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

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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 bacteria 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.

Keywords

Streptococcus pneumoniae; adhesion; microtiter plate; biofilm; virulence

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,

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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 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 (≤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.

MATERIALS AND METHODS

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 Δpln), Choline binding protein A (T4 $\Delta cpbA$), and hydrogen peroxide production (T4 $\Delta spxB$) were made by insertion duplication mutagenesis with the suicide vector pJDC9 using previously described constructs [19]. Mutants were maintained with 1 µg/ml erythromycin.

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 with a spectrophotometer at 540 nm (CV_{540}). Images of the biofilms were captured using a Leica inverted microscope at 15X and 200X magnification prior to drying. Additional wells not inoculated with bacteria served as controls for inadvertent bacterial contamination of the media and served as background levels for CV staining. Experiments were done in triplicate with ≥3 replicate wells tested per strain in each experiment.

Animal infections

Female BALB/cJ mice (4–5 weeks old; The Jackson Laboratory) were maintained in animal biosafety level 2 facilities. For the initial virulence screen of all 52 isolates, a minimum of 2 replicate experiments were performed, each with 2–3 mice, for no less than 5 mice sampled for each clinical isolate tested. Briefly, mice were anesthetized with 2.5% inhaled isoflurane (Baxter Healthcare) and challenged intranasally with 10^7 colony forming units (CFU) suspended in 25 µL of PBS. Post-infection, blood was collected from the tail vein to test for bacteremia and nasal lavage performed to determine bacterial titers in the nasopharynx [20]. For long-term nasopharyngeal colonization studies, cohorts of 6–12 mice were challenged intranasally with 10^5 CFU in 10 µl PBS and examined over a 28-day period. For intratracheal challenge experiments cohorts of 6 mice were used. Mice were anesthetized, hung upright by their incisors, and 10^5 CFU in 100 µL of PBS placed in their throats. Aspiration was induced by gently pulling the tongue outward and covering the nostrils. Lung and blood samples were collected 2 days later. For blood and nasal lavage samples, serial dilution in PBS followed by plating and colony counting was used to determine the bacterial burden. For lung samples, the lungs were weighed and homogenized in 1 ml of PBS. Bacterial titers in the lungs were assessed per gram of homogenized tissue following serial dilution of the homogenate and colony counting. In all instances, the infectious dose administered was confirmed.

Statistical analyses

Differences between the highest and lowest biofilm producers in regards to biofilm production, blood, and nasopharyngeal bacterial titers were tested for significance using a two-tailed Student's *t*-test. Comparisons regarding the incidence of positive blood cultures were done using a two-tailed Fisher's exact test. Simple linear regression analysis was performed using SigmaStat 3.1 software (Systat Software Inc. Point Richmond, CA). For comparison of strains in the long-term nasopharyngeal colonization studies and the intratracheal challenge studies statistical analysis were performed using a One-Way ANOVA.

RESULTS

Early biofilm formation *in vitro*

The static 96-well microtiter plate model tests for bacterial attachment to an abiotic surface and early biofilm formation. All clinical isolates tested were able to attach to the polystyrene surface; however, individual isolates had differential abilities to form early biofilms as measured by CV_{540}, an indicator of biofilm biomass (Figure 1). For example, in serotype 6A the lowest early biofilm producer was 6A16 which had a $CV_{540} = 0.241 \pm 0.047$, whereas the greatest biofilm producer was 6A10 which had a $CV_{540}= 2.64 \pm 0.133$ (6A16 versus 6A10; $p<0.001$). Likewise for 6B the lowest early biofilm producer was 6B26 which had a $CV_{540} = 0.376 \pm 0.060$ and the highest was 6B8 with a $CV_{540} = 1.457 \pm 0.377$ (6B26 versus 6B8; $p=0.03$). Due to the exceptionally high and divergent ability of 6A10 to produce early biofilms, no significant differences in early biofilm production were observed between serotype 6A and 6B when compared as a whole. For all 6A isolates the mean CV_{540} detected was 0.674 ± 0.099; whereas for all 6B isolates the mean CV_{540} was 0.768 ± 0.051 ($p=0.375$). Excluding 6A10, a statistically significant difference was observed between serotype 6A and 6B, with 6B isolates having 31% higher mean CV_{540} values ($p=0.018$).

As exemplified by 6A10, the ability to form biofilms was highly diverse between individual clinical isolates. Closely related strains, including those determined to be clonal (i.e. within the same phylogenetic clade) by previous comparative genomic hybridization studies [17], varied considerably in their attachment and early biofilm forming ability. For example, 6A17 and 6A18 differed by 2.4-fold (6A17 $CV_{540} = 0.873 \pm 0.100$; 6A18 $CV_{540} = 0.365 \pm 0.044$; $p=0.009$) despite belonging to the same phylogenetic clade. Importantly, no significant differences were observed for attachment/early biofilm formation when comparing clinical isolates from individuals with invasive disease versus those collected from healthy carriers (6A non-invasive $CV_{540} = 0.825 \pm 0.217$; 6A invasive $CV_{540} = 0.564 \pm 0.066$; $p=0.199$) (6B non-invasive $CV_{540} = 0.809 \pm 0.098$; 6B invasive $CV_{540} = 0.739 \pm 0.058$; $p=0.513$). Thus, we determined that initial attachment and early biofilm production was not correlated with the disease state of the individuals from whom the isolates were obtained, and that sufficient individual variation existed between clinical isolates (as much as 10-fold) that no statistically significant difference in early biofilm formation occurred between serotype 6A and 6B.

