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Mg^{2+}/Ca^{2+} promotes the adhesion of marine bacteria and algae and enhances following biofilm formation in artificial seawater

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ABSTRACT

Adhesion of microorganisms in the marine environment is essential for initiation and following development of biofouling. A variety of factors play roles in regulating the adhesion. Here we report the influence of Ca^{2+} and Mg^{2+} in artificial seawater on attachment and colonization of *Bacillus* sp., *Chlorella* and *Phaeodactylum tricornutum* on silicon wafer. Extra addition of the typical divalent cations in culturing solution gives rise to significantly enhanced adhesion of the microorganisms. Mg^{2+} and Ca^{2+} affect the adhesion of *Bacillus* sp. presumably by regulating aggregation and formation of extracellular polymeric substances (EPS). The ions alter quantity and types of the proteins in EPS, in turn affecting subsequent adhesion. However, it is noted that Mg^{2+} promotes adhesion of *Chlorella* likely by regulating EPS formation and polysaccharide synthesis. Ca^{2+} plays an important role in protein expression to enhance the adhesion of *Chlorella*. For *Phaeodactylum tricornutum*, Ca^{2+} expedites protein synthesis for enhanced adhesion. The results shed some light on effective ways of utilizing divalent cations to mediate formation of biofilms on the marine structures for desired performances.

1. Introduction

It is virtually known that biofouling of marine organisms on submerged infrastructures is an intricate problem that has caused a series of detrimental consequences [1–3]. Extensive efforts have long been made towards understanding the biofouling for possible prevention [4,5]. Biofouling is usually originated and deteriorated by formation of biofilm on the surfaces of marine structures, while biofilm formation is a complex process influenced crucially by initial adhesion of marine bacteria and diatoms [6]. In this regard, to constrain development of biofilm, it is essential to impede the adhesion in the first place. Yet, control and eradication of the adhesion and following colonization of microorganisms persists as a worldwide problem for varieties of factors, for example characteristics of the species, physiochemical properties of target materials [7,8], and environment variables like ambient temperature, microorganism concentration, time period of exposure and salinity [9,10], affect the behaviors of the microorganisms. These in turn bring about challenges in the research pertaining to biofilm.

Adhesion of microorganisms is usually divided into two stages, namely reversible physical attachment and irreversible molecule and chemical interaction [9]. Attachment is the initial yet essential stage of adhesion, which is decided by Lifshitz-van der Waals attraction force, Brownian motion, electrostatic and acid-base interactions [11]. Many studies have reported the remarkable role of divalent cations, especially Ca^{2+}, on initial adhesion of marine microorganisms [12,13]. Related research efforts were devoted predominately to conventional physicochemical interactions, such as neutralization of the electrical double layer [14], decreased electrostatic repulsion and enhanced compression of the electrical double layer [15], reduction in electron donicity [13], and so on. In addition, it was recently reported that divalent cations not only affect the physical interaction, but also occupy a special place in formation of microorganism-associated biofilm, and affect the adhesion by chemical interactions [16]. Yet knowledge of the mechanisms about how the divalent cations take part in the development of biofilm is insufficient.

There has been no doubt that Mg^{2+}/Ca^{2+} influences biofilm formation. It is known that biofilm has multilayered structure comprising microorganisms and their hydrated extracellular polymeric substances (EPS), which are composed of polysaccharides, proteins, and DNA [16,17]. Usually bacteria-/algae- secreted lectins and lectin-like proteins or carbohydrates in biofilm are primarily

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responsible for adhesion [9]. In general, Ca^{2+} triggers strong biofilm development and bacterial aggregation by binding with the eDNA of several species of bacteria, for example wildtype *P. aeruginosa* PA14, *A. hydrophila*, *E. coli*, *S. aureus*, *S. epidermidis*, and *E. faecalis* [18]. It was realized that Ca^{2+}/Mg^{2+} increased production of glycoproteins as well as exopolysaccharides and consequently enhanced adhesion of *P. mendocina* NR802 [16]. And Ca^{2+} and Mg^{2+} promote cell growth and cell-to-cell adhesion in biofilms by enhancing structural integrity through electrostatic interactions [17]. However, divalent cations might also have negative impact on biofilm formation. Shukla et al. pointed out that Ca^{2+} inhibited biofilm formation of *S. aureus* and presence of Ca^{2+} changed their architecture [6]. There is so far no further investigation on influence of Mg^{2+} and Ca^{2+} on adhesion of diverse microorganisms and related molecular mechanism is still elusive.

This study examined the role the divalent cations, Mg^{2+} and Ca^{2+}, played in adhesion of several microorganisms, *Bacillus* sp., *Phaeodactylum tricornutum* and *Chlorella*, in artificial seawater (ASW). Addition of the divalent cations significantly promotes adhesion of the microorganisms by regulating and controlling cell-to-cell aggregation, EPS formation, and protein and polysaccharide synthesis respectively. Remarkably different influence of the two ions on adhesion of the microorganisms was realized. Results indicate significant impact of Mg^{2+}/Ca^{2+} on microorganism adhesion and give some inspiration on effective ways of utilizing divalent cations to regulate formation of biofilm on marine structures.

