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Note

An improved medium for growing *Staphylococcus aureus* biofilm

Ping Chen, Johnathan J. Abercrombie, Nicole R. Jeffrey, Kai P. Leung *

Microbiology Branch, US Army Dental and Trauma Research Detachment, Institute of Surgical Research, Fort Sam Houston, TX 78234, United States

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ABSTRACT

A medium (Brain Heart Infusion plus 10% human plasma) was developed, tested, and validated for growing *Staphylococcus aureus* biofilm *in vitro*. With this medium, *S. aureus* forms reproducible and robust biofilms in flow chambers under controlled shear flow and with increased viability recovery in static well plates.

Considered one of the leading causes of human bacterial infections, *Staphylococcus aureus*, a gram positive organism, is a ubiquitous opportunistic pathogen that commonly colonizes the anterior nasopharynx of humans. The organism readily produces biofilms, a complex microbial community, on tissues or medical indwelling devices (Higashi and Sullam, 2006). Though little is known about the processes regulating the mature *S. aureus* biofilm, adherence and accumulation of cells are required. The adherence of *S. aureus* to surfaces and a wide range of plasma as well as extracellular matrix proteins (*e.g.*, fibrinogen, collagen, fibronectin, laminin, and elastin) is mediated by adhesion molecules (Higashi and Sullam, 2006). Identified adhesion molecules include the cell wall associated, negatively charged, teichoic acids and a large number of surface proteins belonging to a family of molecules called microbial surface components recognizing adhesive matrix molecules. Cell accumulation of *S. aureus* involves the polysaccharide intercellular adhesin, also known as polymeric N acetylglucosamine (Gotz, 2002; Higashi and Sullam, 2006). The bacteria within the biofilm matrix are protected from the host immune system and are resistant to antibiotic therapy. It has been shown that *S. aureus* biofilms are involved in osteomyelitis; indwelling medical device infections; and peri implantitis, chronic wound infections, chronic rhinosinusitis, endocarditis, and ocular infections (Archer et al., 2011). In addition, emerging evidence indicates that *S. aureus* biofilms cause healing impairment (Gurjala et al., 2011) in wounds, perhaps by altering the proper course of wound healing. Therefore, biofilm clearance is critical to combat the infections caused by this organism.

To improve biofilm treatment strategies and outcomes, we need to develop *in vitro* biofilm models for effective screening of antibiofilm agents in modulating *S. aureus* biofilm development. An important component of an effective anti biofilm screening assay is its ability to generate reproducible robust *S. aureus* biofilms such that the biofilm burden produced can accurately measure the effectiveness of the test agents and present a reasonable chance that the agents will be effective upon evaluation *in vivo*. It is also likely to reduce the chance in identifying false positive(s). In our efforts to develop an assay that can produce a consistent *S. aureus* biofilm burden, we learned that there is a lack of widely accepted media to grow *S. aureus* biofilms. Different media have been used by the research community for studying *S. aureus* biofilms. These media consist mainly of complex medium supplemented with different compound(s), such as, Trypticase Soy Broth (TSB) plus glucose (Shanks et al., 2005; Merino et al., 2009); TSB enriched with glucose and sodium chloride (NaCl) (Beenken et al., 2003; Luong et al., 2009); TSB supplemented with glucose and citrate (Shanks et al., 2008); TSB plus yeast extract, glucose, and citrate (Craigen et al., 2011); and Brain Heart Infusion (BHI) broth with glucose or NaCl (Houston et al., 2011). *S. aureus* formed decent biofilms on microtiter (static well) plates with some of these media. However, in the microfluidic system, only sporadic loose and inconsistent *S. aureus* biofilm was obtained during a 24 hour incubation period with these media. Here, we report a medium that is suitable for use in growing *S. aureus* biofilms under both static and flow (microfluidic) conditions with a consistent test biofilm load.

One key factor that *S. aureus* encounters *in vivo* is blood. Transcriptome analysis of *S. aureus* showed that a number of genes encoding known or putative *S. aureus* virulence factors, such as genes of gamma hemolysin subunits A, B, and C (*hlgA*, *hlgB*, *hlgC*), are up regulated

* Corresponding author at: 3650 Chambers Pass, Bldg 3610, US Army Dental and Trauma Research Detachment, Institute of Surgical Research, Fort Sam Houston, TX 78234, United States. Tel.: +1 210 539 3803; fax: +1 210 539 7566.
E-mail addresses: ping.chen@us.army.mil (P. Chen), Johnathan.abercrombie@amedd.army.mil (J.J. Abercrombie), Nicole.jeffrey@amedd.army.mil (N.R. Jeffrey), kai.leung@amedd.army.mil (K.P. Leung).

