Antibiofilm activity of coral-associated bacteria against different clinical M serotypes of Streptococcus pyogenes

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Abstract

Streptococcus pyogenes is the frequent cause of purulent infections in humans. Formation of a biofilm is one of the important aspects of its pathogenicity. Streptococcus pyogenes biofilm communities tend to exhibit significant tolerance to antimicrobial challenge during infections. Exploring novel targets against biofilm-forming pathogens is therefore an important alternative treatment measure. We attempted to screen marine bacteria, especially coral-associated bacteria (CAB), for antibiofilm activity against streptococcal biofilm formation. The bacterial biofilms were quantified by crystal violet staining. Of 43 CAB isolates, nine clearly demonstrated antibiofilm activity. At biofilm inhibitory concentrations (BIC), biofilm formation was reduced up to 80%, and sub-BIC (0.5 and 0.25 BIC) significantly reduced biofilm formation by up to 60% and 40–60%, respectively. Extracts of Bacillus horikoshii (E6) displayed efficient antibiofilm activity. As quorum sensing (QS) and cell surface hydrophobicity (CSH) are crucial factors for biofilm formation in S. pyogenes, the CAB were further screened for QS inhibition properties and CSH reduction properties. This study reveals the antibiofilm and QS inhibition property of CAB.

Introduction

Biofilm formation is recognized as an important virulence factor for both opportunistic and true pathogens (O’Toole et al., 2000). Bacterial biofilms have a structurally complex and dynamic architecture and develop on many abiotic surfaces (plastic, glass, metal and minerals) and biotic (plants, animals and humans) surfaces (Stoodley et al., 2002; Hall-Stoodley et al., 2004). Biofilm-associated diseases caused by Gram-positive bacteria include caries, gingivitis, periodontitis, endocarditis and prostatitis (Hall-Stoodley et al., 2004). Many forms of streptococcal infections, especially recurrent and chronic infections, are associated with the formation of bacterial biofilms (Lembke et al., 2006).

Streptococcus pyogenes (group A Streptococcus) is an important human pathogen that causes a variety of clinical manifestations ranging from noninvasive diseases, such as pharyngitis and impetigo, to more severe, invasive infections, including necrotizing fasciitis, sepsis and toxic shock-like syndrome (Cunningham, 2000). Biofilm formation by S. pyogenes has been reported in patients with atopic dermatitis and impetigo (Akiyama et al., 2003). Furthermore, S. pyogenes have also been reported to be members of root canal multispecies biofilms (Takemura et al., 2004). This newly emerging virulence trait (biofilm formation) of S. pyogenes potentially renders these pathogens more resistant to antibiotic therapy as well as to immune responses (Lembke et al., 2006).

Penicillin remains the antibiotic of choice for S. pyogenes infections based of its narrow spectrum of effect, efficacy, good safety profile and low cost (Bisno et al., 2002). However, antibiotic treatment failure of S. pyogenes infections has been demonstrated to be associated with biofilm formation. Biofilm formation significantly impairs antimicrobial therapy even in those cases caused by strains that are not resistant to the relevant antibiotics (Macris et al., 1998; Kuhn et al., 2001). The strains may use biofilms as a barrier to survival in the host against the action of antimicrobials (Conley et al., 2003; Baldassarri et al., 2006). As there is an increase in the resistance mechanisms of S. pyogenes to antibiotics, much effort is being exerted to identify novel compounds with effective antibacterial properties. Biofilms
plays a crucial role in the emergence of new pathogens by horizontal gene transfer of genetic elements (Watnick & Kolter, 2000). Hence, there is a critical need for the identification of antibiofilm compounds with potential application against these detrimental pathogens.

