Calcium-mediated modulation of *Pseudomonas fluorescens* biofilm formation

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ABSTRACT

Biofilm formation is usually affected by many environmental factors including divalent cations. The purpose of the current work was to analyze how calcium (Ca\(^{2+}\)) affects the biofilm formation of dairy *P. fluorescens* isolates by investigating their growth, swarming motility, biofilm-forming capacity, EPS production, and biofilm structures. Moreover, the regulation mechanism of Ca\(^{2+}\) involved in its biofilm formation was explored through RNA-sequencing analysis. This work revealed that supplementation of 5, 10, 15, and 20 mM Ca\(^{2+}\) significantly reduced the swarming motility of *P. fluorescens* strains (P.F2, P.F4, and P.F17), but the biofilm-forming ability and polysaccharide production were increased after the supplementation of 5 and 10 mM Ca\(^{2+}\). By the supplementation of Ca\(^{2+}\), complex structures with more cell clusters glued together in *P. fluorescens* P.F4 biofilms were confirmed by scanning electron microscopy, and increased biomass and coverage of *P. fluorescens* P.F4 biofilms were observed by confocal laser scanning microscopy. In addition, RNA-sequencing results showed that *P. fluorescens* P.F4 showed a transcriptional response to the supplementation of 10 mM Ca\(^{2+}\), and a total of 137 genes were significantly expressed. The differential genes were represented in 4 upregulated KEGG pathways (nonribosomal peptide structures, quorum sensing, biosynthesis of siderophore group nonribosomal peptides, and phenylalanine metabolism), and 4 downregulated KEGG pathways (flagellar assembly, amino sugar and nucleotide sugar metabolism, nitrotoluene degradation, and cationic antimicrobial peptide (CAMP) resistance). The results indicate that Ca\(^{2+}\) might serve as an enhancer to substantially trigger the biofilm formation of dairy *P. fluorescens* isolates in the dairy industry.

Key words: Biofilm, calcium, RNA-seq, *Pseudomonas fluorescens*

INTRODUCTION

*Pseudomonas fluorescens* has long been regarded as the predominant bacterium in raw milk by its short generation time, simple nutritional requirements, and good adaptability to environments (Li et al., 2023; McHugh et al., 2020). Despite the pasteurization or ultra-high temperature (UHT) treatment applied in the dairy industry, *P. fluorescens* members are frequently found in dairy processing environments, and may contaminate dairy products predominantly via postpasteurization (Reichler et al., 2018). In addition, their production of heat-stable enzymes could negatively affect the quality of dairy products by causing color defects, undesirable odors, bitterness, and coagulation (Carminati et al., 2019; Zhang et al., 2019).

Biofilms are defined as the complicated community of microorganisms adhering on interfaces, and forming a spatially 3-dimension structure consisting of self-secreted extracellular polymeric substances (EPS) (Yuan et al., 2020). Many studies have confirmed that *P. fluorescens* isolates are quick biofilm formers, and may form thick biofilms on different surfaces of equipment of dairy manufacturing plants, such as in plate heat exchangers and evaporators (Yuan et al., 2018; Zou and Liu, 2018). Once formed, the biofilm cells are difficult to be removed by traditional chemical and physical treatments, such as ultrasound, low-energy x-ray irradiation, UV light, sodium hypochlorite, chlorogenic acid, and quaternary ammonium (Yuan et al., 2021b; Pang et al., 2022; Zekanovic et al., 2022; Sun et al., 2022; Pang et al., 2020). *P. fluorescens* biofilm has notoriously been identified as the potential source of microbial enzymes to shorten the quality of dairy products (Teh et al., 2012). Besides, *P. fluorescens* biofilm may not only cause pipelines corrosion in the dairy industry...
(Aswathanarayan and Vittal 2014), but also provide appropriate substratum for the growth of foodborne pathogens by the enhanced EPS production (Kim et al., 2022). For example, Maggio et al. (2021) confirmed that the EPS produced by *P. fluorescens* could facilitate the attachment, colonization, and biofilm formation of *Listeria monocytogenes*. The mixed-species biofilm by *P. fluorescens* and foodborne pathogens may further protect pathogens from chemical disinfectants in multispecies biofilms, creating serious challenges to the dairy industry (Pang et al., 2020).

