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

Hongyu Chen and Leonardo De La Fuente*
Department of Entomology and Plant Pathology, Auburn University, Auburn, AL 36849, USA.

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.**

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*For correspondence. E-mail lzd0005@auburn.edu; Tel. (334) 844-2582; Fax (334) 844-1947.
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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 (Dominguez, 2004; Dominguez *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, both considered 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 $CaCl_2$-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

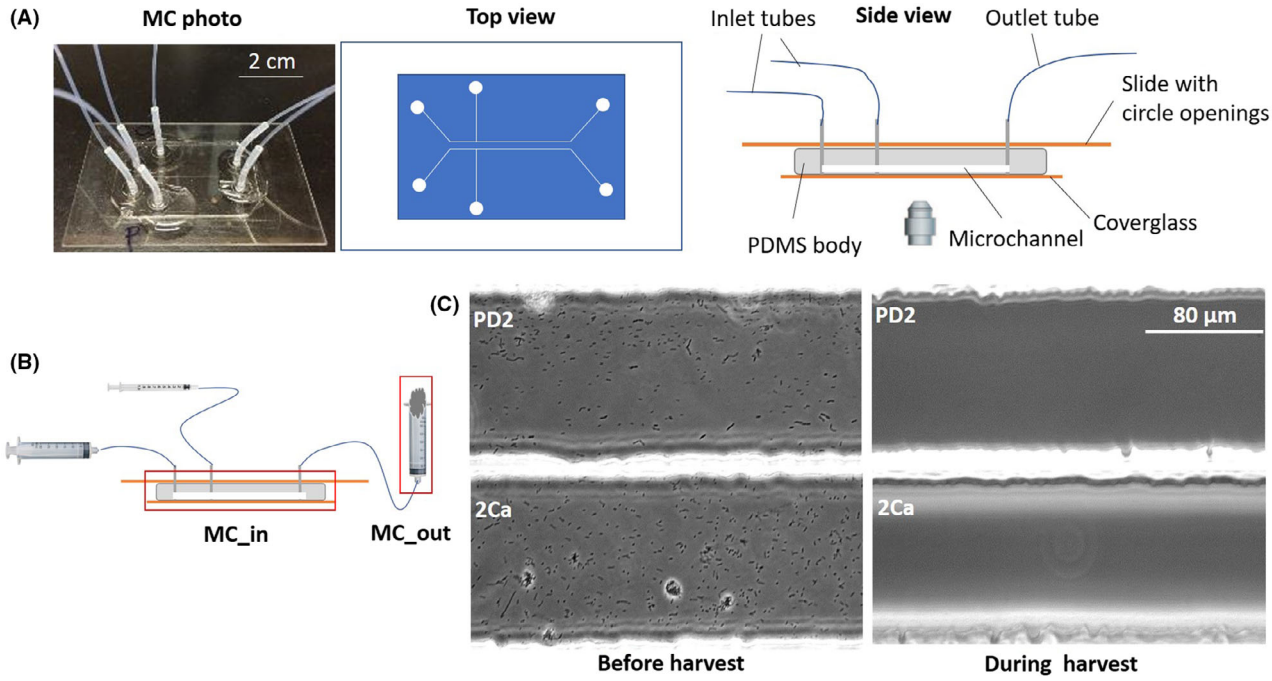

Fig. 1. Preparation of *X. fastidiosa* cultures in microfluidic chamber (MC) for whole transcriptome analysis.
A. MC with dual parallel channel design. Left picture shows an assembled MC, middle diagram shows top view, and right diagram shows side view of the MC.
B. Side view of the MC during *X. fastidiosa* growth experiments. 5 ml glass syringes filled with media were connected to medium inlets, 1 ml plastic syringes filled with *X. fastidiosa* suspension were connected to bacterium inlets, and 10 ml plastic syringes sealed with cotton balls were connected to outlets. During cell harvest, 1 ml plastic syringe filled with RNA/DNA Shield buffer replaced the 5 ml glass syringes, and the 10 ml plastic syringes were replaced by 1.5 ml microcentrifuge tubes.
C. Micrographs of the two parallel channels showing the growth of *X. fastidiosa* and harvest of cells from channels.

