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EFFECTS OF MAGNESIUM AND CALCIUM CATIONS ON BIOFILM FORMATION BY *Sphingomonas paucimobilis* FROM AN INDUSTRIAL ENVIRONMENT

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ABSTRACT

Bacterial biofilms may form on all surface-associated natural and many industrial environments. Biofilm formation requires particular notice due to its associated risks for human health and its impact on environmental contamination and pollution. In this work, we investigated the effects of Mg^{2+} and Ca^{2+} ions on biofilm formation by *Sphingomonas paucimobilis* from an industrial environment. The biofilm formation on coupons within nutrient broth medium was significantly enhanced after addition of 0, 100, 250 and 500 μM Mg^{2+}. Similarly, the addition of Ca^{2+} caused a significant increase in *S. paucimobilis* biofilm formation when the above concentration levels for Ca^{2+} were tested. In contrast, the same concentrations of these ions had no effect on growth of free-living (planktonic) *S. paucimobilis* cells in the medium. Hence, Mg^{2+} and Ca^{2+} increased the biofilm formation as adherent-cells on the coupons. Both ion types were significantly effective on *S. paucimobilis* biofilm formation, particularly at 100 and 250 μM ($P \leq 0.05$). These firstly reported data for *S. paucimobilis* biofilms are important in the elucidation of the roles of divalent cations, such as Mg^{2+} and Ca^{2+}, in bacterial adhesion to the environmental surfaces for biofilm formation, and prevention of environmental contamination by this bacterium.

KEYWORDS: Biofilm, industrial environment, bacterial adhesion, *Sphingomonas paucimobilis*,magnesium ions,calcium ions

1 INTRODUCTION

In environment, bacteria often grow as populations attached to surfaces in complex structures called biofilms. A biofilm is an aggregate of bacteia in which cells adhere to each other and/or to a surface. Bacterial adhesion involves the attachment (or deposition) of bacteria on the surface (solid, gel layer, etc.). In nature, microorganisms usually attach to solid surfaces, especially on the liquid–solid interface. After attachment, they form micro-colonies, and a biofilm which is highly resistant to antimicrobials and some physical treatments [1, 2], and it is also a serious problem for infectious diseases [3].

Biofilm contamination and biofouling occurs on nearly every environmental water-contacted surface [4]. Environmental systems by their nature are comprised of interfaces that provide chemical and mechanical stimuli for microbial attachment and biofilm accumulation. Often, attachment to a surface is the way in which microbes respond to environmental stimuli, and this attachment effects numerous environmental and health problems [5, 6]. Virtually any surface - animal, mineral, or vegetable (i.e., biotic or abiotic) - is fair game for bacterial colonization and biofilm formation, including contact lenses, ship hulls, dairy and petroleum pipelines, rocks in streams, and all varieties of biomedical implants and transcutaneous devices [7-9].

Understanding the environmental (e.g., temperature, pH, ionic strength, electrolyte type), interfacial (e.g., surface charge and hydrophobicity), and physiological (e.g., bacterial growth stage and metabolic activity) factors that govern adhesion mechanisms defines one of the most important challenges in the microbial and interfacial sciences [10, 11]. Environmental factors, such as electrolyte con-centrations [12] and medium composition [13], have important impacts on biofilm formation. Bacterial exopolysaccharides are the main component of the biofilm matrix. Their production and surface adhesion, and, hence, surface colonization are influenced by electrostatic and hydrophobic interactions, steric hindrance, van der Waals forces, temperature, and hydrodynamic forces, to name a few [14, 15].

Divalent cations can potentially initiate bacterial adhesion and biofilm formation directly through effects on electrostatic interactions, and indirectly by affecting physiology-dependent attachment processes [16, 17]. From these divalent cations, magnesium (Mg^{2+}) is the molecular key that activates many important enzymes responsible for diverse biochemical reactions in living cells [18, 19]. Mg is regarded as one of the intracellular bulk elements [20]

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and is involved in enzyme catalysis, which performs a variety of roles, such as structure stabilization, charge neutralization, and control of osmotic pressure [21].

