Microbial Pathogenesis 48 (2010) 124–130

Contents lists available at ScienceDirect

Microbial Pathogenesis

journal homepage: www.elsevier.com/locate/micpath

Early biofilm formation on microtiter plates is not correlated with the invasive disease potential of *Streptococcus pneumoniae*

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ARTICLE INFO

Article history:
Received 15 September 2009
Received in revised form
4 January 2010
Accepted 14 January 2010
Available online 22 January 2010

Keywords:
Streptococcus pneumoniae
Adhesion
Microtiter plate
Biofilm
Virulence

ABSTRACT

Biofilm formation has been suggested to play an important role during *Streptococcus pneumoniae* nasopharyngeal colonization and may facilitate progression to pneumonia. To test whether the ability of *S. pneumoniae* to form biofilms was important for virulence we screened the ability of 30 invasive and 22 non-invasive clinical isolates of serotype 6A and 6B to form early biofilms on polystyrene microtiter plates and infect mice following intranasal and intratracheal challenge. We first determined that no correlation existed between the ability to form early biofilms and whether isolates were collected from healthy carriers or individuals with invasive disease. A disconnect between biofilm forming ability and the capacity to colonize the nasopharynx, cause pneumonia, and enter the bloodstream was also observed in mice. Importantly, *S. pneumoniae* mutants deficient in the established virulence determinants pneumolysin, CbpA, and hydrogen peroxide formed biofilms normally. Incidentally, we determined that robust biofilm production was dependent on the formation and coalescing of bacterial aggregates on a thin layer of bacteria attached to the plate surface. In summary, these studies suggest that the ability to form early biofilms *in vitro* does not reflect virulence potential. More complex studies are required to determine if biofilm formation is important for virulence.

1. Introduction

Since the discovery that *Pseudomonas aeruginosa* forms biofilms in cystic fibrotic lungs, considerable attention has been placed on the role of bacteria biofilms during infectious diseases. To date bacteria in biofilms have been shown to have differences in metabolism, virulence gene expression, and protein production that contribute to surface adhesion and persistence of an infection. Because biofilm bacteria are enmeshed within an extracellular matrix, and in some instances metabolically inert, biofilm bacteria are more resistant to killing by leukocytes and antimicrobials, and serve as an recalcitrant source [of bacteria] during persistent infections [1,2].

Considerable evidence suggests that biofilm formation is the underlying mechanism responsible for chronic otitis media. In regards to *Streptococcus pneumoniae,* the leading cause of otitis media, pneumococci have been detected on the surface of adenoid and mucosal epithelial cells biopsied from children with recurrent middle ear infections [3–5], occluded tympanostomy tubes isolated from the latter [6], and middle ear sections taken from challenged chinchillas [7,8]. More recently, experimental evidence has been collected that suggests a role for pneumococcal biofilms during nasopharyngeal colonization and pneumonia. For example, Sanderson et al. found pneumococcal biofilms in biopsies of sinuses from individuals with chronic rhinosinusitis [9]. Following an *in vitro* screen of transposon mutants, Munoz-Elias et al. identified 23 genes necessary for biofilm formation that were also required for nasopharyngeal colonization of mice [10]. Trappetti et al. found that treatment of mice with sialic acid, a condition that enhanced pneumococcal biofilm formation *in vitro*, increased bacterial counts in the nasopharynx of mice and instigated translocation of the bacteria into the lungs [11]. Finally, Oggioni and colleagues found that biofilm pneumococci had gene expression profiles similar to those of bacteria isolated from the lungs of mice; these profiles were distinct from planktonic bacteria isolated from either blood or culture media [12]. Thus, considerable evidence suggests that biofilms are an important aspect of pneumococcal pathogenesis.

To date many investigators have used the static polystyrene microtiter plate system to examine the molecular mechanisms underlying bacterial attachment to abiotic surfaces and early events

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doi:10.1016/j.micpath.2010.01.002

during pneumococcal biofilm formation [10–16]. Advantages of this model system include that it is easy to establish, is applicable to high-throughput screens, and allows visualization of biofilm structures using an inverted microscope. Based on existing evidence supporting a role for biofilm formation during middle ear infection and nasopharyngeal colonization, we hypothesized that the ability to form biofilms might also contribute towards the ability to cause invasive pneumococcal disease (IPD). To test this hypothesis, we examined the ability of 30 invasive and 22 non-invasive low-passage clinical isolates of serotype 6A and 6B to attach to and form early biofilms ($\leq$18 hours old) on untreated polystyrene 96-well microtiter plates and infect mice. In this manuscript we show that no correlation was found between biofilm production and the source of the clinical isolate, the ability of an isolate to colonize the nasopharynx, or cause invasive disease in mice. We conclude that the ability to form early biofilms *in vitro* had no correlation with virulence in mice and that extrapolations regarding *in vitro* biofilm formation with virulence are tenuous. These findings emphasize the importance of testing suspected pathogenic mechanisms using validated model systems along with a diverse panel of clinical isolates.

