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Differential gene expression in *Xylella fastidiosa* 9a5c during co-cultivation with the endophytic bacterium *Methylobacterium mesophilicum* SR1.6/6

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Xylella fastidiosa, the causal agent of citrus variegated chlorosis (CVC), colonizes plant xylem, reducing sap flow, and inducing internerval chlorosis, leaf size reduction, necrosis, and harder and smaller fruits. This bacterium may be transmitted from plant to plant by sharpshooter insects, including *Bucephalogonia xanthopis*. The citrus endophytic bacterium *Methylobacterium mesophilicum* SR1.6/6 colonizes citrus xylem and previous studies showed that this strain is also transferred from plant to plant by *B. xanthopis* (Insecta), suggesting that this endophytic bacterium may interact with *X. fastidiosa in planta* and inside the insect vector during co-transmission by the same insect vector. To better understand the *X. fastidiosa* behavior in the presence of *M. mesophilicum*, we evaluated the *X. fastidiosa* transcriptional profile during *in vitro* interaction with *M. mesophilicum* SR1.6/6. The results showed that during co-cultivation, *X. fastidiosa* down-regulated genes related to growth and up-regulated genes related to energy production, stress, transport, and motility, suggesting the existence of a specific adaptive response to the presence of *M. mesophilicum* in the culture medium.

Keywords: Biofilm / Citrus variegated chlorosis / Endophyte / Microarray / *Quorum sensing*

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Introduction

Brazil is the world's largest citrus grower. It is responsible for more than 30% of the worldwide production of this commodity [1]; nevertheless, millions of dollars are lost in sweet orange due to citrus variegated chlorosis (CVC) [2], primarily as a result of CVC symptoms, which include internerval chlorosis, reduction of leaf size, necrosis, and harder and smaller fruits [3]. CVC is caused by the phytopathogenic bacterium *Xylella fastidiosa*, which colonizes the xylem vessels of susceptible hosts plants [3, 4]. Although this bacterium is not transmitted from seeds to seedlings [5], it is able to colonize the gut of many sharpshooters, including *Bucephalogonia xanthopis*, transmitting *X. fastidiosa* from plant to plant during the feeding of these insects [6].

The CVC symptoms are a result of vessel interruption caused by *X. fastidiosa* biofilm [7] and, therefore, the intensity of these symptoms is associated with the pathogens ability to colonize and disseminate within the plant. Newman et al. [8] observed that in CVC symptomatic plants, the number of blocked vessels is larger than that observed in asymptomatic plants. In fact, in low number inside the vessels, *X. fastidiosa* behaves as an endophyte, causing no harm to the host plant and, consequently no reduction in productivity [9]. Thus, controlling the bacterial population within the plant is likely to be sufficient to prevent the development of CVC.

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In this sense, Muranaka et al. [10] have recently shown that N-Acetylcysteine (NAC) can inhibit *X. fastidiosa* growth in infected citrus plants, suggesting that this could be a promising alternative to control CVC through a drug-based method. However, biological control may be used as an alternative approach to combat this disease, since specific interactions involving *X. fastidiosa* and other endophytic bacteria have been shown to interfere with the bacterium ability to grow and develop within the plant [11, 12]. Araújo et al. [11] studied the interaction between endophytic communities and *X. fastidiosa* in citrus, observing that endophytes, such as *Curtobacterium flaccumfaciens* was isolated mainly from resistant plants (infected with *X. fastidiosa*, but without CVC symptoms), while other species of the *Methylobacterium* spp. genus (mainly *M. extorquens* species) were isolated mainly from CVC-affected plants. Therefore, it is possible that *M. mesophilicum* (strain SR1.6/6), isolated from a healthy plant, may interact with *X. fastidiosa* in a way that hampers its growth and development, thereby preventing the occurrence of disease [11, 12].

In fact, *Methylobacterium* spp. has been frequently isolated from citrus plants (healthy, asymptomatic, and symptomatic) [11]. However, according to Araújo et al. [11], in CVC-affected plants was observed a higher *Methylobacterium* diversity than in asymptomatic plants, being *M. mesophilicum* the only species able to colonize these asymptomatic plants. In addition, Lacava et al. [13] performed *in plant* interaction between *X. fastidiosa* and *M. mesophilicum*, demonstrated that the population of *X. fastidiosa* was lower in the presence of *M. mesophilicum* SR1.6/6 and the population of *M. mesophilicum* SR1.6/6 was in turn reduced by *X. fastidiosa*, suggesting that these bacteria interact inside the host plant. This could be accomplished, for example, by a diffusible signal and/or toxin produced by *M. mesophilicum*, or may simply result from its capacity to occupy the same niche as the phytopathogen [14]. Thus, these results argue in favor of the possibility that *M. mesophilicum* may behave as a plant-beneficial bacterium that inhibits *X. fastidiosa* growth in xylem vessels, which may contribute to prevent the development of CVC.

