

## Biofilm Formation of *Agrobacterium tumefaciens* on Abiotic Surface is Independent of Cellulose Synthesizing Gene

Reshma Tuladhar

Central Department of Microbiology, Tribhuvan University, Kathmandu, Nepal  
e-mail: resutu@gmail.com

### Abstract

Surface attachment is indispensable in the process of biofilm formation in *Agrobacterium tumefaciens*, which has been attributed to the production of extracellular polysaccharide. Adherence of bacteria on the plant was expected to be strong with cellulose synthesis. However, its role in the biofilm formation on abiotic surface is not known. Biofilm cover-slip assay was carried to estimate the biofilm formation. Biofilm formation was not reduced by truncating cellulose synthesis gene. The *celCD* deleted strain produced biofilm slightly greater than normal strain NTL4 of *A. tumefaciens*. Besides, production of extracellular polysaccharide was not reduced in the cellulose gene deleted strain NTL4 $\Delta$  *celCD*. Quantitative estimation showed increased exo-polysaccharide in *celCD* deleted strain. Abolishing the cellulose synthesis property by truncating the cellulose gene did not affect biofilm formation in abiotic surface. In fact cellulose might be repressing biosynthesis of other polysaccharides responsible in biofilm formation.

**Key words:** biofilm cover-slip assay, exo-polysaccharide, calcofluor assay

### Introduction

*Agrobacterium tumefaciens* is a gram-negative soil bacterium that forms crown gall tumors in most dicotyledonous plants. Crown gall formation is the result of the infections of the wound sites in the plant (Reuhs *et al.* 1997). Pathogenesis involves the horizontal transmission of a segment of *A. tumefaciens* DNA carried on the tumor-inducing (Ti) plasmid into the host plant genome (Gelvin 2003). Genes on the transferred DNA direct a hormonal imbalance in the plant that results in uncontrolled proliferation of plant cells forming the characteristic gall. An early step in tumor formation is the attachment of the bacteria on the plant cell surface (Matthysse 1986). Surface attachment is indispensable in the process of the host plant infection and biofilm formation in *A. tumefaciens*.

Microbial biofilms are defined as surface-associated, multicellular assemblages of organisms (Parsek & Fuqua 2004). Organic compounds released by plant

serve as powerful attractant for *A. tumefaciens* (Binns *et al.* 1992), which initiates the attachment. Formation of biofilm begins with the attachment of individual or small groups of bacteria to the surface. This is followed by bacterial growth and additional attachments to form a biofilm. Large amounts of extracellular matrix materials are often produced during biofilm formation (Matthysse *et al.* 2005). This matrix holds the cells in association with each other and the surface and commonly contains exopolysaccharides (EPS) (Sutherland 2001).

An EPS produced by *A. tumefaciens* and closely related *Rhizobium meliloti* is succinoglycan, which consists of repeating units of linked glucose and galactose with acidic sidegroups. Mutants of *R. meliloti*, which lack this EPS induced ineffective nodules on alfalfa (Cangelosi *et al.* 1987). Besides, several other bacteria are known to produce polysaccharides contributing to the formation of biofilm. Overproduction of *Vibrio* polysaccharide in

*Vibrio cholerae* has been associated with increased biofilm formation and elevated synthesis of EPS in opportunistic human pathogen *Pseudomonas aeruginosa* form dense biofilms on inert surfaces as well as human tissue (Mathee *et al.* 1999, Matthyse *et al.* 2005). However, overproduction of EPS does not result in a hyperadherent phenotype in all biofilm forming bacteria (Parsek & Fuqua 2004).

Cellulose is frequently found component of the biofilm matrix in many organisms including members of the Rhizobiaceae (Flemming & Wingender 2010, Bogino *et al.* 2013). *A. tumefaciens* in presence of cellulose fibrils bind tightly to the plant surface while the mutants show the reduced ability to colonize plants and induce tumor formation (Matthyse 1983). Over production of cellulose enhances attachment to plant roots in *A. tumefaciens* (Matthyse *et al.* 2005). Cellulose synthesis in *A. tumefaciens* requires genes in two operons, *celABCG* and *celDE* (Matthyse 1995, Matthyse *et al.* 2005). It is not confirmed that cellulose overproduction has any effect on biofilm formation on inert material. Thus, this work was carried out to study the effect of cellulose on the biofilm formation on inert material by *A. tumefaciens*.

