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Semi-Automated Method for Multi-Tasking Measurement of Microbial Growth, Capsule, and Biofilm Formation

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ABSTRACT

The majority of bacterial infections involve the formation of biofilms. Biofilm formation is nutrient and growth dependent. Determination of the effects of nutrients on exopolysaccharide production and bacterial growth is labor and time intensive. We tested whether the Bioscreen C (Growth Curves, Inc.) would have utility as a high-throughput tool in the measurement of fundamental phenotype expression, as it relates to growth conditions. Within 48 - 72 hr, reproduceble, statistically significant data on the affects of growth conditions on generation time, capsule production and biofilm formation (maximally for 25 different conditions per 24 hr run cycle; n = 4) were obtained. Although all *S. aureus* strains produced similar amounts of capsule, $sarA^-$ and agr^- strains grew significantly slower than parent strain (1.6 fold slower) and produced significantly ($p < 0.05$) less biofilm (~2 fold). *E. coli* growth rate, biofilm and capsule production in simulated nephropathic urine medium was similar for urine with insulin (20 µU). Addition of insulin to urine medium with proline increased generation time, capsule and biofilm production. Findings from this study show that the Bioscreen C is a rapid, reproducible, and easily manipulated system to concurrently measure bacterial growth, biofilm formation, and capsule production. In addition, there is the potential for further applications of this system by expanding the types of detector dyes used.

Keywords: Biofilm; *Staphylococcus aureus*; *Escherichia coli*; Quorum-Signaling Compounds; Menaquinone; Insulin; Growth

1. Introduction

The formation of biofilms is a major virulence phenotype which is associated with over half of all bacterial infections. Biofilms function to protect bacterial communities from environmental factors, e.g. serum proteases, antibodies and antibiotics, and help to anchor these communities to substrates ranging from industrial plastics and tubing to mucosal surfaces [1-3]. Polysaccharide capsule participates in both the protective and anchoring functions essential for biofilm stability. Biofilm formation and stability is dependent on the environment. Promotion, or inhibition, of biofilm formation occurs in response to available nutrients including pathway metabolites and quorum-signaling compounds. Bacterial growth conditions can affect the levels of biofilm formed. A flaw that can arise when interpreting the data is the assumption that the rate and maximum level of growth achieved is congruent for all growth conditions tested. Unfortunately, such bias can only be eliminated experimentally. To be able to compare biofilm formation in response to various growth conditions all measurements should be performed on cells grown under identical conditions which can be difficult and labor intensive to achieve. The focus of this study is to determine if a high-throughput semi-automated system for measuring growth kinetics (Bioscreen C) could be multi-purposed to accommodate measurement of biofilm formation and capsule production. This was tested by measurement of growth kinetics followed by assessment of biofilm and capsule formation in the same honeycomb microtiter plate wells. Initial measurements were done using a gram positive organism, *Staphylococcus aureus*. We then examined how alteration in growth affected biofilm and capsule formation in a model for *E. coli* colonization in response to the inter-kingdom quorum signal, insulin [4,5].

2. Materials and Methods

2.1. Chemicals and Reagents

Menaquinone (K_2; stored at –20°C; Sigma-Aldrich) was dissolved in dimethyl sulfoxide (DMSO) then diluted in media for use. Humulin-R (Lily) was used for all insulin

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assays [5].

2.2. Bacteria and Medium

Escherichia coli ATCC 25922 and *Staphylococcus aureus* (**Table 1**) were used. For testing, *S. aureus*, strains were grown in Mueller-Hinton broth (MH) and Yeast Nitrogen Base with 1% peptone (YNBP); *E. coli* was grown in YNBP and artificial urine (AU; g/L: $CaCl_2$, 0.65 g; $MgCl_2$, 0.65 g; NaCl, 4.6 g; Na_2SO_4, 2.3 g; $Na_3C_3H_5O(CO_2)_3$, 0.65 g; $Na_2C_2O_4$, 0.02 g; KH_2PO_4, 2.8 g; KCl, 1.6 g; NH_4Cl, 2.0 g; urea, 12.0 g; creatinine, 1.1 g; tryptic soy broth 3.0 g; $NaSO_4$, 2.3 g) [6]. The amino acids tested for their effect on *E. coli* growth, biofilm and capsule formation are listed in **Table 2**.

