Calcium and Magnesium Cations Enhance the Adhesion of Motile and Nonmotile *Pseudomonas aeruginosa* on Alginate Films

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We investigated the impact of calcium and magnesium ions on the deposition kinetics of flagellated and nonflagellated *Pseudomonas aeruginosa* onto an alginate conditioning film in a radial stagnation point flow system. The bacterial deposition/adhesion behavior was related to structural changes of the alginate film in the presence of the divalent cations. Our results showed that adhesion of nonmotile bacteria was governed by cation bridging interactions between high-affinity sites at the bacterial surface and either clean or alginate-conditioned substrate surfaces. For motile bacteria, the adhesion onto clean quartz was governed by electrostatic interactions while adhesion onto alginate-conditioned quartz was dependent on the structure and viscoelastic properties of the alginate film in the presence of calcium or magnesium. We demonstrate that bacterial adhesion behavior is governed both by the effects of divalent cations on the surface properties of the bacteria and the substrate and by the type of specific interactions occurring between these two surfaces.

1. Introduction

Adhesion of microorganisms and the subsequent development of biofilms on substrates remains a crucial problem limiting the efficiency of environmental, industrial, and biomedical systems. 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. Three specific features have been shown to be ubiquitous to all environmental and biomedical systems and are likely to collectively control the interactions between bacterial cells and the solid substrate: (i) the presence of a conditioning film at the substrate surface,[1] (ii) the presence of flagella at the bacterium surface,[2] and (iii) the presence of divalent cations, calcium and magnesium, in the surrounding solution.[3]

Adsorption of dissolved organic matter at the solid/liquid interface creates a polyelectrolyte film known as the conditioning film, which introduces physical, chemical, and biological heterogeneities at the substrate surface.[4] Previously, we successfully incorporated a conditioning film into our bacterial adhesion studies by using a well-defined alginate film.[5] We showed that these alginate films provided variable roughness, viscoelastic properties, and surface charges to the substrate as a function of the medium electrolyte concentration.[6] Because alginates are bacterial exopolymeric products, an alginate conditioning film is likely to exhibit biological characteristics that could induce specific responses from approaching microorganisms.[7] These physicochemical and biological alterations of the substrate surface were demonstrated to be representative of the effects of complex conditioning films encountered in natural aquatic systems and were shown to impact bacterial adhesion.[1,8]

Bacterial transport and adhesion are also influenced by the presence of flagella at the cell surface.[2] The transport of the cells toward the substrate is enabled by the rotation of the flagella, which is driven by coupled electrochemical potentials and electrostatic interactions across the cell membrane.[9] Several studies have proposed that flagella can enhance bacterial adhesion by enabling motile cells to overcome repulsive electrostatic barriers and adhere irreversibly to the substrate.[10,11] Flagellum activity was previously shown to be highly dependent on the electrolyte concentration of the surrounding solution, whereby maximum activity was observed under physiological saline conditions.[5] When highly active, we have shown that the flagellum was the controlling factor in the enhanced adhesion of bacterial cells onto the alginate conditioning film.[5]

Constituents of substrate and bacterial surfaces, such as the conditioning film and flagella, as well as interactions between these components, are highly influenced by the presence of divalent cations in solution. In biomedical systems, free calcium and magnesium cations are major components of human metabolic pathways and are present in most physiological fluids.[12,13] For example, typical concentrations of free calcium and magnesium ions range between 1.6 and 5.0 mM in urine[14] and between 0.7 and 1.4 mM in blood serum.[15,16] However, in environmental systems, calcium and magnesium have been measured in water

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(1) Bakker, D. P.; Klijnstra, J. W.; Busscher, H. J.; van der Mei, H. C. *Biofouling* **2003**, *19*, 391−397.
(2) Camesano, T. A.; Logan, B. E. *Environ. Sci. Technol.* **1998**, *32*, 1699−1708.
(3) Fletcher, M. *J. Bacteriol.* **1988**, *170*, 2027−2030.
(4) Melo, L. F.; Bott, T. R. *Exp. Therm. Fluid Sci.* **1997**, *14*, 375−381.
(5) de Kerchove, A. J.; Elimelech, M. *Appl. Environ. Microbiol.* **2007**, *73*, 5227−5234.
(6) de Kerchove, A. J.; Elimelech, M. *Biomacromolecules* **2007**, *8*, 113−121.
(7) Gomez-Suarez, C.; Pasma, J.; van der Borden, A. J.; Wingender, J.; Flemming, H. C.; Busscher, H. J.; van der Mei, H. C. *Microbiol.-Sgm* **2002**, *148*, 1161−1169.
(8) van Hoogmoed, C. G.; van der Kuijl-Booij, M.; van der Mei, H. C.; Busscher, H. J. *Appl. Environ. Microbiol.* **2000**, *66*, 659−663.
(9) Berg, H. C. *Annu. Rev. Biochem.* **2003**, *72*, 19−54.
(10) Marshall, K. C.; Stout, R.; Mitchell, R. *J. Gen. Microbiol.* **1971**, *68*, 337-&.
(11) Meadows, P. S. *Arch. Mikrobiol.* **1971**, *75*, 374−381.
(12) McLean, R. M. *Am. J. Med.* **1994**, *96*, 63−76.
(13) Ladenson, J. H.; Bowers, G. N. *Clin. Chem.* **1973**, *19*, 575−582.
(14) Taylor, E. N.; Curhan, G. C. *J. Am. Soc. Nephrol.* **2007**, *18*, 654−659.
(15) Reinhart, R. A. *Arch. Intern. Med.* **1988**, *148*, 2415−2420.
(16) Fanconi, A.; Rose, G. A. *Q. J. Med.* **1958**, *27*, 463−494.

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over a large concentration range primarily due to human impacts, such as industrial and agricultural applications.[17] In North American rivers, calcium and magnesium concentrations vary between 0.4 and 1.7 mM and between 0.2 and 0.9 mM, respectively.[18] However, in wastewater-impacted source water and groundwater, free calcium and magnesium were measured in concentrations as high as 10 mM.[19−21] Concentrations of calcium and magnesium of the same order of magnitude have been shown to highly affect bacterial adhesion[3] and the flocculation or gelation of biomacromolecules.[22]

Enhancements in adhesion and aggregation of bacteria and biopolymers by divalent cations rely on screening and neutralization of surface charge[23,24] and on the formation of cationic bridges between specific negatively charged functional groups.[25] However, the simultaneous effects of calcium and magnesium on more intricate systems that include both motile bacterial cells and conditioning films remain unknown. The control of such complex systems, which begin to approach the complexity of conditions encountered in natural and engineered systems, would allow the development of more efficient antifouling substrates and cleaning treatments for bacterial adhesion prevention.