In spite of this potentially important role, the effect of Mg^{2+} or Ca^{2+} concentrations on bacterial growth and adhesion have not been studied in *Sphingomonas paucimobilis* biofilms. The aim of this study was to investigate the effects of Mg^{2+} and Ca^{2+}concentrations on the planktonic and sessile growth in biofilm formation by *S. paucimobilis*. This bacterium was selected because it commonly exists in biofilms in natural, clinical, and industrial environments e.g. [22-24]. *S. paucimobilis* is a yellow-pigmented, aerobic, motile with polar flagellum, non-fermentative, gram-negative bacterium. This organism is widely distributed in various environments, especially in water and soil [25], and has also been recovered from hospital environments [26]. *S. paucimobilis* is an opportunistic pathogen and has been implicated in a variety of community-acquired and nosocomial infections, including bacteremia, catheter-related sepsis, meningitis, peritonitis, cutaneous infections, visceral abscesses, urinary tract infections, adenitis, and diarrheal disease [27]. *S. paucimobilis* has been reported to cause outbreaks of bacteremia; these outbreaks are possibly related to bacterial colonization of water systems [23, 26]. Gellan, an exopolysaccharide (EPS), is produced by *S. paucimobilis* and is effective on its bacterial colonization and biofilm formation [28], but the effects of divalent cations, such as Mg^{2+} and Ca^{2+}, on *S. paucimobilis* biofilms are largely unknown. Attachment and biofilm formation by opportunistic pathogens, such as *S. paucimobilis,* are of public health and cross-contamination concern.

2 MATERIALS AND METHODS

2.1 Bacterium and medium

S. paucimobilis, isolated from the biofilms in the cooling tower water of a petrochemical industrial plant, was used as the test strain in this study. This strain was reported to be a biofilm-forming bacterium [24]. Because of continuous circulation of water, the warm temperatures and continuous scrubbing of nutrients, cooling towers are a prime environment for the build-up of microorganisms [29, 30].

Identification of the isolate was carried out using Standard biochemical tests as outlined in Bergey's Manual [31]. API 20 E and API 20 NE commercial kits (Bio-Me´rieux, France), and 16S rRNA sequencing analysis were used to further identify this isolate [32]. The bacterium was cultured in Modified Tryptone Soya Broth (TSB) (Oxoid CM0989, pancreatic digest of casein 17.0 g/L, papaic digest of soybean meal 3.0 g/L, sodium chloride 5.0 g/L, dipotassium hydrogen phosphate 2.5 g/L, glucose 2.5 g/L, bile salts 1.5 g/L, distilled water 1 L; pH 7.4±0.2.

2.2 Preparation of test surfaces

Bacterial adhesion and biofilm accumulation were performed on stainless steel coupons (30x10x15 mm). Stainless steel coupons were prepared according to Nandakumar *et al.* [21] with modifications. In the study, six stainless steel coupons were used for treatment group (3 different concentrations of $MgCl_2$ and $CaCl_2$), and two coupons were used for control group (without $MgCl_2$ and $CaCl_2$). Prior to the biofilm experiments, the coupons were smoothed with emery papers and washed with a commercial surfactant solution, and then deionized water. They were dried in a Pasteur oven at 50 °C.