Early biofilm formation is not correlated with the ability to colonize the nasopharynx or cause invasive disease in mice

To determine if *in vitro* early biofilm formation was correlated with *in vivo* fitness, we first infected cohorts of Balb/cJ mice with 10^7 CFU of each strain and determined their ability to colonize the nasopharynx and cause bacteremia (Table 1). With exception of 6B10 that failed to colonize mice, we observed stable nasopharyngeal colonization for all isolates on day 7 that was comparable with levels observed in previous studies [20,21]. Between strains, colonization levels were determined to be uniform with no statistical differences between the highest and lowest colonizers of each serotype (6A21, 6A23, respectively; $p=0.111$) (6B29, 6B2, respectively; p=0.194). Likewise no significant differences were observed between serotype 6A and 6B when compared as groups (average value of medians: 6A = $5.04 \pm 0.944 \times 10^5$ CFU/ml nasal elute, 6B =$3.11 \pm 1.47 \times 10^6$ CFU/ml; $p=0.111$). Again, no differences where observed when comparing invasive disease isolates versus asymptomatic carrier isolates (average value of medians: 6A non-invasive = $3.33 \pm 0.843 \times 10^5$ CFU/ml; 6A invasive = $5.82 \pm 1.43 \times 10^5$ CFU/ml nasal elute; 6A invasive vs. noninvasive: $p=0.192$; 6B non-invasive = $9.68 \pm 1.91 \times 10^5$ CFU/ml; 6B invasive = $4.65 \pm 2.54 \times 10^5$ CFU/ml; 6B invasive vs. non-invasive: $p=0.244$). Thus excluding 6B10, clinical isolates from serotype 6A and 6B colonized mice stably and equally despite a differential ability in abiotic surface colonization.

To assess invasiveness, the same mice were tail bled on days 1, 4, and 7 post-challenge and bacteria burden in the blood determined (Table 1). Most mice infected with 6A isolates were not bacteremic during the 7 days of observation (blood culture positive on day 1: 9/23, day 4: 4/23, day 7: 9/23); whereas in contrast the majority of 6B isolates were able to cause bacteremia (day 1: 16/29, day 4: 20/29, day 7 11/29). On day 4, the number of isolates able to cause bacteremia was determined to be statistically significant between the serotypes using a two-tailed Fisher's Exact test ($p < 0.001$). In general bacterial titers in the blood were low with little to no mortality due to infection. Of the 148 mice infected with serotype 6B only 9 died: 2 for 6B6, and 1 for 6B3, 6B14, 6B19, 6B20, 6B25, 6B28, and 6B31. Whereas for 6A, only 15 of the 120 mice infected died: 3 for 6A4 and 6A24, 2 for 6A3 and 6A14, and 1 for 6A7, 6A10 and 6A15. Importantly, the ability to cause bacteremia was not correlated with the source of the clinical isolate (Day 4, invasive vs. asymptomatic carrier: 6A $p=1.0$; 6B $p=0.105$), suggesting that a discrepancy also exists between the disease potential of *S. pneumoniae* in humans and mice. We next directly tested whether early biofilm formation on a microtiter plate was positively correlated with virulence in the mice. To do this, we performed linear regression analysis using CV_{540} biofilm production measures and nasal lavage and blood bacterial titers. For serotype 6A we used bacterial titers from day 7, as more mice were blood culture positive at this later time point. For serotype 6B we used day 4 bacterial titers for the same reason. Figure 2 shows that biofilm formation on microtiter plates *in vitro* was not correlated with the ability to colonize the nasopharynx or cause bacteremia for either serotype 6A or 6B.

To more robustly confirm the observed disconnect between biofilm formation and colonization and invasion, we tested the ability of the 3 highest (6A4, 6A10, 6A13) and 2 lowest (6A16, 6A21) biofilm producers of serotype 6A to colonize the nasopharynx in a long-term model (Figure 3A) and following intratracheal challenge cause pneumonia (Figure 3B). Following intranasal challenge of mice, we observed no significant differences between levels of the 5 isolates on days 1, 3, 7, 14, 21 and 28. Thus the highest biofilm producers colonized the nasopharynx equally to those that formed little to no biofilms. Similarly, no differences were observed in the lungs of mice infected intratracheally with 6A4, 6A10, and 6A21. Of note, mice infected with 6A13 had no bacteria detectable in the lungs, as did the majority of mice infected with the low biofilm producing strain 6A16. Thus, in separate experiments, we confirmed that early *in vitro* biofilm forming capability was not correlated with the ability to colonize or cause pneumococcal disease. Finally, we also tested the reverse; whether early biofilm formation was affected by the deletion of established virulence determinants known to be required in the nasopharynx and lungs [19]. Isogenic mutants deficient in the toxin pneumolysin, the adhesin CbpA, and hydrogen peroxide synthesis, all formed biofilms comparable to the parent strain TIGR4 (T4 WT $CV_{540} = 0.849 \pm 0.121$; T4 Δpln $CV_{540} = 0.964 \pm 0.124$; T4 $\Delta cbpA$ $CV_{540} = 0.737 \pm 0.072$; T4 $\Delta spxB$ $CV_{540} = 1.093 \pm 0.132$). Thus early biofilm formation was determined not to be associated with these virulence determinants.