2. Materials and methods

2.1. Preparation of bacterial strain and bacterial adhesion testing

Gram-positive *Bacillus* sp. bacterium (MCCC1A00791, Marine Culture Collection of China) was chosen for the adhesion testing and was cultured in CM 0471-2216E media. *Bacillus* sp. is a typical rod-shaped Gram-positive bacterium, forming biofilm with its abundant secreted EPS to cause biofouling [19–21]. Adhesion testing was conducted in ASW prepared according to ASTM D1141-98. All the reagents and solvents were used as received without any further purification. CM 0471-2216E media were prepared by dissolving 1 g yeast extract, 1 g beef extract, 0.01 g $FePO_4$ and 5 g peptone in 1000 ml sterile ASW. A single bacterial colony was picked from nutrition agar plate to 50 ml sterile CM 0471-2216E media. The sterile media containing the bacterial strain were shaken at 25 °C at 120 rpm for 24 h *Bacillus* sp. was washed with sterile ASW for 3 times through centrifugation with a rotational speed of 2500 rpm for 5 min then re-suspended in sterile ASW. The *Bacillus* sp. suspension with a concentration of 10^7 ml^{-1} was prepared in sterile ASW. Silicon wafers with three specimens for each testing group were put into 24-well plates. Prior to the testing, the wafers were ultrasonically washed with ethanol and subsequent deionized water, and then dried under a flow of dried air at 37 °C. 2 ml of the *Bacillus* sp. suspension was added into each well for soaking in shaker at 25 °C at 120 rpm for 3 days. To study the influence of Mg^{2+} and Ca^{2+} on *Bacillus* sp. adhesion, extra 10 mM of Mg^{2+} or Ca^{2+} were prepared by adding $MgCl_2$ or $CaCl_2$ into the solution contained in each well. Our previous investigation proved that extra addition of the ions in 10 mM already resulted in significant changes in adhesion behaviors of the microorganisms. And this concentration was also found sufficient for the divalent cations to have pronounced impact on bacterial biofilm growth [16]. After the incubation, the samples were washed with ASW for three times to remove the bacteria that did not adhere onto the samples and then fixed by 2.5% glutaraldehyde in ASW. Morphological features of the samples were characterized by field emission scanning electron microscopy (FESEM, FEI Quanta FEG 250, The Netherlands). For FESEM observation, dehydration of the samples was carried out through the critical point drying using 25%, 50%, 75%, 90%, and 100% ethanol solution successively.

2.2. Preparation of algal strains and algal adhesion testing

Marine strains *Chlorella* (Chlorophyta) and *Phaeodactylum tricornutum* (Bacillariophyta) (Ningbo University, China) were used for the adhesion testing. *Phaeodactylum tricornutum* is coastal marine pennate diatom that is usually considered as a model organism [22,23], and it participates in the early stage of biofouling and often dominates the fouling [24]. *Chlorella* was also employed which has spherical shape and is categorized as one of the colonizers on artificial surface in the marine environment [25,26]. *Chlorella* was cultured in enriched filtered sterilized ASW with Guillard's F/2 growth medium, while *Phaeodactylum tricornutum* was cultured in sterilized ASW with silicate-enriched Guillard's F/2 growth medium. The algae were cultured in an incubator with a 12 h: 12 h light/dark cycle at 22 °C. The algae used in the experiments were in the exponential phase of growth.

2 ml algal suspensions (without extra addition of the divalent cations, or with extra 10 mM Mg^{2+} or 10 mM Ca^{2+}) with a concentration of 10^6 ml^{-1} were used for adhesion testing. Silicon wafers with three specimens for each testing group were soaked by algal suspension in shaker (to avoid deposition) for 7 days with a 12 h: 12 h light/dark cycle at 22 °C. After the incubation, the wafers were washed with sterile seawater to remove the algae that did not adhere and then fixed by 2.5% glutaraldehyde in ASW for 2 h. The samples were characterized by confocal laser scanning microscopy (CLSM, Leica TCS SP5, Germany).

2.3. Characterization of bacterial/algal cell-to-cell aggregation

Bacterial/algal aggregation was examined using the already established protocol [27]. Settling ratio was adopted to characterize cell-to-cell aggregation by a Lambda 950 UV/VIS spectrophotometer (PerkinElmer, USA). The bacterial suspensions (without addition of the divalent cations, or with 10 mM Mg^{2+} or 10 mM Ca^{2+}) were mixed uniformly and then 3 ml suspensions were transferred to cuvette for spectrophotometer testing. OD_{600} was continuously measured for 1 min to obtain the settling ratio, which was calculated as the slope of the linear portion of OD_{600} values changed over time [27]. The procedures for assessing algal aggregation were the same as the one used for characterizing the bacterial aggregation. The optical density was measured at 450 nm. All the settling ratios were measured in triplicate.

