Application of Alkaliphilic Biofilm-Forming Bacteria to Improve Compressive Strength of Cement-Sand Mortar

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The application of microorganisms in the field of construction material is rapidly increasing worldwide; however, almost all studies that were investigated were bacterial sources with mineral-producing activity and not with organic substances. The difference in the efficiency of using bacteria as an organic agent is that it could improve the durability of cement material. This study aimed to assess the use of biofilm-forming microorganisms as binding agents to increase the compressive strength of cement-sand material. We isolated 13 alkaliphilic biofilm-forming bacteria (ABB) from a cement tetrapod block in the West Sea, Korea. Using 16S RNA sequence analysis, the ABB were partially identified as Bacillus algicola KNUC501 and Exiguobacterium marinum KNUC513. KNUC513 was selected for further study following analysis of pH and biofilm formation. Cement-sand mortar cubes containing KNUC513 exhibited greater compressive strength than mineral-forming bacteria (Sporosarcina pasteurii and Arthrobacter crystallopoietes KNUC403). To determine the biofilm effect, Dnase I was used to suppress the biofilm formation of KNUC513. Field emission scanning electron microscopy image revealed the direct involvement of organic–inorganic substance in cement-sand mortar.

Keywords: Exiguobacterium marinum, biofilm formation, halophilic bacteria, cement-sand mortar, compressive strength

Cement concrete is a strong and cheap construction material and is the most widely used construction material [10]. However, cement is prone to the weathering action of several physical, chemical, and biological factors [21]. To remediate the deterioration of degraded cement buildings, a broad range of organic and inorganic products have been proposed; these can be costly and environmentally toxic [6, 14]. Microbiological calcium carbonate precipitation (MCP), which can be used as a binder or sealing agent in building material, has been proposed as an eco-friendly organic material to enhance the durability of cement material [7].

Research to date involving the use of mineral-producing bacteria in remediation and protection have focused on repair of pours and cracks in cement structures [6, 7], improvement of compressive strength of cement mortar [10, 11, 16], and as a coating on the surface of cement material to increase durability [21, 22]. MCP is induced by bacterial urea hydrolysis, which increases the alkalinity of the environment. The induced alkalinity affects as a motive force on calcium carbonate precipitation, and the negatively charged bacterial cell wall favors binding of divalent cations such as Ca²⁺ and Mg²⁺ as a nucleation site of crystal maturation [8, 12, 15, 16].

The main concept behind the use of MCP is that, when poured into the cement structure, the calcium crystals will increase the structural strength of the material [1,3,6,7]. The strength improvement is probably due to deposition on the bacteria cell surface, which plugs the pores within the mortar [10]. The same study also examined the effect of the use of Shewanella, which is not associated with carbonate precipitation, and reported a positive effect on the compressive strength of mortar [10]. The same authors also reported greater strength of mortar containing bacterial protein compared with the control mortar at all ages [11]. This is consistent with the suggestion that bacterial extracellular polymeric substances and biofilms could serve as potential factors for cementing the sand [1]. The collective data to date support the idea that organic substances secreted by bacteria could be used as filling material in poured cement-sand mortar.

In this study, we isolated alkaliphilic biofilm-forming bacteria (ABB) that could endure the high alkalinity environment of cement and examined the effect of the biofilms formation in increasing the compressive strength
of cement-sand mortar. Among the ABB, *Exiguobacterium marinum* KNUC513 was selected for in-depth study. The cement mortar containing *E. marinum* KNUC513 was stronger as compared with cement specimens treated with calcite-forming bacteria such as *Sporosarcina pasteurii* and *Arthrobacter crystallopoietes* KNUC403. There was also a correlation between the formation of the biofilm induced by KNUC513 and the improvement in the compressive strength of cement-sand mortar. This is the first study to directly demonstrate the utility of bacterial biofilms as an organic substance capable of increasing the durability of cement-sand mortar.

**Materials and Methods**

**Isolation of ABB**

ABB were isolated from tetrapod blocks in the West Sea of Korea. The fragments of cement tetrapod blocks were directly suspended in sterilized 0.85% NaCl and incubated for 10 days at 10°C on a shaker operating at 80 rpm. The samples were diluted 1:100 and cultured on a solidified version of marine broth (MB) containing 8% NaCl. The plates were incubated at 30°C under aerobic conditions until colonies were detected. *S. pasteurii* ATCC11859, used as an experimental control, was purchased from the Korean Biological Resource Center (Korea). *S. pasteurii* produces calcite and improves the compressive strength of cement mortar [1, 2]. *A. crystallopoietes* KNUC403, which also forms calcite and which was isolated from concrete structures in our previous study [17], was used.

