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Full Length Research Paper

Growth properties of *Staphylococcus aureus* in biofilm formed on polystyrene plate

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***Staphylococcus aureus* is an opportunistic pathogen. Indwelling device-associated infections commonly involve the formation of *S. aureus* biofilm on metal or polymeric surfaces. Additionally, *S. aureus* biofilm in the food industry poses a serious contamination risk. It is of great importance to investigate the growth properties of *S. aureus* cultivated in biofilm condition for efficiently controlling *S. aureus* biofilm. In this study, a few properties of *S. aureus* in biofilm formed on polystyrene cell culture plate were investigated. The biofilm biomass reached the maximum at 12 h after inoculation, indicating that the development of biofilm got into the mature stage. The architecture of biofilm at different growth stages was illustrated by scanning electron microscopy, which showed that biofilm consisted of multilayered cell clusters and displayed the heterogeneity. *S. aureus* cell in biofilm was nearly colourless because it produced less staphyloxanthin. *S. aureus* in biofilm existed in a poorly acidic environment. The metabolic profile of organic acids was investigated by ^{1}H nuclear magnetic resonance spectroscopy. The organic acids accumulated by *S. aureus* in biofilm growth pattern included acetate, isobutyrate, isovalerate, lactate and pyruvate, especially acetate and lactate.**

Key words: *Staphylococcus aureus*, biofilm, architecture, scanning electron microscopy, staphyloxanthin, organic acid, ^{1}H nuclear magnetic resonance spectroscopy.

INTRODUCTION

Staphylococcus aureus is an opportunistic human pathogen, causing major problems in food sector and medical field (Rode et al., 2007; Rosado et al., 2010). *S. aureus* is a frequent cause of indwelling device-associated infections which commonly involve the biofilm formation on metal or polymeric surfaces (Schlag et al., 2007; Zhu et al., 2007). Additionally, *S. aureus* biofilm on surfaces in the food industry poses a serious risk of food contamination (Gibson et al., 1999). The importance of contaminated surfaces in spreading pathogens to foods is already well established in food processing, catering and domestic environment (Silva-Meira et al., 2012).

Biofilms are complex structures consisting of surface-attached bacteria surrounded by a self-produced extracellular polymer matrix and are currently recognised as the predominant bacterial life-style (Wu et al., 2009; Fagerlind et al., 2012). Biofilm-embedded bacteria are more resistant to antimicrobial agents and the immune defense system than their planktonic counterparts (Schlag et al., 2007). Even bacterial aggregates detached from biofilms also retain the high level of resistance to antimicrobials and may contain enough number of cells to represent a potential infectious dose (Silva-Meira et al., 2012).

A major future task is to find new and effective treatments for *S. aureus* biofilm-associated infections and contaminations. The higher resistance of bacteria in biofilm than their planktonic counterparts suggests that the bacteria in biofilm have altered growth characteristics.

For example, the bacteria in biofilms formed under nutrient limitation exhibit reduced growth rate, which adds to their tolerance to antimicrobial agents (Pagedar and Singh, 2012). Thus, it is of great importance to

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investigate the growth properties of *S. aureus* cultivated in biofilm condition in order to efficiently inhibit or eradicate *S. aureus* biofilm. However, up to now, few comprehensive studies which related to these aspects have been reported.

The biofilm formation is highly influenced by the nature of subtrata, cell surface charge, presence of flagella and microbial growth phase (Pagedar and Singh, 2012). In response to different environmental conditions, biofilms can adopt different structures, which can range from homogenous monolayers to heterogeneous structures including mushrooms and filaments (Fagerlind et al., 2012). The aim of this work was mainly to investigate the growth properties (for example, biomass, coloring, organic acid production, etc.) of *S. aureus* in biofilm formed on polystyrene plate. In addition, the architecture of biofilm formed by *S. aureus* in different growth stages was also investigated by scanning electron microscopy (SEM). It will be a good starting point for future, more intensive studies of the growth properties of *S. aureus* in biofilm formed under various conditions.

