Monosaccharides Composition of Biofilm Produced by Xylella Fastidiosa Wild Type and Rpf Mutants

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Abstract: Xylella fastidiosa is a phytopathogen that produces biofilms, which are involved in its virulence and pathogenicity. Biofilms are composed of diverse compounds, including exopolysaccharides (EPSs). The EPSs are important components of biofilms that play a large role in its stability and structure. Its secretion is strongly connected to expression of the rpf (regulation of pathogenicity factor) gene cluster. To identify the monosaccharides that comprise the EPS of X. fastidiosa Temecula strain ATCC 700964, high performance liquid chromatography (HPLC) was used. The EPS produced by rpfA mutant is absent in galacturonic acid but contains a high proportion of glucose. The rpfB mutants doesn’t present rhamnose and galacturonic acid in its EPS composition. These differences in monosaccharide composition, mainly the compared to the wild type, indicating that the rpf gene cluster is involved in biofilm formation and that these genes are more than simple regulators of the EPS production.

Key words: galacturonic acid, glucose, rhamnose, rpfA, rpfB, HPLC.

INTRODUCTION

Xylella fastidiosa is a phytopathogen, and its virulence and pathogenicity are linked to biofilm formation[21,27]. Biofilms are microbial communities attached to a solid surface[4,22]. Biofilm formation is the result of an initial adhesion event of one or a few cells. These cells, once established, start to form a biofilm through growth, division and exopolysaccharides (EPS) secretion[6,27], which shapes the biofilm structure[34]. In addition to EPSs, a biofilm may also be composed of diverse substances that include polysaccharides, proteins, nucleic acids and lipids[10].

EPSs such as fastidian, xanthan and curdlan gum have attracted great attention from researchers mainly because of their innumerable industrial and medical applications[18,29].

These compounds have beneficial characteristics, including the ability to protect against antibiotics; promote resistance to UV radiation, changes in pH and osmotic shock; increase survival in adverse environments and protect against dehydration, because the EPS matrix is highly hydrated and acts as a physical barrier[2,25,29].

EPSs play an essential role in defining the physical properties of the biofilm[33], in which the distinct biofilm morphotypes formed depend on the different genes expressed[33] and the composition of the biofilm.

In Xanthomonas campestris pv. campestris, some of the rpf genes have been characterized in detail, and they seem to regulate EPS production expression because rpf mutants were shown to have reduced production of these extracellular polysaccharides[9,10,32].

Mutagenesis based on random Tn-5 transposon insertion was used to inactivate certain rpf genes in X. fastidiosa Temecula strain, and the biofilms formed by these mutants were characterized and compared with the wild type by HPLC analysis to elucidate the role of the rpf genes in X. fastidiosa.

The aim of this work was to determine the EPS composition of biofilms formed by the X. fastidiosa wild type strain and to compare it with the EPSs formed by rpf mutants in the same growth conditions.

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Methods:

Bacterial Isolates and Culture: The *X. fastidiosa* Temecula strain ATCC 700964 was cultivated in BCYE media [31] from glycerol culture stocks maintained at –80°C. The colonies that were recovered from mutageneses were transferred to liquid BCYE medium and grown for 5 days at 28°C for DNA isolation and subsequent sequence analyses. Mutants were maintained on modified BCYE agar containing kanamycin (30 µg/mL).

Plasmid Constructions: Genomic DNA from *X. fastidiosa* Temecula strain was amplified with Platinum Taq DNA Polymerase High Fidelity (Invitrogen) and primers for rpfa (R-5'CACGACGCGGTAGACTAGTTGT3' and F-5'ATGCGCGATTCGTTCTCCACC3') and for rpfb (R-5'GTAGTTGGTGTAAGTGTCGAGTTGGGT3' and F-5'CACGACGCGGTAGACTAGTTGT3'). The sequences of the *X. fastidiosa* rpfa genes were obtained from the Genome Project database for *X. fastidiosa* Temecula strain [http://aeg.lbi.ic.unicamp.br/work/xfpd]. PCR products were cloned into the pGEM-T Easy cloning vector (Promega) and transformed into *E. coli* competent cells by chemical transformation [15]. The Electrotransformation was conducted under an electric field of 1.6 kV for 5 ms, with a resistance value of 200 W and a capacitance of 25 mFD. Electrotransformed cells were plated on modified BCYE agar containing kanamycin (30 µg/mL) to select for resistant cells. The colonies that were recovered from mutageneses were transferred to liquid BCYE medium and grown for 5 days at 28°C for DNA isolation and subsequent sequence analyses. The digestion of DNA samples was fixed on the membranes by baking at 85 to 90°C for 2 h.