## Methodology

### Strain and growth condition

The bacterial strains used were i) C58 (Wild type *A. tumefaciens* which extensively forms biofilm), ii) NTL4 (derivative of C58 with deletion in *tet<sub>CS8</sub>* locus in plasmid pTi C58), iii) NTL4 *celCD* ( derived from NTL4 with deletion in *celCD* locus of cellulose biosynthesis gene), iv) *exoR* (positive control for exopolysaccharide producer and v) *exoA* (negative control for exopolysaccharide producer). C58 (Wild type), NTL4 (pTi C58) and NTL4  $\Delta$  *celCD* were generously provided by Farrand SK, University of Illinois, IL. Bacteria were maintained on AT minimal medium supplemented with 0.5% (wt/vol) glucose and 15mM ammonium sulfate (ATGN).

### Analysis of static culture biofilms

Polyvinyl chloride (PVC) coverslips were placed vertically in 12-well polystyrene cell culture plates (Corning Inc.) inoculated with cells in ATGN at an OD<sub>600</sub> of 0.05 and incubated at room temperature for 48 hours. Biofilms were visualized macroscopically by staining with 1% Crystal Violet solution. For assessing biofilm by visualizing Crystal Violet,

coverslips were rinsed in double-distilled water, stained with 0.1% (wt/vol) CV, and rinsed again in double-distilled water. Biomass adhered to the coverslip was quantified by soaking stained coverslips in 1ml dimethyl sulfoxide (DMSO) to solubilize the CV and measuring the absorbance at 600nm (A<sub>600</sub>) in a Bio-Tek synergy HT microplate reader. Absorbance values were normalized to culture growth by dividing the A<sub>600</sub> value for solubilized CV by the OD<sub>600</sub> of the planktonic cells. Each of the experiment was carried out in triplicate.

### Calcofluor assay

*A. tumefaciens* strains were streaked onto ATGN agar containing 0.02% calcofluor (fluorescent brightener 28; Sigma F-3397) and incubated for growth. To determine if a strain produced exopolysaccharide, agar plates were exposed to long-wavelength UV light, and the bacterial streaks on the agar plates were monitored for bright blue fluorescence.

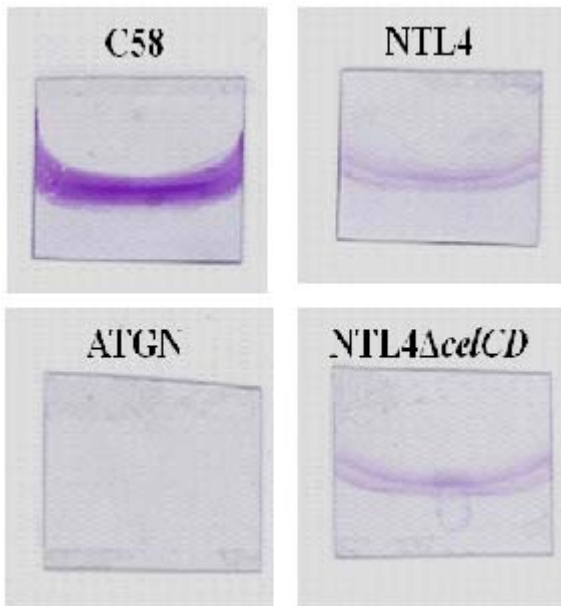
### Estimation of exopolysaccharide

Anthrone method for estimation of glycosidic linked polysaccharide was performed for the estimation of exopolysaccharide. The bacterial cells were grown in ATGN at an OD<sub>600</sub> of 0.08. Four ml of the anthrone reagent was added to 1 ml of the supernatant of cell culture.

