to the proposal of two contrasting models of the pherotype impact on genetic exchange [15]. In the first model, the lack of inter-pherotype communication prevents genetic exchange between phenotypes favoring genetic differentiation [20,21]. The second model is based on the proposal that the absence of inter-pherotype cross-activation would result in a race for competence activation with the winning phenotype inducing the lysis of cells belonging to the other pherotype [22]. The latter would result in a more frequent exchange of genetic information between different pherotype lineages that is assumed to result in enhanced genetic diversity of pneumococci.

The human host is the only natural ecological niche of all pneumococcal strains where they are exposed to the same environmental insults and share very similar lifestyles. We propose that limitations to lateral gene transfer, through a kind of "assortative mating" promoted by the existence of two pherotypes, is creating genetically differentiated subpopulations within *S. pneumoniae*.

Results and discussion

Pherotype distribution among the pneumococcal population

Traditionally, pneumococcal strains have been characterized by their capsular polysaccharide (serotype) of which pneumococci produce 91 chemically and immunologically distinct variants [23]. Although it has been shown that the serotype defines important epidemiological and virulence properties of pneumococcal isolates [24], it is also recognized that each serotype comprises different clones that may present different properties [25].

The collection of 483 invasive pneumococcal isolates was characterized for the *comC* allele (pherotype) carried by each isolate. All isolates could be classified either as CSP-1 or as CSP-2 and, in agreement with previous findings, most presented the CSP-1 pherotype (70.6%) [see Additional file 1 - Table S1]. The data was analyzed to determine if the pherotypes were randomly distributed among the population or if there were associations with particular characteristics of the isolates, namely serotype, antibiotic resistance and the genetic lineages identified by pulsed-field gel electrophoresis (PFGE) profiling and MLST.

As a first approximation we used the Wallace coefficient (W) [26,27]. W provides an estimate of the probability of two strains sharing the same pherotype if they share another characteristic such as serotype or being classified in the same PFGE cluster. Table 1 shows the W values obtained, indicating that isolates sharing the same serotype have a high probability of belonging to the same pherotype (W = 0.730) and this probability is higher if the

Table 1: Wallace's coefficients and respective confidence intervals testing the ability of several methods to predict the pherotype.

Parameter	W (95% CI)	W_i[a]
Serotype	0.730 (0.689;0.772)	0.584
PFGE cluster	0.771 (0.726;0.816)	0.584
Sequence type	0.982 (0.964;1)	0.621
Clonal complex	0.986 (0.961;0.992)	0.621

[a]W_i is the expected Wallace coefficient if the classification method is independent of the pherotype.

isolates belong to the same PFGE cluster (W = 0.771). Both values are significantly different from the expected values in case of a random association between pherotype and either of these two characteristics (W_i = 0.584), demonstrating that pherotypes are not randomly dispersed within the pneumococcal population.

To determine if individual serotypes and PFGE clusters were significantly enriched in isolates presenting each pherotype, odds ratios (OR) were calculated. A total of five serotypes are significantly associated with either one of the pherotypes (Table 2 and see Additional file 1 - Table S1). The high Wallace values suggest that pherotype/serotype association is not only due to these five serotypes. Many serotypes are present in insufficient numbers to reach a significant odds ratio. By simultaneously looking at each pair of strains the Wallace statistic has an increased power to detect associations. Serotypes 1 and 14 are strongly associated with CSP-1 whereas serotypes 3, 6A and 9N show an association with CSP-2. The same approach was used to determine if pherotypes were associated with particular PFGE clusters within each serotype, aiming to subdivide serotypes into closely related genetic lineages. Five PFGE clusters showed association with a particular pherotype [see Additional file 2 - Table S2]. Of these, the largest PFGE clusters within serotypes 1, 3, 9N and 14 maintained the same association found between these serotypes and pherotype. Possibly due to the small number of isolates in each PFGE cluster, none of the clusters expressing serotype 6A was significantly associated with either pherotype, in contrast with the association found between this serotype and CSP-2. On the other hand, serotype 4 presents one PFGE cluster that was significantly associated with CSP-2, whereas no association was found at the serotype level possibly as a consequence of the largest cluster of serotype 4 being mainly CSP-1 [see Additional file 2 - Table S2]. Taken together the data suggest that pherotype is a clonal property that may vary independently of the serotype.

Table 2: Odds ratios measuring significant associations between pherotype and serotype.

Serotype	CSP-1	CSP-2	OR (95%CI)[a]	FDR[b]
1	48	2	11.434 (2.923;98.526)	$< 10^{-4}$
3	23	23	0.375 (0.193;0.729)	0.017
6A	2	11	0.071 (0.007;0.330)	0.001
9N	2	8	0.099 (0.010;0.506)	0.013
14	61	4	7.497 (2.698;28.985)	$< 10^{-4}$

[a]Odds ratio (OR) describes the strength of the association between a pherotype and a particular serotype. In each case, if the OR is significantly > 1, CSP-1 is associated with the serotype and if OR is significantly < 1 means that the serotype is enriched in CSP-2 beyond what would be expected.
[b]Values obtained after false-discovery rate correction for multiple testing

MLST is a sequence based approach that uses the sequence of internal fragments of housekeeping genes for the purpose of characterizing, typing, and classifying members of bacterial populations. The data derived from MLST can also be used to study the population genetics of bacteria such as *Streptococcus pneumoniae* [28]. Applying eBURST to MLST data originates subnetworks of isolates with increased probability of sharing a recent common ancestor. These subnetworks define clonal complexes as groups of isolates that share the alleles at no less than six loci with at least another member of that group [29]. MLST from 90 selected strains [30] revealed 57 different sequence types grouped into 39 distinct clonal complexes. The ability of sequence type and clonal complex to predict the pherotype is remarkably high, both with W > 0.97 (Table 1). PFGE and MLST are widely used tools to define bacterial clones, the fact that the groups defined by both these methods show such strong correspondence with pherotype further strengthen the indication that pherotype is a clonal property within the pneumococcal population.

A consistent hypothesis with pherotype clonality is that the role of CSP in triggering competence and its consequences on lateral gene transfer is itself responsible for the distribution of the pherotypes in the pneumococcal population. If this hypothesis is correct and the pherotype is indeed restricting gene transfer within the pneumococcal population, genes that are under recent strong selective pressure and that are known to be horizontally transferred should be associated with pherotype.

Pherotype and antibiotic resistance

To test our hypothesis, we checked if there was an association between antibiotic resistance and pherotype. Resistance to several antibiotics in pneumococcus was shown to be mediated by the acquisition of foreign DNA that has subsequently spread within the pneumococcal population [31]. Emergence of resistance in pneumococci and its dissemination in the population is postulated to have occurred since their widespread use in clinical practice in the late 1940s. The results in Table 3 indicate that there was an association of most antibiotics (with the exception

Table 3: Association between antibiotic resistance and pherotype.

Antibiotic	CSP-1		CSP-2		OR (95% CI)[a]	FDR[b]
	Resistant	Susceptible	Resistant	Susceptible		
Penicillin[c, d]	92	249	21	121	2.13 (1.24;3.78)	0.012
Erythromycin	32	309	16	126	0.82 (0.42;1.65)	0.611
Clindamycin	22	319	16	126	0.54 (0.26;1.15)	0.141
Tetracycline[d]	18	323	20	122	0.31 (0.16;0.70)	0.010
Chloramphenicol[d]	5	336	9	133	0.22 (0.05;0.75)	0.013
Co-trimoxazole[d]	89	252	17	125	2.59 (1.45;4.86)	0.005
Cefuroxime[d]	68	272	12	129	2.68 (1.38;5.64)	0.010

[a] Odds ratio (OR) measures the strength of the association between a pherotype and resistance to a particular antibiotic. In each case, if OR is significantly > 1, CSP-1 is associated with resistance to that antibiotic and if OR is significantly < 1 this means that CSP-2 is associated with resistance to that particular antibiotic.
[b] Correction for multiple testing performed by the false discovery rate method (FDR)
[c] $p < 0.05$ after FDR correction.
[d] Both penicillin intermediate and fully resistant isolates were considered resistant for this analysis.

of erythromycin) with a particular pherotype. Isolates resistant to penicillin and other β-lactams were associated with CSP-1. It is known that resistance to β-lactams was acquired from closely related species of the mitis complex and that genes encoding resistance are transferred within the pneumococcal population by genetic recombination [31]. The fact that penicillin resistant isolates are more frequently CSP-1 suggests that, in addition to the expansion of resistant clones, current gene flow occurs primarily between isolates that share the same pherotype.

The relationship between pherotype and restriction/modification systems

Another important mechanism of lateral gene transfer is bacteriophage transduction [32]. This is an especially important mechanism for the transfer of large DNA fragments that may be restricted in transformation. This is for instance the case of the locus encoding the capsular polysaccharide biosynthesis machinery and of some of the genetic determinants of resistance to tetracycline, chloramphenicol or erythromycin, that are large composite transposons unable to transfer by conjugation, leaving phage transduction as the most likely mechanism of dissemination in the bacterial population, similarly to what was described in other streptococci [33].

Transduction should be independent of CSP activity, but the presence of restriction/modification (R/M) systems was shown to impair horizontal transfer through this mechanism [34]. Pneumococci are unusual in that they posses either one of two complementary R/M systems located in interchangeable genetic cassettes. Strains of *S. pneumoniae* contain either the *dpn*I cassete, containing an endonuclease that cleaves only the methylated DNA-sequence 5'GmeATC3' or the *dpn*II cassette, which includes an endonuclease that cleaves the same sequence when not methylated, together with the corresponding methylase. These mutually exclusive R/M systems were shown to protect against viral infection by viruses produced in cells of the opposite genotype, reducing infection frequency to $< 10^{-5}$ [35].

The R/M cassette has a size compatible with horizontal transfer by transformation, so we wondered if the distribution of the R/M cassettes could be correlated to the pherotype and thereby contribute to promote asymmetries of horizontal gene transfer within the pneumococcal population.

To pursue this hypothesis, the R/M cassette carried by pneumococcal isolates previously characterized by MLST was determined. The proportion of CSP-2 isolates with the *dpn*II cassette (3/23) is lower than the proportion of CSP-1 isolates with that same cassette (25/67) and the association between pherotype and the R/M system is significant ($p = 0.037$, Fisher exact test), suggesting that phage transduction may be indirectly arbitrated by the pherotype *via* the R/M systems, such that the spread of large genetic elements that rely on this mechanism of horizontal gene transfer could also be limited by pherotype.

Pherotype is a marker of population segregation

MLST data has been used to characterize the clonality of bacterial populations and to explore the impact of recombination and mutation in bacterial evolution [4]. For *S. pneumoniae* the recombination rate has been estimated to be 3-10 times the mutation rate per locus [28,36]. To test if the pherotype could be limiting the genetic exchanges within pneumococci, we took the simple approach of testing among all pairs of sequence types that diverge at the allele of a single locus (single-locus variants - SLV) and that should represent the initial stages of diversification dominated by recombination, if the allele that differed was more frequent among sequence types sharing the same pherotype or among isolates of a different pherotype. Considering the observed SLV pairs in our study, the

probability that the changing allele came from a different pherotype is 0.11. In a panmictic population, the expected probability would be 0.38 ($p < 10^{-4}$, permutation test), again suggesting that recombination between pherotypes is reduced.

To test if the populations defined by each pherotype showed genetic differentiation we analyzed the concatenated sequences of six of the genes used in MLST, excluding *ddl* since it was previously shown that this gene showed a hitchhiking effect with *pbp2b* involved in penicillin resistance[37] and could thus bias the results. Out of 143 mutations in 142 polymorphic sites, 66 were shared between the two pherotype defined populations, 63 were polymorphic in the CSP-1 population but monomorphic in the CSP-2 population and 14 were polymorphic in CSP-2 but monomorphic in CSP-1. To estimate the level of gene flow and whether pherotype defined diverging populations, the classic F_{ST} parameter [38], the K^*_{ST} statistic [39] and the more powerful nearest-neighbor statistic S_{nn} [40] were used. The F_{ST}, K^*_{ST} and S_{nn} statistics are measures of population differentiation based on the number of differences between haplotypes. The statistical significance of both the K^*_{ST} and S_{nn} statistics were evaluated by permutation. The data in Table 4 shows that statistically significant K^*_{ST} values ($p < 0.01$) were obtained not only for the analysis of the concatenated sequences but also for most of the individual genes. The more sensitive S_{nn} statistic presented significant values ($p < 0.01$) for the analysis of the concatenated sequence as well as for all individual genes.

A different approach to test if the pherotype is a marker of genetic isolation consists of calculating the probability that pairs of isolates with increasing levels of genetic divergence have of belonging to different pherotypes. Figure 1 shows that the closest pairs of isolates have a significantly lower probability of having different pherotypes. When genetic divergence increases, the probability of differing in pherotype also increases, reaching the levels expected by chance when isolates differ in more than three alleles. Again, these results show that isolates that are phylogenetically closely linked have an increased likelihood of sharing the same pherotype.

Table 4: Nucleotide variation and population differentiation parameters.

Alleles	π	F_{ST}	K^*_{ST}	p (K^*_{ST})[a]	S_{nn}	p (S_{nn})[a]
aroE	0.005	0.021	0.018	0.022	0.721	$< 10^{-4}$
gdh	0.009	0.025	0.008	0.115	0.706	0.004
gki	0.019	0.134	0.045	$< 10^{-4}$	0.810	$< 10^{-4}$
recP	0.005	0.072	0.039	0.001	0.717	$< 10^{-4}$
spi	0.009	0.190	0.062	$< 10^{-4}$	0.677	0.004
xpt	0.007	0.133	0.042	$< 10^{-4}$	0.790	$< 10^{-4}$
ddl	0.012	0.018	0.012	0.033	0.738	$< 10^{-4}$
Combined[b]	0.009	0.115	0.025	$< 10^{-4}$	0.833	$< 10^{-4}$

[a]Probabilities evaluated by 1,000 permutations.
[b]The results correspond to the analysis of the concatenated sequences of the *aroE*, *gdh*, *gki*, *recP*, *spi* and *xpt* alleles.

