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Non-ureolytic calcium carbonate precipitation by *Lysinibacillus* sp. YS11 isolated from the rhizosphere of *Miscanthus sacchariflorus*

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Although microbially induced calcium carbonate precipitation (MICP) through ureolysis has been widely studied in environmental engineering fields, urea utilization might cause environmental problems as a result of ammonia and nitrate production. In this study, many non-ureolytic calcium carbonate-precipitating bacteria that induced an alkaline environment were isolated from the rhizosphere of *Miscanthus sacchariflorus* near an artificial stream and their ability to precipitate calcium carbonate minerals with the absence of urea was investigated. MICP was observed using a phase-contrast microscope and ion-selective electrode. Only *Lysinibacillus* sp. YS11 showed MICP in aerobic conditions. Energy dispersive X-ray spectrometry and X-ray diffraction confirmed the presence of calcium carbonate. Field emission scanning electron microscopy analysis indicated the formation of morphologically distinct minerals around cells under these conditions. Monitoring of bacterial growth, pH changes, and Ca^{2+} concentrations under aerobic, hypoxia, and anaerobic conditions suggested that strain YS11 could induce alkaline conditions up to a pH of 8.9 and utilize 95% of free Ca^{2+} only under aerobic conditions. Unusual Ca^{2+} binding and its release from cells were observed under hypoxia conditions. Biofilm and extracellular polymeric substances (EPS) formation were enhanced during MICP. Strain YS11 has resistance at high pH and in high salt concentrations, as well as its spore-forming ability, which supports its potential application for self-healing concrete.

Keywords: *Lysinibacillus* sp. YS11, MICP, aeration, urea, aerobic MICP, X-ray diffraction

Introduction

Microbially induced calcium carbonate precipitation (MICP) is an omnipresent process that has contributed to the Earth's geochemical processes since the Archean, shaping the minerals on Earth's surface and participating in global element cycling (Ehrlich, 2002; Falkowski *et al.*, 2008; Hazen *et al.*, 2008). Physiological activities of microorganisms are known to induce carbonate precipitation in the form of calcium carbonate ($CaCO_3$) by altering the parameters involved in mineralization such as pH, concentration of calcium ions, and dissolved inorganic carbon (DIC) (Hammes and Verstraete, 2002). Many ubiquitous bacterial species are capable of carbonate precipitation (Boquet *et al.*, 1973). Various metabolic pathways can produce adequate conditions for carbonate precipitations; such activities include ureolysis, photosynthesis, ammonification, denitrification, sulfate reduction, and methane oxidation (Fujita *et al.*, 2000; Rodriguez-Navarro *et al.*, 2003; Dupraz *et al.*, 2004; Braissant *et al.*, 2007; Reeburgh, 2007; Van Paassen *et al.*, 2010; Zhu and Dittrich, 2016). Aggregation of calcium carbonate precipitates is known to be triggered with the help of components of the microbial cell structure, such as bound extracellular polymeric substances (EPS) or the cell wall itself, which provides nucleation sites (Dupraz *et al.*, 2009), facilitates the absorption of calcium ions, and accelerates MICP by decreasing the activation energy needed (Warren and Ferris, 1997; Warren *et al.*, 2001).

To date, MICP via bacterial ureolysis has been demonstrated extensively at laboratory scale. Urea efficiently induces MICP because it promotes bacterial growth and increases pH with nitrogen, bicarbonate and hydroxides, respectively (Dhami *et al.*, 2013). In fact, many MICP-inducing bacteria are known to produce ureases and are almost ubiquitous in soils (Dhami *et al.*, 2013). Owing to the convenience of their production and their accessibility, calcium carbonate precipitates formed via ureolysis have been deliberately considered for application in environmental engineering fields, including as a plugging and cementing agent and in metal remediation (Ferris *et al.*, 1997; Gadd, 2010; Dhami *et al.*, 2013; Wang *et al.*, 2016). In particular, its ability to plug porous substances has been investigated widely for use in self-healing concrete for the repair of micro-cracks (Hammes *et al.*, 2003; Silva *et al.*, 2015; Zhu and Dittrich, 2016). Despite these advantages, the production of unwanted ammonia from urea hydrolysis could result in unsustainable mechanical properties of building structures and toxicity problems (Dhami *et al.*, 2013; Zhu and Dittrich, 2016).

In this study, we provide new insights in MICP by introducing the non-ureolytic bacterial strain *Lysinibacillus* sp. YS11, isolated from the rhizosphere. Its non-ureolytic MICP was analyzed by field-emission scanning electron microscopy (FE-SEM), energy dispersive X-ray spectrometry (EDS), and X-ray diffraction (XRD). In-depth examination of the MICP process under different degree of aeration via successive monitoring of growth rate, pH changes, and calcium utilization

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confirmed, quantitatively, the MICP capacity of strain YS11. Aerobic MICP with alkali- and halotolerance, as well as the spore-forming ability and anaerobic growth of strain YS11, show the potential for this strain to be applied in self-healing concrete.