Bacteria aggregation contributes to early biofilm biomass

Incidentally, microscopic inspection of the surface attached biofilm biomass determined that the ability to form robust surface attached communities was associated with the formation of large aggregates on the polystyrene surface (Figure 4). Low early biofilm producers (e.g. 6A16, 6B14) formed only a thin layer on the microtiter well bottom composed primarily of individual diplococci, while mid level biofilm producers formed a similar bacterial "carpet" accentuated by the presence of small bacterial aggregates (e.g. 6A3, 6B22). As the respective CV_{540} values increased, the size of the bacterial aggregates also increased, ultimately coalescing into microcolonies ranging in size between 100 and 500 µm (e.g. 6A13, 6B25). Finally in the highest early biofilm producers, the microcolonies converged forming a thick confluent layer of bacteria along the bottom of the well (e.g. 6A10, 6B7). Thus, increased CV_{540} values were

associated with an incremental ability to form bacterial aggregates following initial attachment to the plastic surface.

DISCUSSION

Because biofilm formation is a mechanism for the pneumococcus to evade the host-defense and resist antimicrobials, it is reasonable to hypothesize that strains better able to form biofilms are more virulent. To test this hypothesis, we examined the ability of 32 clinical isolates from individuals with IPD and 20 isolates from healthy asymptomatic carriers to form biofilms *in vitro* as well as cause IPD in mice. If our hypothesis were true, we would have predicted that the clinical isolates that caused invasive disease in humans, as well as in mice, were those that best formed biofilms *in vitro*. In fact, this was not observed and instead we determined that the ability of serotype 6A and 6B isolates to form biofilms *in vitro* was not correlated with the disease condition of the host (i.e. invasive or non-invasive), the ability to colonize the nasopharynx, or the ability to cause invasive disease in mice. Thus our experimental results suggested that the ability to form early biofilms was not important for IPD.

This study has important limitations. Foremost, we examined the ability of *S. pneumoniae* to form biofilms *in vitro* and thus our findings regarding virulence were correlative. Biofilm formation *in vivo* occurs under selective pressure and environmental signals that are distinct from *in vitro*. Environmental signals such as limited nutrients, the presence of antimicrobial host factors, and bacteria density may serve as signals for the expression of biofilm related genes. Previously we have shown that gene expression during bacterial growth in media is distinct from that which occurs *in vivo*, and from bacteria attached to epithelial cells *in vitro* [21]. Thus it is possible that biofilm factors are differentially expressed *in vitro* and that biofilm formation occurs differently *in vivo*. Furthermore, studies have shown that differences in the richness of growth media, bacteria seeding, and strain capsulation, affect biofilm formation on microtiter plates [10,12–14,16]. Had we used different media or altered our growth conditions we may have observed distinct results. Finally, we only tested serotype 6 clinical isolates. Capsule types 6A and 6B are near identical but the linkage between a component rhamnose and ribitol is 1→3 for serotype 6A and 1→4 for serotype 6B [22]. Perhaps for other serotypes, biofilm production under these conditions would have been positively correlated with invasive disease potential.

Currently two model systems are used to grow biofilms *in vitro*, a static microtiter plate system and a continuous flow system. Static systems such as the microtiter plate system permit growth of the bacteria in a vessel without replacement of media. Advantages of this model system are that it is easily amendable to high through-put screens, particularly when using a 96-well polystyrene plates. However, this model is short-term as nutrients are depleted and metabolic wastes accumulate; typically growth of the biofilm stops between 8 to 16 hours followed by loss of adhered bacteria presumably due to autolytic properties [13]. Recent findings also suggest the accumulation of a biofilm dispersion signal, cis-2-decenoic acid [23]; thus, without frequent exchanges of media, the static system does not allow for the formation of mature biofilms. Importantly, differences between the microtiter plate system and continuous flow reactor in pneumococcal biofilm formation have been reported. Studies by Allegruci et al., have shown that the inability to form early biofilms in a microtiter plate is not correlated with the ability to form biofilms in a flow-through reactor over an extended period [24]. Similar observations have been made for various other biofilm forming bacteria including Gram-negative pili mutants. While pili-deficient mutants have been shown to be defective in initial attachment to non-coated abiotic surfaces, most retain the capability to form biofilms (although with altered biofilm architecture compared to wild type) following several days of growth under flowing conditions [27–31]. Thus the continuous flow through model emphasizes distinct physiological properties and may have distinct *in vivo* correlates. It is therefore possible that

had we used the continuous flow through reactor we would have observed distinct results. This possibility highlights the arbitrary nature of the current *in vitro* biofilm models.