We studied the adhesion of flagellated and nonflagellated bacteria on conditioned surfaces as a function of calcium and magnesium concentrations to determine the simultaneous impact of divalent cations on cell adhesion and film complexation. Experiments were conducted using an alginate layer as a model conditioning film and *Pseudomonas aeruginosa* PAO1 as a model bacterial species. PAO1 is a well-characterized pathogen that is extremely active in the biofouling of substrates in natural and engineered aquatic systems.[26] Motile and nonmotile mutant strains were used to study the specific effects of divalent cations on cell adhesion. We demonstrate that specific divalent cation bridging controls the adhesion of nonmotile bacteria on clean and conditioned substrates. However, the adhesion of motile cells is mostly governed by the steric and electrostatic interactions between the flagella and the substrates.

2. Materials and Methods

2.1. Bacterial Cell and Substrate Preparation and Characterization. Motile, PAO1 Δ*pilA*, and nonmotile, PAO1 Δ*fliC* Δ*pilA*, strains of *P. aeruginosa* were used as model bacteria. We have previously shown that both strains are deficient in biosynthesis of type IV pilus protein and lack twitching motility.[5] PAO1 Δ*fliC* Δ*pilA* was shown to be deficient in the biosynthesis of the flagellin protein and lack swimming motility. PAO1 strains were incubated in LB at 37 °C and harvested at midexponential growth phase. The bacterial suspension was centrifuged (Sorvall RC 26 Plus) for 15 min at 1000*g*. The cell pellet was washed once with 100 mM KCl solution, recentrifuged under the same conditions, and resuspended in KCl (100 mM). Carboxylated modified latex (CML) particles (1.2 μm in diameter) were used as model nonbiological particles (Invitrogen, California).

The average bacterial size (over 100 cells) was determined by contrast phase microscopy as a function of divalent cation concentration (0.3 and 3 mM). The major and minor axes were calculated assuming an ellipse with the same second moments as rod-shaped bacteria (Matlab, The MathWorks Inc., Massachusetts).[5] Cell viability was determined as a function of divalent cation concentration using a BacLight Viability kit (Molecular Probes, Oregon), which uses fluorescence to detect stained dead and live cells. The presence of divalent cations could be a potential source of fluorescence quenching; therefore, cell viability was confirmed on independent cell cultures by colony-forming unit (CFU) assays.

Ultrapure quartz coverslips (Electron Microscopy Sciences, Pennsylvania) were cleaned in a three-step procedure to remove organic, metal, and residual carbon impurities. Favorable (nonrepulsive) deposition conditions between the particles and the substrate were induced through charge reversal by adsorption of a layer of poly-L-lysine (PLL) at the quartz surface. Deposition experiments under favorable conditions were performed in the presence of calcium chloride or magnesium chloride (0.3 and 3 mM) at ambient pH (5.4−5.6). Alginate films were formed by adsorption of sodium alginate (0.1 g/L, 10 mM KCl solution) for 15 min on PLL-coated quartz under laminar flow. We used commercial sodium alginate (Sigma, Missouri) with an average molecular weight of 72 700. Alginate films were rinsed prior to bacterial adhesion experiments for 15 min with the electrolyte solution used during the experiment. Details on cleaning and preparation of the substrate and conditioning film are given elsewhere.[5]

Electrophoretic mobility measurements (ZetaPALS, Brookhaven Instruments Corp., New York) of bacterial cells, CML particles, and substrates were obtained as a function of divalent cation concentration. Clean and alginate-coated silica particles measuring 1.6 μm in diameter (Bangs Laboratories, Inc., Indiana) were used as surrogates for clean and modified quartz slides. The size of the clean and coated particles was measured by light scattering (ZetaPALS, Brookhaven Instruments Corp.) as a function of divalent cation concentration.

2.2. Deposition Kinetics in Stagnation Point Flow. Deposition kinetics were studied in a well-controlled radial stagnation point flow (RSPF) system mounted on the stage of an inverted microscope (Axiovert 200, Zeiss) operated in contrast phase. Particle deposition was recorded at regular intervals (10−20 s) for 10 min with a DP70 digital camera (Olympus). The distance between the outlet of the injection capillary (2 mm diameter) and the collector surface was 2 mm. All experiments were conducted under the same hydrodynamic conditions and constant temperature (25 ± 1 °C), maintaining a constant flow of 4.93 mL/min (average capillary velocity 2.65 cm/s). The Reynolds number at the outlet of the capillary is 28.4, resulting in a bacterial cell Peclet number of 0.22.[27]

Deposition kinetics were studied as a function of divalent cation concentration (calcium or magnesium chloride) at ambient pH (5.4−5.6). The cell concentration of each culture was determined in a Buerker−Tuerk cytometer chamber (Marienfeld Laboratory Glassware, Germany). Prior to each experiment, the concentrated cell suspension or the CML particle stock solution was diluted into the electrolyte solution of interest. The transfer rate coefficient, k_{RSPF}, and attachment efficiency, α, were calculated for each deposition experiment.[24] Reported values of attachment efficiency are averages of data taken from 2−4 experiments conducted using discrete cell cultures.

2.3. Quartz Crystal Microbalance with Dissipation. The quartz crystal microbalance with dissipation (QCM-D 300, Q-Sense AB, Sweden) was operated at its fundamental frequency of 5 MHz using AT-cut quartz crystals with SiO_2 coating. Structural and viscoelastic properties of the alginate conditioning film were calculated using data from the fifth and seventh overtones (resonance frequencies of 15, 25, and 35 MHz). All experiments were conducted in continuous-

