Calcium transcriptionally regulates movement, recombination and other functions of Xylella fastidiosa under constant flow inside microfluidic chambers

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Summary

Xylella fastidiosa is a xylem-limited bacterial pathogen causing devastating diseases in many economically important crops. Calcium (Ca) is a major inorganic nutrient in xylem sap that influences virulence-related traits of this pathogen, including biofilm formation and twitching motility. This study aimed to adapt a microfluidic system, which mimics the natural habitat of X. fastidiosa, for whole transcriptome analysis under flow conditions. A microfluidic chamber with two parallel channels was used, and RNA isolated from cells grown inside the system was analysed by RNA-Seq. Ca transcriptionally regulated the machinery of type IV pili and other genes related to pathogenicity and host adaptation. Results were compared to our previous RNA-Seq study in biofilm cells in batch cultures (Parker et al., 2016, Environ Microbiol 18, 1620). Ca-regulated genes in both studies belonged to similar functional categories, but the number and tendencies (up-/downregulation) of regulated genes were different. Recombination-related genes were upregulated by Ca, and we proved experimentally that 2 mM Ca enhances natural transformation frequency. Taken together, our results suggest that the regulatory role of Ca in X. fastidiosa acts differently during growth in flow or batch conditions, and this can correlate to the different phases of growth (planktonic and biofilm) during the infection process.

Introduction

Plant xylem, the system that conveys water and dissolved minerals from the roots to the photosynthetic organs, can serve as a growth niche for microbes including bacterial pathogens. When xylem-residing bacterial pathogens enter the water-conducting xylem vessels through natural openings, wounds or helped by xylem-feeding insects, they proliferate. Inside the xylem, they secrete cell wall-degrading enzymes and obstruct the transportation of water and minerals, which leads to disease of the host plant and eventually death (Yadeta and Thomma, 2013). Since these bacterial pathogens live deep in the interior of plants and can infect a broad range of host plants; cultural and chemical methods to control these pathogens are generally ineffective (Yadeta and Thomma, 2013). Xylella fastidiosa, a xylem-limited bacterial pathogen, is the causative agent of devastating plant diseases in many economically important crops in the Americas, Europe and Asia, including Pierce’s disease on grape, citrus variegated chlorosis, olive quick decline syndrome and others (Almeida and Nunney, 2015; Sicard et al., 2018). Xylella fastidiosa is one of the two plant bacterial pathogens to date that has been reported to be naturally competent (Kung and Almeida, 2011). This ability was hypothesized to confer this pathogen with adaptive advantages (Kandel et al., 2016; Sicard et al., 2018; Potnis et al., 2019).

Calcium is an important inorganic compound in xylem sap, which has multiple functions for both plant host and bacterial pathogens (De La Fuente et al., 2014). The role of Ca in plants has been extensively studied, which not only involves the stabilization of cell wall structures but also acts as a versatile messenger that mediates a sophisticated signalling network (Demarty et al., 1984; Dodd et al., 2010). The role of Ca in bacteria is less studied; however, recent indirect evidence suggests multiple regulatory functions of Ca in bacteria (Domínguez, 2004; Domínguez et al., 2015). Calcium regulates a variety of bacterial behaviours including biofilm formation (Sarkisova et al., 2005; Rinaudi et al., 2006), motility (Gode-Potratz et al., 2010; Guragain et al., 2013), secretion activity (DeBord et al., 2003; Dasgupta et al., 2006), spore germination (Wang et al., 2008) and quorum sensing (Werthén and Lundgren, 2001). A series of studies...
conducted by our group has shown that Ca influences key virulence-related traits of *X. fastidiosa*. An increase of external Ca concentration leads to higher surface adhesion force, twitching speed and more biofilm formation by *X. fastidiosa* (Cruz et al., 2012). Calcium-enhanced twitching motility is associated with the interaction between Ca and the Ca-binding protein PilY1 of the type IV pili (TFP) (Cruz et al., 2014). Whole transcriptome analysis of *X. fastidiosa* biofilm cells in batch culture at different Ca concentration showed that genes involved in attachment and biofilm formation maintained a higher expression level in Ca-supplemented media; in contrast, these gene expression levels were gradually decreased in non-supplemented media, which indicates Ca promotes continued biofilm development in *X. fastidiosa* (Parker et al., 2016). Unravelling the molecular basis of the effect of Ca on virulence-related traits of *X. fastidiosa* is essential for understanding the biology of *X. fastidiosa* in plant xylem.