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

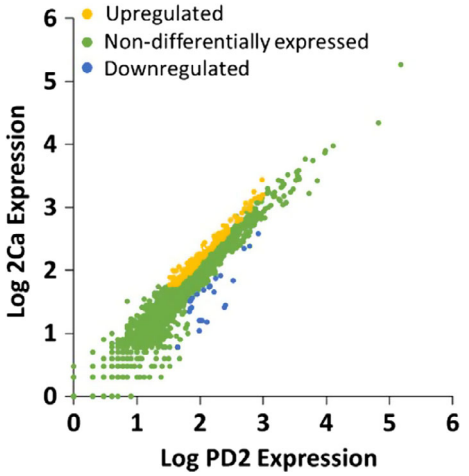

Fig. 2. Scatter plots of log gene expression from *X. fastidiosa* cells cultured in MC (2Ca vs. PD2) as computed from the RNA-Seq data. Genes significantly differentially expressed in 2Ca/PD2 (1.5-fold change; $q < 0.05$) are indicated in yellow (upregulation) or blue (downregulation). Non-differentially expressed genes are indicated in green.

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 *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; Ørtenblad *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.

Differential expression of genes associated with twitching motility in 2Ca compared to PD2

Twitching motility is an important virulence-related trait of *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 *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 *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 pili assembly subcomplex 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 pili 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 *P. aeruginosa* (Jin et al., 1994). Additionally, two of the three predicted *pilA* homologues in *X. fastidiosa* Temecula1, PD1077 and PD1924 (*pilA*1), 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 (*pilA*1) and PD1926 (*pilA*2), 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

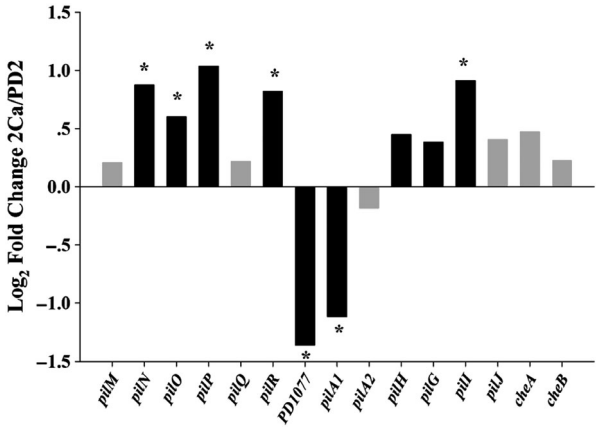

Fig. 3. Expression changes of selected type IV pili genes in *X. fastidiosa* cultured in microfluidic chamber. Each bar shows the log2 fold change of gene expression in 2Ca versus PD2 as computed from the RNA-Seq data. Black bars indicate $q < 0.05$. Asterisk indicates fold changes > 1.5.

Table 1. Selected genes transcriptionally regulated by Ca in MC-RNA-Seq and Flask-RNA-Seq.