(17) Hammes, F.; Seka, A.; de Kniff, S.; Verstraete, W. *Water Res.* **2003**, *37*, 699−704.
(18) Smith, R. A.; Alexander, R. B.; Wolman, M. G. *Science* **1987**, *235*, 1607−1615.
(19) van Langerak, E. P. A.; Hamelers, H. V. M. *Water Sci. Technol.* **1997**, *36*, 341−348.
(20) Awadallah, R. M.; Ismail, S. S.; Soltan, M. E. *J. Environ. Sci. Health, Part A: Environ. Sci. Eng. Toxic Hazard. Subst. Control* **1996**, *31*, 273−282.
(21) DeSimone, L. A.; Howes, B. L.; Barlow, P. M. *J. Hydrol.* **1997**, *203*, 228−249.
(22) Sobeck, D. C.; Higgins, M. J. *Water Res.* **2002**, *36*, 527−538.
(23) Simoni, S. F.; Bosma, T. N. P.; Harms, H.; Zehnder, A. J. B. *Environ. Sci. Technol.* **2000**, *34*, 1011−1017.
(24) Chen, G. X.; Walker, S. L. *Langmuir* **2007**, *23*, 7162−7169.
(25) Davis, T. A.; Llanes, F.; Volesky, B.; Mucci, A. *Environ. Sci. Technol.* **2003**, *37*, 261−267.
(26) Grobe, S.; Wingender, J.; Truper, H. G. *J. Appl. Bacteriol.* **1995**, *79*, 94−102.
(27) Elimelech, M.; Gregory, J.; Jia, X.; Williams, R. A. In *Particle Deposition and Aggregation: Measurement, Modeling and Simulation*; Williams, R. A., Ed.; Butterworth-Heinemann: Newton, MA, 1995.

flow-through mode at a constant flow of 0.2 mL/min. Detailed experimental and analytical procedures can be found in our previous work.[6,28]

Alginate films were formed by adsorption of dissolved sodium alginate (0.1 g/L) for 15 min on PLL-coated quartz in a 10 mM KCl solution. The adsorbed films were rinsed with the electrolyte solution of interest for 15−60 min until the shifts in frequency and dissipation became statistically insignificant. Measurements were carried out at 25 ± 1 °C and ambient pH (5.4−5.6). The film thickness, elastic shear modulus, and shear viscosity were calculated using Q-Tools (Q-Sense AB) and are based on the Sauerbrey equation[29] or the Voigt model[30] assuming a film density of 1030 kg/m^3.[28] The thickness and density of the film were assumed to be uniform.

3. Results and Discussion

3.1. Influence of Divalent Cations on Alginate Films. The structural properties of the alginate conditioning film in the presence of divalent cations were studied using the quartz crystal microbalance with dissipation. The overall film rigidity was determined by the ratio of shifts in dissipation to shifts in frequency.[28] The Voigt model was used to calculate both the thickness and viscoelastic properties of the film; however, this model cannot be successfully applied to very rigid films.[30] In these cases, the thickness was determined by the Sauerbrey equation.[29]

The change in layer thickness, calculated using the Sauerbrey equation, was monitored during initial adsorption of the alginate layer and for the duration of the film rinsing with solutions containing either calcium or magnesium salts (0.1−10 mM) (Figure 1). No significant differences in the alginate layer structure were observed after rinsing with solutions containing less than 1.8 mM calcium (Figure 1A, average thickness of 0.5 ± 0.2 nm) or less than 10 mM magnesium (Figure 1B, average thickness of 0.6 ± 0.1 nm). At higher calcium concentrations (1.8 mM and higher), the layer thickness increased with increasing calcium concentrations, reaching a maximum of 10.6 nm at 10 mM calcium chloride. In addition, the elastic shear modulus and the shear viscosity of the alginate film decreased from 3.62×10^{-5} to 1.21×10^{-5} N m^{-2} and from 2.5×10^{-3} to 1.3×10^{-3} N s m^{-2}, respectively, with calcium concentrations between 1.8 and 10 mM. These values are comparable to our recently published data on alginate films[6] and are indicative of increasing fluidity of the alginate layer. However, at high magnesium concentrations (i.e., 10 mM), only a slight increase in layer thickness (i.e., 1.6 nm) was observed.

Increasing layer thickness and fluidity at calcium concentrations greater than 1.0 mM suggest that alginate complexation does occur in the presence of calcium. Gelation of alginate polysaccharides results from strong and specific interactions between calcium and guluronic acid blocks, which are major constituents of the alginate polymer.[31] The formation of polymeric dimers by sequestration of divalent cations is known to form a highly hydrated matrix.[32] In contrast, the minor increase in layer thickness observed in the presence of 10 mM magnesium chloride can be attributed to film swelling as the total ionic strength of the system increases[6,28] and not to alginate complexation. This further confirms the unique and specific properties of calcium to complex alginate polysaccharides.

(28) de Kerchove, A. J.; Elimelech, M. *Macromolecules* **2006**, *39*, 6558−6564.
(29) Sauerbrey, G. Z. *Z. Phys.* **1959**, *155*, 206−212.
(30) Voinova, M. V.; Rodahl, M.; Jonson, M.; Kasemo, B. *Phys. Scr.* **1999**, *59*, 391−396.
(31) Grant, G. T.; Morris, E. R.; Rees, D. A.; Smith, P. J. C.; Thom, D. *FEBS Lett.* **1973**, *32*, 195−198.
(32) Smidsrod, O.; Haug, A. *Acta Chem. Scand.* **1965**, *19*, 329−340.