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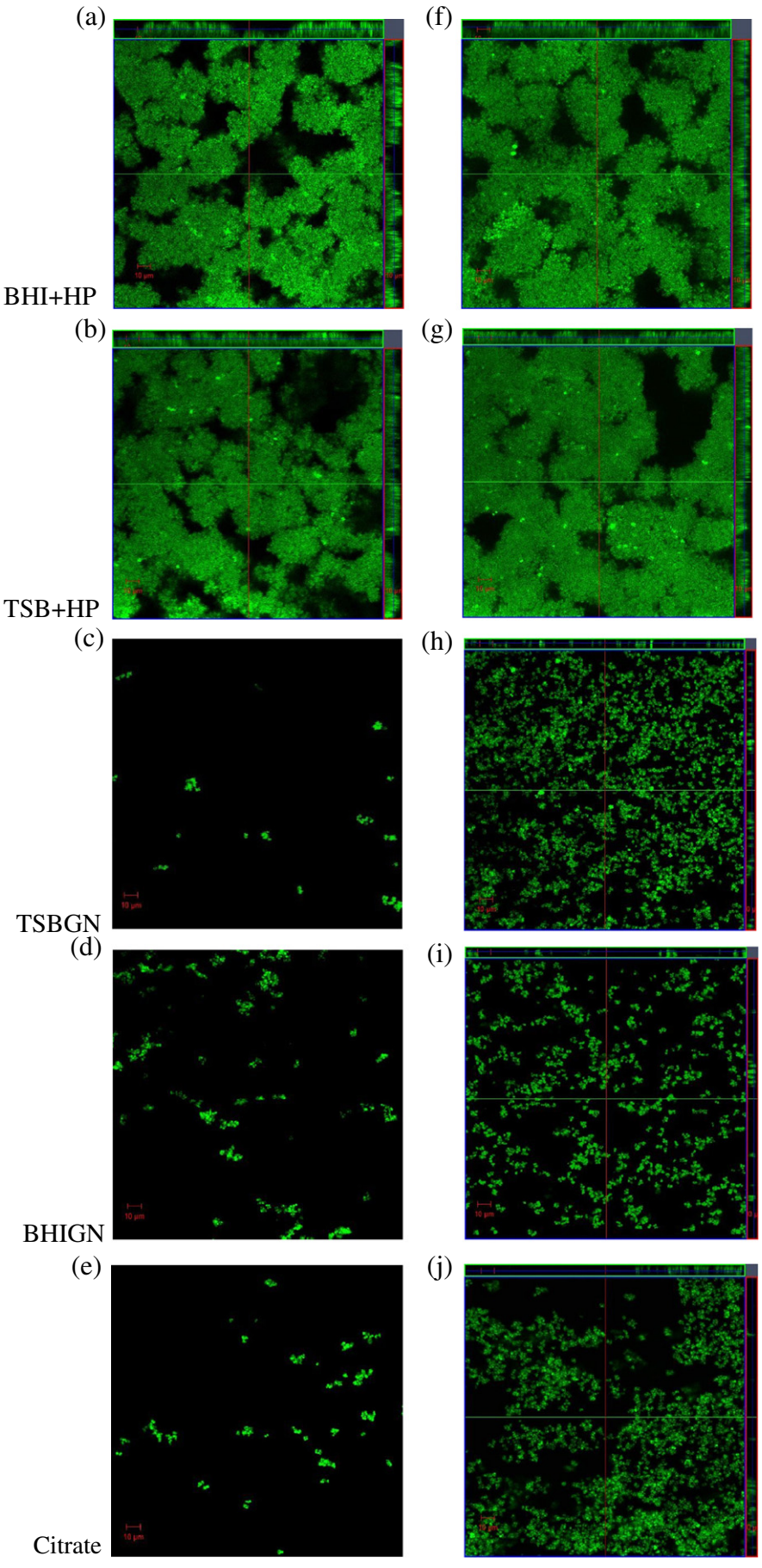
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during culture in human serum or blood (Malachowa et al., 2011). Therefore, we were interested in testing whether any human blood components can also improve the *S. aureus* biofilm formation *in vitro*. For this test, we chose the use of TSB or BHI as the base medium and supplemented it with human plasma (Biological Specialty Corporation, Colmar, PA). We first tested the feasibility of producing *S. aureus* biofilm by using the plasma containing growth medium in an *in vitro* biofilm model based on a microfluidic platform (Fluxion Biosciences, South San Francisco, CA). In essence, the system consists of a network of micro fluidic laminar flow channels integrated into standard well plates suit able for conventional or confocal laser inverted scanning microscopy (LSM). The growth of biofilms is under controlled shear force and temperature mimicking physiologically relevant conditions. To monitor *S. aureus* biofilm formation in real time, we used overnight green fluores cent protein (GFP) tagged clinical isolate *S. aureus* UAMS 1 (University of Arkansas Medical System 1, provided by Dr. S J Hong of Northwestern University) culture to produce biofilms in these channels. The growth properties of GFP tagged and wild type *S. aureus* UAMS 1are similar (data not shown). The inoculum was passed through a 5 μm syringe filter (Pall Corporation, Ann Arbor, MI) to reduce the number of aggregates and adjusted to an optical density at a wavelength of 600 (OD_{600}) of 0.1 [ca. 10^7 colony forming units per milliliter (CFU/ml)] for inoculation. The channels were primed with media, and each channel was inoculated with 50 μl *S. aureus* suspension. After 2 h of attachment, shear flow of 0.6 dyn/cm^2 was initiated, and biofilm formation was monitored in real time using LSM710 (Carl Zeiss MicroImaging, Thornwood, NY). As shown in Fig. 1 left panels, *S. aureus* readily formed a robust compact biofilm (approximately 11 μm thick) with medium containing 10% plasma after 6 h of incubation. Similar results in biofilm formation were obtained by increasing the flow rate up to 1.5 dyn/cm^2. Howev er, the growth of biofilm was affected when the flow rate reached 2.0 dyn/cm^2 or higher. We also tested the effects of different concen trations of plasma on the growth of *S. aureus* biofilms. There was a dose dependent effect of plasma on the biofilm growth up to 10%. No discernible effects were observed when the plasma concentra tions were increased above 10% (data not shown). In contrast, poor biofilm formation was observed using media containing no plasma. Furthermore, it has been reported that pre coating surfaces with plas ma proteins is essential to grow *S. aureus* biofilm (Beenken et al., 2003). To test the effect of pre coating plasma proteins, flow channels in the system were pre coated with 20% human plasma overnight prior to medium priming and bacteria inoculation. Although there was improvement as indicated in Fig. 1 (right panels), *S. aureus* biofilms, formed in medium without supplemented human plasma, were still not robust and were inconsistent. These cell aggregates were often rolled off under the shear flow force.