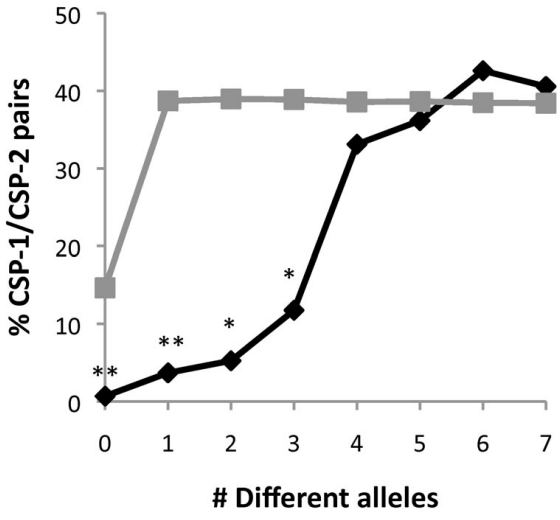

Figure 1
Probability of pairs of isolates with different alleles to belong to different pherotypes. The black line indicates the fraction of observed CSP-1/CSP-2 pairs differing at the indicated number of alleles and the grey line the expected number if there was a random association between pherotype and sequence type. As the allelic differences increase, the probability of diverging in pherotype also increases reaching levels undistinguishable from those expected by chance when strains differ in more than three alleles. One asterisk, $p < 0.01$ and two asterisks, $p < 0.001$.

Infinite allele model

The structured nature of the pneumococcal population and the geographically limited origin of our sample could explain, at least partially, the segregation of pherotypes seen in Figure 1 and the high Wallace indices of Table 1. To address this issue a MLST infinite allele model was used to test the effect of restricting or promoting recombination between the two pherotype defined subpopulations. This modeling approach was previously shown to reproduce the clonal structure of the pneumococcal population [36,41] and provides a possibly more realistic null hypothesis for the distribution of phenotypes in the population. The model was expanded to include a new locus with two possible alleles: CSP-1 and CSP-2. This extra locus recombines with the same rate as the MLST loci and the frequency of each allele is kept constant and equal to 70 and 30% of CSP-1 and CSP-2 respectively, corresponding to the observed values in natural populations. Addi-

tionally, a new parameter IPR was introduced, that controls the probability of inter-pherotype recombination. If pherotype differences would not prevent or promote recombination, the observed frequencies of each pherotype in the population would lead to a probability of inter-pherotype recombination of 0.42. Figure 2A shows that even in the absence of a pherotype effect on recombination, high Wallace values of clonal complex predicting pherotype are expected. This result is intuitive since the recent common ancestry of strains belonging to the same clonal complex would also cause them to share the same pherotype. Still, there is a marked shift to higher Wallace values when the probability of inter-pherotype recombination decreases (IPR = 0.1 in Figure 2A). On the other hand, if genetic exchange between pherotypes is favored, in spite of their different prevalence in the population (IPR = 0.9 in Figure 2A), a shift towards lower $W_{CC \rightarrow ST}$ values is observed. When systematically varying IPR and computing the probability density for the observed Wallace coefficients (Figure 2B), one concludes that a value of 0.2 is 2-3 times more likely to explain the observed values than an IPR of 0.42, expected in case of no CSP effect in recombination. Since the more probable IPR is lower than expected if the two pherotype populations were recombining freely, these results strengthen the proposal that recombination is promoted within individuals sharing the same pherotype, promoting the divergence of two subpopulations of *S. pneumoniae*.

Conclusion

In agreement with previous suggestions [14,20,21], we propose that the specific ComC/ComD match facilitates a form of assortative genetic exchange, which could maintain genetically diverse subpopulations within this species. Although recent studies addressing the phenomenon of fratricide in pneumococci favor the hypothesis of preferential inter-pherotype genetic exchange [42], the data presented here argues that in natural populations intra-pherotype exchanges prevail, creating a barrier to gene exchange. In vitro studies that led to the fratricide hypothesis show that if two pneumococcal strains with different pherotypes are grown together, the one that becomes competent earlier will have a greater probability of being transformed with DNA from the other strain [42]. In order to observe the impact of this admixture promoting event in pneumococcal natural populations, frequent and adequate co-colonization events involving different pherotypes must occur. On the other hand, fratricide has also been observed in experiments with a single strain [13]. Dynamic bi-stable regulatory systems, as described for

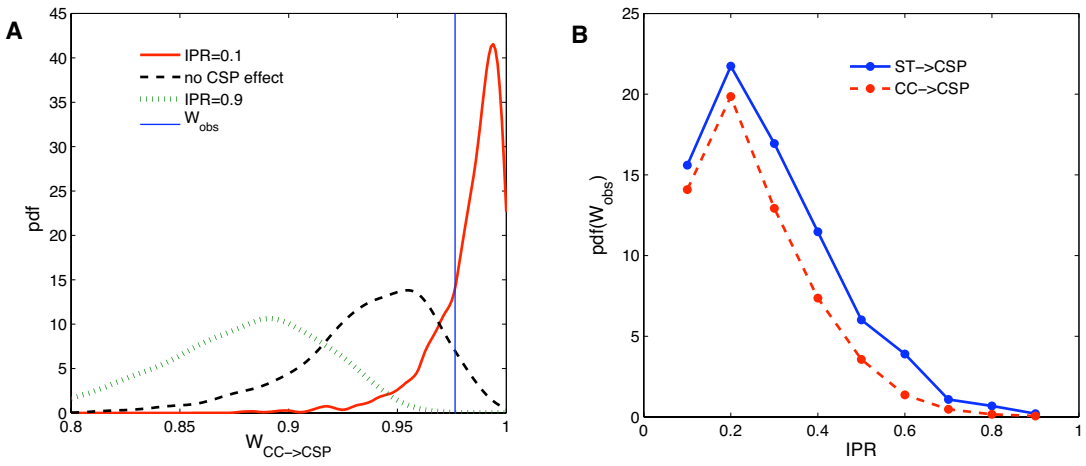

Figure 2

Probability density function of Wallace values for simulated populations. Multilocus sequence types of a pneumococcal population were generated with an adapted infinite allele model [36]. It includes an additional locus for CSP type and a new parameter IPR that, given a recombination event, defines the probability that the two recombining strains have different pherotypes. The prevalence of each pherotype in the population was fixed during the simulation at 70% for CSP-1 and 30% for CSP-2. (A) From 1,000 simulations, the probability density functions of Wallace values for Clonal Complex predicting pherotype were computed for three scenarios: (1) pherotype is a barrier to recombination (IPR = 0.1, red line), (2) pherotype has no impact in gene exchange (equivalent to IPR = 0.42, black dashed line) and (3) inter-pherotype recombination is favored (IPR = 0.9, green dotted line). The vertical blue line indicates the observed Wallace value in the studied sample. (B) To identify the value of the IPR parameter that is in best agreement with the data, the probability density at the observed Wallace values was computed for simulated populations with varying inter-pherotype recombination probabilities (IPR from 0.1 to 0.9), both for Wallace indexes of sequence type (blue line) and of clonal complex (red line) predicting pherotype.

Bacillus subtilis [43], may underlie the mechanism leading to the simultaneous presence of competent and non-competent cells of the same strain or the same pherotype. If natural co-colonization by strains of different pherotypes is rare or inadequate to promote gene exchange, it is possible to reconcile the inter-pherotype fratricide observations with the pherotype defined genetic differentiation identified here. The observed genetic barrier would then be justified if co-colonization events involving different strains of the same pherotype are more frequent or more adequate for recombination, leaving intra-pherotype fratricide and genetic exchange as the most common event in natural populations.

All the isolates analyzed were recovered in Portugal from invasive infections and it is therefore unlikely that geographic or ecological fragmentation could explain the pattern observed. The model simulations also exclude the possibility that our observation results simply from the structure of the pneumococcal population, with multiple isolates sharing the same genotype or with a recent common ancestry. It would also be plausible to assume that the CSP-2 population was recently established by introduction of a novel pherotype into pneumococci. This would result in the genome wide differentiation observed, but if it had occurred recently it would also cause lower haplotype diversity in the CSP-2 population, that the data does not support. Furthermore, the CSP-2 pherotype was found in multiple serotypes and clones, including strains differing in the alleles of up to five of the seven genes used in the pneumococcal MLST scheme. These observations support an ancient origin of the CSP-2 pherotype that would have allowed sufficient time for the coalescence of the two pherotype defined populations due to the high recombination of pneumococci.

Although only invasive strains were used in the present study, a comparison of previous studies [30,44] indicates that clones found causing invasive infections are also found among the most prevalent in carriage, meaning that the results described here are also expected to be valid for the overall pneumococcal population in Portugal.

The concept of allopatric speciation follows the intuitive rationale that genetic divergence subsequent to geographic isolation could lead to the emergence of different species [45]. In bacteria, this has been connected with the concept of ecotypes [46], arising as a consequence of a single clone expanding into a new niche. These events have been implicated in the emergence of human pathogens from environmental or commensal species, such as the rise of *Yersinia pestis* or *Mycobacterium tuberculosis* from within the *Yersinia* and mycobacteria respectively [47]. But genetic differentiation in microorganisms was also shown to occur mainly as a result of geographic barriers, such as that of the wild yeast *Saccharomyces paradoxus* [48].

In the absence of ecological isolation, a process of sympatric speciation, shown to occur in sexual eukaryotes [45], is deemed unlikely in bacteria due to the occurrence of recombination. In fact, theoretical studies have shown that if recombination is more frequent than mutation, the "cohesive force of recombination" is an effective barrier to divergence and to bacterial speciation [49,50]. This received further support from the recent observation of an accelerated convergence of species within the *Campylobacter* genus proposed to be caused by the breakdown of ecological or geographical barriers and the effect of recombination [51].

Pneumococci are generally considered a sexual population due to the dominant role of recombination in the evolution of this species [49]. It was therefore surprising to find that two genetically distinct subpopulations could be identified. Extensive sequence divergence, previously shown to be a major barrier to gene exchange [52], could not be implicated as attested by the low π values and the fact that 66 out of the 143 mutations were shared between the two pherotype populations. Interestingly, the existence of three differentiated subpopulations within pneumococci, with different rates of admixture, was recently inferred using a Bayesian method of population analysis [53], but no explanation for this differentiation was presented.

We propose that "assortative mating" mediated by different pherotypes and ongoing genetic drift may be driving an incipient speciation process within *S. pneumoniae*. Our data support theoretical predictions that the existence of barriers to recombination allow the accumulation of significant genetic drift, even within highly recombinogenic bacterial species. An understanding of these mechanisms and their consequences offer further insights into the evolution of bacterial pathogens and may allow more informed predictions on the consequences of human interventions such as antibiotic use and vaccination on bacterial populations.

Addendum in proof

We recently became aware of a study (Omar Cornejo, personal communication) that has addressed the same issue discussed here. In contrast to our findings, the authors failed to detect any differentiation between the two pherotype defined populations. The reasons behind this discrepancy of results is not clear and further studies are needed to reconcile these apparently contradictory findings.

Methods

Bacterial strains, growth conditions, PFGE and MLST

A collection of 483 invasive pneumococcal isolates recovered during the period of 1999 to 2002 in Portugal were obtained from the Faculdade de Medicina de Lisboa collection. The serotype, PFGE type, MLST characterization and antibiotic susceptibility of these strains were collected from previous studies[25,30,54]. Briefly, all *S. pneumoniae* strains were grown in a casein-based semi-synthetic medium (C+Y) at 37°C without aeration or in tryptic soy agar (TSA) (Oxoid, Hampshire, England) supplemented with 5% (v/v) sterile sheep blood incubated at 37°C in 5% CO_2. Antimicrobial susceptibility, serotyping and PFGE analysis was performed for all isolates. MLST analysis was performed for at least one isolate in each major PFGE cluster (n = 90) and revealed 57 different sequence types (ST) corresponding to 39 different lineages by eBURST analysis.

Detection of the pherotype and endonuclease restriction phenotype by PCR

CSP-1 and CSP-2 gene fragments were amplified using multiplex PCR with primers CSP_up (5'-TGA AAA ACA CAG TTA AAT TGG AAC-3'), CSP1_dn (5'-TCA AGA AAG GAT AAA GGT AGT CCT C-3') and CSP2 _dn (5'-TAA AAA TCT TTC AAT CCC TAT TT-3'), which allowed the amplification of fragments of 620 bp for the CSP-1 allele and 340 bp for the CSP-2 allele. *dpn*I and *dpn*II genotype was also detected by multiplex PCR with primers DpnI_up (5'-GAA GTA GGA GAT AAA TTG CCA GAG), DpnII_up (5'-TAC GAA TGA TGG GAA TAC TGT G-3') and Dpn_dn (5'-TGT CCT CAA TGC CGT ATT AAA TC-3'), with the expected products of 342 bp and 421 bp for *dpn*I and *dpn*II, respectively. Template DNA was prepared by diluting 9 μl of an overnight culture in 441 μl of water and boiling this mixture for 2 minutes. The PCR reactions were performed in 50 μl of final volume containing 20 μl of template solution, 1× reaction buffer (Biotools, Madrid, Spain), 10 mM dNTPs (Fermentas, Vilnius, Lithuania), 20 pmol of each of the primers and 1.25 U GoTaq Polymerase (Invitrogen, Carlsbad, California). The same PCR program was used consisting of 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 sec, and primer extension at 72°C for 1 min. Followed by 10 min incubation at 72°C to complete extension.

Data analysis

Statistical association between serotypes, PFGE clusters, antimicrobial resistance or endonuclease restriction phenotype and pherotype where characterized by odds ratios (OR) with 95% confidence intervals (CI) computed through the Fisher method implemented in the epitools package for the R language. OR significance was evaluated with the Fisher exact test. The resulting p-values were corrected for multiple testing by controlling the False Discovery Rate (FDR) under or equal to 0.05 through the linear procedure of Benjamini and Hochberg [55].

Wallace coefficients (W) and respective 95% confidence intervals were computed as previously described [26,27].

The relationship between cross-pherotype pair frequency and the number of divergent alleles between STs was validated for statistical significance by permutation tests. The latter consisted in repeating the computation of frequencies of cross-pherotype strain pairs for 1,000 times, randomly shuffling the pherotype assignment of the strains before each repetition. The p-values were obtained from the fraction of the 1,000 random runs where the cross-pherotype pair frequency was lower than the respective values with the correct pherotype assignment. A permutation test was also performed to evaluate the significance of the probability that a divergent allele in an SLV pair was donated from a strain with a different pherotype. In this case, in each of the 1,000 runs, the divergent allele was randomly sampled from the corresponding locus in the collection of STs. The determination of π, F_{ST}, K^*_{ST} and S_{nn} for the analysis of sequence data was done using the DNASP v4.50.3 program. The values of K^*_{ST} and S_{nn} were used to assess population differentiation in combination with permutation tests (1,000 permutations).