Wild type rpfa and rpfb genes were digoxigenin (DIG) labeled by PCR using the same primers described above and used as hybridization probes. The probes were detected by the enhanced chemiluminescent detection system with a sheep anti-DIG antibody labeled with alkaline phosphatase (Boehringer Mannheim).

Bacterial Isolates and Culture for HPLC Analysis: The *X. fastidiosa* Temecula strain was cultivated in XDM6 [19]. The ph value was adjusted to between 6.7 and 6.8. The cells were grown at 28°C for 27 days.

Preparation of Standard Monosaccharides: The following monosaccharides were used as standards: mannose, rhamnose, galacturonic acid, glucuronic acid, glucose and galactose. The six monosaccharides were derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) and dissolved in 40 µL of 0.3 M NaOH and adding 40 µL of 0.5 M PMP in methanol. The mixture was kept at 70°C for 2 h. The solution was cooled to 24°C and 40 µL of 0.5 M HCl was added for neutralization. Butyl ether (0.5 mL) was added to the resulting solution, and the mixture was shaken vigorously for 5 s. The tube with the labeled monosaccharides was centrifuged at 5000 x g for 5 min. The organic phase was carefully removed and discarded. This process was repeated twice, and the lower layer was dissolved in 400 µL of distilled water.

Calibration Curve: To generate a calibration curve, all six monosaccharides were mixed and filtered through a 0.45 µm pore membranes. The mixture was diluted and different standard concentrations (6.25, 12.5, 25 and 50 µmol) were injected onto the HPLC column.

The procedure was carried out on a VP CLASS-VP 5.0 HPLC system equipped with a Shimadzu UV/VIS detector that measured absorbance at 245 nm. An ODS-C18 (6.0 x 150) column was used to separate the PMP-labeled monosaccharides. The column was eluted at 0.5 mL/min, and the elute was monitored at 245 nm.

Two mobile phase buffers were used for separation. Buffers A and B were 100 mM ammonium acetate (pH 5.5) containing 10% and 25% acetonitrile, respectively. The retention time of each monosaccharide was used as an internal standard to compare the EPS monosaccharides of the biofilms of the *X. fastidiosa* mutants and wild type.

EPS Preparation: The cells grown for 27 days were harvested and suspended in 1.0 mL of 0.5 M HCl. Neutralization of the mixture was carried out by the addition of 40 µL of 0.5 M PMP in methanol. The mixture was shaken vigorously for 5 s. The tube with the labeled monosaccharides was centrifuged at 5000 x g for 5 min. The organic phase was carefully removed and discarded. This process was repeated twice, and the lower layer was dissolved in 400 µL of distilled water.
removed from the Petri dishes and washed in 2% NaCl. The cells were separated by centrifugation at 10,000 × g and 4°C for 30 min. The upper layer was transferred to a beaker with 50 mL of anhydrous alcohol. The EPS was collected and dried for 7 days at room temperature. Samples with 1 mg of EPS were dissolved in 4 M trifluoroacetic acid (TFA) in glass ampoules sealed under a vacuum. The ampoules were heated for 2 h at 120°C and opened. The solution was evaporated to dryness under a vacuum using a Hetovac vr-1 for 12 h. The residues were re-dissolved in 0.5 mL isopropyl alcohol and dried again by evaporation.

Like the standard monosaccharides, the EPS samples were PMP labeled as previously described. The samples were injected onto the HPLC column, and the monosaccharide compositions were determined.

RESULTS AND DISCUSSION

Mutagenesis and Genetic Analysis of Xylella Fastidiosa Rpf Genes: The EPS rpfA and rpfB mutants generated by Tn-5 transposon and the insertion confirmed by PCR and Southern blot analysis.

The amplified rpfA and rpfB gene products, which exhibited sizes around 2,627 bp and 1,710 bp, respectively, were cloned using the pGEM-T Easy vector (Promega) and transformed into E. coli competent cells. Tn-5 transposon mutants produced amplicons of 3,848 and 2,931 bp corresponding to the rpfA and rpfB genes, respectively, generated by insertion of the 1,221-bp transposon, which were confirmed by restriction digestion using EcoRI for rpfA gene and EcoRI/Psil for rpfB gene (Fig1).

