Bleach-tolerant Bacterial Species Isolated from Potable Water in Hong Kong

Subramanya Rao¹, Mui Kwok Wai², Wong Ling Tim² and Leung Polly¹*

¹Department of Health Technology and Informatics, Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China.
²Department of Building Services Engineering, Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China.

Received: January 18, 2017; Accepted: February 05, 2017; Published: February 26, 2017

*Corresponding author: Polly Leung, Department of Health Technology and Informatics, Hong Kong Polytechnic University, Hung Hom, Kowloon, Hong Kong, China. Email: polly.hm.leung@polyu.edu.hk

Abstract

Sodium hypochlorite (NaOCl) is a common household bleach. The ability of this alkaline salt to kill a wide variety of harmful bacteria upon contact makes it the chemical of choice in treating potable drinking water. Several studies have shown the ineffectiveness of the bleach in killing certain bacteria. We hypothesize that bleach might not be effective to all type of bacteria, especially the one which has the ability to form biofilms. Here we report the isolation and phylogenetic identification of bleach-tolerant bacteria from potable drinking water. The bacterial species isolated from this study phylogenetically affiliated to Ralstonia picketti, usually found in occurrence with biofilm-associated microflora.

Keywords: Biofilm; Disinfectant; Sodium hypochlorite; MALDI-TOF MS; 16S rRNA gene

Introduction

Water is an essential element for life, contamination of tap water as a result of improper water treatment and disposal serves as a route of the transmission of water-borne diseases. Therefore, provision of safe and clean potable water is one of the most important tasks of the public health sectors.

Chlorine is the chemical of choice in treating potable drinking water due to its effectiveness, high solubility and low cost [1,2]. When mixed with water, hypochlorous Acid (HOCI) is formed, which is a strong oxidizing agent that kills bacteria and viruses [3]. It has been documented that exposure to chlorine for about 1 hour achieved a great deal of reduction in the indicator bacterial species in effluent samples [4]. However, this does not necessarily applicable to the entire bacterial population in the water body as the biofilm organisms are resistant to disinfectants [5]. In our experiment, we aimed to test the effectiveness of NaOCl in killing of potable water microorganisms, especially Legionella, in an artificially constructed potable water distribution system. Furthermore, we also attempted to isolate NaOCl-tolerant bacterial strains. Here we report the isolation and phylogenetic identification of bleach-tolerant bacterial genus Ralstonia from potable drinking water.

Method

Construction of Model water systems

A model water distribution system was constructed in order to investigate the ecological relationship between biofilm microorganisms and Legionella in potable water distribution system. Each system comprised an 30-L electric heater, which connected to a potable water circulation system, a makeup water supply system, a duplicate variable speed water pump set (with one pump set served as the standby in case the duty pump was out of order), and control accessories for monitoring temperature, flow rates and other physicochemical properties of water.

In potable water circulation system, the speed pump fed water through the water heater where the water temperature was maintained at 25º C and the pump forced the water through the system at sufficient pressure to ensure appropriate circulation flow rate (0.3 ms⁻¹).

Introduction of water with microbial consortium into the model systems

Twenty litres (20L) of water collected from a water tap within a university campus were fed to the model systems. As the water sample was found to be negative for Legionella pneumophila after screening, Legionella pneumophila (ATCC33152) was then inoculated into the system at a concentration of 10³ colony-forming units (CFU)/L, this concentration was based on the findings of Legionella counts in potable water system [6]. After feeding of water into the model systems, water was circulated at a flow rate of 1.5 ms⁻¹ for 8 hours in order to distribute the microorganisms within the water systems. After that, water flow rate was maintained at 0.3 ms⁻¹ for 4 weeks.

