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# Proteome responses of *Citrobacter werkmanii* BF-6 planktonic cells and biofilms to calcium chloride

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ABSTRACT

Calcium ions are well-known as intracellular second messengers that also have an important extracellular structural role for bacteria. Recently, we found that denser biofilms were formed by *Citrobacter werkmanii* BF-6 in the presence of 400 mM $Ca^{2+}$ than that of 12.5 mM $Ca^{2+}$. Therefore, we employed two-dimensional (2-D) electrophoresis methods to investigate the proteome profiles of planktonic cells and biofilms in BF-6 under different concentrations of $Ca^{2+}$. Meanwhile, BF-6 biofilm architecture was also visualized with confocal laser scanning microscopy (CLSM). The results demonstrated that BF-6 biofilms formed at the bottom of microtiter plates when grown in the presence of 400 mM $Ca^{2+}$. A total of 151 proteins from planktonic cells and biofilms after exposure of BF-6 cells to 12.5 and 400 mM $Ca^{2+}$ were successfully identified. Different gene ontology (GO) and KEGG pathways were categorized and enriched for the above proteins. Growth in the presence of 400 mM $Ca^{2+}$ induced more complex signal pathways in BF-6 than 12.5 mM $Ca^{2+}$. In addition, the biofilm architectures were also affected by $Ca^{2+}$. Our results show two different modes of biofilm enhancement for *C. werkmanii* in the presence of excess $Ca^{2+}$ and provide a preliminary expression of these differences based on proteomic assays.


## 1. Introduction

Bacterial biofilms are communities of bacteria attached to biotic or abiotic surfaces and enclosed by a matrix of extracellular polymeric substance (EPS) [1,2]. Biofilm development is a multi-step process involving an initial adhesion phase, followed by reversible and irreversible attachment, leading to the formation of mature biofilms that can then disperse [3,4]. It has been reported that all steps in the biofilm development process can be affected by one or two or more of the following metal ions: $Na^{+}$ [5,6], $K^{+}$ [6], $Mg^{2+}$ [7], $Cu^{2+}$ [8], $Mn^{2+}$ [9], $Fe^{3+}$ [10], and $Al^{3+}$ [8], resulting in the promotion or inhibition of biofilm formation.

Calcium ions have been implicated in a large number of biological pathways and usually act as a well-known intracellular second messenger [11]. In addition, this cation also has an important role in maintaining the integrity of cellular structures [12,13]. Moreover, calcium can also modulate bacterial biofilm formation and architecture. The addition of millimolar amounts of calcium to the growth media inhibited intercellular adhesion and biofilm formation by biofilm-associated protein (Bap) positive *Staphylococcus aureus* V329 [14]. The mucoid strain of *Pseudomonas aeruginosa* FRD1 formed more dense biofilms in the presence of 1.0 and 10 mM $CaCl_2$ and the structure and extracellular matrix composition of these biofilms could be affected by the added calcium through increased expression and stability of bacterial extracellular products [15]. Two-dimensional (2-D) gel electrophoresis studies indicated that calcium caused global changes in matrix materials, as well as in cellular and extracellular protein profiles of *Pseudoalteromonas* sp. 1389 biofilms [16]. Similar results were also found in both the mucoid *P. aeruginosa* FRD1 and the non-mucoid strain PAO1 [17]. The strongest increase in biofilm formation by *Xylella fastidiosa* was observed when the cultured PD2 growth media was supplemented with at least 1.0 mM $CaCl_2$ and the role of $Ca^{2+}$ in biofilm formation may be related to the initial surface and cell-to-cell colonization stages of biofilm establishment [18]. $Ca^{2+}$ did not influence planktonic growth of *S. aureus* V329; however, it modulated the biofilm architecture of this strain in a dose dependent manner [19]. Binding of $Ca^{2+}$ to extracellular DNA (eDNA) mediates bacterial aggregation and biofilm formation in both Gram-negative and Gram-positive bacteria [20]. The presence of calcium during biofilm development in *Pseudomonas fluorescens* led to higher surface coverage with distinct structural phenotypes [21]. Meanwhile, the production of extracellular polymeric substances (EPS), phenanthrene degradation and biofilm growth were enhanced by $Ca^{2+}$ in a dose dependent manner in *Pseudomonas mendocina* NR802 [22].