![Fig. 1](image)

Fig. 1: Agarose gel electrophoresis showing transposon insertions in rpfA and rpfB genes from Xylella fastidiosa by restriction analysis. Lane 1: Molecular size marker – 1 kb Plus DNA ladder. Lane 2: genes without the insertion. Lane 3: genes with transposon insertions. a: rpfA gene and b: rpfB gene

Plasmids with the Tn-5 transposon disrupting the rpf genes were electroporated into X. fastidiosa Temecula strain.

The amplicons containing the rpfA and rpfB genes disrupted by the transposon insertions are shown in Figure 2 and the bands in Figure 3 correspond to the Southern blot analysis. In both analyses, 1,221 bp increases in size were observed, which correspond to the Tn-5 transposon.

![Fig. 2](image)

Fig. 2: PCR analysis for comparison between Xylella fastidiosa wild type and mutant bacteria, Lane 1: Molecular size marker – 1 kb Plus DNA ladder. (a) Lane 2: wild type bacteria; Lane 3: rpfA mutant (3,848 bp = 2,627 bp gene + 1,221 bp transposon) and (b) Lane 2: wild type bacteria; Lane 3: rpfB mutant (2,931 bp = 1,710 bp gene + 1,221 bp transposon)

To select only the mutants generated by double homologous recombination, the Southern blot analysis was used. These analysis shows that the tested mutants contained a single transposon insertion with the corresponding increase of only 1,221 bp (Fig3). A schematic of the plasmids constructed is shown in Figure 4.

![Fig. 3](image)

Fig. 3: Double homologous recombination, resulting in a transposon integration event in rpfA and rpfB genes from Xylella fastidiosa, shown by an increase of 1,221 bp in the gene sizes. a: Gel electrophoresis of enzyme digestion and b: Membrane of Southern blot analysis. Lane 1: Molecular size marker – 1 kb Plus DNA ladder. Lane 2: wild type bacteria; Lane 3: rpfA mutant

EPS Composition: In the present study, the biofilms secreted by X. fastidiosa wild type and the rpfA and rpfB mutants were harvested and the EPS sugar amounts and compositions were analyzed. The choice of standard monosaccharides was mainly based on the fastididum gum
model and xanthan gum composition (gums formed by the EPS produced by X. fastidiosa and X. campesiris, respectively). The monosaccharides composition was used to compare the biofilms produced by the mutants and wild type.

**Fig. 4:** Scheme of plasmid construction used in mutations in rpfA and rpfB genes from Xylella fastidiosa using the Tn-5 transposon. Δ: rpf primer site; Tn-5: transposon insertion; Gene: rpfA or rpfB gene

Table 1 describes the monosaccharides concentration in mg/mL.

The HPLC analysis of the monosaccharides shows that the EPSs secreted from the rpfA and rpfB mutants presented qualitative and quantitative differences compared to the EPS produced by the wild type grown in the same conditions. The EPS composition analysis revealed the presence of mannose, glucose, and galactose in all samples. The EPS from the rpfA mutant contained a high proportion of glucose (0.141 mg/mL), which was approximately 70 times more than that produced by wild type (0.003 mg/mL), and lacked galacturonic acid. The proportion of the monosaccharides in the EPS formed by the rpfA mutant was 0.141 mg/mL glucose, 0.050 mg/mL mannose, 0.014 mg/mL glucuronic acid, 0.011 mg/mL rhamnose and 0.002 mg/mL galactose (Table 1).

The EPS from the rpfB mutants was appreciably different compared with the EPS from wild type. The rpfB EPS was composed of only four monosaccharides: mannose, glucuronic acid, glucose and galactose. Rhamnose and galacturonic acid were absent. The rpfB mutant produced EPS with very low monosaccharide concentrations, when compared with wild type, in a ratio of 7:3:3:2 for mannose, glucuronic acid, galactose, and glucose, respectively (Table 1). The EPS of the wild type strain showed a subtle predominance of glucuronic and galacturonic acids and was comprised of glucuronic acid, galacturonic acid, mannose, glucose, rhamnose and galactose in a ratio of 25:16:14:3:2:2, respectively (Table 1).