Functional group	Gene name	Locus tag	Product	MC	Flask
Twitching motility	*pilG**	PD0845	Pili chemotaxis CheY homolog PilG	Up	–
	pilI	PD0846	Pili chemotaxis CheW homolog Pil	Up	–
	-	PD1077	Fimbrial protein	Down	–
	*pilH**	PD1632	Regulatory protein pilH family	Up	Down
	pilP	PD1692	Fimbrial assembly protein	Up	–
	pilO	PD1693	Fimbrial assembly membrane protein	Up	–
	pilN	PD1694	Fimbrial assembly membrane protein	Up	–
	*pilA*1	**PD1924**	Fimbrial protein	Down	Down
	*pilA*2	PD1926	Fimbrial protein	-	Down
	pilR	PD1928	Regulatory protein	Up	–
Adhesins and EPS biosynthesis	xanA	PD0213	Phosphoglucomutase	Down	–
	xadA	PD0731	Outer membrane protein XadA	–	Up
	hsf	**PD0744**	Surface protein	Up	Up
	pspA	PD0986	Hemagglutinin-like secreted protein	–	Up
	gumM	PD1387	GumM protein	–	–
	gumJ	PD1389	GumJ protein	–	Up
	gumH	PD1391	GumH protein	–	Up
	gumF	PD1392	GumF protein	–	Up
	hxfA	PD2118	Hemagglutinin-like secreted protein	–	Up
Regulatory function	–	**PD1087**	XRE family transcriptional regulator	Down	Down
	gacA	PD1984	Transcriptional regulator	Down	-
Phage-related proteins	–	**PD0377**	Phage-related protein	Down	Up
	–	**PD0906**	Phage-related protein	Up	Down
	–	**PD0916**	Phage-related protein	Up	Down
	–	**PD0929**	Phage-related protein	Up	Down
	–	**PD0932**	Phage-related protein	Up	Down
	–	**PD0936**	Phage-related protein	Up	Down
	–	**PD0943**	Phage-related protein	Up	Down
Colicin V-like bacteriocins	*cvaC*	**PD0215**	colicin V precursor	Up	Up
Recombination and competence	*recF*	**PD0003**	Recombination protein F	Up	–
	recJ	**PD0402**	ssDNA-specific exonuclease RecJ	–	Up
	recD	**PD1651**	exodeoxyribonuclease V subunit alpha	–	Up
	comA	**PD0358**	DNA uptake protein	–	Up
	comM	**PD0464**	Competence-like protein	Down	–
	comB	**PD1558**	DNA transport competence protein	Down	–
	ruvC	**PD0886**	Holliday junction resolvase	Up	Down
	ung	**PD2049**	Uracil-DNA glycosylase	Up	–
Uncharacterized Proteins		**PD0556**	hypothetical protein	Up	Down
		PD1111	hypothetical protein	Down	Up

–, No changes; Down, downregulated gene expression; Flask, Flask-RNA-Seq (Parker *et al*., 2016); MC, MC-RNA-Seq (this study); and Up, upregulated gene expression.
Locus tag in bold text indicates genes overlapped between MC and Flask.
Asterisk indicates q value of gene is < 0.05, but fold change is not > 1.5.

PD1077 has 45% identity with PilA1 and 36% identity with PilA2. The transcriptome data show that expression of PD1077 was similar to *pilA*1 but much lower than *pilA*2. 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 *pilA*1 but did not affect *pilA*2, 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* mutant were previously studied, concluding that GacA in *X. fastidiosa* 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