Members of the genus *Citrobacter*, which are known to cause significant nosocomial infections [23], are commonly found in water, soil, food and the intestinal tracts of animals and humans [24]. A biofilm-

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immobilized *Citrobacter* sp. has been used for the bioremediation of heavy metals *via* the activity of an acid-type phosphatase enzyme or their ability to accumulate heavy metals [25–27]. Recently, *Citrobacter werkmanii* BF-6, which has a high level of biofilm formation, has been isolated in our laboratory from industrial waste. In the present study, the effects of calcium ions on biofilm formation in *C. werkmanii* BF-6 and relevant proteome profiles were determined. Two different modes for the promotion of biofilm formation induced by different concentrations of calcium ions in *C. werkmanii* BF-6, as well as the corresponding altered proteome profiles, are reported.

## 2. Materials and methods

### 2.1. Bacterial strains, culture conditions and chemicals

*C. werkmanii* BF-6 was identified and stored in our laboratory [6] and was routinely grown in liquid Luria Bertani (LB) medium containing 1% (wt: vol) sodium chloride, 1% peptone powder (Oxoid, Hampshire, England) and 0.5% yeast powder (Oxoid) at 30 °C under shaking or static conditions throughout all the experiments. All chemicals used in this study were reagent grade and purchased from Sigma (St Louis, MO, USA) unless otherwise indicated.

### 2.2. Microtiter plate assay for biofilm formation

Biofilms of *C. werkmanii* BF-6 were formed on commercially available, pre-sterilized polystyrene flat-bottomed 96-well microtiter plates (Corning Incorporated, Corning, NY, USA), based on our previously published methods [6], which are based on a previously described protocol [28]. According to our previous experiments, maximum biofilm formation by *C. werkmanii* BF-6 was observed on the fourth day when cultured under static conditions in 96-well microtiter plates in LB medium at 30 °C [6]. Therefore, all biofilms and corresponding planktonic cells in this paper were harvested on the fourth day.

### 2.3. Effect of $Ca^{2+}$ on biofilm formation

In order to evaluate the effect of $Ca^{2+}$ on the growth of planktonic cells and biofilm formation by *C. werkmanii* BF-6, the microtiter wells were inoculated with bacterial suspensions at an optical density (OD) at 600 nm of 0.10 and the same volume of LB medium supplemented with various concentrations of $CaCl_2$ (6.25, 12.5, 25, 50, 100, 200, 400, 600, 800, 1000 and 2000 mM). After culturing at 30 °C for 4 days under static conditions, planktonic growth and biofilm formation were determined using a Multiskan GO reader at 600 nm ($OD_{600}$) and 595 nm ($OD_{595}$), respectively, according to the methods described above. For each concentration, eight replicate wells were inoculated and cultures without any $Ca^{2+}$ were used as controls.

### 2.4. Aggregation assay

An aggregation assay was performed according to the previously described method with some modifications [29]. Briefly, aggregation of *C. werkmanii* BF-6 cells was determined growing 6 ml static cultures in LB medium, with or without $Ca^{2+}$ (at either 12.5 and 400 mM) at 30 °C for 4 days in 10 × 160 mm test tubes. The upper 1 ml of the cultures was carefully removed and the $OD_{600}$ was measured (recorded as $OD_{600}$ pre-vortex). The culture tube was then vortexed to resuspend the aggregated cells, and 1 ml of this suspension was removed to determine its $OD_{600}$ (recorded as $OD_{600}$ post-vortex). "Percent aggregation" was calculated using the following formula:

$$100\% \times (OD_{600}\ \text{post-vortex} - OD_{600}\ \text{pre-vortex}) / OD_{600}\ \text{post-vortex}.$$

