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Hemolytic activity and biofilm-formation among clinical isolates of group B streptococcus causing acute urinary tract infection and asymptomatic bacteriuria

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ABSTRACT

Streptococcus agalactiae, also known as group B Streptococcus, is an aetiological agent of urinary tract infection (UTI) in adults, including cystitis, pyelonephritis and asymptomatic bacteriuria (ABU). Whereas ABU-causing *S. agalactiae* (ABSA) have been shown to grow and achieve higher culture denstity in human urine compared to uropathogenic *S. agalactiae* (UPSA) other phenotypic distinctions between *S. agalactiae* isolated from different forms of UTI are not known. Here, we define the hemolytic activities and biofilm-formation of a collection of clinical isolates of UPSA, ABSA and recurrent *S. agalactiae* bacteriuria (rSAB) strains to explore these phenotypes in the context of clinical history of isolates. A total of 61 UPSA, 184 ABSA, and 47 rSAB isolates were analyzed for relative hemolytic activity by spot assay on blood agar, which was validated using a erythrocyte lysis suspension assay. Biofilm formation was determined by microtiter plate assay with Lysogeny and Todd-Hewitt broths supplemented with 1% glucose to induce biofilm formation. We also used multiplex PCR to analyze isolates for the presence of genes encoding adhesive pili, which contribute to biofilm formation. Comparing the hemolytic activities of 292 isolates showed, surprisingly, that ABSA strains were significantly more likely to be highly hemolytic compared to other strains. In contrast, there were no differences between the relative abilities of strains from the different clinical history groups to form biofilms. Taken together, these findings demonstrate a propensity of *S. agalactiae* causing ABU to be highly hemolytic but no link between clinical history of UTI strains and ability to form biofilm.

1. Introduction

Urinary tract infections (UTI) are prevalent infections of humans worldwide with more than half of all women developing a UTI at least once in their lifetime; half of these women will experience a recurrent infection within 6 months of antibiotic treatment (Sihra et al., 2018). The spectrum of UTI encompasses acute infection in the bladder (cystitis), kidneys (pyelonephritis) and bloodstream (urosepsis) (Tamadonfar et al., 2019). Infection can be classified as complicated, for cases where predisposing factors such as abnormalities of the urinary tract (anatomic or functional) or co-morbidities are present, or uncomplicated (Bader et al., 2010). Asymptomatic bacteriuria (ABU) is a highly prevalent sub-clinical condition, with a diverse etiology (Ipe et al., 2013). The societal burden of UTI is huge in terms of financial costs to the healthcare system, and societal costs from lost productivity (Foxman, 2014; Francois et al., 2016; Sihra et al., 2018).

Streptococcus agalactiae, also known as group B Streptococcus, causes acute UTI as well as ABU (Anderson et al., 2007; Falagas et al., 2006; Hernaiz et al., 2004; Tan et al., 2012b; Ulett et al., 2009). The overall burden of UTI due to *S. agalactiae* in the US alone is estimated to be 160,000 cases per year (Kline and Lewis, 2016). Generally, most cases of uncomplicated cystitis due to uropathogenic bacteria resolve quickly

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(Foxman, 2014), however, acute *S. agalactiae* UTI can be refractive to antibiotic therapy, particularly in the setting of co-morbidities (Ulett et al., 2012). Globally, *S. agalactiae* bacteriuria is estimated at a prevalence between 1.0–3.5 % (Aungst et al., 2004; Le et al., 2004; McKenna et al., 2003; Whitney et al., 2004). Moreoever, in pregnancy, detection of *S. agalactiae* at any count in urine is considered a risk factor for vertical transmission of the pathogen to the neonate and is routinely treated with antibiotics (Lumbiganon et al., 2010; Schnarr and Smaill, 2008; Verani et al., 2010).

A few studies have examined mechanisms of pathogenesis for *S. agalactiae* UTI, which have been informed by discoveries of virulence factors in other infection settings. For example, expression of bacterial β-hemolysin/cytolysin (β-H/C), adhesive pili and biofilm formation have each been shown to contribute to infection or disease due to *S. agalactiae* in various *in vitro* and *in vivo* models (Konto-Ghiorghi et al., 2009; Landwehr-Kenzel and Henneke, 2014; Okumura and Nizet, 2014; Rosini and Margarit, 2015). Uropathogenic *S. agalactiae* (UPSA) are capable of binding to uroepithelial cells of the bladder, and can induce cytokine production (Kline et al., 2011; Tan et al., 2012a; Ulett et al., 2013, 2010) that is triggered, in part, by the β-H/C (Kulkarni et al., 2013). The β-H/C also elicits a strong neutrophil infiltrate in the bladder during acute UTI in mice, and is cytotoxic to uroepithelial cells, which appears to subvert innate immune-mediated clearance of *S. agalactiae* from the bladder (Leclercq et al., 2016). It has been hypothesized that differential expression of β-H/C might influence the pathogenicity of *S. agalactiae* for causing acute UTI (Leclercq et al., 2016). Sialic acid substructures of the capsular polysaccharide, as well as host-derived cathelicidin also appear to influence the outcomes of UTI due to *S. agalactiae* (Kline et al., 2011; Patras et al., 2020), highlighting the multifactorial nature of acute UTI pathogenesis. Finally, relative to the many studies of gram-negative uropathogens, virulence phenotypes of *S. agalactiae* causing UTI have received little attention.

Conversely, ABU-causing *S. agalactiae* (ABSA) have been shown to utilize human urine for growth, unlike UPSA, which stems partly from malic acid metabolism (Ipe et al., 2015). In *Bacillus*, malate potentiates biofilm formation (Pisithkul et al., 2019); and biofilm formation is of interest for the UTI field due to notable associations with colonization, as reviewed elsewhere (Subashchandrabose and Mobley, 2015). Biofilm formation by *S. agalactiae* requires pili to enhance binding to epithelial cells (Konto-Ghiorghi et al., 2009). Overall, however, many aspects of *S. agalactiae* UTI, including the nature of ABU and biofilm formation, remain unexplored.