Temperature, pH, osmotic pressure, nutrients, and divalent cations are the environmental factors that may affect bacterial biofilm formation (Yuan et al., 2020). Calcium (Ca$^{2+}$) is naturally presented in raw milk, and different milk formulations with a wide range of Ca$^{2+}$ concentrations are processed into dairy products in the dairy industry, and there is potential for different Ca$^{2+}$ concentrations in milk formulations to differentially regulate biofilm formation (Wang et al., 2019; Somerton et al., 2015). In general, calcium affects biofilm formation through assisting in conditioning film formation, modifying cell surface adhesins and proteins, altering properties of cell surface, bridging molecules of surfaces and EPS, and regulating gene expressions (Wang et al., 2019). Thereof, the presence of Ca$^{2+}$ may promote the colonization and biofilm formation of dairy isolates in processing facilities, which facilitates their ability to resist cleaning and sanitation efforts (Wang et al., 2021b). However, Ca$^{2+}$ might also have negative impacts on the biofilm formation and biofilm structure of *Staphylococcus aureus* (Shukla and Rao, 2013). Moreover, Ca$^{2+}$ structurally stabilizes biofilms and prevents the dispersion of biofilm by *Bacillus subtilis* (Nishikawa and Kobayashi, 2021). Understanding how Ca$^{2+}$ regulates the biofilm formation of *P. fluorescens* isolates is vital; however, the effect largely varied among different species and the current knowledge is still limited.

Based on the discussion above, this work aims to examine the biofilm formation by various *P. fluorescens* strains under different Ca$^{2+}$ concentrations by exploring biofilm-related phenotypes including bacterial growth, swarming motility, biofilm-forming capacity, EPS production, and biofilm structures. Moreover, the regulation mechanism of Ca$^{2+}$ in biofilm formation was also explored by RNA-sequencing based transcriptomics analysis. The findings of the current study illustrate that the presence of Ca$^{2+}$ reduced swarming motility, promoted the production of polysaccharide, altered biofilm structures, and induced a transcriptional response that may promote continued biofilm formation of *P. fluorescens* strains, which could provide novel insight into the potential roles of calcium in regulating biofilm formation behaviors, and practical strategies for the control of biofilms in the dairy industry.

**MATERIALS AND METHODS**

**Bacterial Strains and Culture Conditions**

Three *P. fluorescens* strains (P.F2, P.F4, and P.F17) were previously isolated from raw milk collected in Yangzhou, China (Li et al., 2023). The strains were maintained in tryptic soy broth (TSB, Difco, USA) with 20% glycerol (China National Pharmaceutical Group Corporation, China) at −80°C, and resuscitated after streaked onto tryptic soy agar plate and incubated for 48 h at 28°C. Then, the *P. fluorescens* isolates were cultured in either Luria Bertani broth (LB, Huanhai Microbiology Science & Technology, Guangzhou, China) or LB supplemented with 4 different concentrations of Ca$^{2+}$ (5, 10, 15, and 20 mM) at 28°C.

**Growth and Biofilm Formation of *P. fluorescens* Isolates**

Both growth and biofilm-forming ability of 3 *P. fluorescens* isolates were tested in LB (as control), or LB supplemented with 4 different concentrations of Ca$^{2+}$ (5, 10, 15, and 20 mM) as previously described (Mangwani et al., 2014). Briefly, after the incubation at 28°C for 24 h, each bacterial culture was diluted to 10$^4$ cfu/mL, and 200 μL of each bacterial suspension were added into each well of a 96-well plate. Growth of each isolate was measured at regular time intervals of 12, 24, and 48 h at 28°C. Then, crystal violet (CV, Aladdin Industrial Corporation, China) staining method was applied to test the biofilm formation of *P. fluorescens* in 96-well plates (Yuan et al., 2022). The medium in plates was poured, and wells were washed with sterile phosphate buffer saline (PBS, pH 7.2) for 3 times, dried for 20 min at room temperature. After fixing by adding 200 μL of methanol (Aladdin Industrial Corporation) into each well, the wells were stained with 200 μL of 0.05% (wt/vol) CV for 10 min. After a washing step, the adhered CV was re-solubilized in 200 μL of 33% (vol/vol) glacial acetic acid. The OD$_{594nm}$ value was measured using a microplate reader (Xinnao Instrument Co., Ltd., China).

Biofilm formation on stainless steel coupons was also analyzed according to the method described by Wang et al. (2021a). Coupons type 304 (1 cm × 1 cm × 0.1 cm) were immersed in acetone (China National Pharmaceutical Group Corporation) for 30 min to remove grease, washed by 75% ethanol (China National Pharmaceutical Group Corporation), rinsed with MilliQ water, and then sterilized by autoclaving at 121°C for
15 min. Then, each coupon was transferred into each well of 12-well plates (Corning, USA). Five mL of LB (as control) or LB supplemented with Ca²⁺ at 5, 10, 15, and 20 mM were inoculated with overnight bacterial cultures to reach an initial concentration of 10⁴ cfu/mL in each well, and incubated at 28°C for 48 h. Each coupon was aseptically removed, rinsed with PBS (pH 7.2), and then placed into a container containing 10 g of sterile glass beads (5 mm diameter) and 10 mL of sterile PBS (pH 7.2). Biofilm cells were detached by vigorously vortex mixing for 2 min. Finally, the obtained dislodged cell suspension was spread onto LB agar plates followed by the incubation at 28°C for 48 h.