Despite these considerations, our finding that the capability to initiate surface attached growth on microtiter plates was not correlated with disease suggests that for at least serotype 6A and 6B the ability to form early biofilms does not model pneumococcal events necessary for the development of invasive disease in humans or mice. Importantly, biofilm formation on polystyrene [or: in microtiter plates] with media would be distinct from events *in vivo* due to the fact that the bacteria would not be interacting with host components such as mucin, fibronectin, or laminin which may act as bridging molecules between individual bacteria and eukaryotic cells. Of note, finding that TIGR4 mutants deficient in pneumolysin, CbpA, and hydrogen peroxide synthesis were able to form biofilms was consistent with previous findings by Munoz-Elias et al. which showed that only deletion of *lytC*, a gene encoding a murein hydrolase, and *cps4E*, a capsule synthesis enzyme, affected early biofilm production following a screen of 6,500 TIGR4 mutants [10].

Interestingly, Munoz-Elias found 49 genes including *cbpA* that contributed to biofilm production in an acapsular strain. One explanation provided for the discrepancy between capsulated and unencapsulated mutants in biofilm production was increased bacterial interactions in the absence of capsule. Of note, 23 of the 49 genes identified by Munoz-Elias were subsequently shown to be important for nasopharyngeal colonization in TIGR4 [10]. Thus, when using unencapsulated pneumococci and a mutant with its isogenic parent, the early biofilm model provided important information regarding bacteria to bacteria interactions that were pertinent to the disease process. One important caveat is that clinical isolates of *S. pneumoniae* are almost always encapsulated, the exception being those collected from individuals with conjunctivitis [34]. Also that we observed contradictory results, strains unable to form dense biofilms colonized normally and in some instances were able to cause disease in mice. Thus these strains lacked biofilm determinants that were not required *in vivo*; alternatively, the presence of capsule alters the role of surface expressed proteins.

Despite the absence of an invasive disease correlation, our studies were the first to show that strains within a single serotype have a diverse ability to form early biofilms. While numerous studies have shown that the ability to form biofilms is nutrient dependent and enhanced by the absence of capsule [10,13,15,35], our studies are the first to rigorously show that bacterial components other than capsule play important roles. The finding that phylogenetically similar strains (i.e. clonal derivatives), as determined by genetic content using microarrays, have diverse early biofilm forming abilities suggests that the ability to form biofilms may be regulated at the transcriptional level and not due to the presence or absence of genes [17]. Importantly, this study does not attempt to identify which genes are responsible for altered biofilm formation or correlate the serotype 6 clinical isolates with virulence gene expression. Our studies also corroborate earlier work by Hall-Stoodly et al. showing that the formation of microcolonies occurs and that they are important for biofilm biomass accumulation [14]. Examination of biofilm images revealed that early pneumococcal biofilm formation as determined by CV_{540} stating intensity coincided with the formation of bacterial aggregates superimposed on a bacterial "lawn"; suggesting that the formation of pneumococcal aggregates on top of a bacterial layer is a critical event during early biofilm formation.

In summary, it is clear that the ability to form early biofilms *in vitro* under the growth conditions tested were not positively correlated with the ability of clinical isolates to cause invasive disease. This suggests that the ability to form biofilms is not important for invasive disease. However, the discussed limitations in the model system and the fact that these studies are correlative, still leaves open the possibility that biofilm formation *in vivo* is important for

invasive disease. These findings emphasize a need for future studies examining biofilm formation in animals during invasive disease.