2. Materials and methods

2.1. Bacteria strains

Clinical isolates of *S. pneumoniae* were collected at The University of Texas Southwestern Medical Center in Dallas County, Texas, from February 1999 to January 2003. A total of 52 clinical isolates were examined, 23 serotype 6A isolates and 29 serotype 6B isolates. Non-invasive isolates were obtained from nasopharyngeal swabs of healthy carriers (strains 6A1–6A10 and 6B1–6B12). Invasive isolates were obtained from blood, cerebrospinal fluid, or aspirates of normally sterile sites from individuals with invasive disease. Phylogenetic relationships between the clinical isolates were extrapolated using comparative genomic hybridization data previously obtained for these strains [17]. TIGR4 is a virulent laboratory strain [18]. Isogenic mutants of TIGR4 deficient in pneumolysin (T4 Δ*pln*), Choline binding protein A (T4 Δ*cpbA*), and hydrogen peroxide production (T4 Δ*spxB*) were made by insertion duplication mutagenesis with the suicide vector pJDC9 using previously described constructs [19]. Mutants were maintained with 1 μg/ml erythromycin.

2.2. In vitro screening for biofilm formation

We tested biofilm formation in the static polystyrene microtiter well system previously described by Allegrucci et al. [16]. Bacteria were streaked from blood agar plates (Remel, Lenexa, KS) into Todd Hewitt Broth (THB) (Difco, Detroit, MI) and grown at 37 ° C in 5% CO_2. At mid-logarithmic phase growth ($OD_{620} = 0.5$) bacteria were diluted 1:20 in media containing 10% glycerol and frozen stocks were created. 96-well polystyrene microtiter plate (CELLSTAR, Greiner Bio-One, Monroe, NC) wells containing 270 μl of THB were inoculated with 30 μl of thawed stocks. Plates were incubated at 37 °C, 10% CO_2, overnight for 18 hours. The next day plates were washed with phosphate buffered saline (PBS), biofilms stained with 150 μl 0.5% crystal violet (CV) for 30 minutes, washed with PBS, and allowed to air dry. Biofilm formation was quantified by solubilizing the CV stain with 150 μl of 95% ethanol and measuring absorbance

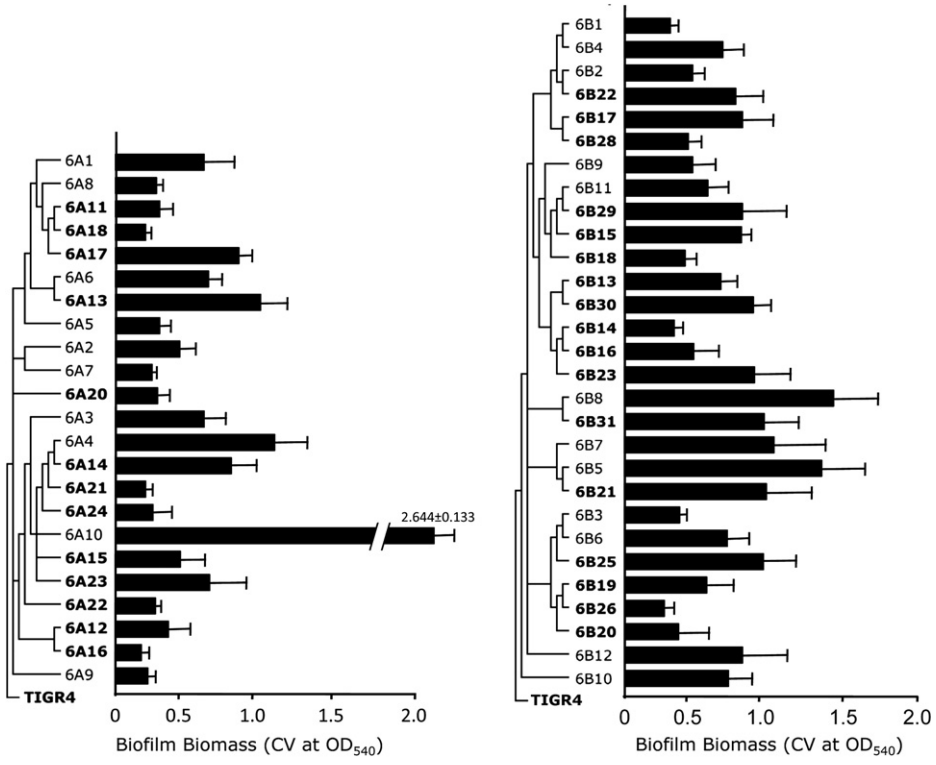

Fig. 1. Biofilm formation on 96-well polystyrene microtiter plates by individual isolates of *S. pneumoniae*. Clinical isolates belonging to serotype 6A and 6B were collected from individuals with invasive disease (bold font) and healthy asymptomatic carriers (regular font). Media in the wells were inoculated with $\sim 10^5$ CFU and incubated overnight at 37 °C at 10% CO_2. Plates were washed, and biofilm formation was assessed by crystal violet staining (CV_{540}) as described in the methods. Shown is the average of three independent experiments, each with >3 replicate wells for each clinical isolate tested. Error bars indicate the standard error of means. Phylogenetic trees on the left of the isolate name are based on comparative genomic analyses done using microarrays [17]. Branches indicate phylogenetic relationships between the clinical isolates in context of TIGR4, a serotype 4 isolate, from whose genomic DNA the microarray was designed.