**Swarming Motility of *P. fluorescens***

Swarming mobility of *P. fluorescens* was measured in dishes (90 mm diameter) containing 20 mL of LB or LB supplemented with Ca²⁺ solidified with 0.5% agar (Wang et al., 2021b). Five μL of each overnight *P. fluorescens* culture were spotted on the swarming plates, and diameters of swarming motility zones were measured after the incubation at 28°C for 48 h.

**Assessment of Biofilm Structures by Scanning Electron Microscopy (SEM)**

After incubation in LB or LB supplemented with Ca²⁺ at 28°C for 48 h, each coupon was washed by PBS (pH 7.2), fixed with 2.5% glutaraldehyde (Sigma, USA) at 4°C for 16 h, and rinsed with PBS (pH 7.2) for 3 times. Then, each coupon was fixed by 2.5% glutaraldehyde (Sigma, USA) for 90 min followed by 3 consecutive washing with PBS (pH 7.2). After that, each coupon was dehydrated with a series of ethanol (15 min each at 30%, 50%, 70%, 80%, 90%, 95%, and 100%), and critical-dried with liquid CO₂ (Leica CPD-300, Germany). After coated with gold in an ion sputter (Hitachi E-1010, Japan), the observation of samples was performed using SEM (GeminiSEM 300, Carl Zeiss, Germany).

**Assessment of Biofilm Structures by Confocal Laser Scanning Microscopy (CLSM)**

Biofilm structures of *P. fluorescens* isolates were also analyzed by CLSM (Lu et al., 2023). After incubation in LB or LB supplemented with Ca²⁺ at 28°C for 48 h, coupons were rinsed by sterile PBS (pH 7.2), and biofilms were stained by 0.3% SYTO-9 (Thermo Fisher, USA) at room temperature for 20 min in the dark according to the manufacturer’s instructions. Fluorescence of SYTO 9 was recorded in the green channel with excitation lasers at 488 nm to identify living cells. Biofilm morphology was obtained by CLSM (Zeiss LSM880, Carl Zeiss, Germany) with an EC Plan-Neofluar 40X/1.3 Oil DIC M27 objective.

**Quantification of Polysaccharide in EPS**

Polysaccharide in EPS of *P. fluorescens* biofilms was extracted according to Li et al. (2020) with minor changes. Briefly, overnight culture of each *P. fluorescens* strain was diluted to 10⁴ cfu/mL, and aliquots of bacterial suspension were added into wells of 12-well plates before the incubation at 28°C for 48 h. Then, medium in each well was poured, and adherent cells were rinsed by PBS. After that, the biofilms were resuspended in 5 mL of 0.01 M KCl (Sinopharm Chemical Reagent Co., Ltd., China), and collected by vortexing and scraping. Biofilm cells of *P. fluorescens* were dispersed in a sonication water bath (Ningbo Scientz, Ningbo, China) for 1 min at 300 w. After centrifugation at 4°C for 10 min at 7,000 g, the supernatant was removed and filtered by using a 0.22-μm filter (Millipore Ltd., Ireland). The concentration of carbohydrate was measured using a phenolsulfuric acid method (Li et al., 2020).

**Genome Sequencing of *P. fluorescens* P.F4**

The genomic DNA of *P. fluorescens* P.F4 was extracted by the Bacterial DNA Kit (Omega, USA) based on the manufacturer’s instructions. The quality of extracted DNA was analyzed by NanoDrop 2000 spectrophotometer (Thermo Scientific, USA) and 1% agarose gel electrophoresis. The fragmentation and library preparation of DNA was carried out using TruSeqTM DNA Sample Prep Kit (Illumina Inc., San Diego, CA, USA) following the manufacturer’s protocol. The next-generation sequencing of the good quality library was performed by paired-end (2 × 250 bp) technology using V2 Illumina chemistry at Illumina NovaSeq platform (Illumina Inc., San Diego, CA, USA) following the manufacturer’s protocol. The next-generation sequencing of the good quality library was performed by paired-end (2 × 250 bp) technology using V2 Illumina chemistry at Illumina NovaSeq platform (Illumina Inc., San Diego, CA, USA). The sequences were assembled by ABYSS, and raw paired-end reads were controlled by Trimmomatic. The genetic prediction was performed using GeneMarkS. The genes were compared and annotated based on 5 databases, including nonredundant protein (NR) database, Gene Ontology (GO), Clusters of Orthologous Groups (COG), Kyoto Encyclopedia of Genes and Genomes (KEGG), and UniProt/Swiss-Prot (Yuan et al., 2023a).