Acknowledgments

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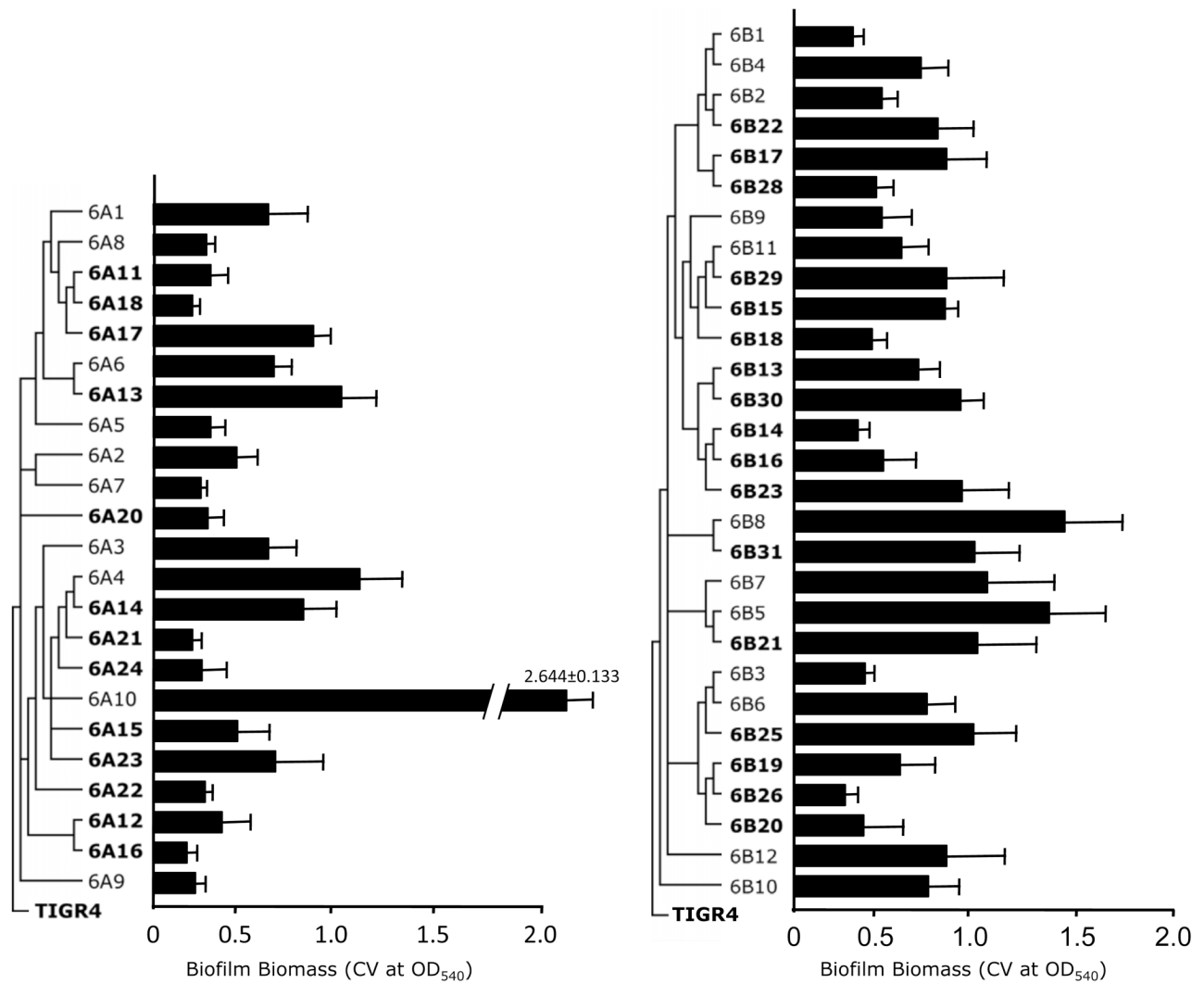
Figure 1. Biofilm formation on 96-well polystyrene microtiter plates by individual isolates of *S. pneumoniae*

