Identification of Factors Involved in Biofilm Formation by a Non-motile Gram-negative Bacterium, *Paracoccus denitrificans*

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Keitaro YOSHIDA

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Keitaro YOSHIDA

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Contents

(Summarized version)

Chapter 1
Introduction

Many bacteria exist as multicellular groups in the environments rather than as planktonic states. The multicellular communities formed on surfaces are predominant bacterial mode of life, called biofilms. Understanding biofilm formation mechanisms is essential to interpret bacterial ecology in the environments and also to control beneficial and harmful biofilms in industrial and medical settings. Biofilm formation is studied in detail in motile bacteria, where it is a sequential, developmental process involving expression of biofilm-specific genes. Cellular motility is one of essential factors to biofilm formation, and swimming motility significantly accelerates the initial step of cell attachment to surfaces. Despite the detailed understanding about motile bacteria, not all bacteria are motile. While non-motile bacteria cannot actively attach to surfaces, they are considered to attach to surfaces passively and form biofilms via the surrounding water flow or settling. However, the understanding of biofilm formation by non-motile bacteria is still poorly understood especially in Gram-negative bacteria. This study focuses on a non-motile Gram-negative bacterium, *Paracoccus denitrificans*, which is a metabolically versatile, soil bacterium belonging to Alphaproteobacteria. Because *P. denitrificans* shows high denitrification ability, it is considered as an important species in wastewater treatments, where *P. denitrificans* is found in biofilms. Despite its importance, how this bacterium attaches to surfaces and organizes themselves into biofilms is poorly understood. The aim of this study is to examine biofilm formation of non-motile Gram-negative bacteria using *P. denitrificans* as a model strain.

Chapter 2
Results

To examine the formation of biofilms in *P. denitrificans*, microtiter plate assays were performed. Despite the lack of motility, *P. denitrificans* formed a biofilm on a microtiter plate at the air-liquid interface under static conditions. Structural analyses of the biofilm by a microscope revealed that the biofilm is very thin with a thickness of ~4 μm, where cells densely compacted within the biofilm. Cells in chain were frequently observed in the biofilm, which was presumably formed by cell division. These observation suggests that *P. denitrificans* forms biofilms by spreading on the surface, which driven by cell growth. Because extracellular components are important to sustain biofilm structure, extracellular components involved in *P. denitrificans* biofilm formation were examined by adding DNase I or proteinase K to the media prior to incubation. While not affected by DNase I, biofilm formation was significantly inhibited by proteinase K, suggesting that extracellular protein components, but not DNA, are necessary for biofilm formation. To identify biofilm-related genes, transposon mutant library was constructed with Tn*5*, and screened for mutants that cannot form a biofilm. Of ~4,500 Tn mutants, 26 mutants showed inability to form a biofilm. Transposon insertion sites in these mutants were identified by sequencing, where transposons were intensively inserted to the genomic region between PDEN_RS11975 and PDEN_RS12005 genes in 11 mutants, indicating that this region encodes genes required for biofilm formation. Because this genomic region contains a large intergenic region of >5000-bp, open reading frames (ORF) were searched for this region. Missing from the genome database, a 6,636-bp ORF was found. This putative gene was likely involved in biofilm formation and thus named biofilm-associated protein (*bapA*). In addition, there are genes encoding the HlyBD-TolC T1SS downstream to *bapA*, that were accordingly named *bapB*, *bapC* and *bapD*. To examine the involvement of these genes in biofilm formation, biofilm formation assays were performed with newly constructed in-frame deletion mutants of *bapA*, *bapB*, *bapC* and *bapD* genes. The *bap* mutants did not form biofilms, indicating that *bap* genes are required for biofilm formation. Moreover, microscopic observation revealed that the Δ*bapA* mutant did not attach, indicating that BapA is

necessary for the initial attachment to the surface. To examine the localization of BapA, the presence of BapA was analyzed in the cell lysate and supernatant by Western blotting in the *bap* mutants. BapA was detected in both cell and supernatant fractions of the WT, which remained in cell fractions in the Δ*bapB*, Δ*bapC* and Δ*bapD* mutants, indicating that BapBCD transporter is involved in BapA secretion to extracellular milieu. To further analyze BapA localization, BapA was visualized by immunofluorescence using super-resolution microscopy. In the WT, BapA was dotted around the cells, indicating that BapA is localized on the cell surface. Cell surface hydrophobicity is an important property for bacterial adhesion. To examine if BapA affects hydrophobic interactions, cell surface hydrophobicity of the *bap* mutants was measured. Deletions of *bap* genes significantly reduced cell hydrophobicity compared with the WT, suggesting that BapA on the cell surface increases cell hydrophobicity. These results indicate that *P. denitrificans* secretes BapA, which makes the cell surface hydrophobic, leading to cell adhesion to surfaces via hydrophobic interactions. In addition, this is the first study to identify a T1SS-dependent adhesin in Alphaproteobacteria.

Chapter 3
Discussion

In Gram-negative bacteria, while biofilm formation has been studied in detail in motile bacteria, much less is understood about non-motile bacteria (1). This study identifies an adhesin BapA, which is essential to biofilm formation in non-motile *P. denitrificans*. BapA is a protein secreted by BapBCD T1SS, which resides on the cell surface and presumably mediates cell adhesion by hydrophobic interactions. During initial attachment to surfaces, motile bacteria sense surface attachment and initiate adhesin production to form biofilms (2). In contrast, this study shows non-motile *P. denitrificans* significantly expresses BapA in planktonic state, suggesting a strategy to form biofilms that *P. denitrificans* produces BapA to passively attach and initiate biofilm formation. *P. denitrificans* has been identified in activated sludge and biofilms in wastewater treatment systems, where cell hydrophobicity is essential to cellular adherence of bacteria in these systems (3, 4). Taken together, the results suggest that BapA plays critical roles in biofilm formation of *P. denirificans* in the environments and wastewater treatment systems. BapA is a large, repetitive protein with the estimated molecular size of 210 kDa, which contains extensive tandem repeats of aspartate and alanine. Although large repetitive proteins involved in biofilm formation have been reported in other Gram-negative bacteria (5), the protein sequence of BapA shows very low sequence similarity to these proteins. It is interesting that bacteria have evolved these large, variable adhesive proteins, which implies their significance for bacterial adaption to various environments.

References

1. **Karatan E, Watnick P**. 2009. Signals, regulatory networks, and materials that build and break bacterial biofilms. Microbiol Mol Biol Rev **73**:310–347.

2. **Luo Y, Zhao K, Baker AE, Kuchma SL, Coggan KA, Wolfgang MC, Wong GCL, O'Toole GA**. 2015. A hierarchical cascade of second messengers regulates *Pseudomonas aeruginosa* surface behaviors. mBio **6**:e02456.

3. **Neef A, Zaglauer A, Meier H, Amann R, Lemmer H, Schleifer KH**. 1996. Population analysis in a denitrifying sand filter: conventional and in situ identification of *Paracoccus* spp. in methanol-fed biofilms. Appl Environ Microbiol **62**:4329–4339.

4. **Zita A, Hermansson M**. 1997. Effects of bacterial cell surface structures and hydrophobicity on attachment to activated sludge flocs. Appl Environ Microbiol **63**:41168–1170.

5. **Satchell KJF**. 2011. Structure and function of MARTX toxins and other large repetitive RTX proteins. Annu Rev Microbiol **65**:71–90.