**RNA-sequencing Analysis of the Induced Biofilm Formation by *P. fluorescens* P.F4**

In this study, the supplementation of Ca²⁺ at 10 mM increased the biofilm formation of *P. fluorescens* P.F4.
the most, and thereof, this concentration was used for the analysis of the induced biofilm formation of \( P. \, \text{fluorescens} \) \( \text{P.F}_4 \) by RNA-sequencing. Total RNA was extracted from \( P. \, \text{fluorescens} \) \( \text{P.F}_4 \) grown in LB broth with or without the 10 mM of \( \text{Ca}^{2+} \) by using the Trizol Reagent (Qiagen, Germany), and was further treated by RNaseasy Mini kit (Qiagen, Germany) for purification. The RNA quality was analyzed by 2100 Bioanalyser (Agilent Genomics, USA) and Nanodrop 2000 (Thermo Scientific, USA). Then, ribosomal RNA (rRNA) from the total RNAs was removed by the Ribo-Zero rRNA Removal Kit (Illumina, USA), and the messenger RNA (mRNA) was fragmented in the fragmentation buffer. Sequencing libraries were constructed by TruSeq Stranded mRNA Library Preparation Kit (Illumina, USA). All the processes were conducted according to the instructions from manufacturers.

The constructed cDNA libraries were sequenced by Illumina HisSeq 4000 platform (Illumina, USA). Low quality bases (Q score <30) and short sequences (<20 bp) from raw RNA-sequencing reads were filtered, and the clean reads were mapped to the sequenced strain \( P. \, \text{fluorescens} \) \( \text{P.F}_4 \) in this study. Gene expression level was measured by fragments per kilobase per million (RPKM) values. Transcripts that showed the fold change of \( \geq 2 \) and false discovery rate (FDR) of <0.05 were significantly differentially expressed genes (DEGs). All the DEGs were enriched on the basis of GO annotations and KEGG pathway.

**Validation by reverse transcription quantitative PCR (RT-qPCR)**

The RNA-sequencing results were validated by RT-qPCR analysis. Total RNA of each strain was extracted as described before, and was used for cDNA synthesis using reverse transcriptase. The 7500 Fast Real-time PCR System (Applied Biosystem, USA) was used to perform thermal cycling and record fluorescence changes. The relative transcription levels of each targeted genes (oprM, hutH, cadA, udq, and ribD) were calculated by the comparative threshold cycle method \( (2^{-\Delta\Delta CT}) \) (Yuan et al., 2023b).

**Statistical Analysis**

All results in this study are expressed as mean ± standard deviation from experiments in triplicate. One way ANOVA (ANOVA) in SPSS (IBM, USA) was applied for the analysis of statistical significance (\( P \) value <0.05).

**RESULTS AND DISCUSSION**

Most \( P. \, \text{fluorescens} \) isolates are strong biofilm formers, and have been frequently isolated in biofilms formed on surfaces during dairy storage and processing (Yuan et al., 2018). Calcium in milk is highlighted for the growth, bacterial attachment, and biofilm formation of microorganisms. The effects of \( \text{Ca}^{2+} \) on biofilm formation can be highlighted in via diverse mechanisms such as physio-chemical interactions and gene regulations (Cruz et al., 2012; Xiong et al., 2022). This study explored the phenotypic changes and mechanisms during the biofilm formation of dairy \( P. \, \text{fluorescens} \) isolates when in the presence of \( \text{Ca}^{2+} \).

**Calcium Affects the Bacterial Growth of \( P. \, \text{fluorescens} \) Isolates**

The ability of \( P. \, \text{fluorescens} \) isolates to use \( \text{Ca}^{2+} \) for growth was tested in this study (Figure 1). The capacity to cope with \( \text{Ca}^{2+} \) varied among different bacterial isolates, and changed over the time, which indicated that the role of \( \text{Ca}^{2+} \) in bacterial growth is rather dynamic and complex. The growth of 3 isolates were not affected (\( P > 0.05 \)) by the presence of \( \text{Ca}^{2+} \) at the beginning (12 h). High concentrations of \( \text{Ca}^{2+} \) (10, 15 and 20 mM) obviously decreased (\( P < 0.05 \)) bacterial growth of 3 \( P. \, \text{fluorescens} \) strains, and the highest inhibition rates were 23.3% (\( P. \, \text{fluorescens} \) \( \text{P.F}_2 \)), 23% (\( P. \, \text{fluorescens} \) \( \text{P.F}_4 \)), and 24.3% (\( P. \, \text{fluorescens} \) \( \text{P.F}_17 \)) at 24 h. This observation may be explained by the hypothesis that a collective effect of the interaction between \( \text{Ca}^{2+} \) and proteins could cause an inhibitory effect on bacterial growth at certain time point (Wang et al., 2021b). However, this assumption still needs to be verified in future. Additional, all the concentrations of \( \text{Ca}^{2+} \) significantly increased (\( P < 0.05 \)) the growth of \( P. \, \text{fluorescens} \) strains at 48 h, with the increase in \( \text{OD}_{594\text{nm}} \) values from 4.6% to 17.4% (\( P. \, \text{fluorescens} \) \( \text{P.F}_2 \)), from 7.3% to 18.5% (\( P. \, \text{fluorescens} \) \( \text{P.F}_4 \)), and from 4.9% to 17.8% (\( P. \, \text{fluorescens} \) \( \text{P.F}_17 \)). Thus, we concluded that adequate concentrations of \( \text{Ca}^{2+} \) increased \( P. \, \text{fluorescens} \) biofilm formation by promoting bacterial growth or the lifestyle switching from planktonic cells.