Although studies have been reported on the differential gene expression of both pathogenic [15] and beneficial bacteria [16], the response of phytopathogen during interaction with endophytes is still unknown. Therefore, this work aims to study the *X. fastidiosa* 9a5c gene expression during co-cultivation with *M. mesophilicum* SR1.6/6, trying to explain, in a molecular level, the *X. fastidiosa* response induced by the endophytic bacterium during the interaction inside the host plant. The results contributed toward the molecular mechanisms that underline the interaction between endophytic and phytopathogen bacteria.

Materials and methods

Bacterial strains and growth conditions

X. fastidiosa strain 9a5c, previously isolated from *C. sinensis* (L.) Osbeck, was grown in PW medium (4 g L^{-1} soy peptone, 1 g L^{-1} tryptone, 1.2 g L^{-1} K_2HPO_4,1 g L^{-1} KH_2PO_4, 0.4 g L^{-1} $MgSO_4.7H_2O$, 0.1% hemine clorinated, 0.2% phenol red, add 0.4% L-glutamine and 0.6% BSA), a medium commonly used to grow *X. fastidiosa*, as described by Davis et al. [17]. *M. mesophilicum* strain SR1.6/6, previously isolated from *C. sinensis* [11], was cultivated on SPW modified medium (PW medium – without hemine chlorinated, phenol red, and BSA), as described by Araújo et al. [11]. Both bacteria were grown at 28 °C, in a rotatory shaker (150 rpm) for 96 h.

Other bacteria was tested to compare with *M. mesophilicum* strain SR1.6/6 results. An *Escherichia coli* DH5α and two citrus endophyte: *M. extorquens* AR1.6/2 and *Curtobacterium flaccumfaciens* ER1/6 also isolated by Araújo et al. [11].

Co-cultivation of *X. fastidiosa* and *M. mesophilicum*

The interaction between the phytopathogen *X. fastidiosa* 9a5c and the endophyte *M. mesophilicum* SR1.6/6 was evaluated in a co-cultivation experiment, with three biological replicates. For this analysis, *X. fastidiosa* cells were grown, separately, in 300 ml of PW medium, and *M. mesophilicum* SR1.6/6 cells were grown in 300 ml of SPW medium; both cultures were incubated for 72 h at 28 °C (150 rpm). On the 3rd day, 100 mL from each individual cell suspension (10^8 CFU ml^{-1}) were mixed together (totaling 200 ml). The remains from each original culture (200 ml) were kept in separate flasks without interaction, to serve as controls. Afterwards, the interaction and control flasks were incubated at 28 °C, in a rotatory shaker (150 rpm) for additional 24 h.

The same experiment was repeated replacing *M. mesophilicum* SR1.6/6 by *E. coli* DH5α or *M. extorquens* AR1.6/2 (citrus endophytes) or *C. flaccumfaciens* ER1/6 (citrus endophyte). Therefore, the experiment was performed in 1/10 of the volume, so 10 ml from each individual cell suspension were mixed together (totaling 20 ml) and 10 ml of the original culture were kept in separate flasks without interaction.

Microarray fabrication, RNA extraction, cDNA labeling, and hybridization

The *X. fastidiosa* microarrays were constructed as previously described [18, 19]. To evaluate the effect of

the *X. fastidiosa* 9a5c and *M. mesophilicum* SR1.6/6 co-cultivation on the gene expression of *X. fastidiosa*, the RNA of the controls and the co-cultivation cell suspensions of three biological replicates were extracted with Trizol reagent (Invitrogen, Foster city, CA) and purified with an RNeasy kit (Qiagen, Redwood City, CA). Thereafter, the RNA samples from the replicates of each treatment were pooled together, labeled by reverse transcription by the incorporation of Cy3- or Cy5-dCTP and hybridized to the Xf-microarrays, as previously described [19, 20]. The RNA of only *M. mesophilicum* SR1.6/6 was also extracted as a negative control. For cDNA hybridizations, two independent experiments were performed with different aliquots of the pooled RNA preparations and with dye swap (controlXf-Cy3 versus co-cultivatedXf-Cy5 and controlXf-Cy5 versus co-cultivatedXf-Cy3). Since each microarray carries two complete copies of the *X. fastidiosa* genome, replicated hybridizations resulted in a series of eight independent readings for each probe spotted in the microarrays.