2.4. Quantification of biofilm

Biofilm formation was assessed by 0.1 wt.% crystal violet binding assay that has been well established [6,16,27,28]. 10 μl *Bacillus* sp. suspension with a concentration of 10^7 ml^{-1} was transferred to pre-sterilized 96-well plate containing 190 μl sterile CM 0471-2216E media. After incubation at 25 °C for 24 h, the solution contained in the 96-well plate was gently removed by pipetting, then rinsed 3 times with sterile ASW and subsequently stained with 0.1% crystal violet for 5 min. The plate was then washed twice with ASW to remove excess crystal violet, and was subsequently air-dried. 95% ethanol solution was used to dissolve the cell bond crystal violet for 30 min. Density of the samples was measured at the wavelength of 570 nm using a microplate reader (SpectraMax 190, Molecular Devices, USA). To form the algal-associated biofilm, 200 μl algae in their respective medium was transferred to 96-well plate and incubated with a 12 h: 12 h light/dark cycle at 22 °C for 7 days. The subsequent steps were the same as the ones used for *Bacillus* sp. To study the effect of Mg^{2+} and Ca^{2+} on biofilm forma-

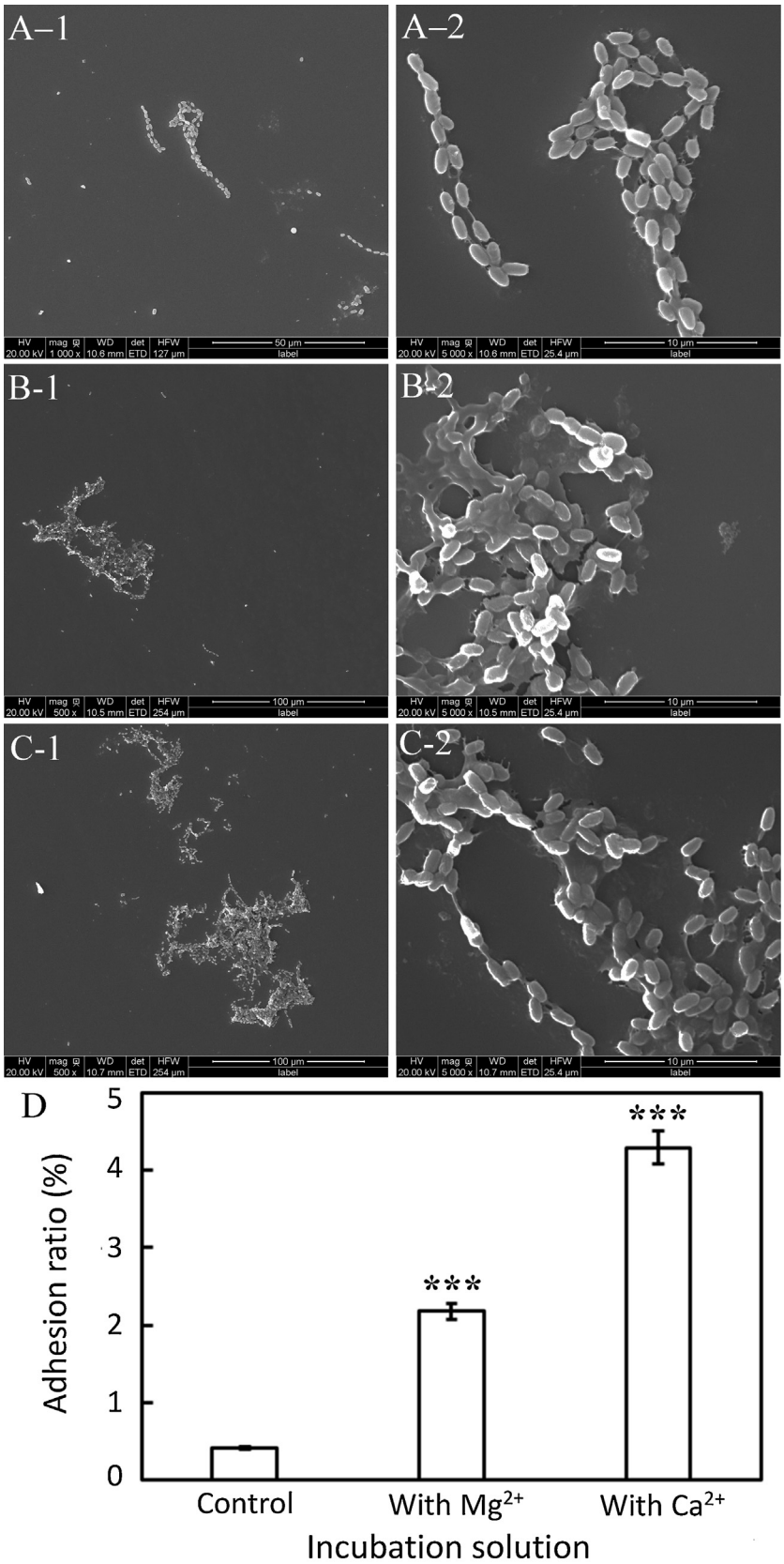

Fig 1. SEM Images of *Bacillus* sp. adhered on silicon wafer without the extra addition (A) and with the extra addition of 10 mM Mg^{2+} (B) and 10 mM of Ca^{2+} (C) in the incubation media (-2 is enlarged view of −1). And (D) statistical analyses of the bacteria adhered on silicon wafer. Error bars are shown as ±SD (n = 10). ***: $p < 0.005$ as compared with the control groups (analyzed by paired Student's *t*-test).

tion, two thirds of the wells contained 10 mM Mg^{2+} or 10 mM Ca^{2+}. All experiments were performed in nonuplicate.