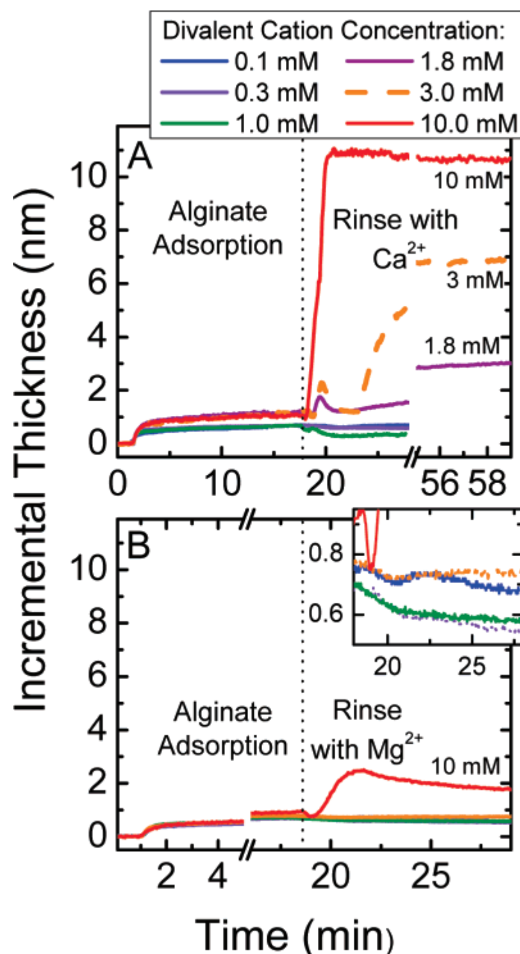

Figure 1. Variation of the thickness of an alginate layer during alginate adsorption and subsequent rinse with a solution containing different concentrations of (A) calcium chloride and (B) magnesium chloride, obtained using the QCM-D. Initial alginate adsorption was conducted using a fixed ionic strength background solution (10 mM KCl). The subsequent rinse solution contained either calcium or magnesium chloride (0.1−10 mM) only. A flow rate of 0.2 mL/min was employed; measurements were carried out at ambient pH (5.5−5.7) and a temperature of 25 ± 1 °C. The thicknesses of gel layers formed at 1.8, 3.0, and 10.0 mM calcium were calculated using the Voigt-based model (considering harmonics 5 and 7, in a single-layer system, and a fixed-layer density of 1030 kg/m^3); the thicknesses of all other layers were calculated with the Sauerbrey equation.

3.2. Electrokinetic Properties of Bare and Alginate-Coated Substrates. To estimate the surface potential of the substrates in the presence of divalent cations, the electrophoretic mobility of bare and alginate-coated silica particles was measured as a function of calcium (Figure 2A) and magnesium (Figure 2B) concentrations. Similar electrophoretic mobilities were observed for bare silica particles in the presence of either divalent salt, with the surface potential becoming less negative with increasing divalent cation concentration. For both divalent salts, in general, significantly more negative electrophoretic mobilities were measured for alginate-coated particles than those measured for bare particles. This suggests that the negative charge was increased at the particle surface as a result of alginate adsorption. Interestingly, at calcium concentrations greater than 1 mM (Figure 2A), an abrupt reduction in the magnitude of the electrophoretic mobility of alginate-coated particles was observed, which was not replicated with magnesium.

To examine whether the observed change in electrophoretic mobility at high calcium concentrations was an artifact of the experimental technique, we measured the size of the suspended

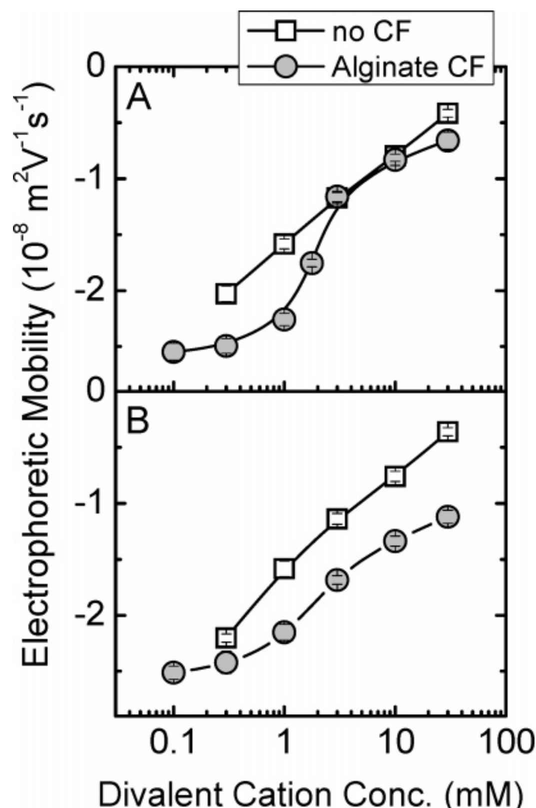

Figure 2. Electrophoretic mobility of clean and alginate-film-coated silica particles as a function of (A) calcium and (B) magnesium chloride concentrations (0.1−30 mM). Measurements were carried out at ambient pH (5.4−5.6) and a temperature of 25 ± 1 °C. Error bars indicate 1 standard error.

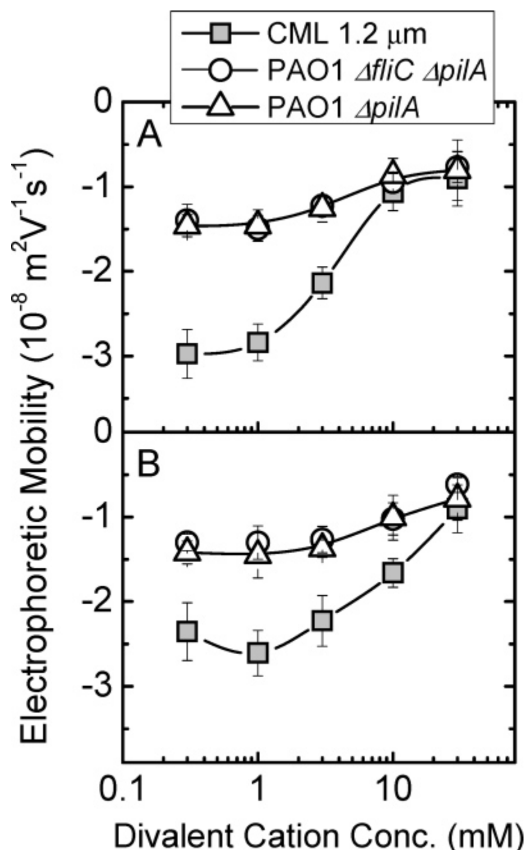

Figure 3. Electrophoretic mobility of CML, PAO1 Δ*fliC* Δ*pilA*, and PAO1 Δ*pilA* as a function of (A) calcium and (B) magnesium chloride concentrations (0.3−30 mM). Measurements were carried out at ambient pH (5.4−5.6) and a temperature of 25 ± 1 °C. Error bars indicate 1 standard error.

alginate-coated particles using dynamic light scattering. At calcium concentrations of less than 1 mM and over the entire range of magnesium concentrations (0.1−10 mM), a constant particle diameter of 1.8 ± 0.2 μm was measured. This diameter is equivalent to the diameter of bare silica particles (i.e., ∼1.6 μm), which suggests that alginate-coated particles do not aggregate under these conditions. However, at 1.8 and 3 mM calcium, the diameter of alginate-coated particles was estimated as 9 ± 2 and 25 ± 5 μm, respectively. This dramatic increase in measured particle size implies that aggregation of the particles through calcium/alginate bridging[33] takes place at calcium concentrations greater than 1 mM. These large aggregates display lower electrophoretic mobility due to the competitive effect of sedimentation, as well as the enhanced shear forces and friction with the surrounding fluid.[34]

