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Influence of environmental and genotypic factors on biofilm formation by clinical isolates of group B *streptococci*

Shuping Nie[a,b], Xuedong Lu[b], Yan-Wei Hu[a], Lei Zheng[a], Qian Wang[a,*]

[a] *Laboratory Medicine Centre, Nanfang Hospital, Southern Medical University, Guangzhou, China*
[b] *Department of Laboratory Medicine, The Eighth Affiliated Hospital, Sun Yat-sen University, Shenzhen, China*

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ABSTRACT

Group B *Streptococcus* (GBS) colonizes the gastrointestinal and urogenital tracts of approximately 30% of women, and it can cause sepsis and meningitis in neonates. GBS has been shown to form biofilms *in vitro*, but the effects of environmental and genotypic factors upon GBS biofilm formation are unclear. The aim of the present study was to optimize culture conditions for enhanced GBS biofilm production. Furthermore, this study also investigated the influences of strain lineage, pilus profile, and isolation source on GBS biofilm formation. The results demonstrate that the fed-batch mode and acidic pH strongly enhanced GBS biofilm formation *in vitro*. These findings suggest that the fed-batch mode may be suitable for both screening and fundamental studies of GBS biofilm formation. Moreover, this study demonstrated a correlation between the hyper virulent clonal complex 17 and a strong biofilm phenotype.

1. Introduction

Group B *Streptococcus* (GBS) colonizes the gastrointestinal and urogenital tracts of approximately 30% of healthy women, and it can cause severe infections in neonates, pregnant women, the elderly, and immunocompromised patients under certain circumstances [1–3]. In previous studies, multilocus sequence typing (MLST) was used to target seven conserved housekeeping genes and classify GBS strains into numerous sequence types (STs) and clonal complexes (CCs) [4,5]. The distribution of CCs varies in colonizing and invasive strains, and strains belonging to the CC17 lineage are associated strongly with invasive infections [6–8].

Biofilms are sessile microbial communities in which bacteria are embedded into a self-produced extracellular polymer matrix [9,10]. This microenvironment may enhance resistance to extreme pH, antimicrobial agents, and immune cells and lead to persistent colonization and infection [9,11]. Several studies have examined biofilm formation by different GBS strains *in vitro*; however, there have been inconsistent results regarding the effects of pH and glucose upon GBS biofilm production [12–17]. These contradictory results could be due to the use of the batch growth mode for screening biofilm forming capacity in most studies because this mode lacks the fluid shear stress encountered *in vivo* [18]. An improved protocol based on the fed-batch growth mode was designed by D'Urzo and colleagues to overcome these problems [12]. According to this improved protocol, the plate is incubated under shaking condition to mimic fluid circulation *in vivo*, and nonattached bacteria are removed by washing, followed by replacement of the culture medium [12].

The capacity of GBS to form biofilms varies across environments, genotypes, and individual isolates [14,19]. Although biofilm formation is highly genotype- and strain-dependent, and can be affected by various environmental conditions [14–16,20,21], data regarding the effects of these environmental and genotypic factors on GBS biofilm formation have been inconsistent [12–14,16,17,22]. In this study, biofilm formation by 111 clinical GBS isolates was assessed under batch and fed-batch growth conditions at different pH values (5.0 and 7.8) and in the presence or absence of glucose to evaluate the effects of these environmental factors on biofilm development on an abiotic surface. Furthermore, the influence of genotypic diversity on GBS biofilm formation was also investigated.

2. Materials and methods

2.1. Bacterial strains

A total of 111 GBS isolates representing 23 STs and five CCs (CC1, n = 3; CC12, n = 26; CC17, n = 31; CC19, n = 23; CC23, n = 11; and singletons, n = 17) were included in this study. Isolates were grouped by clinical outcomes and isolation sites. Invasive strains (n = 50) were isolated from normally sterile body sites, such as blood, cerebrospinal

* Corresponding author. Laboratory Medicine Centre, Nanfang Hospital, Southern Medical University, 1023 Shatai South Road, Guangzhou 510515, Guangdong, China.
E-mail addresses: nfyywangqian@163.com, 793499131@qq.com (Q. Wang).

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fluid, peritoneal fluid, or synovial fluid. Noninvasive isolates (n = 61) were derived from urine, tracheal, and gastric aspirates. Three pilus islands, PI-1, PI-2a, and PI-2b, were previously characterized in these strains (Nies et al., unpublished data). Strains are listed in additional file 1.

2.2. Biofilm assays

2.2.1. Biofilm formation in batch mode

The biofilm formation assay was performed as previously described [22]. In brief, GBS strains were grown at 35 °C under 5% CO_2 overnight on blood agar plates. Bacterial cell density was adjusted to an optical density (OD) at 600 nm (OD600) of 0.05 in Todd-Hewitt broth (THB; Becton Dickinson, Sparks, MD). Growth and biofilm experiments were performed using THB at different pH values (7.8 and 5.0) with or without 1% glucose. An aliquot (200 µl) of the diluted culture was used to inoculate 96-well polystyrene flat bottom microtiter plates (Costar; Corning, Inc., Corning, NY). Plates were incubated under static conditions at 37 °C for 18 h. Following incubation, unattached bacteria were removed by rinsing three times with 200 µl of double distilled H_2O (ddH_2O). Adherent bacteria were stained for 10 min with 200 µl of 0.5% (w/v) crystal violet (CV; Sigma-Aldrich, Inc., St. Louis, MO). After rinsing three times with ddH_2O, unbound CV was removed and bound CV was released from stained cells using 200 µl of 95% ethanol. Biofilm formation was quantified by measuring absorbance readings at 595 nm (OD595) with a microplate reader (Shenzhen Huisong Technology Development Co., Ltd., Shenzhen, China). All assays were performed three times with three technical replicates. Uninoculated THB samples were used as negative controls.