MATERIALS AND METHODS

Bacterial strain and growth conditions

S. aureus ATCC 6538 was used for all tests. The strain was grown in tryptic soy broth supplemented with 0.25% glucose (TSBg) to promote biofilm formation (Beenken et al., 2004; Resch et al., 2005). The same medium was used for typical aerobic and anaerobic cultivation. *S. aureus* was firstly inoculated into TSBg and cultured at 37°C with shaking until optical density at 600 nm (OD600) reached about 1.0. This culture was diluted and used for further inoculation (0.1%) (Resch et al., 2005; Wu, et al., 2009). For biofilm growth condition, *S. aureus* was cultured in polystyrene 6-well flat-bottom cell culture plates (Corning, USA) as 6 mL per well at 37°C without shaking (Schlag et al., 2007). For typical aerobic growth condition, *S. aureus* was cultivated in Erlenmeyer flasks (30 mL medium in a 300 mL Erlenmeyer flask) at 37°C with shaking at 150 rpm (Resch et al., 2005; Wu et al., 2009). For typical anaerobic growth condition, *S. aureus* was cultivated in anaerobic tubes using high purity nitrogen to drive out air (15 mL medium in a 25 mL anaerobic tube) at 37°C without shaking) (Ma et al., 2008).

Sampling methods

S. aureus cultures under biofilm growth condition were harvested at 4 h intervals. The liquid cultures in plate wells were immediately centrifuged for 10 min at 3,000 × g and the supernatants were used for pH assay and metabolite analysis. The adherent cells were used for biofilm biomass measurements, and pigment assay (at 36 h). *S. aureus* cultures under aerobic and anaerobic growth conditions were harvested at 36 h. The cultures were immediately centrifuged for 10 min at 3,000 × g. The cell pellets were stored at -70°C until they were used for pigment assay (Resch et al., 2005).

Biofilm biomass assay

The biomass here represents the total bacteria number, whether living or dead. For biofilm biomass assay, at each sampling point, the medium was gently aspirated from the plate and the wells were rinsed three times with phosphate buffered saline (PBS). Then the plate was dried for 1 h at 60°C. The adherent cells were stained with 6 mL of 0.1% safranin for 1 min, and then washed three times with distilled water and dried. The stained biofilms were resuspended in 6 mL of 30% glacial acetic acid (v/v). The absorbance was measured at 450 nm by spectrophotometer (Model 2800 UV/VIS; UNICO, China) (Schlag et al., 2007).

Visualization of biofilm architecture

For visualization of *S. aureus* biofilm architecture, SEM images were taken (Kwieciński et al., 2009). *S. aureus* biofilms were cultivated as described above in a 6-well tissue culture plate which was placed with coverslips at the bottom beforehand. At the incubation time of 4, 8, 20 and 36 h (according to the results of biofilm biomass measurements), the medium was gently aspirated from the plate and the wells including the slips were rinsed three times with PBS. The biofilm samples adhered to the slips were fixed in 2% glutaraldehyde in cacodylate buffer for 30 min, then washed twice with cacodylate buffer and dehydrated for 10 min using a graded ethanol series. A critical point drying procedure followed and the specimens were then sputter-coated with gold. Samples were examined with a Quanta200 scanning electron microscope (FEI, USA).

Extraction and quantification of pigments in cells

Staphyloxanthin and intermediate carotenoids were extracted and quantified according to Rosado et al. (2010) with minor modifications. Briefly, at 36 h of incubation, *S. aureus* cells cultivated under different conditions were harvested respectively. For harvesting biofilm cells, after discarding the liquid culture, the biofilm was further gently washed once with PBS to remove residual planktonic cells. Then biofilm cells were suspended gently in PBS using a cell scraper, and the resultant suspension acted as the counterpart of cultures from aerobic and anaerobic cultivations. The cell densities of three kinds of cultures were adjusted to obtain comparable values, and the cells were collected by centrifugation (3,000 × g, 10 min) and washed with PBS twice. The packed cells were resuspended in 3 mL methanol, held at 55°C in water bath for 5 min and cooled, and then, the extracts were obtained by centrifugation. The extraction was repeated twice, until no further pigment could be extracted. Carotenoids were estimated quantitatively by measuring absorbance of solutions at 462 nm, the maximum absorbance of the primary carotenoid pigment staphyloxanthin.