Xylem-residing bacterial pathogens grown in the xylem vessels are under continuous flow conditions. However, the majority of in vitro studies of xylem-residing bacterial pathogens have been performed in batch cultures. *X. fastidiosa* only survives in two natural habitats, the plant xylem vessels and the feeding canal of insects, which considers flow channels for xylem fluid (Chatterjee et al., 2008). Microfluidic chambers (MC), an artificial device mimicking the natural environment of plant xylem vessels and insect foreguts, provide a continuous flow condition for growth of xylem-residing bacterial pathogens (Meng et al., 2005; De La Fuente et al., 2007; Bahar et al., 2010). Bacterial cells grown in MC are monitored by time-lapse microscopy, which provides important spatial and temporal information for understanding physiological processes of bacteria that we are unable to observe directly inside the host in vivo. Previously, this MC system has been used to study virulence-related traits of *X. fastidiosa* (De La Fuente et al., 2007; Cruz et al., 2012; Navarrete and De La Fuente, 2014; Chen et al., 2017). In this present study, RNA-Seq was conducted on *X. fastidiosa* Temecula1, the causal agent of Pierce’s disease on grape, grown in MC comparing 2 mM CaCl2-supplemented and non-supplemented media. Results revealed regulation of genes by Ca that are responsible for enhanced twitching motility and are associated with pathogenicity and adaptation to the host. Comparison of these RNA-Seq data with our previous assessment in biofilm cells in batch culture (Parker et al., 2016) suggests that the regulation by Ca in *X. fastidiosa* is different between cells in biofilm and under flow conditions. In addition, phenotypic assessment revealed that Ca enhanced natural transformation of *X. fastidiosa*. These findings support that the regulatory role of Ca in *X. fastidiosa* acts differently depending on the stage of the infection process.

**Results and discussion**

**Adaptation of a microfluidics system for Xylella fastidiosa whole transcriptome analysis**

Both natural habitats of *X. fastidiosa*, that is insect foregut and plant xylem vessels, are under continuous flow conditions. Our MC dual parallel channel design (Fig. 1A, B) not only mimic these natural habitats but also offers the possibility to compare simultaneously bacterial behaviour under two different treatments. To confirm a previously observed phenotype (Cruz et al., 2012), twitching motility of *X. fastidiosa* Temecula1 in PD2 and 2Ca was quantified by tracking cells upstream movement. Cell twitching speed in 2Ca was significantly higher (*P < 0.001*) (0.65 ± 0.3 μm min⁻¹) than that in PD2 medium (0.38 ± 0.2 μm min⁻¹) (Fig. S1). *Xylella fastidiosa* Temecula1 cells were harvested from the MC system 6 days post-inoculation, when biofilm started to form (Fig. 1C). At this time point, the majority of Temecula1 cells in the channel were motile, and enough Temecula1 cells could be collected from the channel with the help of DNA/RNA Shield.

Initially, Temecula1 cells were harvested by increasing media flow rate, which could not remove all cells from the channel, resulting in a low cell concentration (data not shown). RNA isolated from those samples were in low quantity and quality and were not suitable for RNA-Seq analysis. When media was replaced by DNA/RNA Shield for harvest, all cells can be collected in a short time (Fig. 1C and Video S1), and more importantly, composition of mRNA from cells in the RNA protection buffer was not modified during the harvesting process and was protected for further processing.

In total, nine independent MC experiments were conducted, each consisting of two treatments (PD2 and 2Ca); therefore, 18 RNA samples were obtained. Although *X. fastidiosa* cells collected from each microchannel of MC were in a limited amount, RNA isolated from these samples fulfilled the requirements of quantity and quality for RNA sequencing analysis on Illumina HiSeq platform. Three groups of RNA samples with the best quality (Table S1) were used for whole transcriptome sequencing. Six RNA sequencing libraries were constructed and each library generated more than 9.7 million reads (sequencing reads available from NCBI BioProject ID PRJNA556203). After adapter and quality trimming and ribosome RNA filtering, 44–73% clean reads remained. The average length of post-trim reads ranged from 73 to 89 bp, and 92–96% of post-trim reads aligned to the *X. fastidiosa* Temecula1 genome (Data Set S1). Mapped reads were used to determine transcript boundaries and normalized expression for all protein-coding genes (Data Set S2). These results suggest our MC system is suitable for whole transcriptome analysis.
analysis of *X. fastidiosa*. Recently, microfluidic systems have been demonstrated to be suitable diagnostic devices measuring RNA expression of bacteria (by microarray) in human blood (Gandi et al., 2015). High-throughput microfluidics were used to assess the genome of a single bacterial cell (Pamp et al., 2012) and transcriptome of a single mammalian cell (Tang et al., 2009; Streets et al., 2014). These studies suggest the advantage for using a microfluidic system to discover novel finding in genetics. The present study is the first time where MC and RNA-Seq were combined to analyse the whole transcriptome of bacterial cells, which is a suitable system for novel discoveries in bacteriology.

