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Pneumococcal biofilms

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Summary. Over 60% of bacterial infections (and up to 80% of chronic infections) are currently considered to involve microbial growth in biofilms. This peculiar form of life poses an array of problems in human clinical practice, from infections associated with the implant of prosthetic devices and dental plaque formation to diseases such as cystic fibrosis, otitis media, and endocarditis. Biofilms are also at the basis of a variety of problems in industry. This report describes the biofilms produced by *Streptococcus pneumoniae*. This bacterium often colonizes the upper airways in humans as a normal commensal, yet it may spread to other areas of the body, causing otitis media, pneumonia, or invasive diseases such as bacteremia and meningitis. The capacity of *S. pneumoniae* to form biofilms had not been explored until recently. Several newly developed in vitro systems have allowed to test the capacity of *S. pneumoniae* to form biofilms, and to analyze the influence of several factors, including DNA and proteins—which play a role in the virulence of this "supergerm" in the formation and development of biofilms. In this brief review, we update the knowledge available on pneumococcal biofilm formation and the unusual features of this structure. [**Int Microbiol** 2009; 12(2):77-85]

Keywords: *Streptococcus pneumoniae* · biofilms · quorum sensing · otitis media · extracellular matrix

General background

Among the samples routinely used by Anton van Leeuwenhoek to test his newly invented microscopes, were smears he prepared from his own dental plaque, from which he was able to observe bacteria, giving way to the discovery of microorganisms. Van Leeuwenhoek could not have imagined that bacteria of the oral cavity multiply to form the thin layers that we today know as biofilms. In fact, precise knowledge of such bacterial formations was not defined until 1978. Subsequently, this knowledge became organized in the studies of the 1990s conducted mainly by Costerton's group [8]. Biofilms are structured populations of microorganisms adhered to a surface (or interface) and embedded in an extracellular matrix consisting mainly of exopolysaccharides (sometimes bound together by proteins and DNA) [20]. In nature, these communities can be mono- or, more frequently, multispecific [14,52] and show a modified phenotype in terms of their growth rate and different gene expression patterns. Biofilms have been likened in their level of organization to a eukaryotic organism, undermining the frontier between the biology of eukaryotes and prokaryotes [8]. The polysaccharide surrounding the biofilm is frequently composed of one or more anionic uronic acids.

It is currently estimated that more than 60% of all human bacterial infections are the result of microbial growth as biofilms and of the inherent tolerance of these communities to antibiotic agents and host immune defense mechanisms. So far, the most overwhelming evidence of the pathogenic relationship between humans and biofilms is based on microscopy observations that have revealed the presence of these communities at the site of infection (otitis caused by pneumococcus, endocarditis by staphylococci, *Pseudomonas* in the lungs of patients with cystic fibrosis, etc.) or in pros-

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thetic implants recovered from patients. Koch's postulates appear not to be applicable to biofilms given the peculiar features of these cell aggregates [34].

Biofilm formation is in itself a simple strategy of microbial survival that facilitates the spread of pathogens by providing a stable protective environment and acting as a reservoir for the dissemination of a large number of microorganisms to new surfaces. We also know today that pathogenic bacteria form biofilms in the lungs of patients with cystic fibrosis and that a greater resistance to antibiotics seems to be a characteristic feature of cells that live in a sessile state (forming communities) compared to cells that adopt a planktonic lifestyle (not attached to a surface) [6,9,34]. Microorganisms in biofilms can be up to 1000 times more resistant to antibiotics than the same free-living microorganisms. This greater resistance has been attributed to the difficulty of antimicrobial agents to penetrate a biofilm, to the short replication time of bacteria in this biological state, to the appearance of modified microenvironments within the biofilm, or to the above-mentioned antibiotic tolerance, although this last property requires further investigation [4]. Some biofilms play a protective role in humans; this is the case of vaginal communities formed by lactobacilli that ferment glycogen and reduce the vaginal pH, preventing its colonization by diverse pathogens [13].