In this study, we investigated a collection of UPSA, ABSA and recurrent clinical isolates from different forms of UTI to explore hemolytic activities, biofilm formation and the repertoire of pilin-encoding adhesin genes among UTI isolates to investigate potential disease associations.

2. Materials and methods

2.1. Bacterial strains

The bacterial strains used in this study, including the genome-sequenced reference *S. agalactiae* strains NEM316 and 874391, are listed in Table S1. All 292 test isolates were cultured from urine of patients with acute UTI or ABU during the 48-month period between August-2007 and July-2011, as described elsewhere (Ipe et al., 2015; Tan et al., 2012b; Ulett et al., 2009). UPSA isolates (n = 61) were from acute UTI, as defined by positive urinary leukocyte esterase, pyuria and symptoms; ABSA isolates (n = 184) were from cases of single-organism bacteruria (any count) in the absence of symptoms in addition to negative findings for esterase and pyuria in cases where urinalysis was undertaken (Tan et al., 2012b). A collection of 47 additional isolates were recovered from repeat urine cultures of individual patients, and categorized as recurrent *S. agalactiae* bacteriuria (rSAB) for inclusion in this study. The details of the rSAB isolates (n = 47) are provided in Table S1. Two gene-deficient derivatives of wild-type (WT) *S. agalactiae* 874391, namely a non-hemolytic *cylE*$^{-}$ mutant (Ulett et al., 2003), and a hyper-hemolytic *covR*$^{-}$ mutant (Sullivan et al., 2017) were used in hemolytic activity assays as negative and positive control strains, respectively.

2.2. Hemolytic activity assays

The relative levels of hemolytic activity among the bacterial isolates were determined using a spot assay on blood agar. For spot assay, overnight THB cultures were diluted 1/100 into fresh media and 2 µl inocula were spotted onto the surface of freshly prepared 5% horse-blood agar in triplicate. Each assay included negative and positive control strains of mutant 874391 deficient in *cylE*$^{-}$ (non-hemolytic) and *covR*$^{-}$ (hyper-hemolytic), respectively. Data are reported using a scale of no hemolytic activity (-), low hemolytic activity (+), and high hemolytic activity (++). The spot assays were repeated three independent times for all strains. The semi-quantitative nature of the spot assay was validated against the control strains in analogous erythrocyte lysis suspension assays, as described elsewhere (Sullivan et al., 2017). Briefly, overnight THB cultures grown at 37 °C were resuspended in PBS plus 0.2 % glucose and added to a volume of 1% (v/v) horse erythrocytes. During a 2 h incubation at 37 °C, supernatants were diluted 1:5 in PBS, and hemoglobin release was measured at an optical density (OD) of 420 nm. For ABSA strains 1014, 834 and 729 (which grow in human urine (Ipe et al., 2015, 2021)), additional erythrocyte lysis suspension assays were performed using cultures of the bacteria in synthetic human urine (SHU) to examine hemolytic activity in a medium more relevant to the urinary tract niche. For this, the strains were grown in SHU as described previously, with 40 mM malic acid used to support the growth of ABSA 729 (Ipe et al., 2015). Following overnight growth, the bacteria were resuspended in PBS plus 0.2 % glucose and used as described above.

2.3. Measurement of biofilm formation

The relative levels of biofilm formation by the strains were measured using a microtiter plate assay, essentially as described elsewhere (Mabbett et al., 2009), with minor modifications. Briefly, overnight THB cultures grown at 37 °C were diluted 1:20 into fresh media, and 150 µl were used to inoculate the wells of a 96-well microtitre plate (tissue culture treated polystyrene, round bottom (Corning, CLS3799)). Initial optimization experiments were performed to compare biofilm formation in different media, including Lysogeny broth (LB) and Todd-Hewitt broth (THB), with 1% glucose supplemention, based on previous studies (Konto-Ghiorghi et al., 2009; Rinaudo et al., 2010). Following 24 h of static or shaking growth at 37 °C, the wells were washed five times with water to remove unbound cells, and stained with 1% crystal violet in water for 30 min at 4 °C. Bound bacteria, representing the amount of biofilm formed, were quantified by the addition of 150 µl of acetone–ethanol (20:80); the amount of dissolved crystal violet was measured at an OD of 595 nm. The data are shown as means of three independent assays, each comprising eight technical replicates for each strain in each assay. For categorical analysis, the biofilm forming ability of each isolate was classified as either low ($OD_{595} < 0.5$), medium ($0.5 < OD_{595} < 1.0$) or high ($OD_{595} > 1.0$).

2.4. Multiplex PCR screen for pilin-encoding genes and dot-blot

We developed a multiplex PCR to determine the presence of genes encoding pili, which contribute to *S. agalactiae* biofilm formation, among the isolates used in this study. Genomic DNA was extracted from overnight bacterial cultures, as described elsewhere (Ipe et al., 2015) and used as template for PCR assays with MyTaq Mix (Bioline). The primers were Pil1-scrn-F ATTGTTTTCGGCTGCTGTTT, Pil1-scrn-R CTTCGCCGTCTTTATTCTCG (209 bp); Pil2a-scrn-F CCAATCAACCCATCAGAACC, Pil2a-scrn-R CACCGGCGTTAGAGATCAAT (382 bp);

Pil2b-scrn-F GGGCAGCTACCAATACTCCA, Pil2b-scrn-R CACCTGTTGAAGGCAACTCA (579 bp). The cycling conditions were as follows: initial denaturation (95 °C, 1 min), denaturation/annealing/extension (95 °C, 15 s/ 60 °C, 15 s/ 72 °C, 20 s; 30 cycles), final extension (72 °C, 1 min), and hold (10 °C). A dot-blot assay was used to detect the expression of pili protein Spb1 using anti-sera as described elsewhere (Chattopadhyay et al., 2011) using equal amounts of bacteria for each strain grown overnight in THB cultures.