Neutral Multilocus Infinite Allele Model

The model presented by Fraser et al. [36] was expanded to include an additional CSP locus and a new IPR parameter. The CSP locus has only two possible alleles, CSP-1 and CSP-2 that can interchange by recombination but are not affected by mutations. The parameter IPR defines the inter-pherotype recombination probability. The model was simulated with the parameter values determined in [36] for the pneumococcal population. Namely, the population size was 1,000, the population mutation and recombination rates were 5.3 and 17.3, respectively. All the analyses were repeated with a population recombination rate reduced in 50% and the results were qualitatively similar. All simulations were run for 1,000 generations, after which the sequence type diversity was stable, as measured by the Simpson's index of diversity [56]. At each generation, 70% of the selected individuals were CSP-1 and 30% were CSP-2. For each value of parameter IPR, 1,000 independent simulations were carried out. Wallace coefficients for ST and CC predicting CSP type were calculated for each of the final 1,000 populations. Probability density functions for the Wallace distributions were determined by kernel density estimation with a Gaussian kernel function. All simulations and computations were done in Matlab version 7.7.

Authors' contributions

MC, FRP, JMC and MR designed research; MC performed research; FRP and MR analyzed data; MC, FRP, JMC and

MR wrote the paper. All authors read and approved the final manuscript.

Additional material

Additional file 1

Table S1 - Pherotype distribution arranged by serotype in the pneumococcal collection. *Odds ratios (OR) represent the strength of the association between a pherotype and a particular serotype. In each case, if the OR is significantly > 1, CSP-1 is associated with the serotype and if OR is significantly < 1 means that the serotype is enriched in CSP-2.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-191-S1.pdf]

Additional file 2

Table S2 - Pherotype distribution arranged by PFGE cluster in the pneumococcal collection. *Odds ratios (OR) represent the strength of the association between a pherotype and a particular PFGE cluster. In each case, if the OR is significantly > 1, CSP-1 is associated with the PFGE cluster and if OR is significantly < 1 means that the PFGE cluster is enriched in CSP-2.*
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2180-9-191-S2.pdf]

Acknowledgements

This work was partially supported by Fundação para a Ciência e Tecnologia, Portugal (PTDC/SAU-ESA/64888/2006, PTDC/SAU-ESA/71499/2006 and PIC/IC/83065/2007), Fundação Calouste Gulbenkian, the European Union (CAREPNEUMO - Combating antibiotic resistance pneumococci by novel strategies based on in vivo and in vitro host-pathogen interactions, FP7-HEALTH-2007-223111) and an unrestricted grant from Glaxo Smithkline Portugal. MC is supported by a research grant from Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/35854/2007).

References

1. Maynard Smith J, Dowson CG, Spratt BG: **Localized sex in bacteria.** *Nature* 1991, **349:**29-31.
2. Maynard Smith J: **The role of sex in bacterial evolution.** *J Hered* 1993, **84:**326-327.
3. Gogarten JP, Doolittle WF, Lawrence JG: **Prokaryotic evolution in light of gene transfer.** *Mol Biol Evol* 2002, **19:**2226-2238.
4. Feil EJ: **Small change: keeping pace with microevolution.** *Nat Rev Microbiol* 2004, **2:**483-495.
5. Kilian M, Poulsen K, Blomqvist T, Havarstein LS, Bek-Thomsen M, Tettelin H, Sorensen UB: **Evolution of *Streptococcus pneumoniae* and its close commensal relatives.** *PLoS ONE* 2008, **3:**e2683.
6. Griffith F: **The significance of pneumococcal types.** *The Journal of Hygiene* 1928, **27:**113-159.
7. Tomasz A: **Control of the competent state in pneumococcus by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria.** *Nature* 1965, **208:**155-159.
8. Waters CM, Bassler BL: **Quorum sensing: cell-to-cell communication in bacteria.** *Annu Rev Cell Dev Biol* 2005, **21:**319-346.
9. Claverys JP, Havarstein LS: **Extracellular-peptide control of competence for genetic transformation in *Streptococcus pneumoniae*.** *Front Biosci* 2002, **7:**d1798-1814.
10. Pozzi G, Masala L, Iannelli F, Manganelli R, Havarstein LS, Piccoli L, Simon D, Morrison DA: **Competence for genetic transformation in encapsulated strains of *Streptococcus pneumoniae*: two allelic variants of the peptide pheromone.** *J Bacteriol* 1996, **178:**6087-6090.
11. Ramirez M, Morrison DA, Tomasz A: **Ubiquitous distribution of the competence related genes *comA* and *comC* among isolates of *Streptococcus pneumoniae*.** *Microb Drug Resist* 1997, **3:**39-52.
12. Whatmore AM, Barcus VA, Dowson CG: **Genetic diversity of the streptococcal competence (*com*) gene locus.** *J Bacteriol* 1999, **181:**3144-3154.
13. Guiral S, Mitchell TJ, Martin B, Claverys JP: **Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements.** *Proc Natl Acad Sci USA* 2005, **102:**8710-8715.
14. Claverys JP, Martin B, Havarstein LS: **Competence-induced fratricide in streptococci.** *Mol Microbiol* 2007, **64:**1423-1433.
15. Claverys JP, Havarstein LS: **Cannibalism and fratricide: mechanisms and raisons d'être.** *Nat Rev Microbiol* 2007, **5:**219-229.
16. Gilmore MS, Haas W: **The selective advantage of microbial fratricide.** *Proc Natl Acad Sci USA* 2005, **102:**8401-8402.
17. Havarstein LS, Martin B, Johnsborg O, Granadel C, Claverys JP: **New insights into the pneumococcal fratricide: relationship to clumping and identification of a novel immunity factor.** *Mol Microbiol* 2006, **59:**1297-1307.
18. Gray BM, Converse GM 3rd, Dillon HC Jr: **Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life.** *J Infect Dis* 1980, **142:**923-933.
19. Brugger SD, Hathaway LJ, Muhlemann K: **Detection of *Streptococcus pneumoniae* strain cocolonization in the nasopharynx.** *J Clin Microbiol* 2009, **47:**1750-1756.
20. Havarstein LS, Hakenbeck R, Gaustad P: **Natural competence in the genus *Streptococcus*: evidence that streptococci can change pherotype by interspecies recombinational exchanges.** *J Bacteriol* 1997, **179:**6589-6594.
21. Tortosa P, Dubnau D: **Competence for transformation: a matter of taste.** *Curr Opin Microbiol* 1999, **2:**588-592.
22. Claverys JP, Prudhomme M, Martin B: **Induction of competence regulons as a general response to stress in Gram-positive bacteria.** *Annu Rev Microbiol* 2006, **60:**451-475.
23. Park IH, Pritchard DG, Cartee R, Brandao A, Brandileone MC, Nahm MH: **Discovery of a new capsular serotype (6C) within serogroup 6 of *Streptococcus pneumoniae*.** *J Clin Microbiol* 2007, **45:**1225-1233.
24. Hausdorff WP, Feikin DR, Klugman KP: **Epidemiological differences among pneumococcal serotypes.** *Lancet Infect Dis* 2005, **5:**83-93.
25. Aguiar SI, Serrano I, Pinto FR, Melo-Cristino J, Ramirez M: **The presence of the pilus locus is a clonal property among pneumococcal invasive isolates.** *BMC Microbiol* 2008, **8:**41.
26. Pinto FR, Melo-Cristino J, Ramirez M: **A confidence interval for the wallace coefficient of concordance and its application to microbial typing methods.** *PLoS ONE* 2008, **3:**e3696.
27. Carrico JA, Silva-Costa C, Melo-Cristino J, Pinto FR, de Lencastre H, Almeida JS, Ramirez M: **Illustration of a common framework for relating multiple typing methods by application to macrolide-resistant *Streptococcus pyogenes*.** *J Clin Microbiol* 2006, **44:**2524-2532.
28. Feil EJ, Enright MC, Spratt BG: **Estimating the relative contributions of mutation and recombination to clonal diversification: a comparison between *Neisseria meningitidis* and *Streptococcus pneumoniae*.** *Res Microbiol* 2000, **151:**465-469.
29. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG: **eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data.** *J Bacteriol* 2004, **186:**1518-1530.
30. Serrano I, Melo-Cristino J, Carrico JA, Ramirez M: **Characterization of the genetic lineages responsible for pneumococcal invasive disease in Portugal.** *J Clin Microbiol* 2005, **43:**1706-1715.
31. Bergmann C, Chi F, Rachid S, Hakenbeck R: **Mechanisms for penicillin resistance in *Streptococcus pneumoniae*: penicillin binding proteins, gene transfer and cell wall metabolism.** In *The pneumococcus* Edited by: Toumanen EI, Mitchell TJ, Morrison DA, Spratt BG. Washington, D.C.: ASM Press; 2004:339-349.
32. Canchaya C, Fournous G, Chibani-Chennoufi S, Dillmann ML, Brussow H: **Phage as agents of lateral gene transfer.** *Curr Opin Microbiol* 2003, **6:**417-424.
33. Ubukata K, Konno M, Fujii R: **Transduction of drug resistance to tetracycline, chloramphenicol, macrolides, lincomycin and**

clindamycin with phages induced from *Streptococcus pyogenes*. *J Antibiot (Tokyo)* 1975, **28:**681-688.
34. Jeltsch A: **Maintenance of species identity and controlling speciation of bacteria: a new function for restriction/modification systems?** *Gene* 2003, **317:**13-16.
35. Lacks SA, Mannarelli BM, Springhorn SS, Greenberg B: **Genetic basis of the complementary DpnI and DpnII restriction systems of S. pneumoniae: an intercellular cassette mechanism.** *Cell* 1986, **46:**993-1000.
36. Fraser C, Hanage WP, Spratt BG: **Neutral microepidemic evolution of bacterial pathogens.** *Proc Natl Acad Sci USA* 2005, **102:**1968-1973.
37. Enright MC, Spratt BG: **Extensive variation in the ddl gene of penicillin-resistant *Streptococcus pneumoniae* results from a hitchhiking effect driven by the penicillin-binding protein 2b gene.** *Mol Biol Evol* 1999, **16:**1687-1695.
38. Hudson RR, Slatkin M, Maddison WP: **Estimation of levels of gene flow from DNA sequence data.** *Genetics* 1992, **132:**583-589.
39. Hudson RR, Boos DD, Kaplan NL: **A statistical test for detecting geographic subdivision.** *Mol Biol Evol* 1992, **9:**138-151.
40. Hudson RR: **A new statistic for detecting genetic differentiation.** *Genetics* 2000, **155:**2011-2014.
41. Turner KM, Hanage WP, Fraser C, Connor TR, Spratt BG: **Assessing the reliability of eBURST using simulated populations with known ancestry.** *BMC Microbiol* 2007, **7:**30.
42. Johnsborg O, Eldholm V, Bjornstad ML, Havarstein LS: **A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species.** *Mol Microbiol* 2008, **69:**245-253.
43. Dubnau D, Losick R: **Bistability in bacteria.** *Mol Microbiol* 2006, **61:**564-572.
44. Nunes S, Sa-Leao R, Carrico J, Alves CR, Mato R, Avo AB, Saldanha J, Almeida JS, Sanches IS, de Lencastre H: **Trends in drug resistance, serotypes, and molecular types of *Streptococcus pneumoniae* colonizing preschool-age children attending day care centers in Lisbon, Portugal: a summary of 4 years of annual surveillance.** *J Clin Microbiol* 2005, **43:**1285-1293.
45. Savolainen V, Anstett MC, Lexer C, Hutton I, Clarkson JJ, Norup MV, Powell MP, Springate D, Salamin N, Baker WJ: **Sympatric speciation in palms on an oceanic island.** *Nature* 2006, **441:**210-213.
46. Cohan FM: **What are bacterial species?** *Annu Rev Microbiol* 2002, **56:**457-487.
47. Feil EJ, Spratt BG: **Recombination and the population structures of bacterial pathogens.** *Annu Rev Microbiol* 2001, **55:**561-590.
48. Koufopanou V, Hughes J, Bell G, Burt A: **The spatial scale of genetic differentiation in a model organism: the wild yeast *Saccharomyces paradoxus*.** *Philos Trans R Soc Lond B Biol Sci* 2006, **361:**1941-1946.
49. Fraser C, Hanage WP, Spratt BG: **Recombination and the nature of bacterial speciation.** *Science* 2007, **315:**476-480.
50. Hanage WP, Spratt BG, Turner KM, Fraser C: **Modelling bacterial speciation.** *Philos Trans R Soc Lond B Biol Sci* 2006, **361:**2039-2044.
51. Sheppard SK, McCarthy ND, Falush D, Maiden MC: **Convergence of *Campylobacter* species: implications for bacterial evolution.** *Science* 2008, **320:**237-239.
52. Majewski J: **Sexual isolation in bacteria.** *FEMS Microbiol Lett* 2001, **199:**161-169.
53. Hanage WP, Fraser C, Tang J, Connor TR, Corander J: **Hyper-recombination, diversity, and antibiotic resistance in pneumococcus.** *Science* 2009, **324:**1454-1457.
54. Serrano I, Ramirez M, Melo-Cristino J: **Invasive *Streptococcus pneumoniae* from Portugal: implications for vaccination and antimicrobial therapy.** *Clin Microbiol Infect* 2004, **10:**652-656.
55. Benjamini Y, Hochberg Y: **Controlling the false discovery rate - a practical and powerful approach to multiple testing.** *J R Stat Soc Ser B Statistical Methodology* 1995, **57:**289-300.
56. Simpson EH: **Measurement of diversity.** *Nature* 1949, **163:**668.

Chapter 4

Pherotype influences biofilm growth and recombination in *Streptococcus pneumoniae*

Running title: Impact of competence stimulating peptide on biofilm

Abstract: In Streptococcus pneumoniae the competence-stimulating peptide (CSP), encoded by the comC gene, controls competence development and influences biofilm growth. In the present study, we explored the influence of pherotype, defined by the two major comC allelic variants (comC1 and comC2), on biofilm development and recombination efficiency. Isolates recovered from infections and presenting comC1 show a higher capacity to form biofilms. The influence of pherotype on biofilm growth was confirmed by experiments with isogenic strains differing in their comC alleles. Biofilm architecture evaluated by confocal laser scanning microscopy showed that strains carrying comC1 form biofilms that are denser and 35% thicker than those carrying the comC2 allele. Evaluation of competence in liquid culture and in biofilms of isogenic strains of different pherotypes showed that strains carrying the comC1 allele yielded more transformants than those carrying the comC2 allele. In contrast to mixed planktonic growth, within mixed biofilms inter-pherotype genetic exchange is less frequent than that occurring between bacteria of the same pherotype. Since biofilms are a major bacterial lifestyle, these observations may explain the genetic differentiation between populations with different pherotypes reported previously. Considering that biofilms have been associated with colonization our results suggest that strains carrying the comC1 allele may be more transmissible and more efficient at persisting in carriage. Both effects may help explain the higher prevalence of the comC1 allele in the pneumococcal population.