Quorum-sensing (QS) molecules have been shown to be essential for biofilm formation (Hornby & Nickerson, 2004) in many pathogens. QS is a strategy of cell-to-cell communication favouring the biofilm community by regulating unnecessary overpopulation and nutrient competition with important implications for the infectious process (Davey & O’Toole, 2000). As QS plays an important role in biofilm formation, the inhibition of QS is a possible novel target to control biofilm-associated infections in human pathogens. The hydrophobic property of bacterial surfaces is a major determinant in the adhesion of bacteria and in the formation of biofilms by bacteria on animate and inanimate surfaces (Doyle & Rosenberg, 1990). It has previously been reported that extracts of many species of sponges, tunicates and corals contain QS inhibitors (Dobretsov et al., 2009). As it is now recognized that bacteria may be the true producers of many bioactive compounds isolated from marine invertebrates such as sponges and corals (Fenical, 1993; Kelman et al., 2006; Ritchie, 2006; Hamann et al., 2007), we envisage that coral-associated bacteria (CAB) might also produce QS inhibitors. Here we investigated the bacteria associated with the coral Acropora digitifera for QS inhibition.

Combinations of different chemicals (Gawande et al., 2008) and essential oils from plants (Nostro et al., 2007) have been reported to demonstrate antibiofilm activity against Streptococcus mutans and staphylococcal biofilm infections. Yet only a few antibiofilm agents have been reported against S. pyogenes biofilms. The essential oils of certain plants such as Rosmarinus officinalis and Trachyspermum copticum (Rasooli et al., 2008) and extracts of certain Thai plant species (Limswan & Voravutkunchai, 2008) are known to possess antibiofilm activity against S. pyogenes biofilms. Marine resources have seldom been targeted for antibiofilm agents against S. pyogenes. Taxonomically diverse marine bacteria have proven to be a rich resource for the discovery of structurally unique and bioactive secondary metabolites (Teasdale et al., 2009). The use of marine bacteria to inhibit biofilms is gaining importance. As an example, marine Actinomycetes have shown to inhibit biofilm formation by pathogenic Vibrio sp. (Yoo et al., 2007). Recently, CAB have been reported to exhibit antibacterial activity against human pathogens (Nithyanand & Karutha Pandian, 2009; Shnit-Orland & Kushmaro, 2009). However, the ability of CAB to inhibit biofilm formation of various pathogens has yet to be investigated. In the present study, we investigated the antibiofilm activity of CAB against different biofilm-forming M serotypes of S. pyogenes. M protein is a major antigen in S. pyogenes. The N-terminal region of M protein is highly variable between S. pyogenes strains. So each variable M protein forms a different M serotype (there are > 150 M serotypes). Characterization of S. pyogenes has been based on serological identification of each M protein (Yoonim et al., 2005). As QS and cell surface hydrophobicity (CSH) are crucial factors for biofilm formation in S. pyogenes, the CAB were further screened for QS inhibition properties and cell surface hydrophobicity reduction properties.

Materials and methods

Bacterial strains

Forty-three CAB were screened for antibiofilm activity against different biofilm-forming M serotypes of S. pyogenes isolated from throat swabs. All six S. pyogenes isolates were identified to the species level based on 16S rRNA gene sequencing. Their GenBank accession numbers are FJ662827, EU660335, EU660337, FJ662828, EU660341 and FJ662845. The M serotypes (M56, st38, M89, M65, M100 and M74) of S. pyogenes isolates were determined by emm gene sequencing and their accession numbers are EU636227, EU636229, EU660375, EU660377, EU660379 and EU660380, respectively. CAB were isolated from the mucus and tissue of the coral A. digitifera from the Gulf of Mannar (Nithyanand & Karutha Pandian, 2009). Reporter strains Chromobacterium violaceum ATCC 12472 (wild type) and C. violaceum CV026 (mutant) were used to determine QS inhibitory activity. These strains were cultured in Luria-Bertani (LB) broth at 33 °C.