2.5. Statistical analysis

Growth curve data were compared using area-under-the-curve and students *t* test. Biofilm data were compared using Kruskall-Wallis ANOVA followed by Dunn's post-hoc analysis. The proportional distributions of categorical variables for hemolytic phenotypes were compared by Fisher's exact test. Statistical significance was accepted with P values of ≤ 0.05. All data were analysed using GraphPad Prism V8.

3. Results

3.1. Hemolytic activities of UTI isolates of S. agalactiae

Hemolysin expression is associated with processes of *S. agalactiae* UTI (Kulkarni et al., 2013; Leclercq et al., 2016) and we assessed the hemolytic activities of 292 clinical isolates of UPSA, ABSA and rSAB to explore potential disease associations. The isolates were compared to control strains of non-hemolytic 874391Δ*cylE* (-), wild-type (WT) 874391 (+), and a hyper-hemolytic 874391Δ*covR* mutant (++). The control strains exhibited the expected phenotypes in each independently performed spot assay (Fig. 1A), which was validated against the erythrocyte lysis suspension assay (Fig. 1B). Assessment of all 292 test isolates showed an overall distribution of no hemolytic activity for a total of 6 strains (2.1 %), low hemolytic activity (+) for 252 strains (86.3 %), and high hemolytic activity (++) for 34 strains (11.6 %) (Fig. 2A). Separating these data according to the clinical history of isolates to analyse the proportional distributions revealed that ABSA strains were significantly more likely to be highly hemolytic compared to either UPSA or rSAB strains (Fig. 2B). Surprisingly, among the total 34 strains identified as highly hemolytic were 28 ABSA strains, accounting for 82.4 % of all highly hemolytic strains ($p = 0.009$). Interestingly, all of the 6 non-hemolytic strains were also ABSA strains. Thus, overall, among this collection, isolates classed as highly hemolytic by standard hemolysis assays are more likely to be of an ABU clinical history. Next, to examine whether the hemolytic activity of ABSA strains might be affected by urine, we tested the relative hemolytic activity of ABSA strains 1014, 834, and 729 grown in SHU compared to THB. Surprisingly, ABSA 1014 and 834 grown in SHU were less hemolytic than THB-grown cultures; but ABSA 729 was more hemolytic (Fig. 3). Thus, the hemolytic activity of ABSA strains *in vitro* can be affected by growth of the bacteria in urine.

3.2. Biofilm formation by UPSA, ABSA and rSAB

Biofilms can be important for the development of UTI (Subashchandrabose and Mobley, 2015) prompting us to assess the ability of all 292 test isolates to form biofilms on abiotic surface. Initial assays confirmed that *S. agalactiae* NEM316, previously shown to form biofilms *in vitro* (Konto-Ghiorghi et al., 2009), produced substantial biofilm in the microtitre assay; in contrast, genome-sequenced *S. agalactiae* 874391 did not form any detectable biofilm (Fig. 4A). Notably, supplementation of THB and LB media with 1% glucose was essential for significant biofilm formation by the positive control NEM316 strain in both shaking and static culture conditions (Fig. 4A); a finding consistent with prior reports (Konto-Ghiorghi et al., 2009; Rinaudo et al., 2010). The divergent biofilm forming phenotypes of the NEM316 and 874391 control strains developed despite equivalent growth rates in the culture conditions tested (*i.e.* media with 1% glucose); specifically, the average doubling times of the control strains were 0.59−0.82 for NEM316 and 0.63−0.88 for 874391 (calculated at 2−3 h) (Fig. 4B). Based on this, we chose to use LB with 1% glucose in subsequent assays of test strains, and included NEM316 and 874391 in each assay.

Comparisons of all 292 test isolates for biofilm formation were performed in both shaking and static growth conditions; both shaking and static growth conditions could be encountered in the urinary bladder, whereas the latter is often used for microtitre plate biofilm assays. Overall, we detected no clear differences in biofilm formation between the groups of isolates; each of the different groups comprised many isolates that formed substantial biofilms (OD_{595nm} values >1.5) as well as many that failed to make biofilm (OD_{595nm} values <0.5) (Fig. 5A). Interestingly, many isolates of each different clinical history group showed substantially higher biofilm formation compared to the positive control NEM316, with peak OD_{595nm} values >1.5, compared to an average peak of <1.0 for the control, indicating very strong biofilm formation *in vitro* by multiple *S. agalactiae* UTI isolates.

Sorting the isolates into phenotypic categories of high, medium and low biofilms formers identified 113 isolates as low (38.7 %), 105 as medium (36.0 %), and 74 as high (25.3 %) biofilm formers in shaking culture conditions (Fig. 5B). In comparison, static culture conditions identified 116 isolates as low (39.7 %), 118 as medium (40.4 %), and 58 as high biofilm formers (19.9 %). However, analyzed according to clinical history, we found no significant differences in the proportions of strains that exhibited low, medium, or high biofilm formation for UPSA, ABSA, or rSAB strains under shaking or static conditions (Fig. 5B). We also tested the biofilm forming ability of ABSA strains 1014, 834, and 729 in SHU with 1% glucose and compared this to the results obtained using LB media with 1% glucose. This showed strong biofilm formation by ABSA strains 1014, 834 in SHU (Fig. S1), suggesting that the phenotype may occur in the urinary niche. Together, these data establish that biofilm formation is variable among *S. agalactiae* UTI isolates, and independent of the clinical history of strains, according to *in vitro* biofilm analysis.