A multitude of factors regulate biofilm formation. Many bacteria, including streptococci, are known to regulate diverse physiological processes through a mechanism called "quorum sensing" (QS). The QS, or autoinducer system, was early described in *Pseudomonas aeruginosa* biofilms. In gram-negative bacteria, the best known autoinducer is acyl homoserine lactone, which can diffuse through the membrane, while in gram-positive bacteria, signaling usually involves the production of small linear or cyclic peptides that are translated as a larger pro-peptide within the cell and then processed during secretion [8]. Other QS systems participate in interspecies cross-talk based on a signaling molecule originally described as autoinducer-2 (AI-2) and subsequently identified as a furanone (furanosyl borate diester) (see below and [29] and references therein). AI-2 is biosynthesized by LuxS, the product of the *luxS* gene, in the *S*-adenosylhomocysteine pathway [50]. It is noteworthy that the *luxS* gene appears to be conserved in most gram-positive and gram-negative bacteria. These external signal regulators allow for the modulation inside the bacterial cell of the expression of genes required for adaptation from the planktonic to the sessile state. The control of these regulators is considered a good target to avoid the formation of bacterial cell aggregates. In fact, some years ago a study reported the isolation of an analogue of homoserine lactone from the marine red alga *Delisea pulchra*. This compound was designated "furanone" [36] and was shown to interfere with bacterial communication by blocking the metabolic cascade that generates the QS signal. Laboratory tests in which furanone was used to combat *P. aeruginosa* infection in a mouse model have been successful [63]. However, given the toxicity of furanone, other types of molecules capable of inhibiting biofilm formation need to be identified. *P. aeruginosa* biofilms grown in the presence of synthetic furanone C-30 are significantly more susceptible to tobramycin, an aminoglucoside antibiotic used to treat cystic fibrosis [23]. Although the structure of the AI-2 produced by streptococci is still unknown, a synthetic bromated furanone inhibits biofilm formation in several oral streptococci, presumably through interference with the AI-2 signaling pathway [29].

Here, we briefly review the systems recently developed for the analysis of biofilms produced by *Streptococcus pneumoniae*, the main causal agent of several serious diseases, including pneumonia and otitis media. Special emphasis is placed on the importance of visualizing biofilms using new electron microscopy approaches designed to observe hydrated structures in their natural state.

Pneumococcal biofilms

Streptococcus pneumoniae is a gram-positive facultative anaerobe that often colonizes the nasopharynx of healthy individuals. Nasopharyngeal colonization in the absence of invasive disease provides *S. pneumoniae* with a stable environment, from which it may be propagated to other hosts via sneezing or mucus exchange. Thus, any microbial factor that contributes to the in vivo growth and survival of *S. pneumoniae*, such as nasopharyngeal colonization, could be considered a virulence factor [32]. Note that the carrier state makes biofilm development a "persistence factor," as opposed to a virulence factor per se [20]. Respiratory infections are responsible for the death of 4 million people each year, and *S. pneumoniae* is the predominant species in these infections [39]. *S. pneumoniae* is also responsible for diseases such as chronic otitis media (COM, accounting for 40% of these infections), pneumonia, bacteremia, and meningitis. During their first three years of life, approximately a third of all children suffer three or more episodes of COM, possibly leading to hearing loss. Pneumococcal biofilms have been detected in the mucosa of the middle ear in children with COM [18] as well as in a chinchilla model of otitis [46]. It has been proposed that biofilm formation in vivo is intertwined with the formation of extracellular neutrophil traps [56]. Until recently, information on pneumococcal biofilms at the structural or

genetic level was hardly available. The first system worked out used continuous cultures in Sorbarods (cellulose) filters, and was described in 1997 [7].

