Biofilm formation in *Streptococcus pneumoniae*

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Summary

Biofilm-grown bacteria are refractory to antimicrobial agents and show an increased capacity to evade the host immune system. In recent years, studies have begun on biofilm formation by *S. pneumoniae*, an important human pathogen, using a variety of *in vitro* model systems. The bacterial cells in these biofilms are held together by an extracellular matrix composed of DNA, proteins and, possibly, polysaccharide(s). Although neither the precise nature of these proteins nor the composition of the putative polysaccharide(s) is clear, it is known that choline-binding proteins are required for successful biofilm formation. Further, many genes appear to be involved, although the role of each appears to vary when biofilms are produced in batch or continuous culture. Prophylactic and therapeutic measures need to be developed to fight *S. pneumoniae* biofilm formation. However, much care needs to be taken when choosing strains for such studies because different *S. pneumoniae* isolates can show remarkable genomic differences. Multispecies and *in vivo* biofilm models must also be developed to provide a more complete understanding of biofilm formation and maintenance.

Introduction

It is well recognized that ‘wild-living’ bacteria organize themselves within biofilms and that their growth rate, metabolism, gene expression and protein production are different to those of planktonic cultures. Biofilms are sessile microbial communities in which cells are attached to a surface or an air–liquid interface, and enveloped within an extracellular polymeric matrix (Costerton et al., 1995). From a medical perspective the importance of biofilms lies in the reduced susceptibility of the participating bacteria to antimicrobial agents (Lewis, 2008) and their ability to evade host immune defence systems (Jensen *et al.*, 2010). Biofilm-associated growth has been associated with a high percentage of patients with chronic and persistent infections, the biofilms acting as pathogen reservoirs (Wolcott and Ehrlich, 2008).

*Streptococcus pneumoniae* is an important human respiratory pathogen that causes a variety of serious diseases such as community-acquired pneumonia, meningitis and sepsis. It is also the main causal agent of otitis media in children. Several authors have recently detected pneumococcal biofilms on the surface of adenoid and mucosal epithelial tissues in children with recurrent middle-ear infections and otitis media with effusion (Hall-Stoodley *et al.*, 2006; Coates *et al.*, 2008; Hoa *et al.*, 2009; Nistico *et al.*, 2011), as well as on the sinus mucosa of patients with chronic rhinosinusitis (Sanderson *et al.*, 2006). Biofilm-like structures detected in the lungs of mice infected with *S. pneumoniae* are similar to those produced in a continuous flow-through biofilm model (Sanchez *et al.*, 2010).

Although the earliest reports on pneumococcal biofilms go back 10 years or more, the last 5 years have seen an increase in the number of studies examining pneumococcal biofilms at the structural and genetic level. Different laboratories have used different approaches for growing biofilms of human pathogens *in vitro* with the aim of producing an appropriate model that mimics *in vivo* environments. The first system designed for pneumococcal biofilms, described in 1997 and developed as a means of assessing susceptibility to antibiotics, was based on steady-state growth on cellulose Sorbarod filters (Budhani and Struthers, 1997). It was later shown that pneumococcal growth on these filters in a continuous-culture background filters (Budhani and Struthers, 1997). It was later shown that pneumococcal growth on these filters in a continuous-culture-like system resembles the nasopharyngeal carriage of the pathogen (Waite *et al.*, 2001). Other research groups developed biofilm reactor systems for analysing pneumococcal biofilms and studying biofilm processes *in situ* and in real time (Donlan *et al.*, 2004; Goeres *et al.*, 2005). Using a continuous-culture...
one-through flow cell, Allegrucci and colleagues (2006) showed that S. pneumoniae adopts multiple phenotypes over the course of biofilm development. Our group has been involved in developing an in vitro biofilm model for S. pneumoniae using polystyrene microtitre plates or glass-bottom dishes as a support. This system allowed the effects of several factors (e.g. nutrients, pH changes, osmolarity, temperature) on biofilm development to be examined (Moscoso et al., 2006), as well as the rapid screening of mutants defective in biofilm formation (Muñoz-Elías et al., 2008).

The present review summarizes the recent genetic, biochemical and structural data reported for S. pneumoniae biofilms.

Environmental factors affecting biofilm formation

The carbon source, the flow velocity and the physical properties of the surface to which bacteria adhere, such as its hydrophobicity and roughness, can lead to differences in the structure and composition of the biofilms produced (O’Toole and Kolter, 1998; Stoodley et al., 1999). Our group analysed the influence of several environmental factors in pneumococcal biofilm formation (Moscoso et al., 2006). The ability of S. pneumoniae to form biofilms on abiotic surfaces was tested on a range of materials including glass, polyvinylchloride and polystyrene, the last of which was associated with the strongest biofilm formation. The intense biofilm production observed on chemically defined (CDen or CDM) and semisynthetic (C) media indicates that biofilm formation represents a survival strategy in a nutritionally limited environment; pneumococcal cells growing in rich media showed poor biofilm formation in polystyrene (or glass) dishes. Enriching C medium with additives such as yeast extract or bovine serum albumin led to no significant change in biofilm formation on glass dishes following 10–12 h of incubation at 34°C (Moscoso et al., 2006; Domenech et al., 2009). These studies detected the presence of small voids and channels separating the microcolonies within the pneumococcal biofilm. Using a continuous-flow biofilm reactor system, Allegrucci and colleagues (2006) reported that the architecture of mature biofilms (those grown at 37°C in 5% CO₂ for 6–9 days) differed significantly among the serotypes tested.

Biofilm ultrastructure

Biofilms formed by a non-encapsulated pneumococcal strain on abiotic surfaces were found to have a three-dimensional organization with complex structures about 25–30 μm in thickness, as revealed by confocal laser scanning microscopy (CLSM) (Moscoso et al., 2006). The spatial distribution of the adherent bacteria was later examined by CLSM using a non-encapsulated S. pneumoniae strain that synthesizes green fluorescent protein, or by staining the bacteria with fluorescent dyes after biofilm formation on glass dishes following 10–12 h of incubation at 34°C (Moscoso et al., 2006; Domenech et al., 2009). These studies detected the presence of small voids and channels separating the microcolonies within the pneumococcal biofilm. Using a continuous-flow biofilm reactor system, Allegrucci and colleagues (2006) reported that the architecture of mature biofilms (those grown at 37°C in 5% CO₂ for 6–9 days) differed significantly among the serotypes tested.