1.1. Introduction

Although planktonic bacteria are the most frequently studied microbial lifestyle in vitro, it is comparatively rare in vivo, where most prokaryotes grow in matrix-enclosed biofilms. Life within a biofilm matrix, wherein oxygen is limited and the metabolic rate is altered, results in several benefits, including protection from environmental threats such as host immune defenses, antibiotics, and surfactants. Thus, bacteria in biofilms are characteristically highly resistant to immune-mediated clearance (3, 28, 51) and, in some studies, these biological structures have been proposed as the way bacteria organize themselves when colonizing asymptomatically their human hosts (23, 32, 41).

Bacteria are known to communicate with each other within biofilms in order to set up mutually beneficial associations and coordinate protection measures. Communication flows through quorum sensing system (QS), where diffusible chemical signals (autoinducers) interact with specific receptors on the surface or in the cytoplasm of bacteria, regulating the expression of specific target genes (1, 20).

Induction of the competent state in streptococci belonging to the mitis phylogenetic group is controlled by the extracellular concentration of a secreted peptide pheromone, called the competence-stimulating peptide (CSP) the processed product of the *comC* gene (17, 48, 49). In several streptococci, the functioning of the QS system depends on the products of two distinct genetic loci, *comAB* and *comCDE* (17, 22). The operon *comAB* encodes the secretion apparatus necessary for CSP maturation and export whereas the *comCDE* operon encodes the immature signal (ComC) and the necessary sensing machinery. The signaling cascade, which leads to the induction of competence, begins with the interaction of CSP with its cognate receptor, ComD. When CSP reaches an extracellular concentration of 1-10 ng/ml, as a result of a basal expression of both operons and corresponding to a cell density of about 10^7 cells/ml (17), it binds to the membrane-bound histidine kinase receptor ComD, causing its autophosphorilation. Phosphorylated ComD transfers the phosphate group to an intracellular response regulator ComE, which then upregulates the transcription of the *comAB* and

comCDE operons. Phosphorylated ComE also drives the expression of *comX* that encodes an alternative sigma factor, so that in response to the signal-peptide, not only more CSP is produced but a relatively large number of genes are induced (aprox. 6% of total genome) (36). Only a small part of these genes have direct roles in DNA uptake, suggesting that the QS system of *S. pneumoniae* plays a larger role in the biology of this species. A set of genes induced at competence functions to trigger the lysis of a fraction of the bacteria within a population (8, 30, 43, 44). This process of "fratricide" has been postulated to be an important source of DNA for competent cells.

Several allelic variants of the *comC* gene have been identified in *S. pneumoniae*, although the vast majority of isolates carries one of two variants: *comC1* or *comC2* resulting in the production of two distinct peptides CSP1 and CSP2 (38, 39). Each different CSP variant identifies a pherotype and strains genetically carrying one of the variants are mostly unable to respond to the presence of other signaling peptide, possibly due to receptor specificity (38, 39).

The existence of two predominant pherotypes in *S. pneumoniae* and the documented occurrence of co-colonization (5, 14), led to the proposal of two contrasting models regarding the pherotype impact on genetic exchange (8). In the first model, absence of inter-pherotype cross-activation favors the induced lysis of bacteria belonging to the other pherotype (10). Consequently, DNA of bacteria not sharing the same pherotype would be released and become available for transformation, resulting in higher genetic diversity within the pneumococcal population (11). The second model is based on the premise that the lack of inter-pherotype communication prevents genetic exchange and favors differentiation (18, 50). Recently, we have reported that the two predominant pherotypes define genetically differentiated subpopulations of pneumococci consistent with the existence of limitations to gene flow between different pherotypes. We proposed that this "assortative mating" mediated by the pherotypes may be driving an incipient speciation process within *S. pneumoniae* (7).

In streptococci, several reports have shown a link between CSP production and biofilm formation (25-27, 35, 45). In *S. gordonii* a mutant where *comD* was

inactivated presented impaired biofilm growth (27). Further evidence comes from a report in *S.mutans* where the inactivation of any component of the *comCDE* operon resulted in a phenotype of defective competence and biofilm formation (25, 26). An alternative approach showed that the addition of synthetic CSP favored the biofilm mode of growth of *S.intermedius* without affecting the rate of culture growth (35). A previous study in *S. pneumoniae* supports the link between competence and biofilm formation showing that CSP receptor mutants are incapable of forming biofilms (33). We have recently shown that spontaneous phage-mediated lysis, through the release of eDNA, promotes pneumococcal biofilm formation (6). This observation raises the possibility that competence induced fratricide may also be an important mechanism contributing to the accumulation of this important biofilm matrix component.

Given the described links between CSP signalling, biofilm growth and genetic recombination, we pursued the hypothesis that different pherotypes could vary in their capacity for biofilm growth, and that these differences could influence the recombination efficiency between strains with different pherotypes. Oggioni and colleagues had already reported some preliminary data showing that strains carrying the *comC1* allele had higher numbers of cells attached to a plastic surface (33). Here we show that among strains recovered from human infections, those carrying the *comC1* allele are better biofilm producers an observation that was corroborated with isogenic strains differing only in their pherotypes. In contrast to what happens in planktonic growth, when strains of different pherotypes are grown together in biofilms, less genetic exchange occurs than when the biofilm is formed by cells sharing the same pherotype. These observations have important consequences for the population biology of *S. pneumoniae*.

1.2. Materials and Methods

1.2.1. Bacterial strains and growth conditions

The 90 invasive pneumococcal isolates were recovered in Portugal during the period of 1999 to 2002 and are part of the Faculdade de Medicina de Lisboa collection. Bacterial strains R36A and TIGR4 were obtained from the Rockefeller

University collection (A. Tomasz). All *S. pneumoniae* strains were grown in a casein-based semi-synthetic medium (C+Y) at 37ºC without aeration (24) or in tryptic soy agar (TSA) (Oxoid, Hampshire, England) supplemented with 5% (v/v) sterile sheep blood incubated at 37ºC in 5% CO_2.

1.2.2. Liquid medium growth curves

An inoculum of 50 µl of rapidly thawed bacteria was added to a glass tube with C+Y to reach a total volume of 5 ml. Bacteria were incubated at 37ºC for 340 minutes and growth was monitored by OD_{600nm} every 30 minutes. For each strain, 3 replicates of 3 independent experiments were obtained.

1.2.3. Pherotype detection by PCR

The *comC1* (CSP1) and *comC2* (CSP2) gene fragments were amplified as previously described (7). Briefly, a multiplex PCR with primers CSP_up (5'-TGA AAA ACA CAG TTA AAT TGG AAC-3'), CSP1_dn (5'-TCA AGA AAG GAT AAA GGT AGT CCT C-3') and CSP2 _dn (5'-TAA AAA TCT TTC AAT CCC TAT TT-3') was used to amplify fragments of 620 bp for the *comC1* allele and 340 bp for the *comC2* allele. Template DNA was prepared by diluting 9 µl of an overnight culture in 441 µl of water and boiling this mixture for 2 minutes. The multiplex PCR reactions were performed in 50 µl of final volume containing 20 µl of template solution, 1× reaction buffer (Biotools, Madrid, Spain), 10 mM dNTPs (Fermentas, Vilnius, Lithuania), 20 pmol of each of the primers and 1.25 U GoTaq Polymerase (Invitrogen, Carlsbad, California). The PCR program consisted of 30 cycles of denaturation at 95°C for 1', annealing at 55°C for 30'', primer extension at 72°C for 1' and a final 10' incubation at 72°C to complete extension.

1.2.4. Altered pherotype strain construction

To construct strain R36A-CSP2, the operon *comCDE* of TIGR4 strain (CSP2 pherotype) was amplified by PCR reaction. Likewise, construction of strain TIGR4-CSP1 started with the amplification of the operon *ComCDE* of R36A (CSP1 pherotype). In both cases, primers ComCDE_fw (5'-

GCTGACCGTGATTTCGTTATTGTG-3') and ComCDE_rev (5'-AGGATAAGTATGATATGATTGAGC-3'), were used allowing the amplification of fragments with 3415 bp encompassing the entire *comCDE* operon. Template DNAs were prepared by diluting 9 µl of an overnight culture in 441 µl of water and boiling this mixture for 2'. In both cases, the PCR reaction was performed in 50 µl of final volume containing 20 µl of template DNA solution, 1× reaction buffer (Invitrogen, Carlsbad, California), 10 mM of dNTPs (Invitrogen, Carlsbad, California), 2 mM of $MgCl_2$ (Invitrogen, Carlsbad, California), 0,4 pmol of each of the primers and 1.25 U GoTaq Polymerase (Invitrogen, Carlsbad, California). The PCR program consisted of an initial 10 cycles of 10'' denaturation at 94ºC, 30'' of annealing at 53ºC and 30'' of primer extension at 68ºC. A second run of 10 cycles started with 15'' of denaturation at 94ºC, 30'' of annealing at 53ºC and primer extension of 2' 30'' at 68ºC, with a 20'' increase in each successive cycle. The amplification ends with 7' incubation at 68°C to complete extension. The resulting fragments were purified with the High Pure PCR Product Purification kit (Roche Applied Sciences, Germany) and used directly for transformation with R36A or TIGR4, as previously described (37), briefly a 5 ml culture of each strain was grown to an optical density at 600nm of 0.06 and inducing development of competence with $CaCl_2$ (0.5mM), bovine serum albumin (0.002%) and CSP1 (250 ng/ml) in case of strain R36A or CSP2 (250 ng/ml) for TIGR4. After 5 minutes, 1 ml of induced cells were incubated with 100 ng of donor DNA for 70 min. To select R36A-CSP2 and TIGR4-CSP1 mutants, colony pooling was performed using PCR for pherotype identification.

1.2.5. Antibiotic resistant strains

The following antibiotic resistant strains were constructed: R36A(NovR) (novobiocin resistant, streptomycin sensitive), R36A(StrepR) (streptomycin resistant, novobiocin sensitive), R36A-CSP2(NovR) (novobiocin resistant, streptomycin sensitive), R36A-CSP2(StrepR) (streptomycin resistant, novobiocin sensitive). These strains were constructed by transforming R36A and R36A-CSP2 with genomic DNA from the novobiocin and streptomycin resistant strain CP1500. Transformants were selected by plating on tryptic soy agar (Oxoid, Basingstoke, United Kingdom) supplemented with 5% sheep blood (blood agar), containing

5µg/ml of novobiocin or 100µg/ml of streptomycin. Transformants resistant to one antibiotic were tested for growth on blood agar with the other antibiotic. Only transformants that presented resistance to one of the antibiotics were selected. Antibiotic resistant strains TIGR4 and TIGR4-CSP1 were constructed similarly.

1.2.6. Biofilm growth and quantification

Biofilm formation was determined in 96-well flat-bottom polystyrene (PST) microtiter dishes (Nunc™, Roskilde, Denmark). Cells were grown in C+Y medium to an OD_{600nm} between 0.5 and 0.6 and then diluted 1:4 to a final volume of 200 µl per well. Plates were incubated at 37ºC and biofilm mass was determined using the crystal violet staining protocol (31). Biofilm mass was measured at OD_{595nm} using a plate reader (Bio-Rad Model 680, Microplate Manager™ software V5.2.1). For the evaluation of viable cells in biofilms, a previously described procedure was adopted (46). Biofilms were grown in 96-well plates for 24h at 37ºC in 5% CO_2. Culture medium was aspirated from the wells to remove planktonic bacteria. Wells were washed with isotonic phosphate buffer (0.15M, pH 7.2) twice. Biofilms were scraped thoroughly from each well and well contents were aspirated and placed in 1 ml of isotonic phosphate buffer. Total CFU number was determined by serial dilution method and plating on appropriate media. For studying the CSP-impact on biofilm formation, CSP1 (NH2-EMRLSKFFRDFILQRKK-COOH) and CSP2 (NH2-EMRISRIILDFLFLRKK-COOH) (CASLO Laboratory, Lyngby, Denmark) were added to the biofilm growth medium at t=0. Biofilm mass quantification was performed at t=24h corresponding to maximal biofilm growth as determined previously (6).

1.2.7. Transformation within and between strains of different pherotypes

For the biofilm model, an equal number of isogenic *comC1* and *comC2* bacteria, each strain carrying a different antibiotic resistance marker, was seeded in 96-well plates and after 24h of biofilm growth, transformants within these mixed strain biofilms were evaluated. For evaluating transformation in the liquid culture, isogenic *comC1* and *comC2* strains, each carrying a different antibiotic resistance

marker, were individually cultured until OD_{600nm}= 0.3 was reached. Equal volumes of both cultures were mixed, diluted 3 times and incubated until OD_{600nm}= 0.8. In both biofilm and liquid culture, similar mixtures of two strains with the same pherotypes but different antibiotic resistance markers were used as control. The proportion of doubly resistant CFUs was indicative of transformation.

1.2.8. Confocal laser scanning microscopy

Biofilms were stained by using the Live/Dead BacLight kit (Invitrogen, Carlbad, USA) and examined by confocal laser scanning microscopy. Images were acquired on a Zeiss LSM510 META confocal microscope (Carl Zeiss, Jena, Germany) using a PlanApochromat 63x/1.4 objective for cell viability assays and a C-AproChromat 40x/1.2. SYTO 9 fluorescence was detected using the 488 nm laser line of an Ar laser (45 mW nominal output) and a BP 505-550 filter. Propidium iodine fluorescence was detected using a DPSS 561 nm laser (15 mW nominal output) and a LP 575 filter. For imaging, the laser power was attenuated to 1 – 2% of its maximum value. The pinhole aperture was set to 1 Airy unit.

1.2.9. Sequence analysis

To determine the level of identity between the operon *comCDE* of strains R36A and TIGR4, an alignment of the amino acid sequences of the products of both operons was performed using the software Geneious Pro 4.0.4 (Biomatters, Auckland, New Zealand). The sequences with the following Genbank accession numbers were used: NC_003094 and NC_003028. To compare the promoter region of the operon *comCDE* of strains CSP1 [already described in (16)] and TIGR4 (CSP2), an alignment of nucleotide sequences was performed using the same software.