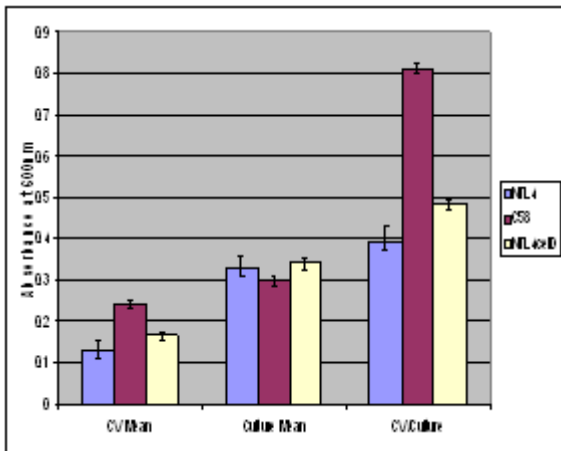
## Results and Discussion

### Biofilm formation

Wildtype and cellulose mutants were monitored by crystal violet staining for the attachment and static biofilm formation on PVC coverslips in ATGN. Visually the crystal violet stained in the biofilm formed by NTL4 parent strain which has intact cellulose biosynthesis gene and NTL4  $\Delta$  *celCD* cellulose mutant were similar. The wildtype C58 form dense biofilms (Fig. 1). The CV staining was normalized for culture growth (CV absorbance divided by planktonic culture turbidity). The comparisons were made from the Crystal violet A<sub>600</sub>/OD<sub>600</sub> data ( Fig. 2). The normalized data showed the overall biofilm formation capacity. The highest biofilm formation ability was found in the wildtype. The cellulose mutant NTL4  $\Delta$  *celCD* showed a slight increase in biofilm formation compared to the the NTL4 strain. The deficiency in an intact cellulose biosynthesis gene did not affect the ability in the formation of biofilm.



**Fig. 1.** Static biofilm formation and surface attachment (Crystal violet staining for the attachment on PVC coverslips in ATGN).

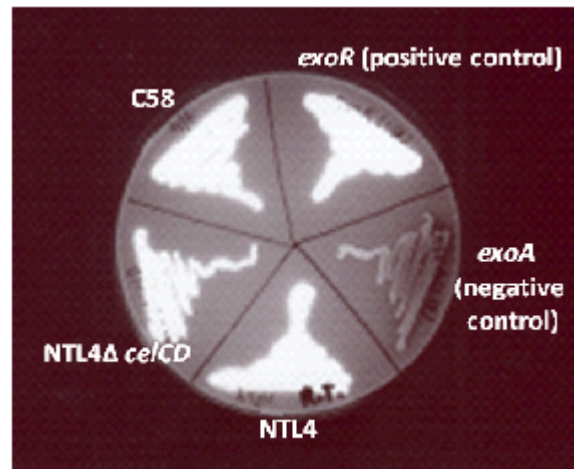


**Fig. 2.** Biofilm formation by different strains: Biofilms formed on PVC coverslip at 72 hpi were stained with CV and quantified from DMSO solubilized CV. CV mean (Adherent biomass  $CVA_{600}$ ), Culture mean (cell biomass OD600) and CV/Culture (biomass normalized for growth  $CVA_{600}/OD_{600}$ ).

### Calcofluor assay for detection of exopolysaccharide

In order to assess the production of exopolysaccharide, the calcofluor assay was performed. Calcofluor binds polysaccharide, and the colonies producing polysaccharide

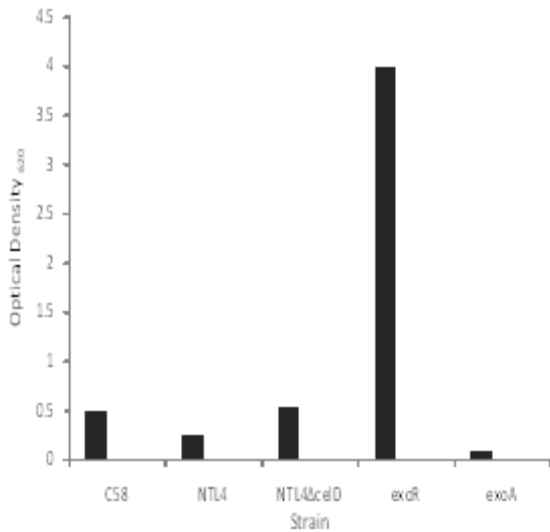
emit bright blue fluorescence when they are exposed to long-wavelength UV light. On ATGN agar containing 0.02% calcofluor, the wild type C58, NTL4, NTL4  $\Delta$  *celCD* and *exoR* (positive control for exopolysaccharide) emitted bright blue fluorescence when exposed to long-wavelength UV light. The negative control *exoA* showed the reduced fluorescence and almost appeared non fluorescent (Fig. 3). The cellulose deficient strain NTL4  $\Delta$  *celCD* showed fluorescence intensity identical to the wild type (C58) and positive control (*exoR*). This shows that polysaccharide other than cellulose is produced by cellulose deficient strain.



**Fig. 3.** Exopolysaccharide production in *A. tumefaciens* C58 (wt), NTL4, NTL4  $\Delta$  *celCD*, *exoR* and *exoA*. Strains were streaked on ATGN supplemented with 0.02% calcofluor. The organisms were grown on plates and exposed to long-wavelength UV light. Bright fluorescent was observed in all the strains except for the negative control *exoA*.