The honeycomb-like structures observed by low-temperature scanning electron microscopy (Moscoso et al., 2006) may provide mechanical stability to pneumococcal biofilms and might serve as an important virulence factor, helping to ward off host defences, as described for other microbial communities (Schaudinn et al., 2007; Moscoso et al., 2009). Figure 1 shows pneumococcal cells associated with the walls of these honeycomb-like structures, as well as their connection to the wall and one another by thin filaments (Fig. 1). Some areas free of bacterial cells may represent channels between cell clusters.

The extracellular matrix of pneumococcal biofilms

Within a biofilm, bacterial cells are embedded in an extracellular matrix composed of different extracellular...
polymeric substances (EPS), including exopolysaccharides, proteins, nucleic acids and lipids. EPS often determine the scaffold for the three-dimensional architecture of the biofilm and provide it structural integrity and cohesion. They also contribute to antimicrobial resistance and host defences mediated by the biofilm, and allow the accumulation of nutrients from the environment and the release of post-death cellular material. The presence of extracellular DNA in the matrix may facilitate horizontal gene transfer (HGT) between biofilm cells (for a review, see Flemming and Wingender, 2010).

Nucleic acids

Extracellular DNA (but apparently not RNA) and extracytoplasmic and surface-exposed proteins appear to be critical elements of the matrix required for the initial attachment and maintenance of pneumococcal biofilms. They also contribute to antimicrobial resistance and host defences mediated by the biofilm, and allow the accumulation of nutrients from the environment and the release of post-death cellular material. The presence of extracellular DNA in the matrix may facilitate horizontal gene transfer (HGT) between biofilm cells (for a review, see Flemming and Wingender, 2010).

Extracellular polysaccharides

It is generally thought that polysaccharides make up a major fraction of the extracellular matrix, providing mechanical stability to the biofilm. Extracellular polysaccharides have been classified as capsular polysaccharides (CPS) when closely associated with the cell surface, and exopolysaccharides when loosely associated (Branda et al., 2005). However, this distinction is inappropriate for biofilms because many of the extracellular polysaccharides they contain are insoluble and cannot be easily separated from cells. In fact, the presence of CPS reduces pneumococcal biofilm development; both clinical pneumococcal isolates and isogenic

Fig. 1. Low-temperature scanning electron micrographs of a S. pneumoniae R6 biofilm.
A. General view of the biofilm formed on the surface of a glass coverslip (g).
B. In the magnification, arrows pointed to filamentous material linking pneumococcal cells to each other and to the intercellular matrix. Bar, 20 μm. Reprinted from Moscoso et al. (2006) with permission.

Fig. 2. Inhibition of biofilm development in S. pneumoniae cultures in the presence of nucleases or proteases.
A. S. pneumoniae R6 was distributed in the wells of a microtitre plate, which was then incubated for 6 h at 34°C (cross-hatched bars). Other samples received either RNase (stippled bars), DNase I (hatched bars), trypsin (blackened bars) or proteinase K (open bars) at the indicated concentrations and were incubated as above. B. After biofilm development, nucleases or proteases were added at 100 μg ml⁻¹ and incubation allowed for an additional 1 h at 34°C before staining with crystal violet to quantify biofilm formation. Slightly modified and reprinted from Moscoso et al. (2006) with permission.

been independently confirmed (Hall-Stoodley et al., 2008; Carrolo et al., 2010). It has recently been proposed that the spontaneous induction of temperate bacteriophages might constitute an important source of extracellular DNA for the pneumococcal biofilm matrix (Carrolo et al., 2010). This agrees with an early report showing that lysogenized S. pneumoniae strains are better biofilm formers than the corresponding cured strains (Loeffler and Fischetti, 2006). Other authors, however, report that DNase treatment does not significantly affect biofilm formation in vitro and suggest that DNA is likely not an essential constituent of biofilms formed under the experimental conditions they used (Muñoz-Elias et al., 2008). It should be mentioned that competence induction and concomitant DNA release in pneumococcus strongly depends on the medium (Moscoso and Claverys, 2004).
encapsulated transformants form significantly less biofilm than non-encapsulated strains (Moscoso et al., 2006). With only one exception (Oggioni et al., 2006) the adherence of non-encapsulated pneumococcal mutants to human bronchial epithelial cells or to abiotic surfaces is reported more efficient than that of encapsulated parent cells. Thus, non-encapsulated pneumococci show a high capacity to form in vitro biofilms (Waite et al., 2001; 2003; Allegrucci and Sauer, 2007; Hiller et al., 2010; Camilli et al., 2011). Whether the discrepancy reported by Oggioni and colleagues (2006) is related to the particular conditions these authors used for growing their biofilms, i.e. tryptic soy broth, incubation of microplates in a CO2-enriched atmosphere, and the addition of competence stimulating peptide, is not known. It has been shown that the emergence of non-encapsulated genotypic and phenotypic variants enhance S. pneumoniae biofilm development. Different types of mutation (single nucleotide polymorphisms, deletions, tandem sequence duplications, etc.), mainly involving the cap3A/cps3A gene, have been seen among spontaneous capsular mutants of S. pneumoniae type 3 biofilms grown on microtitre plates, Sorbarod filters, flow cells and membrane filters (Waite et al., 2001; Allegrucci and Sauer, 2007; McEllistrem et al., 2007; Domenech et al., 2009). The existence of an inverse relationship between the ability of the non-encapsulated variants to form biofilms and the amount of CPS has also been found (Domenech et al., 2009) (Fig. 3). Thus, the non-encapsulated mutants of S. pneumoniae type 3, as good biofilm formers, might be essential in the attachment stage of biofilm formation, and that those variants producing reduced quantities of CPS might only appear in later stages (Allegrucci and Sauer, 2007; Domenech et al., 2009). These results are in keeping with the proposal that pneumococci regulate capsule expression in the transition from nasopharyngeal carriage associated with biofilm development to invasive disease (Waite et al., 2003), as recently shown in Neisseria meningitidis (O’Dwyer et al., 2009). In addition to one study showing that phenotypic variation of the polysaccharide capsule occurs in the initial phase of pneumococcal infections (Hammerschmidt et al., 2005), real-time quantitative PCR results have indicated that cpsA, the first gene of the pneumococcal capsule operon, is downregulated (by up to 10-fold) during biofilm growth compared with that seen in planktonic cultures (Hall-Stoodley et al., 2008). Further, in situ capsule immunofluorescence staining is brighter in biofilm towers of encapsulated S. pneumoniae strains that in adherent cells, suggesting that surface-attached pneumococci have a reduced amount of capsule (Hall-Stoodley et al., 2008). It should be noted here that the factors involved in the regulation of S. pneumoniae CPS biosynthesis remain essentially unknown (Moscoso and García, 2009).