Preparation of CAB extracts

CAB were cultured in Zobell marine broth (Himedia Laboratories, Mumbai, India) and incubated for 48 h. Cells were pelleted and the culture supernatant was filtered through a 0.2-μm filter. The cell-free supernatant was extracted twice with an equal volume of ethyl acetate. The solvent extracts were combined and evaporated to dryness under reduced pressure at room temperature to yield crude extracts and each crude extract obtained was weighed (Nithyanand & Karutha Pandian, 2009). The crude extracts were dissolved in double distilled water (MilliQ, Millipore) and used for antibiofilm screening.

Antimicrobial activity test

Antimicrobial activity of the extracts was assayed by the disc diffusion susceptibility test [Clinical and Laboratory Standards Institute, 2006]. The disc diffusion test was performed in Muller–Hinton agar (MHA) (Himedia Laboratories) supplemented with 5% sheep blood. Overnight cultures of S. pyogenes were subcultured in Todd–Hewitt broth (THB)
until a turbidity of 0.5 McFarland \( (1 \times 10^8 \text{CFU mL}^{-1}) \) was reached. Using a sterile cotton swab, the culture was uniformly spread over the surface of the agar plate. Absorption of excess moisture was allowed to occur for 10 min. Then sterile discs with a diameter of 10 mm were placed over the swabbed plates and 50 \( \mu \text{L} \) of the extracts was loaded on to the disc. MHA plates were then incubated at 37 °C and the zone of inhibition was measured after 24 h.

**Minimal inhibitory concentration (MIC)**
The MIC of the extracts was determined as per Clinical and Laboratory Standards Institute (2006) guidelines. The bacterial suspension \( (5.0 \times 10^6 \text{CFU mL}^{-1}) \) was added to THB supplemented with the serially diluted twofold bacterial extracts to yield final concentrations ranging from 5 to 2000 \( \mu \text{g mL}^{-1} \) and incubated at 37 °C for 24 h. The MIC was recorded as the lowest concentration that produced complete suppression of visible growth.

**Effect of CAB extracts on biofilm formation**
The effect of the CAB extracts against biofilm-forming *S. pyogenes* was tested on 24-well polystyrene plates (Limsuwan & Voravuthikunchai, 2008). CAB extracts at concentrations of 5–2000 \( \mu \text{g mL}^{-1} \) were added to THB containing the bacterial suspension at \( 10^6 \text{CFU mL}^{-1} \). The plates were incubated for 24 h at 37 °C. After incubation, biofilm was stained with 0.4% crystal violet. The biofilm inhibitory concentration (BIC) was determined as the lowest concentration that produces visible disruption in biofilm formation and significant reduction in the readings when compared with the control wells at \( OD_{570 \text{nm}} \) (Baldassarri et al., 2006). Wells containing the media and with extracts were used as blank. Thus, the BIC was determined by both spectrophotometric quantification and also by microscopic visualization. Subinhibitory concentrations (0.5 and 0.25 BIC) of the extracts were also tested against biofilm formation by performing the same protocol. Simultaneously, unstained biofilm and planktonic bacteria were mixed by vigorous vortexing, and bacterial growth was quantified spectrophotometrically at 600 nm.

**Growth curve analysis**
The effect of the CAB extracts was also checked by growth curve analysis against a strain of M56 alone. The BIC of the extracts was added to a conical flask containing 50 mL THB, to which a 1% inoculum from the overnight culture was introduced into the media. The flask was incubated at 37 °C. Growth medium with the addition of bacteria (*S. pyogenes* M56 serotype) and without the addition of the extract was used as a control. Readings were observed for up to 24 h at 1-h intervals.

**Microscopic techniques**
For visualization of biofilms by light microscopy, the biofilms were allowed to grow on glass pieces (1 × 1 cm) placed in 24-well polystyrene plates (Greiner Bio-One) supplemented with the CAB extracts (5–2000 \( \mu \text{g mL}^{-1} \)) and incubated for 24 h at 37 °C. Crystal violet staining was performed as described above. Stained glass pieces were placed on slides with the biofilm pointing up and were inspected by light microscopy at magnifications of × 40. Visible biofilms were documented with an attached digital camera (Nikon Eclipse Ti 100).