To further characterize the use of these media containing plasma for growing *S. aureus* biofilms, we also tested other *S. aureus* strains. Similar results of producing robust biofilms were obtained when other *S. aureus* strains (ATCC 6538, ATCC 33591, and ATCC 43300) were used (data not shown). These results suggest that both TSB and BHI supplemented with 10% human plasma are good media for growing *S. aureus* biofilm under fluid dynamic conditions. These human plasma supplemented media also improved the biofilm growth of *S. epidermidis*, but have adversary effects on the biofilm formation of *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Klebsiella pneumoniae* (data not shown).

To determine whether these two media are also good for *S. aureus* biofilm formation under static condition(s), we used two different systems: the microtiter plate system for measuring the formation of

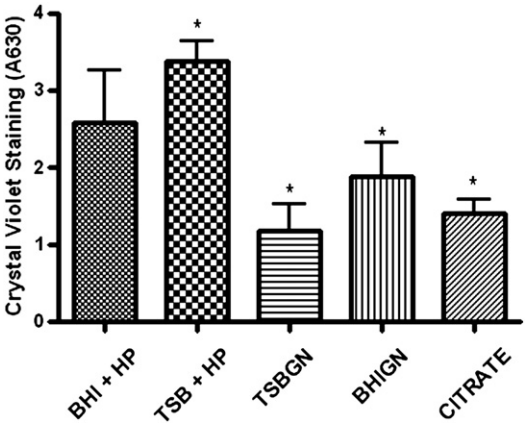

Fig. 2. 24-hour wild type *S. aureus* UAMS-1 biofilms formed in an uncoated 96-well titer plate with different media at 37 °C measured by crystal violet staining (Jackson et al., 2002). Crystal violet was solubilized by 33% acetic acid and read with the BioTek (Winooski, VT) plate reader at the wavelength of 630 nm. The results are representative of those from four independent experiments. Statistics were performed using analysis of variance (one-way) software in which all groups were compared with BHI + plasma resulting in a *P value < 0.001. Error bars represent standard deviation. See Fig. 1 legend for media description.