3.3. Properties of Bacterial Cells and Carboxyl-Modified Latex (CML) Particles. Electrophoretic mobilities of motile and nonmotile cells and CML particles were also measured as a function of calcium (Figure 3A) and magnesium (Figure 3B) concentrations. Both bacterial strains had comparable electrophoretic mobilities with both divalent cations over the entire concentration range studied. The electrophoretic mobility of CML particles was significantly more negative than that of both bacterial strains, which indicates that CML particles have a higher surface potential than bacterial cells. For all particle types, the magnitude of the electrophoretic mobility decreased with increasing salt concentration due to compression of the particle diffuse layer and charge neutralization.[27]

Because deposition kinetics are sensitive to the size and physiological state of cells,[35] we characterized the variation in size and viability of PAO1 Δ*pilA* and PAO1 Δ*fliC* Δ*pilA* as a function of calcium and magnesium concentrations. In both divalent salts, PAO1 Δ*pilA* and PAO1 Δ*fliC* Δ*pilA* cells had major and minor axes of 2.60 ± 0.11 and 0.94 ± 0.01 μm, respectively, which are equivalent to a volumetric spherical diameter of 1.32 ± 0.02 μm. No significant differences in size over a range of divalent cation concentrations (0.3−3 mM) were observed. Viability tests (i.e., BacLight Viability kit and CFU assays) demonstrated that PAO1 Δ*pilA* and PAO1 Δ*fliC* Δ*pilA* cells were equally viable in divalent salts, maintaining a viability averaging 92 ± 4% over cation concentrations ranging from 0.1 to 10 mM.

3.4. Deposition Kinetics under Favorable (Nonrepulsive) Conditions. Particle transfer rates were measured under favorable conditions (i.e., on PLL-coated substrate) as a function of divalent cation concentrations (Table 1). Nonmotile PAO1 Δ*fliC* Δ*pilA* and motile PAO1 Δ*pilA* bacteria exhibited constant transfer rates of $(2.0 \pm 0.1) \times 10^{-7}$ and $(4.6 \pm 0.1) \times 10^{-7}$ m/s, respectively, in the presence of either magnesium or calcium (0.3 and 3 mM). The higher transfer rates for PAO1 Δ*pilA* are attributable to the ability of the cell to swim toward the substrate.

Previously,[5] we demonstrated that the favorable transfer rate for nonmotile bacteria in the presence of monovalent salt (1−100 mM KCl) was $(1.84 \pm 0.07) \times 10^{-7}$ m/s under similar hydrodynamic conditions, which is similar to the favorable rate

(33) Chen, K. L.; Mylon, S. E.; Elimelech, M. *Environ. Sci. Technol.* **2006**, *40*, 1516−1523.
(34) Ottewill, R. H.; Shaw, J. N. *J. Electroanal. Chem.* **1972**, *37*, 133.
(35) Walker, S. L.; Hill, J. E.; Redman, J. A.; Elimelech, M. *Appl. Environ. Microbiol.* **2005**, *71*, 3093−3099.

Table 1. Deposition Kinetics of CML Particles, PAO1 Δ*fliC* Δ*pilA* Cells, and PAO1 Δ*pilA* Cells under Favorable (Nonrepulsive) Conditions in the Presence of Divalent Cations (0.3 and 3 mM)[a]

	k_{RSPF} (10^{-7} m/s)			
	calcium chloride		magnesium chloride	
	0.3 mM	3 mM	0.3 mM	3 mM
PAO1 Δ*fliC* Δ*pilA*	2.0 ± 0.0	1.9 ± 0.2	2.0 ± 0.3	1.9 ± 0.1
PAO1 Δ*pilA*	4.5 ± 0.5	4.5 ± 0.3	4.6 ± 0.3	4.6 ± 0.2
CML	3.5	3.2	3.8	3.1

[a] Deposition kinetics are expressed as the cell transfer rate coefficient, k_{RSPF}. The capillary flow rate in the RSPF system was fixed at 4.93 mL/min (average velocity of 26.5 cm/s), resulting in a capillary Reynolds number of 28.4 and a particle (cell) Peclet number of 0.22. Other experimental conditions employed were an ambient pH (5.5−5.7) and a temperature of 25 ± 1 °C.

measured in the presence of divalent salts. The similarity of these transfer rates indicates that divalent cation salts have no significant impact on the transport rates of nonmotile bacteria. In contrast, favorable transfer rates for motile bacteria in the presence of divalent salts were always significantly higher than transfer rates observed in the presence of monovalent salts at equivalent ionic strengths (i.e., $(3.4 \pm 0.3) \times 10^{-7}$ m/s[5]). In addition, the transfer rates measured in the presence of divalent cations approached the rate obtained previously[5] at physiological saline conditions (i.e., $(5.5 \pm 0.2) \times 10^{-7}$ m/s). These two factors strongly indicate the importance of calcium and magnesium on the physiological mechanisms of cell transport, such as motility and chemotaxis.

Favorable transfer rates of CML particles were intermediate to those of the bacterial strains (Table 1); however, these transfer rates were observed to slightly decrease with increasing divalent cation concentrations. Decreasing transfer rates for CML particles, compared to constant transfer rates for the bacterial strains, may be attributed to the high negative surface potential of latex particles. Our results suggest that CML particles are more sensitive than bacteria to electrostatic shielding by divalent cations, which can subsequently affect the range of attractive electrostatic interactions of these particles with the PLL-coated substrate.[36]

3.5. Deposition Kinetics of Carboxyl-Modified Latex Particles. The impact of the conditioning film on particle deposition was studied by measuring the deposition or attachment efficiency, α, of CML particles onto either bare or alginate-coated quartz in the presence of calcium (Figure 4A) or magnesium (Figure 4B). The attachment efficiency of particles is the ratio of the transfer rate coefficient normalized by the favorable transfer rate at a given solution condition.