**Calcium Affect the Biofilm-forming Capacity of \( P. \, \text{fluorescens} \) Isolates**

In this work, the impact of \( \text{Ca}^{2+} \) on the biofilm-forming capacity of the \( P. \, \text{fluorescens} \) strains were measured by both CV staining and cell enumeration, and the capacity to cope with \( \text{Ca}^{2+} \) varied highly among different bacterial isolates (Figure 2). For the CV method, supplementation of \( \text{Ca}^{2+} \) promoted the biofilm-forming
capacity of the 3 P. fluorescens isolates in both dose-dependent and strain-dependent patterns. In brief, moderate concentrations (5, 10, and 15 mM) of Ca\(^{2+}\) significantly increased \((P < 0.05)\) the biofilm formation of 3 strains, and the highest effect was found in biofilms grown in 10 mM of Ca\(^{2+}\), with 70.5%, 40%, and 48.1% increase for P. fluorescens P.F2, P. fluorescens P.F4, and P. fluorescens P.F17, respectively. However, no significant enhancement \((P > 0.05)\) of the biofilm formation was found when the concentration of Ca\(^{2+}\) reached to a higher level at 20 mM.

The effects of different concentrations of Ca\(^{2+}\) on the biofilm formation of P. fluorescens isolates on stainless steel coupons were different from those obtained from the CV method, mainly due to the different properties of contact surfaces (Wang et al., 2019). Supplementation of Ca\(^{2+}\) at 5, 10, and 15 mM significantly \((P < 0.05)\) increased the biofilm-forming capacity of P. fluorescens P.F2 and P.F17, and the highest increase in biofilm cells was 0.43 Log cfu/cm\(^2\) for P. fluorescens P.F2 at 10 mM, and 0.51 Log cfu/cm\(^2\) for P. fluorescens P.F17 at 15 mM. Meanwhile, the biofilm formation of P. fluorescens P.F4 was significantly enhanced \((P < 0.05)\) by the presence of 10 and 15 mM of Ca\(^{2+}\), with the biofilm cells increased from 5.39 Log cfu/cm\(^2\) to 5.69 Log cfu/cm\(^2\) at 10 mM, and to 5.68 Log cfu/cm\(^2\) at 15 mM.

Previous studies have also concluded that calcium promote the biofilm formation by a variety of bacteria, such as Xylella fastidiosa (Cruz et al., 2012), Bacillus sp. (He et al., 2016), and Pseudomonas sp. (Xiong et al., 2022). The specific-strain response to calcium during the formation of biofilms was also found in other microorganisms such as G. stearothermophilus and Cronobacter sakazakii (Wang et al., 2021b; Ye et al., 2015). In addition, the induction effect is generally in a concentration-dependent way, and high concentrations of calcium may inhibit bacterial biofilm formation. For example, the increasing concentration of Ca\(^{2+}\) in milk significantly promoted the biofilm cell numbers of G. stearothermophilus (Somerton et al., 2015). Shukla et al. (2013) even revealed that the presence of Ca\(^{2+}\) inhibited the biofilm formation S. aureus and modified its biofilm structures. These results of this work indicated that the sensitivity of P. fluorescens to calcium may be different from that of other bacterial species, and this observation needs to be particularly considered when

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Figure 1. Effect of different concentrations of calcium (5, 10, 15, and 20 mM) on the growth of 3 Pseudomonas fluorescens strains (P.F2, P.F4, and P.F17) at 28°C for 12, 24, and 48 h. Data are expressed by the mean of 3 biological repeats with standard deviations. The asterisk (*) and different letters indicate significant difference \((P < 0.05)\) among the growth of strains at 28°C for 48 h and 24 h, respectively.
applying calcium-mediated biofilm control strategies to avoid *P. fluorescens* biofilms in the dairy industry.

**Calcium Affects the Swarming Ability of *P. fluorescens* Isolates**

Three *P. fluorescens* isolates were measured for their swarming ability in Ca²⁺-supplemented LB medium. In general, the swarming ability of the 3 *P. fluorescens* isolates were significantly \( (P < 0.05) \) decreased after the supplementation of Ca²⁺, and the highest inhibition was observed when Ca²⁺ reached 15 mM, with the diameters of the swarming motility zones decreasing from 17.50 mm (P.F₂), 19.00 mm (P.F₄), and 17.17 mm (P.F₁₇) to 6.67 mm, 7.00 mm, and 7.17 mm, respectively (Figure 3).