Disinfection procedures

Ten 1-ml aliquots of water samples from the model water system were obtained at the end of the 4th week. The samples were placed in 1.5-ml tubes and were then treated with 0 ppm, 0.1 ppm, 0.5 ppm and 50 ppm of bleaching solution for 5, 10 and 30 minutes. One hundred microliters of water sample from each tube were neutralized with 100 µl of sodium thiosulphate
(10% weight/volume). The neutralized sample was spread on to Buffered Charcoal Yeast Extract (BCYE) agar plates (Oxoid Microbiology products, UK), which is a selective growth media used to isolate gram-negative bacterial species, particularly *Legionella pneumophila* [7]. These plates were then incubated at 37°C in 5% carbon dioxide for 3 days (2-5 days depended on the growth of bacteria). After incubation, the bacterial colonies were counted. Identities of the bacterial colonies were analyzed using matrix-assisted laser desorption-ionization-time of flight mass spectrometry (MALDI-TOF MS). MALDI-TOF MS is a new technology for rapid identification of bacterial species based on protein molecules [8, 9]. Bacterial identities were further confirmed using PCR amplification and sequencing of the 16s rRNA genes as described below.

**DNA extraction, PCR, and sequencing**

DNA was extracted from the bacterial isolates using PowerWater culture DNA isolation kit according to the manufacturer’s protocol (MO BIO Laboratories Inc., Carlsbad, CA, USA). DNA amplification was performed by PCR. Forward primer 27F (AGAGTTTGATCMTGGCTCAG) and reverse primer 907R (CCGTCAATTCMTTTRAGTTT) were used to amplify the 16s rRNA gene region [10]. The PCR profile included an initial denaturation step at 95°C for 1 min, followed by 30 cycles of amplification which consisted of denaturation at 95°C for 1 min, primer annealing at 52°C for 50 s and extension at 72°C for 1 min. This was followed by a final extension at 72°C for 10 min. The presence of PCR products was confirmed by electrophoresis in 1% agarose gels. Purification of PCR product was carried out using GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, UK). Automated DNA sequencing was performed using dye-terminator cycle sequencing. After cycle sequencing, the purified product was analyzed by capillary electrophoresis on a ABI3730 Genetic Analyzer (Applied Biosystems, US).

**Phylogenetic analysis**

The DNA sequences obtained were aligned with reference to the selected GenBank sequences using ClustalW (http://www.genome.jp/tools/clustalw/). Maximum likelihood analysis was conducted using PAUP* 4.0b8 [11]. Phylogenetic trees were drawn using Fig Tree. Bootstrap values (1,000 replications) were shown for branch nodes supported by more than 70%. All sequences have been deposited in the NCBI GenBank database, all 11 bacterial isolates were identified to be *Ralstonia picketti* or *Ralstonia species* (Table 1). Using 16S rRNA PCR and sequencing, followed by BLAST search matching against the GenBank database, all 11 bacterial isolates were identified to be *Ralstonia picketti* or *Ralstonia species* (Table 1).

**Results and Discussion**

The results revealed that a total of 11 bacterial taxa comprised the *Ralstonia* taxa. *Ralstonia* is usually associated with biofilms in water samples. The strains grew on BCYE after treatment with bleach solution at all three concentrations and at different exposure durations. The total bacterial count for the untreated water sample at T= 0 min was 3.5X10⁴/L. At 0.1 ppm chlorine concentration, the viable bacterial counts under 5, 10 and 30 min treatment duration were 8.0X10⁴/L, 5.4X10⁴/L, 4.9X10⁴/L, respectively. For the chlorine concentration at 0.3 ppm, the viable bacterial counts under 5, 10 and 30 min treatment duration were 3.2X10⁴/L, 4.1X10⁴/L, 7.8X10⁴/L, respectively. For the chlorine concentration at 50 ppm, the bacterial counts under 5, 10 and 30 min treatment duration were 4.4X10⁴/L, 7.9X10⁴/L, 6X10⁴/L, respectively. According to the results, there was less than 10-fold reduction in bacterial counts under various treatment conditions. Since the bacterial loads were still high even after 50 ppm NaOCl treatment for 30 min, we investigated the Identities of the bacterial species that were resistant to NaOCl treatment.