Materials and Methods

Isolation and culture conditions of non-ureolytic calcium carbonate-precipitating bacteria

Environmental samples were collected from the rhizosphere of *Miscanthus sacchariflorus* near an artificial stream (37°34′44.9″N 127°01′28.8″E) and limestone area (37°03′23.87″N 128°07′05.28″E) to isolate calcium carbonate-precipitating bacteria. 0.1 g of soil sample was washed with phosphate-buffered saline (PBS, pH 7.5) and spread onto three different agar media: Luria-Bertani (LB) agar, Tryptic Soy (TSB) agar, and Tryptone Yeast Glucose (TYG) agar. After overnight culture at 30°C, colonies were isolated and streaked onto biomineralization-inducing B4 medium (Boquet *et al*., 1973; Barabesi *et al*., 2007) to screen for possible calcium carbonate-precipitating bacteria.

The surfaces of limestone samples were gently sterilized with 70% alcohol to remove surface bacteria. Then, the limestone was aseptically hammered to obtain 0.1 g and 1 g mass of finely ground limestone sample. All samples were washed with PBS prior to incubation in the designated media. The samples (0.1 g) were then spread onto LB and mineral salt (Atlas, 2005) agar plates containing pyruvate to decrease the impact of oxidative stress on bacteria (Kim *et al*., 2016b). After heating at 60°C for 6 h, the 1 g samples were cultured in LB supplemented with 3% (w/v) NaCl (pH 9) to screen for heat-, alkali-, and halotolerant calcium carbonate-precipitating bacteria. The agar plates were incubated at 30°C for 5 days, and colonies that grew in these media were isolated. Thirteen colonies were isolated from the soil and named strains YS01 to YS13, and seven colonies isolated from the limestone samples were named strains YL01 to YL07.

Examination of MICP by bacterial isolates

The colonies isolated from the environment were incubated overnight in LB broth at 30°C prior to an examination for MICP. The isolates were inoculated into 48-well microtiter plates (BD Biosciences) containing B4 medium composed of 0.25% calcium-acetate, 0.4% yeast extract, and 0.5% glucose. After incubation at 30°C for 5 days, bromothymol blue was added to each well to determine whether bacteria could generate alkaline conditions. The experiment was carried out in duplicate, and *Escherichia coli* K-12 MG1655 was used as MICP negative control, respectively. Crystal formation by strains that were capable of inducing a high pH was observed using an Imager A1 phase-contrast microscope (Zeiss).

Phylogenetic analysis of the 16S rRNA gene sequence

The 16S rRNA gene of verified calcium carbonate-precipitating strain YS11 was amplified using the universal bacterial primers 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). Polymerase chain reaction (PCR) was performed with the following cycling conditions: 94°C for 90 sec, followed by 25 cycles at 94, 58, and 72°C for 45 sec each, and then a final extension step at 72°C for 5 min. Sequence similarity was measured using EzTaxon (Chun *et al*., 2007). A neighbor-joining tree was drawn based on a distance matrix calculated in MEGA 7. Bootstrapping was performed with 1,000 iterations. The 16S rRNA gene sequence of strain YS11 was deposited in the GenBank database under accession number KY575121. A strain YS11 culture was deposited in the Korean Agricultural Culture Collection (KACC) under number KACC 19227.

Growth, pH change, and calcium utilization monitoring for testing non-ureolytic MICP under aerobic, hypoxia, and anaerobic conditions

To provide aerobic, hypoxia, and anaerobic conditions for the examination of non-ureolytic MICP, 10^6 CFU/ml of an overnight culture in LB broth was inoculated into B4 medium. Cultures in B4 medium were incubated at 30°C for 5 days to observe bacterial MICP. Aerobic conditions were created by vigorous agitation at 220 rpm. For hypoxia conditions, the culture medium was covered with paraffin oil to create low oxygen penetration just after the calcium carbonate-precipitating strains were inoculated. Anaerobic conditions were created using vials full of broth which was crimped by closed top cap. An obligate aerobic *Pseudomonas putida* KT2440 was also cultured under anaerobic conditions as a negative control.

The growth rate of strain YS11 was determined by counting the colony-forming units (CFU) under aerobic, hypoxia, and anaerobic conditions. In addition, pH changes and the calcium ion utilization rate were monitored periodically using a pH electrode (Thermo Fisher Scientific) and a calcium-ion selective electrode (ISE) (Thermo Fisher Scientific). This experiment was conducted in triplicate.