1.2.10. Data analysis

One-way and two-way analysis of variance (ANOVA) were used to test effects of CSP level and pherotype on biofilm development or transformation efficiency, followed, when appropriate, by pairwise Tukey's post tests, using

GraphPad Prism version 4.0a for Macintosh (GraphPad Software, San Diego, California USA). In the figures presented, error bars represent 95% Student's *t* confidence intervals for the sample mean.

1.3. Results

1.3.1. Pherotype influences *in vitro* biofilm growth

A total of 90 invasive pneumococcal isolates, representing 26 serotypes and 57 sequence types by multilocus sequence typing (2, 42) and screened for the *comC* allele carried by each isolate (7), were tested for their capacity to form *in vitro* biofilms in 96-well plates. Overall, isolates presenting the CSP2 pherotype showed a lower capacity to form biofilms (Figure 1). Since these strains represent diverse serotypes and genetic lineages this observation suggested that the pherotype impact on biofilm formation was independent of the genetic background and other strain characteristics.

In order to better understand the influence of the two major pherotypes on biofilm growth, we constructed two strains where the pherotypes were switched relative to the ones presented by the wild-type (R36A-CSP2 and TIGR4-CSP1) with the objective of comparing their biofilm formation capacity with that of the isogenic wild-type strains. To do so, we extracted the operon *comCDE* of the donor strain (TIGR4 or R36A) and replaced it by recombination in the genome of the receptor strain. We also compared the DNA sequence of the previously described promoter region for operon *comCDE* in *comC1* strains (16) with the nucleotide sequence of a *comC2* strain and found that both promoter regions are identical, suggesting a similar regulation of expression of each of the pherotype variants.

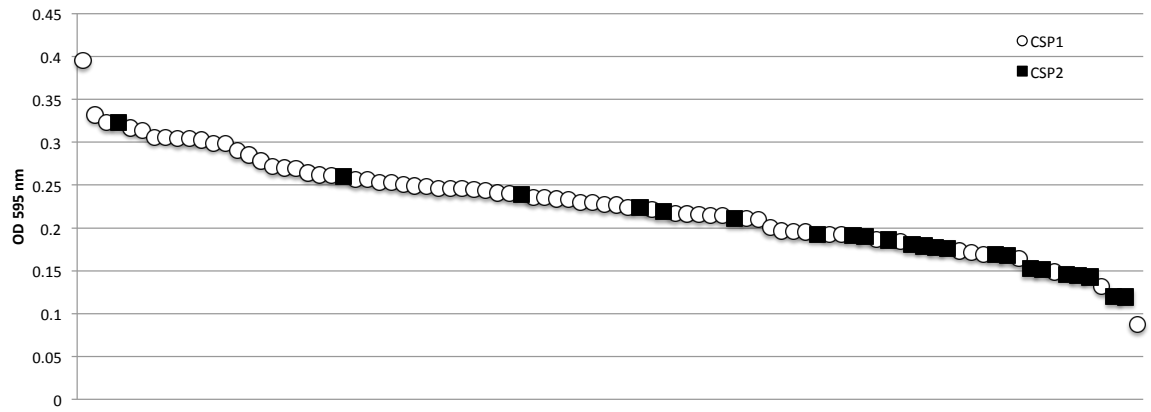

FIGURE 1 – **Distribution of pherotype-characterized strains according to the ability to form *in vitro* biofilms.** A total of 90 strains with pherotype CSP1 (n=67) or CSP2(n=23) were screened for their ability to form biofilms in 96-well plates. Each plotted value is an average of 9 replicates.

The identity of ComC, ComD and ComE of R36A and TIGR4 was determined by performing an alignment of the amino acid sequences of each protein of both strains. ComC includes 41 amino acids with an overall 81% identity between the two variants. However, all of the 8 differences are concentrated in its mature form (found in the extracellular medium), i.e. the 17 amino acid peptide that constitutes the CSP. The 441 amino acids of each of the ComD variants share 97% identity and ComE presents 250 amino acids that are 100% identical in the two strains. This indicates that the differences observed between the mutants with the replaced pherotype and their isogenic counterparts must be caused by the alterations in ComC and ComD.

In liquid growth, the resulting strain R36A-CSP2 shows a similar generation time in the exponential growth phase (70.5 minutes; 95% confidence interval 68.4-72.6 minutes) than the wild-type strain R36A (69.3 minutes; 95% confidence interval 66.8-71.7minutes). The same pattern was observed with TIGR4 (81.4 minutes; 95% confidence interval 78.4-84.3 minutes) and TIGR4-CSP1 (79.0 minutes; 95% confidence interval 75.7-82.2 minutes).

It had been previously shown that the addition of synthetic CSP to biofilms resulted in increased biofilm mass (33). We decided to evaluate the capacity to form biofilms of both pherotype variants and of the wild-type strains. Additionally,

we added synthetic CSP1 and CSP2 to each strain to verify if each one responded exclusively to its cognate CSP. The impact on biofilm formation was measured by the determination of CFUs detached from the 96-well-plates after 24h of incubation (Figure 2). Two-way ANOVA of the effects of the amount of cognate CSP and pherotype on biofilm viable counts shows that both factors have significant effects ($p<10^{-4}$ in both cases) and there is also a significant interaction effect ($p<10^{-4}$). The later results from the amplification of the difference between pherotypes in the presence of cognate CSP. The same analysis applied to the effects of non-cognate CSP level and pherotype reveals that only the latter retains a significant effect.

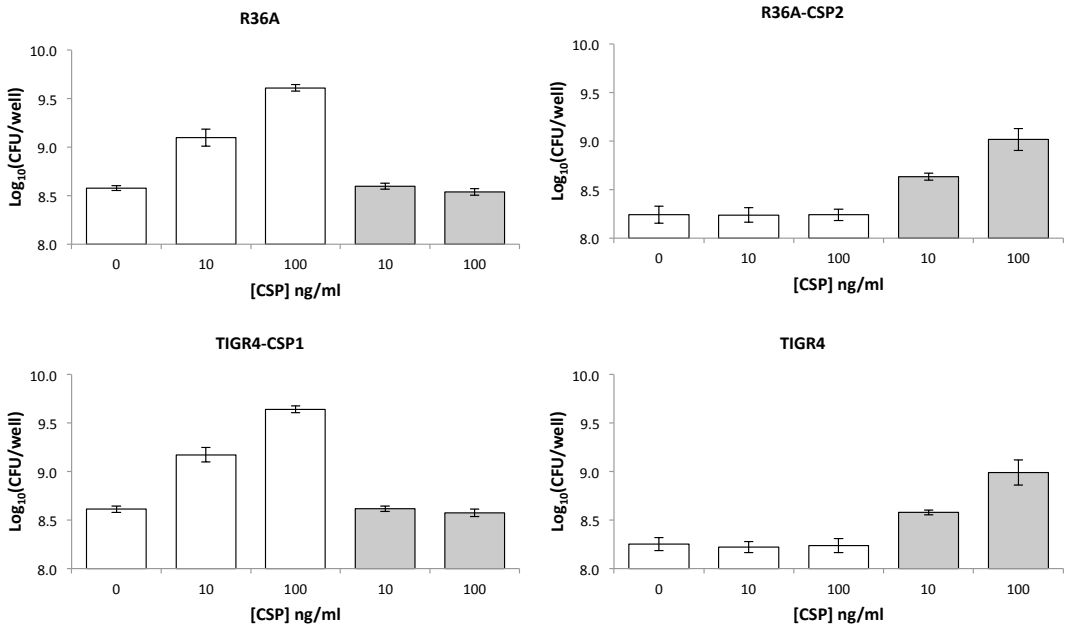

FIGURE 2 –**R36A and R36A-CSP2 mutant biofilms respond to cognate CSP in a dose-dependent manner.** R36A and R36A-CSP2 were incubated with synthetic CSP1 (white bars) and CSP2 (grey bars) during a biofilm growth experiment. Biofilm response to CSP was evaluated by determination of CFUs detached from the 96-well-plates after 24h of incubation. Error bars represent 95% confidence intervals for the mean of 3 independent experiments each with 3 technical replicates. Both strains were responsive to their cognate CSP but not to their heterologous CSP. R36A-CSP1 forms more biofilm than R36A-CSP2 and this observation is repeated for the synthetic CSP dosages used (10, 100 ng/ml).

The results show that R36A-CSP2 responds in a dose-dependent manner to synthetic CSP2 and not to CSP1 as did the wild type and the reverse is true for

TIGR4-CSP1. This non-promiscuous sensing of each peptide (CSP1 and CSP2) by their cognate receptors was also noted previously (38) and can be used as a functional assay to detect the pherotype of a given strain (39). These results also show that isogenic CSP1 strains form better biofilms than their CSP2 counterparts and that this effect is amplified for the several synthetic CSP dosages used.

The architecture of the biofilms formed by R36A and its isogenic mutant was evaluated by confocal laser scanning microscopy (CLSM). As can be seen in Figure 3 the wild-type strain (CSP1) forms biofilms that are 35% thicker than R36A-CSP2 biofilms. Figure 3 also shows that R36A biofilms are more densely packed when compared to R36A-CSP2. Taken together these data justify the differences observed in terms of CFUs described above.

1.3.2. Pherotype influences pneumococcal transformation in planktonic growth

We decided to test if the observed differences in the capacity of biofilm formation for each pherotype would also be reflected in transformation efficiency in different growth conditions since CSP is the key trigger of the competence activating cascade. We started by testing transformation of R36A, R36A-CSP2, TIGR4 and TIGR4-CSP1 in liquid culture. DNA from strain CP1500, which carries resistance genes for both streptomycin and novobiocin (29), was used in the transformation assay. Transformants were identified by the acquisition of the streptomycin resistance phenotype (Figure 4). The strains carrying the *comC1* allele yielded 50% and 37% (for R36A and TIGR4, respectively) more transformants than their isogenic *comC2* strains (Student's *t* test, $p<10^{-4}$ for both R36A and TIGR4 genetic backgrounds). A similar result was obtained previously (21) when transformation in liquid culture from both *comC1* and *comC2* strains was compared. These strains had their *comC* locus deleted and competence was induced by artificial CSP addition. In that experiment, performed in a genetic background similar to R36A, the *comC1* strain yielded 56% more transformants than the *comC2* strain, a comparable result to ours.

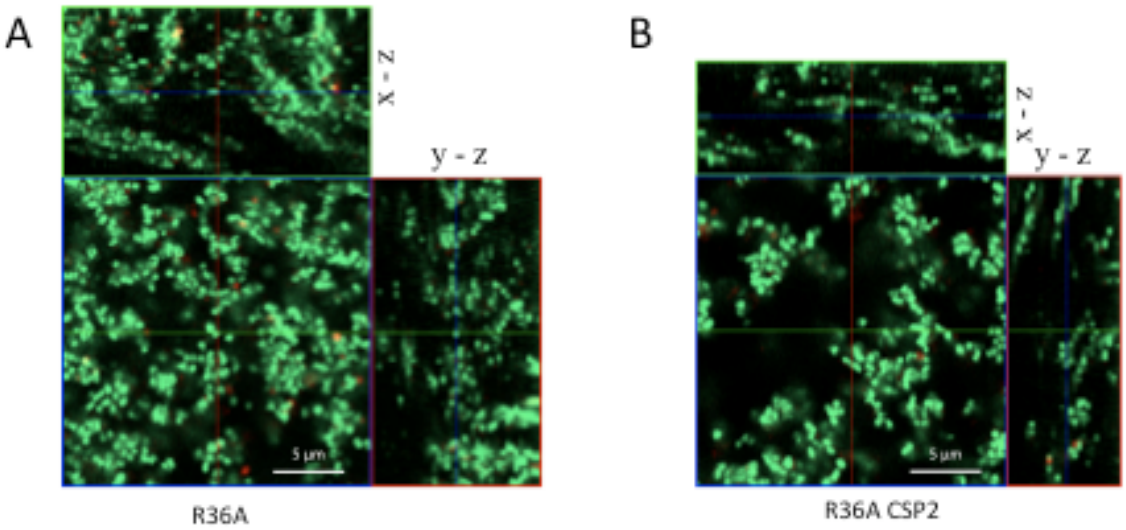

FIGURE 3 – **Confocal laser scanning microscopy (CLSM) images of R36A and R36A-CSP2 mutant biofilms.** R36A and R36A-CSP2 were grown for 24 h at 37ºC and examined by CLSM. Live cells are stained in green and dead cells in red. Images show that R36A forms biofilms that are around 30% thicker and more densely packed than R36A-CSP2 biofilms.

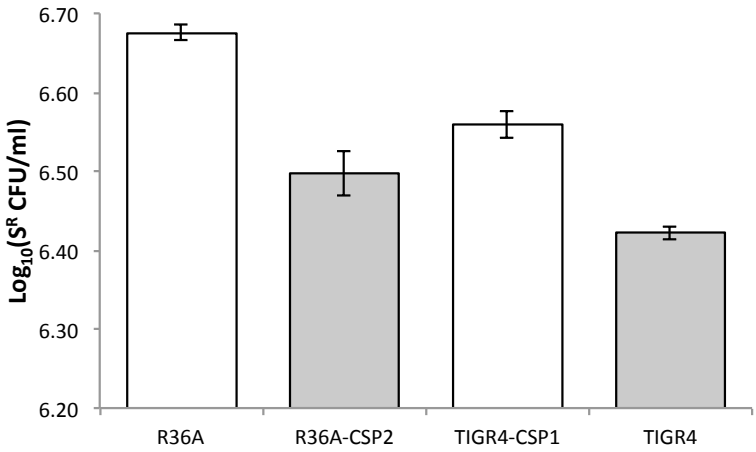

FIGURE 4 – **Transformation experiment of R36A and R36A-CSP2 mutant in liquid culture.** DNA from strain CP1500 (29) was used for the transformation and transformants with streptomycin and novobiocin resistance phenotypes were screened for both pherotypes and no differences were noted. Results are a total of the observed transformants resistant to any of the two antibiotics, subtracting transformants with double antibiotic resistance. R36A-CSP1 yields 1.4× more transformants than R36A-CSP2. Error bars represent the 95% confidence interval for the mean of 9 replicate experiments.