Functional classification of differentially expressed genes (DEGs)

A total of 122 protein-coding genes were differentially expressed (*q* < 0.05, FC ≥ 1.5) when comparing 2Ca treatment to control. These DEGs represent 5% of the annotated genes of *X. fastidiosa* Temecula1. Among the 122 genes, 85 genes were upregulated, and 37 genes were downregulated (Fig. 2 and Data Set S3). To reveal the putative function of the DEGs, they were annotated and classified using Gene ontology (GO) analysis. The 225 GO terms were annotated and classified into three main categories: 82 biological processes (BP), 93 molecular functions (MF) and 50 cellular components (CC) (Fig. S2). Most of the DEGs were mainly related to cellular and metabolic process in BP, binding and catalytic activity in MF, and component of the membrane in CC. According to the GO classification of the DEGs, 56 genes could not be annotated with any GO term and were annotated as unknown function genes. In addition, all DEGs were searched against the KEGG database using a pathway mapping tool to obtain an overview of the metabolic pathways and other functional systems of *X. fastidiosa* Temecula1 regulated by external Ca supplementation. A total of 30 genes were mapped to different pathways involved in a series of compound metabolism and biogenesis, two-component system, homologous recombination, ABC transporters and bacterial secretion system (Data Set S4).

According to the GO classification and KEGG analysis of potential function of DEGs, Ca may be involved in many aspects of *X. fastidiosa* including nutrient metabolism, stimulus response, signal transduction and cellular component biosynthesis. Around 18% of DEGs encode enzymes with functions in metabolism of sugars, nucleic
acids, and amino acids, and biosynthesis of secondary metabolites and antibiotics. Interestingly, some of these enzymes are required for the glycolysis pathway (Data Set S4), indicating Ca could regulate glycolysis of \textit{X. fastidiosa}. Former studies in eukaryotes have shown that glycolysis is regulated by Ca via its effect on activities of glycolytic enzyme (Schönekess et al., 1995; Örtenblad et al., 2009; Nichols et al., 2017), suggesting this role of Ca may be conserved in prokaryotes and eukaryotes. Our results also suggest that Ca is an important signal messenger in this bacterium since many DEGs were associated with signal binding, transport and transcriptional regulation. Similar results were observed in other transcriptome analysis of bacterial responses to Ca (Oomes et al., 2009; Gode-Potratz et al., 2010; Parker et al., 2016; Zhu et al., 2017).

RT-qPCR was used to validate the RNA-Seq differential gene expression results. Expression levels of six selected genes related to pili, surface proteins and virulence were assessed using the same RNA samples that were used in the RNA-Seq analysis. Pearson's correlation coefficient for RT-qPCR and RNA-Seq results was 0.941 ($P = 0.005$, Fig. S3), which indicate a positive correlation and significant similarity between these two experiments.

\textbf{Differential expression of genes associated with twitching motility in 2Ca compared to PD2}

Twitching motility is an important virulence-related trait of \textit{X. fastidiosa} (Mattick, 2002; Chatterjee et al., 2008; Chen et al., 2017). This ability is mediated by extension and retraction of TFP that contributes to upstream movement of \textit{X. fastidiosa}, which is very important for this pathogen to colonize the plant xylem system (Meng et al., 2005; De La Fuente et al., 2007). TFP is complex surface appendages, at least 30 genes in \textit{X. fastidiosa} are involved in biogenesis and function of TFP (Wang et al., 2012). Our data have shown that nine genes involved in TFP assembly and functional regulation were differentially expressed between 2Ca and PD2 treatments (Fig. 3, Table 1). Genes encoding PilN, PilO and PilP for TFP pilus assembly sub-complex were upregulated. These proteins, together with PilQ and PilM, constitute the secretion complex of the pilin protein that is the basic unit for the extracellular part of TFP (Goosens et al., 2017). Mutation of this complex leads to absence of TFP (Li et al., 2007). Three components of the chemosensory system, pilI, pilG and pilH were upregulated as well. This chemosensory system is known to play a role in pil formation, extension and retraction (Bertrand et al., 2010; Cursino et al., 2011). pilR, encoding the response regulator of the two-component system (TCS) PilS-PilR, was upregulated by 2Ca. PilS-PilR TCS plays an important regulatory role in TFP biosynthesis (Hobbs et al., 1993; Kilmury and Burrows, 2016). It has been reported that the regulator PilR can bind to an enhancer sequence associated with the promoter of the pilin gene in \textit{P. aeruginosa} (Jin et al., 1994). Additionally, two of the three predicted pilA homologues in \textit{X. fastidiosa} Temecula1, PD1077 and PD1924 (pilA1), were downregulated. The pilA gene encodes pilin, which is the basic component of the TFP filaments (Mattick, 2002). Proteins encoded by the three pilA homologue genes, PD1077, PD1924 (pilA1) and PD1926 (pilA2), contain conserved protein domains related to PilA. Comparison of amino acid sequences of these three proteins indicated that PilA1 has 65% identity with PilA2, but