FE-SEM, EDS, and XRD analyses of MICP

The strain YS11 was incubated in B4 medium under both aerobic and hypoxia conditions prior to FE-SEM and EDS analyses. The cells were first fixed with low-strength Karnovsky's solution (2% paraformaldehyde, 2.5% glutaraldehyde, and 0.1 M phosphate buffer, with a final pH of 7.2) for 2 h, and additionally fixed with 2% osmium tetroxide solution for 2 h. The fixed samples were gradually dehydrated with ethanol (30, 50, 70, 100%) for 10 min at each gradient and placed onto an aluminum stub for 4 days to be dried. The samples were coated with platinum and analyzed using a Quanta 250 FEG field-emission scanning electron microscope (FEI) and EDS mapping. The lyophilized powder of precipitated calcium carbonate-cell composites from MICP observed aerobic cultures was examined by XRD for mineral identification using Dmax2500/PC (Rigaku).

Examination of alkali- and halotolerance, urease activity, and endospore formation

Growth under alkali and saline conditions was measured at various pH (6–12) and NaCl concentrations (0–7%) in LB broth at ambient temperature, using a microplate absorb-

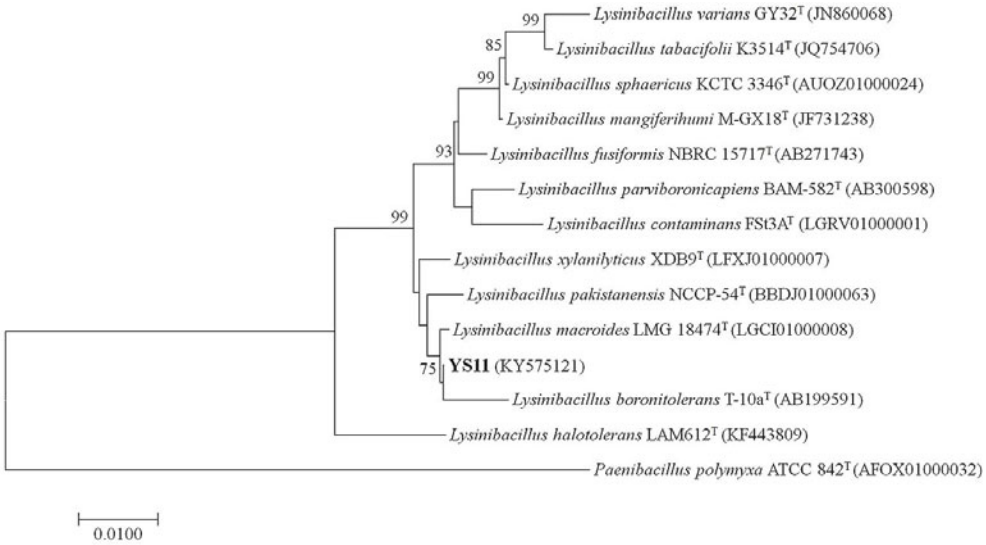

Fig. 1. A neighbor-joining tree based on the 16S rRNA gene sequence of the bacterial strain (YS11) capable of calcium carbonate precipitation. The strain is shown in bold. Node numbers indicate bootstrap values based on 1,000 replicates. Accession numbers are provided in brackets.

ance reader (Tecan). Urease activities of strain YS11 together with urease negative control *Escherichia coli* K-12 MG-1655 and urease positive control *Bacillus* sp. JH7 (Kim *et al.*, 2016a) were tested using phenol-hypochlorite method, diluted overnight samples (20 µl) were mixed with 80 µl of phenol nitroprusside and alkaline hypochlorite solutions and incubated at 37°C for 20 min in the dark and optical density at 640 nm was measured. Standard curve was constructed with different concentrations of NH_4Cl. To detect spore formation by strain YS11, cells were cultured in B4 medium for 10 days and the induced spores were observed under a phase-contrast microscope (Zeiss).

Biofilm and EPS formation assays

An aliquot containing 10^6 CFU/ml of strain YS11 was washed twice with PBS and inoculated into 48-well microtiter plates containing B4 medium. Microtiter plates were incubated at 30°C for 24 h in static conditions. By the crystal violet staining method, biofilms were quantified by measuring the optical density at 595 nm (OD_{595}) using a multi-detection microplate reader (HIDEX Sense), and these values were normalized by the cell density (CFU). EPS was quantified using the Congo red (CR) staining method. Briefly, 1 ml of strain YS11 incubated in B4 medium was treated with 3.5 mg/L of CR for 30 min at 30°C. Then, the cells were centrifuged (10 min, 10,000 × *g*) to clarify the supernatant for measurement at A_{480}. Based on the standard curve obtained, the EPS quantity was calculated as described in a previous report (Kim *et al.*, 2016a).