total biofilm mass and the Minimum Biofilm Eradication Concentra tion (MBEC) device for studying biofilm formation and determining total viable cells in the biofilm (Ceri et al., 1999). Mid log growth phase bacteria were harvested, re suspended in corresponding test media, and adjusted to an OD_{600} of 0.05 (ca. 5×10^6 CFU/ml) for use as inoculum. For the microtiter plate, 200 μl of bacteria suspension per well was added to the desired wells. After approximately 20 h of incubation at 37 °C, media were removed, and biofilms were quantified using the crystal violet staining method and measured spectrophotomet rically at a wavelength of 630 nm (Jackson et al., 2002). For the MBEC system, the MBEC plate was inoculated with 150 μl of bacteria suspen sion per well in the desired wells and incubated for approximately 20 h at 37 °C. The pegs were then rinsed with phosphate buffered saline (PBS) and broken off from the MBEC lid. The biofilms formed on each peg were dislodged into 1 ml of sterile PBS by sonication, and the viable bacterial number was determined by spiral plating. As indicated in Fig. 2, *S. aureus* formed significantly more biofilms with the media containing supplemented plasma than other media tested. The recovered viability cell numbers from biofilms formed with media containing plasma sup plement are more than 2 log higher than with media without the plasma supplement (Fig. 3). These differences are statistically significant with a P value < 0.001. These differences are not resulted simply from increased growth of *S. aureus* in the presence of the plasma supplement. Planktonic growth experiments showed similar growth yields with or without the supplement of the plasma (data not shown).

Our results showed that *S. aureus* formed reproducible and robust biofilms with either TSB or BHI plus 10% human plasma media under both fluid dynamic conditions (Fig. 1) and static conditions (Figs. 2 and 3). It has been shown that, compared with TSB medium, BHI sup ports optimum expression of gamma hemolysin *hlgA* transcript (Malachowa et al., 2011). Therefore, BHI plus 10% human plasma medi um is proposed for use as the medium for growing *S. aureus* biofilms under both static and flow conditions. The inclusion of plasma in the me dium takes the *in vitro* biofilm assays one step closer to mimicking *in vivo* environments. At present, the mechanisms by which plasma proteins stimulate the growth of *S. aureus* biofilms are uncertain. Plasma proteins

Fig. 1. 6-hour GFP-labeled *S. aureus* UAMS-1 biofilms formed with different media at 37 °C with a shear flow of 0.6 dyn/cm^2 in a 48-well microplate Bioflux system (Fluxion Biosciences, South San Francisco, CA). Images were captured with the LSM710 (Carl Zeiss MicroImaging, Thornwood, NY) using a 488-nm excitation wavelength. Left panels (a–e) and right panels (f–j) are representative confocal LSM images of two independent experiments without and with precoating the channels with 20% human plasma, respectively. Side panels in each confocal LSM biofilm image indicate the biofilm's thickness along the x (top side panels) and y (right side panels) axes. BHI + HP: BHI + 10% human plasma. TSB + HP: TSB + 10% human plasma. TSBGN: TSB enriched with 0.5% glucose and 3% NaCl (Beenken et al., 2003; Luong et al., 2009). BHIGN: BHI supplemented with 1% glucose and 2% NaCl. Citrate: TSB plus 0.6% yeast extract and 0.8% glucose and 0.2% sodium citrate (Craigen et al., 2011). Scale bars = 10 μm.

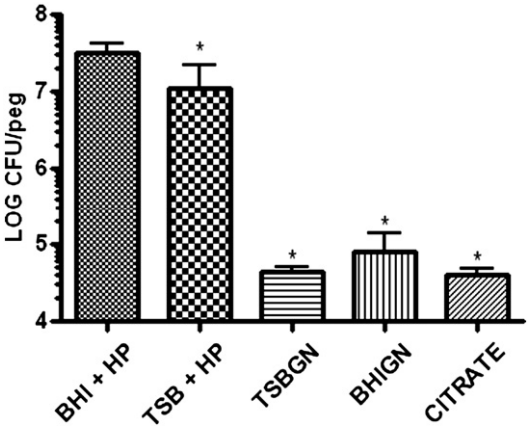

Fig. 3. 20-hour wild type *S. aureus* UAMS-1 biofilms formed with different media at 37 °C in an uncoated minimum biofilm eradication concentration (MBEC) device with different media measured by total viable cell recovering (Ceri et al., 1999). The pegs were broken off from the MBEC lid, and each peg was placed in a microcentrifuge tube containing 1 ml of fresh sterile PBS and sonicated (Misonix Microson XL 2000, Newtown, CT) for 2 min each to dislodge the biofilm from the pegs. The bacteria from the biofilm were then diluted and spiral plated (WASP 2, Don Whitley Scientific, Frederick, MD) onto sheep blood agar plates and incubated overnight at 37 °C for viability determination. The results are representative of those from four independent experiments. Statistics were performed using analysis of variance (one-way) software in which all groups were compared with BHI + plasma resulting in a *P value < 0.001. Error bars represent standard deviation. See Fig. 1 legend for media description.

could represent an excellent nutrient source of nitrogen and iron. Alternatively, some of the plasma proteins, including fibrinogen, could interact with the specific surface adhesins present in *S. aureus*. These interactions might lead to up regulation of biofilm essential genes such as *icaADBC* (polysaccharide biosynthesis). Although the mechanism remains to be determined, our data strongly suggest that *S. aureus* forms robust biofilms with the presence of plasma under both static and fluid dynamic conditions.

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