Deposition of CML particles onto clean quartz was comparable in the presence of either calcium or magnesium over the entire concentration range studied. Nearly a 2 order of magnitude increase in attachment efficiency (∼0.002−0.2) was observed with an increase in divalent cation concentration from 0.3 to 3 mM. This increase in attachment efficiency results from the screening/neutralization of the particle and substrate surface charges and the subsequent reduction in electrostatic repulsion between the particle and the substrate.[27] In the presence of an alginate conditioning film, we measured particle attachment efficiencies 4−6 times higher than those onto clean quartz at divalent cation concentrations less than 1 mM. However, at higher divalent cation concentrations (>1 mM), deposition enhancements in the presence of the alginate film were significantly reduced and attachment efficiencies began to approach those observed

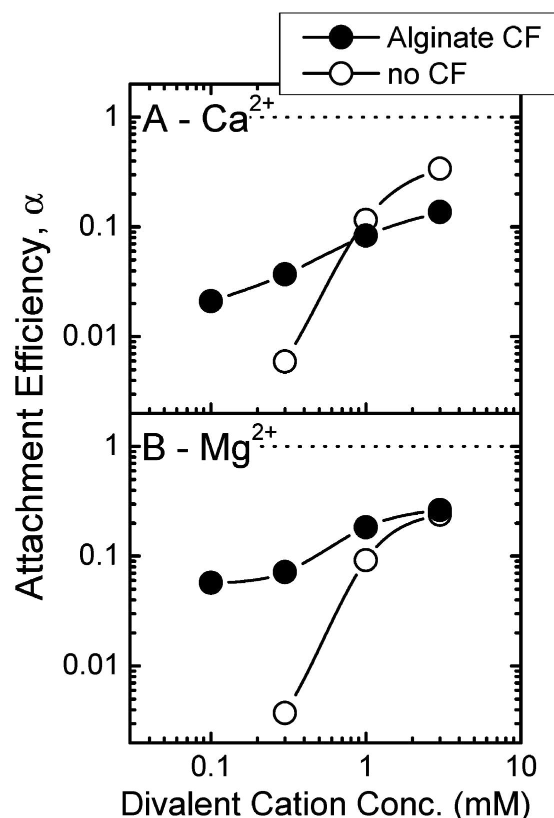

Figure 4. Deposition kinetics of CML particles onto alginate-coated (alginate CF) and clean (no CF) quartz surfaces as a function of (A) calcium and (B) magnesium chloride concentrations (0.1−3 mM). Deposition kinetics are expressed in terms of the attachment efficiency, α. The capillary flow rate in the RSPF system was fixed at 4.93 mL/min (average velocity of 26.5 cm/s), resulting in a capillary Reynolds number of 28.4 and a particle (cell) Peclet number of 0.22. Other experimental conditions employed were an ambient pH (5.5−5.7) and a temperature of 25 ± 1 °C.

onto clean quartz. Interestingly, at high calcium concentrations (i.e., 3 mM), attachment efficiencies onto the conditioning film were even lower than those measured onto clean quartz.

Deposition enhancement onto alginate films may result from surface heterogeneities present on the conditioned substrate surface which facilitate favorable interactions (i.e., hydrophobic interactions, hydrogen bonding, and local favorable electrostatic interactions) with the particles.[37] However, at higher divalent cation concentrations (>1 mM), we observed a change in the film structure (Figure 1) as a result of complexation with calcium or swelling with magnesium. We propose that these structural changes could hinder the ability of the particle to access local heterogeneities present on the film surface, thereby reducing particle deposition. This implies that at higher calcium and magnesium concentrations, only electrostatic interactions and van der Waals dispersion forces govern particle deposition.

3.6. Bacterial Deposition Kinetics. The effects of cell motility and the alginate conditioning film on cell deposition kinetics of the nonmotile (Figure 5A,B) and motile (Figure 5C,D) bacteria onto either clean or alginate-coated quartz were studied in the presence of calcium or magnesium. Attachment efficiencies of nonmotile PAO1 Δ*fliC* Δ*pilA* bacteria onto clean and alginate-coated substrates were similar in the presence of calcium (Figure 5A) and magnesium (Figure 5B) salts. Attachment efficiencies were on average 2 times higher onto the conditioning film than

(36) Elimelech, M. *J. Colloid Interface Sci.* **1994**, *164*, 190−199.

(37) Xu, L. C.; Vadillo-Rodriguez, V.; Logan, B. E. *Langmuir* **2005**, *21*, 7491−7500.

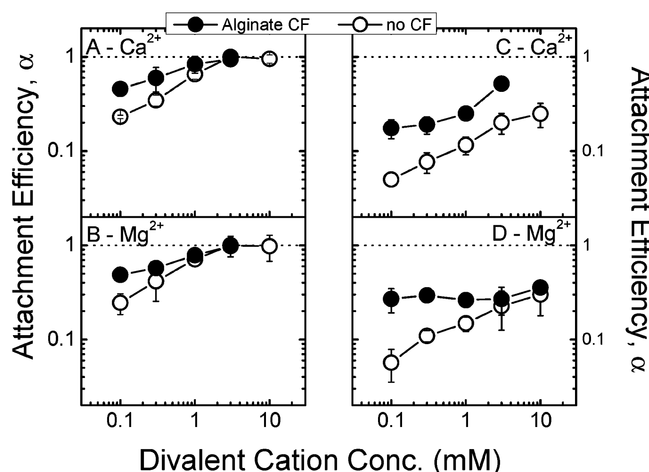

Figure 5. Deposition kinetics of (A, B) PAO1 Δ*fliC* Δ*pilA* and (C, D) PAO1 Δ*pilA* onto alginate-coated (alginate CF) and clean (no CF) quartz surfaces as a function of (A, C) calcium and (B, D) magnesium chloride concentrations (0.1−10 mM). Deposition kinetics are expressed in terms of the attachment efficiency, α. The capillary flow rate in the RSPF system was fixed at 4.93 mL/min (average velocity of 26.5 cm/s), resulting in a capillary Reynolds number of 28.4 and a particle (cell) Peclet number of 0.22. Other experimental conditions employed were an ambient pH (5.5−5.7) and a temperature of 25 ± 1 °C. Error bars indicate 1 standard deviation.