Bacterial isolation using BCYE culture media yielded a total of 11 isolates from 11 samples. These isolates were subjected to MALDI-TOF MS-based identification and further confirmed by 16S rRNA PCR and sequencing. Using MALDI-TOF MS, seven isolates were identified as *Ralstonia picketti* and four bacterial isolates were not identified (Table 1). Using 16S rRNA PCR and sequencing, followed by BLAST search matching against the GenBank database, all 11 bacterial isolates were identified to be *Ralstonia picketti* or *Ralstonia species* (Table 1). Phylogenetic analysis revealed that these cultivated isolates resistant to disinfection were *Ralstonia picketti* isolates (Figure 1).

<table>
<thead>
<tr>
<th>Treatment condition (chlorine concentration/ Time of exposure)</th>
<th>Bacterial identity based on MALDI-TOF MS</th>
<th>Bacterial identity based on 16S DNA sequencing and BLAST match (Accession number)</th>
<th>BLAST Identity</th>
<th>Accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 ppm/ T=5 mins</td>
<td>No Organisms Identified</td>
<td><em>Ralstonia picketti</em> strain CP12 (KF378754)</td>
<td>99%</td>
<td>KX025094</td>
</tr>
<tr>
<td>0.1 ppm/ T=10 mins</td>
<td>No Organisms Identified</td>
<td><em>Ralstonia picketti</em> strain CP12 (KF378754)</td>
<td>99%</td>
<td>KX025085</td>
</tr>
<tr>
<td>0.3 ppm/ T=5 mins</td>
<td>No Organisms Identified</td>
<td><em>Ralstonia picketti</em> isolate DiSc07</td>
<td>96%</td>
<td>KX025086</td>
</tr>
<tr>
<td>0.3 ppm/ T=10 mins</td>
<td>No Organisms Identified</td>
<td><em>Ralstonia sp. PH-S1</em> (JN543508)</td>
<td>99%</td>
<td>KX025087</td>
</tr>
<tr>
<td>0.3 ppm/ T=30 mins</td>
<td>No Organisms Identified</td>
<td><em>Ralstonia sp. PH-S1</em> (JN543508)</td>
<td>99%</td>
<td>KX025089</td>
</tr>
<tr>
<td>50 ppm/ T=5 mins</td>
<td><em>Ralstonia picketti</em></td>
<td>*Uncultured Ralstonia sp. Clone M_KL_110_14 (KX067487)</td>
<td>99%</td>
<td>KX025091</td>
</tr>
<tr>
<td>50 ppm/ T=10 mins</td>
<td><em>Ralstonia picketti</em></td>
<td>*Uncultured Ralstonia sp. Clone M_KL_110_14 (KX067487)</td>
<td>99%</td>
<td>KX025092</td>
</tr>
<tr>
<td>50 ppm/ T=30 mins</td>
<td><em>Ralstonia picketti</em></td>
<td>*Uncultured Ralstonia sp. Clone M_KL_110_14 (KX067487)</td>
<td>99%</td>
<td>KX025093</td>
</tr>
</tbody>
</table>

Table 1: Bacterial identities based on MALDI-TOF MS and 16S rDNA sequencing.
Earlier studies showed that conditions, even at 50 ppm concentration for 30 minutes, that the isolates could survive various NaOCl treatment biofilms in aquatic environments [12,13]. Our findings showed frequently found in water and known for its ability to form biofilms which favors the survival of Legionella. Ralstonia picketti is a Gram-negative bacterial species frequently found in water and known for its ability to form biofilms in aquatic environments [12,13]. Our findings showed that the isolates could survive various NaOCl treatment conditions, even at 50 ppm concentration for 30 minutes. Earlier studies showed that Ralstonia picketti had the ability not only to survive but also to thrive in oligotrophic conditions [12, 13] and its ability to form biofilm which favors the survival of Legionella.

References