2.3. Growth, Biofilm and Capsule Studies

Overnight cultures of *E. coli* and *S. aureus* were inoculated into homologous media (2×10^4 CFU/mL final concentration). Various concentrations of menaquinone or insulin were added to the bacterial suspensions (*S. aureus* and *E. coli*, respectively) prior to incubation (24 or 48 hrs; 37˚C, continuous shaking). Generation times were calculated per formula (Bioscreen C). For *E. coli*, controls consisted of organisms grown in medium alone; *S. aureus* controls were organisms grown in medium with the highest concentration of DMSO used (1% v/v DMSO in MH). Experiments were performed in quadruplicate or octuplicate and repeated once. Biofilm and capsule determinations were done post-growth by staining washed (3×, PBS, pH 7.0) then dried honeycomb plates with crystal violet (biofilm; $Abs_{595\ nm}$) or Alcian blue (capsule; 1% w/v DDI), respectively [10]. For menaquinone, parallel biofilm and capsule formation determinations in 96 well flat bottom uncoated microtiter plates were handled similarly. Absorbance was determined with the Bioscreen C (honeycomb plates) and a Beckman Coulter DTX 880 multimode spectrophotometer (96 well plates).

2.4. Stain Decolorization

To determine if either crystal violet or Alcian blue could be completely removed from the stained biofilm or capsule, respectively, the wells of honeycomb plates (n = 6/stain/treatment) with adherent biofilm (*S. aureus*) were stained, washed, dried, then treated at least 3 times (300 µL/well) with each of the following: acetone, methanol, xylene, absolute ethanol or gram decolorizer (50/50 acetone/ethanol).

2.5. Statistics

Data were evaluated by ANOVA (GraphPad InStat 3.06 for Windows, GraphPad Software Inc.). Mean values were considered significantly different at $p < 0.05$.

3. Results and Discussion

Staphylococcus aureus. Menaquinone (vitamin K_2) is produced via the mevalonate pathway by various bacteria, including *S. aureus* [11]. In *S. aureus* menaquinone has

Table 1. *Staphylococcus aureus* strains tested.

Strains	Comments	Ref
S. aureus ATCC25923	MSSA type strain	
S. aureus 8325-4	Parent strain (Foster)	[7]
S. aureus sarA⁻	Decreased RNAIII production, fibronectin binding and virulence	[8]
S. aureus agr⁻	Increased production of surface proteins, decreased production of exo-proteins and reduced virulence	[7]
S. aureus agr⁻-sarA⁻	Increased production of surface proteins, decreased production of RNAIII and exo-proteins, decreased fibronectin binding and especially decreased virulence	[9]

Table 2. Concentrations (M) of amino acids tested for their effect on *E. coli* ATCC 25922 growth, biofilm and capsule formation.

Amino Acids	M^a	Amino Acids	M^a	Amino Acids	M^a	Amino Acids	M^a
Alanine	10^{-2}	Asparagine	10^{-1}	Aspartic Acid	10^{-2}	Tryptophan	$>10^{-1b}$
Isoleucine	10^{-1}	Cysteine	10^{-2}	Glutamic Acid	10^{-2}	Tyrosine	$>10^{-3b}$
Leucine	10^{-1}	Glutamine	10^{-1}	Histidine	10^{-1}	Proline	10^{-2}
Methionine	10^{-1}	Glycine	10^{-1}	Arginine	10^{-1}	Threonine	10^{-3}
Phenylalanine	10^{-1}	Serine	10^{-3}	Lysine	10^{-1}	Valine	10^{-1}

[a]Values for concentrations tested were based on peak chemotaxis response of *E. coli* as established by Mesibov and Adler [13]. [b]Tryptophan and tyrosine were tested at the limits of their solubility in the YNBP solution at room temperature (28˚C); tryptophan (5.6×10^{-2}), tyrosine (2.5×10^{-3}).

been found to play a key role in the electron transport chain [12]. The effect of exogenous menaquinone on staphylococcal growth, capsule and biofilm formation was determined. Regardless of strain or vitamin concentration present, no significant difference was measured for total absorbance achieved as compared to the control (1.7 $Abs_{420 - 590}$). The effect of menaquinone on generation time was strain and menaquinone concentration specific with no discernable pattern detected (**Table 3**). Of the strains tested, *S. aureus agr⁻-sarA⁻* showed the slowest rate of growth and most variable doubling time in response to menaquinone. *S. aureus* ATCC 25923 exhibited the shortest doubling time regardless of menaquinone concentration. These findings are consistent with reports on the effects of exogenous hormones, *i.e.*, the impact of menaquinone on growth is both strain and hormone concentration specific [5,14]. To determine if measurement of biofilm formation using the Bioscreen C is comparable to that measured for the standard microtiter plates method [10], parallel assays were performed (**Figure 1**). There was no significant difference between biofilm measurements regardless of the methodology (Bioscreen C vs. spectrophotometer) used. Both techniques indicated that menaquinone concentration tested had no effect on biofilm formation. In addition, menaquinone had no effect on capsule formation (Alcian Blue staining material).