### Estimation of exopolysaccharide

The exopolysaccharide produced by different strains was qualitatively estimated by the anthrone method. It was found that the cellulose mutant NTL4  $\Delta$  *celCD* produced nearly equal amount of polysaccharide as the wild type C58. However the polysaccharide concentration was lower in cellulose intact strain NTL4 from which the mutant was derived. Fairly small amount of carbohydrate was present in exopolysaccharide mutant while as the positive control showed the presence in excessively high amount (Fig. 4). The data for the carbohydrate shown over here are not only contributed by the exopolysaccharides but part of free sugars can also be added up. But this amount should be very low.



**Fig. 4.** Estimation of exopolysaccharide of reducing sugar by anthrone

The involvement of cellulose in bacterial biofilms has long been known for *Acetobacter xylinum*, which forms a biofilm at air-water interfaces and on the surface of decaying fruit (Cannon & Anderson 1991). More recently, cellulose has been observed to be involved in biofilm formation by *Escherichia coli*, *Salmonella enteritidis* and *S. typhimurium* (White *et al.* 2003, Zogaj *et al.* 2001). *P. fluorescens* forms a biofilm containing acetylated cellulose (Spiers *et al.* 2003). Previous studies have shown that the absence of cellulose reduces that ability of *A. tumefaciens* to bind to plant surfaces and to colonize roots and severely attenuates virulence (Matthysse 1983, Minnemeyer *et al.* 1991). Mutants of *P. fluorescens* and *S. enteritidis* that are unable to make cellulose also fail to form a biofilm (Spiers *et al.* 2003, White *et al.* 2003).

In this study the biofilm formed on the abiotic surface by *A. tumefaciens* wildtype was compared with that formed by the cellulose deficient mutant. The cellulose deficient mutant NTL4Δ *celCD* showed slight increased biofilm formation in PVC coverslips compared to NTL4 strain when grown in static culture. The NTL4 strain differs from the mutant strain in the cellulose biosynthesis gene only. The cellulose mutant strain has deletion in the *celC* and *celD* region of the cellulose biosynthesis gene. The wild type C58 however showed excessive biofilm formation.

Thus the results from biofilm assay demonstrated that in spite of abolishing the cellulose synthesis property by truncating the cellulose synthesis gene, the biofilm formation has not been affected.

This might indicate that cellulose is not an obligatory compound for biofilm formation. Since various exopolysaccharides are secreted by bacteria contributing to biofilm formation, polysaccharide other than cellulose might be playing important role in biofilm formation.

The results from Calcofluor plate assay and anthrone assay illustrated the presence of extracellular polysaccharide in cellulose mutant strain of *A. tumefaciens*. In fact, the slight increase in biofilm formation and extracellular polysaccharide in NTL4Δ *celCD* compared to NTL4 strain that is not deficient in cellulose biosynthesis must be related with gene for polysaccharide other than cellulose might have been better expressed in absence of cellulose gene

The major component for the biofilm formation is still unknown since several different compounds are associated with the formation of biofilm. There are several exopolysaccharides released by bacteria known to facilitate in the formation of biofilm. The *A. tumefaciens* genome encodes for production of at least six polysaccharide species, several of which play roles in attachment and biofilm formation. These include the UPP adhesin, cellulose, succinoglycan, cyclic  $\alpha$ -1,2-glucans,  $\beta$ -1,3-glucan (curdlan), and outer membrane lipopolysaccharide (LPS) (Heindl *et al.* 2014). Though synthesis of cellulose fibrils have been associated with root colonization of *A. tumefaciens* (Matthysse 1983), it is still unknown if cellulose has important role in the biofilm formation in an inert material.

Outer membrane protein OmpA of *E. coli* decreased cellulose production which increased biofilm formation on polystyrene surface (Ma & Wood 2009). Increasing cellulose production by overproducing AdrA was found to decrease biofilm formation on hydrophobic PS plates by Ma & Wood (2009). Several factors can be associated with biofilm formation. Cellulose was found to decrease biofilm formation in curli-mediated biofilm formation on PP microtitre plates (Gualdi *et al.* 2008). Curli help adherence of *E. coli* to intestinal epithelial cells and coexpression of

curli and cellulose decreases the adherence (Wang *et al.* 2006).

Though production of cellulose enhances attachment to plant root (Matthysse *et al.* 2005) the mechanism of binding on PVC surface may not be identical. Cellulose being hydrophilic in nature the attachment might be enhanced on hydrophilic plant root surface but reduced in hydrophobic PVC. Identifying the factors that reduce the biofilm formation in inanimate object will have implication in overcoming the problems of biofilm formation in medical devices such as catheters.

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