Several methods have been used to try to identify the EPS putatively forming the structural scaffolding of the pneumococcal biofilm matrix. In one experiment, real-time monitoring of S. pneumoniae in a biofilm reactor system led to the spectroscopic detection and quantification of proteins and polysaccharides during biofilm formation (Donlan et al., 2004). In addition, ‘EPS clouds’ were observed in some thick biofilm areas after staining with fluorescently labelled wheat germ agglutinin, suggesting that N-acetylglucosamine residues are one of the biofilm matrix components. However, a clinical, encapsulated pneumococcal isolate was used and its serotype not specified (Donlan et al., 2004). Thus, whether the N-acetylglucosamine residues belong to a previously unidentified polysaccharide or to the cell wall peptidoglycan and/or the CPS remains to be determined. More recently,
strain-related variability in the EPS distribution of pneumococcal biofilms was demonstrated using a cocktail of five fluorescently conjugated lectins (Hall-Stoodley et al., 2008). However, further experiments are required to determine the composition and distribution of the carbohydrate(s) in the matrix.

The use of calcofluor white M2R to stain non-encapsulated pneumococcal cells has revealed that only biofilm-growing cells (Fig. 4), but not planktonic cells (not shown), were able to bind calcofluor. This indicates that S. pneumoniae biofilms are composed of aggregates of microbial cells encased in an extracellular polysaccharide matrix (different to the CPS) that contains – at least – β-linked D-glycopyranosyl units (M. Domenech, M. Moscoso, E. García, in preparation), because calcofluor white M2R is a compound that binds to β-1,3 and β-1,4 polysaccharides (Harrington and Hageage, 2003).

Gene expression patterns and protein production

It is well known that bacteria growing in biofilms show physiological and metabolic differences to their planktonic counterparts. The different stages of biofilm development, such as initial attachment and biofilm maturation, likely require the expression of genes different to those expressed by planktonic cells. Gene expression patterns of S. pneumoniae strain TIGR4 recovered from the tissues of mice with pneumonia or meningitis are similar to that of pneumococci growing in biofilms for nearly all the genes studied (Oggioni et al., 2006). An increase in the expression of neuraminidase-coding genes (nanA/SP_1693 and nanB/SP_1687), competence genes (comA/SP_0042 and comX/SP_0014) and the virulence gene regulator mgrA/SP_1800 in lung and brain tissue isolates and biofilm bacteria was also reported. These authors also assert that sessile cells grown in a biofilm were more effective at inducing meningitis and pneumonia than planktonic cells (Oggioni et al., 2006). Certainly it has been reported that biofilm formation occurs at a slightly higher frequency ($P = 0.04$) among S. pneumoniae isolated from respiratory samples provided by patients with cystic fibrosis than among those from blood provided by subjects without cystic fibrosis (Garcia-Castillo et al., 2007). However, a correlation between the ability to form in vitro biofilms and the origin of pneumococcal isolates (either from the nasopharynx, middle-ear effusion or blood) (Tapiainen et al., 2010), or the clinical presentation of pneumococcal disease (Lizcano et al., 2010), does not appear to exist.

Proteomic studies have revealed an increase in the number of proteins synthesized de novo and differences in protein production patterns over the course of S. pneumoniae biofilm development (Allegrucci et al., 2006). A number of proteins differentially produced during biofilm development were identified by mass spectrometry as proteins involved in virulence, adhesion and resistance. Pneumolysin and pyruvate oxidase, two proteins associated with virulence, were the most abundant S. pneumoniae serotype 3 proteins obtained from 3- and 6-day-old biofilms. A discrepancy noted in the overexpression of pneumolysin in pneumococcal biofilms, possibly due to the production methods used, has been discussed elsewhere (Moscoso et al., 2009). In addition, high concentrations of the α-subunit of ATP synthetase $F_0$, of fructose-stimulated pyruvate kinase and of several surface-associated proteins (such as enolase, peptide methionine sulfoxide reductase MsrA and glyceraldehyde-3-phosphate dehydrogenase) were found after 3 days of biofilm growth (Allegrucci et al., 2006). Enzymes involved in glycolysis, gluconeogenesis and starch metabolism, such as NADP-specific glutamate dehydrogenase, glucose-6-phosphate isomerase and phosphoglycerate kinase, were the most abundant under planktonic growth conditions (Allegrucci et al., 2006).

Muñoz-Elías and colleagues (2008) used a collection of transposon insertion S. pneumoniae mutants to identify pneumococcal genes required for the initiation of biofilm development, and, in some cases, for the nasopharyngeal colonization of mice. The ability of these mutants to form...
biofilms was determined by their attachment to polystyrene plates. Mutations in the genes coding for the choline-binding proteins (lytC/SP_1593, cbpA/SP_2190, cbpF/SP_0391), the neuraminidases (nanB/SP_1687), a putative cardiolipin synthase (SP_0199), the synthases of membrane and cell wall components (fibA/SP_0615, murE/SP_1531, murB/SP_1390), the ABC and PTS transporters (ailB/SP_1527, SP_1682, SP_0137), the proteolytic and ATP-binding subunits of the Clp proteases family (clpP/SP_0746, clpX/SP_1569, clpC/SP_1294), the components of the shikimate pathway for synthesis of isochorismate (aroK/SP_1370, SP_1745), and other conserved proteins of unknown function, all contributed towards biofilm formation (Muñoz-Elías et al., 2008). Moreover, the RrgA subunit of the pili (which are present in some but not all pneumococcal strains), but not the pilus structure per se, was reported to function as an adhesin in biofilm formation. Two genes involved in signal transduction (i.e. SP_2192 and ciaH/SP_0799) were also found to affect biofilm growth, as were two insertions in putative transcriptional regulators (SP_2131, LacR2/SP_1182), which led to biofilm hyperformation (Muñoz-Elías et al., 2008; Trappetti et al., 2011b). In addition, pneumococcal rgg mutants, which are deficient in the putative transcriptional regulator Rgg, are reported more susceptible to oxidative stress and to show a reduced ability to form biofilms (Bortoni et al., 2009). The latter authors found this mutant to be sensitive to oxygen and paraquat, but not to H2O2. Interestingly, as mentioned above, they also described a role for pyruvate oxidase SpxB and its product, hydrogen peroxide, in the emergence of biofilm-derived variants of S. pneumoniae type 19.