These results confirmed our hypothesis that the same plates used for Bioscreen C growth measurements could also be subsequently used for measurement of biofilm or capsule production. We further tested to determine if the utility of the honeycomb plates could be expanded by using multiple stains/well. Wells prestained with gram's crystal violet or Alcian Blue were treated with a variety of stain solvents, *i.e.*, acetone, methanol, xylene, absolute ethanol or gram decolorizer (50/50 acetone/ethanol). None of the solvents tested completely destained the plate wells. Thus, with regards to the two standard stains used for biofilm and capsule determination the plates are single use.

E. coli. Communication between individual bacterial cells and recognition of chemical signals in the environment are necessary for population survival and biofilm formation [1,4,15-20]. *In vivo*, pathogens are exposed to hormones and other micronutrients that are shared across the taxonomic kingdoms. Human recombinant insulin has been shown to function as an interkingdom signal for *E. coli* [4,5]. We tested a range of rh-insulin levels (2 - 200 μU/mL), that correspond to the human physiological range of insulin, to determine if this methodology also has utility in measurement of a gram negative's growth, capsule and biofilm formation in response to a quorum signaling compound. The effect of insulin on growth in response to various amino acids in two different media (YNBP and artificial urine) was measured using amino acid concentrations (**Table 1**) previously reported to affect another behavioral phenotype, chemotaxis [4,5,13]. Of the amino acids tested in YNBP, insulin only affected the growth profile of *E. coli* grown in the presence of proline. *E. coli* exhibited biphasic growth in proline alone; however, with the addition of rh-insulin (2, 20, or 200 μU/mL) *E. coli*'s growth was monophasic (**Figure 2**). Growth of *E. coli* in YNBP-proline with 2 μU or
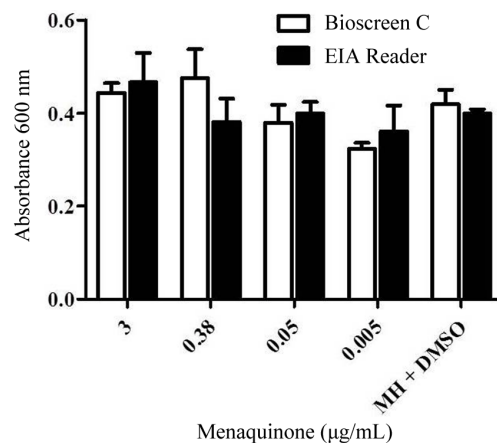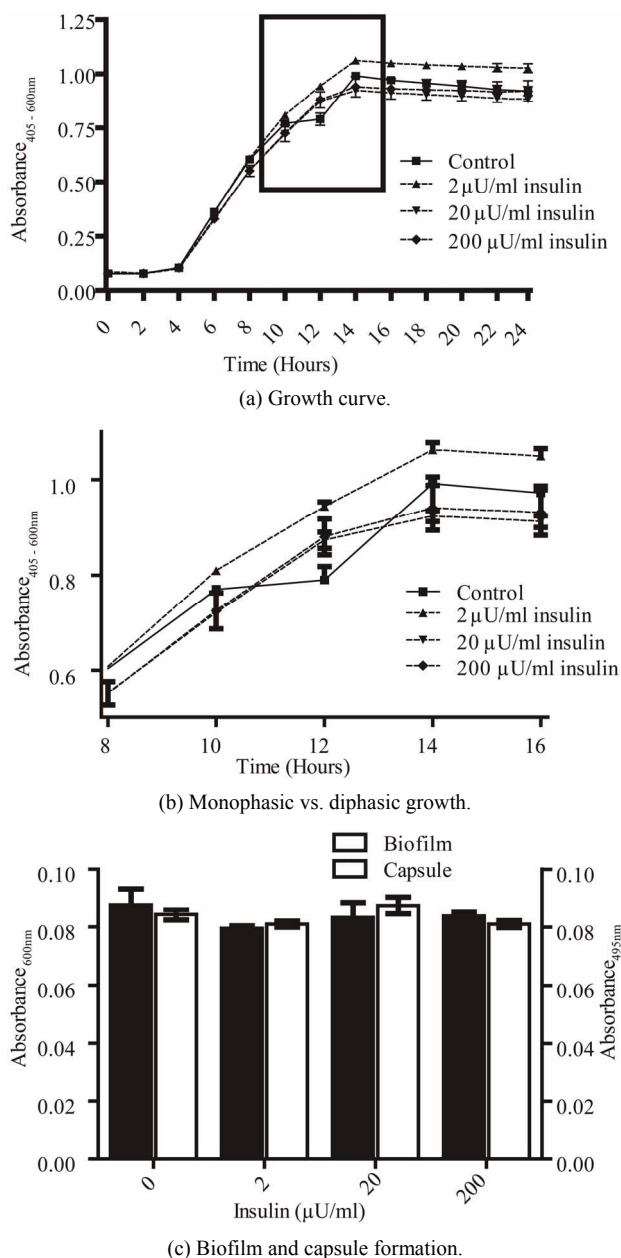

Figure 1. Comparison of the levels of biofilm after growth in 100 well honeycomb plates to levels in 96 well microtiter plates. Levels of *S. aureus* ATCC 25923 biofilm formation after growth in Mueller Hinton medium (48 hrs. 37°C, shaking; pH 7) with various levels of menaquinone (0.05 - 3 μg/mL) were measured in either the Bioscreen C or Beckman EIA reader.