2.3 Formation of biofilms and cultural conditions

Bacterial cells were grown in TSB supplemented with 0, 100, 250, 500 µM of $MgCl_2$, or the same concentrations of $CaCl_2$. Bacterial growth of the cells was carried out in shaken flasks at 100 rpm for 24 h at 30 °C. The flasks with 200 ml of the experimental medium, a flask with 200 ml of deionized water, and a flask with 200 ml of TSB were taken and sterilized. One ml of late exponential phase *S. paucimobilis* culture (at an optical density of 1.0, corresponding to ca. $1.0x10^7$ cells/ml) was inoculated into each of these flasks. The coupons were sterilized by autoclaving for 20 min at 121 °C and transferred into the flasks under aseptic conditions. These flasks were incubated at 30 °C for 7 days. The coupons were removed after 7-days incubation, and the adherent cells on them were finally investigated for biofilm formation. The experiment was not continued further because the number of bacteria in the medium with coupons was reduced considerably by this time so that the experiment could not be continued. Three replicates were tested for each experiment.

2.4 Determination of cell counts in the medium and biofilm

In this study, free-living biomass (planktonic) was considered as cell count (colony forming units (CFU)) in TSB (cell count medium), and adherent-living biomass (sessile) on the coupons was regarded as cell count of biofilms (cell count biofilm). Free-living and sessile bacteria were collected simultaneously with retrieval of coupons. Determinations of the biofilm counts were achieved on the coupons in TSB supplemented separately with Mg^{+2} and Ca^{+2}. The flasks with 200 ml of TSB each were inoculated with *S. paucimobilis* as mentioned above. After 7 days, each coupon was rinsed with 100 ml of sterile 0.1% peptone water to remove unattached cells. It was then placed in a sterile glass jar containing 250 ml TSB. After that, the side of the coupon in contact with the product was repeatedly scraped about 120 times by using a sterile spatula in order to recover attached cells. The cells were placed in 250 ml of TSB and the resulting attached cell suspension, which was later also used for pre-enrichment of cells, was thoroughly shaken and decimal dilutions were immediately prepared with sterile 0.1% peptone water. Dilutions were plated using TSB + 1.5% Tryptone Soya Agar (TSA) (pH 7.4±0.2) for the biofilm forming total viable counts and incubated at 35 °C for 48 h. After the incubation period, colonies were enumerated and the number of biofilm-forming cells per cm^2 was calculated (modified, Gunduz and Tuncel [33]). Sterile TSB was

used as negative control. A minimum of three assays was performed in each experiment.

To control the scraping technique, replicate coupons were examined before and after scraping by fluorescent microscopy to get a second estimate of cell numbers, and the percentage removed. The detection limit for removal of cells was determined to be 99.4± 0.5 %.

2.5 Standard plate count

Cell counts medium and cell counts biofilm were performed by a modified standard plate count method [34]. One ml of the sample was placed on the centre of a sterile Petri dish (100 mm diameter) by using a sterile pipette. Sterile, molten (44-46 °C) TSA was added and mixed with the sample by swirling the plate. The samples were allowed to cool at room temperature until being solidified, and then were inverted and incubated at 35 °C for 48 h. Colonies formed in or on the TSA within 48 h were counted as described in this standard method, and the results were reported as CFU/ml. Where applicable, this value was multiplied by the dilution factor to obtain the corrected CFU/ml.

2.6 Statistical analysis

Statistical analysis was performed using SPSS software (version 16.0). One-way variance analysis (ANOVA) was used to test the differences among all effects of Mg^{+2} and Ca^{+2} concentrations on biofilm formation. Levene`s test was used to examine homogeneity of variances. Fisher's least significant difference (LSD) test was performed for multiple comparisons. All tests were considered to be significant at the level $P<0.05$. Error bar plots in figures are shown as one standard deviation. The values for each experiment are the mean values of results from three experiments unless otherwise stated.

3 RESULTS AND DISCUSSION

A number of researches have demonstrated that environmental conditions play an important role for biofilm formation [35]. Biofilm development is a complex process and can be regulated by different factors, such as cell surface structure, growth medium, oxygen limitation, or substratum [36]. However, it has not been obtained any data with *S. paucimobilis* among previous works on the effecting factors of adhesion to surfaces and the end of this biofilm formation, except from the study of Venugopalan *et al*. [37]. These researches declared that changing hydrodynamic conditions had discernible influence on the characteristics of *Sphingomonas* biofilms during development. In our study, evaluation of the effect of Mg^{+2} and Ca^{+2} separately was done using an assay based on cell counts in medium (TSB) and biofilm by *S. paucimobilis*. In the second part of the study, the effects of these cations on bacterial growth and biofilm development were evaluated statistically.