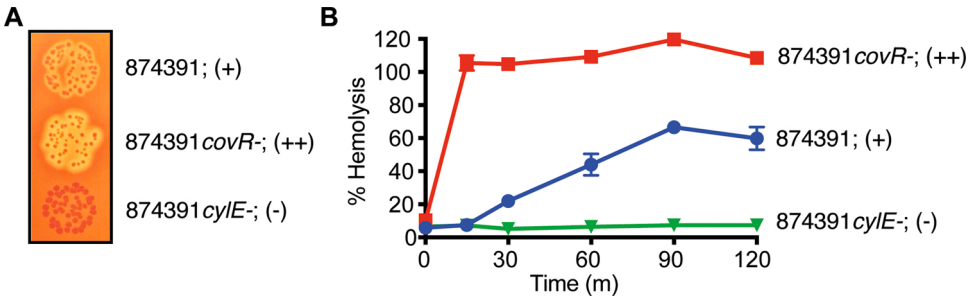

Fig. 1. Semi-quantitative hemolytic activity spot assay. (A) Bacteria were scored using a scale of non-hemolytic (-), low (+), and high (++) levels of hemolysis on blood agar. The control *S. agalactiae* strains of wild-type 874391, hyper-hemolytic 874391*covR*$^{-}$ and the non-hemolytic 874391*cylE*$^{-}$ mutant are shown. (B) The semi-quantitative nature of the assay was validated using controls in a erythrocyte suspension assay; reproduced with permission (Sullivan et al., 2017).

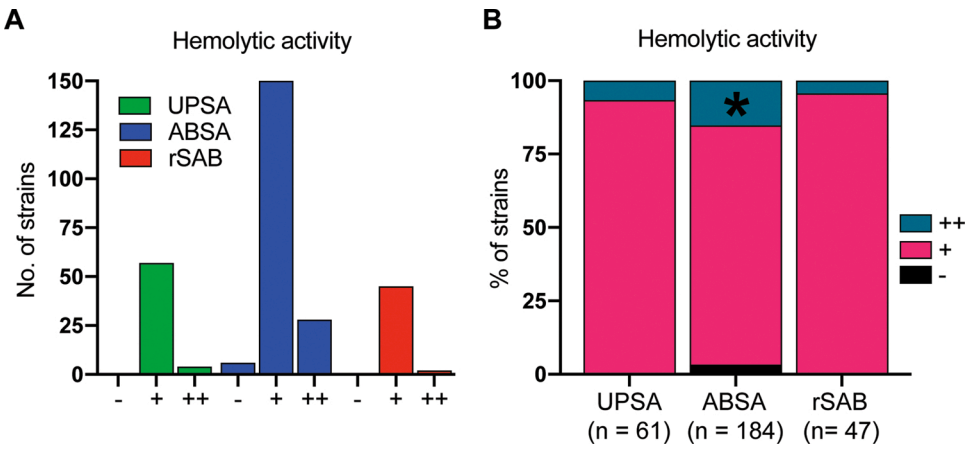

Fig. 2. Comparative hemolytic activities of *S. agalactiae* UTI isolates. (A) Relative hemolytic activities of 292 clinical isolates, as determined by spot assay and grouped according to clinical presentation with acute, UPSA (n = 61); asymptomatic, ABSA (n = 184); and recurrent bacteriuria, rSAB (n = 47) strains. Key is non-hemolytic (-), low (+), and high (++) levels of hemolysis. (B) Comparing the distributions between clinical history groups shows a significantly higher proportion of ABSA strains are highly hemolytic compared to other groups (*p = 0.009).

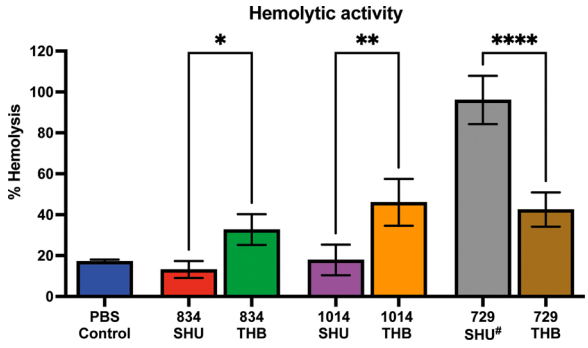

Fig. 3. Hemolytic activity of ABSA strains 1014, 834 and 729 grown in either synthetic human urine (SHU) or THB media. Bacteria were grown in SHU or THB overnight, and hemolytic activity quantified using a erythrocyte suspension assay. Strains 834 and 1014 exhibited higher hemolytic activity when grown in THB as compared to SHU. Conversely, strain 834 exhibited higher hemolytic activity when grown in SHU as compared to THB. SHU# denotes 40 mM supplemental malic acid in SHU required to support the growth of 729. Bars represent % lysis of erythrocytes at 30 min. *p = 0.0337; **p = 0.0026; ****p < 0.0001.