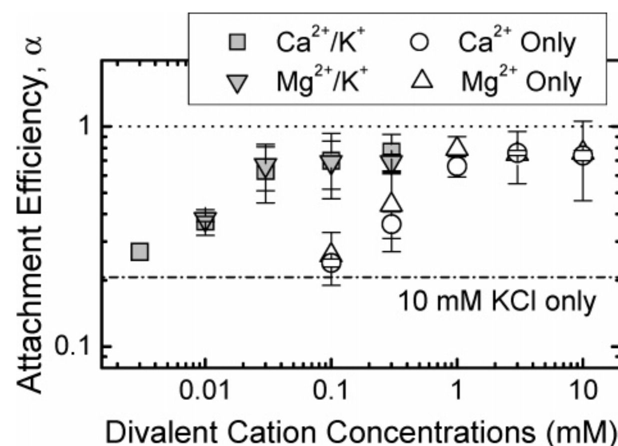

Figure 6. Deposition kinetics of PAO1 Δ*fliC* Δ*pilA* onto clean quartz in a divalent and monovalent salt mixture (solid symbols) and in divalent salts only (open symbols). Deposition kinetics are expressed in terms of the attachment efficiency, α, as a function of divalent cation concentrations. The ionic strength of the salt mixture was maintained constant at 10 mM by addition of KCl. The attachment efficiency obtained in 10 mM KCl solution (i.e., with no divalent cations) is shown as the dashed−dotted line. The capillary flow rate in the RSPF system was fixed at 4.93 mL/min (average velocity of 26.5 cm/s), resulting in a capillary Reynolds number of 28.4 and a particle (cell) Peclet number of 0.22. Other experimental conditions employed were an ambient pH (5.5−5.7) and a temperature of 25 ± 1 °C. Error bars indicate 1 standard deviation.

those onto clean quartz at divalent ion concentrations lower than 1 mM. However, at divalent concentrations greater than 1 mM, maximum attachment efficiencies (α = 1) were quickly attained for both the clean and alginate-coated system. Attachment efficiencies of nonmotile bacteria were greatly enhanced (i.e., 2−5 times higher) compared to attachment efficiencies measured in the presence of monovalent salts at the same ionic strengths (data previously published[5]) for both surface types. In addition, the maximum attachment efficiency in the presence of monovalent cations was reached at concentrations more than 100 times higher than concentrations observed for divalent cations in a clean quartz system.

Attachment efficiencies of motile PAO1 Δ*pilA* bacteria onto clean quartz were comparable in the presence of calcium (Figure 5C) and magnesium (Figure 5D). Attachment efficiencies gradually increased over an order of magnitude with increasing divalent cation concentration (0.1−10 mM). However, these efficiencies were always at least 3 times lower than those observed for nonmotile bacteria. Attachment efficiencies of motile bacteria on clean quartz in the presence of divalent cations were unexpectedly similar to those obtained previously in the presence of monovalent salts at similar ionic strengths.[5] This similarity in deposition kinetics between divalent and monovalent salts strongly suggests that the valence of the cation does not directly affect deposition of motile cells on clean quartz. Instead, cell deposition appears to be mainly governed by the total ionic strength of the system.

For motile bacteria, attachment efficiencies were generally enhanced in the presence of an alginate conditioning film compared to clean quartz for both divalent cations (Figure 5C, D). At low divalent cation concentrations (i.e., 0.1 mM), attachment efficiencies were more than 5 times higher than those measured on clean quartz. Similar attachment efficiencies of 0.24 ± 0.05 were observed for calcium concentrations of less than 1 mM and over the entire range of magnesium concentrations studied (0.1−10 mM). However, at high calcium concentrations (i.e., 3 mM), the cell attachment efficiency increased to 0.52 ± 0.1, which is about 2.5 times higher than that of deposition observed onto clean quartz. In contrast, constant attachment efficiencies, observed with increasing magnesium concentrations, approached deposition values similar to those measured on clean quartz at high magnesium concentrations (3−10 mM). This suggests that structural changes in the alginate layer as a result of calcium specifically impact the attachment of swimming bacteria.

3.7. Impact of Divalent Cations on Adhesion of Nonmotile Bacteria. Our results demonstrate the paramount importance of calcium and magnesium in the attachment of nonmotile PAO1 Δ*fliC* Δ*pilA* bacteria to clean and conditioned substrates. Experimental observations indicated that electrostatic interactions only partially control the adhesion of nonmotile cells and that other specific biological interactions involving calcium and magnesium[22] are significant to cell adhesion. For example, the enhanced deposition of nonmotile bacteria in the presence of divalent cations compared to monovalent cations was significantly underpredicted by the Schulze−Hardy rule,[38] which only considers the efficiencies at which these cations can shield particle charge. The Schulze−Hardy rule[38] states that divalent cations more effectively destabilize colloidal particles than monovalent cations. The theory predicts that a concentration of monovalent cations between 4 and 64 times higher than that of divalent cations is required to completely destabilize colloidal particles. This suggests that the differences in mono- and divalent cation concentrations observed for maximum attachment efficiencies on clean quartz cannot be entirely explained by the higher efficiency of divalent cations in screening charges.

We propose that the enhanced adhesion of nonmotile bacteria in the presence of divalent cations is governed by additional strong interactions between specific features/macromolecules on the bacterial surface and divalent cations. To confirm the impact of biospecific interactions on adhesion of nonmotile bacteria, attachment efficiencies of PAO1 Δ*fliC* Δ*pilA* (Figure 6) were measured on clean quartz in a mixture of monovalent

(38) Overbeek, J. T. G. *Pure Appl. Chem.* **1980**, *52*, 1151−1161.

(potassium) and divalent (calcium or magnesium) salts or in divalent salts only (data reprinted from Figure 5). A fixed total ionic strength of 10 mM was maintained for all salt mixture experiments. We can expect that the partial reduction of the surface potential of both bacteria and substrate at this ionic strength emphasized the effects of the cation valence on adhesion. Attachment efficiencies are expressed as a function of the divalent cation concentration, not ionic strength. Divalent cation concentrations of 0.003−0.3 mM were employed for salt mixtures, and concentrations of 0.1−10 mM were employed for divalent cation solutions only.

In the presence of a salt mixture, enhanced cell adhesion of nonmotile bacteria was once again observed to be similar in the presence of calcium and magnesium salts. However, the maximum attachment efficiency was obtained at a 0.3 mM divalent cation concentration compared to 1 mM previously measured in divalent salts only. We believe that complete elimination of electrostatic repulsion is unrealistic given the low concentrations of divalent salts employed (i.e., 0.003−0.3 mM). In addition, at a 0.003 mM divalent cation concentration, the attachment efficiencies for salt mixtures containing calcium or magnesium were comparable to those measured in 10 mM KCl only (dashed−dotted line, Figure 6). These findings suggest the presence of additional biospecific interactions involving both calcium and magnesium. The results also indicate that a threshold concentration of divalent cations is needed to enhance the adhesion of nonmotile bacteria.