2.5. EPS extraction and quantitative analyses

Bacillus sp. suspensions with concentration of 10^9 ml^{-1} without/with addition of the divalent cations (10 mM Mg^{2+} or 10 mM Ca^{2+}) were put into a shaker at 25 °C for 3 days. The algal suspensions were put into an incubator with a 12 h: 12 h light/dark cycle at 22 °C for 7 days. The EDTA method was employed to extract EPS, which has been proven to be effective [15]. The solution was treated with EDTA to dissociate EPS by disrupting the crosslinking of negatively charged compounds connected by the divalent cations in the EPS. Subsequently, the EPS were detached from cell surface and dissolved into solution by high-speed centrifugation at 13,000*g* for 30 min [15,29]. The supernatant was filtered through 0.22 μm Supor®Membrane.

Content of polysaccharides in the EPS was measured using the phenol-sulfuric acid method [30,31]. Briefly, 1 ml of 6% phenol solution in ultrapure water were added into 2 ml of the supernatant, and immediately 5 ml 98% H_2SO_4 were added. After incubation for 20 min at room temperature, optical density of the samples was acquired at 490 nm wavelength using the microplate reader. Proteins contained in the EPS were examined using a MicroBCA protein assay reagent kit detected at 562 nm wavelength [29,32]. For the testing, the supernatant was transferred to dialysis bag (MWCO: 8 KDa-14 KDa, MD: 44 mm, Beijing Solarbio Technology Co. Ltd., China) for dialysis against 10 mM EDTA solution (pH 8.2) twice and then exhaustively against distilled water. Subsequently, the extracts were concentrated. The protein extracts were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE, Bio-Rad, USA). The protein extracts with loading buffer were boiled for 10 min. 20 μl supernatants were loaded on the gel and run at 70 V in the stacking gel and 120 V in the separating gel. Molecular mass markers (Bio-Rad, USA) were run in the same gel. Protein spots were detected by staining with Coomassie blue.

2.6. Statistical analysis

All of the data were expressed as means ± standard deviation (SD). Statistical analysis of the *Bacillus* sp. adhering on silicon wafers was made from their SEM images. Statistical analysis of the algae adhering on silicon wafers was made by examining their CLSM images by ImageJ software. Standard paired Student's *t*-test was used to determine the differences between control and experiment groups. For statistical analysis data (n = 10), *p*-values less than 0.05 were considered statistically significant while *p*-values less than 0.005 were considered statistically very significant.

3. Results and discussion

It is realized that both Mg^{2+} and Ca^{2+} affect remarkably adhesion of *Bacillus* sp. After 3 days incubation in ASW without the concentrated divalent cations, the silicon wafers already exhibit clear adhesion of *Bacillus* sp. on their surfaces (Fig. 1A). Interestingly, SEM pictures of the samples obviously suggest that the addition of Mg^{2+}/Ca^{2+} in the solution promotes adhesion of the bacteria (Fig. 1B, C). Ca^{2+} triggers more pronounced colonization of *Bacillus* sp. In addition, formation of the bacterial biofilm is clearly seen from enlarged FESEM views (Fig. 1A-2, B-2, C-2). Statistical analysis of the *Bacillus* sp. adhesion was made from at least ten SEM images for each sample using ImageJ software (Fig. 1D). Adhesion ratio is defined here as the ratio of adhesion area of the bacteria to the total surface area of the substratum. After 3 days incubation in ASW, adhesion ratio of *Bacillus* sp. is ~0.41%, while in the media with