Determination of organic acids concentrations

Organic acids were determined by ^{1}H nuclear magnetic resonance (NMR) spectroscopy according to Wu et al. (2009). Briefly, each culture supernatant was mixed with an equal volume of loading buffer and centrifuged at 16060 g at 4°C. Next, 200 µL of the mixture was transferred to a 3 mm NMR tube. All ^{1}H NMR spectra were measured at 300 K using an Avance III 600 NMR spectrometer (proton frequency = 600.45 MHz, 14.1 T; Bruker, Rheinstetten, Germany) with a cryogenic NMR probe. Organic acids were identified and quantified with Chenomx software (version 6.1; Chenomx, Edmonton, Canada) with the reference of internal standard TSP.

Measurement of ammonia concentration

Ammonia concentration was determined by indophenols blue

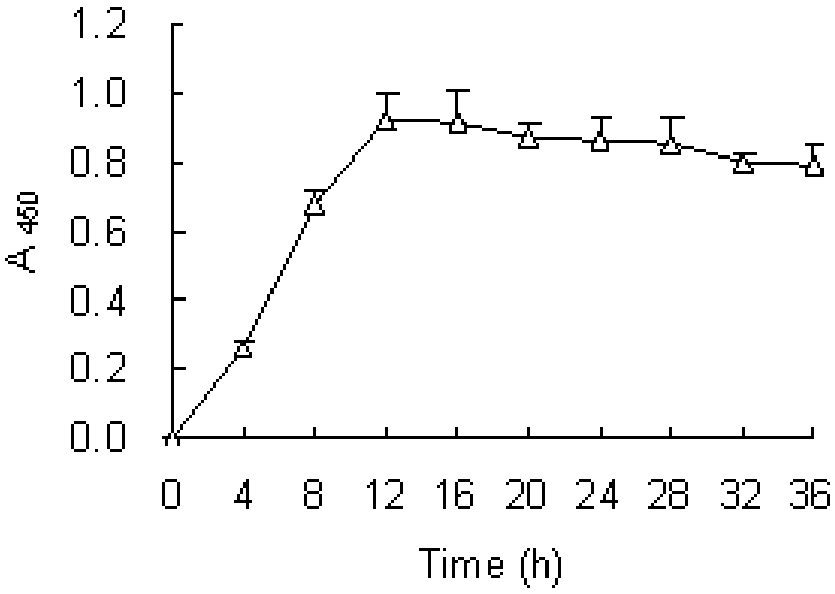

Figure 1. Change of *S. aureus* biofilm biomass during cultivation. Data represent the means ± standard errors of the means (n = 8) of one representative experiment.

method (Van Staden and Taljaard, 1997) with minor modifications. Briefly, 100 µL of culture supertanent was mixed sequentially with 5 mL phenol reagent and 5 mL hypochlorite reagent. The mixture was vortexed for 30 s and incubated at 37°C for 20 min. The absorbance at 637 nm was measured, and the unknown ammonia concentrations were determined with a standard curve generated by known ammonia concentrations.

RESULTS

Change of the biofilm biomass of *S. aureus* during cultivation

The biofilm biomass represents the total bacteria number in biofilm. The change curve of *S. aureus* biofilm biomass during cultivation actually exhibited the time course of biofilm formation. According to Figure 1, *S. aureus* biofilm biomass rapidly increased after inoculation, showing that more and more cells adhered and accumulated in polystyrene plate surfaces. It reached the maximum at 12 h, and then it basically remained unchanged, showing that the development of biofilm got into the mature stage. This result suggested that biofilm formation was a dynamic process.

