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Antibacterial activity of crude extracts of cyanobacteria Phormidium and Microcoleus species

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Cyanobacteria is a group of prokaryotic organisms that occurred originally in the world. Thailand is well known as an area of a high biological diversity. In our study, eighteen cyanobacteria from the natural area were isolated and screened for their antibacterial activities. Of these, two isolated cyanobacteria (NCI1 and NCI4) with positive antibacterial activities were identified by their morphology and 16S rRNA sequences as Phormidium and Microcoleus group in family Oscillatoriaceae. The ethanol extracts of Phormidium sp. and Microcoleus sp. at various concentrations (0.2, 0.06, 0.03 and 0.015 g/ml) showed the antibacterial activity against Streptococcus enteritidis and Escherichia coli on the media. Each cyanobacterial extract showed more inhibition zones against S. enteritidis and E. coli by increasing their concentrations, especially those of Phormidium extracts. At the highest concentration 0.2 g/ml, the maximum diameter of inhibition were observed in the extracts of Phormidium sp. and Microcoleus sp., 11 and 10 mm against S. enteritidis, and 10 and 10 mm against E. coli, respectively. As results, the extracts of Phormidium sp. and Microcoleus sp. showed potentially interesting antibacterial activities against the two pathogens. Therefore, the two cyanobacteria may be useful in various applications and used as basic knowledge for further investigations.

Key words: Phormidium sp., Microcoleus sp., antibacterial activity.

INTRODUCTION

Cyanobacteria are morphologically diverse group of Gram-negative eubacteria. It is able to perform oxygenic photosynthesis and used as important food for other organisms. Moreover, it is widely found in various locations such as pond, soil, rock, bark, sea and fresh water (Carr and Whitton, 1982; Castenholz and Waterbury, 1989). Cyanobacteria are several potential benefits to study on bioactive compounds from these organisms. Although, antibacterial, antiviral, algicide, antifungal and cytotoxic activities have been many researched in these organisms (Rao, 1994; Issa, 1999; Pushparaj, 1999; Schlegel et al., 1999; Schaeffer, 2000), the properties of the bioactive compounds are still not completely understood (Inderjit and Dakshini, 1994). However, cyanobacteria is able to produce many bioactive compounds both intra- and extracellular to survive in extreme environmental sources (Dvornyk and Nevo, 2003; Kulik, 1995; Kreitlow et al., 1999; Patterson et al., 1994). Some properties of bioactive compounds from several cyanobacteria are useful in medicine and agriculture applications (Borowitzka, 1995; Kulik, 1995), such as cryptophycin 1 agent with anticancer (Moore,
1996; Patterson et al., 1991). The discovery of several bioactive compounds demonstrated the important development of new organic agricultures because using the natural compounds can be self-degraded and less toxic than chemicals (Saxena and Pandey, 2001).

However, there are limited studies on certain cyanobacteria such as Phormidium and Microcoleus. Nowadays, the valuable natural resources are still remaining abundance in Thailand. Therefore, the survey of cyanobacteria from the natural regions may increase the opportunities to find cyanobacteria with antibacterial activity. Our goal is to determine antibacterial activity of cyanobacteria isolated from the natural regions and phylogenetic analysis of 16S rRNA sequences.

MATERIALS AND METHODS

Isolation and culture conditions

Samples were collected from natural different locations in Bangkok and Kanchanaburi provinces of Thailand and carried to the laboratory and kept under 4°C until use. Samples were cultured directly in BG-11 agar media and isolated by using streak plate method (Richmond, 1986). Each isolated cyanobacteria was cultured in a 500 ml flask containing 200 ml of BG-11 medium without shaking, for 20-40 days incubated at room temperature and illumination at 3000 lux with a white continuous light.

Preparation of cyanobacterial extracts

Cyanobacterial cells were dried at 60°C, and then the cells were grinded in sterile tubes. The cells were mixed with 80% ethanol for 1 ml with shaking for 5 min and kept in room temperature for 8 h. After that, the solvent was removed by incubation at 60°C and redissolved in water (ratio 0.2; 0.06; 0.03 and 0.015 g/ml) and kept at 4°C until use for further assay.

Antibacterial test of cyanobacterial extracts

The antibacterial activities of cyanobacterial extracts were assayed by agar disc diffusion (Bauer et al., 1966). Gram-positive bacteria, Streptococcus enteritidis and Gram-negative bacteria, Escherichia coli were used to as tested pathogens. Filter paper disks (6 mm) were saturated with 10 μl of each extract and placed on nutrient agar plates with a lawn of the tested pathogens. Plates were incubated at 37°C for 24-28 h and inhibition zones were determined. Ampicillins (100 μg) was used as positive control. The plates were incubated at 37°C for 24-28 h and the inhibition zones were measured as described above.