Image acquisition and analysis

The images were analyzed with the TIGR Spotfinder program (v.2.2.4). All spots with median values lower than the local background median plus two standard deviations were flagged and excluded from further analyses. The results from each hybridization were subjected to a series of mathematical transformations with the aid of the software TIGR MIDAS v.2.19. These included filtering out all spots with integrated intensities below 10,000 a/d units, normalization between the two channels with the aid of the Lowess algorithm, and SD regularization of the Cy5/Cy3 ratios across all sectors (blocks) of the array. Finally, the results from each individual experiment were loaded into the software TIGR Multi-Experiment Viewer (TMEV), v.3.01. The experiments were then normalized, and the genes that displayed statistically significant modulation were identified with the aid of the one-class mode of the Significance Analysis of Microarrays (SAMs) test, described by Tusher et al. [21]. The sigma (σ) factor of the SAM test was adjusted to 0.69, resulting in a median false discovery rate (FDR) of 0.163. For more details in the use of the TIGR microarray software suite (TM4), see Saeed et al. [22]. Raw and normalized data from all microarray hybridizations, as well as the microarray complete annotation file have been submitted, in MIAME-compliant format, to NCBÍs Gene Expression Omnibus (GEO) and can be assessed through series number GSE56901.

Real-time qPCR

For the analysis of gene expression by real-time qPCR, RNA samples were prepared as described above, with three independent biological replicates. SuperScriptII (Invitrogen, Foster city, CA) was used for the RT-PCR reactions, according to the manufacturer's instructions, by using 2 μg of total *X. fastidiosa* RNA. The thermocycling conditions were comprised of an initial step at 50 °C for 2 min, followed by 30 min at 60 °C for reverse transcription to occur. SYBR Green PCR Reagent kits (Applied Biosystems, Foster city, CA) were then used for the qPCR reactions using 10 ng of the resulting cDNA. The detection of PCR products was measured by monitoring the increase in fluorescence emitted by SYBR Green Reagent. The primers for the randomly selected genes were designed using Primer3 software (http://www.genome.wi.mit.edu/genome_software/other/primer3.html) and are listed in Table 1. ORF XF1311, which encodes a rod-shaped determining protein (MreD), was used as an endogenous control for experimental normalization because the microarray hybridization experiments showed that this ORF is constitutively expressed both with and without *M. mesophilicum*. All the real-time qPCR reactions were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystems). The default thermocycler program was used for all genes and qPCR assays were performed in triplicate for each primer pair. For all amplifications performed in real-time qPCR, dissociation curves were produced to check for nonspecific amplification and negative control reactions were done to check for possible contamination. The change in the expression

Table 1. List of *X. fastidiosa* 10 primers pairs used in real-time qPCR.

ORF number		Primer sequence 5′-3′
XF0233	forward	CAGTGGCAATTACACGTTGG
	reverse	GGACAATCCGAGCCTTATCA
XF2451	forward	CTTTGTTGCAGACCAGACCA
	reverse	TTCTTCAGCGGTGAACAGTG
XF0110	forward	GCCAGGGTGACACTATCGTT
	reverse	TGGGACTATGGGTCTGGAAC
XF0511	forward	GTCATGCACAAAATGCTGCT
	reverse	GACTTCTGGGGTGATCTGGA
XF1224	forward	CCAGATGGAGACCGGTAAGA
	reverse	TTATCCCGATTGGTGTTGGT
XF1344	forward	GATCCGACCCGTGAGTTTTA
	reverse	GATCAACCTCGACCTTTCCA
XF2385	forward	GGAGCACGTCAAATTGGTTT
	reverse	GCTATCACTTTCGGGCAGAG
XF1827	forward	CAGCGTTGTATCTTCGGACA
	reverse	TACGACTAGGCCGAAACCAC
XF2237	forward	GCCGTTCCAAGTACGATGTT
	reverse	ACACTGTGCCTGAGTGAACG
XF0128	forward	GGGAAGCGATCATAGGAACA
	reverse	ACCCACCATATTGGTTCCAG

of each gene was calculated using the $2^{-\Delta\Delta Ct}$ method, with the control treatment as the calibrator.

Results

The microarray hybridization analysis showed that 2.18% (49) of the *X. fastidiosa* ORFs were statistically differentially expressed during co-cultivation with *M. mesophilicum* SR1.6/6 (Table 2), negative control (*M. mesophilicum* SR1.6/6) did not hybridize in *X. fastidiosa* chip. To confirm the reliability of the microarray experiments, 10 genes were randomly selected, and their transcription modulations were verified by real-time qPCR. As observed in Fig. 1, data obtained by real-time qPCR for all tested genes showed that transcriptional modulations were in according to the values obtained by the microarray hybridizations, with 0.85 correlation coefficient. Thus, we grouped the differentially expressed genes in eight functional clusters, following the categorization originally proposed by Simpson et al. [23]. As observed in Fig. 3, most differentially expressed ORFs grouped in the categories associated with (III) macromolecule metabolism, (VIII) hypothetical proteins, and (VI) pathogenicity, virulence, and adaptation.

