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Minireview

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 *Streptococcus 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-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

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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 polystyrene microplates. Increasing the osmolarity above 0.2 M, however, drastically inhibited pneumococcal growth and biofilm formation. Variations in the starting pH also influenced biofilm formation; optimal development was seen when the initial pH of the culture medium was 7.0–8.0 (Moscoso *et al*., 2006).

Sialic acid (at a concentration equivalent to that of free sialic acid in human saliva) enhances pneumococcal biofilm formation *in vitro*, and a causal association has been established between free sialic acid and nasopharyngeal colonization and spread to the lungs in mice (Trappetti *et al*., 2009). Preliminary data from our group support the notion that the addition of some sugars, such as melicitose, tagatose, melibiose or pullulan can improve *in vitro* biofilm formation by *S. pneumoniae* (M. Domenech, unpublished). The oxidative stress caused by the production of hydrogen peroxide through the activity of pneumococcal SpxB pyruvate oxidase (Pericone *et al*., 2003) appears to be responsible for the development of non-phase-variable colony variants that appear consistently when encapsulated pneumococci are incubated under biofilm-forming conditions (Allegrucci and Sauer, 2008). It is well known that non-encapsulated *S. pneumoniae* mutants are better biofilm formers than their encapsulated parental strains (see below). Moreover, a TIGR4 Δ*spxB* mutant showed no variation in biofilm formation capacity compared with the wild-type progenitor (Lizcano *et al*., 2010).

It has recently been reported that biofilm formation by pneumococci is favoured by a CO_2-enriched atmosphere (Camilli *et al*., 2011). It should be mentioned, however, that although the growth rate of *S. pneumoniae* was reported higher under anaerobic conditions than in ambient air enriched with 5% CO_2, biofilm formation was less strong [although this could not be confirmed in our laboratory (M. Moscoso, unpublished)].

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% CO_2 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 show 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

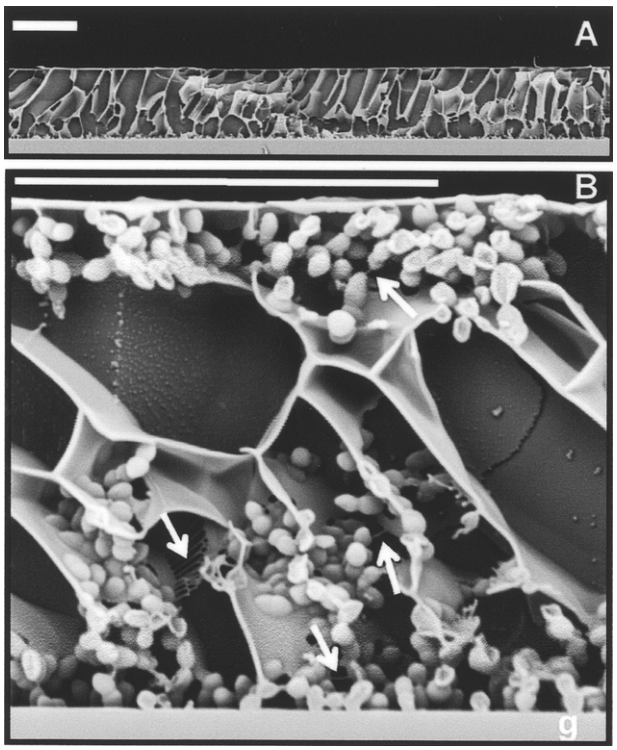

Fig. 1. Low-temperature scanning electron micrographs of a *S. pneumoniae* R6 biofilm.
A. General view of the biofilm formed on the surface of a glass coverslip (g).
B. In the magnification, arrows pointed to filamentous material linking pneumococcal cells to each other and to the intercellular matrix.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

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. Significant inhibitory and disintegrating effects on pneumococcal biofilms are seen when DNase I or proteases is added before or after biofilm development respectively (Fig. 2) (Moscoso *et al.*, 2006). These findings have 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-Elías *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).

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

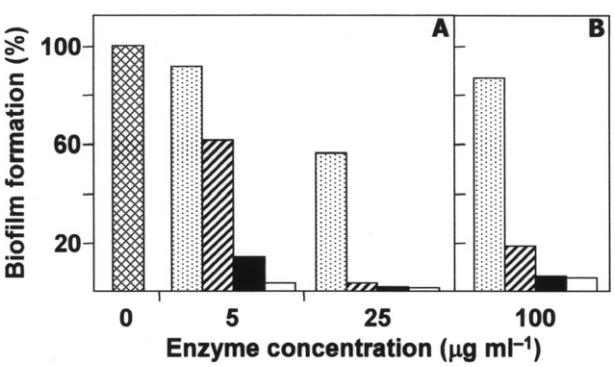

Fig. 2. Inhibition of biofilm development in *S. pneumoniae* cultures in the presence of nucleases or proteases.
A. *S. pneumoniae* R6 was distributed in the wells of a microtitre plate, which was then incubated for 6 h at 34°C (cross-hatched bars). Other samples received either RNase (stippled bars), 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.