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 ~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.
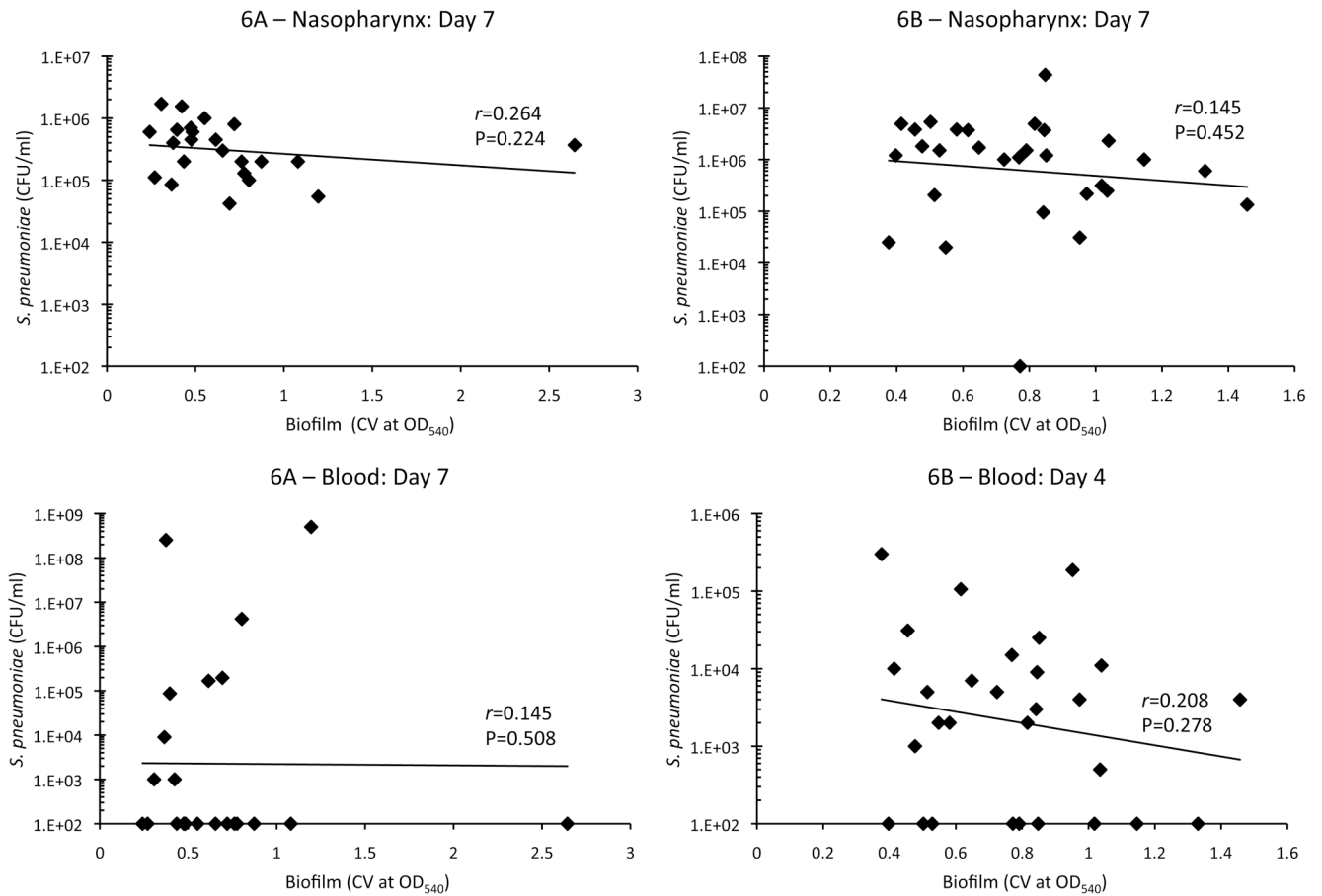
Figure 2. Biofilm formation is not correlated with nasopharyngeal colonization or bacteremia Linear regression analysis was used to determine if biofilm formation was correlated with the ability to colonize the nasopharynx and cause invasive disease. For nasopharyngeal colonization, CV_{540} values were plotted against median bacterial titers in nasal lavage elutes from day 7. For 6A invasive disease, CV_{540} values was plotted against median bacteremia titers at day 7, the day the majority of mice had bacteria in the blood. For 6B invasive disease, CV_{540} values was plotted against median bacteremia titers at day 4 for the same reason. For both 6A and 6B no correlation was found between biofilm formation and invasive disease on days 1, 4 or 7 (data not shown).
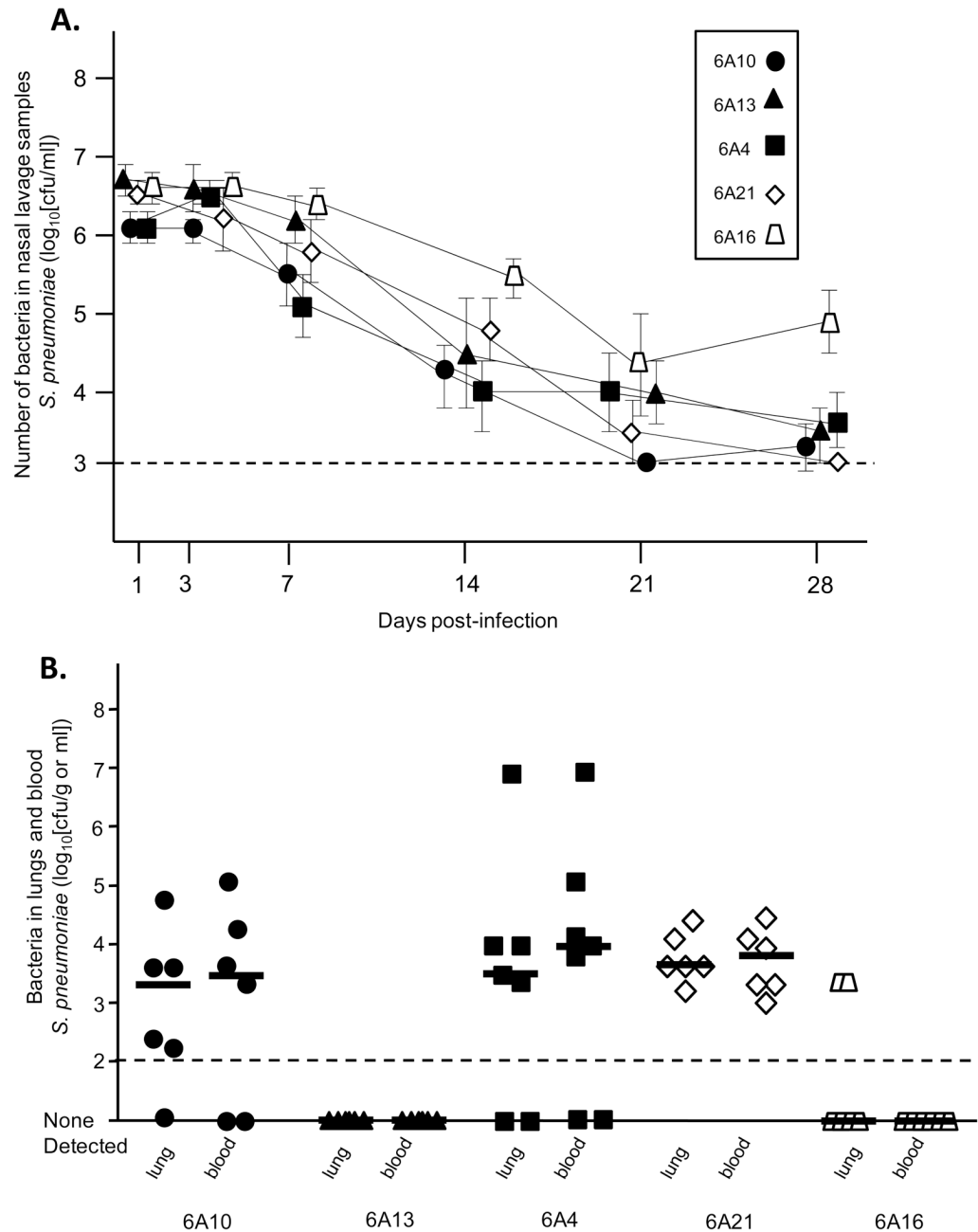
Figure 3. Ability of high and low serotype 6A biofilm producers to colonize the nasopharynx long-term and cause IPD