**Calcium Affects the EPS Production by *P. fluorescens* Isolates**

Bacteria generally create a matrix that hold the mature biofilm together, and enhance the biofilm persistence in environments by the increased production of EPS in the early stages of biofilm formation (Yuan et al., 2021b). In this study, the polysaccharide in EPS varied among different *P. fluorescens* isolates, and the highest content of polysaccharide was produced by *P. fluorescens* P.F₄ (Figure 4). Both 5 and 10 mM Ca²⁺ significantly \( (P < 0.05) \) promoted the amount of polysaccharide of all *P. fluorescens* isolates. However, the difference in polysaccharide was not found \( (P > 0.05) \) when high concentrations of Ca²⁺ (15 and 20 mM) were supplemented. Results from this work suggest that *P. fluorescens* is a strong biofilm former with high exopolysaccharides-producing ability, and the addition of calcium promote the production of polysaccharide, and increase the strength of bacterial attachment to the surfaces. A similar observation in was found in *G. stearothermophilus* where the distribution of polysaccharides in biofilms was influenced by the presence of calcium (Wang et al., 2021b). He et al. (2016) also reported that an additional 10 mM of Ca²⁺ significantly promoted the production of polysaccharide in EPS by *Bacillus* sp. However, the results are inconsistent with a
previous study, which reported that the exogenous Ca$^{2+}$ significantly reduced the polysaccharide content by 10.2–87.7% in the biofilm EPS while increasing biofilm biomass of *Pseudomonas* sp. ZX01 (Xiong et al., 2022).

**Calcium Affects the Structure of *P. fluorescens* Biofilms**

Ca$^{2+}$-induced phenotype changes in biofilm formation may also be influenced by altering cell aggregation, adhesion between biofilm cells and the substratum, and biofilm 3D structures. In this study, notable difference in biofilm morphology and structure of *P. fluorescens* P.F$_4$ after the supplementation of Ca$^{2+}$ was visualized by SEM and CLSM analysis, which were in consistent with the results obtained from the crystal violet and cell enumeration assays. SEM results displayed a complex structure with more cell clusters glued together in biofilms formed on stainless steel coupons after the supplementation of Ca$^{2+}$ (Figure 5). CLSM images also showed the increased biomass, average coverage and thickness of *P. fluorescens* P.F$_4$ biofilms by the supplementation of Ca$^{2+}$ (Figure 6). The role of Ca$^{2+}$ has also been found in consolidating the biofilm structures of many bacterial species. For example, clusters of *Pseudomonas mendocina* cells were glued together in biofilms when supplemented with Ca$^{2+}$ (Mangwani et al., 2014). Sarkisova et al. (2005) found that the supplementation of Ca$^{2+}$ affects both extracellular matrix composition and biofilm structure of *Pseudomonas aeruginosa*. Divalent cations may assist in aggregation of bacteria and EPS and promote the biofilm structural stability by binding to negatively charged functional groups between EPS chains (Sobeck and Higgins, 2002).

**Genome Properties of *P. fluorescens* P.F$_4$**

Whole-genome sequencing is a useful tool to comprehensively analyze the molecular biology features of bacteria at the genetic level, and is important for assessing biofilm formation (Mgomi et al., 2022; Yuan et al., 2023a). *P. fluorescens* P.F$_4$ genome was successfully sequenced in this study. In total, 5391 protein-coding genes were assigned putative functions, which consisted 5282334 bp of its genome with a total length of 6056891 bp, while the others were hypothetical proteins (Figure 7A). Moreover, the results also predicted its G-C content of 60.22%, and 51 tRNA genes, 3 rRNA genes, and 186 non-coding RNAs.

GO functional annotation was performed on the genome of *P. fluorescens* P.F$_4$ (Figure 7B). In total, 5460 genes were annotated into 79 GO items, with 42, 12, and 25 terms corresponding to biological processes (BP), cellular components (CC), and molecular functions (MF), respectively. In BP categories, genes annotation was mainly involved in biological processes (3715 genes), cellular nitrogen compound metabolic process (1336 genes), and biosynthetic process (1322 genes). In CC categories, genes annotation mostly involved in cell (1332 genes), cellular component (1216 genes), and...
intracellular (766 genes). In MF categories, genes annotation mainly involved in molecular function (3554 genes), followed by ion binding (1136 genes), and DNA binding (607 genes).

The COG annotation of *P. fluorescens* P.F4 genome was shown in Figure 7C, and a total of 5461 genes were annotated into 20 groups. The largest functional group was function unknown prediction with 628 genes, followed by 416 genes for amino acid transport and metabolism, and 317 genes for inorganic ion transport and metabolism, which was similar to the COG annotation of *P. fluorescens* previously reported by Biessy et al. (2019).