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig2}
\caption{Scatter plots of log gene expression from \textit{X. fastidiosa} cells cultured in MC (2Ca vs. PD2) as computed from the RNA-Seq data. Genes significantly differentially expressed in 2Ca/PD2 (1.5-fold change; $q < 0.05$) are indicated in yellow (upregulation) or blue (downregulation). Non-differentially expressed genes are indicated in green.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Expression changes of selected type IV pilin genes in \textit{X. fastidiosa} cultured in microfluidic chamber. Each bar shows the log2 fold change of gene expression in 2Ca versus PD2 as computed from the RNA-Seq data. Black bars indicate $q < 0.05$. Asterisk indicates fold changes > 1.5.}
\end{figure}
PD1077 has 45% identity with PilA1 and 36% identity with PilA2. The transcriptome data show that expression of PD1077 was similar to pilA1 but much lower than pilA2. The function of PD1077 has not been characterized yet; however, pilA1 and pilA2 were studied by our group, and we demonstrated that pilA1 affects the number and position of TFP, while pilA2 is needed for twitching movement (Kandel et al., 2018). This suggests that PilA1 may be a regulator protein rather than the major pilin of TFP, but this needs to be further studied. 2Ca suppressed pilA1 but did not affect pilA2, which could contribute to a more effective twitching motility of X. fastidiosa. According to these results, we hypothesize that Ca transcriptionally regulated TFP assembly and function to enhance twitching motility of X. fastidiosa.

Results in this study have shown that more than ten genes encoding response and transcriptional regulators were regulated by 2Ca in X. fastidiosa (Data Set S3). A key transcriptional regulator downregulated by 2Ca is gacA, which is a global activator in many plant-associated gram-negative bacteria (Heeb and Haas, 2001). The phenotype and transcriptome of X. fastidiosa gacA contributes to cell adhesion and surface attachment (Shi et al., 2009). In P. aeruginosa, GacA is a key component in the Gac/Rsm pathway which mediates Ca signaling to regulate motility and biofilm formation of this pathogen (Broder et al., 2017). Since our previous results (Cruz et al., 2012) showed that Ca regulates attachment and motility of X. fastidiosa, we...
speculate that gacA may be part of the regulatory cascade involving Ca.

Comparison of gene expression profiles of X. fastidiosa in microfluidic chamber and batch cultures

The whole transcriptome of X. fastidiosa biofilm cells grown in flasks with PD2 and 4Ca (PD2 supplemented 4 mM CaCl₂) for 72 h was previously studied by our group using RNA-Seq (Parker et al., 2016), which is referred here as ‘Flask-RNA-Seq’. For comparisons, results from this present study are referred as ‘MC-RNA-Seq’. Here, the two RNA-Seq experiments were compared (Fig. 4, Table 1). Even considering that many parameters between these two experiments were different, including culture conditions (MC and Flasks), cell physiological state (all cells and biofilm cells) and Ca concentrations (2 and 4 mM); we believe that comparison of these two studies has significance for understanding the molecular basis of the role of Ca during the X. fastidiosa infection process.

The overall distribution of DEGs based on functional annotation in both RNA-Seq studies in X. fastidiosa was similar, which confirmed the regulatory function of Ca in X. fastidiosa. Around 40% DEGs were classified as unknown function genes and phage-related genes (Fig. 4A), suggesting the Ca-regulated genetic network in X. fastidiosa remains elusive. According to this distribution, the percentage of genes belonging to the motility functional group in MC-RNA-Seq was much higher than that in Flask-RNA-Seq. The majority of DEGs belonging to motility functional group in MC-RNA-Seq were upregulated by Ca, while genes belonging to this group in Flask-RNA-Seq were downregulated (Table 1). However, in Flask-RNA-Seq, the percentage of DEGs belonging to afimbrial adhesins and exopolysaccharides (EPS) biosynthesis functional groups was higher than that in MC-RNA-Seq (Fig. 4A). Adhesion and EPS production are two critical aspects for biofilm formation in many bacteria (Marques et al., 2002; Castiblanco and Sundin, 2015). Genes belonging to these two groups in Flask-RNA-Seq were upregulated, but genes belonging to EPS biosynthesis functional groups in MC-RNA-Seq were downregulated (Table 1). These results corresponded with the physiological states that were used in these two studies: MC included both planktonic and biofilm cells, but most cells were actively moving against the flow (Fig. 1C and Video S2); while cells in flasks were collected exclusively from biofilms (Parker et al., 2016).