Deoxyribonucleic acid (DNA) extractions

Genomic DNA was extracted from isolated cyanobacteria cells following standard methods (Sambrook et al., 2001) with slight adjustments. Then, DNA was checked by running on a 0.7% agarose gel (Sambrook et al., 2001).

PCR amplification and 16S rRNA sequencing

The purified DNA was used as a template for PCR amplification. The 16S rRNA gene was amplified using specific primers CYA359F and CYA781R (Nübel et al., 1997). The 50 µl of mixtures contained 1.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 µM of each primer, 0.1 mg/ml bovine serum albumin, 1.5 U Tag polymerase, 1× buffer (Promega) and 15 ng DNA. The PCR was carried out by an initial predenaturation at 95°C for 5 min, followed by 35 cycles consisting 1 min at 94°C denaturation, 1 min at 60°C and 30 sec at 72°C. The final extension was 7 min at 72°C. The PCR products were checked on 1% agarose gel by comparing with 100 bp standard DNA marker.

PCR products were purified by PCR purification kit (QIAGEN) and directly sequenced at Macrogen Inc., Korea. The nucleotide sequences (partial 16S ribosomal RNA sequences) were analyzed by using BLAST program at http://www.ncbi.nlm.nih.gov and aligned using BioEdit program version 7.0.9.0 (Hall, 1999).

Phylogenetic analyses

Phylogenetic tree was constructed using the neighbor-joining method which is implemented MEGA 4.0.2 (Tamura at al., 2007). The tree topology was evaluated by 10,000 resampling bootstrap.

RESULTS

In the present study, eighteen cyanobacteria from the natural areas were isolated and screened for their anti-bacterial activities. Of these, two genera of cyanobacteria (NCI1 and NCI4) with positive antibacterial activities obtained from each fresh water pond in Bangkok and Kanchanaburi province of Thailand, respectively. Cyanobacteria, NCI1 and NCI4 were basically identified by external morphology according to Peerapornpisal (2005) and Anagnostidis and Komárek (1988). It indicated that these two isolates belonged to the family Oscillatoriaceae (Figure 1). Likewise, they were confirmed by 16S rRNA sequences. The 16S rRNA sequences of the two isolates were analyzed by using BLASTN program (www.ncbi.nlm.nih.gov) that provides the highest similarity scores for cyanobacteria. It was found that the 16S rRNA sequences of NCI1 and NCI4 cyanobacteria resemble to Phormidium sp. for 98 and 100% in Microcoleus sp., respectively. 16SrRNA sequences of Phormidium sp. and Microcoleus sp. were aligned with those of other cyanobacteria from the GenBank by using BioEdit 7.0.4.1 (data not shown). Phylogenetic tree was constructed using the neighbor-joining method which is implemented MEGA 4.0.2 and supported by 10,000 bootstrap replications. The result indicated that the NCI1 and NCI4 could be recognized to phylogenetically deferent clusters. The NCI1 and NCI4 were recognized in group Phormidium sp. and Microcoleus sp., respectively (Figure 2). The effect of ethanol extractions of cyanobacteria Phormidium sp. and Microcoleus sp. on the inhibition of tested pathogens was shown in Table 1. The results showed that ethanol extracts of Phormidium sp. and Microcoleus sp. at various concentrations 0.2, 0.06, 0.03 and 0.015 g/ml exhibited the antibacterial activities against Streptococcus enteritidis and E. coli. Inhibition activities...
Figure 1. Light photomicrographs of NCI1 (A) and NCI4 (B) corresponding to *Phormidium* sp. and *Microcoleus* sp., respectively.

Figure 2. Neighbor-joining phylogenetic tree based on analysis of cyanobacterial 16S rRNA sequences with other sequences obtained from GenBank. *Phormidium* sp. and *Microcoleus* sp. were underlined and asterisked, respectively. Numbers near the point at nodes indicate bootstrap values.
Table 1. Antibacterial activity of *Phormidium* sp. and *Microcoleus* sp. extracted with 80% ethanol (diameter of inhibition zone in mm).