Results

Isolation and identification of non-ureolytic calcium carbonate-precipitating bacteria

Bacterial isolates were tested for their ability to promote an alkaline environment under biomineralization-inducing conditions to screen for non-ureolytic calcium carbonate-precipitating bacteria. B4 medium culture was used to induce non-ureolytic MICP conditions. After incubation, bromothymol blue was dropped into cell cultures as a pH indicator for screening. Only strain YS11, isolated from the rhizosphere of *Miscanthus sacchariflorus*, showed an increase in pH. Crystal formation on cell surfaces was also observed. The phylogenetic analysis of strain YS11 was evaluated based on the 16S rRNA gene sequences (Fig. 1). Strain YS11 was phylogenetically affiliated with the genus *Lysinibacillus*, and its nucleotide sequence showed the highest similarity (99.64%) with that of *Lysinibacillus macroides* LMG 18474^T. Strain YS11 also shared high similarity with *Lysinibacillus fusiformis* NBRC 15717^T (98.77%) and *Lysinibacillus sphaericus* KCTC 3346^T (98.41%). Different strains of these species have been reported to be calcium carbonate-precipitating bacteria (*L. fusiformis* KNUC404 and *L. sphaericus* INQCS 414) that tolerate up to 7% NaCl and grow at pH 6.0–9.5 (Priest *et al.*, 1988; Ahmed *et al.*, 2007; Park *et al.*, 2010; Wan *et al.*, 2010; Shirakawa *et al.*, 2011). Along with phylogenetically similar strains that are capable of MICP and are alkaline- and halotolerant, strain YS11 was found to be a calcium carbonate-precipitating bacterium.

pH alteration and calcium utilization during biomineralization

Growth, pH changes, and calcium ion utilization were analyzed under varying oxygen concentrations to verify whether strain YS11 could also precipitate calcium carbonate under oxygen deprived conditions (Fig. 2). MICP only occurred in aerobic conditions where the patterns of growth, pH, and unbound calcium ion concentrations showed tight correlations with one another (Fig. 2A) as the values changed significantly during the shift from the exponential to stationary growth phase of strain YS11 between 12 and 24 h. The pH of the culture increased along with bacterial growth and the unbound calcium ion concentration started to decrease from the entrance of stationary phase. Strain YS11 entered the stationary phase at 12 h, and its maximum cell density was 1.78×10^8 CFU/ml ($\pm 0.44 \times 10^8$ CFU/ml). The concentration of unbound calcium ions gradually increased from 0 h to its

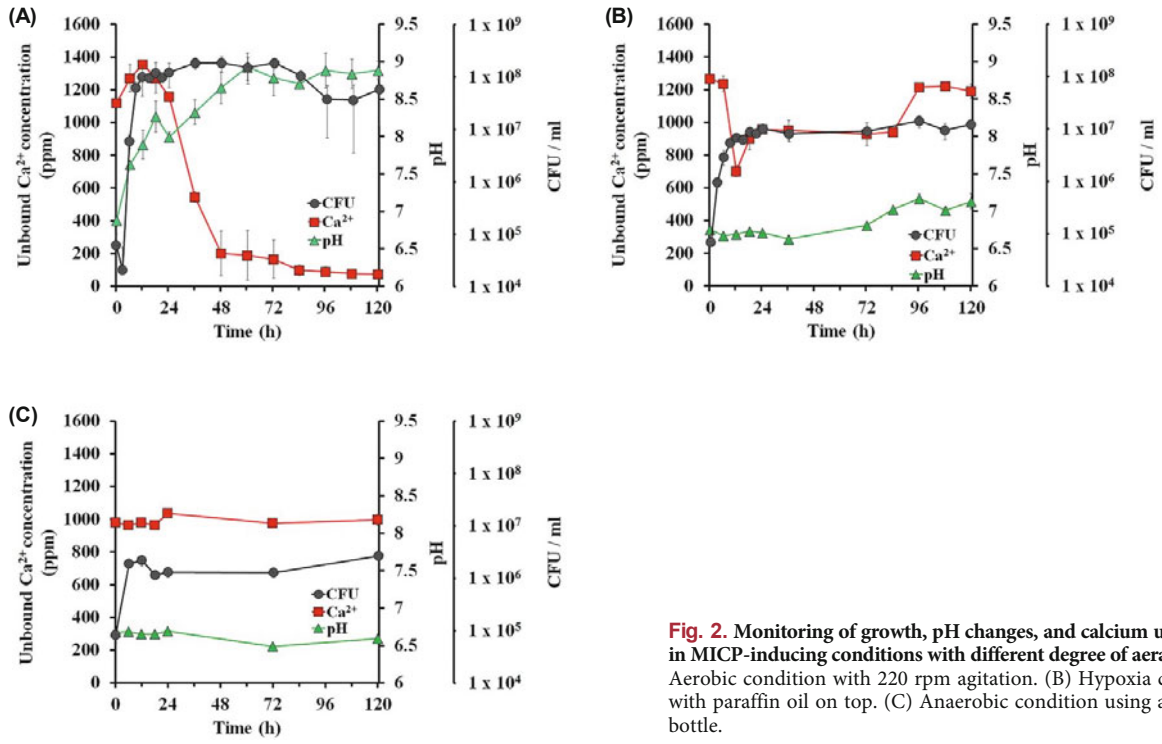

Fig. 2. Monitoring of growth, pH changes, and calcium utilization in MICP-inducing conditions with different degree of aeration. (A) Aerobic condition with 220 rpm agitation. (B) Hypoxia condition with paraffin oil on top. (C) Anaerobic condition using anaerobic bottle.