A) Average bacterial titers in nasal lavage samples collected from mice colonized with the high biofilm producing strains (black shapes) 6A10, 6A13 and 6A21 as well as the low biofilm producing strains (white shapes) 6A21 and 6A16. Mice were challenged with 10^5 CFU in 10 µl PBS and bacterial titers in the nasopharynx determined on days 1, 3, 7, 14, 21 and 28. No statistical differences were observed between the groups using One-Way ANOVA. Error bars indicate ± SEM. **B)** Bacterial titers in the lungs and blood 48 hours after intratracheal challenge of mice with the same 6A isolates. Mice were infected with 10^5 CFU in 100 µl PBS. Individual

shapes represent distinct mice. Horizontal bars designate the median bacterial value. Statistical analyses between the groups were performed using One-Way ANOVA.
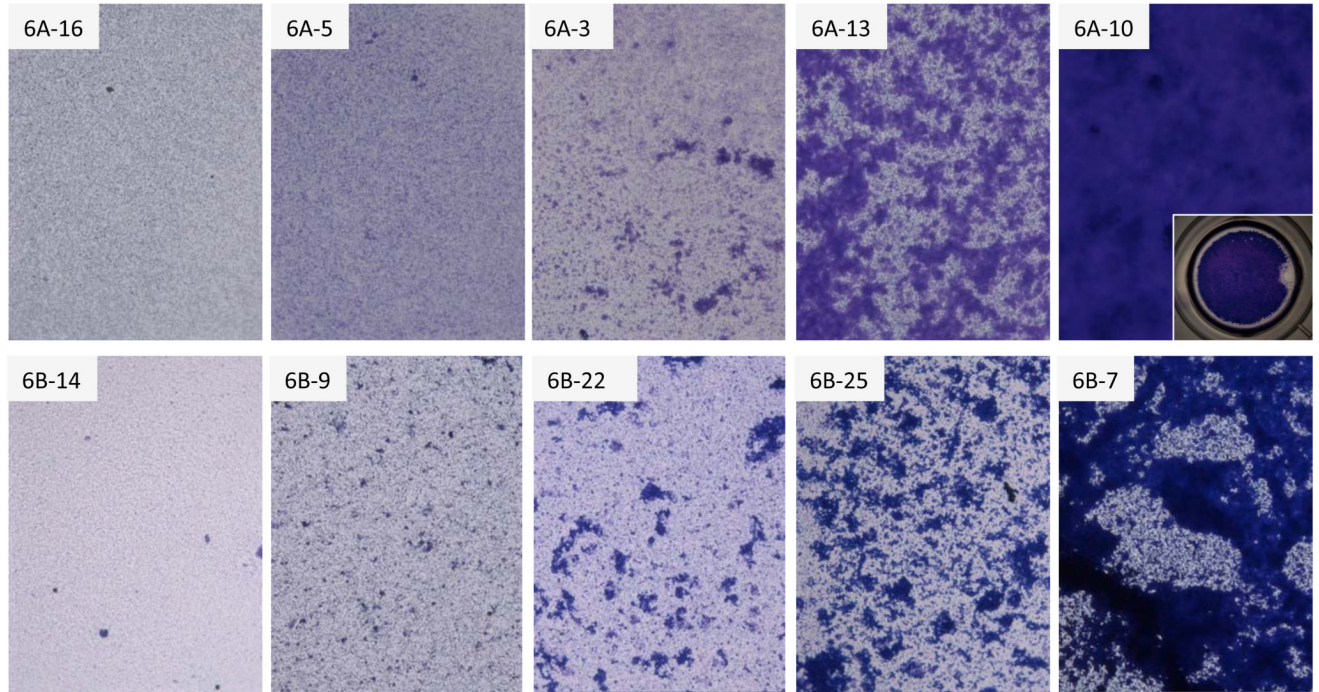
Biofilm Biomass (CV$_{540}$)

Figure 4. Increased biofilm biomass corresponds to the formation of increasingly larger bacterial aggregates
Representative micrographs of pneumococcal biofilms forming on the bottom of the polystyrene microtiter plate wells. Images were taken with at 200X with an inverted microscope. For 6A10 we include an insert of a well taken at 15X magnification. This is to demonstrate that the 6A10 biofilm has completely covered the well bottom. Strains were selected based on their increasing CV$_{540}$ value.