To confirm if these results were due to the interaction of *X. fastidiosa* 9a5c with any other bacteria, just by joining growth, similar experiment was repeated replacing *M. mesophilicum* SR1.6/6 with other three bacteria (Fig. 2). A non-environmental strain *E. coli* DH5α broadly used in the laboratory essays, a citrus endophyte from the same genera *M. extorquens* AR1.6/2, which does not inhibit *X. fastidiosa* growth *in vitro* [12] and another citrus endophyte *C. flaccumfaciens* ER1/6, which also reduce *X. fastidiosa* growth *in vitro* as *M. mesophilicum* SR1.6/6 [12].

Growth-related genes including the regulator of the carbon storage gene (XF0125), two 50S ribosomal proteins (XF0110 and XF0739), DNA polymerase III (XF1807), and the enzyme topoisomerase (XF1847) were down-regulated in *X. fastidiosa*, during co-cultivation with *M. mesophilicum.* One of the 50S ribosomal proteins (XF0110), responsible for protein synthesis, was also down-regulated in the qPCR experiment. While in *X. fastidiosa* in co-cultivation with *C. flaccumfaciens* ER1/6 (that also inhibits *X. fastidiosa* growth) this gene was up-regulated (Fig. 2). The topoisomerase enzyme is responsible for preventing the supercoiling generated by the replication fork during DNA duplication. In contrast, during co-cultivation with *M. mesophilicum*, we observed an increase in the expression of genes related to energy generation in *X. fastidiosa*, including the genes that encode fumarate hydratase (XF1554) and dihydrolipoamide dehydrogenase (XF1548) of the Krebs cycle (Table 2).

Different genes that respond to environmental stress, such as *pil*Y (XF1224), *clp*P peptidase (protease) (XF0511), acriflavin resistance (XF2385), and toluene tolerance (XF0418 and XF0420), were all up-regulated in *X. fastidiosa* in the presence of *M. mesophilicum* and confirmed by qPCR. This indicates that the presence of *M. mesophilicum* may generate a stressful environment for *X. fastidiosa. X. fastidiosa* in co-cultivation with *C. flaccumfaciens* ER1/6 also up-regulated acriflavin resistance genes (XF2385), however, down-regulated other stress genes such as *pil*Y (XF1224) (Fig. 2).

The ABC transporter superfamily (XF1344 and XF2455) and transporter membrane (*ton*B) (XF2237) were also 1.6–2.6 times up-regulated in *X. fastidiosa* during co-cultivation with *M. mesophilicum. X. fastidiosa* during co-cultivation with other tested strains presented a different regulation of these transporter genes, *X. fastidiosa* in the presence of *C. flaccumfaciens* ER1/6 down-regulated ABC transporter (XF1344), while in the presence *E. coli* DH5α and *M. extorquens* AR1.6/2 *ton*B gene (XF2237) was down-regulated. This ABC transporter system is presented in different ways and is dependent on ATP hydrolysis.

The phosphotransferase system (XF1402) and other transporters related to the phosphate ligation (XF0420, XF0418, XF1827, and XF2385) were also (approximately 2X) up-regulated in co-cultivation with the endophytic bacteria (*M. mesophilicum*). Similar gene regulation of these transporter genes (XF1827 and XF2385) was observed in co-cultivation with all other tested bacteria, suggesting that different molecules could be translocated during the *X. fastidiosa–M. mesophilicum* interaction.

Discussion

X. fastidiosa causes CVC and Pierce's disease in grapevines; besides being the causal agent of CVC in citrus, the existence of many asymptomatic plants infected by *X. fastidiosa* has also been reported [9]. In this sense, interactions involving *X. fastidiosa* and other endophytic bacteria from the xylem microbiome has been suggested to be a determining factor toward the development of disease symptoms [11], since endophytic community is reported to prevent pathogens infection [24, 25]. In host plants, *X. fastidiosa* colonizes the xylem vessels, from which sharpshooter vectors may acquire this bacterium, while feeding on the infected plant and may, thus, transmit the bacterium from plant to plant [6]. Previous studies have shown that *M. mesophilicum* (SR1.6/6) colonizes the xylem vessels of citrus plants [13, 26] and, more importantly, can be transmitted by *B. xanthopis*, a sharpshooter vector [13], suggesting that

Table 2. List of 49 genes with significative statistic variation in *X. fastidiosa* in co-cultive with *M. mesophilicum*classified in eight genic categories according to http://www.lbi.ic.unicamp.br/xf/ (continues).