Observation of *S. aureus* biofilm by SEM

The architectures of *S. aureus* biofilms formed on polystyrene plate at 4, 8, 20 and 36 h during cultivation were investigated by SEM. For a more comprehensive and intensive understanding of the architecture of *S. aureus* biofilm, SEM images were acquired at 100, 2,000 and 20,000 magnification from relatively macroscopic to relatively microscopic, illustrated in Figures 2, 3 and 4, respectively.

In Figure 2, *S. aureus* biofilm could hardly be seen at 4 h (Figure 2a), and became to be apparent until 8 h (Figure 2b), which further confirmed that biofilm formation was a dynamic process as showed in Figure 1. In addition, the biofilm displayed the heterogeneity, which meant that the biofilm was not evenly distributed in polystyrene plate surface (Figures 2b, c and d).

At the higher magnification (2,000×), it was showed more distinctly that biofilm consisted of multilayered cell clusters and the architecture of biofilm was three-dimensional (Figures 3b, c and d). Figure 3a corresponded to the attachment phase, in which *S. aureus* mostly existed in monolayer.

At the highest magnification (20,000×) used in this study, a few fragments were seen in the biofilms (Figures 4b, c and d). They were supposed to be the residues of biofilm matrix after the pretreatment procedures for SEM sample preparation.

Pigmentation of *S. aureus* in biofilm

The yellow-to-orange colony color of *S. aureus* is one of the classical criteria for identification of the species. However, the pigmentation of *S. aureus* is not a very stable character. In this study, *S. aureus* grown in biofilm pattern was nearly colorless. Meanwhile, *S. aureus* grown in anaerobic and aerobic patterns was colorless and yellow, respectively (Figure 5a).

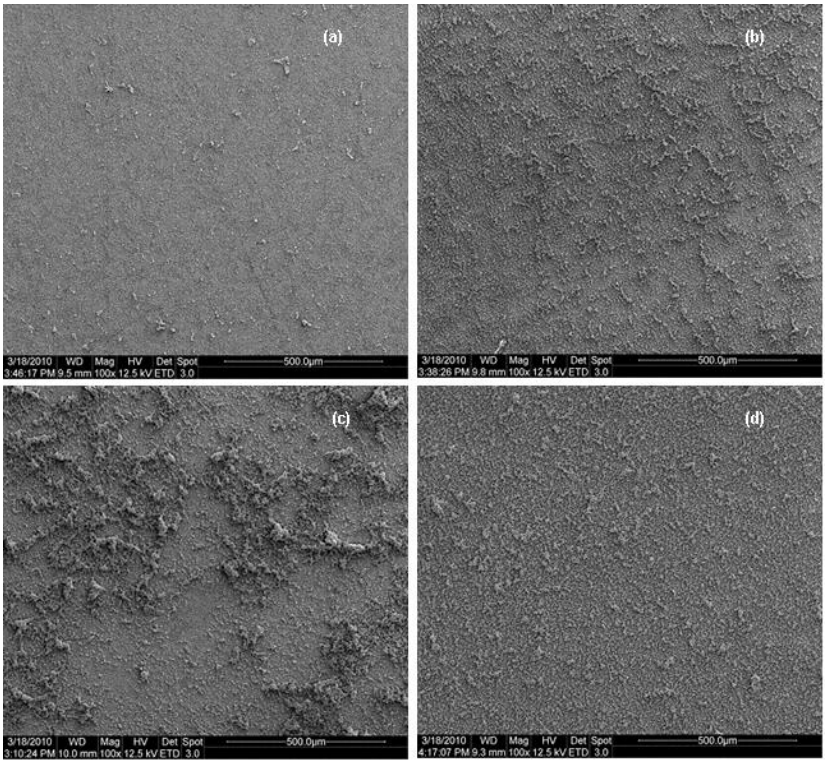

Figure 2. SEM images (100×) of *S. aureus* biofilm at different growth stages: (a) 4 h, (b) 8 h, (c) 20 h, (d) 36 h.