Biofilm structure and the intercellular matrix. The initial stages of pneumococcal biofilm production on an abiotic substrate such as polystyrene or glass have revealed that, in its unencapsulated form, *S. pneumoniae* generates on these surfaces three-dimensional structures some 25 μm in depth, observable both by confocal laser scanning microscopy (CLSM) (Fig. 1) and by low-temperature scanning electron microscopy (LTSEM) (Fig. 2) [41]. In a recent fascinating observation, bacterial aggregates, i.e., biofilms, were found to adopt regular shapes to form honeycomb-like structures [51]. This conclusion has been reached by examining microcolonies by transmission electron microscopy, which entails subjecting samples to freezing under high pressure to keep the samples in a fully hydrated state. Our team has used a similar method (LTSEM) [41] to obtain microscopy images of *S. pneumoniae* yielding images comparable to those of *Staphylococcus epidermidis* and *P. aeruginosa*, published by Schaudinn et al. [51]. These structures, which are highly organized (Fig. 2), occasionally disassociate from the cells responsible for their formation. It seems that we have only just begun to unveil the complexity of biofilms but we expect to localize the genes involved in forming this type of structure, which is thought to help biofilms withstand the force of the fluids that surround them. The elasticity of this structure allows deformation in response to stress applied along any of its six axes of symmetry in addition to providing mechanical stability, which could be a major factor for virulence [51].

The presence of both extracellular DNA and certain proteins (e.g., extracytoplasmic and surface-exposed proteins) was found to influence biofilm formation in *S. pneumoniae* [19,41]. However, Muñoz-Elías et al. did not detect any effect of DNase I treatment on biofilm formation [42]. This discrepancy could have resulted from the use of media, with different effects on competence induction (Fig. 3) in pneumococcus and thus on the amount of DNA released [40,41]. The importance of extracellular DNA in biofilm formation has also been demonstrated in *P. aeruginosa*. Moreover, it has been suggested that this bacterium undergoes programmed lysis and cell death depending on its spatial orientation in the biofilm [57]. This in turn supports the idea that cell lysis, as a matrix event, contributes to the biofilm's structural stability [5,22]. Likewise, for *S. pneumoniae* it is known that both competent and non-competent cells intervene in its tendency to form aggregates under certain conditions, and the capacity for aggregation is determined by DNA release into the medium. A prerequisite for this process is the activity of the lytic enzymes LytA or LytC and CbpD. LytA and CbpD also play a role in the lysis of non-competent cells in a process known as "microbial fratricide," which provides their competent "sibling" cells with a pool of released DNA [12,22,40]. Competent cells are protected against lysis by a regulated immune function expressed during the state of competence and mediated by the product of the gene *comM*. This set of observations regarding death and cell lysis in *S. pneumoniae* has prompted the study of biofilm maturation, an issue that deserves particular attention in future studies on the formation of biofilms in this model.

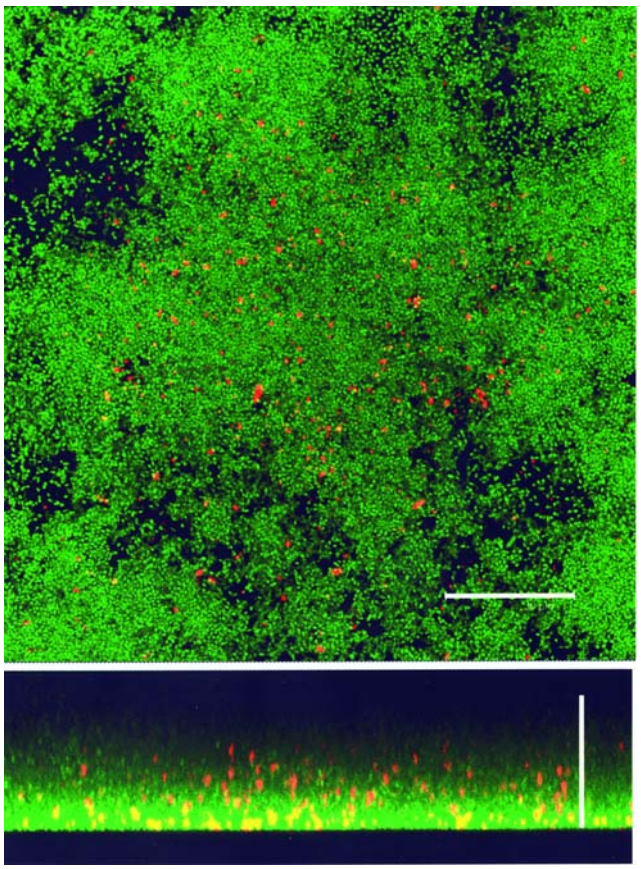

Fig. 1. Confocal laser scanning microscopy image of the viability of biofilm-grown *S. pneumoniae* R6 in C medium. After incubation at 34°C for 12 h, the cells in the biofilms were stained with the *Bac*Light kit, showing living (green fluorescence) and dead (red fluorescence) bacteria. Bar = 30 μm.