Only 14 DEGs overlapped between MC-RNA-Seq and Flask-RNA-Seq (Fig. 4B, Table 1), and only 4 of the 14
genes regulated by Ca showed the same tendency (viz., upregulated or downregulated in both cases), including PD1087, pilA1, cvaC and hsf (Table 1). PD1087 and pilA1 were downregulated by Ca in both RNA-Seq experiments. PD1087 encodes an XRE family transcriptional regulator and is located upstream of multiple phage-related gene operons. Regulators of the XRE family are widely distributed and regulate functions such as oxidant tolerance and virulence in Streptococcus suis (Hu et al., 2018); repression of a defective prophage in Bacillus subtilis (Wood et al., 1990; McDonnell and McConnell, 1994) and resistance of antibiotics in Lactococcus lactis (McAuliffe et al., 2001). However, the role of PD1087 in X. fastidiosa is unclear and needs further characterization. pilA1, as mentioned above, is associated with the number and location of TFP. Deletion of pilA1 led to an increase in biofilm formation by X. fastidiosa but did not affect its twitching motility (Kandel et al., 2018). This protein seems to be an important component for both stages of X. fastidiosa growth, planktonic and biofilm, and this can explain the same regulatory tendency in both transcriptome studies discussed here.

The cvaC and hsf were upregulated by Ca in both experiments. cvaC is predicted to encode a colicin V-like bacteriocin, an antimicrobial peptide first reported in E. coli (Waters and Crosa, 1991). Genes required for production and secretion of colicin V-like bacteriocin were found among the genome of X. fastidiosa, including cvaC (PD0215), cvaA (PD0496), cvaB (PD0499) and cvpA (PD0852). Here, we found that in addition to cvaC, cvpA was also upregulated by 2Ca. In host plant xylem systems, X. fastidiosa is not the only bacterium to reside in that environment, previous studies showed that there is an endophytic bacterial community, and the communities are different between healthy and X. fastidiosa infected hosts (Araújo et al., 2002). Xylella fastidiosa should have some arsenals to compete with other endophytic bacteria, enabling the colonization of xylem vessels. According to previous transcriptome analysis of X. fastidiosa under other xylem-related environmental contexts, including basic medium supplemented with the inorganic nutrient iron (Fe) (Zaini et al., 2008), organic nutrient glucose (Pashalidis et al., 2005) and grape sap (Ciraulo et al., 2010), cvaC was upregulated in all of these xylem-related conditions, suggesting that this gene may play an important role in the environmental adaptation of X. fastidiosa. The hsf gene encodes a surface protein similar to Hsf (Haemophilus surface fibril), and the C-terminal region of this protein shares characteristics with autotransporter proteins (de Souza et al., 2003). Homologues of this protein in other bacteria are important for cell adhesion and attachment to the host cell (de Souza et al., 2003; Singh et al., 2015). The hsf gene expression level in recently isolated X. fastidiosa 9a5c was significantly higher than that in 9a5c after many passages in axenic culture (de Souza et al., 2003), suggesting hsf could be a pathogenicity-related factor in X. fastidiosa. Interestingly, expression of hsf was also upregulated in grape sap-supplemented media (Ciraulo et al., 2010) but suppressed by Fe (Zaini et al., 2008), and did not change in basic medium supplemented with glucose (Pashalidis et al., 2005). Accordingly, modification of hsf gene expression is associated with increased concentrations of mineral nutrients Ca and Fe and grape sap; therefore, we speculate that hsf encodes a type of adhesin that may be involved in ion bridge-mediated adherence. The function of hsf in X. fastidiosa still needs further studies to be elucidated.

Fig. 5. Effect of 2 mM external Ca on natural recombination in X. fastidiosa Temecula1.

A. X. fastidiosa was grown in microfluidic chambers. Plasmid pAX1.Cm was used as donor DNA.

B. X. fastidiosa was grown on agar media plates. Plasmids pAX1.Km and pAX1.Cm were used as donor DNA. For each growth condition, three independent experiments were conducted. Data represent means (A; MC: n = 6, B, plates: n = 9), and error bars represent standard errors form different experiments. Asterisk indicates significant difference as analysed by H-test (P < 0.05).
Another interesting finding among these 14 overlapped DEGs was that the regulation of 7 phage-related genes by Ca that is exactly opposite between MC and flasks (Table 1). PD0906, PD0916, PD0926, PD0932, PD0936 and PD0943 were upregulated in MC and downregulated in flasks. These genes are located in a putative genomic island comprising PD0906-PD0943 (Parker et al., 2016). 21 out of the 38 genes in this island do not have homologues in the closely related avirulent strain X. fastidiosa EB92-1 (Zhang et al., 2011; Parker et al., 2016). These findings suggest that the role of these phage-related genes may be associated with twitching motility, biofilm formation and virulence of X. fastidiosa, but to confirm this, further investigation is needed.

