of the biofilm of the control, nonencapsulated strain M11. In contrast, strains belonging to serotypes 19B and 19C showed a drastic reduction in the number of biofilm-associated sessile cells. Similar results were observed with the corresponding isogenic capsular transformants producing serogroup 19 capsules, generated to reduce the genetic variability among strains.

A close comparison of the primary structures of the repeating units of the CPS of types 19F/A on one hand, and 19B/C on the other, suggested that the disaccharides α-D-Glcp-(1→2)-α-L-Rhap-(1→ and α-D-Glcp-(1→3)–α-L-Rhap-(1→ (present in serotypes 19F and 19A CPSs, respectively, but not in the 19B and 19C capsules) may be important for promoting biofilm formation in the pneumococcus.

The disaccharide α-D-Glcp-(1→3)-α-L-Rhap-(1→ is also present in the CPS of the four serotypes of the serogroup 6. Previous studies of the biofilm-forming capacity showed that serotype 6B isolates formed 70% of the biofilm of strain M11 [6]. The analysis of others members of this group, namely serotypes 6A and 6C, showed that both, clinical and M11 transformants expressing these capsules, were able to form more than 50% of the biofilm of the nonen-capsulated control strain.

Biofilm-formation capacity was also studied in serotypes 18A and 18C. Serotype 18C has a glycerol-phosphate substituent that must be preserved for conserving the adequate anti-genicity of the 18C capsular polysaccharide [7]. Serotypes 18A and 18C have in their structure the disaccharides α-D-GlcpNAc-(1→3)-β-L-Rhap-(1→ and α-D-Glcp-(1→3)-β-L-Rhap-(1→, respectively, very similar to the ones mentioned above. However, a single isolate of either serotype able to form a significant biofilm was not found.

3. Conclusion

The results presented in this study indicate that, in addition to its genetic background, the chemical composition/structure of CPS is crucial to define the biofilm-forming capacity of a particular *S. pneumoniae* isolate.

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Insight into the composition of the intercellular matrix of *Streptococcus pneumoniae* biofilms

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ABSTRACT:

Biofilm matrices consist of a mixture of extracellular polymeric substances synthesized in large part by the biofilm-producing microorganisms themselves. These matrices are responsible for the cohesion and three-dimensional architecture of biofilms. The present study demonstrates the existence of a matrix composed of extracellular DNA, proteins and polysaccharides in the biofilm formed by *Streptococcus pneumoniae*. Extracellular DNA, visualized by fluorescent labeling, was an important component of the matrix. The existence of DNA–protein complexes associated with bacterial aggregates and other polymers was hypothesized based on the unexpected DNA binding activity of lysozyme LytC. The presence of intercellular DNA-LytC protein complexes in pneumococcal biofilms was demonstrated by confocal laser scanning microscopy. Evidence of extracel-lular polysaccharide different from the capsule was obtained by staining with Calcofluor dye and four types of lectin conjugated to Alexa fluorophores, and by incubation with glycoside hydrolases. The presence of residues of Glcp(1,4) and GlcNAc(1,4) in the pneumococcal biofilm was confirmed by GC-MS techniques.

Keywords: biofilm, matrix composition, eDNA, polysaccharide, EPS.

1. Introduction

Recent reports have shown the in vivo for-mation of *S. pneumoniae* biofilms on adenoid and mucosal epithelial tissues in children with recurrent or chronic ear infections (for a recent review, see ref. 1). Using low-temperature scanning electron microscopy (LTSEM) techniques, we reported a biofilm matrix containing an intercellular, fiber-like material that linked the pneumococcal cells to one another and to the glass substrate on which they were grown [2]. The presence of extracellular proteins in this matrix was inferred from the biofilm-disaggregating activity of proteolytic enzymes [2].

Several authors have reported extracellular DNA (eDNA) to be an important extracellular polymeric substance (EPS) in pneumococcal biofilms, based on the dramatic disappearance and inhibited formation of biofilms following treatment with DNase [2].