In addition to DNA and proteins, increasing evidence suggests that polysaccharides also form part of the extracellular matrix of *S. pneumoniae* biofilms. Staining of the biofilms with wheat germ agglutinin, which binds *N*-acetylglucosamine, conjugated with a fluorescent compound, provided some evidence of the presence of an extracellular polysaccharide [11], although the authors could not determine whether the label was due to *N*-acetylglucosamine residues

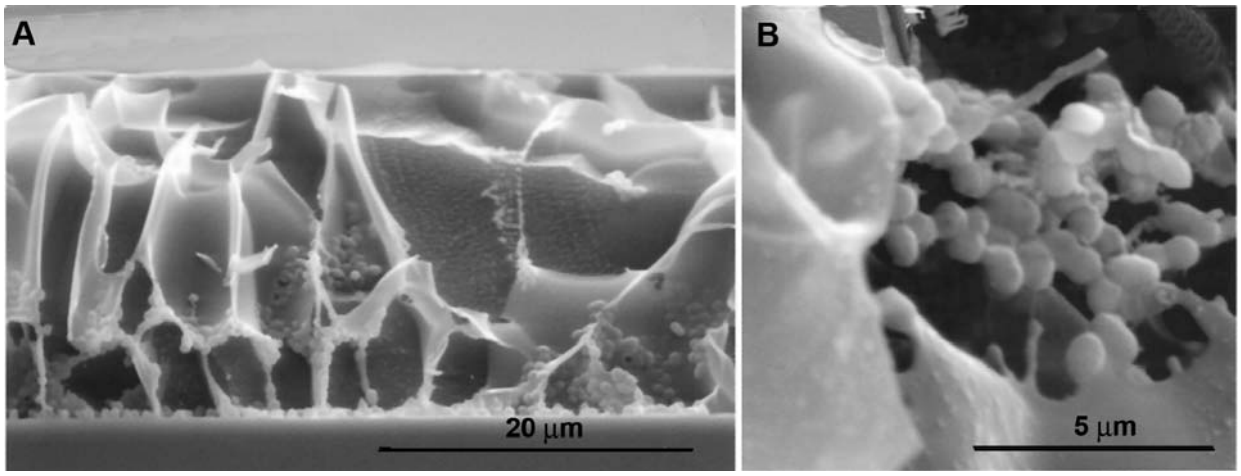

Fig. 2. Low-temperature scanning electron microscopy analysis of pneumococcal biofilms. (**A**) Honeycomb-like structures produced by *Streptococcus pneumoniae* biofilms, in which some cells are associated with the walls of these structures. (**B**) Detail of another area of the same biofilm, in which the bacterial cells are inside a honeycomb structure; clumps of cells bound by filamentous material can be seen.

of the glycan chains of the cell-wall peptidoglycan, to the capsular polysaccharide, or to a different extracellular compound. More recently, Hall-Stoodley et al. used a nonencapsulated pneumococcal strain and confirmed that biofilms of this strain also bind lectins [19]. Nevertheless, the precise composition of the extracellular material remains to be determined.

Colonization, biofilm formation and capsular polysaccharide. A minimum amount of the capsular polysaccharide (CPS) is absolutely required for efficient nasopharyngeal colonization in mice [35], although the capsule must be subsequently recovered for survival during systemic invasion of the host [31]. More recently and also using a mouse model, it was reported that nonencapsulated pneumococcal mutants retain their capacity for nasal colonization but at a reduced density and duration compared to their encapsulated parent strains [43]. This impaired colonization could be attributed to the fact that nonencapsulated mutants remain agglutinated within luminal mucus and are thus less likely to reach the epithelial surface, where stable colonization occurs.