3.3. Multiplex PCR for pilin-encoding genes

The expression of pili is associated with the ability of *S. agalactiae* to form biofilms (Konto-Ghiorghi et al., 2009; Rinaudo et al., 2010) and we examined the prevalence of the three *S. agalactiae* pilus islands (PI; PI-1, PI-2a and PI-2b) among the test isolates by utilizing a novel multiplex PCR targeting each pili gene cluster (Fig. 6). We found that PI-1 was the predominant pilus type, with the correct sized fragment amplified from 205 isolates (70.2 %). PI-2a was the next most common pilus type (117 isolates; 40.1 %), followed by PI-2b (30 isolates; 12.3 %) (Table 1). Of the strains that screened positive for the different pili, 80, 7 and 15 isolates possessed only PI-1, PI-2a or PI-2b alone respectively. A total of 65 isolates did not screen positively for any of the three pili. 110 isolates screened positively for both PI-1 and PI-2a, and 15 for both PI-1 and PI-2b (Table 1). Analyses of potential correlation between the level of biofilm formation and the presence of pilin-encoding genes revealed that a significant higher proportion of isolates categorized as low biofilm formers (OD_{595} < 0.5) possessed one of the three pili, as compared to high biofilm formers (OD_{595} > 1.0), across both static and shaking conditions. In other words, high biofilm forming strains had the lowest proportion of strains that possessed any of the three pilin-encoding genes, compared to low and medium biofilm formers (Table 1). Expression of Pil2B in strains that screened positive by PCR for pilin-encoding genes was detected using antisera against the backbone protein and confirmed expression but no correlation with the degree of biofilm formation (Fig S2).

4. Discussion

In the absence of any established phenotypic distinctions between *S. agalactiae* isolates from different forms of UTI, excepting a capacity of some ABU strains to grow in human urine, we undertook this study to explore virulence-associated phenotypes in the context of clinical history. We examined hemolytic activity and biofilm formation of a collection of UPSA, ABSA and rSAB isolates because these traits in bacteria that cause UTI are, in general, associated with disease pathogenesis and virulence. The key findings of this study are (i) the identification of a disproportionately higher number of highly hemolytic *S. agalactiae* UTI isolates among ABSA isolates, (ii) an absence of any association between biofilm formation by *S. agalactiae* UTI isolates and the clinical history of a given strain, and (iii) a mixed distribution of pilin-encoding genes among *S. agalactiae* UTI isolates. These findings are surprising in the discovery of a significant association between hyper-hemolytic activity in *S. agalactiae* and ABU, and a lack of correlation between biofilm formation and acute or asymptomatic infection for this pathogen.

The association between ABU and a higher probability of a *S. agalactiae* isolate exhibiting a hyper-hemolytic phenotype is unexpected in the context of a prior finding that the pathogenesis of acute UTI in mice depends on the β-H/C that mediates cytotoxicity, inflammatory cytokine synthesis, and virulence (Leclercq et al., 2016). A separate study also reported that β-H/C enhances host inflammation in the bladder of mice infected with *S. agalactiae* with but is dispensable for establishment of UTI (Kulkarni et al., 2013). If we accept that the β-H/C causes inflammation in the urinary tract, how then do we reconcile that finding that ABSA isolates are more likely to be hyper-hemolytic than strains that cause acute UTI? This is an intriguing finding and one that we cannot readily explain. At the cellular level, β-H/C strongly dictates the intracellular survival of *S. agalactiae* in human macrophages, and tightly controlled regulation of β-H/C expression is required for the successful establishment of *S. agalactiae* in different host niches (Sagar et al., 2013). Perhaps such tightly controlled expression might also be operative in the host environment of the urinary tract; indeed, in one study of uropathogenic *Escherichia coli* (UPEC), the gene encoding hemolysin A was found to be not highly expressed in the bacteria during UTI in humans (Subashchandrabose et al., 2014). Additionally, UPEC (the most common causative agent of UTIs in humans) possess a plethora of factors which aid in colonization and survival in the host (Brzuszkiewicz et al., 2006; Wiles et al., 2008). UPEC pathogenesis cannot be universally linked to any single factor, and disease severity/progression is largely associated not only with the factors the invading strain possesses, but also host factors and immune responses (Johnson, 1991; Maslow et al., 1993). This suggests that other factors independent of