Sample preparation for scanning electron microscopy (SEM) studies was performed as described by Lembke et al. (2006). The biofilms on the glass pieces were fixed for 1 h in a solution containing 2.5% glutaraldehyde. The glass pieces were washed in 0.1 M sodium acetate buffer (pH 7.3). Samples were dehydrated through a graded series of ethanol, critical-point dried, gold sputtered and examined with a Hitachi S-3000 H (Japan).

**Screening for QS inhibition by quantification of violacein production**
*Chromobacterium violaceum* ATCC 12472 was preliminarily used for screening CAB for QS inhibition properties. Only extracts that showed QS inhibition properties against *C. violaceum* ATCC 12472 were further examined for quantification of violacin reduction using *C. violaceum* (CV026). The effects of the CAB extracts on inhibition of violacin were quantified using *C. violaceum* CV026 by spectrophotometric analysis. *Chromobacterium violaceum* (CV026) was incubated for 16–18 h and inoculated to \( OD_{600 \text{nm}} = 0.1 \) in test tubes with LB supplemented with N-hexanoyl-l-homoserine lactone (HHL, Sigma) at a working concentration of 1 \( \mu \text{mol mL}^{-1} \), which acted as a control, and LB supplemented with HHL and CAB extract at various concentrations (500–1200 \( \mu \text{g mL}^{-1} \)). The test tubes were further incubated at 33 °C for 24 h. From the test tubes, 1 mL of culture was centrifuged at 15 870 \( g \) for 10 min to precipitate the insoluble violacin. The culture supernatant was discarded and 1 mL of dimethyl sulfoxide was added to the pellet. The solution was vortexed vigorously for 30 s to completely solubilize violacin and centrifuged at 15 870 \( g \) for 10 min to remove the cells. The cells were removed and the supernatant was quantified spectrophotometrically (Hitachi U-2800, Japan) at 585 nm (Choo et al., 2006).

**Microbial adhesion to hydrocarbon (MATH) assay**
The effect of *S. pyogenes* on CSH was measured by MATH as described by Courtney et al. (2009). Briefly, 1 mL of bacteria \( (OD_{530 \text{nm}} = 1.0) \) was placed into glass tubes and 100 \( \mu \text{L} \) of
toluene along with the CAB extracts at their BIC was added. The mixtures were vigorously vortexed for 2 min, followed by a 10-min incubation at ambient temperature to allow phase separation, and then the OD_{530} nm of the lower, aqueous phase was recorded. Controls consisted of cells incubated with toluene. The percentage hydrophobicity was calculated according to the formula: % hydrophobicity = [(OD_{530} nm after vortexing/OD_{530} nm before vortexing)] × 100.

**Statistics**

Statistical analysis was performed using *spss*. Values were expressed as mean ± SD. A Duncan–ANOVA test was used to compare parameters between groups and a Dunnett–ANOVA test to compare between tests and control.

**Results**

**Evaluation of *S. pyogenes* biofilm formation**

Among the six biofilm-forming M-serotypes of *S. pyogenes*, M56 was found to be a potential biofilm former, and M89 and M65 to have the least potential (data not shown). *Streptococcus mutans* UA159 (ATCC 700610), a well-documented biofilm former, was used as a positive control along with *S. pyogenes*. *Streptococcus mutans* UA159 (ATCC 700610) was provided by Dr Gilad Bachrach (Institute of Dental Sciences, Faculty of Dentistry, Hebrew University-Hadassah, Jerusalem, Israel).

**Effect of extracts on *S. pyogenes* growth**

All CAB extracts (2000 μg mL⁻¹) were evaluated for their antibacterial activity by disc diffusion assay. The CAB extracts did not show any antibacterial activity as there was no zone of inhibition around the discs. This was further confirmed by spectrophotometric analysis and the extracts did not show any antibacterial activity as was evident from the growth curve analysis. Even after the addition of the extracts at BIC the growth of *S. pyogenes* was at the same level as that of control.