As shown in Figure 7D, a total of 5459 genes were further annotated into 51 KEGG pathways belonging to 8 groups, including 3 brite hierarchies pathways (2023 genes), 12 metabolism pathways (1796 genes), 4 cellular processes pathways (309 genes), 4 genetic information processing pathways (225 genes), 12 human diseases pathways (221 genes), 9 organismal systems pathways (103 genes), and 4 pathways not included in pathway or brite (445 genes). In particular, several biofilm formation related pathways, such as ABC transporters, quorum sensing (QS), 2-component system, flagellar assembly, biofilm formation, bacterial chemotaxis, were annotated in *P. fluorescens* P.F4 genome.

**Molecular Mechanism of the Induced Biofilm Formation by Ca²⁺**

The role of Ca²⁺ has been established; however, there is no further work on the influence of Ca²⁺ on the biofilm formation by a variety of microorganisms, and relevant molecular mechanism is still elusive. In recent years, advanced omics tools have opened new perspectives for elucidating the key regulators for biofilm formation at levels of genes, RNA, proteins and metabolites (Yuan et al., 2021a).

In this study, RNA-sequencing based transcriptomics tool revealed that extensive reprogramming of gene expression across 8 essential pathways occurred for the involvement of calcium in biofilm formation by *P. fluorescens* P.F4. Overall, RNA-seq analysis showed the significant regulation of 137 genes in *P. fluorescens*.
Figure 5. Scanning electron microscopy observation of biofilm structures of *Pseudomonas fluorescens* P.F4 on stainless steel coupons under different concentrations of calcium (5, 10, 15, and 20 mM).
Figure 6. Confocal laser scanning microscopy observation of biofilm structures of *Pseudomonas fluorescens* P.F4 on stainless steel coupons under different concentrations of calcium (5, 10, 15, and 20 mM).
Figure 7. Whole genome sequencing and annotation of *Pseudomonas fluorescens* P.F4. (A) Genomic circle map of *Pseudomonas fluorescens* P.F4. (B) Gene Ontology (GO) classification of gene function annotation. (C) Cluster of orthologous groups (COG) classification of protein functions. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) classification of gene function annotation. The numbers on the bar chart represent the number of genes annotated.
Figure 7 (Continue). Whole genome sequencing and annotation of *Pseudomonas fluorescens* P.F.4. (A) Genomic circle map of *Pseudomonas fluorescens* P.F.4. (B) Gene Ontology (GO) classification of gene function annotation. (C) Cluster of orthologous groups (COG) classification of protein functions. (D) Kyoto Encyclopedia of Genes and Genomes (KEGG) classification of gene function annotation. The numbers on the bar chart represent the number of genes annotated.
P. F. 4 induced by the supplementation of 10 mM Ca\(^{2+}\), among which 88 and 49 genes were downregulated and upregulated, respectively (Figure 8A). The DEGs more than 2 times as compared with the P. fluorescens P. F. 4 growth in LB without Ca\(^{2+}\) were listed in Table S1. The distribution of DEGs induced by the supplementation of 10 mM Ca\(^{2+}\) was shown in a volcano map (Figure 8B).

GO annotation was further conducted with DEGs from the transcriptomes of Ca\(^{2+}\) treated and control groups (Figure 9A). These DEGs were classified into 86 significantly enriched GO terms (\(P < 0.05\)), with 38 terms corresponding to BP, 6 terms belonging to CC, and 42 terms corresponding to MF. Most of the DEGs were involved in MF related to binding (22 DEGs), and in BP related to biosynthetic process (40 DEGs). These findings are in consistent with the results from phenotype changes induced by Ca\(^{2+}\). Therefore, we speculated that 10 mM of Ca\(^{2+}\) obviously altered the process of biofilm formation by P. fluorescens by regulating cell surface properties. Similar results were also observed in a previous study, which reported that different types and concentrations of ions in the growth environments modulated surface charge and membrane composition of bacteria, and significantly changed genes involved in ion binding and biosynthetic process (Wang et al., 2015).