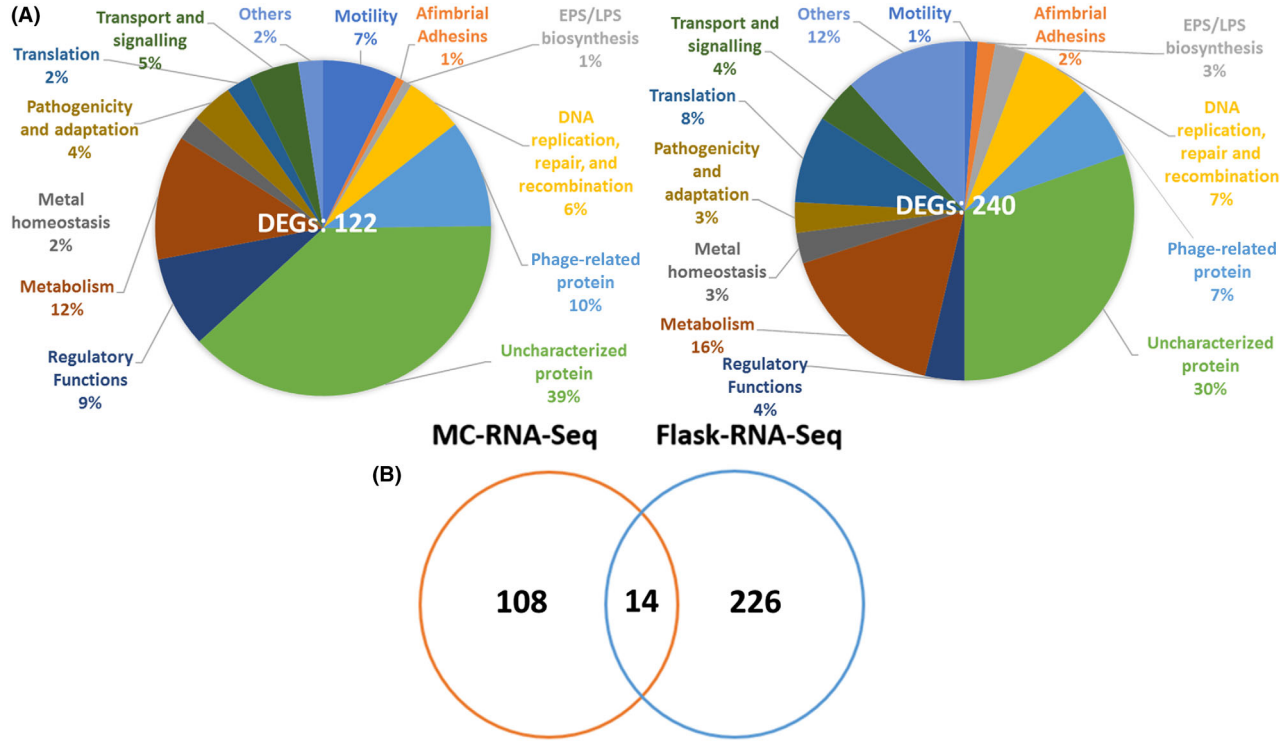

Fig. 4. Comparison of the effect of Ca on gene expression profiles of *X. fastidiosa* grown in microfluidic chamber (MC-RNA-Seq) and batch cultures (Flask-RNA-Seq, Parker et al., 2016).A. Pie charts for distribution of differentially expressed genes (DEGs) in different functional categories between MC-RNA-Seq and Flask-RNA-Seq.B. Venn diagram showing number of DEGs overlapping between the two RNA-Seq experiments.

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 $CaCl_2$) 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 *pil*A1 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. *pilA*1, as mentioned above, is associated with the number and location of TFP. Deletion of *pilA*1 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. *cva*C 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.