**Determination of BIC and sub-BIC**

Among the 43 CAB isolates, nine showed positive results by inhibiting biofilm formation (Table 1). To determine the MIC of the CAB extracts on *S. pyogenes*, extracts with concentrations of 5–2000 μg mL⁻¹ were assessed. Surprisingly, none of the extracts showed antibacterial activity against the *S. pyogenes* strains. As shown in Table 2, all nine extracts inhibited biofilm formation against the different serotypes of *S. pyogenes* at concentrations ranging from 10 to 500 μg mL⁻¹ (BIC). Interestingly, the CAB extracts E1 (*Vibrio natrigens*), E2 (*Bacillus pumilus*) and E6 (*Bacillus horikoshii*) showed a pronounced effect on inhibition of biofilm formation at low concentrations. It is evident from Table 2 that E6 shows promising antibiotic activity against all the *S. pyogenes* strains at the lowest concentration of 50 μg mL⁻¹, whereas E1 and E2 exhibit activity at 100 μg mL⁻¹. E15 and E16 showed antibiotic activity at concentrations of 500 and 400 μg mL⁻¹, respectively. All the CAB extracts showed effective antibiotic activity against the least biofilm formers M89 and M65 at a very low concentration. The CAB extracts efficiently disintegrated the biofilms formed by M56 (Fig. 1a–i) and also against other five M serotypes (data not shown).

To analyse the efficiency of the extracts that produce significant reduction in biofilm formation, biofilm

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**Table 1. Status of the CAB extracts exhibiting antibiofilm activity**

<table>
<thead>
<tr>
<th>Coral bacterial extract</th>
<th>CAB isolate</th>
<th>GenBank accession number</th>
<th>Yield of extract (%)</th>
<th>Extract exhibiting antibiofilm activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>E1</td>
<td><em>Vibrio parahaemolyticus</em></td>
<td>EU636230</td>
<td>0.17</td>
<td>+</td>
</tr>
<tr>
<td>E2</td>
<td><em>Bacillus pumilus</em></td>
<td>EU660356</td>
<td>0.14</td>
<td>+</td>
</tr>
<tr>
<td>E6</td>
<td><em>Bacillus horikoshii</em></td>
<td>EU660327</td>
<td>0.21</td>
<td>+</td>
</tr>
<tr>
<td>E9</td>
<td><em>Vibrio natrigens</em></td>
<td>EU660320</td>
<td>0.53</td>
<td>+</td>
</tr>
<tr>
<td>E10</td>
<td><em>Bacillus firmus</em></td>
<td>EU660344</td>
<td>0.09</td>
<td>+</td>
</tr>
<tr>
<td>E15</td>
<td><em>Vibrio natrigens</em></td>
<td>EU660325</td>
<td>0.14</td>
<td>+</td>
</tr>
<tr>
<td>E16</td>
<td><em>Halomonas salaria</em></td>
<td>EU660353</td>
<td>0.17</td>
<td>+</td>
</tr>
<tr>
<td>E17</td>
<td><em>Bacillus pumilus</em></td>
<td>EU660365</td>
<td>0.59</td>
<td>+</td>
</tr>
<tr>
<td>E31</td>
<td><em>Bacillus subtilis</em></td>
<td>EU660358</td>
<td>0.13</td>
<td>+</td>
</tr>
</tbody>
</table>

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**Table 2. BIC of CAB extracts against the different biofilm-forming M serotypes of *Streptococcus pyogenes* strains at 24 h**