Table 1

Percentage of mice with positive blood cultures following intransal challenge and bacterial burden in the blood and nasopharynx[*]

	n=	day 1		day 4		day 7		
		% bact.	blood (cfu/ml)	% bact.	blood (cfu/ml)	% bact.	blood (cfu/ml)	nasal lavage (cfu/ml)
6A1	5	0%	$<10^3$	0%	$<10^3$	20%	$<10^3$	8.00E+05
6A2	5	0%	$<10^3$	40%	$<10^3$	20%	$<10^3$	3.00E+05
6A3	5	60%	7.00E+03	100%	4.40E+06	80%	1.97E+05	4.20E+04
6A4	5	80%	3.00E+03	100%	1.00E+06	60%	5.00E+08	5.45E+04
6A5	5	0%	$<10^3$	20%	$<10^3$	20%	$<10^3$	6.00E+05
6A6	5	0%	$<10^3$	0%	$<10^3$	0%	$<10^3$	2.00E+05
6A7	5	0%	$<10^3$	20%	$<10^3$	60%	8.70E+04	6.50E+05
6A8	5	0%	$<10^3$	40%	$<10^3$	0%	$<10^3$	2.00E+05
6A9	5	0%	$<10^3$	0%	$<10^3$	0%	$<10^3$	1.11E+05
6A10	5	60%	4.00E+03	20%	$<10^3$	20%	$<10^3$	3.68E+05
6A11	5	60%	2.00E+03	60%	2.00E+03	20%	$<10^3$	7.00E+05
6A12	5	0%	$<10^3$	0%	$<10^3$	0%	$<10^3$	1.00E+06
6A13	5	20%	$<10^3$	0%	$<10^3$	0%	$<10^3$	2.00E+05
6A14	5	40%	$<10^3$	40%	$<10^3$	80%	4.20E+06	1.00E+05
6A15	5	60%	2.00E+03	40%	$<10^3$	60%	1.68E+05	4.50E+05
6A16	5	0%	$<10^3$	0%	$<10^3$	0%	$<10^3$	6.00E+05
6A17	5	60%	1.00E+03	20%	$<10^3$	0%	$<10^3$	2.00E+05
6A18	5	80%	1.50E+04	80%	5.70E+04	80%	9.00E+03	8.50E+04
6A20	8	0%	$<10^3$	0%	$<10^3$	13%	$<10^3$	4.50E+05
6A21	5	40%	$<10^3$	20%	$<10^3$	60%	1.00E+03	1.70E+06
6A22	6	50%	3.00E+03	17%	$<10^3$	50%	1.00E+03	1.55E+06
6A23	5	40%	$<10^3$	20%	$<10^3$	20%	$<10^3$	1.29E+05
6A24	6	67%	9.34E+05	23%	$<10^3$	83%	2.53E+08	4.00E+05
6B1	5	80%	4.00E+03	40%	$<10^3$	20%	$<10^3$	1.20E+06
6B2	5	40%	$<10^3$	100%	2.00E+03	20%	$<10^3$	2.00E+04

	n=	day 1		day 4		day 7		
		% bact.	blood (cfu/ml)	% bact.	blood (cfu/ml)	% bact.	blood (cfu/ml)	nasal lavage (cfu/ml)
6B3	5	100%	3.40E+04	80%	1.00E+03	40%	<10³	1.80E+06
6B4	5	60%	1.20E+04	20%	<10³	20%	<10³	1.50E+06
6B5	5	60%	1.00E-03	40%	<10³	60%	2.00E+03	6.00E+05
6B6	5	60%	1.00E+03	100%	1.50E+04	100%	7.70E+04	1.10E+06
6B7	5	40%	<10³	40%	<10³	20%	<10³	1.00E+06
6B8	5	60%	1.10E+04	80%	4.00E+03	20%	<10³	1.34E+05
6B9	5	40%	<10³	40%	<10³	0%	<10³	1.50E+06
6B10	5	0%	<10³	0%	<10³	0%	<10³	<10³
6B11	5	20%	<10³	60%	7.00E+03	40%	<10³	1.70E+06
6B12	5	40%	<10³	60%	3.00E+03	60%	6.00E+03	9.50E+04
6B13	5	40%	<1O3	100%	5.00E+03	80%	1.00E+04	1.00E+06
6B14	5	60%	1.00E+06	100%	1.00E+04	40%	<10³	4.90E+06
6B15	5	80%	4.30E+04	100%	2.50E+04	40%	<10³	1.20E+06
6B16	5	80%	1.00E+04	60%	2.00E+03	80%	4.00E+03	3.80E+06
6B17	5	40%	<10³	60%	9.00E+03	0%	<10³	3.70E+06
6B18	6	50%	4.50E+04	40%	<10³	17%	<10³	5.35E+06
6B19	5	80%	1.00E+04	100%	1.06E+05	80%	1.00E+03	3.70E+06
6B20	7	29%	<10³	100%	3.10E+04	86%	5.30E+04	3.80E+06
6B21	5	80%	1.00E+03	100%	1.10E+04	40%	<10³	2.30E+06
6B22	5	40%	<10³	80%	2.00E+03	40%	<10³	4.90E+06
6B23	5	20%	<10³	100%	1.87E+05	80%	2.00E+03	3.10E+04
6B25	5	80%	1.35E+04	60%	5.00E+02	80%	2.50E+03	2.49E+05
6B26	5	60%	2.00E+03	100%	3.00E+05	80%	6.00E+03	2.50E+04
6B28	5	80%	2.80E+04	80%	5.00E+03	60%	6.00E+03	2.05E+05
6B29	5	60%	1.00E+03	40%	<10³	0%	<10³	4.34E+07
6B30	5	40%	<10³	100%	4.00E+03	0%	<10³	2.17E+05
6B31	5	20%	<10³	40%	<10³	20%	<10³	3.13E+05

*
Median values are shown