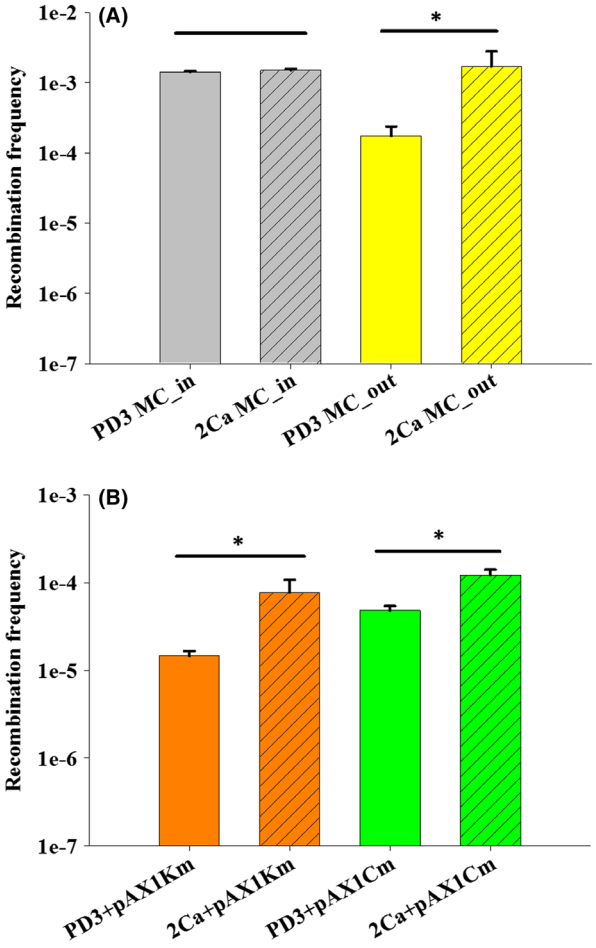

Fig. 5. Effect of 2 mM external Ca on natural recombination in *X. fastidiosa* Temecula1.
A. *X. fastidiosa* was grown in microfluidic chambers. Plasmid pAX1.Cm was used as donor DNA.
B. *X. fastidiosa* was grown on agar media plates. Plasmids pAX1.Km and pAX1.Cm were used as donor DNA. For each growth 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 *t*-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 Nunney, 2015; Kandel *et al*., 2016; Sicard *et al*., 2018; Potnis *et al*., 2019). The differential gene expression data in this study showed that genes associated with homologous recombination, including *recF, ruvD and ung*, were upregulated in 2Ca (Table 1). Interestingly, in Flask-RNA-Seq, genes *recJ*, *recD* and *comA* involved in recombination and competence were also upregulated in 4Ca (Table 1) (Parker *et al*., 2016). Moreover, twitching motility of *X. fastidiosa*, which has been reported to be important for natural transformation (Kandel *et al*., 2017), was enhanced in 2Ca. Taking these results into consideration, we hypothesized that 2 mM external Ca contributes to natural competence of *X. fastidiosa*.

To determine whether natural competence of *X. fastidiosa* is influenced by Ca, *X. fastidiosa* Temecula1 was incubated with plasmid pAX1.Cm or pAX1.Km and cultured in MC or on plates with and without amendment of 2 mM $CaCl_2$ (2Ca vs. PD3), and the frequency of acquisition of antibiotic resistance marker by *X. fastidiosa* Temecula1 was assessed (Fig. 5). Under MC conditions, using pAX1.Cm as donor DNA, the recombination frequency (recombinant/total cells) in PD3 and 2Ca was 1.42×10^{-3} and 1.50×10^{-3} respectively. For MC_in fraction (*X. fastidiosa* cells collected from inside the microchannels of MC, Fig. 1B), no significant differences ($P = 0.287$) between treatments were found. However, for MC_out fraction (*X. fastidiosa* cells collected from the outlet containers of MC, Fig. 1B), the recombination frequency in PD3 and 2Ca was significantly different ($P = 0.026$) with 1.74×10^{-4} and 1.70×10^{-3} respectively. We also observed that the recombination frequency in PD3 for MC_in fraction was higher than that for MC_out fraction, similar to that in previous studies (Kandel *et al*., 2016), which confirmed flow conditions in MC contributes to natural competence of *X. fastidiosa*. Under agar plate conditions, using pAX1.Km plasmid as donor DNA, the recombination frequency on 2Ca plates (7.7×10^{-5}) was significantly higher ($P < 0.001$) than that on PD3 plates (1.5×10^{-5}). Similarly, using pAX1.Cm as donor DNA, the recombination frequency in 2Ca treatment and control (1.22×10^{-4} and 4.80×10^{-5}) was significant different ($P = 0.014$). These results suggest that 2 mM external Ca increased 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 *Azotobater 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. 10^8 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 our 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., Newark, NJ, USA) to reduce background noise. Gene expression analysis was conducted as previously described using ROCKHOPPER version 2.03 (McClure *et al*., 2013; Parker *et al*., 2016). In this study, q-value < 0.05 and fold change (FC) ≥ 1.5 were considered as the threshold values for selecting DEGs. GO annotation of DEGs was performed using BLAST2GO 5.0. DEGs were searched against the Kyoto Encyclopedia of Gene and Genomes (KEGG) database using KEGG mapper.

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). TaqMan qPCR 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 SuperMix Low-ROX (Quanta Biosciences), 0.4 μM forward and reverse primers and 0.2 μM TaqMan probe. The following cycling parameters were used 95°C for 1 min, followed by 40 cycles of 95°C for 15 sand 60°C for 1 min. Fold change in expression of target genes was calculated by the $2^{-\Delta\Delta C}{}_{T}$ method (Livak and Schmittgen, 2001). Transcripts of the *nuoA* gene were used as internal control to perform normalization (Cruz *et al*., 2014).

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 $CaCl_2$ (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 OD_{600} 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 ml^{-1}. Cell suspensions of OD_{600} 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. Log_2 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[a] used in qPCR analysis.