A biofilm can be defined as a sessile bacterial community of cells that live attached to each other and to surfaces [38]. Hence, we detected the numbers of sessile cells for biofilm formation. Significant differences between planktonic counts in medium and sessile counts on the coupons at a given point ($P\leq0.05$) are shown in Table 1. Asterisks indicate a significant ($P\leq0.05$) reduction or enhancement in mean CFU counts of planktonic *S. paucimobilis* in liquid culture when Mg^{+2} and Ca^{+2} was supplemented on the coupons.

After 7 days, biofilm development with a significant enhancing in *S. paucimobilis* log CFU/cm^2 was noted at 100 µM ($3.1x10^7$, P = 0.000), at 250 µM ($10.4x10^7$, P = 0.000), at 500 µM ($6.5x10^7$, P = 0.000) of Mg^{2+} compared with cell counts at 0 µM ($1.2x10^7$). Conversely, non-significant enhancing planktonic *S. paucimobilis* counts/ml of the medium containing Mg^{2+} was noted at 100 µM ($1.7x10^7$, P = 0.866), at 250 µM ($2.3x10^7$, P = 0.092), at 500 µM ($2.2x10^7$, P = 0.120) compared with planktonic cell counts at 0 µM ($1.6x10^7$) (Figs. 1 and 2, Table 2). Similarly, biofilm development with Ca^{+2} was significantly enhanced in log CFU/cm^2 at 100 µM ($2.3x10^7$, P = 0.000), at 250 µM ($8.3x10^7$, P = 0.000), at 500 µM ($5.2x10^7$, P = 0.000) compared with cell counts at 0 µM ($1.2x10^7$). On the contrary, non-significant enhancing planktonic *S. paucimobilis* counts/ml of the medium containing Ca^{+2} was noted at 100 µM ($1.6x10^7$, P = 0.866), at 250 µM ($2.0x10^7$, P = 0.092), at 500 µM ($2.0x10^7$, P = 0.120) with regard to planktonic cell counts at 0 µM ($1.6x10^7$) (Table 2). In other two works, Tamura *et al.* [39] showed that magnesium had no significant effect on adherence of Streptococci at physiological concentrations (2mM Mg^{2+}) while higher concentrations enhanced adherence to a small degree. Dunne and Burd [40] found that magnesium (as low as 16 mM) significantly enhanced the *in vitro* adhesion of *Staphylococcus epidermidis* to plastics.

TABLE 1 - The results of ANOVA and LSD multiple comparison test over cell counts in medium supplemented with Mg^{2+} or Ca^{2+}, and mean counts of *S. paucimobilis* cells in biofilms formed in the presence of Mg^{2+} or Ca^{2+}.

	Concentrations of Mg^{2+} and Ca^{2+} (µM)			
Statistics	0	100	250	500
P value of ANOVA	0.029*	0.000*	0.000*	0.000*
Mean±SE for Mg^{2+}-Medium	1.567 ± 0.067^{bd}	1.633 ± 0.088^{bd}	2.200 ± 0.115^{bd}	2.233 ± 0.208^{bd}
Mean±SE for Mg^{2+}-Biofilm	1.200 ± 0.115^{ac}	3.167 ± 0.088^{acd}	10.533 ± 0.088^{acd}	6.500 ± 0.115^{acd}
Mean±SE for Ca^{2+}-Medium	1.633 ± 0.145^{bd}	1.167 ± 0.186^{bd}	2.000 ± 1.000^{bd}	1.967 ± 0.088^{bd}
Mean±SE for Ca^{2+}-Biofilm	1.200 ± 0.058^{ac}	2.367 ± 0.120^{abc}	8.267 ± 0.033^{abc}	5.467 ± 0.088^{abc}