Class	Genic category	Genes	Ratio Xf + Mm/Xf
I intermediary metabolism			
carbon metabolism	TCA cycle	FUMC – fumaratehydratase (50.5 kDa) – XF1554	1.6194202
	TCA cycle	LPD, dihydrolipoamide dehydrogenase (52.1 kDa) – XF1548	0.6590899
	electron transport	CYOB – cytochrome O ubiquinol oxidase (75.4 kDa) – XF1389	3.5746431
	electron transport	SPAC977.08 – oxidoreductase (29.0 kDa) – XF2082	−1.2750703
regulatory function		carbon storage regulator – csrA (8.3 kDa) – XF0125	−4.954483
		nitrilehydrataseactivator (49.4 kDa) – XF1830	−2.4510627
II biosynthesis of small molecules			
cofactor	biotin	adenosylmethionine-8-amino-7-oxononanoate aminotransferase-BIOA (53.9 kDa) – XF0189	−1.1853067
	pyridoxine	PDXA – pyridoxal phosphate biosynthetic protein (34.6 kDa) – XF0839	−1.4434927
amino acids biosynthesis	aromatic amino acid family	AROC – chorismate synthase (40.2 kDa) – XF1369	−1.3695219
	glycine–serine family/sulfur metabolism	Y4XP, cysteine synthase (42.5 kDa) – XF0128	−1.9135294
nucleotides biosynthesis	purine ribonucleotides	PRSA or PRS – phosphoribosyl pyrophosphate synthetase (33.8 kDa) – XF2644	−0.94446343
III macromolecule metabolism			
DNA metabolism	restriction, modification	HI1201 – adenine-specific methylase (34.1 kDa) – XF1368	−2.148348
	replication	DNAG or DNAP or PARB, DNA primase (65.7 kDa) – XF0430	0.9398372
	replication	segregation and condensation protein A scpA (37.9 kDa) – XF2451	1.3481789
	replication	DNA topoisomerase III (traE) (3.8 kDa) XF1847	−1.171989
	replication	DNAX ou DNAZ ou DNAZX, DNA polymerase III (66.4 kDa) – XF1807	−0.78765035
RNA metabolism	ribosomal protein	RPLS – 50S ribosomalprotein L19 (15.0 kDa) – XF0110	−1.8595717
	ribosomal protein	RPMI, 50S ribosomalprotein L35 (7.6 kDa) – XF0739	−2.8989055
	DNA transcription	NmrA transcripcional regulator (31.0 kDa) – XF0241	−1.5789075
	ribossomos maturation and modification	rimP ribosomal maturation factor – HI1282 (23.8 kDa) – XF0233	0.9591459
protein metabolism	translation and modification	peptide chain release factor 3 – PRFC OR HI1735 (60.9 kDa) – XF0174	1.4547925
	protein degradation	clpP peptidase (80.4 kDa) – XF0511	1.3658125
	protein degradation	dipeptidyl-peptidase (91.1 kDa) – XF0015	−1.408684
IV cell structure			
surface structures	membrane	pilY1 (132.4 kDa) 1224	1.6189611
	outer membrane constituents	SLT ou SLTY – soluble lytic mureintransglycosylase precursor (80.0 kDa) – XF1363	2.0029955
	outer membrane constituents	peptidoglycan-associated outer membrane lipoprotein precursor – pcp or lpp (15.7 kDa) – XF1547	−1.8802352
	outer membrane constituents	outer membrane hemin receptor (74.1 kDa) – XF0384	−0.6212459
	inner membrane	60 kDa inner-membrane protein (64.1 kDa) – XF2780	−1.5237219
V cellular processes			
transport	anions	ABC transporter sulfate binding protein – sbp (38.0 kDa) – XF1344	1.6055375
	protein, peptide secretion	heme ABC transporter ATP-binding protein – ccmA (24.3 kDa) – XF2455	0.77919966

(*Continued*)

Table 2. (*Continued*)

Class	Genic category	Genes	Ratio Xf + Mm/Xf
	carbohydrates, organic acids, alcohols	PHBI/phosphotransferase system enzyme I (65.3 kDa) – XF1402	0.511184
cell division		ZIPA – cell division protein (27.3 kDa) – XF2557	0.6199535
VI mobile genetic elements			
function related to phago and prophago		phago integrase (5.9 kDa) – XF1789	−1.2154113
VII pathogenicity, virulence, and adaptation			
toxin production and detoxification	transport others	YEGN/acriflavin resistance protein D (116.1 kDa) – XF2385	1.3020734
		FRPC, hemolysin-type calcium binding protein (173.0 kDa) – XF1011	1.5402875
		organichydroperoxide resistance protein – ohr – (14.9 kDa) – XF1827	1.6493855
		TonB receptor dependent – (103.0 kDa) – XF2237	2.6398678
adaptation, atypical conditions		toluene tolerance protein – ttg2B (26.3 kDa) – XF0420	1.75569
		toluene tolerance protein – ttg2D (24.5 kDa) – XF0418	2.017376
other		virulence factor (26.4 kDa) – XF0591	0.81298685
VIII hypothetical protein			
		hypothetical protein- (7.6 kDa) – XF0195	−2.9974034
		hypothetical protein (11.1 kDa) – XF1057	−5.2903037
		hypothetical protein (12.1 kDa) – XF1056	−5.0500865
		hypothetical protein (49.1 kDa) – XF2034	−0.85833573
		hypothetical protein (9.5 kDa) – XF0028	1.4203246
		hypothetical protein (18.7 kDa) – XF0700	1.010623
		hypothetical protein (19.4 kDa) – XF0058	3.4253116
		hypothetical protein (22.0 kDa) – XF0054	1.2025653
		hypothetical protein (11.6 kDa) – XF1808	0.85570693

Methylobacterium and *X. fastidiosa* may cohabit the host plant and the insect vector, interacting in different ways.