KEGG pathway analysis was further applied to obtain deep insights into the biological functions of the DEGs (Figure 9B). KEGG analysis results showed that the DEGs were significantly enriched for those involved in 4 upregulated pathways (nonribosomal peptide structures, phenylalanine metabolism, biosynthesis of siderophore group nonribosomal peptides, and QS). In this study, 2 genes (pvdA and pvdQ) involved in the biosynthesis of siderophores were upregulated by the presence of 10 mM Ca\(^{2+}\). The role of siderophore in the biofilm formation by P. fluorescens has been confirmed. For example, pvdA is involved in the biosynthesis of siderophores, and a constructed \(\Delta\)pvdA mutant of P. fluorescens has been shown to have lower ability of EPS production, bacterial motility, and biofilm formation (Chen et al., 2023). QS is a cell density-dependent regulating process, which is employed by microorganisms to communicate each other by the exchange of autoinducers. Increasing evidence have confirmed that functions of QS include bacterial motility, environmental signal sensing, and biofilm formation (Li and Zhao, 2020). In this work, 2 genes (ribD, lgrC) involved in QS were upregulated. Bifunctional pyrimidine deaminase/reductase encoded by ribD plays an important role during riboflavin biosynthesis in a variety of microorganisms, and riboflavin has been found to be involved in biofilm formation (Edel et al., 2021). Gene lgrC is involved in both QS and nonribosomal peptide structures KEGG.
Figure 9. Gene Ontology (GO) annotation (A) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (B) of the differentially expressed genes induced by the supplementation of 10 mM Ca^{2+}.

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pathways, and non-ribosomal peptide synthetases from *P. aeruginosa* play a role in QS regulation (Gonzalez et al., 2017).

In addition, the DEGs involved in 4 KEGG pathways (flagellar assembly, amino sugar and nucleotide sugar metabolism, nitrotoluene degradation, cationic antimicrobial peptide (CAMP) resistance) were down-regulated. The first step during biofilm formation is bacterial attachment, and this process is usually mediated by fimbriae, pili, and flagella. Existing evidence demonstrated that flagellar assembly is required for many bacteria in the early stage of biofilm formation. However, the biofilm-forming capacity is not always in consistent with its swimming or swarming motility (Tang et al., 2019). In consistent with the swarming motility results, 5 genes (*fliL*, *flgB*, *flgN*, *rpoN*, and gene5032) involved in flagellar assembly were down-regulated in this study. Meliani and Bensoltane (2015) proved that one of the first steps of bacterial attachment is to slow down or even stop its flagella rotation. The weak motility in *Pseudomonas* isolates increased the transition from planktonic status to biofilm mode, which resulted in the promotion of initial adhesion and subsequent biofilm formation (Guttenplan and Kearns, 2013). Moreover, the link between biofilm formation and bacterial motility is rather complex, which was associated with certain bacterial strains, and specific conditions (Rossi et al., 2018). Similar results were also observed by Xiong et al. (2022), which proved that Ca$^{2+}$ may neutralize the surface charge of bacterial cells, and further decrease the initial attachment of *Pseudomonas* sp. ZX01 to the positive-charged substratum, but its biofilm-forming ability was promoted. Therefore, we inferred that Ca$^{2+}$ and flagellar assembly may exert a joint regulatory effect on bacterial biofilm formation. Ca$^{2+}$ may indeed promote the formation of biofilms by bacteria; however, the flagella mediated motility still exerts effects on biofilm structures.

The above results clearly revealed the molecular mechanism of how 10 mM Ca$^{2+}$ regulates the biofilm formation of *P. fluorescens*; however, how other concentrations of Ca$^{2+}$ regulating biofilm formation of *P. fluorescens* P.F4 are not included in this work, and need to be clarified in the future.

In addition, the expression of the 5 genes from RT-qPCR analysis showed good consistency with those from RNA-seq analysis, which suggests that the RNA-seq results were credible (Figure 10).

**CONCLUSION**

The influence of Ca$^{2+}$ on biofilm formation was explored for 3 *P. fluorescens* isolates in this study. A strict strain-specific response was found in bacterial mobility, biofilm-forming ability, EPS production, and biofilm architecture. Collectively, Ca$^{2+}$ reduced swarming motility, while moderate concentrations of Ca$^{2+}$ increased the biofilm-forming capacity, the production of EPS of all *P. fluorescens* strains. Increased biomass and coverage, and more complicated structures of *P. fluorescens* biofilms were also observed after the supplementation of Ca$^{2+}$. The mechanism of calcium involved in promoting the continued biofilm formation by *P. fluorescens* was also investigated, and a large number of genes were significantly expressed. The significantly expression of genes suggest the involvement of Ca$^{2+}$ in the biofilm formation of *P. fluorescens* through different pathways (nonribosomal peptide structures, quorum sensing, biosynthesis of siderophore group nonribosomal peptides, and phenylalanine metabolism, flagellar assembly, etc.).
amino sugar and nucleotide sugar metabolism, nitrotoluene degradation, and cationic antimicrobial peptide (CAMP) resistance. Despite the above results, the role of calcium in gene regulation is equivocal, and well-depicted regulatory mechanisms of different bacterial species are still required to generate a comprehensive view of different dairy isolates. In the near future, it would be more interesting to explore how different concentrations of Ca^{2+} regulate the biofilm formation of a more diverse *P. fluorescens* strains with different genetic backgrounds under more realistic dairy processing conditions.

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