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Genomics and X-ray microanalysis indicate that Ca^{2+} and thiols mediate the aggregation and adhesion of *Xylella fastidiosa*

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Abstract

The availability of the genome sequence of the bacterial plant pathogen *Xylella fastidiosa*, the causal agent of citrus variegated chlorosis, is accelerating important investigations concerning its pathogenicity. Plant vessel occlusion is critical for symptom development. The objective of the present study was to search for information that would help to explain the adhesion of *X. fastidiosa* cells to the xylem. Scanning electron microscopy revealed that adhesion may occur without the fastidium gum, an exopolysaccharide produced by *X. fastidiosa*, and X-ray microanalysis demonstrated the presence of elemental sulfur both in cells grown *in vitro* and in cells found inside plant vessels, indicating that the sulfur signal is generated by the pathogen surface. Calcium and magnesium peaks were detected in association with sulfur in occluded vessels. We propose an explanation for the adhesion and aggregation process. Thiol groups, maintained by the enzyme peptide methionine sulfoxide reductase, could be active on the surface of the bacteria and appear to promote cell-cell aggregation by forming disulfide bonds with thiol groups on the surface of adjacent cells. The enzyme methionine sulfoxide reductase has been shown to be an auxiliary component in the adhesiveness of some human pathogens. The negative charge conferred by the ionized thiol group could of itself constitute a mechanism of adhesion by allowing the formation of divalent cation bridges between the negatively charged bacteria and predominantly negatively charged xylem walls.

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Xylella fastidiosa is a major problem in Brazilian citrus production areas. The estimated annual losses due to citrus variegated chlorosis (CVC) are up 100 million dollars, affecting over 70 million sweet orange trees (1). Some other countries in Latin America are also affected. In the United States, pathovars of *X. fastidiosa* have caused severe economic losses in grapevines due to Pierce's disease (2). Vessel occlusion seems to be critical for the development of symptoms caused by *X. fastidiosa* aggregation

inside the xylem. The fastidium gum (3) released by *X. fastidiosa* cells has been reported to play a central role in the clumping of these bacteria and has been previously assumed to be directly involved in adhesion (4). The role of fastidium gum in adhesion was investigated by examining occluded vessels by scanning electron microscopy (SEM). The images generated revealed that most cells are not immersed in the fastidium gum (Figure 1A). SEM examination of the surface of a microscope slide that was immersed in a culture flask and kept there for 20 days under constant shaking showed similar results (Figure 1B), i.e., one cell layer adhered to the glass surface without the fastidium gum. This was considered to be the beginning of a biofilm formation.