**Effect of 2 mM external Ca on natural competence of Xylella fastidiosa**

Xylella fastidiosa is naturally competent, with the ability to take environmental DNA fragments and integrate them into its genome via homologous recombination (Lorenz and Wackernagel, 1994; Kung and Almeida, 2011); this was hypothesized to impact adaption of this pathogen to new hosts and cause disease emergence (Almeida and Wackernagel, 1994; Kung and Almeida, 2011); this was hypothesized to impact adaption of this pathogen to new hosts and cause disease emergence (Almeida and Wackernagel, 1994; Kung and Almeida, 2011). This may be one explanation for the finding that Ca increased natural recombination frequency in MC_out fraction but not in MC_in fraction. Our transcriptome data support this concept. In MC-RNA-Seq, 2Ca enhanced natural recombination frequency in X. fastidiosa, confirming our hypotheses.

Ca has been reported to be necessary for natural competence in some bacteria: 1 mM Ca^{2+} was critical for competence induction in Streptococcus pneumoniae (Trombe, 1993); 1–2 mM Ca^{2+} was enough to induce Escherichia coli to natural competence, which has been originally thought to be incapable of this process (Baur et al., 1996); and in Azotobacter vinelandii, 0.5 and 1 mM Ca^{2+} was required to optimally trigger natural competence (Page and Doran, 1981). Similarly, contribution of Ca to natural competence has been reported in other bacteria. In the human pathogen Acinetobacter baumannii, a naturally competent strain, its natural transformation frequency was increased upon the addition of Ca (Traglia et al., 2016). In another naturally competent pathogen Aggregatibacter actinomycetemcomitans, 1 mM external Ca enhanced its cell aggregation and natural competence (Hisano et al., 2014), and the author of that study postulated Ca-promoted biofilm may increase the ability of the bacteria to uptake environmental DNA (Hisano et al., 2014). This may be one explanation for the finding that Ca increased natural recombination frequency in MC_out fraction but not in MC_in fraction. Ca enhanced natural competence is associated with Ca-promoted biofilm (accumulation of biofilm in MC_out fraction is more than that in MC_in fraction). Our transcriptome data support this concept. In MC-RNA-Seq, 2Ca can increase expression of twitching motility and recombination-related genes; however, competence-related genes comM and comE were downregulated (Table 1). For batch cultures (Flask-RNA-Seq), where biofilms cells were sampled, 4Ca increased expression of competence-related gene comA (Table 1) (Parker et al., 2016).

**Conclusions**

Although it is important to study the whole transcriptome of bacteria by RNA-Seq in planta, as it was done in a few cases (Ailloud et al., 2016; Lee et al., 2017; Nobori...
et al., 2018), this is technically challenging with X. fastidiosa. The main limitation is the small amount and uneven distribution of bacteria found in planta (e.g. $10^5$ to $10^7$ CFU g$^{-1}$ for X. fastidiosa vs. $10^6$ to $10^9$ CFU g$^{-1}$ for Ralstonia sp.) (Hill and Purcell, 1995; Jacobs et al., 2012). Another limitation is that woody plants are hosts for X. fastidiosa, therefore, making it harder to extract the relatively small amount of bacteria inside xylem vessels. However, our MC provides a uniform, precisely controlled growth environment close to the natural conditions for X. fastidiosa. Through time-lapse microscopy, MC also provides critical spatial and temporal information regarding physiological processes of bacteria that we are unable to observe directly in planta. Therefore, we feel that our contribution of the system combining MC with RNA-Seq is a valid approximation to understand gene regulation under flow conditions mimicking the natural habitats of these bacteria. In susceptible grapes, the host of X. fastidiosa Temecula1, Ca concentration in its xylem sap is approximately 2–3 mM (Cobine et al., 2013), the same concentration we used in this present study. We conclude that under flow conditions Ca transcriptionally regulates the machinery of TFP to enhance twitching motility and other key genes involved in pathogenicity and adaptation of X. fastidiosa to the host environment. The transcriptome profile described in this study represents that of X. fastidiosa during the initial stages of colonization when they were either recently injected into the host xylem vessels by insect vectors, or they broke free from mature biofilms, and they are moving in the xylem. Through comparisons with our previous transcriptome study with mature biofilms (Parker et al., 2016), we conclude that Ca influences gene expression differently at different growth stages. Nevertheless, we found a few common regulation patterns between both studies; in particular, we found that Ca can modulate expression of genes related to natural competence and increase natural competence of X. fastidiosa. Collectively, this study suggests the Ca concentration in susceptible grape contributes for pathogenicity, natural competence and host adaptation of X. fastidiosa Temecula1.