1.3.3. Intra-pherotype recombination prevails within biofilms

We then performed experiments to explore intra- and inter-pherotype recombination in both biofilm and liquid culture without exogenously added DNA (Figure 5). Analysis of variance indicates significant differences between the mean proportion of doubly resistant bacteria (transformants) in biofilms ($p<10^{-4}$ for both R36A and TIGR4 genetic backgrounds) and in liquid culture ($p<10^{-4}$ for both R36A and TIGR4 genetic backgrounds). *Post-hoc* analysis reveals that the proportion of transformants is higher for a mixture of R36A (CSP1) carrying different resistance markers when compared with an R36A-CSP2 pair of strains in both biofilm ($p<10^{-3}$) and liquid culture ($p<10^{-2}$). The same is observed for TIGR4-CSP1 and TIGR4 (CSP2) comparison in biofilms ($p<10^{-3}$), but statistical significance is not reached in liquid culture. The higher transformation efficiency of CSP1 strains, already detected in liquid culture, was also observed in biofilms (Figure 5, panel A). Mixed cultures of CSP1 and CSP2 strains do present statistically different transformation efficiencies when compared with transformation occurring between strains producing the same CSP, in both biofilm and liquid culture and independently of the genetic background ($p<10^{-3}$ in all four cases). Since mixtures of CSP1 and CSP2 strains with reversed antibiotic resistance markers do not present statistically different proportions of transformants, this effect cannot be attributed to a different behavior of the two antibiotic resistance markers used (Figure 5, hatched bars). However, the observed differences have opposite signs in biofilm and in liquid culture. In the latter, inter-pherotype transformation is more efficient and that can be interpreted as a consequence of a directional fratricide effect (9, 15). By directional we mean that one of the pherotypes is preferentially lysed during fratricide. CSP1 and CSP2 strains can reach the competent state at different times, due to the non-responsiveness of the ComD1 and ComD2 receptors towards their heterologous CSPs (38, 39). The first pherotype to achieve competence can then induce the lysis of bacteria with the other pherotype. We do not exclude that some lysis of bacteria with the same pherotype may occur, but we expect this to happen with lower frequency, since competent cells activate the expression of a lysis immunity protein ComM (19) and the majority of non-competent cells will carry the opposite *comC* allele.

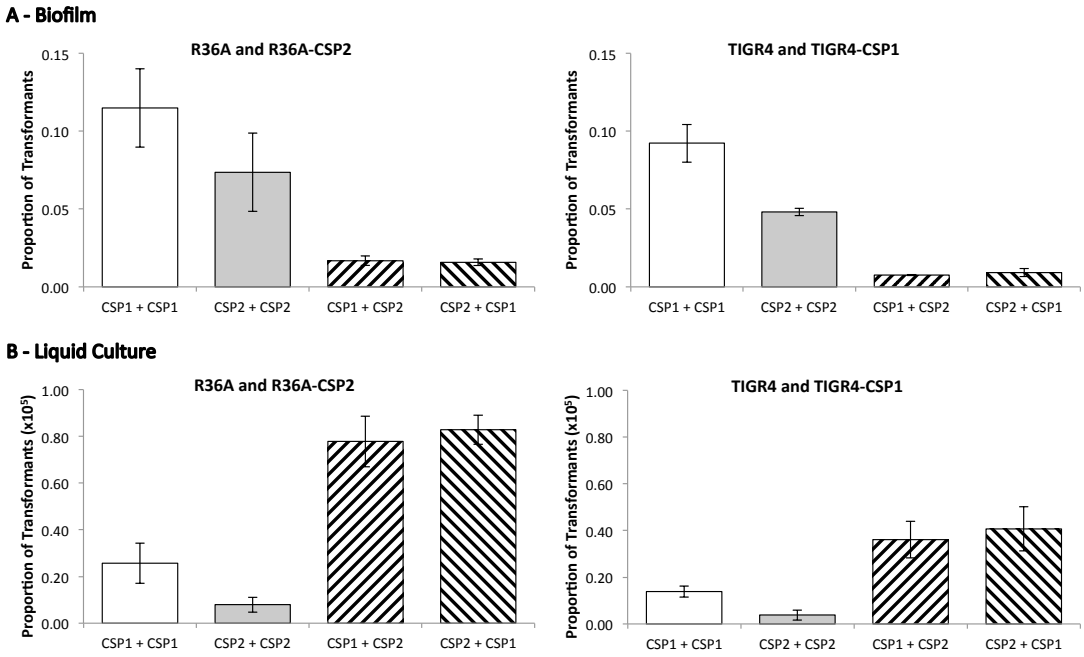

FIGURE 5 – **Comparison of intra-pherotype and inter-pherotype recombination in *in vitro* biofilms and liquid cultures.** Each bar represents the fraction of double resistant CFUs in the total number of CFUs. Experiments were performed both in biofilm (panel A) and in liquid culture (panel B) by mixing equal amounts of two strains, each with one antibiotic resistance marker (novobiocin and streptomycin). In each experiment, the two leftmost bars represent mixtures of strains with the same pherotype and on the right bars pherotypes were mixed to evaluate inter-pherotype recombination. White bars represent mixtures of CSP1 strains, grey bars CSP2 strains and hatched bars mixtures of CSP1 and CSP2 strains. In the left slanted hatched bars the CSP1 strain carried the novobiocin resistance marker, while the CSP2 strain carried the streptomycin resistance marker. In the right slanted hatched bars the resistance markers on the strains were reversed. Results show that in biofilms intra-pherotype recombination is more frequent than inter-pherotype recombination. The opposite relationship is observed in liquid culture. Error bars represent the 95% confidence intervals of the mean of 3 or 9 (biofilms and liquid culture, respectively) independent experiments. Each independent biofilm experiment consisted of 3 technical replicates.

While in an homogeneous bacterial culture only the small fraction of non-competent cells are susceptible to lysis, in mixtures of CSP1 and CSP2 strains in liquid culture the entire population carrying the *comC* allele that "lost" the competence race will be susceptible to lysis. Our data are compatible with this

hypothesis, resulting in more bacterial lysis and higher amounts of free DNA for subsequent recombination.

In contrast to planktonic growth, in biofilms intra-pherotype recombination is more frequent than inter-pherotype recombination. The lower number of inter-pherotype transformants in biofilms could be biased by a different representation of each pherotype in the mixed strain biofilm. Different frequencies of each pherotype in the final biofilm population could be due to different biofilm growth rates or to a directional fratricide effect. Considering the separate biofilm growth of each pherotype, we estimated a proportion of 68.5% and 70.9% of bacteria bearing CSP1 (for R36A and TIGR4 genetic backgrounds, respectively) in the mixed biofilm in the absence of directional fratricide or other interference in biofilm growth rate due to the presence of bacteria with the CSP2 pherotype. To compare these values with what was happening in our experiments, 48 colonies representing the population constituting a biofilm with 24h of growth that had been seeded with CSP1 and CSP2 strains, were randomly picked and pherotyped. We observed proportions of colonies of the CSP1 pherotype of 72.9% (CI 95% 60.3-85.5%) and 62.5% (CI 95% 48.8-76.2%) (for R36A and TIGR4 genetic backgrounds, respectively). These are not significantly different from the predicted values, indicating that the difference in biofilm growth capacity of CSP1 and CSP2 strains may be the sole cause of the frequency imbalance between the two pherotypes in the final bacterial population forming the mixed biofilm.

Considering that transformation can occur randomly between any two bacteria, with one acting as DNA donor and the other as acceptor independently of pherotype, the measured proportion of doubly resistant bacteria would be maximal if half of the total bacteria had a different antibiotic resistance marker. If one marker is more frequent than the other, more transformation events will occur between bacteria with the same marker resulting in fewer doubly resistant transformants. Accordingly, we used the expected frequencies of each pherotype and estimated that a reduction of 13.6% (95% CI 10.8-16.4%) for the R36A and 17.5% (95% CI 11.9-23.2%) for the TIGR4 genetic backgrounds, respectively, in the proportion of doubly resistant transformants can be attributed solely to the frequency imbalance of the pherotypes in the mixed biofilm. However, when we

compared the proportion of doubly resistant transformants obtained in intra-pherotype or inter-pherotype transformation in biofilms, we observed reductions of 77 to 90%. This means that the lower inter-pherotype transformation efficiency is not exclusively due to the pherotype frequency imbalance. In other words, there is an effective reduction in the probability of inter-pherotype transformation within biofilms.

We also tested if transformation was as likely to occur with CSP1 or CSP2 bacteria acting as DNA acceptors. For that purpose, 48 doubly resistant colonies (transformants) obtained after 24h of growth of a biofilm seeded with CSP1 and CSP2 strains, were randomly picked and pherotyped. We observed that these transformants were enriched in the CSP1 pherotype: 91% (95% CI 83.8-99.5%) of CSP1 bacteria for mixed biofilms of strains with the R36A genetic background and 83.3% (95% CI 72.8-93.9%) for mixed biofilms of strains with the TIGR4 genetic background. The proportions of CSP1 bacteria among transformants are significantly higher than those found among all bacteria in the biofilm (Fisher exact test, $p<0.05$ for both R36A and TIGR4 genetic backgrounds). These data are consistent with the higher transformation efficiency of isogenic CSP1 strains when compared with CSP2 strains and are compatible with limited localized directional fratricide, as documented in a larger scale in the case of planktonic growth.

Globally, these results suggest that, within biofilms, the inter-pherotype fratricide phenomenon is less frequent, eventually due to a more restricted spatial organization of bacteria with different pherotypes. Spatial restrictions may impact on DNA diffusion within the biofilm and, since it is also believed that physical contact between competent and non-competent cells may be required to induce lysis (19), hamper the capacity of the highly competent CSP1 bacteria inducing lysis of their CSP2 counterparts.

1.4. Discussion

Evolution of a new pherotype requires matched mutations in both the *comC* and *comD* sequences that encode the CSP and its cognate receptor, respectively. Only

then bacteria of the new pherotype will be able to sense the altered CSP produced by themselves and by other bacteria with the same pherotype, but not from bacteria with the original pherotype.

These changes can potentially alter the efficiency of the competence activation pathway. An important end point of this activation is the induced lysis of a fraction of the pneumococcal population, a phenomenon known as fratricide (15). This targeted lysis is essential for the release of DNA to the environment, where it can participate in transformation events. Additionally, as it has been recently proposed in *S. pneumoniae* (6) and in close related species (34, 47), released DNA can be incorporated into the biofilm extracellular matrix, enhancing biofilm development.

Comparison of isogenic CSP1 and CSP2 strains resulted in three observations supporting the existence of differences in competence of the two pherotypes and of their impact on biofilm growth: 1) transformation of CSP2 strains with exogenously provided DNA in liquid medium is less efficient than that of CSP1 strains, 2) biofilm growth of CSP2 strains is hampered relatively to CSP1 strains, 3) addition of synthetic cognate CSP amplifies the differences in biofilm growth between pherotypes.

Biofilms may be the preferred lifestyle of bacteria during asymptomatic colonization of the nasopharynx (4, 23). If this is so, then the higher capacity of CSP1 strains to develop biofilms can lead to higher transmissibility or longer duration of colonization. Both effects can help explain the higher prevalence of the CSP1 pherotype in pneumococcal populations (7).

Besides biofilm development, different pherotypes also influenced transformation frequency within biofilms. This effect could have a larger impact on the biology of pneumococcal colonization. Unlike when causing infections, multiple pneumococcal strains simultaneously colonizing one individual may be the norm (5, 40). Co-colonization offers the opportunity for gene exchange that can occur through several mechanisms such as conjugation, transduction or, particularly in the case of pneumococci, through competence mediated transformation. The later is considered the major mechanism of generating genetic diversity in this species, driving the evolution of *Streptococcus pneumoniae* (12, 13).

In a recent population genetic study, we suggested that a predominance of intra-pherotype recombination is driving genetic differentiation between pneumococcal strains with different pherotypes (7). The results presented here clarify the apparent conflict between a higher inter-pherotype transformation efficiency due to fratricide suggested previously (19) and the results of the population study. The data reported in this paper supports the population study indicating that genetic exchange between strains with different pherotypes was less frequent than intra-pherotype gene exchange within biofilms that are likely to be associated with colonization where most genetic exchange is expected to occur.

By showing that pherotypes differ both in their biofilm formation and transformation efficiency, with a clear advantage of CSP1 strains, we provide a powerful example of the possibly broad impact of limited genetic differences (in this case restricted to the *comCDE* operon) on the bacterial phenotype that may be reflected in host-pathogen interactions and on the population biology of a given species. Taken together, these results provide new insights towards elucidating the biological significance of pherotypes in *S. pneumoniae*.

1.5. Acknowledgments

Margarida Carrolo is supported by a research grant from Fundação para a Ciência e Tecnologia, Portugal (SFRH/BD/35854/2007). This work was partially supported by Fundação para a Ciência e Tecnologia, Portugal (PTDC/SAU-ESA/64888/2006 and PTDC/BIA-MIC/119709/2010), Fundação Calouste Gulbenkian and an unrestricted grant from Glaxo Smithkline Portugal.

1.6. Bibliography

1. Abraham WR: **Controlling biofilms of gram-positive pathogenic bacteria.** *Curr Med Chem* 2006, **13:**1509-24.

2. Aguiar SI, Serrano I, Pinto FR, Melo-Cristino J, Ramirez M: **The presence of the pilus locus is a clonal property among pneumococcal invasive isolates.** *BMC Microbiol* 2008, **8:**41.

3. Anderson GG, O'Toole GA: **Innate and induced resistance mechanisms of bacterial biofilms.** *Curr Top Microbiol Immunol* 2008, **322:**85-105.

4. Bogaert D, De Groot, R Hermans PW: ***Streptococcus pneumoniae* colonisation: the key to pneumococcal disease.** *Lancet Infect Dis* 2004, **4:**144-54.

5. Brugger SD, Hathaway LJ, Muhlemann K: **Detection of *Streptococcus pneumoniae* strain cocolonization in the nasopharynx.** *J Clin Microbiol* 2009, **47:**1750-6.

6. Carrolo M, Frias MJ, Rodrigues FP, Melo-Cristino J, Ramirez M: **Prophage spontaneous activation promotes DNA release enhancing biofilm formation in *Streptococcus pneumoniae*.** *PLoS One* 2010, **Dec 20;5(12):e15678.**

7. Carrolo M, Pinto FR, Melo-Cristino J, Ramirez M: **Pherotypes are driving genetic differentiation within *Streptococcus pneumoniae*.** *BMC Microbiol* 2009, **9:**191.

8. Claverys JP, Havarstein LS: **Cannibalism and fratricide: mechanisms and *raisons d'etre*.** *Nat Rev Microbiol* 2007, **5:**219-29.