Specific interactions could result from the strong affinity of calcium and magnesium for multiple functional sites at the bacterial surface. For example, calcium and magnesium have been shown to bind to several constituents of lipopolysaccharides, such as mono-, di-, and triphosphates, phosphatidic acid, and 2-keto-3-deoxyoctulosonic acid,[39−41] and to constituents of the bacterial cytoplasmic membrane, such as inorganic phosphate[42] and teichoic acid.[43] These specific associations of cations at the bacterial surface involve specific binding of ions to the functional groups and are not based on nonelectrostatic chemical interactions. The presence of bound calcium and magnesium at the bacterial surface could enhance the adhesion of the cell by (i) creating local charge heterogeneities that could favor electrostatic attractions with the substrate and (ii) forming covalent bridges with sites at the substrate surface that have high affinity for divalent cations.

3.8. Impact of Divalent Cations on Adhesion of Motile Bacteria. Our results indicate that divalent cations significantly enhance the cell swimming motility, which increases the transport of motile cells, but only partially enhances their adhesion. Very low concentrations of divalent cations (i.e., 0.3 mM) were required to reach a favorable transfer rate 2.3 times higher than the rate for nonmotile bacteria (Table 1) and of a magnitude similar to that of cells in physiological saline conditions (i.e., ionic strength of about 150 mM). Divalent cations, which are known for their involvement in biochemical pathways of cell chemotaxis,[44,45] are therefore likely to be of paramount importance in the activation of flagellar rotor mechanisms which govern the swimming ability of the cell.

In contrast with adhesion of nonmotile cells onto clean quartz, the equivalence in attachment efficiencies of motile cells in the presence of divalent (Figure 5C,D) or monovalent (data previously published[5]) salts strongly suggests the absence of direct specific interactions between the bacterial surface of motile cells and the substrate. This result confirms our previous discussion on the adhesion of motile cells onto clean quartz in monovalent salts.[5]

On conditioned quartz, the initial adhesion of motile cells at low divalent cation concentrations (i.e., <1 mM) was significantly and consistently enhanced by the presence of an alginate layer (Figure 5C,D). However, in contrast to nonmotile bacteria, our results did not indicate the importance of specific interactions between the motile bacteria and divalent cations. Therefore, it is likely that, similar to CML particle adhesion, the enhanced attachment of motile cells onto alginate films can be attributed to the presence of local heterogeneities at the film surface; local heterogeneities can directly interact with the cell flagella. At these low ionic strengths (<1 mM), the alginate film is compact and rigid (Figure 1) and therefore is not expected to sterically hinder the approach of motile cells.

As the divalent cation concentration increases above 1 mM, adhesion of motile bacteria was shown to be strongly influenced by specific structural changes in the alginate layer as a result of magnesium or calcium (Figure 1). Similarly to CML particles (Figure 4B), the swelling of the alginate layer at high magnesium concentrations reduced the initial enhanced adhesion of motile bacteria observed at lower magnesium concentrations (Figure 5D). The extension of adsorbed polysaccharides into solution likely creates a steric barrier which limits the approach of motile bacteria into the vicinity of the substrate[46] and minimizes access to local heterogeneities. Eventually, these extensions could also result in the reversal of the cell's swimming direction.[47]

In the presence of high calcium concentrations (>1 mM), the complexation of the alginate layer reduced the attachment efficiency of CML particles (Figure 4A) but significantly enhanced the adhesion of motile cells (Figure 5C). The complexation of alginate by calcium significantly increased both the thickness and the fluidity of the conditioning film. Such viscoelastic conditions have been previously shown to increase the fragility of the film.[28] Fragile films are susceptible to fragmentation and can quickly develop rough irregular surfaces under flow conditions.[6] Entrapment of swimming bacteria in cavities of the hydrogel film resulting from their stochastic enhanced transport could significantly contribute to increased cell adhesion on the substrate.[48] In addition, initial cell attachment to the film could also be enhanced by specific interactions between calcium and high-affinity sites at the surface of the alginate film and bacterial cells. In biofilms, *P. aeruginosa* was shown to naturally produce alginates as exopolymeric substances to maintain the cohesion of the biofilm through complexation with calcium cations.[49] Therefore, it is likely that, in suspension, alginates also partially cover the cell surface and participate in the formation of calcium bridging interactions with the alginate conditioning film.

4. Concluding Remarks

Our study provides new insights into the impact of calcium and magnesium cations on the adhesion of motile and nonmotile *P. aeruginosa* onto clean and alginate-conditioned quartz surfaces.

(39) Rivera, M.; Bryan, L. E.; Hancock, R. E. W.; McGroarty, E. J. *J. Bacteriol.* **1988**, *170*, 512−521.
(40) Amro, N. A.; Kotra, L. P.; Wadu-Mesthrige, K.; Bulychev, A.; Mobashery, S.; Liu, G. Y. *Langmuir* **2000**, *16*, 2789−2796.
(41) Schindler, M.; Osborn, M. J. *Biochemistry* **1979**, *18*, 4425−4430.
(42) van Veen, H. W. *Antonie Van Leeuwenhoek* **1997**, *72*, 299−315.
(43) Heptinst, S.; Archibal, A.; Baddiley, J. *Nature* **1970**, *225*, 519.
(44) Shemarova, I. V.; Nesterov, V. P. *J. Evol. Biochem. Physiol.* **2005**, *41*, 12−19.
(45) Ferrandez, A.; Hawkins, A. C.; Summerfield, D. T.; Harwood, C. S. *J. Bacteriol.* **2002**, *184*, 4374−4383.
(46) Biggs, S. *Langmuir* **1995**, *11*, 156−162.
(47) Cisneros, L.; Dombrowski, C.; Goldstein, R. E.; Kessler, J. O. *Phys. Rev. E* **2006**, *73*.
(48) Rasmussen, K.; Ostgaard, K. *Water Res.* **2003**, *37*, 519−524.
(49) Korstgens, V.; Flemming, H. C.; Wingender, J.; Borchard, W. *Water Sci. Technol.* **2001**, *43*, 49−57.

Because of their valence and diverse biochemical functions, calcium and magnesium cations are of paramount importance to the conditioning of substrates by macromolecules and the subsequent attachment of bacteria onto the conditioning film. Divalent cations regulate electrostatic repulsive interactions and initiate strong cation bridging interactions. The specific responses of our model bacterial strains during adhesion onto clean and conditioned substrates allowed us to develop a foundation for the understanding of the effects of the presence of divalent cations and a conditioning film, both of which are commonly encountered in complex environmental and biomedical aquatic systems.

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