**Experimental procedures**

**Bacterial strains and culture conditions**

*Xylella fastidiosa* subsp. *fastidiosa* type strain Temecula1 was used throughout this study. Temecula1 was cultured on PW (Davis et al., 1980) or PD3 (Davis et al., 1981a) plates at 28°C. Cell suspensions of Temecula1 used for MC experiments were prepared as follows: Temecula1 was recovered from glycerol stocks on PW plates and grown for 7 days. Temecula1 cultures were streaked to new PW plates, and seven-day-old Temecula1 subcultures were scraped from PW plates, suspended in PD2 (Davis et al., 1981b) or PD2 supplemented with 2 mM CaCl$_2$ (2Ca) liquid media and diluted to an OD$_{600}$ of 1.0.

*Xylella fastidiosa* cultures in microfluidic chambers for transcriptome analysis

The MC with dual parallel channel design (Fig. 1A) used in this study was fabricated as previously described (De La Fuente et al., 2007). The major portion of MC consisted of a moulded polydimethylsiloxane (PDMS) body with two parallel microchannels on a surface that was sealed by a cover glass (Fig. 1A). The dimensions of these microchannels were 80 µm wide, 50 µm deep and 3.7 cm long. For each microchannel, there are two inlets for introduction of liquid medium and bacteria suspension, and an outlet for collection of fluid flow.

The Temecula1 suspension was used to fill a pair of 1 ml plastic syringes (Becton Dickinson & Company, Franklin Lakes, NJ, USA) that were, respectively, connected to the bacterial inlet of each microchannel (Fig. 1A). Another pair of 5 ml glass syringes (Hamilton Company, Reno, NV) was filled with PD2 liquid medium (PD2 treatment) or 2Ca (2Ca treatment) and connected to the media inlet of each microchannel (Fig. 1B). Media and Temecula1 suspension were injected into microchannels by two programmable dual channel syringe pumps (Pico Plus; Harvard Apparatus, Holliston, MA, USA). Fluid flow was collected in two sealed containers connected to the outlets (Fig. 1B). To allow Temecula1 cells to attach to the inside of the microchannels, the flow rates of media and suspension were 1 µl min$^{-1}$ and 0.6 µl min$^{-1}$ respectively. After 1 h, cell suspension injection was stopped, and the flow rate of media was adjusted to 0.25 µl min$^{-1}$, which is close to average xylem sap flow rate inside grapevines (Andersen and Brodbeck, 1989; Greenspan et al., 1996). MC was monitored under a Nikon Eclipse Ti inverted microscope (Nikon, Melville, NY, USA), using Nomarski differential interference contrast optics and phase contrast. Time-lapse images were acquired every 30 s, using a Nikon DS-Q1 digital camera (Nikon) controlled by NIS-Elements Advanced Research 3.01 (Nikon). After 6 days, in order to harvest X. fastidiosa cells inside microchannels, 1.5 ml Eppendorf tubes replaced the collection containers. Media was changed to DNA/RNA Shield™ (Zymo Research, Irvine, CA, USA), used to preserve nucleic acids and help remove X. fastidiosa cells attached to surfaces inside the MC. Flow rate during cell removal using DNA/RNA Shield™ was adjusted to 20 µl min$^{-1}$. Finally, each X. fastidiosa sample in 500 µl DNA/RNA Shield was stored at −80°C. Samples from three independent MC experiments were used for sequencing analysis.
Library construction and sequencing analysis

RNA preparation, cDNA library construction and sequencing were performed as previously described (Parker et al., 2016). Briefly, total RNA was extracted and purified using Quick-RNA™ MiniPrep and RNA Clean and Concentration™ (Zymo Research). Purity, concentration and integrity of RNA samples were measured by using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). RNA samples with a RNA:DNA ratio > 15 and RNA integrity number (RIN) > 7 were treated with Ribo-Zero™ rRNA Removal Kit (Gram-Negative Bacteria) (Epicentre, Madison, WI, USA) to deplete ribosomal RNA. Libraries were prepared with the TruSeq Stranded mRNA Sample Prep Kit (Illumina, Inc., San Diego, CA, USA), and paired-end sequencing was performed on an Illumina HiSeq™ 1500 system at the Auburn University Genomics and Sequencing Laboratory.