Controversy exists over whether extracellular polysaccharide is a component of the *S. pneumoniae* biofilm matrix. Using encapsulated *S. pneumoniae* cells to demonstrate the existence of a polysaccharide among the EPS is, however, problematic since some lectins also bind the capsular polysaccharide, as demonstrated more than 30 years ago [3].

The present work provides evidence that the biofilm formed by *S. pneumoniae* R6 contains a matrix made up of eDNA, protein and polysaccharide components.

2. Results

2.1. DNA as a component of EPS

Extracellular DNA, visualized by fluorescent labeling with DDAO [7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)], was an important component of the matrix (Fig. 1).

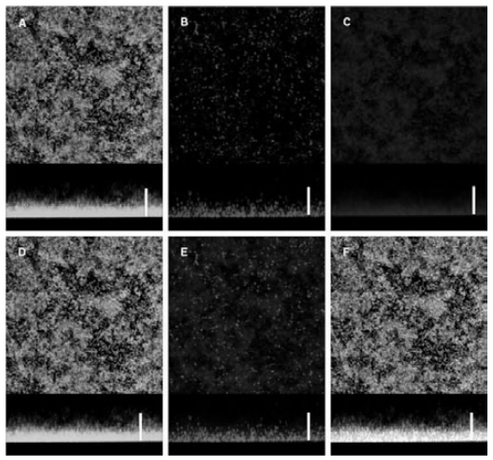

Fig. 1: CLSM evidence of eDNA in pneumococcal biofilms. A biofilm of the S. pneumoniae strain R6 was stained with a combination of SYTO 9 (A, green), propidium iodide (B, red) and DDAO (C, blue). Image (D) is a merger of the channels (A) and (B). Image (E) is a merger of the channels (B) and (C). Image (F) is a merger of the three fluorophores. Scale bars=25μm.

2.2. Evidence of protein-DNA interactions

The existence of DNA-protein complexes associated with bacterial aggregates and other polymers was hypothesized based on the unexpected DNA binding activity of lysozyme LytC, a novel moonlighting protein. Actually, a 25 amino acid-long peptide derived from LytC (positions 408 to 432 of the ma-ture LytC) was also capable of efficiently binding to DNA. Moreover, the presence of intercellular DNA-LytC protein complexes in pneumococcal biofilms was demonstrated by confocal laser scanning microscopy (CLSM).

2.3. The polysaccharide component of the pneumococcal biofilm matrix

Evidence of an extracellular polysaccharide component different to the capsule was obtained by staining R6 biofilms with calcofluor dye and four types of lectin conjugated to Alexa fluorophores, and by treatment with sodium metaperiodate and incubation with various glycoside hydrolases.

Biofilm (but no planktonic) growing non-encapsulated pneumococcal cells, were able to bind calcofluor indicating that *S. pneumoniae* biofilms are composed of aggregates of microbial cells encased in an extracellular polysaccharide matrix that contains at least ß-linked D-glycopyranosyl units. Alexa-conjugated lectins, i.e., wheat germ aggluti-nin (specific for N-acetylglucosamine and N-acetylneuraminic acid) and soybean agglutinin (specific for galactose and N-acetylgalactosamine) clearly stained the pneumococcal biofilm. Moreover, the presence of residues of Glcp(1→4) and GlcNAc(1→4) (in its deacetylated form) in the pneumococcal biofilm was confirmed by GC-MS techniques.

3. Conclusion

The present results provide compelling ultra-structural evidence of a complex, web-like matrix linking biofilm growing pneumococcal cells to one another and to their underlying substrate.

The LytC protein does not recognize any specific DNA sequence or topology, something that may be important in the formation of nasopharyngeal pneumococcal biofilms.

Incubation with NaIO4 or glycoside hydrolases and staining with calcofluor and the lectins WGA and SBA showed that the biofilms formed by strain R6 contain one (or more) polysaccharides.

R6 pneumococcal cells growing as biofilms synthesize anexopolysaccharide containing residues of Glc β(1→4) and GlcNAc β(1→4) with possible Glc/Gal α(1→6) branches.

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