The noticeable differences in the subset of biofilm-related genes identified by mutagenic approaches and proteomic analysis may be due to differences in the sensitivity of these systems, in the levels of transcription and translation, the genetic background of the strains used and/or the biofilm model used.

The roles of pneumococcal surface proteins in biofilm formation have been investigated, especially of those involved in nasopharyngeal colonization and adherence to the host cell (Hammerschmidt, 2006). Choline-binding proteins (López and García, 2004; López et al., 2004), which bind the choline residues in cell wall teichoic acids, cell wall hydrolase LytA (the major autolysin), LytB (a glucosaminidase involved in daughter cell separation) and LytC (a lysozyme acting as an autolysin at 30°C) were all shown to contribute to S. pneumoniae biofilm formation by non-encapsulated strains. Moreover, the inactivation of the genes coding for pneumococcal surface protein A or of the putative adhesins PcpA and CbpA leads to reduced biofilm formation on polystyrene plates (Moscoso et al., 2006). Although the implication of CbpA in biofilm formation has been confirmed in non-encapsulated laboratory mutants, cpbA mutants in an encapsulated background showed levels of biofilm formation comparable with that of the parental wild-type strain (Muñoz-Elías et al., 2008; Lizcano et al., 2010). The reasons for the discrepancies between encapsulated and non-encapsulated strains remain unclear.

The choline residues in cell wall teichoic acids were found to play an essential role in pneumococcal biofilm development when, after incubating pneumococci in the presence of high concentrations of choline or ethanolamine – at which some choline-binding proteins are inhibited or released from the surface of pneumococcal cells (López et al., 2004) – a notable reduction in biofilm formation was observed (Moscoso et al., 2006). In addition, Trappetti and colleagues (2011b) recently proposed that the lic operon involved in choline metabolism (Hakenbeck et al., 2009) also contributes to the formation of the matrix.

Roles for neuraminidase NanA and the pneumococcal serine-rich repeat protein PsrP in biofilm maturation have also been proposed. Using a modified in vitro biofilm model in which pneumococci had previously interacted with human airway epithelial cells, it was shown that NanA neuraminidase, which is conserved in all pneumococcal strains tested, albeit with a high level of diversity (King et al., 2005), releases terminal sialic acid residues from glycoconjugates, thus contributing to biofilm formation (Parker et al., 2009). Similarly, the Basic Region domain of PspR, located in a pathogenicity island present in a number of strains of S. pneumoniae, is reported involved in mature biofilm formation and promotes the formation of bacterial aggregates in the nasopharynx and lungs of infected mice (Sanchez et al., 2010).

Prevention of biofilm formation and therapy

One of the most important and persistent problems posed by biofilms is the inherent tolerance of their associated communities to antibiotic therapy and host defence mechanisms. Different strategies have been developed for the prevention and treatment of biofilm-related infections, such as the use of enzymes that degrade the biofilm matrix, inhibitors of quorum-sensing signals, antimicrobial and anticoagulant agents, surfactants and specific bacteriophages (Kaplan, 2010). Many bacteriophages produce depolymerases, i.e. enzymes that hydrolyse the polysaccharides of the biofilm matrix. The topical application of a mixture of phages on the surface of medical devices to prevent biofilm formation has also been proposed (Azeredo and Sutherland, 2008; Fu et al., 2010). In addition, phages (or their depolymerases) followed by disinfectants may be more effective in the control of biofilm formation than either alone (Flemming and Wingender, 2010).
The literature only contains a few reports on the activity of antibiotics against pneumococcal biofilms, and the data are inconclusive. No protection against the activity of benzylpenicillin, ampicillin, amoxicillin-clavulanic acid or cefuroxime was found when a penicillin-susceptible *S. pneumoniae* isolate was grown as a biofilm on Sorbarod filters (Budhani and Struthers, 1997). However, it has been reported that amoxicillin, erythromycin and levofloxacin at supra-MIC concentrations are less active against biofilm-associated pneumococci than against planktonic cells (del Prado et al., 2010), and it has been suggested that isolates forming biofilms from patients with cystic fibrosis may have become highly adapted to the presence of antibiotics such as penicillin and tetracycline (Garcia-Castillo et al., 2007). In addition, cefditoren, an oral third-generation cephalosporin, better interferes with *S. pneumoniae* biofilm development than does amoxicillin-clavulanic acid (Maestre et al., 2010). Interestingly, moxifloxacin, a fourth-generation oral fluoroquinolone, can inhibit the formation of, and indeed disrupt, biofilms produced by respiratory pathogens, including *S. pneumoniae*, at concentrations easily achieved in the bronchial mucosa (Roveta et al., 2007).

The information on the effect of other agents such as *N*-acetyl-L-cysteine (NAC) or xylitol on pneumococcal biofilm formation is also limited. NAC, a thiol-containing antioxidant that disrupts disulfide bonds in mucus, is used in the treatment of chronic bronchitis, cancer and paracetamol poisoning, but it also has antibacterial properties. Contrary to previous findings with staphylococci (O’Dwyer et al., 2009), it has been reported that NAC alone has very little activity against either planktonic (MIC 4–10 mg ml⁻¹) or biofilm-grown clinical *S. pneumoniae* isolates (del Prado et al., 2010). Combining NAC with amoxicillin, erythromycin and/or levofloxacin barely enhanced the antibacterial activity of either compound. However, intramuscular-to-aerosol sequential therapy using NAC plus thiamphenicol had a noticeable clinical success rate (84–100%) in patients with recurrent rhinosinusitis and other upper respiratory tract infections in which biofilms have been proven present (Macchi et al., 2006). At our laboratory, NAC inhibited the formation a biofilm by a non-encapsulated *S. pneumoniae* strain and partially disintegrated a previously formed biofilm at concentrations around the MIC (Fig. 5A and B). As discussed elsewhere (Riise et al., 2000), the concentrations of NAC that showed inhibition in these assays came close to that which theoretically can be obtained in oropharyngeal secretions when a normal dose of oral NAC medication is administered.