<table>
<thead>
<tr>
<th>M serotypes</th>
<th>E1</th>
<th>E2</th>
<th>E6</th>
<th>E9</th>
<th>E10</th>
<th>E15</th>
<th>E16</th>
<th>E17</th>
<th>E31</th>
</tr>
</thead>
<tbody>
<tr>
<td>M56</td>
<td>100</td>
<td>100</td>
<td>25</td>
<td>50</td>
<td>50</td>
<td>250</td>
<td>250</td>
<td>500</td>
<td>500</td>
</tr>
<tr>
<td>M58</td>
<td>25</td>
<td>50</td>
<td>50</td>
<td>15</td>
<td>200</td>
<td>200</td>
<td>200</td>
<td>500</td>
<td>500</td>
</tr>
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<td>M89</td>
<td>25</td>
<td>10</td>
<td>10</td>
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<td>50</td>
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<tr>
<td>M65</td>
<td>10</td>
<td>15</td>
<td>10</td>
<td>25</td>
<td>50</td>
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<td>M100</td>
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<td>400</td>
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<tr>
<td>M74</td>
<td>50</td>
<td>50</td>
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<td>250</td>
<td>500</td>
<td>300</td>
<td>150</td>
<td>150</td>
</tr>
</tbody>
</table>
Fig. 1. Microscopic visualization (×40) of antibiofilm activity of CAB extracts against the *Streptococcus pyogenes* serotype M56: (a) E1 (100 μg); (b) E2 (50 μg); (c) E6 (50 μg); (d) E9 (250 μg); (e) E10 (200 μg); (f) E15 (500 μg); (g) E16 (400 μg); (h) E17 (200 μg); (i) E31 (250 μg); and (j) control.
susceptibility testing was also carried out at subinhibitory concentrations by diluting the extracts to two-, four- and eightfold. The inhibition of biofilm formation at subinhibitory concentrations is shown in Fig. 2. Biofilm formation was inhibited up to 70–80% at BIC (Fig. 2a). With an increase in dilution, the extracts displayed significant reduction ($P < 0.05$) in biofilm inhibition by 40–70% at 0.5 BIC and 10–60% at 0.25 BIC (Fig. 2b and c). Among the CAB extracts, E6 showed effective antibiofilm activity at 0.5 BIC, with 60% inhibition of biofilm formation in M56. The efficiency of the extracts were also confirmed by microscopic visualization; 0.5 and 0.25 BIC showed appreciable antibiofilm activity whereas 0.125 BIC did not show any significant reduction in biofilm formation when compared with the control (Fig. 3). From the above results it is apparent that all the extracts showed efficient antibiofilm activity against the poor biofilm formers (M65 and M89) as compared with the good biofilm formers (M56, st38, M100 and M74).

### SEM

To ascertain the structures visualized by light microscopy that exhibited antibiofilm activity, we used SEM to elucidate the potential of CAB extracts against *S. pyogenes* biofilm formation. SEM revealed the primary adhesion of the bacterium, leading to dense biofilm formation on the

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**Fig. 2.** Effect of CAB extracts on biofilm formation of *Streptococcus pyogenes* strains as quantified by crystal violet staining and measuring $A_{570 \text{nm}}$. Percentage inhibition of biofilm formation at (a) BIC, (b) 0.5 BIC and (c) 0.25 BIC. Mean values of triplicate independent experiments and SD are shown. Dunnett's test demonstrates significant difference between the tests and the control ($P < 0.05$)
surface of the glass piece by strain M56 (Fig. 4a), whereas Fig. 4b depicts the antibiofilm activity of the CAB extract E6 at 0.25 BIC against the biofilm formation of strain M56. The image reveals the effectiveness of the extract E6 by disintegrating biofilm architecture, which might be the result of the disturbance caused by interbacterial binding.