In the present differential gene expression study, *X. fastidiosa* was co-cultivated with the endophytic bacterium *M. mesophilicum* strain SR1.6/6 and other citrus endophytes as a control. Results indicate that a few important genes, directly related to bacterial growth – notably with DNA replication and protein synthesis (50S ribosome protein and topoisomerase enzyme genes) – have their transcription down-regulated when *X. fastidiosa* is grown in the presence of this endophyte indicating that the presence of *M. mesophilicum* could be related to growth inhibition of *X. fastidiosa* cells, since in previous studies, Lacava et al. [14] showed that the population of *X. fastidiosa* was reduced by *M. mesophilicum* during co-inoculation *in plant*. Other citrus endophyte also able to *in vitro* inhibit *X. fastidiosa* growth (*C. flaccumfaciens* ER1/6) [12] presented opposite results, up-regulating protein synthesis genes, inducing other specific *X. fastidiosa* response to this endophyte.

In addition, *in vitro* experiments, the *M. mesophilicum* exsudates inhibited the *X. fastidiosa*, suggesting that this endophytic bacterium could be able to reduce the CVC symptoms by suppressing *X. fastidiosa* growth [12]. Other interaction studies have also reported the down-regulation of bacterial growth genes in the presence of the plant-specific factors, including a study by Ciraulo et al. [20], in which *X. fastidiosa* was grown in media under xylem-based chemical conditions. Under other stress conditions, such as copper and tetracycline exposure, the same growth gene down-regulation patterns was observed in *X. fastidiosa* [40]. Despite growth reduction, genes related to energy generation, such as fumarate hydratase and dihydrolipoamide dehydrogenase (Krebs cycle) had their expression increased in *X. fastidiosa*, suggesting that although the bacterium is not growing, energy is necessary to keep the interaction profile, including genes related to stress responses and membrane transporters. This gene expression profile is similar to that observed in *Pseudomonas putida* exposed to toluene [27], in which the