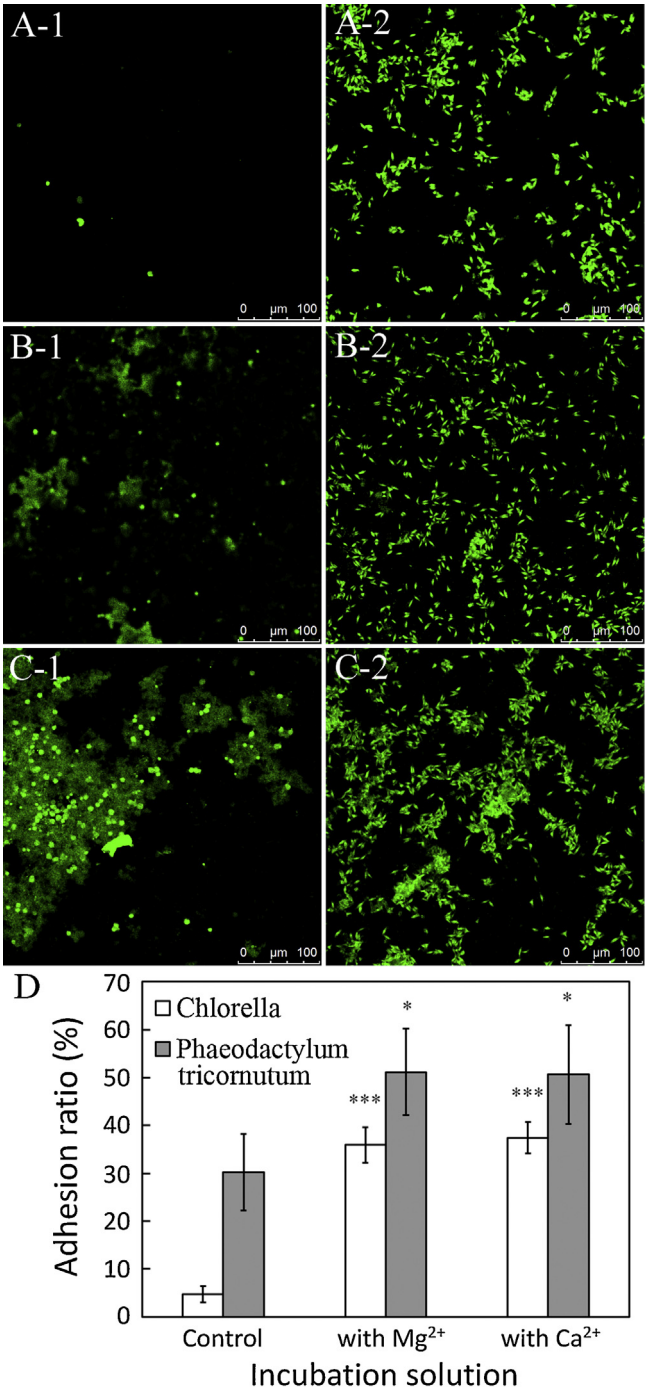

Fig. 2. CLSM imaging characterization of *Chlorella* (-1) and *Phaeodactylum tricornutum* (-2) adhered on silicon wafer without extra addition of the divalent cations (A) and with 10 mM Mg^{2+} (B) or 10 mM Ca^{2+} (C). D: Statistical results of adhesion of *Chlorella* and *Phaeodactylum tricornutum*. Error bars are shown as ±SD (n = 10). *: $p < 0.1$ and ***: $p < 0.005$ as compared with the control groups (analyzed by paired Student's *t*-test).

the extra addition of Mg^{2+}/Ca^{2+}, the value significantly increases to 2.17% and 4.30% ($p < 0.005$, n = 10), respectively. It should be noted that the low adhesion ratios are likely due to the short incubation period (3 days) and smooth nature of the surface of silicon wafer.

Similar trend is also realized for the adhesion of algae. Mg^{2+}/Ca^{2+} promotes the adhesion of *Chlorella* and *Phaeodactylum tricornutum* (Fig. 2). CLSM images show apparent aggregation of algae in the cations-added media. Statistical analysis suggests that after 7 days incubation in the media without addition of the divalent cations,

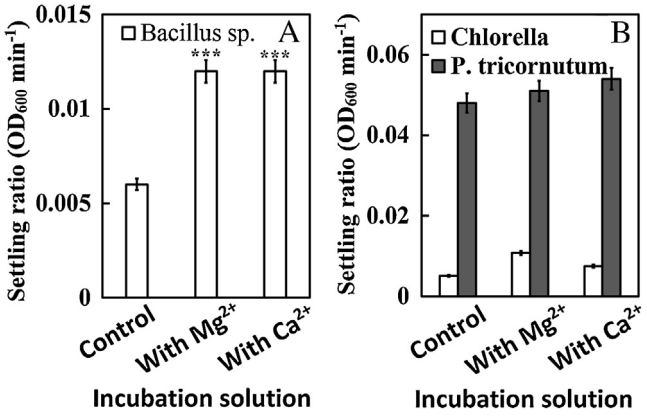

Fig. 3. Evaluation of cell-to-cell aggregation by settling ratio of *Bacillus* sp. (A) and the algae (B). Error bars are shown as ±SD (n = 3). ***: $p < 0.005$ as compared with the control groups (analyzed by paired Student's *t*-test).

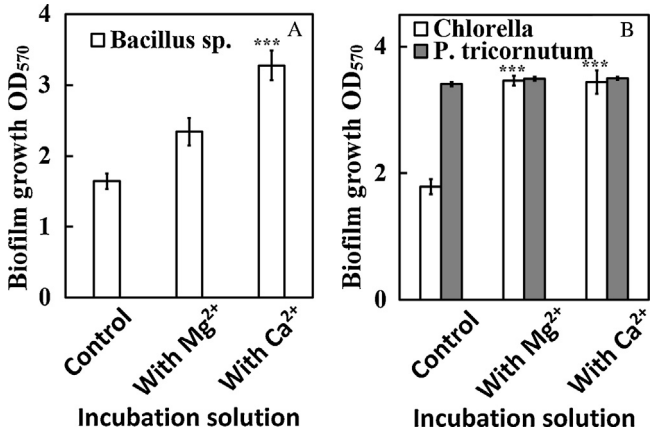

Fig. 4. A Assessment of *Bacillus* sp. bacterial biofilm grown on silicon wafers after 3 days incubation in the solutions with/without the extra addition of 10 mM Mg^{2+}/Ca^{2+}, and B: assessment of the algae biofilm grown on the wafers after 7 days incubation in the solutions with/without the extra addition of 10 mM Mg^{2+}/Ca^{2+}. Error bars are shown as ±SD (n = 9). ***: $p < 0.005$ as compared with the control groups (analyzed by paired Student's *t*-test).

adhesion ratio of *Chlorella* is ~4.70%, while in the media containing the extra cations, the value is significantly increased to 35.9% for Mg^{2+} solution and 37.4% for Ca^{2+} solution ($p < 0.005$, n = 10), respectively. Furthermore, cations-enhanced adhesion is also seen for *Phaeodactylum tricornutum* (30.2% verus 51.1% and 50.7%). Both Mg^{2+} and Ca^{2+} show significant difference from the control experiment ($p < 0.005$, n = 10). It is clear that the addition of Mg^{2+}/Ca^{2+} facilitates recruitment and enhances subsequent affinity of both *Chlorella* and *Phaeodactylum tricornutum* on silicon wafer. Moreover, Mg^{2+}/Ca^{2+} plays significant roles in *Chlorella* adhesion. It is noted that the two cations showed similar effect on the adhesion.