Pneumococcal strains of serotypes showing greatest adhesion to polystyrene have a higher capacity to produce otitis media in animal models, such as gerbils, than serotypes poorly adhering to polystyrene [24]. However, several reports have documented that nonencapsulated pneumococcal mutants show increased adhesion properties and are therefore more prone than their encapsulated parents to form biofilms in vitro [2,38,41,42,58,59]. This observation is directly related to the fact that the capsule is a limiting factor for pneumococcal adhesion to the cells of the nasopharynx, while it plays an essential protective role against phagocytosis during invasive infection (see above). Several different mutations were found among the type 3 capsular mutants that appeared in biofilms formed on polystyrene plates. Most strains contained single nucleotide polymorphisms in *cap3A*, the first gene of the type 3 capsular operon [16], one had a mutated –10 promoter hexamer (CATAAT), and three had large deletions affecting *cap3A* and, in one case, also *cap3B* (Domenech et al., submitted for publication). This contrasts with previously described phase variants containing reversible tandem sequence duplications within *cap3A*; these variants were obtained when biofilms were allowed to form under continuous culture using Sorbarod filters [59]. Similar results were later reported when type 8 or type 37 pneumococci were analyzed using the same Sorbarod system [58]. However, when biofilms were produced as a continuous flow tube reactor biofilm [2], the nonmucoid variants that appeared lacked most of the *cap3* operon because of a lengthy (>7 kb) deletion. Moreover, plate-based biofilms on membrane filters gave rise to different kinds of type 3 capsular mutants upon extended growth (4–7 days) [38]. These results strongly suggest that the method chosen for biofilm formation modulates, in some unknown way, the kinds of capsular mutations that will appear in the culture. Indeed, experimental evidence suggests that environmental conditions specific to biofilm growth substantially increase mutation frequencies [3]. In addition, in an elegant electron microscopy study [21] on cultured epithelial cells, neither serotype 3 pneumococci in close contact with the host cell membrane nor invading pneumococci had a visible capsular structure, whereas pneumococci not in close contact with the