**Discussion:** A strategy to introduce mutations based on in vitro transposon insertion into plasmids or chromosome sites has been widely employed. In our study, the X. fastidiosa mutants were obtained by transposon Tn-5 insertions into the two rpf genes. Tn-5 insertions are stable and apparently unaffected by the activities of any potential chromosomally encoded enzymes.

Other methodologies involving the assembly of mutated genes into plasmids using amplicons have already been employed by Guilhabert et al. to generate a large number of mutant clones. Therefore, before insertion of the transposon, plasmids with the respective amplicons were easily generated by PCR. Because the terminal transferase activity of Taq DNA polymerase adds a deoxyadenosine overhang to the ends of the amplicons, PCR products can be directly ligated into vectors that contain a 3’ deoxythymidine overhangs. This strategy resulted in an easier, quicker and more efficient methodology compared to strategies that use restriction enzymes.

Double homologous recombination is used to introduce the disrupted genes or deletions into specific locations, using a construct with two regions of sequence identity with the bacterial genome. Although difficulties in the transformation of X. fastidiosa have been reported, excellent results that showed double recombination were obtained. Even bacteria such as Rhodococcus rhodochrous, Rhodococcus equi and Mycobacterium smegmatis that are also known to exhibit transformation problems have been successfully mutated using this method.

The production of mutants is very important because it can reveal the function of each gene, allowing us to understand how bacteria produce pathogenicity factors and the roles of these factors within the metabolic pathways. This is also known as site-directed mutagenesis and is currently used to show the involvement of some genes in a number of cell processes and whether these functions are similar among different organisms of interest.

Studying mechanisms of biofilm formation in pathogenesis may reveal the role of diffusible molecules in various physiological processes including cell agglutination and the dispersal of bacterial cells from a biofilm. These processes have been shown to be important for the pathogenicity of a number of bacteria, such as Pseudomonas fluorescens, P. aeruginosa and X. fastidiosa. However, in X. fastidiosa, its action is felt on a slower fashion when compared to other organisms.

It has been previously demonstrated that X. fastidiosa is able to form biofilms and that its EPSs are involved in pathogenicity, which is beneficial for the cells and has an enormous impact on important crops, such as grapevine and sweet oranges.

Only a few studies about biofilm composition have been reported, possibly due to the difficulty in producing biofilms in vitro and extracting sufficient amounts for analysis, especially for biofilm produced by X. fastidiosa. Information pertaining to EPS composition could thus help us design more efficient cleaning procedures to prevent or control diseases caused by this bacterium.
Based on the data shown above, we can deduce that the mutant is impaired in successful infection thread formation involving the galacturonic acid residues in the outer membrane structure. Nodulation studies showed the involvement of the galacturonic acid residues in this process.

The EPS compositions of the biofilms secreted by Xylella fastidiosa Temecula wild type and mutants grown on agar medium for 20 days at 28°C are presented in Table 1.

<table>
<thead>
<tr>
<th>Monosaccharides</th>
<th>Wild type (mg/mL)</th>
<th>rpfA mutant (mg/mL)</th>
<th>rpfB mutant (mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mannose</td>
<td>0.014</td>
<td>0.05</td>
<td>0.007</td>
</tr>
<tr>
<td>rhamnose</td>
<td>0.003</td>
<td>0.011</td>
<td>absent</td>
</tr>
<tr>
<td>galacturonic acid</td>
<td>0.016</td>
<td>absent</td>
<td>absent</td>
</tr>
<tr>
<td>glucuronic acid</td>
<td>0.025</td>
<td>0.014</td>
<td>0.003</td>
</tr>
<tr>
<td>glucose</td>
<td>0.003</td>
<td>0.141</td>
<td>0.002</td>
</tr>
<tr>
<td>galactose</td>
<td>0.002</td>
<td>0.002</td>
<td>0.003</td>
</tr>
</tbody>
</table>

Furthermore, if biofilm production is defective, such as the shown in this work, only the inoculation of these mutants into a host and the manifestation of symptoms could evaluate this hypothesis because defective EPS production may play a main role in biofilm protection of the bacteria and especially in the ability to attack and colonize a host. Therefore, preventing biofilm formation or disrupting these biofilms could be an effective approach to prevent or treat diseases caused by X. fastidiosa.

This is the first study that characterizes the monosaccharide composition of the biofilms formed by X. fastidiosa and the deficient biofilms formed by rpf mutants.

The continued analysis of the mutants described in this study will increase our understanding of the connection between basic metabolic pathways and aspects related to pathogenicity.

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