RNA-Seq data processing and analysis

Adapter and quality trimming of the raw sequencing data was conducted using TRIMMOMATIC version 0.33 (Bolger et al., 2014). Reads aligning to Ribosomal RNA were filtered using GENEIOUS VERSION 10.0 (Biomatters Ltd., New-

Quantitative PCR (qPCR)

The remaining total RNA from the three MC experiments was adjusted to 1 ng µl⁻¹ and reverse-transcribed to cDNA for qPCR analysis using the qScriptTM cDNA Supermix (Quanta Biosciences, Beverly, MA, USA). Taq-Man qpcrcein primer and probe (labelled with 5’ 6-carboxyfluorescein [FAM] and 3’ Black Hole 588 Quencher-1 [BHQ-1]) sets were used from previous publications or designed using PrimerQuest (Integrated DNA Technologies, Coralville, IA, USA). The primer/probe sets targeted 7 genes (2 upregulated, 3 downregulated, 1 no differentially expressed and 1 internal control; Table S2), qPCR was conducted on a CFX96™ Real-Time System (Bio-Rad Laboratories, Hercules, CA, USA) in 20 µl reactions contains: 1 µl cDNA, 1 × PerfeCTa MultiPlex qPCR Sup-

Effect of calcium on natural competence of X. fastidiosa

Plasmids pAX1.Km and pAX1.Cm from a previous study (Matsumoto et al., 2009) were used as donor DNA for natural transformation of X. fastidiosa Temecula1. Experiments were performed under two growth conditions: solid agar plates (plates) and MC. PD3 medium (PD3) was used as control, and PD3 supplemented with 2 mM CaCl² (2Ca) was used as treatment. Natural competence assays under different growth conditions were conducted as previously described (Kandel et al., 2016). Briefly, for agar plate conditions 10 µl of X. fastidiosa Temecula1 cell suspension at OD₆₀₀ of 0.25 was spotted on top of PD3 or 2Ca plates, and 1 µg of plasmid (pAX1.Km/ pAX1.Cm) in a 10 µl volume was added to the spots. Following incubation at 28°C for ~ 3 days, spots were suspended in 500 µl PD3 and serial dilutions were plated on PW plates with or without the respective antibiotics: 30 µg ml⁻¹ kanamycin (Km) and 10 µg ml⁻¹ chloramphenicol (Cm). After 10–14 days of incubation at 28°C, CFUs on all plates were enumerated for recombinants and total viable cells. Recombination frequency was calculated as a ratio of the number of recombinants to total viable cells (recombinant/ total cells) (Kandel et al., 2016). MC recombination experiments were performed as described before (Kandel et al., 2016). PD3/2Ca media with 1 µg ml⁻¹ pAX1.Cm plasmid flowed through the microchannel with a rate of 0.25 µl min⁻¹. Cell suspensions of OD₆₀₀ of 0.25 were inoculated into the microchannel. After 7 days, the fraction of cells collected in the outlet containers (MC_out, Fig. 1B) was harvested; then, the fraction inside the microchannel (MC_in, Fig. 1B) was detached and harvested. Serial dilution, plating, CFU counts and recombination frequency calculations were performed following standard procedures (Kandel et al., 2016). For agar plates condition, three independent experiments were performed, and three replications were included in each experiment (n = 9). For MCs condition, two independent experiments were performed with six replicates in total (n = 6). Recombination frequency data were analysed by Students’ t-test (P < 0.05) using R Project 3.4.3 for Windows.

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Conflict of interest
None declared.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1. Twitching speed assessments of *X. fastidiosa* Temecula1 in microfluidic chambers comparing PD2 and 2Ca treatments. Different letters on top of the bars indicate significant differences (*P* < 0.05) according to Student’s *t* test. Data presented was obtained from six independent experiments. Error bars correspond to SE of the mean (*n* = 18).

Fig. S2. Gene Ontology classification of the differentially expressed genes in *X. fastidiosa* microfluidic transcriptome study. Gene Ontology (GO) term assignment to the 122 DEGs in this study were summarized into three main GO categories (biological process, cellular component, molecular function) and 28 sub-categories (Level 2).

Fig. S3. RNA-Seq confirmation using qPCR. Log2 fold change of qPCR data is plotted against that of RNA-Seq data for 6 genes. Equation of the line and correlation coefficients are presented.

Video S1. Harvest of *X. fastidiosa* Temecula1 cells in microfluidic chambers. DNA/RNA shield buffer constantly flows from left to right in both channels. All Temecula1 cells grown in the channel were removed quickly.

Video S2. *X. fastidiosa* Temecula1 cells grown in microfluidic chambers. PD2 and 2Ca media constantly flows from left to right in both channels.

Data Set S1. RNA-Seq read and assembly statistics for the 6 *X. fastidiosa* samples.

Data Set S2. RNA-Seq data analysis for all *X. fastidiosa* protein-coding genes. Includes transcript boundaries, normalized expression levels and statistical significance of differential gene expression for 2Ca and PD2 treatments.

Data Set S3. Functional categorization of *X. fastidiosa* protein-coding genes differentially expressed in 2Ca/PD2. The fold changes are shown for these genes.

Data Set S4. Mapping of *X. fastidiosa* protein-coding genes differentially expressed in 2Ca/PD2 to the KEGG pathways.

Table S1. Summary of purified total RNA samples.

Table S2. Primers and probes used in qPCR analysis.

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