**Polymerase Chain Reaction (PCR) Amplification of 16S rRNA Genes**

Isolates were identified based on 16s rRNA sequence analyses. The 16S rRNA gene fragments were obtained by PCR amplification using a QIAamp DNA Mini Kit 250 (Qiagen Co., USA) according to the manufacturer’s instructions and amplified with the primers H+ (5'-GAGTTTGACATGCTGTCAG-3') and SE- (5'-TTACACCACTATTCCATATG-3'). DNA sequencing of the PCR amplicon was carried out by Cosmogenetech (Korea). The multiple alignments for the 16S rRNA full gene sequence of isolated strains were performed using an EzTaxon search (http://147.47.212.35:8080). The identified strains are listed in Table 1.

**Bacterial Growth in Alkaline Environment**

The ability of the identified strains to grow in an alkaline environment was tested from pH 7.0 to pH 12.0. The ABB were cultured in 5 ml of MB at 30°C and 150 rpm for 24 h. One ml of each cell culture was inoculated in 100 ml of MB in which the pH was controlled by addition of 6 N HCl and 10 N NaOH. The samples were incubated for 2 days at 30°C and 150 rpm. After 2 days, 200 µl of the cell culture was added to a 96-well plate and the cell growths were measured using a microtiter plate reader at 580 nm (Merck Co., USA).

**Adhesion Assay**

Biofilm formation was semiqualitatively determined as described previously [19]. The isolates were individually precultured in 5 ml of tryptic soy broth (TSB) aerobically for 24 h and harvested by centrifugation at 8,000 rpm. The harvested cells were suspended in TSB containing glucose (TYG, containing 30 g/l of TSB, 6 g/l of yeast extract, and 8 g/l of glucose). Two hundred µl of the suspension was added to each well of a 96-well culture plate. The plates for KNUC strains were incubated for 24 h at 30°C on a shaker at 50 rpm [4]. After inoculation, each microtiter plate was washed three times by tap water and then 200 µl of 99% methanol was added to each well. The wells were emptied after 15 min at room temperature and air-dried for 15 min. The plate was stained with 200 µl of crystal violet and rinsed by placing the plate under running tap water. Finally, 200 µl of 99% ethanol was added to each well. The 96-well plate was measured using a microtiter plate reader at 580 nm [4, 19].

**Table 1. Identification and characterization of isolates from cement tetrapod block in the West Sea of South Korea.**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Closest relative (percentage identity)</th>
<th>NaCl (%) (optimal growth)</th>
<th>pH range (optimal growth)</th>
<th>GenBank Accession No. of 16S rDNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>KNUC501</td>
<td><em>Bacillus algicola</em> (98.4%)</td>
<td>0 ~ 18 (2)</td>
<td>7 ~ 8 (9)</td>
<td>JN195783</td>
</tr>
<tr>
<td>KNUC502</td>
<td><em>Exiguobacterium marinum</em> (99.9%)</td>
<td>0 ~ 16 (4)</td>
<td>7 ~ 12 (8)</td>
<td>JN195784</td>
</tr>
<tr>
<td>KNUC503</td>
<td><em>Jeotgalibacillus soli</em> (99.1%)</td>
<td>0 ~ 16 (10)</td>
<td>7 ~ 11 (11)</td>
<td>JN195785</td>
</tr>
<tr>
<td>KNUC504</td>
<td><em>Halomonas aquamarina</em> (99.6)</td>
<td>0 ~ 20 (12)</td>
<td>7 ~ 12 (8)</td>
<td>JN195786</td>
</tr>
<tr>
<td>KNUC505</td>
<td><em>Halomonas venusta</em> (99.4%)</td>
<td>2 ~ 20 (8)</td>
<td>7 ~ 11 (11)</td>
<td>JN195787</td>
</tr>
<tr>
<td>KNUC506</td>
<td><em>Alkalibacterium putridalgicola</em> (100%)</td>
<td>0 ~ 10 (8)</td>
<td>7 ~ 12 (11)</td>
<td>JN195788</td>
</tr>
<tr>
<td>KNUC507</td>
<td><em>Alkalibacterium putridalgicola</em> (98.3%)</td>
<td>2 ~ 10 (8)</td>
<td>7 ~ 11 (11)</td>
<td>JN195793</td>
</tr>
<tr>
<td>KNUC508</td>
<td><em>Planococcus maritimus</em> (99.9%)</td>
<td>0 ~ 16 (8)</td>
<td>7 ~ 12 (11)</td>
<td>JN195790</td>
</tr>
<tr>
<td>KNUC509</td>
<td><em>Halomonas hydrothermalis</em> (98.2%)</td>
<td>2 ~ 20 (10)</td>
<td>7 ~ 12 (8)</td>
<td>JN195791</td>
</tr>
<tr>
<td>KNUC510</td>
<td><em>Staphylococcus sciuiri</em> (99.7%)</td>
<td>0 ~ 20 (2)</td>
<td>7 ~ 12 (9)</td>
<td>JN195793</td>
</tr>
<tr>
<td>KNUC511</td>
<td><em>Bacillus vietnamensis</em> (98.7%)</td>
<td>0 ~ 14 (2)</td>
<td>7 ~ 9 (9)</td>
<td>JN195794</td>
</tr>
<tr>
<td>KNUC512</td>
<td><em>Exiguobacterium profundum</em> (99.6%)</td>
<td>0 ~ 16 (2)</td>
<td>7 ~ 11 (8)</td>
<td>JN195795</td>
</tr>
<tr>
<td>KNUC513</td>
<td><em>Exiguobacterium marinum</em> (98.1%)</td>
<td>0 ~ 16 (2)</td>
<td>7 ~ 12 (8)</td>
<td>JN195792</td>
</tr>
</tbody>
</table>