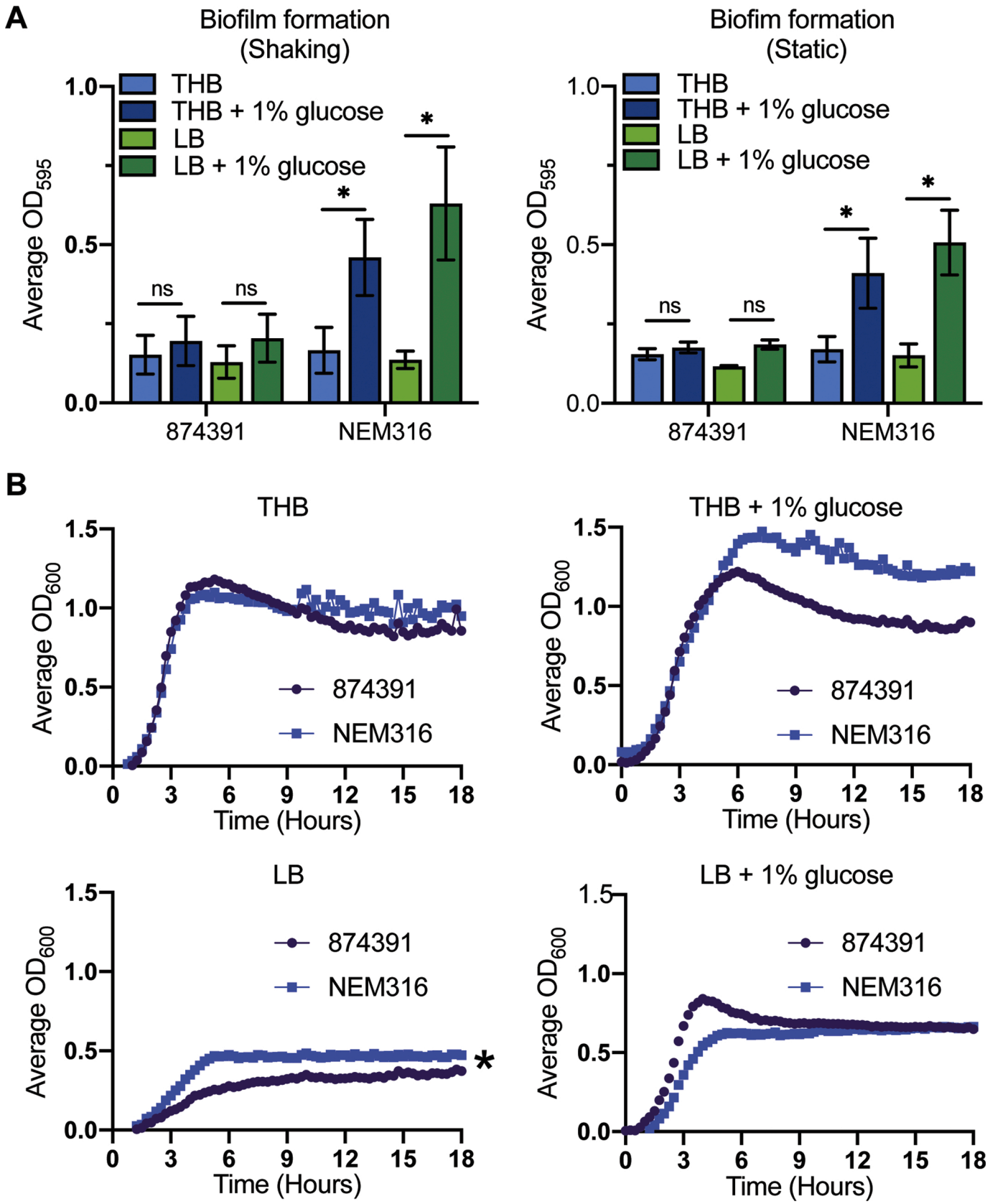

Fig. 4. Biofilm formation and growth of control strains 874391 and NEM316. (A) Results of microtitre plate assay under shaking and static conditions shows biofilm formation by positive control NEM316 but no biofilm formation by negative control 874391. Supplementation of THB or LB with 1% glucose enhanced biofilm formation; *$p < 0.05$. (B) Growth curves show equivalent growth rates of the two strains overall, excepting in LB. Strains were grown in THB, THB + 1% glucose, LB, or LB + 1% glucose. Graphs show average absorbance values at OD_{600} from three independent assays. Mean absorbances compared using AUC and *t* test; * $p < 0.05$.