9. Claverys JP, Martin B, Havarstein LS: **Competence-induced fratricide in streptococci.** *Mol Microbiol* 2007, **64:**1423-33.

10. Claverys JP, Prudhomme M, Martin B: **Induction of competence regulons as a general response to stress in gram-positive bacteria.** *Annu Rev Microbiol* 2006, **60:**451-75.

11. Cornejo OE, McGee L, Rozen DE: **Polymorphic competence peptides do not restrict recombination in *Streptococcus pneumoniae*.** *Mol Biol Evol* 2010, **27:**694-702.

12. Feil EJ, Enright MC, Spratt BG: **Estimating the relative contributions of mutation and recombination to clonal diversification: a comparison between *Neisseria meningitidis* and *Streptococcus pneumoniae*.** *Res Microbiol* 2000, **151:**465-9.

13. Fraser C, Hanage WP, Spratt BG: **Neutral microepidemic evolution of bacterial pathogens.** *Proc Natl Acad Sci U S A* 2005, **102:**1968-73.

14. Gray BM, Converse GM, Dillon HC: **Epidemiologic studies of *Streptococcus pneumoniae* in infants: acquisition, carriage, and infection during the first 24 months of life.** *J Infect Dis* 1980, **142:**923-33.

15. Guiral S, Mitchell TJ, Martin B, Claverys JP: **Competence-programmed predation of noncompetent cells in the human pathogen *Streptococcus pneumoniae*: genetic requirements.** *Proc Natl Acad Sci U S A* 2005, **102:**8710-5.

16. Halfmann A, Hakenbeck R, Bruckner R: **A new integrative reporter plasmid for *Streptococcus pneumoniae*.** *FEMS Microbiol Lett* 2007, **268:**217-24.

17. Havarstein LS, Coomaraswamy G, Morrison DA: **An unmodified heptadecapeptide pheromone induces competence for genetic transformation in *Streptococcus pneumoniae*.** *Proc Natl Acad Sci U S A* 1995, **92:**11140-4.

18. Havarstein LS, Hakenbeck R, Gaustad P: **Natural competence in the genus Streptococcus: evidence that streptococci can change pherotype by interspecies recombinational exchanges.** *J Bacteriol* 1997, **179:**6589-94.

19. Havarstein LS, Martin B, Johnsborg O, Granadel C, Claverys JP: **New insights into the pneumococcal fratricide: relationship to clumping and identification of a novel immunity factor.** *Mol Microbiol* 2006, **59:**1297-307.

20. Hoch JA, Varughese KI: **Keeping signals straight in phosphorelay signal transduction.** *J Bacteriol* 2001, **183:**4941-9.

21. Iannelli F, Oggioni MR, Pozzi G: **Sensor domain of histidine kinase ComD confers competence pherotype specificity in *Streptoccoccus pneumoniae*.** *FEMS Microbiol Lett* 2005, **252:**321-6.

22. Johnsborg O, Blomqvist T, Kilian M, Havarstein LS: Biologically active peptides in streptococci, p. 25-59. *In* R. Hakenbeck and S. Chhatwal (ed.), Molecular Biology of Streptococci. Horizon bioscience; 2007.

23. Johnsborg O, Eldholm V, Bjornstad ML, Havarstein LS: **A predatory mechanism dramatically increases the efficiency of lateral gene transfer in *Streptococcus pneumoniae* and related commensal species.** *Mol Microbiol* 2008, **69:**245-53.

24. Lacks S, Hotchkiss RD: **A study of the genetic material determining an enzyme activity in pneumococcus.** *Biochim. Byophys. Acta* 1960, **39:**508-17.

25. Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG: **Natural genetic transformation of *Streptococcus mutans* growing in biofilms.** *J Bacteriol* 2001, **183:**897-908.

26. Li YH, Tang N, Aspiras MB, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG: **A quorum-sensing signaling system essential for genetic competence in *Streptococcus mutans* is involved in biofilm formation.** *J Bacteriol* 2002, **184:**2699-708.

27. Loo CY, Corliss DA, Ganeshkumar N: ***Streptococcus gordonii* biofilm formation: identification of genes that code for biofilm phenotypes.** *J Bacteriol* 2000, **182:**1374-82.

28. Mah TF, O'Toole GA: **Mechanisms of biofilm resistance to antimicrobial agents.** *Trends Microbiol* 2001, **9:**34-9.

29. Morrison, D. A., S. A. Lacks, W. R. Guild, and J. M. Hageman: **Isolation and characterization of three new classes of transformation-deficient mutants of Streptococcus pneumoniae that are defective in DNA transport and genetic recombination.** *J Bacteriol* 1983, **156:**281-90.

30. Moscoso M, Claverys JP: **Release of DNA into the medium by competent *Streptococcus pneumoniae*: kinetics, mechanism and stability of the liberated DNA.** *Mol Microbiol* 2004, **54:**783-94.

31. Moscoso M, Garcia E, Lopez R: **Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion.** *J Bacteriol* 2006, **188:**7785-95.

32. Neil RB, Shao JQ, Apicella MA: Biofilm formation on human airway epithelia by encapsulated *Neisseria meningitidis* serogroup B. *Microbes Infect* 2009, **11:**281-7.

33. Oggioni MR, Trappetti C, Kadioglu A, Cassone M, Iannelli F, Ricci S, Andrew PW, Pozzi G: **Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis.** *Mol Microbiol* 2006, **61:**1196-210.

34. Perry JA, Cvitkovitch DG, Levesque CM: **Cell death in *Streptococcus mutans* biofilms: a link between CSP and extracellular DNA.** *FEMS Microbiol Lett* 2009, **299:**261-6.

35. Petersen FC, Pecharki D, Scheie AA: **Biofilm mode of growth of *Streptococcus intermedius* favored by a competence-stimulating signaling peptide.** *J Bacteriol* 2004, **186:**6327-31.

36. Peterson SN, Sung CK, Cline R, Desai BV, Snesrud EC, Luo P, Walling J, Li H, Mintz M, Tsegaye G, Burr PC, Do Y, Ahn S, Gilbert J, Fleischmann RD, Morrison DA: **Identification of competence pheromone responsive**

genes in *Streptococcus pneumoniae* by use of DNA microarrays. *Mol Microbiol* 2004, **51:**1051-70.

37. Piotrowski A, Luo P, Morrison DA: **Competence for genetic transformation in *Streptococcus pneumoniae*: termination of activity of the alternative sigma factor ComX is independent of proteolysis of ComX and ComW.** *J Bacteriol* 2009, **191:**3359-66.

38. Pozzi G, Masala L, Iannelli F, Manganelli R, Havarstein LS, Piccoli L, Simon D, Morrison DA: **Competence for genetic transformation in encapsulated strains of *Streptococcus pneumoniae*: two allelic variants of the peptide pheromone.** *J Bacteriol* 1996, **178:**6087-90.

39. Ramirez M, Morrison DA, Tomasz A: **Ubiquitous distribution of the competence related genes comA and comC among isolates of *Streptococcus pneumoniae*.** *Microb Drug Resist* 1997, **3:**39-52.

40. Sa-Leao R, Tomasz A, Santos-Sanches I, de Lencastre H: **Pilot study of the genetic diversity of the pneumococcal nasopharyngeal flora among children attending day care centers.** *J Clin Microbiol* 2002, **40:**3577-85.

41. Satorres SE, Alcaraz LE, Cargnelutti E, Di Genaro MS: **IFN-gamma plays a detrimental role in murine defense against nasal colonization of *Staphylococcus aureus*.** *Immunol Lett* 2009, **123:**185-8.

42. Serrano I, Melo-Cristino J, Carrico JA, Ramirez M: **Characterization of the genetic lineages responsible for pneumococcal invasive disease in Portugal.** *J Clin Microbiol* 2005, **43:**1706-15.

43. Steinmoen H, Knutsen E, Havarstein LS: **Induction of natural competence in *Streptococcus pneumoniae* triggers lysis and DNA release from a subfraction of the cell population.** *Proc Natl Acad Sci U S A* 2002, **99:**7681-6.

44. Steinmoen H, Teigen A, Havarstein LS: **Competence-induced cells of *Streptococcus pneumoniae* lyse competence-deficient cells of the same strain during cocultivation.** *J Bacteriol* 2003, **185:**7176-83.

45. Suntharalingam P, Cvitkovitch DG: **Quorum sensing in streptococcal biofilm formation.** *Trends Microbiol* 2005, **13:**3-6.

46. Tetz GV, Artemenko NK, Tetz VV: **Effect of DNase and antibiotics on biofilm characteristics.** *Antimicrob Agents Chemother* 2009, **53:**1204-9.

47. Thomas VC, Hiromasa Y, Harms N, Thurlow L, Tomich J, Hancock LE: **A fratricidal mechanism is responsible for eDNA release and contributes to biofilm development of *Enterococcus faecalis*.** *Mol Microbiol* 2009, **72:**1022-36.

48. Tomasz A: **Control of the competent state in pneumococcus by a hormone-like cell product: an example for a new type of regulatory mechanism in bacteria.** *Nature* 1965, **208:**155-9.

49. Tomasz A, Mosser JL: **On the nature of the pneumococcal activator substance.** *Proc Natl Acad Sci U S A* 1966, **55:**58-66.

50. Tortosa P, Dubnau D: **Competence for transformation: a matter of taste.** *Curr Opin Microbiol* 1999, **2:**588-92.

51. Xu KD, McFeters GA, Stewart PS: **Biofilm resistance to antimicrobial agents.** *Microbiology* 2000, **146 (Pt 3):**547-9.

Chapter 5

Final Remarks and Future Perspectives

For over a century, *Streptococcus pneumoniae* strains have been categorized by serology, with distinct serotypes identified on the basis of ninety-four immunologically and chemically distinct polysaccharide capsules [1, 2]. More recently, molecular typing, such as pulse-field gel electrophoresis of endonuclease digestion fragments and multilocus sequence typing has refined the observation of the diversity in the pneumococcal population showing that even within serotypes different clones exist [3, 4]. Moreover, during the last decade, it has been shown that pneumococcal diversity is, in fact, high as determined by its large genome plasticity [5-7].

In this thesis we decided to explore the mechanisms that generate pneumococcal genetic diversity and explored the impact that biological variants implicated in the major process of gene exchange could have on biofilms, which are the structures where bacteria spend most of their lifetimes [8].

Biofilms are in themselves strategies of microbial survival providing a protective environment and acting as a reservoir for dissemination between hosts. Considering this fact and adding the physical proximity of bacteria within biofilms, it is probable that genetic exchange will occur preferentially within these bacterial structures. Thus biofilms may play a central role in pneumococcal biology.

For the last decade, eDNA has been increasingly recognized as a critical element of the biofilm structure, providing structural stability and protection against antimicrobial agents [9-12]. One strategy for eDNA to become available for incorporation into the biofilm structure is through prophage host-mediated lysis. The large majority of pneumococcal clinical isolates carry prophages [13, 14], which upon induction will shift to the lytic mode resulting in host lysis and consequent eDNA release. An *in vitro* system was used to understand the effect of lysogeny and its spontaneous-mediated host lysis in pneumococcal biofilm

formation. Our data provides evidence that prophage carriage has a positive impact on biofilm formation and that, although spontaneous phage induction results in the death of their bacterial hosts, the bacterial population as a whole can benefit from these events. It had already been shown that the mobilization of the host autolytic enzyme guaranteed an optimized lysis with the release of a higher proportion of phage progeny relative to sole dependence the on phage encoded lysin [15]. Interestingly, we found that host lysis in biofilms is also accomplished by the combined action of both these lytic activities, despite cell behavior in this structured lifestyle being often distinct from that of planktonic cells. Thus, an optimized lytic process underlies a very favorable phage-bacteria cooperative relationship. Moreover this work provides evidence for the link between the DNA released through phage mediated-lysis and the observed enhancement in pneumococcal biofilm formation.

Another factor that is known to be able to enhance streptococcal biofilm growth is genetic competence [16-20]. We decided to explore the effect of competence, through the contribution of the two major pherotypes, firstly on the pneumococcus ppulation genetics and afterwards on the ability of this bacterium to form biofilms.

We started by designing and performing a molecular epidemiology study with pneumococcal invasive isolates [21]. The first result documented the dominance of CSP1 over CSP2 within the population: more than 70% of analyzed strains presented the CSP1 pherotype. It was also shown that pherotypes are not randomly distributed among the population, as several associations were detected. For example, serotypes 1 and 14 were found to be strongly associated with CSP1 whereas serotypes 3, 6A and 9N showed a clear association with CSP2. Five PFGE clusters were associated with a particular pherotype and the ability of the sequence type and clonal complex defined by MLST to predict the pherotype was remarkably high. We also detected an association with some antibiotics, as isolates resistant to penicillin and other β-lactams were associated with CSP1. Moreover, we found that phage transduction may be indirectly arbitrated by the pherotype *via* the restriction/modification systems, such that the spread of large genetic elements that rely on this mechanism of horizontal

gene transfer (e.g. the locus encoding the capsular polysaccharide biosynthesis machinery and some of the genetic determinants of resistance to tetracycline, chloramphenicol or erythromycin), could be limited by the pherotype. Statistical analysis of genetic differentiation within the pneumococcal population also showed that strains phylogenetically close have an increased likelihood of sharing the same pherotype. As a whole these data suggest that the presence of the two pherotypes and its failure to communicate with each other was having a detectable impact in the genetic diversity of the pneumococcal population [21].

We then moved forward to explore if strains with different pherotypes could vary in their capacity for biofilm formation and if these putative differences could influence the interpherotype recombination efficiency. The study design was a comparison of the behavior between R36A and TIGR4, two pneumococcal laboratory reference strains that produce respectively, CSP1 and CSP2, and their isogenic mutants that differed only on the fact that their CSP alleles were switched (R36A-CSP2 and TIGR4-CSP1). We observed that biofilms of strains presenting CSP1 had increased biofilm mass and are more densely packed as shown by CLSM. Moreover, the addition of synthetic cognate CSP amplifies the observed differences in biofilm growth between pherotypes. Since CSP is a key trigger of the competence-activating cascade, we then checked if transformation efficiency was also being affected by the different pherotypes. Our data showed that CSP2 strains, with exogenously provided DNA transforms less efficiently than the CSP1 strains both in liquid medium and within biofilms. So the differences in biofilm formation and transformation efficiency caused by pherotypes, with a clear advantage of CSP1 strains, are an example of the possibly broad impact of limited genetic differences, in this case restricted to a single gene (*comC*), on the *S. pneumoniae* phenotype.