There are possibly two regimes for Mg^{2+}/Ca^{2+} to affect the bacterial/algal adhesion, either physically or biologically. To date, most of the research efforts have been devoted to clarifying the effect of physical interactions of the divalent cations with bacteria, for example neutralized electrical double layer [14], compressed electrical double layer [15], and reduced electron donicity [13]. It is established that the thickness of electrical double-layer is inversely proportional to square root of ionic strength of bulk aqueous medium [13]. Electrostatic repulsion would be reduced when the ionic strength is high. In this study, the influence of Mg^{2+}/Ca^{2+} on the adhesion of the bacteria/diatoms was examined in respective ASW-based culture media. Salinity of the ASW is about 50-fold of that of the solution containing extra 10 mM Mg^{2+}/Ca^{2+}. Therefore, the impact of the cations on compression of electrical double layer can be ignored. Mg^{2+}/Ca^{2+} presumably play a minor role in influencing electrical double layer and electrostatic repulsion. This in turn raises the probability that the biological effect of Mg^{2+}/Ca^{2+} likely significantly affect the microorganisms' adhesion.

Biological assessment using the settling ratio (aggregation) assay reveals that Mg^{2+}/Ca^{2+} promotes significantly aggregation of the Gram-positive bacterium *Bacillus* sp. ($p < 0.005$, n = 3) (Fig. 3A) and algae *Chlorella* ($p < 0.005$, n = 3) (Fig. 3B), while slightly enhances the aggregation of *Phaeodactylum tricornutum* (Fig. 3B). The results are consistant with the characterization by CLSM and FESEM (Figs. 1 and 2). It is noted that Mg^{2+} and Ca^{2+} showed similar impact on the aggregation of the bacteria. However, Mg^{2+} plays more important role in the aggregation of *Chlorella* than Ca^{2+} (Fig. 3B). It is known that there are a lot of negatively charged functional groups in EPS, for instance carboxyl, phosphoric, sulfhydryl, and hydroxyl groups, which can bind with divalent cations, such as Mg^{2+} and Ca^{2+} in this case, mainly by intermolecular interactions [15]. The interaction between the cations in particular Ca^{2+} [33] and EPS can improve the flocculation of microbial aggregates and maintain the microbial aggregate structure [15,27]. The species of negatively charged functional groups in EPS secreted by the microorganisms might be different. This likely results in altered affinities of Mg^{2+} and Ca^{2+} with the negatively charged functional groups, in turn giving rise to the different flocculation of the microbial aggregates. Yet, to understand how the binding affinities are affected, the EPS secreted by the microorgnisms needs to be further characterized.

Growth of the biofilm resulted directly from the adhesion of the microorganisms was measured by micro-plate spectrophotometer. Positively charged crystal violet can easily combine with negatively charged components in EPS. After the combination, the linked crystal violet was dissolved in ethanol and was then assessed by OD_{570} values. The amount of EPS can be defined as $k_1 OD_{570}$. It is realized that formation of the bacterial biofilm is enhanced by the metal ions, showing an increase of 42.3% and 99.1% for Mg^{2+} and Ca^{2+}, respectively (Fig. 4A). In addition, more remarkably promoted biofilm growth is revealed for *Chlorella* ($p < 0.005$, n = 3) (Fig. 4B). Biofilm growth is comparatively high in Mg^{2+}/Ca^{2+}-containing media compared to Guillard's F/2 growth media. The functional groups, for example carboxylate, phosphate, amine, and so on, in EPS were found to accelerate the adhesion of bacteria/algae to solid surfaces and enhance the interaction of the species with substrate [15,34,35]. Increasing EPS secretion by the divalent cations could be one of the approaches to promote adhesion of microorganisms. Surprisingly, however, formation of the *Phaeodactylum tricornutum*-associated biofilm does not show correlation with the cations (Fig. 4B), suggesting selective influence of the metal ions on biofilm formation. These are in agreement with other findings that divalent cations enhance some bacterial biofilm formation [15,16,28].