peak at 12 h, probably because of the release of calcium ions at the initial attachment to negatively charged cell walls. Subsequently, the culture reached the stationary phase, and the unbound calcium concentration showed a dramatic decrease between 12 and 48 h, which was then followed by a slow, gradual decrease to 120 h. The efficiency of calcium ion consumption by strain YS11 was approximately 95% (1280.85 ppm), as the final concentration of calcium ions in the medium was 74.15 ppm (± 21.57 ppm). The pH of the medium increased to pH 8.9 at 120 h of incubation, and no considerable pH change occurred after 48 h. However, a slight decrease in pH was observed from 18 to 24 h of incubation at pH 8.

In hypoxia conditions (Fig. 2B), the growth rate of strain YS11 was congruous with that in the aerobic B4 medium culture, reaching the stationary phase at 12 h. However, maximal growth was 1.42×10^7 CFU/ml (± 0.38 $\times 10^7$ CFU/ml) in hypoxia conditions which is substantially less than that in aerobic conditions (1.78×10^8 CFU/ml). The initial calcium ion concentration decreased, however, release of bound calcium ions from cell walls was detected beginning at 12 h after the cells reached the stationary phase. The calcium concentration surged from then with a final concentration of 1190 ppm (± 43.59 ppm). The pH value also differed from that in aerobic conditions, where the pH showed only minor increase from pH 6.8 to pH 7.1. In anaerobic conditions (Fig. 2C), the maximal growth was counted as 2.65×10^6 CFU/ml (± 0.35 $\times 10^6$ CFU/ml) which was the smallest value above all conditions. The pH did not increase, but stayed around pH 6.6 to 6.7 and unbound calcium concentration was consistent with the initial concentration. Inability of *Pseudomonas putida* KT2440 to grow under the same condition proved that desired anaerobic conditions were provided (data not shown).

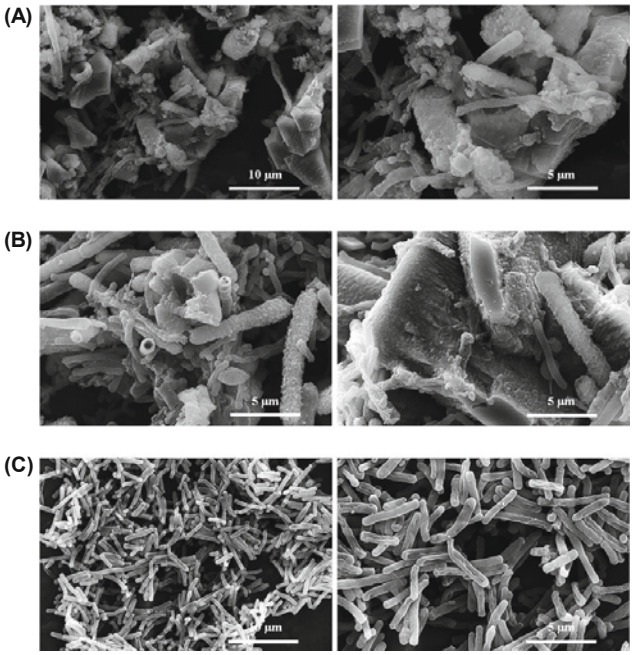

Fig. 3. Field emission scanning electron microscopy (FE-SEM) of strain YS11 in non-ureolytic biomineralization conditions after 5 days of incubation in B4 medium. (A) Microbially induced calcium carbonate precipitation (MICP) of strain YS11 in aerobic non-ureolytic condition (5,000 × and 10,000 × magnification). (B) Images of other encrusted cells under aerobic non-ureolytic conditions (10,000 × magnification). (C) FE-SEM (5,000 × and 10,000 × magnification) shows no calcium carbonate mineral formation under hypoxia conditions.

Non-ureolytic MICP of strain YS11 in aerobic conditions

The aerobic and hypoxia B4 medium cultures of strain YS11 were analyzed by FE-SEM for the observation of precipitated minerals. Anaerobic cultures were excluded because the absence of MICP was obvious from the monitoring experiments. The minerals of strain YS11 were precipitated under aerobic conditions whereas only cells were observed under hypoxia conditions (Fig. 3A and C). In aerobic conditions with B4 medium, minerals were formed as bacteria-calcium carbonate precipitate clusters. The MICP induced by strain YS11 resulted in two different forms of mineral aggregates; some had sharp calcite-like edges, whereas others had an irregular amorphous vaterite-like shape (Fig. 3A). In addition, the surface of some individual cells of strain YS11 were fully encrusted with minerals (Fig. 3B). Minerals precipitated in aerobic conditions were verified to be calcium carbonates, as the EDS analysis indicated the presence of calcium, carbon, and oxygen (Fig. 4) and this mineral was confirmed to be calcium carbonate by XRD (Fig. 5). Thus, strain YS11 was able to precipitate calcium carbonate under aerobic conditions without urea hydrolysis.