Xylitol, which is non-fermentable by oral bacteria, is known to inhibit growth, metabolism and polysaccharide production in cariogenic *Streptococcus mutans*. In addition to growth inhibition, the reduction of insoluble extracellular polysaccharides is probably important in xylitol-induced reductions of both bacterial numbers and transmission (Söderling, 2009). Xylitol may also be useful for the prophylaxis of acute otitis media in children, although in tests it did not reduce the nasopharyngeal carriage of pneumococci (reviewed by Danhauer et al., 2010). When clinical *S. pneumoniae* isolates were incubated with xylitol at concentrations of up to 50 mg ml⁻¹ the inhibition of biofilm formation was reported as either insignificant (Ruiz et al., 2011) or only small (Kurola et al., 2011). In contrast, when the growth medium was supplemented with xylitol plus glucose or fructose (5 mg ml⁻¹), biofilm formation was enhanced (Kurola et al., 2011). A significant reduction in biofilm formation by non-encapsulated, laboratory strains of *S. pneumoniae* has been observed in our laboratory at concentrations of ≥ 50 mg ml⁻¹ (Fig. 5C). However, biofilm disintegration was not observed when xylitol was added at concentrations of < 50 mg ml⁻¹ (Fig. 5D).

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**Fig. 5.** Inhibition of biofilm development and biofilm dispersal in *S. pneumoniae* cultures in the presence of *N*-acetyl-cysteine or xylitol.

A. *S. pneumoniae* R6 was distributed in the wells of a microtitre plate, which was then incubated for 6 h at 34°C in the presence of different concentrations of NAC. Biofilms were washed with fresh CPsh 8 medium and incubated with NAC for 2 h at 37°C. C and D. As in (A) and (B) respectively, but with xylitol instead of NAC. Biofilm formation was quantified by staining with crystal violet. Open and blackened bars indicate growth and biofilm formation respectively. In all panels the results represent the mean ± standard error of at least four independent experiments, each performed in triplicate. Asterisk-marked results are statistically significant (*P < 0.05; **P < 0.01) compared with the control.
Ceragenin CSA-13, a cholic acid derivative that mimics the activity of antimicrobial peptides, is capable of actively destroying the biofilms formed by *S. pneumoniae* (M.M. Esteban, M. Moscoso, E. García, in preparation), in a manner similar to that previously reported for young and mature *Pseudomonas aeruginosa* biofilms (Nagant et al., 2010).

An alternative strategy for eradicating pneumococcal biofilms is the use of cell wall hydrolases encoded by *S. pneumoniae* and its phages. The antimicrobial activity of these enzymes on *S. pneumoniae* planktonic cultures, as well as its therapeutic effects in animal models, have been reported (reviewed by Hermoso et al., 2007). Recently, promising results regarding the destruction of pneumococcal biofilms in vitro have been obtained (M. Domenech, E. García, M. Moscoso submitted).

**Future perspectives**

Although great advances in understanding *S. pneumoniae* biofilm development and biofilm-related infections have been made in the last 5 years, many basic aspects of biofilm formation remain to be investigated. The genetic grounds upon which pneumococcal biofilm formation and maturation is based are still largely unknown, probably because the genes essential for biofilm formation are yet to be identified. Further, the diversity of the polysaccharide and protein components of the pneumococcal biofilm matrix still needs to be characterized. Future studies should investigate the molecular mechanisms underlying the regulation of the synthesis and/or degradation of the matrix.

Upper respiratory tract infections are caused by the synergistic and/or antagonistic interactions between the commensal microbiota, respiratory viruses and potential pathogens such as *S. pneumoniae, Haemophilus influenzae* or *Moraxella catarrhalis* (Murphy et al., 2009; Laufer et al., 2011). However, most of our current knowledge about biofilm-related infections is derived from monospecific studies. It would be very interesting to use simple continuous culture biofilm systems to investigate the potential indirect pathogenicity of mixed *S. pneumoniae* and *M. catarrhalis* infections (Budhani and Struthers, 1998) and to study bacterial interactions under, for example, antibiotic stress. It has recently been reported that the presence of *H. influenzae* increases pneumococcal biofilm formation in vitro as well as the persistence of pneumococci on the mucosal surface of the middle ear (Weimer et al., 2010). Non-typeable *H. influenzae* appear to provide passive protection against pneumococcus in the chinchilla model through two mechanisms: the production of β-lactamase and the formation of biofilm communities (Weimer et al., 2011). Studying interspecies interactions in biofilms may be a new way to gain insight into the events underlying the formation and maintenance of mixed biofilms and pneumococcal disease such as otitis media, pneumonia and meningitis. Moreover, it would be interesting to test the capacity of *S. pneumoniae* to form biofilms, ideally using more than one model system. In this context, efforts to develop *in vivo* models of pneumococcal biofilms may represent an important technical step forward (Chaney et al., 2011).

Many bacteria use intercellular, cell density-dependent communication systems (quorum sensing systems) to coordinate the expression of genes involved in the regulation of their interactions with one another and their environment. Information on the communication systems used by *S. pneumoniae* is limited. It has been shown that the induction of the pneumococcal competence system by competence-stimulating (quorum sensing) peptide (CSP) promotes stable biofilm formation in vitro (Oggoni et al., 2006), although its impact varies depending on the experimental biofilm model used (Trappetti et al., 2011a). Therefore, in the search for new therapies, the inter- and intracellular signals that regulate the formation and/or dispersal of *S. pneumoniae* biofilms need to be identified.

Few studies have documented strategies to prevent biofilm development in human infections and any benefits it might bring. Given the important role of NanA neuraminidase in biofilm formation and nasopharyngeal colonization, efforts must be made to identify inhibitors targeting pneumococcal neuraminidase. Competition experiments using neuraminidase inhibitors have been performed (Trappetti et al., 2009), and the *in silico* docking studies reported by Parker and colleagues (2009) look promising. These authors identified a potent inhibitor of NanA neuraminidase activity (known as XX1) that acts at concentrations in the low-micromolar range. It also inhibits biofilm formation. Studies to characterize the dispersal mechanisms of *S. pneumoniae* biofilms would also help in the development of agents that promote their eradication. Finally, biofilms show resistance to host phagocytic defences (Bryers, 2008), but at the moment this has received little research attention in *S. pneumoniae*. Most of our information on the immune response to bacteria has been obtained using planktonic cultures; studies focused on the interaction between biofilm-associated pneumococci and the host immune system are therefore necessary.