Mg^{2+}/Ca^{2+} promote secretion of EPS from the microorganisms. Further investigation shows that the proteins in the EPS might also be affected by the metal ions. Protein content in EPS was examined using a MicroBCA protein assay reagent kit detected at 562 nm wavelength. The protein content is represented by $k_2 OD_{562}$. OD_{562} value of the attached *Bacillus* sp. is 0.28, while it increases to 0.34 and 0.44 for the bacteria cultured in the extra Mg^{2+}- and Ca^{2+}-containing solution respectively (Fig. 5A-1). Only the value for the bacteria cultured in the media containing extra Ca^{2+} shows significant difference ($p < 0.05$, n = 3). Percentage of the proteins in EPS can be calculated as $k_2/k_1 \times OD_{562}/OD_{570}$. It is clear that the extra addition of Mg^{2+} and Ca^{2+} results in increased amount of the proteins. However, the percentage of the proteins in EPS drops when the microorganisms were cultured in the cations-added media

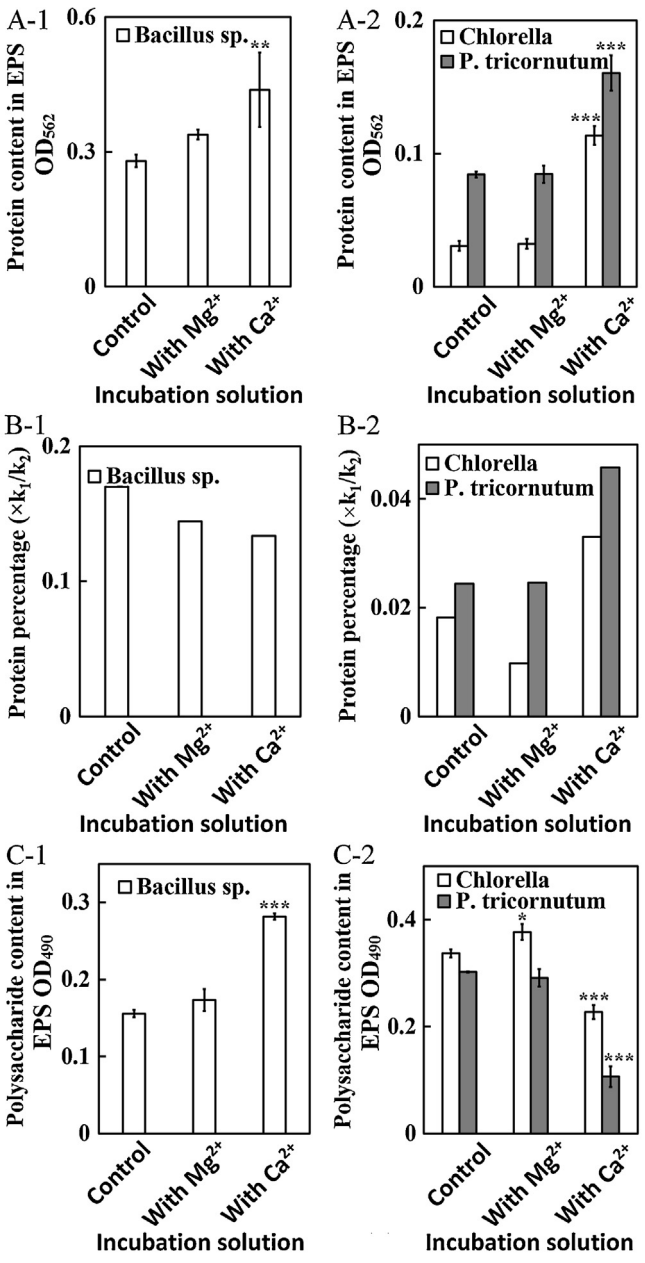

Fig. 5. Influence of the extra addition of the divalent cations on synthesis of proteins and polysaccharides of EPS of *Bacillus* sp. (A-1, B-1, C-1), and *Chlorella* and *Phaeodactylum tricornutum* (A-2, B-2, C-2). Error bars are shown as ±SD (n = 3). *: $p < 0.1$, **: $p < 0.05$ and ***: $p < 0.005$ as compared with the control groups (analyzed by paired Student's *t*-test).

(0.14 (k_2/k_1) and 0.13 (k_2/k_1) versus 0.17 (k_2/k_1), Fig. 5B-1). This indicates that Mg^{2+} and Ca^{2+} probably intend to increase synthesis of polysaccharides instead of proteins. This is consistent with the results obtained for the polysaccharides that both Mg^{2+} and Ca^{2+} promote expression of polysaccharides in the bacterial EPS (Fig. 5C-1). The increased protein content might be attributed to the significantly enhanced EPS secretion (Fig. 4).

Similar phenomena of enhanced protein synthesis are seen for *Chlorella* and *Phaeodactylum tricornutum* adhered on the wafers (Fig. 5A-2). OD_{562} value of the EPS secreted by *Chlorella* and *Phaeodactylum tricornutum* is 0.031 and 0.084 for the control sample, 0.032 and 0.085 for the Mg^{2+}-containing solution, and 0.114 and 0.16 for the Ca^{2+}-containing solution, respectively. Obviously, Mg^{2+} in the solution shows minor influence on protein expression of the EPS secreted from *Chlorella* and *Phaeodactylum tricornutum*