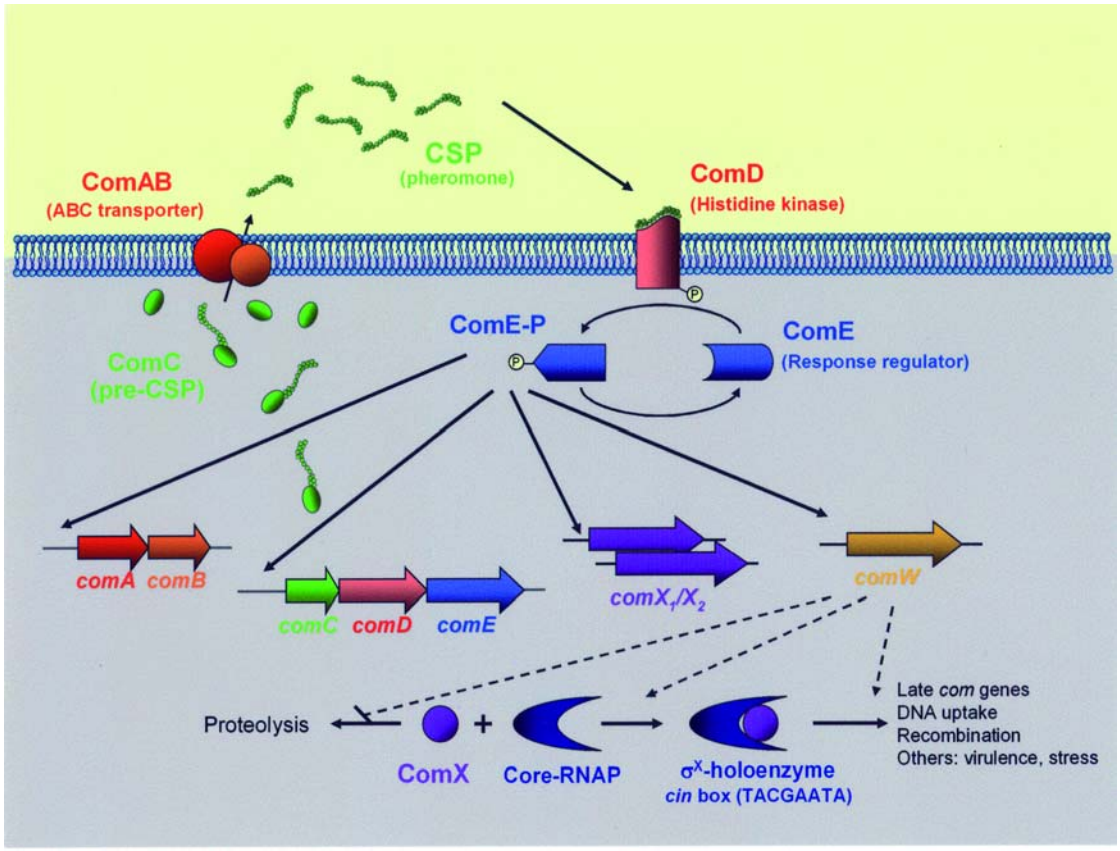

Fig. 3. The induction of competence for genetic transformation in *Streptococcus pneumoniae*. Competence in pneumococcus is regulated by a QS system via CSP. CSP is derived from the precursor protein ComC and it is cleaved and exported by the ATP-binding cassette transporter system, ComAB. When a critical extracellular concentration of CSP is reached, the signaling cascade begins with interaction of CSP with its histidine kinase receptor, ComD. It is likely that ComD passes on the signal by transferring a phosphate group to the cognate response regulator, ComE. Phosphorylated ComE activates the transcription of early *com* genes: *comCDE, comAB, comX*, and *comW*. ComX, encoded by two identical genes, acts as an alternative sigma factor that initiates transcription of the genes required for DNA uptake and recombination, and of late *com* genes. ComW is a positive regulator of competence that could promote the assembly of ComX with RNA polymerase by protection from proteolysis, by modification of σ^X to an active form, or by stabilization of the σ^X-holoenzyme.

host membrane had a typical capsule. In addition, *S. pneumoniae* cells expressed CPS in the lungs of infected mice, whereas bacteria in contact with lung epithelial tissue showed a drastic reduction in the density of the CPS layer.

Proteins involved in biofilm formation. Several recent works have established the basis to explore the gene products involved in the formation of biofims of *S. pneumoniae*. In biofilms produced using a continuous-culture system, proteomic analysis showed that biofilm development correlated not only with the differential production of proteins but also with a dramatic increase in the number of detectable proteins [1]. Protein identification revealed that several proteins involved in virulence, adhesion, and resistance were more abundant under biofilm growth conditions. Oggioni and collaborators reported that, in the blood of mice inoculated intravenously (septic mice), pneumococci showed a pattern of gene expression very similar to that in mid-exponential phase of growth in liquid culture whereas, for nearly all the genes studied, the expression pattern was identical in pneumococci recovered from tissue and from biofilm [44]. Furthermore, whereas pneumolysin was overexpressed in biofilms formed under continuous culture conditions [1], the pneumolysin-coding gene (*ply*) was repressed in polystyrene-grown biofilms [44]. Whether this (and other) discrepancies are related to differences in the strains used and/or to the biofilm-forming conditions remains to be determined.

The role in biofilm formation of choline binding proteins, which anchor to the choline residues of the cell-wall teichoic acids, was studied using unambiguously characterized

mutants. The results showed that LytA amidase, LytC lysozyme, LytB glucosaminidase, CbpA adhesin, PcpA putative adhesin, and PspA (pneumococcal surface protein A) mutants had a decreased capacity to form biofilms, whereas no such reduction was observed in Pce phosphocholinesterase or CbpD putative amidase mutants [41]. More recently, 69 mutants with insertions in 42 different genes and eight promoters, showing altered biofilm formation, were studied [42]. It was found, for instance, that *lytC* or *cbpA* mutants were poor biofilm formers, in agreement with previous findings [41]. Moreover, an additional choline binding protein (SP0391) was required for optimal biofilm formation [42], whereas interruption of the gene encoding SP1538, a putative protein/peptidyl-prolyl *cis-trans* isomerase, led to drastic inhibition of biofilm formation [42]. SP1538 was reported to be overexpressed by *S. pneumoniae* forming biofilms [1]. Interestingly, encapsulated serotype 2 pneumococci having mutations in SP1538, *cbpD* or SP0391 (among other genes) were severely compromised in their ability to colonize the nasopharynx in a mouse model of infection [42].