The data reported in this thesis work indicates that genetic exchanges between strains with different pherotypes are less frequent that intra-pherotype gene exchanges. These observations where obtained with bacteria living within biofilms, which are structures likely to be associated with colonization where most genetic exchange is expected to occur. So, we propose that the higher prevalence of the CSP1 pherotype observed in the pneumococcal population, can

eventually be supported by the higher capacity of these strains to develop biofilms, which could lead to a higher transmissibility and longer duration of colonization.

Throughout the studies performed, the variants observed lead to binary genotypes: a) to present the CSP1 or CSP2 pherotype and b) to carry or not to carry a prophage. In both cases there is one genotype that favors biofilm formation: a) to present CSP1 and b) to carry a prophage. We detected that the biofilm-favoring genotype is the most prevalent among the clinical invasive isolates studied, which could be an indicative that the ability to form biofilms is important for the success of pneumococcal strains in causing invasive disease. To test this hypothesis it would be necessary to perform a comparative study between invasive and colonization strains to determine if there are differences in the dominance of the biofilm-favoring genotype.

Another remark that results from the collected data is that both studied processes that enhance biofilm formation, the CSP signalling and the spontaneous prophage release, have in common the involvement of lysis of a fraction of the bacterial cells inside the biofim. We showed that LytA was implicated in the enhancement of biofilm through prophage-mediated lysis and it had previously been documented that LytA resulting from the competence activaction (via CSP signaling) was important for biofilm development. In both processes, the bacterial major autolysin, LytA, plays a relevant role and eDNA is implicated. The recruitment of LytA yielding eDNA through cell lysis of a fraction of the population may be a property that is being favored within clinical isolates. Two proposals can then be established: a) the same level of partial population lysis found in clinical isolates is also prevalent among colonization strains: if so, this property may be important for the complete pneumococcal lifestyle or b) levels of population lysis found among colonization strains are smaller, which could indicate that limited lysis is specifically promoting the invasion process.

Another important contribution of the present work is that combining the obtained results a set of interesting questions can be raised. Those questions can be separated into two major paths:

1. from biofilms towards pneumococcal population and clinically important issues;
2. from biofilms towards the cellular and molecular aspects.

In the first path, the most obvious question to be raised concerns the comparison of the frequency of CSP1 and CSP2 obtained in our study among a population of isolates causing human infections with a population of colonization strains. As an association between the pherotype and the serotype was observed in our study and because the frequency of serotypes is different among colonization and clinical isolates, we expect to observe differences in the frequency of CSP1/CSP2 in colonization versus the frequency observed among isolates causing infections. Nevertheless, it persists among the scientific community the uncertainty that when a strain with a given serotype and a given genetic typing profile is detected both in colonization and in invasion this happens due to molecular differences that are not associated with the serotype and with the genetic typing method or, on the other hand, this dual ability to colonize and invade is common among the pneumococci with a more accentuated preference of strains for one of the processes.

An additional aspect that could also be explored relates to the prophage distribution within the pneumococcal population. Prophages have a high prevalence in isolates associated with infection [13, 14]. In this thesis work, we showed that a bacterial community benefits from the spontaneous prophage induction in a fraction of its members since it promotes biofilm renewal and may improve biofilm architecture increasing the overall competitive fitness of the bacterial population. It would now be interesting to test if the prevalence of prophages varies between colonization and invasion strains and to see if the advantage on biofilm formation favours any of these stages.

Another aspect that could be exploited is to study the impact of CSPs and spontaneous prophage-mediated lysis in continuous biofilms. The well-established *in vitro* system used within this thesis work is ideal to study adhesion and early biofilm development of bacteria. So it would now be interesting to observe if the same conclusions obtained with this study are

conserved in a more dynamic system where biofilms are allowed to grow for a broaden time scale. If results are similar to those obtained in the *in vitro* system used or if the effect of biofilm enhancement is amplified in the dynamic system it supports the idea that CSP and prophage carriage are important throughout biofilm full development. On the other hand, if significant less biofilm enhancement effect is observed with the dynamic system, it may indicate that the biofilm enhancement caused by both CSP and prophage-mediated lysis is more important during the initial steps of biofilm formation.

Moving beyond the *in vitro* systems, it would be important to know if the effects of both CSP and prophage carriage on biofilm formation are extensible to *in vivo* animal models. If such a study corroborates the differences observed between the two pherotypes as well as the impact resulting from prophage carriage, it would be a more direct evidence for the implication of these parameters on pneumococcal lifestyle. By doing so, these results could provide a mechanism to explain the distribution asymmetries of pherotypes and prophage carriage (among invasive isolates or possibly between invasive and colonization isolates and within the latter) in the pneumococcal population.

The frequencies of intra- and interpherotype that were measured during this thesis work could be explored to estimate the evolutionary dynamics of the pneumococcal population. These frequencies could globally explain the differences in genetic diversity within each pherotype. Ultimately, these studies could justify the coexistence of the two major pherotypes within the pneumococcal population.

In the second path, towards the cellular and molecular aspects of pneumococcal biofilms, several aspects deserve further elucidation. To begin with, it is presently unknown what are the dynamics of competence activation within biofilms. In particular, it would be enriching to find out what fraction of cells enter the competent state and if these cells form a spacially organized pattern within the structure of the biofilm. This information could allow a deeper understanding of the impact of DNA release on biofilm development as well as

provides new hypothesis to explain intra- and interpherotype transformation frequencies.

The understanding of prophage-mediated lysis on pneumococcal biofilms could also be enhanced by a spatial model of biofilm architecture. Prophage lysis could be preferentially induced in cells under stress, namely by nutrient deprivation. If so, besides DNA release, the lytic event could generate a structural renovation of the biofilm in critical regions.

Finally, the molecular determinant of the phenotype differences found between CSP1 and CSP2 could be further explored through binding and structural biochemistry assays of the receptor ComD for both pherotypes and their cognate signal peptides. ComC includes 41 aminoacids with 81% identity. Nevertheless, in its mature form (found in the extracellular medium), the peptide presents 17 aminoacids with 53% of identity. The 441 aminoacids of ComD share a 97% identity and ComE presents 250 aminoacids with 100% identity. This indicates that the differences observed between the mutants with the replaced pherotype and their isogenic counterparts must be caused by ComC and/or ComD. Sequence modifications can yield differences in affinity and/or kinetics of the ComC/ComD binding. These differences would impact on the remaining downstream signaling competence cascade, resulting in the observed phenotypic differences in biofilm development and transformation efficiency.

Throughout this thesis work, we found that within the pneumococcal population, two distinct subpopulations can be segregated based on their pherotype. We also found that one of the pherotypes, CSP2, is less efficient at transforming and at forming biofilms. This information could eventually be used to favor this subpopulation and, by doing so, decreasing the transmission of antibiotic resistance genes and other genes capable of improving the bacterial pathogenic potential. Aiming for the total eradication of *S. pneumoniae* would rather result in the vacancy of its ecological niche that would be probably occupied by other species potentially capable of causing disease in the human host. So favoring the existence of the individuals that respond less promptly to

new environmental challenges is probably a safer way of controlling the co-habitation of humans and the pneumococcus.

In 2007, Fraser and colleagues [22] observed that *S. pneumoniae* is highly recombinogenic, behaving like a sexual population, capable of merging genetically distant subpopulation clusters through recombination. Nevertheless, the pneumococcal genetic structure is not as homogeneously shuffled as it would be expectable [23]. The data obtained during this thesis on the dominance of intra-pherotype genetic exchanges in biofilms, contributes with a putative mechanism to explain the apparent disagreement existing between the high levels of recombination detected in *S. pneumoniae* and the limited impact of that recombination events on the genetic population structure. Interestingly, the interpherotype recombination barrier is itself codified within the molecular system that promotes recombination.

One question that remains is to know if these effects have been selected or if they were historical incidents in evolution that are maintained by chance alone.

At the origin of this story could be a random mutation on ComC or ComD gene that originated a compensatory mutation on ComD or ComC, respectively, in order to yield a functional signaling pair. This pair would have a different signaling efficiency. The impact of this difference, which has been studied throughout this thesis, could elicit fitness effects that would promote the selection of one of the variants. As a result, it would be expected that with time one of the variants would extinguish.

However, the fact that both sup-populations have limited genetic exchange can facilitate the subsistence within the population of both variants, by decreasing the probability of pherotype conversion, even though one is potentially advantageous over the other. Time will confirm us if what we observe presently is pherotype co-existence or a path to the extinction of one of the pherotypes.

This thesis works unveils a paradigm of a small molecular difference with a significant impact on the pneumococcal population biology. A few aminoacid exchanges modify the interaction between two proteins. This modification impacts on the efficiency of a signaling cascade, a sub-cellular phenomena, which

in turn affects the communication and the global behavior of a population of independent organisms colonizing the same human host. As the referred signaling cascade modulates the process of recombination, the few aminoacid exchanges influence the genetic flow between different pneumococcal strains co-colonizing the same host. As these strains are transmitted from host to host, the impact of the few aminoacid exchanges is propagated throughout the whole pneumococcal population colonizing and infecting the whole human population. It is the communication and interdependence between these different hierarchical organizational levels (molecular, cellular, subpopulation and population levels) that make biological systems highly complex, but at the same time more passionate and challenging objects of scientific inquiry.

Bibliography References

1. Calix JJ, Nahm MH: **A new pneumococcal serotype, 11E, has a variably inactivated wcjE gene.** *J Infect Dis*, **202:**29-38.
2. Song JH, Baek JY, Ko KS: **Comparison of capsular genes of Streptococcus pneumoniae serotype 6A, 6B, 6C, and 6D isolates.** *J Clin Microbiol*.
3. Mizrachi Nebenzahl Y, Porat N, Lifshitz S, Novick S, Levi A, Ling E, Liron O, Mordechai S, Sahu RK, Dagan R: **Virulence of Streptococcus pneumoniae may be determined independently of capsular polysaccharide.** *FEMS Microbiol Lett* 2004, **233:**147-152.
4. Sandgren A, Sjostrom K, Olsson-Liljequist B, Christensson B, Samuelsson A, Kronvall G, Henriques Normark B: **Effect of clonal and serotype-specific properties on the invasive capacity of Streptococcus pneumoniae.** *J Infect Dis* 2004, **189:**785-796.
5. Bruckner R, Nuhn M, Reichmann P, Weber B, Hakenbeck R: **Mosaic genes and mosaic chromosomes-genomic variation in Streptococcus pneumoniae.** *Int J Med Microbiol* 2004, **294:**157-168.
6. Obert C, Sublett J, Kaushal D, Hinojosa E, Barton T, Tuomanen EI, Orihuela CJ: **Identification of a Candidate Streptococcus pneumoniae core genome and regions of diversity correlated with invasive pneumococcal disease.** *Infect Immun* 2006, **74:**4766-4777.
7. Hiller NL, Janto B, Hogg JS, Boissy R, Yu S, Powell E, Keefe R, Ehrlich NE, Shen K, Hayes J, et al: **Comparative genomic analyses of seventeen Streptococcus pneumoniae strains: insights into the pneumococcal supragenome.** *J Bacteriol* 2007, **189:**8186-8195.
8. Hall-Stoodley L, Costerton JW, Stoodley P: **Bacterial biofilms: from the natural environment to infectious diseases.** *Nat Rev Microbiol* 2004, **2:**95-108.
9. Whitchurch CB, Tolker-Nielsen T, Ragas PC, Mattick JS: **Extracellular DNA required for bacterial biofilm formation.** *Science* 2002, **295:**1487.
10. Mulcahy H, Charron-Mazenod L, Lewenza S: **Extracellular DNA chelates cations and induces antibiotic resistance in Pseudomonas aeruginosa biofilms.** *PLoS Pathog* 2008, **4:**e1000213.
11. Vilain S, Pretorius JM, Theron J, Brozel VS: **DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms.** *Appl Environ Microbiol* 2009, **75:**2861-2868.
12. Guiton PS, Hung CS, Kline KA, Roth R, Kau AL, Hayes E, Heuser J, Dodson KW, Caparon MG, Hultgren SJ: **Contribution of autolysin and Sortase a during Enterococcus faecalis DNA-dependent biofilm development.** *Infect Immun* 2009, **77:**3626-3638.
13. Ramirez M, Severina E, Tomasz A: **A high incidence of prophage carriage among natural isolates of Streptococcus pneumoniae.** *J Bacteriol* 1999, **181:**3618-3625.
14. Severina E, Ramirez M, Tomasz A: **Prophage carriage as a molecular epidemiological marker in Streptococcus pneumoniae.** *J Clin Microbiol* 1999, **37:**3308-3315.

15. Frias MJ, Melo-Cristino J, Ramirez M: **The autolysin LytA contributes to efficient bacteriophage progeny release in Streptococcus pneumoniae.** *J Bacteriol* 2009, **191:**5428-5440.
16. Li YH, Lau PC, Lee JH, Ellen RP, Cvitkovitch DG: **Natural genetic transformation of Streptococcus mutans growing in biofilms.** *J Bacteriol* 2001, **183:**897-908.
17. Li YH, N. , Tang MB, Aspiras PC, Lau JHL, Ellen RP, Cvitkovitch DG: **A quorum-sensing signaling system essential for genetic competence in Streptococcus mutans is involved in biofilm formation.** *J Bacteriol* 2002, **184:**2699-2708.
18. Loo CY, Corliss DA, Ganeshkumar N: **Streptococcus gordonii biofilm formation: identification of genes that code for biofilm phenotypes.** *J Bacteriol* 2000, **182:**1374-1382.
19. Petersen FC, Pecharki D, Scheie AA: **Biofilm mode of growth of Streptococcus intermedius favored by a competence-stimulating signaling peptide.** *J Bacteriol* 2004, **186:**6327-6331.
20. Suntharalingam P, Cvitkovitch DG: **Quorum sensing in streptococcal biofilm formation.** *Trends Microbiol* 2005, **13:**3-6.
21. Carrolo M, Pinto FR, Melo-Cristino J, Ramirez M: **Pherotypes are driving genetic differentiation within Streptococcus pneumoniae.** *BMC Microbiol* 2009, **9:**191.
22. Fraser C, Hanage WP, Spratt BG: **Recombination and the nature of bacterial speciation.** *Science* 2007, **315:**476-480.
23. Serrano I, Melo-Cristino J, Carrico JA, Ramirez M: **Characterization of the genetic lineages responsible for pneumococcal invasive disease in Portugal.** *J Clin Microbiol* 2005, **43:**1706-1715.