*Significantly different ($P\leq0.05$). Each assay was carried out in triplicate (n=3). [a] There is a significant difference from Mg^{2+}-Medium. [b] There is a significant difference from Mg^{2+}-Biofilm. [c] There is a significant difference from Ca^{2+}-Medium. [d] There is a significant difference from Ca^{2+}-Biofilm

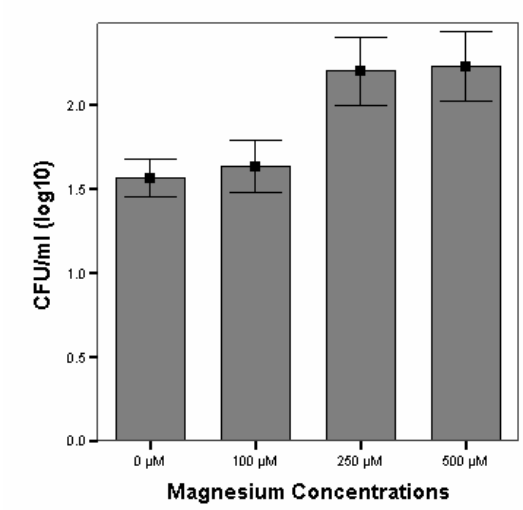

FIGURE 1 - Effects of Mg^{2+} concentrations on the cell counts in medium (Error bars indicate one standard deviation).

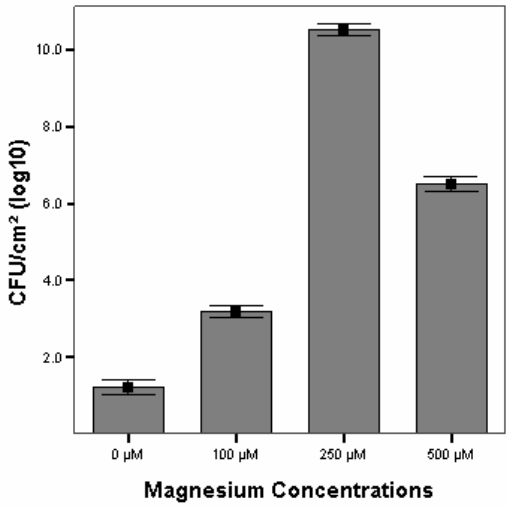

FIGURE 2 - Effects of different Mg^{2+} concentrations on the cell counts of biofilms formed on the coupon (Error bars indicate one standard deviation).

TABLE 2 - The results of ANOVA and LSD multiple comparison tests over mean counts of *S. paucimobilis* cells in the different concentrations of Mg^{2+} or Ca^{2+}.

Statistics	MgM	MgB	CaM	CaB
P value of ANOVA	0.002*	0.000*	0.187	0.000*
Mean±SE for 0 µM	1.567 ± 0.067^{cd}	1.200 ± 0.115^{bcd}	1.633 ± 0.145	1.200 ± 0.058^{bcd}
Mean±SE for 100 µM	1.633 ± 0.088^{cd}	3.167 ± 0.088^{acd}	1.667 ± 0.186	2.367 ± 0.120^{acd}
Mean±SE for 250 µM	2.200 ± 0.115^{ab}	10.533 ± 0.088^{abd}	2.000 ± 0.100	8.267 ± 0.033^{abd}
Mean±SE for 500 µM	2.233 ± 0.120^{ab}	6.500 ± 0.115^{abc}	1.967 ± 0.088	5.467 ± 0.088^{abc}

*Significantly different (P≤0.05). Each assay was carried out in triplicate (n=3). [a] There is a significant difference from 0 Mm. [b] There is a significant difference from 100 µM. [c] There is a significant difference from 250 µM. [d] There is a significant difference from 500 µM.