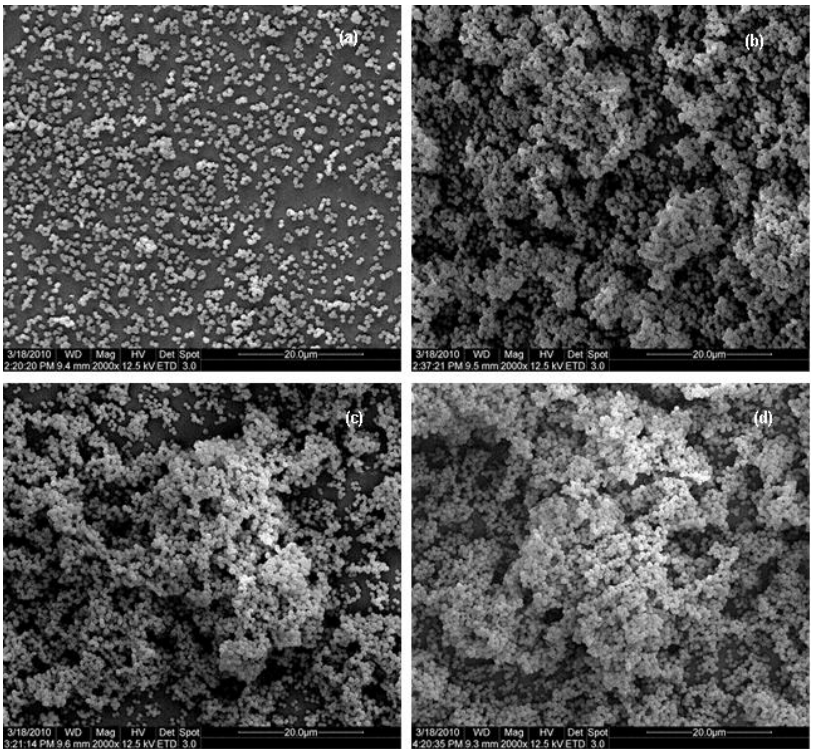

Figure 3. SEM images (2,000×) of *S. aureus* biofilm at different growth stages: (a) 4 h, (b) 8 h, (c) 20 h, (d).