*The multiple alignments for the 16S rRNA full gene sequence of isolated strains were performed using an EzTaxon search.*

*The salt concentration for growth of these isolates was measured from 0% to 20% in MB.*

*The pH ranges for growth of these isolates were measured from pH 7 to pH 12 in MB medium; pH was controlled by addition of HCl and NaOH.*

*The sequences for the 16S rRNA genes in the four CFB have been deposited in the GenBank database under accession numbers JN195783 to JN195792.*
Microbiologically Induced CaCO₃ Precipitation
For qualitative analysis of calcium carbonate precipitation, test strains were cultured on urea-CaCl₂ medium (3 g/l of nutrient broth, 10 g/l of ammonium chloride, 2.12 g/l of sodium hydrogen carbonate, 3.17 g/l of calcium chloride, and 20 g/l of urea) and incubated at 30 °C for 5 days [12, 16]. The crystals precipitated on the bacterial colony were captured by a Zentech digital camera equipped with a stereomicroscope (Sw 804425; Samwon, Seoul, Korea) established with 40× magnifications.

Compressive Strength Test
E. marinum KNUC513, S. pasteurii, and A. crystallopoietes KNUC403 were inoculated on TSB and cultured for 24 h at 30 °C. Cell cultures were centrifuged at 8,000 rpm and washed twice with 50 mM sodium phosphate buffer (pH 7.5). Each bacterial suspension prepared to the same cell density was prepared using 50 mM sodium phosphate buffer and adjusted to an optical density (OD) at 600 nm of 0.8. A mixture of 240 g cement, 660 g sand, and 116.4 ml of the bacterial suspension was used to cast three cement-sand mortars with dimensions of 50.8 mm × 50.8 mm × 50.8 mm. All samples were prepared in sets of six. Cell-free phosphate buffer was used as a control. The mortars were demolded after 48 h and were cured in 1 l of urea-CaCl₂ medium at 30 °C. The urea-CaCl₂ medium was changed every 10 days. After removing the medium, the surface of each cube was completely dried at 25 °C prior to a compressive strength test. The compressive strengths of the specimens were measured after 3, 7, and 28 days [16, 18].

Inhibition of Biofilm Formation
For biofilm inhibition assay of E. marinum KNUC513, Dnase I was used as described previously to prevent biofilm formation [23]. The strain was inoculated in TSB and cultured for 24 h at 30 °C at 180 rpm. The cell pellet was harvested by centrifugation (8,000 rpm) and suspended in 5 ml of TYG containing Dnase I. Quantification of biofilm inhibition was measured by the aforementioned adhesion assay.

Effect of Biofilm Formation of E. marinum KNUC513 on Compressive Strength
Isolate KNUC513 was cultured in TSB at 30 °C for 24 h and the cell pellet was harvested by centrifugation (8,000 rpm). The concentration of sample was controlled to 0.8 at OD 600 nm and suspended with 232.8 ml of TYG containing 400 U/ml of Dnase I. A mixture of 480 g cement, 1,320 g sand, and 232.8 ml of TYG containing KNUC513 and Dnase I was used to cast six cement-sand mortars with dimensions of 50.8 mm × 50.8 mm × 50.8 mm. TYG treatment containing no cells and TYG with incorporated Dnase I were used as a control. The mortars were demolded after 48 h and were cured in 1 l of distilled water (DW) at 30 °C. The DW was changed every 10 days. After 7 and 28 days, the cement-sand cubes were completely air-dried at 25 °C and the compressive strength of the specimens were measured by a universal test machine (Shimadzu Co., Japan) [16, 17].

Field Emission Scanning Electron Microscopy (FE-SEM)
The morphology of bacterial biofilm in the cement-sand specimens was examined by FE-SEM using S-4300 and EDX-350 microscopes (Hitachi Co., Japan). Test samples were gathered and suitably clashed after compressive strength test.