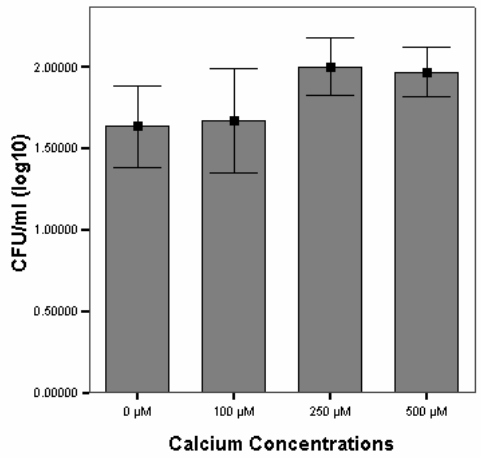

FIGURE 3 - Effects of Ca^{2+} concentrations on the cell counts in medium (Error bars indicate one standard deviation).

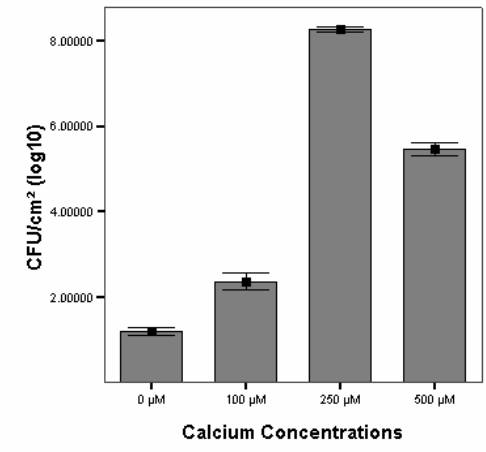

FIGURE 4 - Effects of different Ca^{2+} concentrations on the cell counts of biofilms formed on the coupon (Error bars indicate one standard deviation).

Previous researches with the gram-negative bacteria *P. aeruginosa* and *Escherichia coli* and the gram-positive bacteria *S. epidermidis* and *S. aureus* revealed that divalent cations are stimulating attachment to surfaces, especially at low or moderate concentrations of them [41-44]. Similarly, in this study, the mean CFU values of *S. paucimobilis* in biofilm cultures were $3.1x10^7$ and $2.3x10^7$, respectively, with Mg^{+2} and Ca^{+2}. The mean CFU of *S. paucimobilis* increased dramatically within the biofilm with 250 µM of Mg^{+2} ($10.4x10^7$, P = 0.000; Table 2, Fig. 2)

but only moderately with 500 µM ($6.5x10^7$, P = 0.000) (Table 2, Fig. 2). Moreover, the mean of bacterial counts increased also dramatically in the biofilm with 250 µM of Ca^{+2} ($8.3x10^7$, P = 0.000) (Table 2, Figs. 4 and 5). Conversely, this effect on planktonic bacterial growth in liquid culture was not seen at these concentrations of Mg^{+2} or Ca^{+2} (Table 2, Figs. 1, 3 and 5). High levels of these cations might screen cross-linking electrostatic interactions [45], and have resulted in a reduction of attached cells in 500 µM. In addition, these cations might enhance the production of EPS by the bacterium [41]. EPS are often associated to bacteria growing in biofilms in which they play a key role in the architecture [46].

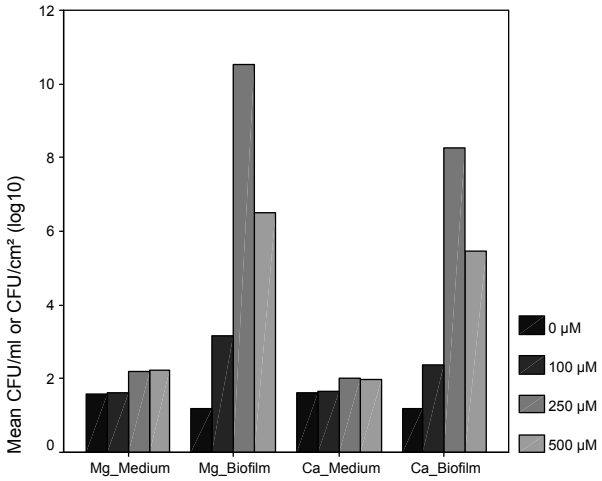

FIGURE 5 - Comparison of cell counts in Mg^{2+}-Medium, Mg^{2+}-Biofilm, Ca^{2+}-Medium, and Ca^{2+}-Biofilm.