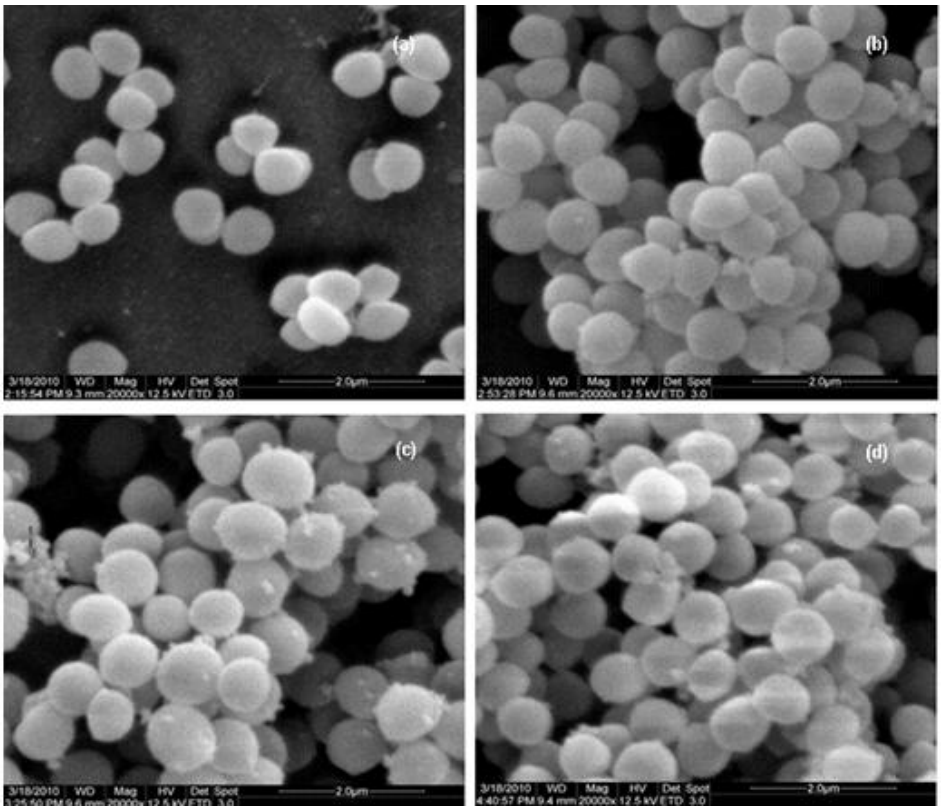

Figure 4. SEM images (20,000×) of *S. aureus* biofilm at different growth stages: (a) 4 h, (b) 8 h, (c) 20 h, (d) 36 h.

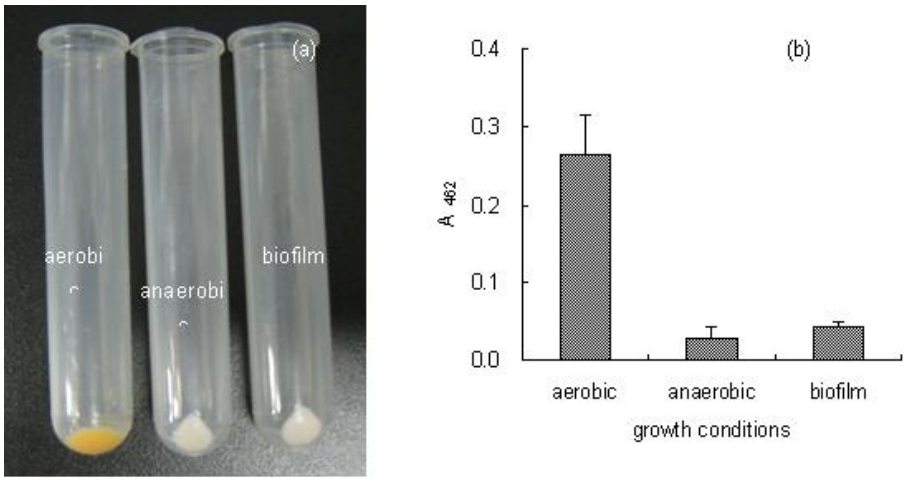

Figure 5. Pigmentation of *S. aureus* in different growth patterns. (a) Colour of the packed cells of *S. aureus* in aerobic, anaerobic and biofilm cultures after 36 h of incubation. (b) Staphyloxanthin production of *S. aureus* grown under different conditions. Data represent the means ± standard errors of the means (n=3) of one representative experiment.

S. aureus produces carotenoid pigments that impart the yellow-orange color to colony. The main pigment is staphyloxanthin, which is located in the cell membrane. In this study, staphyloxanthin was extracted by methanol and measured at 462 nm by spectrophotometry. According to Figure 5b, *S. aureus* produced less