**Anti-QS activity**

The CAB extracts that showed antibiofilm activity were further screened for anti-QS activity. The inhibitory activity of the CAB extract was determined by violacein production using *C. violaceum* CV026. In preliminary screening, extract E6 showed significant reduction of about 80% in violacein production against *C. violaceum* ATCC 12472 (Fig. 5a). Hence, extract E6 extract was further tested against *C. violaceum* CV026. Extract E6 exhibited concentration-dependent inhibitory activity, which showed a reduction in violacein production with increasing concentration (Fig. 5b). In parallel experiments, the E6 extract was checked for antibacterial activity against *C. violaceum* CV026, by measuring the cell density spectrophotometrically at 600 nm after 24h incubation at applied concentrations of 500–1200 μg mL⁻¹. There was no significant reduction in cell density when compared with that of control (Fig. 5c). This indicates that the E6 extract has no effect on the growth of *C. violaceum* CV026. Therefore, it is concluded that extract E6 possesses considerable QS inhibition activity.

**Effect of various CAB extracts on CSH**

The adhesion of streptococci to toluene was used to measure the hydrophobicity of *S. pyogenes*. The effect of BIC and sub-BIC of CAB extracts on CSH of *S. pyogenes* was investigated. Among the nine extracts screened, extract E1 significantly reduced CSH at 0.5 and 0.25 BIC against most of the M serotypes. Although E6 was found to be efficient extract in anti-QS activity, surprisingly, it showed no reduction in...
CSH. Other extracts such as E31, E16 and E9 showed reduction in CSH only at 0.5 but not at 0.25 BIC.

Discussion

Only medicinal plants have previously been reported to inhibit the biofilm formation of *S. pyogenes* (Limsuwan & Voravuthikunchai, 2008). Here, we show that CAB are promising candidates with potential antibiofilm activity. The metabolites of CAB extracts inhibited biofilm formation by reducing the formation of microcolonies by *S. pyogenes* (Fig. 1). This indicates that biofilm formation was possibly inhibited at the beginning of the attachment stage. The bioactive compounds present in the extracts...
might have interfered with the adherence of *S. pyogenes* by releasing the adhesion compound lipoteichoic acid (LTA) from the streptococcal cell surface (Sun *et al*., 1988).

An important step in biofilm development is the formation of the characteristic biofilm architecture (You *et al*., 2007). Figure 1 shows that the architecture of the *S. pyogenes* microcolonies that were treated with CAB extracts was looser than that of the control. It is envisaged that the natural products of CAB possibly interfered at any step of the *S. pyogenes* biofilms but evidently did not inhibit the growth of *S. pyogenes* at all BICs and sub-BICs tested (Limsuwan & Voravuthikunchai, 2008). The architecture of *S. pyogenes* by SEM further confirmed the antibiofilm activity of the extracts. When compared with the control (Fig. 4a), the samples treated with a subinhibitory concentration of the extracts showed absence of dense biofilm layers (Fig. 4b). Thus, the CAB extracts disintegrate the architecture of the *S. pyogenes* microcolonies, suggesting a possible approach to reducing the resistance of sessile cells (which form a dense layer of biofilm) to antibiotics (You *et al*., 2007).

It is evident from Table 1 that extracts of both Gram-positive and Gram-negative bacteria inhibited the biofilm formation of *S. pyogenes*. Among Gram-positive bacteria, several *Bacillus* sp. such as *Bacillus firmus*, *B. horikoshii* and *Bacillus subtilis* showed good antibiofilm activity. Several studies report the antibiofilm activity of *Bacillus* species isolated from different environments. *Bacillus firmus* and *Bacillus licheniformis* isolated from an oil reservoir inhibited biofilm formation in oil pipelines (Korenblum *et al*., 2008). Thus, it is not surprising that *Bacillus* sp. were the predominant members that inhibited biofilms of *S. pyogenes*. Among the several *Bacillus* sp. screened in this study, an extract of *B. horikoshii* (E6) was found to be the most potent biofilm inhibitor of *S. pyogenes*, and inhibited biofilm formation against all the serotypes of *S. pyogenes* at very low concentrations of the extract, i.e. 10–50 μg mL⁻¹. Limsuwan & Voravuthikunchai (2008) report that three plant extracts, namely of *Boesenbergia pandurata*, *Eleutherine americana* and *Rhodomyrtus tomentosa*, showed antibacterial activity at high concentrations and only the subinhibitory concentrations of the plant extract inhibited antibiofilm activity against *S. pyogenes*. The bacterial extracts used in the present study did not possess antibacterial activity even at high concentrations (Fig. 5a and b) and solely inhibited biofilm formation of *S. pyogenes*. This property of inhibiting only biofilm formation and not the bacterial growth of a pathogen is considered a hallmark of a good antibiofilm compound and from our results we envisage that the coral bacterial extracts used in this study can be developed as antibiofilm compounds against *S. pyogenes*.