Physical and chemical environment has a significant impact on the way of biofilm formation and its persistence. Divalent cations are regarded to be important constituents of microbial aggregates, since they bind to negatively charged groups present on bacterial surfaces, in EPS, and on inorganic particles entrapped in biofilms [41]. It has been reported that extraction of calcium (Ca^{2+}) from biofilms by displacement with monovalent cations or by chelators, such as EDTA and EGTA, resulted in the destabilization of biofilms. These observations suggest that divalent cations may be important in maintaining the biofilm structure, acting in the bridging of a 3-D polymeric matrix [15]. Similar with these investigations, in the absence of Mg^{+2} and Ca^{+2} in TSB, sessile bacterial counts on the coupons did not increase significantly (P = 1.000) in this study (Table 1, Figs. 6 and 7). On the other hand, the mean number of *S. paucimobilis* CFU on the coupons increased significantly in the presence of Mg^{+2} or Ca^{+2} ($P<0.05$) (Table 1, Fig. 7), whilst Mg^{+2} or Ca^{+2}, at all concentrations examined, had no significant effects on *S. paucimobilis* numbers in the medium ($P>0.05$) (Table 1, Fig. 7). When all corresponding concentrations of these cations were compared, the statistical analyses showed highly significant differences between attached cell counts by adding Mg^{+2} and attached cell counts by adding Ca^{+2} into TSB (Table 1, Figs. 5-8). According to

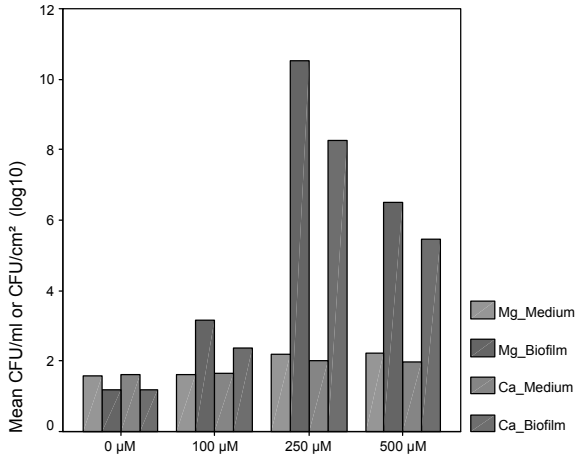

FIGURE 6 - Comparison of cell counts in medium or biofilm when different concentrations of Mg^{2+} and Ca^{2+} are considered.

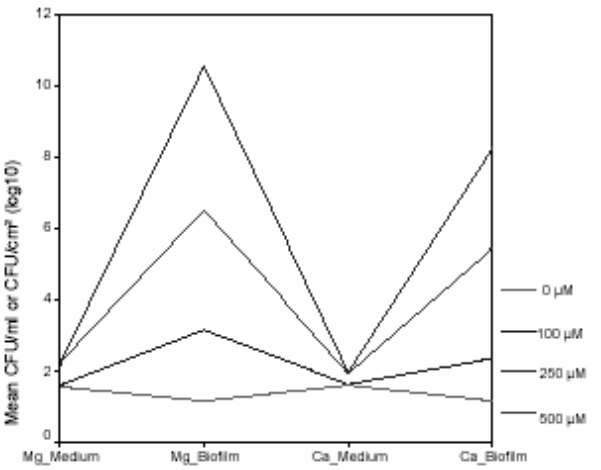

FIGURE 7 - Comparison of cell counts determined in different concentrations of Mg^{2+}-Medium, Mg^{2+}-Biofilm, Ca^{2+}-Medium, Ca^{2+}-Biofilm with each other.