Physiological characteristics of non-ureolytic calcium carbonate-precipitating strain YS11

The ability to endure alkalinity, a high salt concentration, and extreme temperatures is crucial for applying calcium carbonate-precipitating bacteria to concrete, because the manufacturing process and interior of concrete are harsh conditions (Dhami *et al.*, 2013; Phillips *et al.*, 2013; Wang *et al.*, 2016). Strain YS11 was capable of growing in an alkaline medium up to a pH of 11 and in salt (NaCl) concentrations above 6% (Fig. 6A), although the growth rate and lag phase were slower and longer, respectively, as the medium was adjusted to relatively alkaline or halo-environments. In addition, the similar maximum optical density was achieved at alkaline pH range from pH 6 to 9 (OD_{600} ~ 1.1), whereas various maximum OD_{600} was observed with different salinity.

To test surface chemical properties and attachment ability, EPS and biofilm formation analyses were conducted using Congo red and crystal violet, respectively. EPS and biofilm production were higher in both aerobic and hypoxia MICP-inducing conditions (Fig. 6B and C). Interestingly, however, both EPS and biofilm formation increased when calcium was provided in the medium. This suggests that EPS and biofilm formation are altered during the MICP process of strain YS11. Since bacterial species belonging to the genus *Lysinibacillus* are known to form endospores (Ahmed *et al.*, 2007), the induced endospores of strain YS11 were examined by phase-contrast microscopy. Strain YS11 produced central endospores after 3 days of incubation and readily formed seed-like spores in 5 days (data not shown). The non-ureolytic calcium carbonate-precipitating *Lysinibacillus* sp. YS11 bacterium showed both alkali- and halotolerance, with growth in alkaline (pH 11) and high salt (above 6% NaCl) conditions. Strain YS11 also formed spores and showed higher EPS and biofilm production under aerobic and hypoxia MICP conditions. Also, the urease activity of strain YS11 was compared with that of urease positive *Bacillus* sp. JH7 which indicated low level of urea degradation by strain YS11 similar to that of urease negative *Escherichia coli* K-12 MG1655 (Fig. 6D) (Kim *et al.*, 2016a).

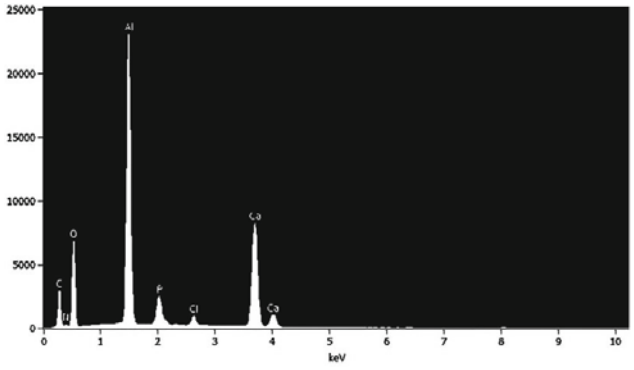

Fig. 4. Energy dispersive X-ray spectrometry (EDS) of strain YS11 in aerobic non-ureolytic biomineralization conditions. Calcium peaks indicate the presence of calcium in a mineral form as a result of MICP by strain YS11.

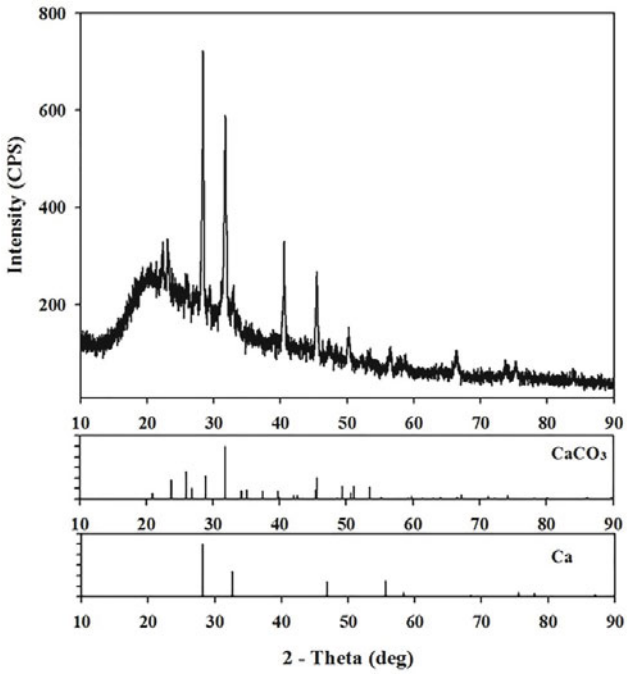

Fig. 5. X-ray diffraction (XRD) of strain YS11-$CaCO_3$ composites in aerobic non-ureolytic biomineralization conditions. Several peaks verify the formation of calcium carbonates.