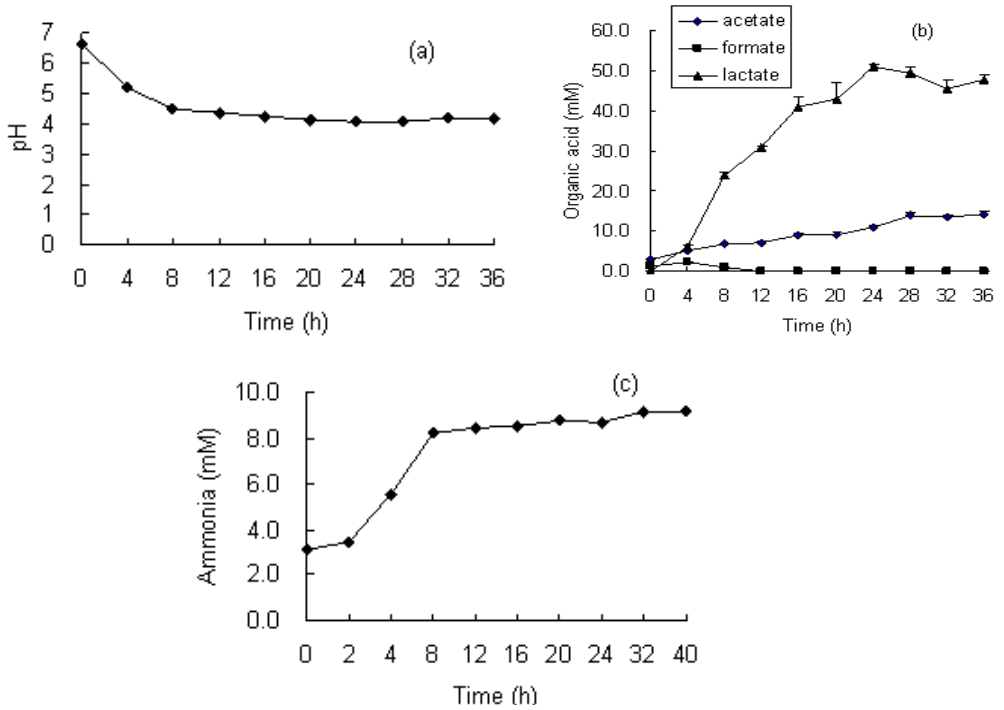

Figure 6. The pH change, representative organic acids and ammonia accumulation in *S. aureus* biofilm cultures. (a) Change in pH of the cultures. (b) Representative organic acids accumulation in the cultures. (c) Ammonia accumulation in the cultures.

staphyloxanthin when grown under biofilm condition. Meanwhile, *S. aureus* grown under anaerobic and aerobic conditions produced the least and the most staphyloxanthin, respectively. These data could explain the phenomenon shown in Figure 5a. In addition, it was suggested that oxygen availability had an important effect on the pigmentation of S. aureus, and *S. aureus* grown in biofilm mode might be in oxygen-limited or relatively anaerobic state.

Change in pH of *S. aureus* culture and its organic acid profile in biofilm growth pattern

According to Figure 6a, pH of *S. aureus* biofilm cultures decreased rapidly from inoculation to 8 h. After that, it continued to decrease slowly. These data showed that *S. aureus* in biofilm existed in a poorly acidic environment.

The change in pH of *S. aureus* culture must be resulted from the change of components in it. Therefore, the organic acids profile was determined by ^{1}H NMR, and the ammonia concentration was analyzed by indophenol blue method in this study. About 8 organic acids were identified in TSBg medium and supernatants of *S. aureus* biofilm cultures at 20 h (Table 1). These organic acids included 2-hydroxybutyrate, acetate, formate, isobutyrate, isovalerate, lactate, pyruvate and succinate. The 20 h time point was chosen because it was in the mature phase for *S. aureus* growing in biofilm condition (Figure 1). Most secondary metabolites could be produced and/or accumulated in the phase. *S. aureus* in biofilm growth pattern mainly accumulated lactate and acetate. Meanwhile, it consumed a large amount of formate. The change of representative organic acids and ammonia concentrations in the cultures could largely explain the change of pH (Figures 6b and c).

DISCUSSION

Biofilm was a complex aggregation of bacteria commonly encapsulated by an adhesive matrix (Zhu et al., 2009). In this study, the biofilm formation of *S. aureus* was a dynamic process, which was in accordance with the previous reports (Beenken et al., 2004; Rode et al., 2007). It has been suggested that biofilm development is influenced by a number of different processes such as adhesion, detachment, mass transport, quorum sensing, cell death and active dispersal (Fagerlind et al. 2012).