Quorum sensing systems and biofilms. Loo et al. [30] first reported that biofilm formation in one streptococcal species (*Streptococcus gordonii*) involves QS. Afterwards, several studies confirmed that the QS system promotes the formation of biofilms in streptococci, and that, in a biofilm, *Streptococcus intermedius* takes up tenfold more DNA from the medium than its free-living counterparts [55]. More recently, it was proposed that the system regulating the development of competence for the bacterial transformation process in *S. pneumoniae*, made up of phosphotransfer signals involving two proteins that detect an environmental signal (in this case a so-called competence-stimulating peptide, or CSP) (Fig. 3), plays a role in colonization of the respiratory tract [27]. Some authors have noted that induction of the competence system by the QS CSP signal promotes biofilm formation in vitro, and pneumococcal cells recovered from these biofilms show enhanced virulence in models of pneumonia and meningitis [44]. In contrast, planktonic cells tested in animal models were more efficient at inducing sepsis. These data suggest that during host infection invasive pneumococci should exist in two physiological states. In addition, during tissue infection *S. pneumoniae* were visualized in a state resembling a biofilm [44].

As mentioned above, the LuxS protein is required for the biosynthesis of AI-2, which is involved in QS in a wide range of bacterial species. Although not yet confirmed in biofilms, it has been reported that, although a *luxS* mutant was able to colonize the nasopharynx of the mouse as efficiently as the wild-type strain, it was less able to spread from the nasopharynx to the lungs or the blood [54]. Moreover, a profound defect of the mutant in its ability to persist in the nasopharyngeal tissues was noted [26]. Lastly, increased expression of *luxS* under all in vivo conditions (including biofilms) has been observed [44].

Future prospects

According to a recent report by the WHO, pneumonia is the main cause of death of children less than 5 years of age, surpassing deaths caused by AIDS, malaria, and measles together. Two-thirds of these more than 2 million children die in 15 countries, with *S. pneumoniae* and *Haemophilus influenzae* type Ib identified as the main causal agents of acute pneumonia in children in developing countries [60]. The paucity of research devoted to these bacteria has precluded their better control.

Biofilms are now recognized as etiological agents in many chronic infections affecting tissues and surgical implants. Estimates of the clinical costs generated by infections involving biofilms are already high. Thus, in bacteremia that develops in adults dependent on hemodialysis, these costs have been calculated to range from US$ 17,000 in mild cases to US$ 32,000 in bacteremia with complications. Costs for surgical infections in prosthetic implants can, for example, in the case of orthopedic implants for fracture fixation, amount to US$ 50,000 per patient [34]. As already discussed, preventing or limiting colonization by biofilms is likely to be the most effective procedure for the control of this type of infection. Arguably, the routine use of antibiotics to eliminate biofilms is, to say the least, a dubious strategy, and this practice could further aggravate the already worrisome situation of hospital-acquired infections. Antibiotics usually fail in certain infections caused by pneumococcus, such as otitis media. This type of recurrent infection involves the formation of a biofilm in the mucosa by the same strain of the microorganism that we thought had been eradicated by antibiotics, and not by a second infective agent as has been postulated [37].