The bacterial biofilm is a community of microorganisms mobilized on a given sur-

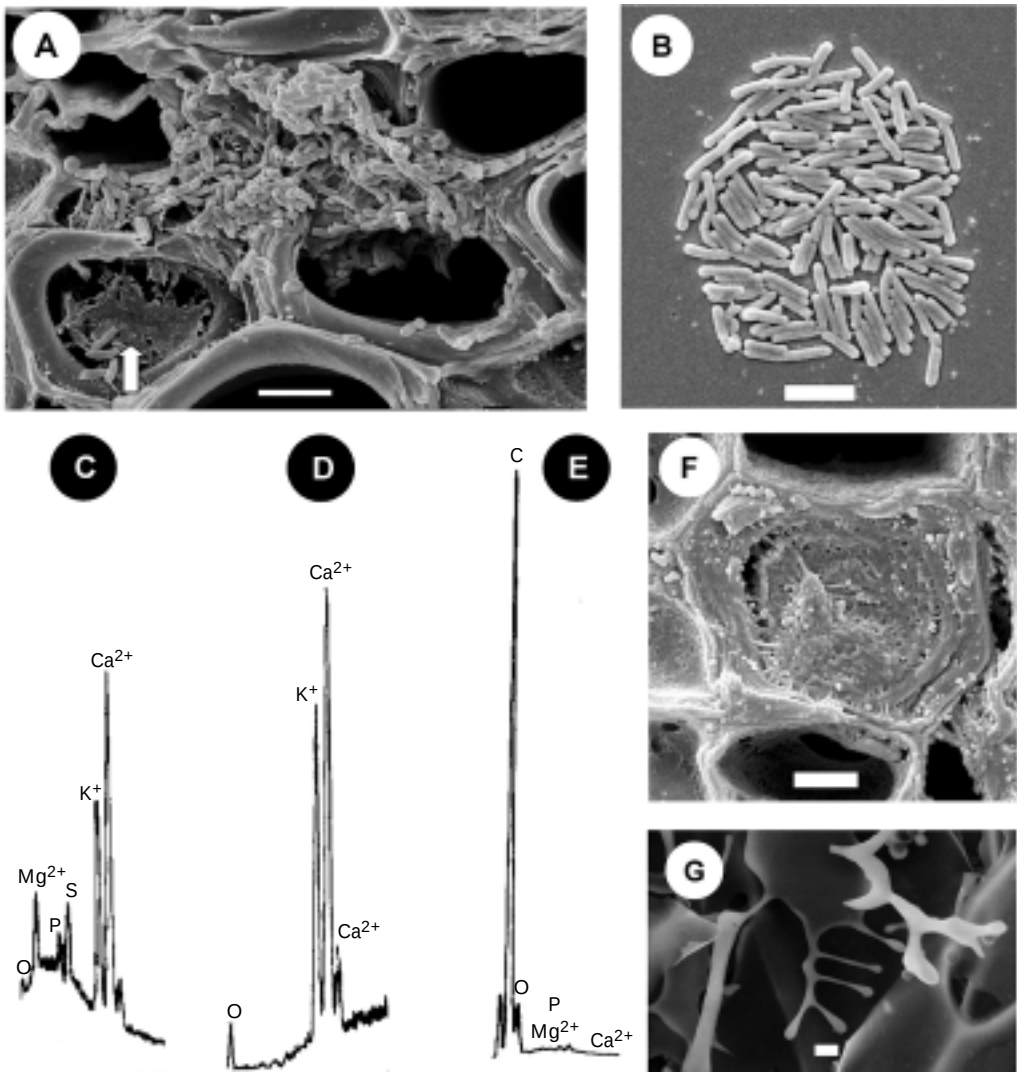

Figure 1. *A*, *Xyllela fastidiosa* occluding citrus xylem vessels. Scanning electron microscopy of citrus xylem vessels partially occluded by the plant pathogenic bacterium *X. fastidiosa*, the causal agent of citrus variegated chlorosis. Notice the presence of extracellular polysaccharides (fastidium gum) in the vessel lumen (arrow). Bar = 5 µm. *B*, *X. fastidiosa* adhered to a glass slide. The figure shows a group of *X. fastidiosa* cells on an artificial surface, a standard glass microscope slide. Slides were inserted into culture flasks for 20 days. *X. fastidosa* spontaneously formed colonies on the glass surface, even though the flask was permanently under shaking. Different degrees of biofilm development can be visualized in this image, a single cell layer on the border and double-layered cells in the center. It is interesting to notice the absence of extracellular polysaccharide surrounding the bacterial cells. Bar = 3 µm. Energy dispersive X-ray microanalysis of the citrus vessel occluded by *X. fastidiosa* and the isolated fastidium gum. The X-ray probe was pointed at an occluded vessel (F) and the spectrum was recorded (C). A very strong calcium signal was detected, accompanied by sulfur and by the divalent ions calcium and magnesium. Points selected away from the occluded vessel (D) did not exhibit the sulfur signal, but calcium was frequently present. Graphs *C* and *D* have the same ordinate scale, 40 counts per second. The fastidium gum (G) shows strong carbon and oxygen signals and in graph *E* the carbon peak reaches 1300 counts per second. Bar (F) = 2 µm. Bar (G) = 6 µm. Methodology: X-ray microanalysis was performed in cross sections of lyophilized leaf petioles coated with carbon and observed under a ZEISS 940 A microscope equipped with an EDX Oxford system for X-ray detection and spot analysis. The data are representative of repeated preparations performed with tissues from distinct infected plants. Uninfected xylem vessels were used as control specimens.

face, which improves their chances for survival in the environment. The biofilm begins with a single cell layer followed by the deposition of additional layers and by an increase in exopolysaccharide (EPS) production (5). Our findings agree with the predicted stages and suggest that the fastidium gum is not essential in the preliminary steps of *X. fastidiosa* biofilm formation. In a recent study, Danese and collaborators (6) showed that the adhesion of *Escherichia coli* K12 was not influenced by the lack of EPS production. A mutant of *E. coli* incapable of producing colanic acid, an EPS, was tested for biofilm formation against the wild type. Both the mutant and the wild type *E. coli* adhered to a polyvinylchloride surface. However, only the wild type developed a multilayered biofilm. These investigators concluded that the lack of EPS production affected the biofilm architecture, but not the cell adhesion. Similarly, our results indicate that *X. fastidiosa* adhesion is primarily dependent on the ability of the bacterium to get in contact with other cells and substrates relying solely on their surface characteristics.