The accumulation of multilayered cells can lead to an oxygen-limited microenvironment inside the biofilm. Anaerobic growth has already been found to induce expression of the ica operon and production of polysaccharide intercellular adhesin (PIA) in *S. aureus* (Cramton et al. 2001). PIA is one of primary determinants of the accumulation phase of staphylococcal biofilm

Table 1. Presumptive organic acids and their contents (mM) in TSBg medium and supernatants of *S. aureus* biofilm cultures at 20 h by means of Chenomx. Mean ± standard error of the mean for three independent experiments performed in duplicate.

Compound	TSBg medium	Biofilm culture
2-hydroxybutyrate	0.30 ± 0.03	0.27 ± 0.02
acetate	2.78 ± 0.50	9.06 ± 0.73
formate	1.15 ± 0.20	nd [a]
isobutyrate	nd	0.04
isovalerate	0.11 ± 0.01	0.21 ± 0.02
lactate	0.10 ± 0.02	43.05 ± 3.99
pyruvate	0.35 ± 0.02	0.61 ± 0.07
succinate	0.08 ± 0.01	0.08 ± 0.01

[a] nd, not detected.

formation. It is synthesized when the tricarboxylic acid (TCA) cycle is repressed (Zhu et al., 2009). The inverse correlation between PIA synthesis and TCA cycle activity leads to a hypothesis that increasing TCA cycle activity would decrease PIA synthesis, and be a potential measure for controlling biofilm. The accumulation of multilayered cells can also impair the nutrient availability inside the biofilm, which is hypothesized to be one factor of a multi-layered defense for antibiotics (Stewart, 2002).

The pigmentation of *S. aureus* is not a very stable character. For example, pigmentation is usually apparent after 18 to 24 h of growth at 37°C but is more pronounced when cultures are held at room temperature for 24 to 48 h longer (Wieland et al., 1994). In this study, *S. aureus* grown under different conditions also exhibited different pigmentation.

Staphyloxanthin is a triterpenoid esterified with a C15 fatty acid (Pelz et al., 2005). The biosynthesis pathway of staphyloxanthin has been elucidated (Wieland et al., 1994; Pelz et al., 2005), in which five genes and enzymatic reactions were involved. One step in staphyloxanthin biosynthesis is the oxidization of the terminal methyl group of 4,4′-diaponeurosporene to form 4,4′-diaponeurosporenic acid, catalyzed by a mixed-function oxidase CrtP. This oxidization step needs the direct participation of molecular oxygen. Therefore, it might explain why *S. aureus* hardly produced staphyloxanthin when grown under biofilm and anaerobic conditions.

Staphyloxanthin is a typical secondary metabolite. It might serve a role in survival in infected hosts and in combating the immune system (Pelz et al., 2005). It is well known that carotenoids function as antioxidants, and it has been suggested that staphyloxanthin can protect *S. aureus* against oxidative stress (Clauditz et al., 2006; Rosado et al., 2010). Mutants unable to synthesize these carotenoids molecules have impaired survival within neutrophils and are less pathogenic (Liu et al., 2005; Clauditz et al., 2006).

As far as carotenoid pigment biosynthesis is only concerned, the resistance of *S. aureus* in biofilm seems to be weaker than that of *S. aureus* grown under aerobic condition. However, the resistance of bacteria in biofilm comes from a complex defense system, including poor antibiotic penetration, nutrient limitation and slow growth, adaptive stress responses, formation of persister and so on. For example, the reduced growth rate of bacteria in biofilm limits the efficacy of antibiotics that target cell wall biosynthesis, while the reduced oxidative metabolism limits the uptake of aminoglycosides (Stewart, 2002).

In this study, the pH of *S. aureus* culture grown in biofilm pattern maintained within the acid range, which was probably resulted from fermentation in the anoxic areas of the biofilm (Resch et al., 2005). *S. aureus* is a facultative anaerobic bacterium. It has been known that enzymes belonging to the glycolysis and the mixed-acid and butanediol fermentation pathways were induced in *S. aureus* under anaerobic condition (Fuchs et al., 2007), which could partly explain the organic acid profile of *S. aureus* grown under biofilm condition.

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