The available data clearly show that many genes fulfilling quite diverse functions are involved in the formation and dispersal of *S. pneumoniae* biofilms. It is currently recognized that pneumococcus is a genetically diverse species capable of evolving over short-time scales, mainly by intra- and inter-species HGT (Donati et al., 2010). Biofilms provide the ideal environment for facilitating HGT in *S. pneumoniae* as this naturally transformable bacterium may easily encounter extracellular DNA that forms part of the biofilm matrix (Moscoso et al., 2006). Ehrlich
and colleagues (2010) indicated that to be capable of promoting HGT, biofilms should be polyclonal in nature, being formed by different strains of the same species and/or different species. The real-time in vivo generation of pneumococcal genetic diversity has recently been documented (Hiller et al., 2010). Over a period of 7 months, a high degree of HGT was found within the S. pneumoniae strains isolated from a child suffering from chronic upper respiratory and middle-ear infections. Interestingly, sequencing showed four of the six isolates to have non-typeable genomes, which were all capable of forming more biofilm than their encapsulated progenitor(s) (Hiller et al., 2010). This appears to be important for the establishment and maintenance of chronic otitis media. Given the enormous variability seen among S. pneumoniae isolates, studies on pneumococcal biofilms should be performed with strains differing in just one gene (or only a few) for reliable conclusions to be drawn.

Acknowledgements

We thank R. López and P. García for helpful comments and for critically reading the manuscript, and A. Burton for revising the English version. Partial support for this research was provided by Grant SAF2009-10824 from the Dirección General de Investigación Científica y Técnica of Spain. Centro de Investigación Biomédica en Red de Enfermedades Respiratorias (CIBERES) is an initiative of the ISCIII. M.D. was supported by an FPI fellowship from the Spanish Ministerio de Ciencia e Innovación.

References

Allegrucci, M., and Sauer, K. (2007) Characterization of colony morphology variants isolated from Streptococcus pneumoniae biofilms. J Bacteriol 189: 2030–2038.

Allegrucci, M., and Sauer, K. (2008) Formation of Streptococ- cus pneumoniae non-phase-variable colony variants is due to increased mutation frequency present under biofilm growth conditions. J Bacteriol 190: 6330–6339.

Allegrucci, M., Hu, F.Z., Shen, K., Hayes, J., Ehrlich, G.D., Post, J.C., and Sauer, K. (2006) Phenotypic characterization of Streptococcus pneumoniae biofilm development. J Bacteriol 188: 2325–2335.

Azeredo, J., and Sutherland, I.W. (2008) The use of phages for the removal of infectious biofilms. Curr Pharm Biotechnol 9: 261–266.

Bortoni, M.E., Terra, V.S., Hinds, J., Andrew, P.W., and Yesilkaya, H. (2009) The pneumococcal response to oxidative stress includes a role for Rgg. Microbiology 155: 4123–4134.

Brandá, S.S., Vik, Á., Friedman, L., and Kolter, R. (2005) Biofilms: the matrix revisited. Trends Microbiol 13: 20–26.

Bryers, J.D. (2008) Medical biofilms. Biotechnol Bioeng 100: 1–18.

Budhani, R.K., and Struthers, J.K. (1997) The use of Sor- barod biofilms to study the antimicrobial susceptibility of a strain of Streptococcus pneumoniae. J Antimicrob Chemother 40: 601–602.

Budhani, R.K., and Struthers, J.K. (1998) Interaction of Strepto- cococcus pneumoniae and Moraxella catarrhalis: investigation of the indirect pathogenic role of β-lactamase-producing moraxellae by use of a continuous-culture biofilm system. Antimicrob Agents Chemother 42: 2521–2526.

Camilli, R., Pantosti, A., and Baldassarri, L. (2011) Contribution of serotype and genetic background to biofilm forma- tion by Streptococcus pneumoniae. Eur J Clin Microbiol Infect Dis 30: 97–102.

Carrolo, M., Frias, M.J., Pinto, F.R., Melo-Cristino, J., and Ramirez, M. (2010) Prophage spontaneous activation promotes DNA release enhancing biofilm formation in Streptococcus pneumoniae. PLoS ONE 5: e15678.

Chaney, E.J., Nguyen, C.T., and Boppart, S.A. (2011) Novel method for non-invasive induction of a middle-ear biofilm in the rat. Vaccine 29: 1628–1633.

Coates, H., Thomson, R., Langlands, J., Filon, P., Keil, A.D., Vijayasekaran, S., and Richmond, P. (2008) The role of chronic infection in children with otitis media with effusion: evidence for intracellular persistence of bacteria. Otolaryngol Head Neck Surg 138: 778–781.

Costerton, J.W., Lewandowski, Z., Caldwell, D.E., Korber, D.R., and Lappin-Scott, H.M. (1995) Microbial biofilms. Annu Rev Microbiol 49: 711–745.

Danhauer, J.L., Johnson, C.E., Corbin, N.E., and Bruccheri, K.G. (2010) Xylitol as a prophylaxis for acute otitis media: systematic review. Int J Audiol 49: 754–761.

Domenech, M., García, E., and Moscoso, M. (2009) Versatility of the capsular genes during biofilm formation by Streptococcus pneumoniae. Environ Microbiol 11: 2542–2555.

Donati, C., Hiller, N.L., Tettelin, H., Muzzi, A., Croucher, N., Anguoli, S., et al. (2010) Structure and dynamics of the pan-genome of Streptococcus pneumoniae and closely related species. Genome Biol 11: R107.

Donlan, R.M., Pide, J.A., Heyes, C.D., Sanii, L., Murga, R., Edmonds, P., et al. (2004) Model system for growing and quantifying Streptococcus pneumoniae biofilms in situ and in real time. Appl Environ Microbiol 70: 4980–4988.

Ehrlich, G.D., Ahmed, A., Earl, J., Hiller, N.L., Costerton, J.W., Stoodley, P., et al. (2010) The distributed genome hypothesis as a rubric for understanding evolution in situ during chronic bacterial biofilm infectious processes. FEMS Immunol Med Microbiol 59: 269–279.

Flemming, H.-C., and Wingender, J. (2010) The biofilm matrix. Nat Rev Microbiol 8: 623–633.

Fu, W., Forster, T., Mayer, O., Curtin, J.J., Lehman, S.M., and Donlan, R.M. (2010) Bacteriophage cocktail for the prevention of biofilm formation by Pseudomonas aeruginosa on catheters in an in vitro model system. Antimicrob Agents Chemother 54: 397–404.

García-Castillo, M., Morosini, M.I., Valverde, A., Almaraz, F., Baquero, F., Cantón, R., and del Campo, R. (2007) Differ- ences in biofilm development and antibiotic susceptibility among Streptococcus pneumoniae isolates from cystic fibrosis samples and blood cultures. J Antimicrob Chemother 59: 301–304.

Goeres, D.M., Loetterle, L.R., Hamilton, M.A., Murga, R., Kirby, D.W., and Donlan, R.M. (2005) Statistical assessment of a laboratory method for growing biofilms. Microbi- ology 151: 757–762.