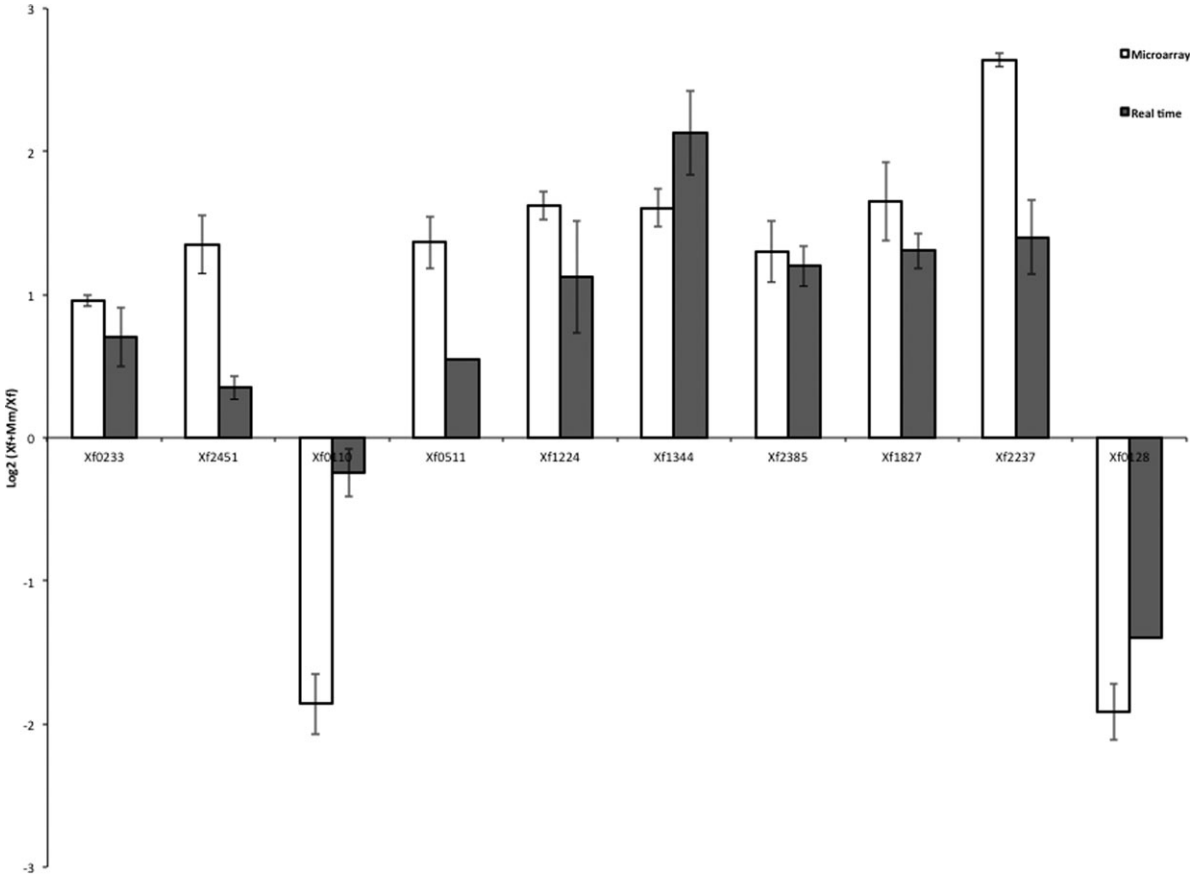

Figure 1. Evaluation of transcriptional modulation of selected genes by real-time qPCR. In order to confirm the reliability of the microarray experiments, 10 genes have been randomly selected and their transcription modulation was verified by real-time qPCR. The same RNA samples used in the microarray hybridizations were converted to cDNA and the relative expression ratios (RQ) of these genes have been measured. XF1311, which encodes a rod-shaped determining protein (MreD), has been used as an endogenous control for experimental normalization. Experiments were performed in triplicate and graphic shows the average values and their respective standard deviations. XF0233–rimP ribosomal maturation factor, XF2451–segregation and condensation protein A (scpA), XF0110–50S ribosomal protein, XF0511–clpP peptidase, XF1224–pilY, XF1344–ABC transporter sulfate binding protein, XF2385–acriflavin resistance protein D, XF1827–organic hydroperoxide resistance protein, XF2237–TonB receptor dependent, XF0128–cysteine synthase.

authors observed that this organic compound increased the energy demand and stress response, while down-regulating genes related to sugar storage.

Investigating the function of some differentially regulated stress genes (*pil*Y, transporter, clpP peptidase, acriflavin resistance, and toluene tolerance), we observed that *pil*Y was involved in the long pili type IV formation and was responsible for bacterial motility [28]. Moreover, the genome analysis of *X. fastidiosa* revealed the presence of orthologous genes that encode proteins involved in the biogenesis and function of type IV pili, which is responsible for a system of chemotaxis-related motility control, in response to variations in environmental conditions [29]. The activation of this gene could be an indicator of the pathogen dispersion throughout of the xylem vessels, which does not occur during co-cultivation with *C. flaccumfaciens* ER1/6, indicating that each endophytic strain presents different strategies to inhibit the pathogen *X. fastidiosa*.

Another gene that can affect motility is *tonB*, which in activation in *X. fastidiosa* resulted in the motility loss and significant decrease on biofilm formation when compared to wild type [30]; this mutation also affected the bacteria virulence. TonB protein is also known to be related to iron homeostasis, an important factor that regulates the gene expression involved in bacterial pathogenicity [29]. Moreover, TonB protein can act as a membrane receptor for the *cell-surface signaling* (CSS) system [31], a regulatory mechanism of *quorum sensing* used by the bacteria to perceive signals from the extracellular medium. Furthermore, bacteria from different genera can communicate using *quorum sensing*