Discussion

Recent studies of MICP have focused on a single mechanism or metabolic pathway of a single strain (Zhu and Dittrich, 2016). In contrast, our data tried to show that microorganisms could induce mineralization through processes other than urea hydrolysis and its capability in MICP depends on a sufficient aeration. Microorganisms alter their natural microenvironments during growth and development (Riding, 2000; Zhu and Dittrich, 2016), and the requirements for

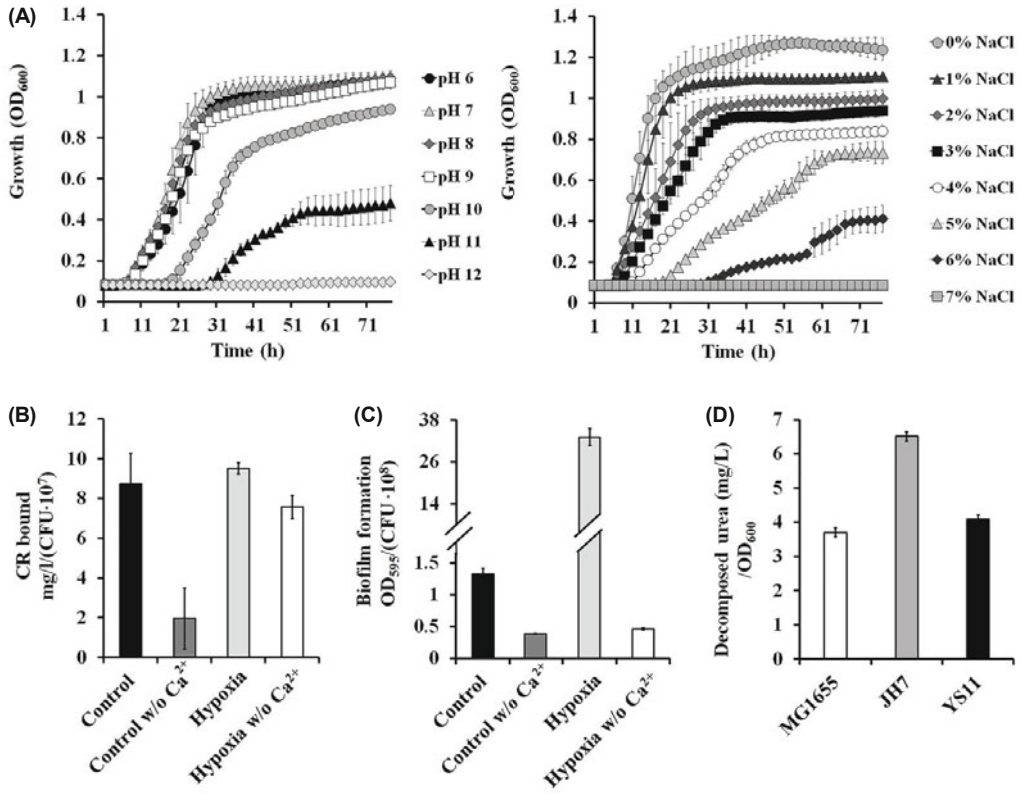

Fig. 6. Physiological characterization of non-ureolytic calcium carbonate-precipitating strain YS11. (A) Growth in alkaline and high salt conditions. (B) Extracellular polymeric substances (EPS) and biofilm formation with/without the presence of calcium in microbially induced calcium carbonate precipitation (MICP)-inducing media under aerobic and hypoxia conditions. (C) Urease activities of *Escherichia coli* K-12 MG1655 (urease-negative), *Bacillus* sp. JH7 (urease-positive), and *Lysinibacillus* sp. YS11.

MICP may be met when the surrounding pH and DIC are increased with calcium ions (Hammes and Verstraete, 2002). Owing to their shared carbonate-forming properties, MICP, especially by bacteria, have been applied for the last decade to environmental engineering of self-healing concrete, metal remediation, and soil strengthening materials (Dhami *et al*., 2013; Phillips *et al.,* 2013). MICP by ureolysis have been vigorously studied because of its ease of use in examinations and applications (Dhami *et al*., 2013; Wang *et al*., 2016). However, urea is not ubiquitous in natural environments; thus, ureolysis alone is insufficient to understand the versatility of calcium carbonate-precipitating bacteria in nature (Zhu and Dittrich, 2016). In addition, when applied to concrete structures, ureolytic calcium carbonate-precipitating bacteria produce the unwanted by-product ammonia (Dhami *et al*., 2013; Zhu and Dittrich, 2016). For this reason, our study aimed to identify a novel model bacterium for non-ureolytic MICP. Here, we suggest *Lysinibacillus* sp. YS11 as that model, because it demonstrates a non-ureolytic MICP mechanism that is dependent on aerobic conditions.