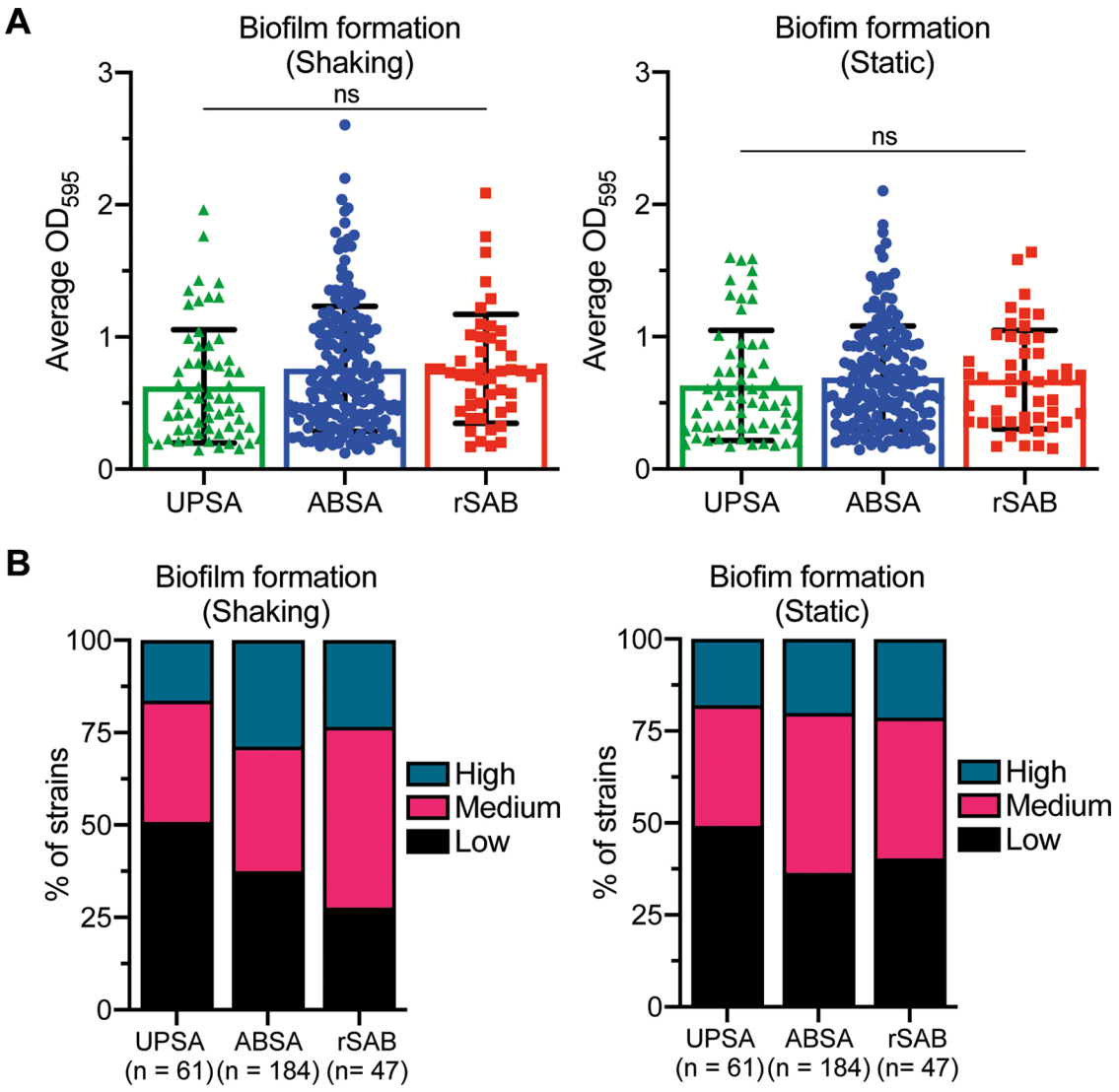

Fig. 5. Biofilm formation by *S. agalactiae* UTI isolates. (A) Biofilm formation by the 292 clinical isolates according to clinical history (*i.e.* acute, asymptomatic, or recurrent), grown in LB + 1% glucose under either shaking or static conditions at 37 °C. Each point represents the mean absorbance value of one strain at OD_{595nm} calculated from three independent experiments, and error bars show the standard deviation of the OD_{595nm} of the strains within the same clinical presentation. (B) Categorizing the biofilm forming ability of each isolate as low ($OD_{595} < 0.5$), medium ($0.5 < OD_{595} < 1.0$) or high ($OD_{595} > 1.0$) demonstrates no significant difference in the proportions of strains between clinical histories to form the different biofilm phenotypes.

β-H/C may have contributed to the ability of the UPSA isolates to cause acute infections. Intriguingly, we also found that the level of hemolytic activity in some ABSA strains grown in urine differ compared to the same strains grown in THB; for some, we observed less hemolytic activity after growth in urine, but for others, growth in urine led to higher hemolytic activity. This suggests that host factors might effect hemolysin expression *in vivo*. A limitation of this part of the study is that many *S. agalactiae* isolates are unable to grow in human urine (Ipe et al., 2015, 2021), which precludes equivalent assays for a broad range of strains in human urine.

In the broader context of disease due to *S. agalactiae*, a previous study correlated the severity of arthritis with β-H/C expression (Puliti et al., 2000), which highlights the role of this factor in host inflammatory processes. Clinical isolates of *S. agalactiae* cultured simultaneously from a single tissue sample can also exhibit heterogeneous hemolysin expression, according to concurrent isolation of hemolytic and nonhemolytic colonies from blood cultures of an infant with sepsis (Sigge et al., 2008). This suggests differential levels of β-H/C can exist in a population of *S. agalactiae* cells within infected tissue. Among several dozen isolates identified as hyper-hemolytic in the current study (most of which were cultured from individuals with ABU), we observed a variety of serotypes and distinct patterns of antibiotic resistance according to disc diffusion assay. These observations argue against the idea that these hyper-hemolytic isolates are of a single clone. However, how β-H/C expression might vary during different stages of UTI and in different tissue contexts during infection remains unknown.

The expression of β-H/C in *S. agalactiae* is repressed by the CovR/S two component system (Jiang et al., 2005), which itself affects the pathogenesis of acute UTI (Sullivan et al., 2017). In group A streptococci, the parallel two component system enables gene expression to be regulated to confer adaptation to changing conditions in the host, such as at different infection stages (Hertzen et al., 2012). Taken together, these observations highlight the overarching governance by a global virulence regulator over multifactorial inputs into pathogenesis, including hemolysin toxin expression. Finally, it is important to emphasize the limits of the *in vitro* assay used in this study to examine β-H/C activity of the *S. agalactiae* isolates; similar assays are extensively applied as a routine and convenient measure of hemolytic activity in bacteria but are not reflective of β-H/C expression in a setting of infection. Thus, divergent β-H/C expression or distinct biological effects

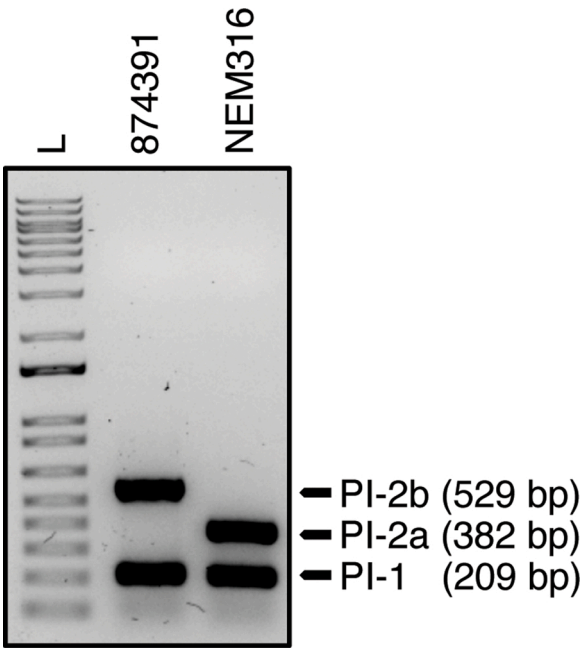

Fig. 6. Multiplex PCR for pilin-encoding genes PI-1, PI-2a and PI-2b in *S. agalactiae*. Results for the control strains 874391 and NEM316 show a band indicating the presence of PI-1 (209 bp) in both strains, a band indicating the presence of PI-2a (382 bp) only in NEM316, and a band indicating the presence of PI-2b (529 bp) only in 874391. L: 1 Kb Plus DNA ladder.

might be specific to different host niches at the cellular and/or tissue level, and further examination of β-H/C expression in *S. agalactiae* cultured in urine, or isolated from different urinary tract niches would be of interest.