<table>
<thead>
<tr>
<th>Species</th>
<th>Pathogen bacteria</th>
<th>Phormidium sp. (g/ml)</th>
<th>Microcoleus sp. (g/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.015</td>
<td>0.03</td>
</tr>
<tr>
<td><em>Streptococcus enteritidis</em></td>
<td></td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td></td>
<td>8</td>
<td>8</td>
</tr>
</tbody>
</table>

Figure 3. Antibacterial activity of *Phormidium* sp. and *Microcoleus* sp.

of the two cyanobacterial extracts were more effective at high concentration against the tested pathogens at the low concentration, especially those of the *Phormidium* extracts (Figure 3). At the highest concentration 0.2 g/ml, the maximum diameter of inhibition was noticed in the extracts of *Phormidium* sp. and *Microcoleus* sp., 11 and 10 mm against *Streptococcus enteritidis*, and 10 and 10 mm against *Escherichia coli*, respectively. As results, the extracts of *Phormidium* sp. and *Microcoleus* sp. showed potentially interesting antibacterial activities against the tested pathogens.

DISCUSSION

Cyanobacteria are groups of photosynthetic eubacteria with diversity, approximately 7500 species (Chapman and Chapman, 1975). In the present study, eighteen cyanobacteria of Thailand were selected from the natural area. Among them, two genera of cyanobacteria (NCI1 and NCI4) which are shown in positive antibacterial activities were obtained. Although, cyanobacteria are widely distributed in various natural regions (Carr and Whitton, 1982; Castenholz and Waterbury, 1989), there are limitations in the study of microorganisms from the environments because some of them are not able to grow on synthesized mediums in the laboratory. Therefore, the selection and study of microorganisms from natural sources may provide less species (Embry and Stackebrandt, 1997).

In the present study, NCI1 and NCI4 with positive antibacterial activity were first identified by morphology and confirmed by 16S rRNA sequence analysis. The 16S rRNA sequences of the two isolates were analyzed by using BLASTN program and aligned with other cyanobacteria sequences from the GenBank. Honda et al. (1999) reported about the phylogenetic analysis of several cyanobacteria from 16S rRNA sequences.

In this study, phylogenetic tree was constructed using the neighbor-joining method which is implemented MEGA 4.0.2 and supported by 10,000 bootstrap replication. The result showed that, NCI1 and NCI4 were recognized in
group *Phormidium* sp. and *Microcoleus* sp., respectively. The species *Phormidium* and *Microcoleus* demonstrated the effective extracts for antibacterial activity against *Streptococcus enteritidis* and *Escherichia coli*. The extracts of *Phormidium* sp. shows highest inhibition zone to *Streptococcus enteritidis* (11 mm) at 0.2 g/ml and *Streptococcus enteritidis* showed more sensitivity to the cyanobacteria extracts than those of *Escherichia coli*. Moreover, the cyanobacterial extracts showed more effective inhibition activities by increasing their concentration. It was reported that correlation between the extracts concentration and the inhibition zone sizes in the logarithm revealed the linear relationship (Crosby, 1991).

For other cyanobacteria species, *Anabaena* sp., it have been reported that antibacterial properties against *S. aureus*, *E. coli*, *P. aeruginosa*, *S. typhi* and *K. pneumoniae* (Kaushik et al., 2009). It was imply that the extracts of cyanobacteria may present diverse bioactive compounds responsible for the antibacterial activity (Kempf and Bremer, 1998; Schwartz et al., 1990). Moreover, many bioactive compounds may be excreted into environment due to stress to survival of cyanobacteria (Martin and Maris, 1995; Nicholson et al., 2000; Soltani et al., 2006). It hypothesized that bioactive compounds can damage microorganisms by attacking the cytoplasmic membrane and penetrate the cell (Lampe et al., 1998; Maillard, 2002; Ultee et al., 2000). The bioactive compounds were able to disturb the Gram-posi-tive bacteria by destabilizing the cytoplasmic membrane and led to cell death (Ultee et al., 2000). In the Gram-negative bacteria, the outer membrane of the bacteria showed the hydrophilic surface on the side chains of lipopolsaccharides which restrict the entrance of hydrophobic substances to the cellular membrane (Bergsson, 2005). However, certain molecules can destabilize the lipopolysaccharide layer, such as terpenoid and phenolic compounds (Helander et al., 1998).

In the present study, it was concluded that the extracts of *Phormidium* and *Microcoleus* species indicated the potential of antibacterial activity against *S. enteritidis* and *E. coli*. Therefore, the basic knowledge may useful in various applications such as pharmaceutics and agricultures, and for further investigations.

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