Subsequently, SEM coupled with X-ray microanalysis (EDS = energy dispersive X-ray spectrometry) was performed to compare the elemental composition of the fastidium gum obtained from cultures of *X. fastidiosa* and the elemental composition of occluded vessels (Figure 1C-G). EDS allows the detection and identification of the X-rays produced by the impact of the electron beam on the sample, thereby allowing qualitative and quantitative elemental analysis. The analysis is limited to the electron beam penetration, which is usually 1 to 5 micra, and therefore only the surface elemental constitution is determined. As expected, most of the fastidium gum (Figure 1G) exhibited carbon and oxygen as major peaks (Figure 1E). Occluded vessels (Figure 1F) consistently exhibited calcium, magnesium and sulfur (Figure 1C), contrary to surrounding vessel areas, in which the sulfur signal was absent (Figure 1D). Since the occluded vessel contained cells and a previous experiment showed that *X. fastidiosa* cells alone exhibited sulfur (data not shown), we think it is reasonable to conclude that the sulfur signal is generated on the bacterial surface.

Two questions were raised by these results: i) If the adhesive material from these two different organisms has the presence of sulfur in common, what is the role of sulfur

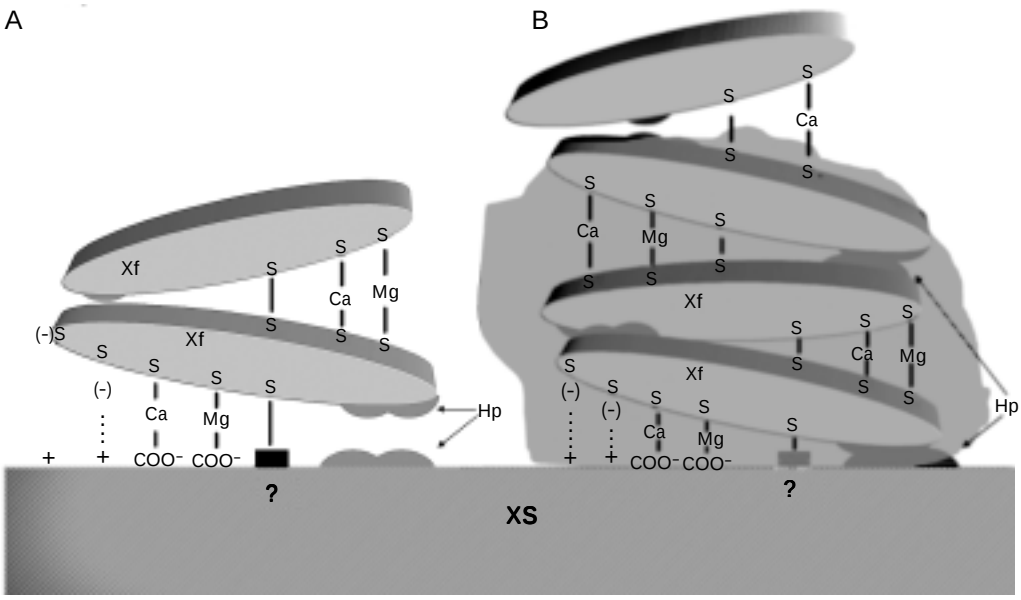

Figure 2. Model proposed to explain adhesion and aggregation of *Xylella fastidiosa*. In scheme *A* an *X. fastidiosa* cell (Xf) is placed in contact with the xylem surface (XS) and with another bacterial cell. All potential points of adhesion are represented. In the interaction between *X. fastidiosa* cells and the XS hydrophobic interactions occur between hydrophobic (Hp) portions on both sides and additional interactions occur between the surface sulfur (sulfhydryl groups) and charges localized on the xylem side (S-? S-+) directly or bridged by calcium and magnesium (S-Ca-COO^-, S-Mg-COO^-). In contrast, cell-to-cell adhesion is believed to be mediated by hydrophobic interactions as just described, by the formation of disulfide bonds (S-S) and by sulfur groups bridged by divalent ions (S-Ca-S, S-Mg-S). The situation described in *B* involves multilayered deposition of cells supported by the architecture provided by exopolysaccharides, with the fastidium gum being represented by the shadowed area.

in adhesion? ii) In addition to giving a negative charge to the bacterial surface, characteristic of several microorganisms (7,8), how would sulfur provide adhesive properties to a microorganism surface?