Biofilm formation by *S. agalactiae* depends on pili expression (Konto-Ghiorghi et al., 2009; Rosini and Margarit, 2015) and is suggested to enhance colonization and persistence in the host by conferring resistance to host defences and nutrient deprivation (Rosini and Margarit, 2015). Environmental factors influence biofilm formation by *S. agalactiae* (Rosini and Margarit, 2015); among such factors, acid pH was shown to promote biofilm formation by some serotype III strains (D'Urzo et al., 2014), and changes in pH altered biofilm formation by *S. agalactiae* in another study (Borges et al., 2012). Interestingly, of the ten capsular serotypes of *S. agalactiae*, serotype III is among the most frequently isolated from acute UTI patients (along with serotypes V and Ia) (Ulett et al., 2009). In serotype III *S. agalactiae*, the sialic acid-containing capsular polysaccharide enhances virulence during UTI in mice (Kline et al., 2011). Also in serotype III *S. agalactiae*, mutation of the capsule-encoding *cpsE* gene leads to enhanced bacterial infection of the bladder in mice (Sullivan et al., 2017). Thus, the nature of *S. agalactiae* capsule (structure and expression) affects the pathogenesis of UTI; however, there are no reports to our knowledge that have explored possible role(s) for biofilm formation by *S. agalactiae* in the context of UTI.

In the current study, our finding of varying degrees of biofilm formation among *S. agalactiae* UTI isolates independent of clinical history offers a parallel to a prior description of variable biofilm formation among clinical *S. agalactiae* isolates from India (Kaur et al., 2009). In that study, the capability of *S. agalactiae* to form a biofilm was more characteristic of strains cultured from asymptomatic carriers (pregnant women) *versus* symptomatic patients of varied disease presentation. Our findings support the notion that biofilm formation is no more typical of asymptomatic (ABU) strains of *S. agalactiae* as compared to symptomatic (uropathogenic) strains. Unexpectedly, multiplex PCR screening of pilin-encoding genes showed that high biofilm forming strains had the lowest proportion of strains that possessed any of the three genes, noting that while we confirmed expression of the pilus backbone protein in the strains tested, we found no correlation with biofilm formation. Thus, there remains no established role for biofilm formation in models of *S. agalactiae* UTI, nor any associations with clinical history of strains.

It is important to consider the inclusion criteria for the bacterial isolates used in this study for the categorization and interpretation of the results relating to hemolytic activity and biofilm formation. The UPSA isolates used in this study were cultured from individuals with acute cystitis and pyelonephritis, as defined by case definitions described elsewhere (Tan et al., 2012a). This grouping encompasses a broad range of UTI disease severity and duration of disease, with diversity in host co-morbidities and demographic characteristics; we chose to not subgroup these isolates into further subdivisions of cystitis and pyelonephritis to maintain simplicity in our goal of comparing uropathogenic isolates with those from cases of ABU. Previous studies of a small number of UPSA strains identified phenotypes of adherence to human urothelial cells, elicitation of inflammatory cytokine responses, and an ability to colonize the bladder in mice. Interestingly, a recent study showed that *Candida* can promote the pathogenesis of acute *S. agalactiae* UTI by increasing the ability of the bacteria to colonize the bladder (Shing et al., 2019). Similarly, the ABSA isolates used in this study were cultured from individuals with varying degrees of ABU of diverse duration, that might contribute to the phenotypic variability observed in this strain group. For these strains, we used a previously accepted definition of ABU (Tan et al., 2012b). In individuals with recurrent infections from whom multiple isolates were cultured from urine, we noted isolates of distinct serotypes, hemolytic levels and antibiotic resistance patterns suggesting separate occurrences of infection with unrelated *S. agalactiae* isolates; sequencing of the genomes of such isolates will be important in future to provide further detailed insight into this aspect. In considering further work, noting the limitations of this study is useful to rationally inform future undertakings that will focus on different types of *S. agalactiae* isolates recovered from UTI. Genomic characterization of the strains in this study would be of exceptional value to provide insight into clonal complex distributions and the genetic relatedness of groups of isolates.

Table 1
Number of strains that screened positively for PI-1, PI-2a and/or PI-2b. % indicates the % of isolates that screened positively for that particular subset of genes (left) and % of isolates that screened positively for that subset of genes within a given clinical presentation (right).

	No. of strains	UPSA (n = 61)	ABSA (n = 184)	rSAB (n = 47)
PI-1	205	43 (21 % / 70.5 %)	135 (65.9 % / 73.4 %)	27 (13.2 % / 57.4 %)
PI-2a	117	26 (22.2 % / 42.6 %)	72 (61.5 % / 39.1 %)	19 (16.2 % / 40.4 %)
PI-2b	30	10 (33.3 % / 16.4 %)	17 (56.7 % / 9.2 %)	3 (10 % / 6.4 %)
PI-1 only	80	16 (20 % / 26.2 %)	57 (71.3 % / 31 %)	7 (8.8 % / 14.9 %)
PI-2a only	7	4 (57.1 % / 6.6 %)	3 (42.9 % / 1.6 %)	0 (0% / 0%)
PI-2b only	15	5 (33.3 % / 8.2 %)	8 (53.3 % / 4.3 %)	2 (13.3 % / 4.3 %)
PI-1 & 2a	110	22 (20 % / 36.1 %)	69 (62.7 % / 37.5 %)	19 (17.3 % / 40.4 %)
PI-1 & 2b	15	5 (33.3 % / 8.2 %)	9 (60 % / 4.9 %)	1 (6.7 % / 2.1 %)

In conclusion, this study shows that isolates of *S. agalactiae* that are cultured from ABU are more likely to be highly hemolytic compared to isolates causing acute UTI. Furthermore, we conclude that the ability to form biofilm *in vitro* is unrelated to the clinical history of UTI strains analyzed in this study. Further examination of the expression of hemolysin by *S. agalactiae* in human urine and during infection in the urinary tract is warranted.

Author contributions

G. Ulett and D. Chattopadhyay conceived the study. G. Ulett, D. Desai, K. Goh, and M. Sullivan designed the research and analyzed the data. D. Desai and K. Goh performed the research. All authors contributed to writing of the paper and approved the final manuscript.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ijmm.2021.151520.

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