Recent studies have shown that the combined action of *S. pneumoniae* and a respiratory virus variant contributes to the formation of these bacterial aggregates. The recurrent infections produced in many of the 24 million people diagnosed with otitis media every year in the USA are currently treated with antibiotics; however, a novel experimental system has been developed using a mouse model in which intranasal infection with *S. pneumoniae* is induced. In the presence of an influenza virus, 70% of the animals develop otitis media. The most revealing finding of this study was

that the administration of a phage lytic enzyme (Cpl-1) before provoking the middle ear infection in the presence of the virus prevented biofilm formation [37]. Phage lytic enzymes are murein hydrolases responsible for the release of phage progeny after bacterial infection. In our laboratory, a series of pneumococcal lytic enzymes has been analyzed in detail [17]. These enzymes have been used in in vitro studies in murine models developed to eliminate both nasal and systemic infections [25,47] and were originally designed as "enzybiotics" [15,28]. However, while this model to eliminate otitis media is based on preventive treatment with a phage lysin, another model has been developed in which already formed biofilms are eliminated using bacteriophages that have been genetically modified to express an enzyme capable of degrading the biofilm. This phage therapy resulted in the elimination of 99% of the biofilms formed by *Escherichia coli* [33]. It has also been reported that an organic compound identified as *cis*-2-decenoic acid induces the dispersion of biofilm microcolonies of *P. aeruginosa* and other microorganisms, including several gram-positive species [10]. Note that *cis*-2-decenoic acid is known to be involved in interspecies communication in bacteria [49]. These alternative or complementary treatments to antibiotic therapy acquire special relevance if we consider that, in addition to the passive resistance to antibiotics of biofilm-residing cells, in *P. aeruginosa* a genetically programmed flow pump that confers resistance to tobramycin, gentamicin, and ciprofloxacin has been newly identified. While the genes involved in this process are strongly expressed in biofilm cells, they are very weakly expressed in their planktonic counterparts [64].

The isolation of axenic bacterial cultures has played a key role in the development of microbial chemotherapies. However, in view of our current understanding of the significance of biofilms, this simple analysis is rendered largely incomplete. The complex world of biofilms co-inhabited by polymicrobial communities reflects a more complex reality that requires new experimental rationales. We are just beginning to appreciate that extensive, thorough knowledge of these pathogens is critical to designing treatments and to adopting new health control measures [60]. The availability of experimental models to examine biofilm formation by a human pathogen of the significance of pneumococcus would help to advance our knowledge of cell-to-cell communication. An understanding of QS systems and concomitant DNA transfer via genetic transformation, both of which have been well studied in planktonic cultures [40], would enable the analysis of issues such as the passive resistance to antibiotics of these cell communities, including the phenomenon of antibiotic tolerance. In contrast, it is thought that the honeycomb structure of extracellular polymeric substances is a peculiar feature of each microorganism, implying that their formation is subject to genetic control. Moreover, when the culture is transferred, the gene cycle responsible for the development of each community is repeated, which suggests that the ontogeny of these structures is as reproducible as that of the embryos of higher organisms [51].

Finally, a subject of particular interest is the mechanism that underlies biofilm dispersion. A mechanism proposed early on is that of mechanical transmission provoked by shear forces. In *P. aeruginosa,* the presence of a bacteriophage (Pf4) in the prophage state leads to phenotypic variations in this bacterium. This suggests programmed mobilization of live cells, so-called *persisters*, that subsequently establish a new colony in the biofilm at a new site with the colonizers, or *settlers* [61]. In addition, several studies have shown that these temperate phage genes are among those most up-regulated genes in biofilm formation [53,62]. Considering that over 70% of recent clinical isolates of pneumococcus are lysogenic [45,48], the study in this system of the relationship between biofilm development and bacteriophages is of special interest.

Infectious diseases are still leaders in the number of deaths they cause and tragedies they provoke [39]. Along with the appearance of new causal agents of infectious diseases (AIDS, Lyme, Ebola), old acquaintances, such as *P. aeruginosa*, *Staphylococcus*, pneumococcus and many more, as well as apparently innocuous bacteria, such as *Moraxella* and *Legionella*, have started to reveal their extraordinary versatility in giving rise to complex structures such as biofilms. It now emerges that biofilms have been and still are today the most common mode of initiating an infection. Clearly, we need to improve our understanding of the dynamic relationship among the bacterium, the host, and the environment. The issues discussed here document new compelling research lines in the study of these cellular aggregates in a system of such major clinical importance as pneumococcus infections.

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