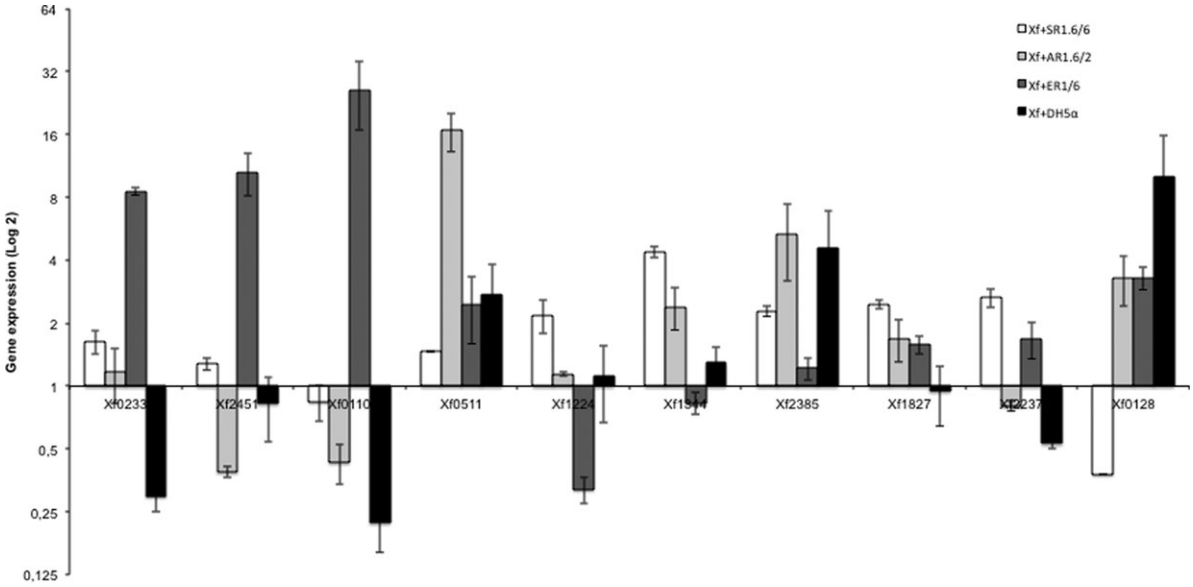

Figure 2. Transcription modulation of 10 randomly selected genes used for microarray validation of three endophyte bacteria (*M. mesophilicum* SR1.6/6, *M. extorquens* AR1.6/,2 and *Curtobacterium flaccumfaciens* ER1/6) was also verified by real-time qPCR.

molecules like Diffusible Signaling Factor (DSF) [32] because the signaling DSF system is conserved between different genera of bacteria [33].

In this context, other cellular transport genes that were up-regulated in *X. fastidiosa* during co-cultivation with *M. mesophilicum* were phosphotransferase system gene (XF1402) and the ABC transporter genes (XF1344 and XF2455), which are related to carbohydrate uptake [34]. Similar cell transport genes include the acriflavin resistance gene (YEGN protein), the toluene genes (*ttg2*B and *ttg2*D), and the organic hydroperoxide (*ohr*) gene, which are all grouped as transporters in the pathogenicity, virulence, and adaptation class.

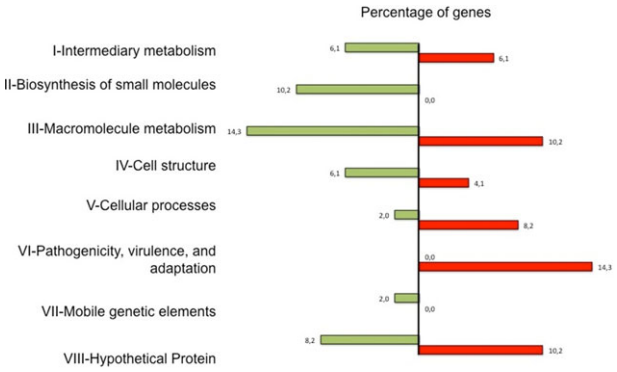

Figure 3. Distribution of functions of genes statistically differential expressed (green down-regulated and red up-regulated) of *X. fastidiosa* in the presense of *M. mesophilicum.*

In the present study, we did not investigate the expression of *M. mesophilicum* genes during the interaction. However, Pomini et al. [35] described six quorum sensing molecules (homoserine lactone – AHL) produced by *M. mesophilicum* SR1.6/6. The presence of these molecules induces in *M. mesophilicum* the expression of different genes involved in bacteria–bacteria or bacteria–plant interaction, such as the carbon metabolism gene (*mxa*F) and stress genes (carotenes and ethylene) [36]. In addition, although *X. fastidiosa* is not able to synthesize AHL, this bacterium presents two LuxR regulators (XF2608 and XF0972) [23], suggesting that *X. fastidiosa* could recognize the AHL produced by *M. mesophilicum* during the interaction.

Therefore, this study evaluated the response of *X. fastidiosa* (9a5c) to *M. mesophilicum* (SR1.6/6) in order to understand genetic mechanisms involved in their interaction, which is different from the response to other citrus endophytes, even other endophyte that also inhibt *X. fastidiosa* growth. *X. fastidiosa* pathogenesis is associated with the interruption of water and ion flow in the host plant xylem, which is, probably, caused by biofilm formation [2, 27, 37]. Araújo et al. [11] and Lacava et al. [12] suggested that *M. mesophilicum* may be associated with the development of CVC symptoms, and based on the present results, *X. fastidiosa* responds to the presence of this endophytic bacterium by down-regulating the genes related to growth, increasing the genes related to energy generation (genes of cellular respiration), and directing part of its energy to transport

and the stress response. There are few studies that have evaluated the gene expression of bacteria–bacteria interactions, and the present analysis showed, for the first time, that *X. fastidiosa* genes are regulated by the presence of the endophytic bacteria *M. mesophilicum.* However, there is much more to be discovered about the interactions and communication that occur among bacteria and other microorganisms present in the plant microbiome.

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Conflict of interest

The authors are not aware of any conflicts of interest related to this article.

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