2.2.2. Biofilm formation in fed-batch mode

The fed-batch growth mode for biofilm formation was performed as described previously [12]. GBS strains were grown overnight on blood agar plates and diluted to an OD600 of 0.05 in different media as described above for batch mode. Culture samples (200 µl) were added to Costar plates, which were sealed to limit oxygen exchange and shaken at 60 revolutions per minute (rpm) at 37 °C for 8 h. Subsequently, nonattached GBS cells were removed by washing with phosphate buffered saline (PBS; Sigma-Aldrich, Inc., St. Louis, MO), and 200 µl of fresh media was added. After incubation for 15 h at 37 °C, the plates were washed three times with 200 µl PBS. Then, the plates were air dried at room temperature for 30 min. Biofilm biomass was quantified using the CV staining method, as described above.

2.3. Growth experiments

Four clinical isolates (two strong and two weak biofilm-forming strains) were grown overnight at 37 °C in THB at pH 7.8. The samples were then diluted to an OD600 of 0.1 with 10 ml of different types of THB media, as described above. An aliquot (200 µl) of this working culture was used to inoculate Costar plates. The plates were then incubated under static conditions at 37 °C. OD600 was measured after an 18-h incubation period. Each experiment was performed in triplicate. Results are presented as $\ln(N/N_0)$, where N_0 and N are the mean OD600 values measured at time 0 and t, respectively, for each of the four representative strains. Maximum specific growth rates (μ_{max}, h^{-1}) were calculated as slopes of the growth curve in the exponential phase with the following equation:

$$\mu max = \frac{\ln\ OD2 - \ln\ OD1}{t2 - t1}$$

Where OD_1 and OD_2 are the two OD values on the line corresponding to time points t_1 and t_2, respectively [23,24].

2.4. Statistical analysis

The optical density cut-off (ODc) was calculated as the mean OD value of the negative control and was equal to 0.21. Based on the cut-off, GBS isolates were classified as strong (OD595 > 0.84), moderate (0.84 ≥ OD595 > 0.42), weak (0.42 ≥ OD595 > 0.21), and non-(OD595 ≤ 0.21) biofilm producers. A generalized linear mixed model (GLMM) with ordinal data was used to determine the fixed effects of pH, glucose, and the growth mode as well as the interaction of pH and glucose on biofilm formation. The GLMM is an extension of the generalized linear model in which the linear predictors contain random effects in addition to the usual fixed effects [25]. The chi-square, Fisher's exact, and Wilcoxon rank sum tests were used to compare groups. Univariate and multivariate ordinal logistic regression model analyses were performed to determine genotypic predictors of enhanced biofilm production. A p-value < 0.05 was considered significant. All calculations were analyzed with SPSS software version 20.0 (SPSS Science, Chicago, IL).

3. Results

3.1. Impact of pH and glucose on biofilm formation

Concerning the effect of pH on biofilm formation, in the absence of glucose, GBS isolates achieved significantly greater biomass amounts in THB at pH 5.0 than in THB at pH 7.8, independent of the growth mode (Table 1). The average CV assay values at pH 5.0 were 0.54 (range: 0.19 to 2.67) and 1.02 (range: 0.22 to 3.83) for the batch and fed-batch growth modes, respectively. In contrast, the average CV assay values at pH 7.8 were 0.24 (range: 0.18 to 0.87) and 0.25 (range: 0.17 to 1.52) for the batch and fed-batch growth modes, respectively. In THB at pH 7.8 without glucose, only one ST529 strain tested was observed to form strong biofilm in both growth modes. On the other hand, in THB at pH 5.0 (growth condition without glucose), 17 strains were classified as strong biofilm formers in the batch mode and 33 strains were classified as strong biofilm formers in the fed-batch mode (Table 2).

Glucose supplementation at neutral pH led to a significant increase in the mean CV assay value. The average CV assay values in THB at pH 7.8 with glucose were 0.57 (range: 0.22 to 3.50) and 1.01 (range: 0.23 to 4.87) for the batch and fed-batch growth modes, respectively (Table 1). There were 13 strains in the batch mode and 26 strains in the fed-batch growth mode that formed strong biofilms in THB at pH 7.8 with glucose. In contrast, whatever the growth mode, there was only one strong biofilm producer in THB at pH 7.8 without glucose (Table 2). Although an equal number of strong biofilm-forming strains were found in THB at pH 5.0 with or without glucose for the two growth modes, differences were noted in the CV assay values. Overall, the highest mean CV assay value and the highest number of strong biofilm-forming strains were those grown in THB supplemented with 1% glucose at pH 5.0, independent of growth mode (Fig. 1).

However, the results of the growth experiments revealed that

Table 1
Effects of pH, glucose and growth mode on biofilm formation.

Medium	CV assay value		Mean/mean$_{THB\ pH\ 7.8}$ (p)		Mean$_{Fed\text{-}batch}$/ mean$_{Batch}$ (p)
	Batch	Fed-batch	Batch	Fed-batch	
THB pH 7.8	0.24	0.25	–	–	1.04 (0.035)
THB pH 7.8 + 1% glucose	0.57	1.01	2.38 (< 0.0001)	4.04 (< 0.0001)	1.77 (< 0.0001)
THB pH 5.0	0.54	1.02	2.25 (< 0.0001)	4.08 (< 0.0001)	1.89 (< 0.0001)
THB pH 5.0 + 1% glucose	0.62	1.16	2.58 (< 0.0001)	4.64 (< 0.0001)	1.87 (< 0.0001)