Table 3. Effect of menquinone on generation times of *Staphylococcus aureus* strains.

Menaquinone (μg/mL)	ATCC 25923	Parent 8325-4	*agr⁻*	*sarA⁻*	*agr⁻-sarA⁻*
Control	2.6[b]	2.7	3.4	2.9	4.2
4	2.6	3.0[a]	3.4	3.1[a]	4.3[a]
2	2.5[a]	3.3[a]	3.4	3.1[a]	3.9[a]
1	2.7[a]	3.0[a]	3.5[a]	2.9	4.1[a]
0.5	2.6	2.6[a]	3.4	2.9	3.8[a]
0.05	2.6	3.2[a]	3.4	2.9	4.2

[a]Significance from medium + DMSO control; [b]Mean (n = 4); SEM for all measurements ≤ 0.003.

Table 4. Effect of rh-insulin on *E. coli* ATCC 25922 generation time in YNBP and artificial urine with proline.

rh-Insulin (µU/mL)	YNBP + Proline			Artificial Urine + Proline		
	min[a]	% Control	Δ[c]	min[a]	% Control	Δ[c]
0 (Control)	161	100	-	58.8	100	-
2	157[b]	98	−4	129[b]	210	70.2
20	156[b]	97	−5	92[b]	160	33.2
200	162	101	1	70.9[b]	120	12.1

[a]Mean (n = 4); SEM for all measurements ≤ 0.003; [b]Significant from control ($p < 0.05$); [c]Δ (delta) column values are the differences between the adjacent row values and the respective insulin control (0 µU/mL) value.
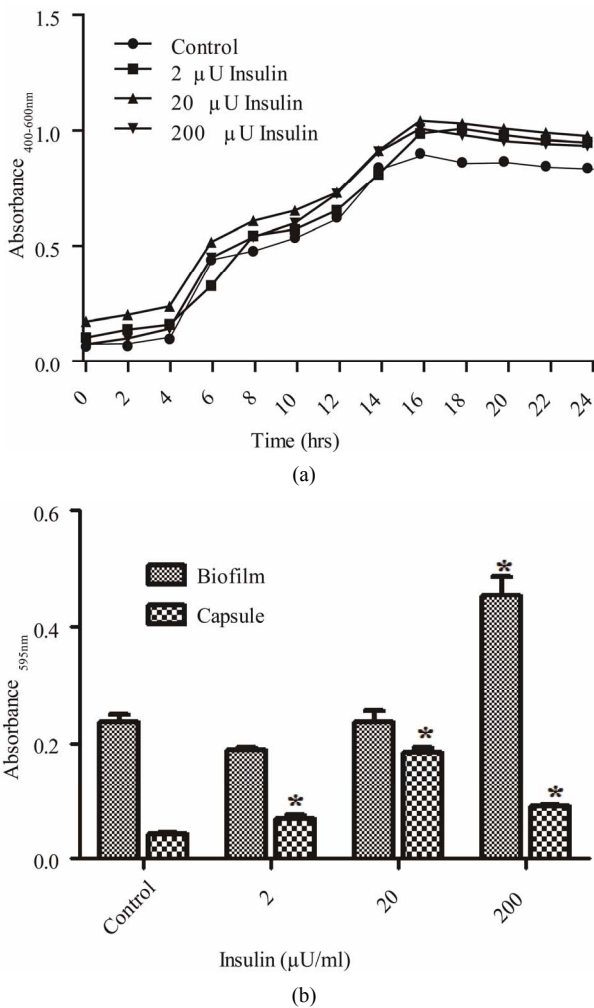
Figure 3. Effect of rh-insulin on *E. coli* ATCC 25922 growth, biofilm, and capsule production at 37°C in artificial urine with proline (10^{-2} M). (a) Organisms grown (37°C, 24 hours, shaking) in artificial urine with proline (10^{-2} M) at various levels of rh-insulin (2, 20, 200 µU/mL) were evaluated for growth and generation time by an automated system (Bioscreen C; (n = 6); absorbance reading: growth—$Abs_{405 - 600 nm}$); (b) Biofilm (crystal violet stained material) and capsule production (Alcian blue stained material) was evaluated after growth as described in A for growth determinations. Significant ($p < 0.05$) differences between test and medium alone control are designated by asterisk.

4. Acknowledgements

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