QS is a mechanism of release and reception of signalling molecules produced by bacteria and thereby exchanges the signals for the release of chemical substances termed autoinducers. The signal is then transduced into an intracellular biochemical signal that induces a variety of adaptive physiological changes (Raffa *et al*., 2004). QS inhibitors are attractive small molecules and many naturally occurring QS inhibitors are known (Kaplan, 2005; Rasmussen *et al*., 2005). Streptococci use QS systems to adapt themselves and in the regulation of virulence factors to promote their pathogenicity, including biofilm formation (Cvitkovitch *et al*., 2003). The production of QS signals has been much less studied in marine bacteria other than *Vibrio* sp. (Dobretsov *et al*., 2009). On screening the several extracts that inhibited biofilm formation for QS inhibition activity, anti-QS activity was observed in extract E6. This extract reduced the production of violacein dramatically, which was therefore not due to the reduction of ‘quorum’ but due to the interruption of ‘sensing’ (Choo *et al*., 2006). Teadale *et al.* (2009) reported for the first time a marine bacterium *Halobacillus salinus* that secretes secondary metabolites capable of quenching QS-controlled behaviours in several Gram-negative strains. Along similar lines, we envisage that our extract E6 from *B. horikoshii* might possess the chemical ability to compete with the microbial assemblages of *S. pyogenes* strains by disrupting the proper signalling, either diminishing or abolishing subsequent gene transcription and thereby preventing biofilm formation.

Cell surface charge and CSH play a crucial role in bacterium–host cell interactions (Swiatlo *et al*., 2002). There are several reports regarding plant extracts interfering in the CSH against Gram-negative bacteria (Turi *et al*., 1997; Annuk *et al*., 1999) and Gram-positive bacteria (Nostro *et al*., 2004; Prabu *et al*., 2006; Rahim & Khan, 2006; Razak *et al*., 2006) and thereby inhibiting biofilm formation. Limsuwan & Voravuthikunchai (2008) reported that none of the plant extracts they investigated showed a reduction in CSH against *S. pyogenes* biofilms. In contrast, the CAB extracts showed a reduction in hydrophobicity at sub-BICs. Sub-BICs of the CAB extracts are the optimum concentration of CAB extracts that reduce hydrophobicity. This phenomenon is also observed in antibiotics where the subinhibitory concentrations (sub-MICs) of antibiotics, although not able to kill bacteria, can modify their physico-chemical characteristics and the architecture of their outermost surface and may interfere with some bacterial functions, thereby reducing CSH (Fonseca *et al*., 2004). LTA is a major hydrophobin that contributes to the hydrophobicity of a variety of Gram-positive bacteria (Doyle & Rosenberg, 1990; Courtney *et al*., 1990, 2009; Fedtke *et al*., 2007). In *S. pyogenes*, LTA functions not only as a hydrophobin but also mediates adhesion of the organism to a variety of host cells. A possible mechanism by which the CAB extracts reduce CSH might be by affecting hydrophobins such as LTA on the bacterial structures, thus affecting
the initial step of colonization and most probably the development of infection.

We conclude that CAB have the ability to effectively inhibit the biofilms of *S. pyogenes*. As the extract of *B. horikoshii* (E6) efficiently inhibited QS (reduced the production of violacein by 80%), *B. horikoshii* should be explored further as a means to control biofilm-associated infections caused by other pathogens.

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**References**