Assays with Sephadex ion-exchange resins, DEAE (positive charges) and carboxymethyl (negative charges) indicated that *X. fastidiosa* cells are more attracted to positively charged DEAE (data not shown). These results are consistent with our hypothesis that the majority of charges on the surface of *X. fastidiosa* are negative. Other pathogens, such as *E. coli* K12 have negatively charged surfaces (8). Cooper and collaborators (9), also using X-ray microanalysis to study cacao (*Theobroma cacao*) vessels occluded by *Verticillium dahlia*, observed peak patterns similar to those obtained for *X. fastidiosa*-occluded vessels. These investigators concluded that sulfur was involved in the resistance response of cacao plants to *V. dahlia* by assuming that the plant produced and accumulated sulfur in the form of cyclooctasulfur (S_8) to resist the fungal attack. However, in the case of *X. fastidiosa*, the sulfur signal is due to the physical presence of the pathogen and is not a localized plant response. If the sulfur were part of a general metabolic stress response it would also be present in vessels in which the pathogenic organism could not be seen *in situ*. With proper stimulation, a general response would affect both infected and non-infected areas through signaling and sulfur would be detected in the surrounding vessel areas. Recently, the adhesive material obtained from *Colletotrichum graminicola* conidia was also shown to exhibit calcium and sulfur, even after extensive dialysis (10).

In support of these data, the study of the *X. fastidiosa* genome revealed the presence of open reading frames with high homology to several proteins involved in adhesion (4). The enzyme denoted methionine sulfoxide reductase (MsrA; EC 1.8.4.6) may be of particular importance. MsrA is an adhesion maintenance enzyme that helps maintain the adhesiveness of some human pathogenic bacterial cells. MsrA is recognized as having a broad substrate specificity and a general mechanism of action controlling a variety of proteins (11). However, the best known substrate for MsrA is oxidized methionine (12).

The influence of thiol (SH) groups on the adhesiveness of some human pathogenic bacteria has been evaluated. Strains of *Streptococcus pneumoniae*, *Neisseria gonorrhoeae* and *E. coli* mutants, that lack the capacity to produce MsrA, exhibited reduced ability to adhere when compared to their respective wild type strains (13). Many research groups have been working with the adhesive properties of thiols such as the generation of mucoadhesive polymers with thiol groups (14) and adherence of human polymorphonuclear leukocyte (15). The enhancement of human polymorphonuclear leukocyte adhesion was demonstrated to be dependent on constitutive peripheral SH groups, CD11/C18 integrins (adhesins) and extracellular calcium. The bacterium *Thiobacillus ferrooxidans* was also shown to adhere by means of thiol groups. A 40-kDa surface protein, which strongly binds elemental sulfur, was isolated from the bacterial flagella (16). The *X. fastidiosa* genome contains some surface proteins (Hsf-like) and type 4 fimbriae which could be the targets for MsrA activity.

A model to explain the adhesion of *X. fastidiosa* to xylem vessels was elaborated and is summarized in Figure 2. The first step of adhesion occurs when the *X. fastidiosa* cell surface and xylem cell wall are attracted only by surface characteristics. In this manner, the presumed negatively charged surface of *X. fatidiosa* could be attributed to the presence of sulfur, as suggested for *Mycobacterium bovis* (17). In this context, calcium and magnesium (divalent cations) would be able to bridge negatively charged substrates on the xylem wall and on the *X. fastidiosa* surface. It is not surprising that

diseased leaves of *Vitis vinifera* were found to have accumulated Ca^{2+} and Mg^{2+} (18), similar to what was demonstrated for occluded citrus vessels (Figure 1C and F). The high density of negative charges may attract several cations such as Ca^{2+} and Mg^{2+}, which are more tightly associated with the negative charges on the cell walls than monovalent cations, such as K^+ and Na^+ (19). Zinc is sometimes detected in the elemental profile of *X. fastidiosa* aggregates (data not shown). Zinc sequestration may possibly explain the symptoms of zinc deficiency in the CVC syndrome. The putative existence of an adhesion maintenance enzyme such as MsrA that would maintain the thiol groups with active adhesive properties is another facet of the adhesion model, explaining how cells adhere to a substrate or to other cells, while calcium would form bonds between negative surfaces and negatively charged pathogen cells (20). In addition, negative sulfur moieties may directly form bonds with positively charged portions of the host tissue. This hypothesis and/or the existence of other forces (such as hydrophobicity) or other charge sources may also be true.

We propose a detailed investigation of the nutrient status of the xylem fluid, which may be interfering with disease development. This proposition seems to be highly justified and is supported by the fact that xylem cell walls contain fixed negative charges resulting from dissociated polygalacturonic acid, yielding COO^- groups (19). The extent and importance of each adhesion/aggregation component has yet to be evaluated.

In summary, our results open an avenue of investigation concerned with the adhesion of plant pathogens involving sulfur, calcium, magnesium and MsrA. The study of xylem chemistry may be of significant importance to understand resistance and/or susceptibility to *X. fastidiosa*. The combination of genomic information and classical research methodologies should uncover the mechanisms of pathogenicity.

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