Nucleotide Sequence Accession Numbers
The GenBank accession numbers for the sequences of the rRNA genes cloned in this study are JN195783 to JN195792.

RESULTS AND DISCUSSION

The idea that prompted this study was that bacterial biofilm could be used as a binder for the improvement of compressive strength in cement-sand mortar. We hypothesized that alkaliphilic bacteria would be useful for this purpose as they would endure the high internal pH (>12) of the cement. The mature biofilm could cover the mineral particles in the cement construction structure, and so the strengthened deposition of the biofilm could induce improved strength of the cement [10].

Test strains were isolated from cement tetrapod blocks, an extremely alkalophilic and halophilic environment in the West Sea of Korea. Twelve strains belonging to 7 genera were identified using MB amended with 8% NaCl and the pH ranges were determined. In the result, the strains required a relatively high concentration of NaCl and pH level for cell growth (Table 1). Among them, the KNUC502, 504, 506, 508, 509, 510, and 513 strains could grow at pH 12 (Table 1). These strains could be more efficient as an organic material in the cement environment to increase the durability of the cement material. To quantify bacterial biofilm formation, the microtiter-plate test [19] was carried out. Fig. 1 summarizes the biofilm assay results of the identified strains. KNUC513 showed significantly higher biofilm formation than S. pasteurii and TYG treatment (Fig. 2). KNUC513 was selected for further study.

Compressive strength was increased by the addition of biofilm-forming bacteria (Fig. 2). In the experiment, S. pasteurii and KNUC403, which are calcite-forming bacteria

**Fig. 1.** Quantification assay of biofilm formation of identified strains isolated from cement tetrapod blocks. Biofilm formation was determined using a microtiter plate reader at 580 nm.
and which have been suggested as sealing and self-healing agents in construction materials [1, 3, 15], were used to compare the effect of strength improvement of cement-sand mortar. KNUC513 showed the greatest increase in strength (20.3%) compared with other S. pasteurii and KNUC403 (increase of 5.4% and 8.8%, respectively) (Fig. 2). The results are consistent with the view that strength is increased by the plugging of pours in the cement-sand mortar by the calcite crystals formed at biofilm nucleation sites [6, 10, 15, 16]. However, KNUC513 did not produce calcium carbonate when cultured in urea-CaCl$_2$ medium (Fig. 3). These results indicate that the biofilm formed by bacteria could serve as potential factors and can be more efficient compared with calcite formation for improvement of strength in cement-sand material [1]. We then investigated the effect of the inhibition of KNUC513 biofilm by Dnase I, which has been used as a negative control to verify the effect of biofilms on strength improvement [23]. Samples treated with 400 U/ml of Dnase I produced the least biofilm (Fig. 4). This concentration was used for further strength tests.

In the compressive strength tests, cement-sand mortar containing KNUC513 without Dnase I produced greater strength improvement than samples treated with KNUC513 and Dnase I (Fig. 5). Microscopy examination revealed that mature KNUC513 biofilm widely covered the cement-sand matrix (Fig. 6D), obliterating the particle morphology (Fig. 6A). In the presence of Dnase I, KNUC513 did not grow as biofilms (Fig. 6C).

The results indicate that cement-sand mortar may be strengthened by mature biofilms formed by E. marinum KNUC513. The deposition of biofilm substances and cell structures in the cement matrix could increase the compressive strength of cement-sand material [9]. We conclude that bacterial biofilm can be used as a binder to improve the durability of cement material.

Fig. 2. Compressive strength induced by bacteria. The microorganisms were suspended with PBS and mixed with cement-sand materials.

Fig. 3. Microbiologically induced CaCO$_3$ precipitation. Strains were inoculated on urea-CaCl$_2$ plates for CaCO$_3$ precipitation. Calcium crystals were observed around the colonies of S. pasteurii (A) and A. crystallopoietes KNUC403 (B). However, E. marinum KNUC513 did not show any calcium carbonate precipitation (D). Escherichia coli K-12 was used as negative control (C).

Fig. 4. Inhibition of biofilm formation of E. marinum KNUC513. Each point represents the average of six assays. Abbreviations: PBS, phosphate buffered saline; KNUC403, A. crystallopoietes.

Fig. 5. Compressive strength improvement of cement-sand mortar containing calcite and biofilm-forming bacteria. Dnase I was used for the inhibition of KNUC513 biofilm in cement-sand mortar. A, DW treatment; B, DW + Dnase I; C, DW + Dnase I + KNUC513; D, DW + KNUC513.
Panels A–D show results of DW treatment, DW + Dnase I, DW + Dnase I + KNUC513, and DW + KNUC513, respectively.

Fig. 6. FE-SEM images of cement-sand mortar containing Dnase I and KNUC513.

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