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Hakenbeck, R., Madhour, A., Denapaite, D., and Brückner, R. (2009) Versatility of choline metabolism and choline-binding proteins in *Streptococcus pneumoniae* and commensal streptococci. *FEMS Microbiol Rev* 33: 572–586.

Hall-Stoodley, L., Hu, F.Z., Gieske, A., Nistico, L., Nguyen, D., Hayes, J., *et al.* (2006) Direct detection of bacterial biofilms on the middle-ear mucosa of children with chronic otitis media. *JAMA* 296: 202–211.

Hall-Stoodley, L., Nistico, L., Sambanthamoorthy, K., Dice, B., Nguyen, D., Mershon, W.J., *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.

Hammerschmidt, S. (2006) Adherence molecules of pathogenic pneumococci. *Curr Opin Microbiol* 9: 12–20.

Hammerschmidt, S., Wolff, S., Hocke, A., Rosseau, S., Müller, E., and Rohde, M. (2005) Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. *Infect Immun* 73: 4653–4667.

Hall-Stoodley, L., and Hageage, G.J. (2003) Calcofluor white: a review of its uses and applications in clinical mycology and parasitology. *Lab Med* 34: 361–367.

Hermoso, J.A., García, J.L., and García, P. (2007) Taking aim on bacterial pathogens: from phage therapy to enzybiotics. *Curr Opin Microbiol* 10: 461–472.

Hiller, N.L., Ahmed, A., Powell, E., Martin, D.P., Eutsey, R., Earl, J., *et al.* (2010) Generation of genic diversity among *Streptococcus pneumoniae* strains via horizontal gene transfer during a chronic polyclonal pediatric infection. *PLoS Pathog* 6: e1001108.

Ho a, M., Tomovic, S., Nistico, L., Hall-Stoodley, L., Stoodley, P., Sachdeva, L., *et al.* (2009) Identification of adenoid biofilms with middle ear pathogens in otitis-prone children utilizing SEM and FISH. *Int J Pediatr Otalaryngol* 73: 1242–1248.

Jensen, P.O., Givskov, M., Bjarnsholt, T., and Moser, C. (2010) The immune system vs. *Pseudomonas aeruginosa* biofilms. *FEMS Immunol Med Microbiol* 59: 292–305.

Kaplan, J.B. (2010) Biofilm dispersal: mechanisms, clinical implications, and potential therapeutic uses. *J Dent Res* 89: 205–218.

King, S.J., Whatmore, A.M., and Dowson, C.G. (2005) NanA, a neuraminidase from *Streptococcus pneumoniae*, shows high levels of sequence diversity, at least in part through recombination with *Streptococcus oralis*. *J Bacteriol* 187: 5376–5386.

Kurola, P., Taiapainen, T., Sevander, J., Kajialainen, T., Leinonen, M., Uhari, M., and Saukkoriipi, A. (2011) Effect of xyitol and other carbon sources on *Streptococcus pneumoniae* biofilm formation and gene expression in vitro. *APMIS* 119: 135–142.

Lafer, A.S., Metlay, J.P., Gent, J.F., Fennie, K.P., Kong, Y., and Pettigrew, M.M. (2011) Microbial communities of the upper respiratory tract and otitis media in children. *mBio* 2: e00245–10.

Lewis, K. (2008) Multidrug tolerance of biofilms and persistier cells. *Curr Top Microbiol Immunol* 322: 107–131.

Lizcano, A., Chin, T., Sauer, K., Tuomanen, E.I., and Orihuela, C.J. (2009) Early biofilm formation on microtiter plates is not correlated with the invasive disease potential of *Streptococcus pneumoniae*. *Microb Pathog* 48: 124–130.

Lofeffler, J.M., and Fischetti, V.A. (2006) Lysogeny of *Streptococcus pneumoniae* with MM1 phage: improved adherence and other phenotypic changes. *Infect Immun* 74: 4486–4495.

López, R., and García, E. (2004) Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. *FEMS Microbiol Rev* 28: 553–580.

López, R., García, E., García, P., and García, J.L. (2004) Cell wall hydrolases. In *The Pneumococcus*. Tuomanen, E.I., Mitchell, T.J., Morrison, D.A., and Spratt, B.G. (eds). Washington, DC, USA: ASM Press, pp. 75–88.

Macchi, A., Ardito, F., Marchese, A., Schito, G.C., and Fadda, G. (2006) Efficacy of N-acetyl-cysteine in combination with thiamphenicol in sequential (intramuscular/aerosol) therapy of upper respiratory tract infections even when sustained by bacterial biofilms. *J Chemother* 18: 507–513.

McEllistrem, M.C., Ransford, J.V., and Khan, S.A. (2007) Characterization of in vitro biofilm-associated pneumococcal phase variants of a clinically relevant serotype 3 clone. *J Clin Microbiol* 45: 97–101.

Maestre, J.R., Mateo, M., Méndez, M.L., Aguilar, L., Gimenez, M.J., Alou, L., *et al.* (2010) In vitro interference of β-lactams with biofilm development by prevalent community respiratory tract isolates. *Int J Antimicrob Agents* 35: 274–277.

Moscoso, M., and Claverys, J.P. (2004) Release of DNA into the medium by competent *Streptococcus pneumoniae*: kinetics, mechanism and stability of the liberated DNA. *Mol Microbiol* 54: 783–794.

Moscoso, M., and García, E. (2009) Transcriptional regulation of the capsular polysaccharide biosynthesis locus of *Streptococcus pneumoniae*: a bioinformatic analysis. *DNA Res* 16: 177–186.

Moscoso, M., García, E., and López, R. (2006) Biofilm formation by *Streptococcus pneumoniae*: role of choline, extracellular DNA, and capsular polysaccharide in microbial accretion. *J Bacteriol* 188: 7785–7795.

Morris, M., García, E., and López, R. (2009) Pneumococcal biofilms. *Int Microbiol* 12: 77–85.

Muñoz-Elias, E.J., Marcano, J., and Camilli, A. (2008) Isolation of *Streptococcus pneumoniae* biofilm mutants and their characterization during nasopharyngeal colonization. *Infect Immun* 76: 5049–5061.

Murphy, T.F., Bakaletz, L.O., and Smeesters, P.R. (2009) Microbial interactions in the respiratory tract. *Pediatr Infect Dis J* 28: S121–S126.