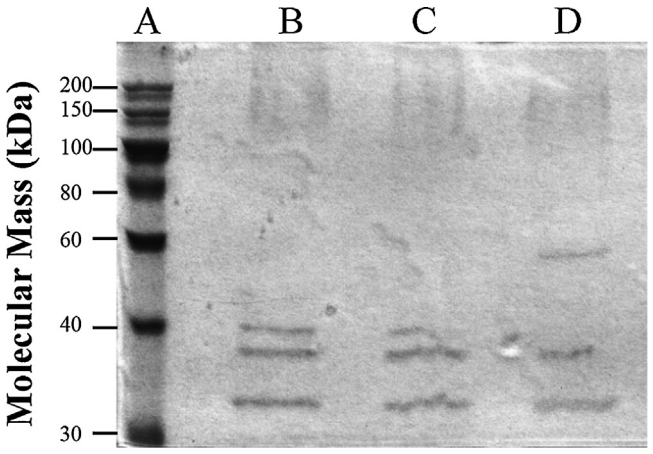

Fig. 6. Electrophoresis detection by staining with Coomassie Blue of the proteins extracted from the EPS of *Bacillus sp.*, lane A: marker, lane B: the bacteria were incubated in original ASW, lane C: the bacteria were incubated in extra 10 mM Mg^{2+}-added ASW, and lane D: the bacteria were incubated in extra 10 mM Ca^{2+}-added ASW.

while Ca^{2+} has significant influence ($p < 0.005$, n = 3) (Fig. 5B-2). Interestingly, Ca^{2+} accelerates the protein synthesis (0.046 (k_2/k_1) versus 0.024 (k_2/k_1)). In addition, further analyses suggest that Mg^{2+} increases expression of polysaccharides in the EPS of *Chlorella* ($p < 0.1$, n = 3), while Ca^{2+} significantly constrains the polysaccharide synthesis ($p < 0.005$, n = 3) (Fig. 5C-2). It seems clear that Mg^{2+} does not work in polysaccharide synthesis, but Ca^{2+} rapidly reduces the polysaccharide synthesis in the EPS of *Phaeodactylum tricornutum* (Fig. 5C-2). It therefore indicates that Mg^{2+} might not take part in EPS production, protein synthesis and polysaccharide formation of *Phaeodactylum tricornutum*. The reason why Mg^{2+} increases adhesion of *Phaeodactylum tricornutum* is still unknown and further investigation is needed. In addition, it seems that Ca^{2+} does not play a role in formation of *Phaeodactylum tricornutum* EPS, while it greatly promotes synthesis of proteins rather than polysaccharides in the EPS.

The results are consistent with other reported findings that protein content in EPS changed at high concentrations of Mg^{2+}or Ca^{2+} [15], and our findings provide direct evidences of related mechanisms. The question as to whether protein species change with addition of Mg^{2+} or Ca^{2+} needs to be further explored. To elucidate this, SDS-PAGE was run to separate the EPS extracts (Fig. 6). The gel for the bacterial EPS extracts shows no marked differences between the samples incubated in the solutions with/without the extra addition of the cations. Three different fragments with the size less than 40 kDa are the major components in the *Bacillus* sp. EPS. However, a new protein fragment located at ~60 kDa is replaced by another fragment of ~40 kDa in the bacterial EPS formed in the media containing extra Ca^{2+}. Further analyses of the bands on the gels were carried out by determining the molecular mass of the proteins by calculating relative electrophoretic mobility. It is realized that the band close to 60 kDa (lane D, Fig. 6) refers to the protein with the molecular weight of 58.5 kDa. It is speculated that Ca^{2+} could facilitate synthesis of some proteins while inhibit that of others simultaneously. It is conjectured that the enhanced bacterial adhesion might be closely related to the altered protein synthesis. Further characterization of the proteins is yet to be carried out. Due to the difficulties of collecting sufficient amount of proteins from the algal EPS, related protein analysis by the SDS-PAGE was not made yet. Nevertheless, based on the results on the polysaccharides, it is likely that the divalent cations affect the proteins of the algal EPS in a similar way.

It is therefore clear that both Mg^{2+} and Ca^{2+} affect the adhesion of *Bacillus* sp. and Ca^{2+} plays more important roles. This effect seems

to prevail prominently by mediating the secretion of proteins and polysaccharides. Mg^{2+} and Ca^{2+} play similar roles in aggregation of *Bacillus* sp. and both the cations could influence EPS formation and protein expression. The role of Ca^{2+} is much more pronounced. It is known that the unique properties of Ca^{2+} benefit EPS formation and protein expression and in turn promote the interactions between protein/polysaccharide and surface to increase bacterial adhesion [14]. Our results indicate that EPS plays a crucial role in the bacterial adhesion. In addition, Mg^{2+} and Ca^{2+} also give rise to enhanced algal adhesion. For *Chlorella*, Mg^{2+} promotes adhesion of *Chlorella* as a result of possibly regulated EPS formation in particular polysaccharide synthesis. Meanwhile, proteins in EPS seem to perform a greater role in adhesion of *Chlorella* than other macromolecules in EPS. For *Phaeodactylum tricornutum*, the reason why Mg^{2+} promotes adhesion is not clear yet. Extensive biochemical examination for instance catalytic actions of various enzymes of the microorganisms related to the cations is needed.

4. Conclusions

As typical divalent cations, Ca^{2+} and Mg^{2+} significantly promote adhesion of typical marine microorganisms, *Bacillus* sp., *Chlorella* and *Phaeodactylum tricornutum*. Results suggest that the cations influence the adhesion of the species presumably by regulating protein expression and polysaccharide synthesis in their secreted EPS. The regimes operated by the ions for affecting the adhesion of the microorganisms are remarkably different depending presumably on either altering EPS protein expression or mediating EPS formation in particular polysaccharide synthesis. Both the effects give rise to alternations in adhesion of the microorganisms for consequent biofilm formation.

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