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 CO_2-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,

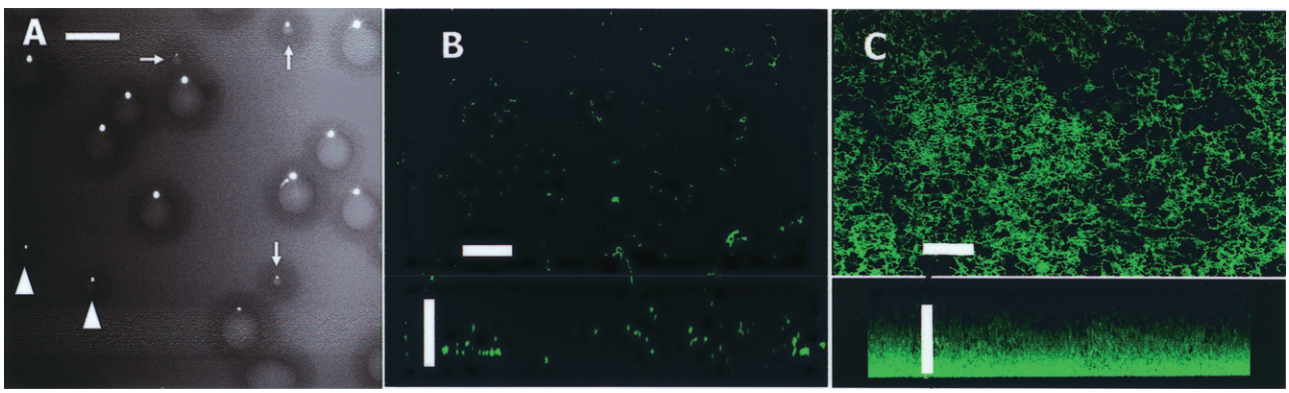

Fig. 3. Colony morphology and biofilm-forming capacity of a type 3 encapsulated *S. pneumoniae* strain and of non-encapsulated mutants appearing in biofilm-grown cultures.
A. Colonies of the encapsulated M23 strain and of several non-mucous colonies of different morphology (indicated by arrows or triangles). Bar, 2.5 mm.
B and C. CLSM of the biofilms formed by the M23 strain and one of the non-encapsulated mutants respectively. Bar, 20 μm. Reprinted from Domenech *et al.* (2009) with permission.

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 (García-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.

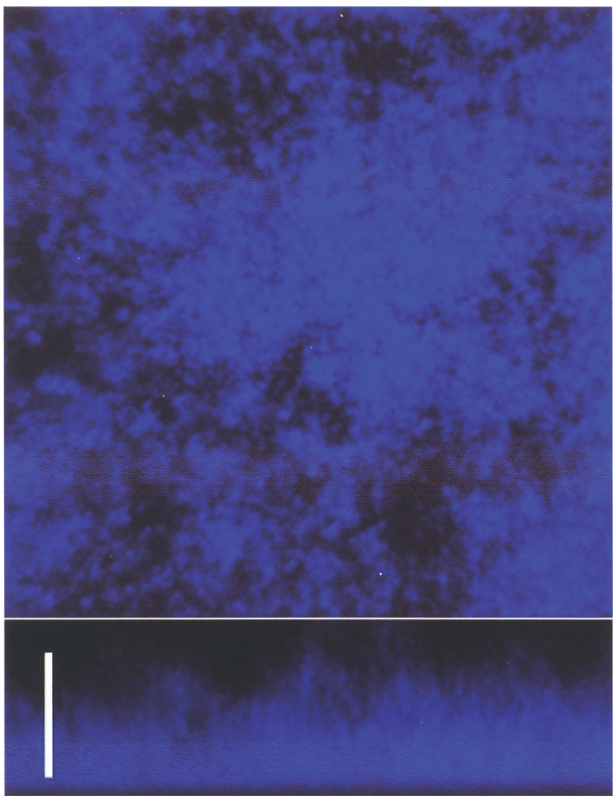

Fig. 4. CLSM of the biofilm formed by the *S. pneumoniae* R6 strain stained with calcofluor white. Bar, 25 μm.

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_1, 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 (*aliB*/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 H_2O_2. 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/or 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 (García-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

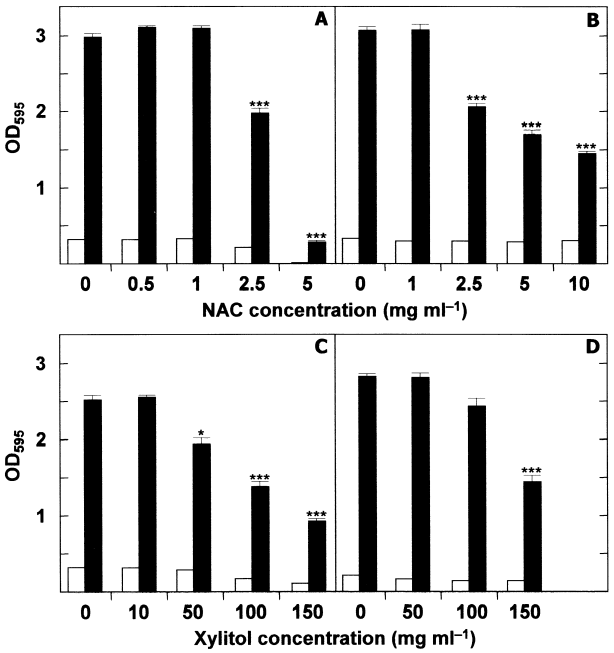

Fig. 5. Inhibition of biofilm development and biofilm dispersal in *S. pneumoniae* cultures in the presence of *N*-acetyl-cysteine or xylitol.
A. *S. pneumoniae* R6 was distributed in the wells of a microtitre plate, which was then incubated for 6 h at 34°C in the presence of different concentrations of NAC.
B. *S. pneumoniae* R6 was distributed in the wells of a microtitre plate, which was then incubated for 6 h at 34°C. Biofilms were washed with fresh CpH 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 (*$P < 0.05$; ***$P < 0.001$) compared with the control.

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 $\geq$ 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).

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 aeruginos*a 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* (Oggioni *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 nontypeable 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.

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