Nagat, C., Tré-Hardy, M., El-Ouaaliti, M., Savage, P., Deleeschouwer, M., and Dehaye, J.P. (2010) Interaction between tobramycin and CSA-13 on clinical isolates of *Pseudomonas aeruginosa* in a model of young and mature biofilms. *Appl Microbiol Biotechnol* 88: 251–263.

Nistico, L., Kreft, R., Gieseke, A., Coticchia, J.M., Burrows, A., Kham pang, P., et al. (2011) Adenoid reservoir for pathogenic biofilm bacteria. *J Clin Microbiol* 49: 1411–1420.

O’Dwyer, C.A., Li, M.-S., Langford, P.R., and Kroll, J.S. (2009) Meningococcal biofilm growth on an abiotic surface – a model for epithelial colonization? *Microbiology* 155: 1940–1952.

O’Toole, G.A., and Kolter, R. (1998) Initiation of biofilms formation in *Pseudomonas fluorescens* WCS365 proceeds
via multiple, convergent signalling pathways: a genetic analysis. Mol Microbiol 28: 449–461.

Oggoni, M.R., Trappetti, C., Kadioglou, A., Cassone, M., Iannelli, F., Ricci, S., et al. (2006) Switch from planktonic to sessile life: a major event in pneumococcal pathogenesis. Mol Microbiol 61: 1196–1210.

Parker, D., Soong, G., Planet, P., Brower, J., Ratner, A.J., and Prince, A. (2009) The NanA neuraminidase of Streptococcus pneumoniae is involved in biofilm formation. Infect Immun 77: 3722–3730.

Pericone, C.D., Park, S., Imlay, J.A., and Weiser, J.N. (2003) Factors contributing to hydrogen peroxide resistance in Streptococcus pneumoniae include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the Fenton reaction. J Bacteriol 185: 6815–6825.

del Prado, G., Ruiz, V., Naves, P., Rodriguez-Cerrato, V., Soriano, F., and Ponte, M.C. (2010) Biofilm formation by Streptococcus pneumoniae strains and effects of human serum albumin, ibuprofen, N-acetyl-L-cysteine, amoxicillin, erythromycin, and levofloxacin. Diagn Microbiol Infect Dis 67: 311–318.

Riise, G.C., Qvarfordt, I., Larsson, S., Eliasson, V., and Andersson, B.A. (2000) Inhibitory effect of N-acetylcysteine on adherence of Streptococcus pneumoniae and Haemophilus influenzae to human oropharyngeal epithelial cells in vitro. Respiration 67: 552–558.

Roveta, S., Schito, A.M., Marchese, A., and Schito, G.C. (2007) Activity of moxifloxacin on biofilms produced in vitro by bacterial pathogens involved in acute exacerbations of chronic bronchitis. Int J Antimicrob Agents 30: 415–421.

Ruiz, V., Rodriguez-Cerrato, V., Huelves, L., del Prado, G., Naves, P., Ponte, C., and Soriano, F. (2011) Adherence of Streptococcus pneumoniae to polystyrene plates and epithelial cells and the antiadhesive potential of albumin and xylitol. Pediatr Res 69: 23–27.

Sanchez, C.J., Shivshankar, P., Stol, K., Trakhtenbroit, S., Sullam, P.M., Sauer, K., et al. (2010) The pneumococcal serine-rich repeat protein is an intra-species bacterial adhesin that promotes bacterial aggregation in vivo and in biofilms. PLoS Pathog 6: e1001044.

Sanderson, A.R., Leid, J.G., and Hunsaker, D. (2006) Bacterial biofilms on the sinus mucosa of human subjects with chronic rhinosinusitis. Laryngoscope 116: 1121–1126.

Schaudinn, C., Stoodley, P., Kainović, A., O’Keefe, T., Costerton, B., Robinson, D., et al. (2007) Bacterial biofilms, other structures seen as mainstream concepts. Microbe 2: 231–237.

Söderling, E.M. (2009) Xylitol, mutants streptococci, and dental plaque. Adv Dent Res 21: 74–78.

Stoodley, P., Dodds, I., Boyle, J.D., and Lappin-Scott, H.M. (1999) Influence of hydrodynamics and nutrients on biofilm structure. J Appl Microbiol 85: 19S–28S.

Tapiainen, T., Kujala, T., Kajalainen, T., Ikaheimo, I., Saikkonen, A., Renko, M., et al. (2010) Biofilm formation by Streptococcus pneumoniae isolates from paediatric patients. APMIS 118: 255–260.

Trappetti, C., Kadioglou, A., Carter, M., Hayre, J., Iannelli, F., Pozzi, G., et al. (2009) Sialic acid: a preventable signal for pneumococcal biofilm formation, colonization, and invasion of the host. J Infect Dis 199: 1497–1505.

Trappetti, C., Gualdi, L., Di Meola, L., Jain, P., Korir, C., Edmonds, P., et al. (2011a) The impact of the competence quorum sensing system on Streptococcus pneumoniae biofilms varies depending on the experimental model. BMC Microbiol 11: 75.

Trappetti, C., Oggunniyi, A.D., Oggoni, M.R., and Paton, J.C. (2011b) Extracellular matrix formation enhances the ability of Streptococcus pneumoniae to cause invasive disease. PLoS ONE 6: e19844.

Waite, R.D., Struthers, J.K., and Dowson, C.G. (2001) Spontaneous sequence duplication within an open reading frame of the pneumococcal type 3 capsule locus causes high-frequency phase variation. Mol Microbiol 42: 1223–1232.

Waite, R.D., Penfold, D.W., Struthers, J.K., and Dowson, C.G. (2003) Spontaneous sequence duplications within capsule genes cap8E and its control phase variation in Streptococcus pneumoniae serotypes 8 and 37. Microbiology 149: 497–504.

Weimer, K.E., Armbruster, C.E., Juneau, R.A., Hong, W., Pang, B., and Swords, W.E. (2010) Coinfection with Haemophilus influenzae promotes pneumococcal biofilm formation during experimental otitis media and impedes the progression of pneumococcal disease. J Infect Dis 202: 1068–1075.

Weimer, K.E., Juneau, R.A., Murrah, K.A., Pang, B., Armbruster, C.E., Richardson, S.H., and Swords, W.E. (2011) Divergent mechanisms for passive pneumococcal resistance to β-lactam antibiotics in the presence of Haemophilus influenzae. J Infect Dis 203: 549–555.

Wolcott, R.D., and Ehrlich, G.D. (2008) Biofilms and chronic infections. JAMA 299: 2682–2684.