### 2.5. Assessment of biofilm structures using confocal laser scanning microscopy (CLSM) and image analysis

Biofilms formed on pre-sterilized glass microscope slides were analyzed using CLSM as described previously, with slight modifications [19]. Briefly, a portion of a coverslip was placed at the bottom of one well in 24-well microtiter plate. Another portion of a coverslip was placed in another well at a 45 degree angle. Aliquots of 1.5 ml *C. werkmanii* BF-6 overnight culture ($OD_{600} = 0.05$) supplemented with 12.5 or 400 mM $CaCl_2$ were inoculated into each well. Control wells contained only bacterial suspension without any $CaCl_2$. The microtiter plates were incubated statically at 30 °C. Four days later, the cultured slides were gently removed from the wells and carefully washed three times with deionized water to remove loosely attached cells. The biofilms grown on the coverslips were stained with SYTO9 dye (5 μM; Invitrogen, Carlsbad, CA, USA) in the dark for 15 min at room temperature. The stained coverslips were then washed again at least three times with deionized water and were then visualized by CLSM (LSM 710 Zeiss, Jena, Germany). Finally, quantification of biofilm structures was evaluated using COMSAT software [30,31] based on the obtained serial CLSM figures.

### 2.6. Protein extraction and two-dimensional (2-D) gel electrophoresis

Planktonic cells and biofilms grown in the presence of different concentrations of $Ca^{2+}$ (0, 12.5, or 400 mM) were harvested by suspending and washing with PBS buffer (pH 7.0). Total proteins from planktonic cells and biofilms were extracted using the Bacterial Total Protein Extraction Kit (2-D) (BestBio, Shanghai, China) according to the manufacturer's instructions. Protein concentration was determined using the Bradford Protein Assay Reagent Kit (BestBio) with bovine serum albumin as a standard according to the manufacturer's instructions. Isoelectric focusing (IEF) and subsequent sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) were performed essentially according to previously described methods with minor modifications [32]. Briefly, 300 μg of the extracted total proteins from planktonic cells and biofilms were used to rehydrate ReadyStrip IPG Strips (GE Healthcare), containing a nonlinear gradient from pH 3 to 10 (17 cm) in 0.2% Bio-Lyte ampholyte, 8 M urea, 65 mM dithiothreitol (DTT), 0.2% ampholytes (pH 3–10), 4% CHAPS and trace amounts of 0.001% bromophenol blue. Following overnight rehydration at room temperature, the strips were focused in a PROTEAN isoelectric focusing system (GE Healthcare) according to the manufacturer's instructions, using the following conditions: step 1, 250 V for 20 min; step 2, ramping up to 8000 V over 2.5 h; and step 3, 8000 V for a total of 30 000 V/h. Prior to SDS-PAGE, the strips were transferred into equilibration buffer consisting of 50 mM Tris–HCl, 2% SDS, 30% glycerol, 2 mM DTT and 6 M urea, and subsequently equilibrated with the same solution as above with the DTT replaced by 2.5% iodoacetamide for 2 × 15 min. The strips were loaded on top of 12% acrylamide resolving gels in low-melting-point agarose and the second dimensional separation were performed on an IPGphor electrophoresis system (GE Healthcare) using a vertical SDS-PAGE system. Gels were stained with Coomassie Bright Blue R-250 and scanned by Image Scanner (GE Healthcare) at 600 dpi. The above 2-D gel electrophoresis was performed in triplicate and the best images were recorded. All spots were matched and analyzed by gel-to-gel comparison using Image-Master 2D Elite 5.0 software (GE Healthcare). The spots with statistically significant (Student's *t*-test with a $p$-value $<0.05$) and reproducible changes in abundance were considered to be differentially expressed and were used for subsequent analyses.

### 2.7. Protein identification by MALDI-TOF-MS assay

Differentially expression protein spots were manually excised from 2-D gels, in-gel digested with trypsin (Promega, Madison, WI), and analyzed using MALDI-TOF-MS (Voyager-DE, Perseptive Biosystems, Framingham, MA). Mono-isotopic peptide masses obtained from the

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