Journal of Microbiology, Immunology and Infection (2016) 49, 988–991

Available online at www.sciencedirect.com

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journal homepage: www.e-jmii.com

CASE REPORT

Quantitative study on the effect of calcium and magnesium palmitate on the formation of *Pseudomonas aeruginosa* biofilm

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Received 6 February 2014; received in revised form 2 September 2014; accepted 12 June 2015
Available online 30 June 2015

KEYWORDS
Adhesion force;
Biofilm;
Biomass;
Extracellular polysaccharide;
Pseudomonas aeruginosa

Abstract Calcium palmitate and magnesium palmitate (which are major constituents of waste water) are insoluble precipitates that accumulate in bodies of water. This leads to the formation of biofilms because bacterial cells can use these fatty acid salts as a carbon source. It is important to study the formation of biofilms because they cause corrosion of pipelines and water contamination. In this study, the effect of calcium palmitate and magnesium palmitate on *Pseudomonas aeruginosa* biofilm formation has been evaluated. In the presence of calcium palmitate, the biofilm biomass, extracellular polysaccharide, and adhesion force were 3.45 ± 0.06 (A_{590}), 1810 ± 47 μg, and 14.5 ± 0.9 nN, respectively. In the presence of magnesium palmitate, the biofilm biomass, extracellular polysaccharide, and adhesion force were 2.72 ± 0.03 (A_{590}), 1370 ± 56 μg, and 8.0 ± 0.2 nN, respectively. The results suggest that biofilm biomass, extracellular polysaccharide, and adhesion force were higher in the presence of calcium palmitate.

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http://dx.doi.org/10.1016/j.jmii.2015.06.001

Introduction

Calcium palmitate or magnesium palmitate are formed when calcium or magnesium ions (present in hard water) react with the palmitate anion (sodium palmitate is the main constituent of soaps) to form insoluble calcium palmitate or magnesium palmitate. This insoluble precipitate accumulates in pipelines thereby resulting in the formation of biofilms as bacterial cells use these salts as a carbon source for their growth. Calcium palmitate and magnesium palmitate are also constituents of gallstones. This could lead to the growth of microorganisms and subsequently result in the formation of biofilm. This causes drug resistant infections in humans.

Biofilms consist of bacterial cells immobilized in a matrix of extracellular polymeric substances.[1] This matrix helps in immobilization of the cells and also functions as a protective barrier against antibiotics and disinfectants. The resulting biofilms may increase pipe and machine corrosion leading to economic losses, and some biofilm material may also be released into flowing water, thereby resulting in its contamination. This contaminated water could cause infections in humans.

Hence, there is a need to study the factors that are crucial for biofilm formation because they have been reported to cause losses in industry and also cause infections in humans. In this study, the effect of calcium palmitate and magnesium palmitate on the formation of *Pseudomonas aeruginosa* biofilm has been examined.

Materials and methods

Culture media

The organism *P. aeruginosa* was chosen for this study on biofilm formation because it is a model organism used in biofilm research. The strain (MTCC 2297) was cultured in nutrient broth (Hi Media, Mumbai, India) at 37°C prior to inoculation.

Biofilm production media

M9 minimal media with 1% (w/w) calcium palmitate (Media M1) or 1% (w/w) magnesium palmitate (Media M2) was used for the growth of biofilms. It was found that there was no formation of biofilm in M9 minimal media (without calcium and magnesium palmitate). Bacterial cells can use magnesium palmitate and calcium palmitate as a carbon source (for their growth). Hence they were used in the biofilm production media. The pH of the mineral medium was adjusted to 7.2. After inoculation (with *P. aeruginosa* cells), samples were incubated at 37°C for 5 days.

Synthesis of magnesium and calcium palmitate

Magnesium palmitate and calcium palmitate were prepared according to the procedure given by Quraishi et al.[2] Equimolar concentrations of palmitic acid and potassium hydroxide were mixed. The suspension was heated and stirred continuously to yield potassium palmitate. Magnesium chloride, in slight excess of what was required for the stoichiometric reaction, was added dropwise to the solution. The product was then cooled and the precipitate was filtered and washed with water and acetone to remove any unreacted fatty acids or reactants used. Magnesium palmitate thus obtained was dried to constant weight. Calcium palmitate was prepared in a similar manner.

Analysis using an atomic force microscope

The atomic force microscope (AFM) image of polished silicon substrate was resolved in intermittent contact mode using JPK Instruments AG, Berlin, Germany Nanowizard II and the surface roughness parameters were evaluated. The cantilevers used for this purpose were purchased from Applied Nanostructures Inc., Mountain View, CA, USA. The biofilm samples (for measurement of adhesion force) were prepared according to the procedure given by Oh et al.[3] Silicon nitride probes were used for this study and were calibrated before use. A total of 64 force—distance curves were generated to evaluate the adhesion force of the biofilm.

Sample preparation for inductively coupled plasma analysis

Inductively coupled plasma (ICP) analysis is used to determine the concentration of metal ions (which are present at very low concentrations) with high sensitivity. In this study, this method was used to analyze the concentration of calcium and magnesium ions in the biofilm matrix. The samples for ICP analysis were prepared according to the protocol given by Cruz et al.[4] Cells were collected by centrifugation and washed in double distilled water. They were then acid digested in 2 mL of nitric acid. The digested samples were then centrifuged and the supernatant diluted prior to ICP analysis.

Quantification of biofilm formation

The formation of biofilm was estimated using the protocol of Merritt et al.[5] The biofilm samples were first rinsed with ethanol to remove the loosely bound planktonic cells. They were then incubated in 0.1% crystal violet solution for 10 minutes before rinsing with water to remove any unbound crystal violet. The bound crystal violet from the samples was removed using 95% ethanol (solubilizing buffer). The absorbance of the solubilizing buffer was measured at 590 nm. All experiments were conducted in triplicate.

Isolation and quantification of extracellular polysaccharides

Isolation of extracellular polysaccharides was based on the procedure given by Forde and Fitzgerald.[6] Cells were harvested by centrifuging the sample at 12,500*g* for 15 minutes. They were then suspended in 5 mL of sodium hydroxide and boiled for 15 minutes. After boiling, the samples were centrifuged at 12,500*g* for 30 minutes to remove the cells. Ethanol, 99.9% (v/v), was added to the