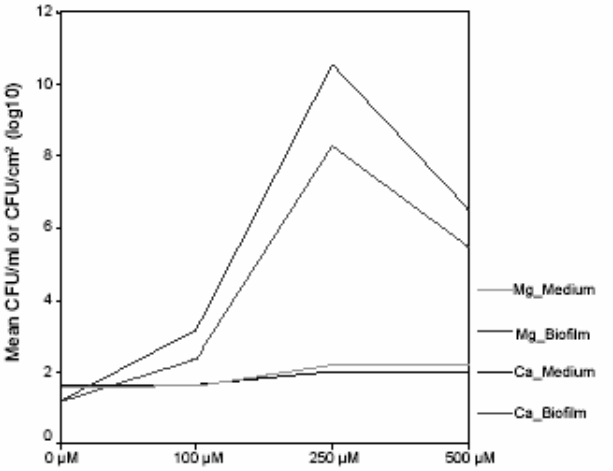

FIGURE 8 - Comparison of cell counts in each concentration of Mg^{2+}-Medium, or Mg^{2+}-Biofilm, or Ca^{2+}-Medium, or Ca^{2+}-Biofilm with others.

these findings, *S. paucimobilis* increased attachment and biofilm formation.

Also in other studies, divalent cations have been shown to influence adherence to surfaces (specifically, using *Pseudomonas* sp. and sand columns) [47]. Furthermore, for *Aeromonas hydrophilia*, mutations in Mg^{2+} transport systems result in reduction of swarming and biofilm formation [48]. For *Pseudomonas fluorescens*, Mg^{2+} increased initial attachment and altered subsequent biofilm formation and structure [49]. Dunne and Burd [40] demonstrated that increasing Mg^{2+} levels enhanced biofilm production by *S. epidermidis*, while EDTA caused a dose-dependent decrease in the accumulation of cells on a plastic surface. Additionally, although Ca^{2+} had a much stronger affinity towards alginate produced by *P. aeruginosa* than Mg^{2+} [50], *P. fluorescens* produces an acidic galactoglucan, and its surface colonization and biofilm depth increased with increasing Mg^{2+} concentrations [49]. All these studies have found that divalent cations have varying effects on bacterial adhesion [38, 40, 49], which might be due to the difference in bacterial species and cation concentrations.

Furthermore, increase in surface hydrophobicity of *E. coli* in the presence of Mg^{2+} excess was found by Latrache *et al.* [51]; on the other hand, antimicrobial effects of Ca^{2+}against various microorganisms isolated from oral cavity were described by Pezelj-Ribari *et al.* [52].

4 CONCLUSION

We detected significant effects of Mg^{2+} and Ca^{2+} on biofilm formation activity of *S. paucimobilis*. These divalent cations showed significant stimulation of the adhesion of *S. paucimobilis* cells onto coupons. The numbers of *S. paucimobilis* cells on the stainless steel coupons were nearly $1.0x10^7$ CFU/cm^2, and these numbers increased significantly ($P \leq 0.05$) after addition of Mg^{2+} and Ca^{2+}, particularly at 100 and 250 µM levels. This influence might contribute to an increase in the hydrophobicity of planktonic cells, which may have altered their ability to attach to surfaces [53]. Moreover, *S. paucimobilis* in a variety of environments is frequently mentioned. Its survival in an environment could be associated with its capacity to colonize on abiotic/biotic surfaces because of its production of gellan [37]. In industrial or other environments, biofilm formation may be undesirable for contamination and safety reasons concerning the attachment of opportunistic pathogenic microorganisms, such as *S. paucimobilis*, to surfaces.

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Received: February 29, 2012
Accepted: June 13, 2012

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