Soil and limestone environments have shared chemical properties because they hold calcium ions in the form of minerals. Owing to the diverse microenvironments of soils and the calcium carbonate rich limestone area, two types of environmental samples were obtained from these sources in an attempt to isolate calcium carbonate-precipitating bacteria that used pathways other than urea hydrolysis for MICP: soil from the rhizosphere of *Miscanthus sacchariflorus* near an artificial stream in Seoul and limestone from Wonju. As a result, strain YS11 was isolated from the rhizosphere of *Miscanthus sacchariflorus* near an artificial stream. Its MICP formed morphologically distinct minerals. Calcium carbonates formed in aerobic non-ureolytic conditions showed a calcite-like shape made of layers of calcium carbonate, indicating that the crystals enlarge by stacking of micro-calcium carbonates on top of the initially formed calcium carbonate. This suggests that the time and protein-specific development of calcium carbonates may be influenced by EPS (Braissant *et al*., 2003; Ercole *et al*., 2007). In addition, the remains of microorganisms as fossils showed the full encrustation of individual strain YS11 cells with calcium carbonates, as if whole cells were crystals. Aeration is known to be one of the main influential parameters in biomineralization of calcium carbonates (Seifan *et al*., 2017). The precipitation of calcium carbonates did not occur in our hypoxia and anaerobic experimental conditions, which supports the importance of aeration in MICP. However, the benefits and impacts of precipitated carbonates on bacteria, and specifically the communication of microbes with minerals, is far from clear (Farhangi *et al*., 2013).

The bacterial cells seemed to interact with calcium during the exponential growth phase by binding positively charged calcium ions on net negative charged cell walls and then gradually releasing them (Thomas *et al*., 2014; Mishra, 2015).

The initial concentration of unbound calcium steadily increased through the exponential phase and then rapidly diminished as cells entered the stationary phase (Fig. 2A). Full-scale MICP appeared to begin as strain YS11 reached its stationary phase, when the cell density was maintained by equal growth and death rates. Interestingly, there was more biofilm and EPS formation in the presence of calcium ions in MICP-inducing medium, suggesting that calcium ions might alter bacterial surface properties and EPS-mediated bacteria-calcium carbonate composites. What factors allow strain YS11 to induce MICP? Although determining the exact metabolic activity that drives MICP will require further examination, the pH in the aerobic non-ureolytic culture increased from pH 6.9 to 8.9 over time. One possible explanation is the deamination of amino acids from the yeast extract (YE) that was present. To test whether the changes in pH and calcium concentrations were similar to that in other amino acid-containing media, YE in non-ureolytic medium was replaced by casamino acid, which contained higher levels of amino acids (Nolan, 1971). However, the results showed unconverted values in both parameters, compared to the values at 0 h after inoculation of strain YS11 (data not shown). Thus, the metabolic activity that triggers MICP in strain YS11 in aerobic non-ureolytic conditions remains to be investigated.

The majority of studies on calcium carbonate-precipitating bacteria applied to self-healing materials have not examined whether the bacteria could survive in the harsh environments of building structures such as concrete. The general conditions inside of concrete possess high alkalinity, with a pH as high as 12, a high concentration of mineral salts, and a minuscule amount of incoming oxygen. For these reasons, strain YS11 was tested for its ability to survive and thrive in alkaline and high salt environments. Strain YS11 showed growth development up to a pH of 11 and at salt concentrations above 6%. Although there was no sign of growth above these limits, strain YS11 may survive in viable but non-culturable (VBNC) state or through spore formation; indeed, strain YS11 could form spore after long starvation. The influx of water and nutrients in an aqueous state can neutralize the pH and nourish the spores, promoting germination. Induction of EPS by Ca^{2+} appeared to promote biofilm formation on hydrophobic surfaces, which might change the MICP rate under aerobic conditions. Further detailed mechanisms for understanding the role of EPS involved in calcium carbonate formation remain to be established. In addition, because strain YS11 is capable of hypoxia and anaerobic growth, the chance that strain YS11 would survive as a crack-healing agent inside of concrete where low concentrations of oxygen are expected seems high. Thus, results of this study suggest the potential for application of strain YS11.

Acknowledgements

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Author Contributions

Y.S.L and W.P. designed the study. Y.S.L. performed all experiments and analyzed the data. Y.S.L., H.J.K., and W.P. drafted the manuscript. Y.S.L., H.J.K., and W.P. participated substantially in discussions and manuscript modifications. All authors contributed to and approved the final version of this manuscript.

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