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Research Article

Transcriptome Analysis of the Phytobacterium *Xylella fastidiosa* Growing under Xylem-Based Chemical Conditions

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Xylella fastidiosa is a xylem-limited bacterium responsible for important plant diseases, like citrus-variegated chlorosis (CVC) and grapevine Pierce's disease (PD). Interestingly, *in vitro* growth of *X. fastidiosa* in chemically defined media that resemble xylem fluid has been achieved, allowing studies of metabolic processes used by xylem-dwelling bacteria to thrive in such nutrient-poor conditions. Thus, we performed microarray hybridizations to compare transcriptomes of *X. fastidiosa* cells grown in 3G10-R, a medium that resembles grape sap, and in Periwinkle Wilt (PW), the complex medium traditionally used to cultivate *X. fastidiosa*. We identified 299 transcripts modulated in response to growth in these media. Some 3G10R-overexpressed genes have been shown to be upregulated in cells directly isolated from infected plants and may be involved in plant colonization, virulence and environmental competition. In contrast, cells cultivated in PW show a metabolic switch associated with increased aerobic respiration and enhanced bacterial growth rates.

1. Introduction

The phytobacterium *Xylella fastidiosa* was described by Wells et al. [1] and has been found to be associated with the development of a wide variety of plant diseases, such as Citrus-Variegated Chlorosis (CVC) in orange trees, Pierce's disease (PD) in vineyards, Phony Peach disease (PP), Periwinkle Wilt and leaf scorch diseases in plum, elm, maple, pecan, oak, sycamore, and coffee ([2, 3], reviewed in [4]). Due to the presence of economically important crops in this list, *X. fastidiosa* has been the subject of intensive research over the past years [5, 6] and the genome sequencing of four different strains has been accomplished: the 9a5c isolate (causative agent of CVC) was the first phytopathogenic bacterium completely sequenced in 2000 [7]. A few years later, two strains isolated from oleander and almond trees had their genomes partially sequenced and annotated [8]. Finally, a fourth strain, Temecula 1, isolated from grapevines and responsible for PD in California has also been sequenced to completion [9].

The elucidation of the complete genomic sequence of *X. fastidiosa* strains was followed by an extensive *in silico* evaluation of the bacterium's presumed proteome, allowing the formulation of a virtual metabolome that provided a comprehensive view of the major biochemical processes that occur in this microorganism [7]. Nonetheless, the exact mechanism(s) involved in the process of host infection and colonization, as well as with the onset of CVC, are yet to be identified and characterized in the *X. fastidiosa* genome [7]. Important information regarding the functionality of different gene products and pathogenicity mechanisms in *X. fastidiosa* could be obtained through the evaluation of differential gene expression using cells submitted to variable culturing conditions, especially those that resemble

the environment found inside the plant. Xylem-inhabiting microorganisms normally display a fastidious nature and cannot be cultured in conventional bacteriological media. Thus, a series of specially formulated media were developed for their axenic cultivation. The most widely employed, such as PD2 [10], PW [11], SPW [12], PYE, GYE [13] and BCYE [14], are complex media, which include peptone, tryptone, soytone, and yeast extract from various sources, as well as hemin chloride or ferric pyrophosphate (as iron sources), aminoacids, inorganic salts, citrate, succinate, starch, BSA, or activated charcoal. However, given the general characteristics of plant sap, xylem-dwelling endophytes are likely to thrive in nutrient-limiting conditions and must be able to adapt accordingly [15]. A few years ago, Leite et al. [16] have described the development of a xylem-based, chemically defined medium (called 3G10R), which supports *in vitro* growth of *X. fastidiosa* strains. Moreover, *X. fastidiosa* cells grown in this medium present some important characteristics that may be associated with colonization and pathogenicity, such as increased aggregation capacity and biofilm formation. This medium provided a new tool that may allow the *in vitro* study of some important characteristics presented by the bacteria during the infection process *in planta*.

Thus, we have employed competitive hybridizations on microarrays to evaluate the global transcriptional profile of *X. fastidiosa* cells grown in 3G10R, when compared to cells grown in PW, the standard complex medium used to cultivate this bacterium under laboratory conditions. These experiments allowed the identification of 299 genes that displayed statistically significant transcription modulation in response to growth in the two media. Some 3G10R-upregulated genes had their expression profiles confirmed by Real-Time qPCR and are likely to be relevant to bacterial adaptation to the plant xylem, such as adhesion to the substrate and competition with other microorganisms. Incidentally, independent studies have confirmed the specific upregulation of some of these genes in *X. fastidiosa* cells that display increased infective capacity and in bacteria directly isolated from plants, reinforcing the idea that the chemical characteristics of 3G10R are likely to induce genes that are naturally expressed by *X. fastidiosa* during the process of xylem colonization [17]. Other transcriptional alterations seem to correlate with significant changes in the cell's overall energetic metabolism and growth rate, as a reduction in the respiratory activity is observed when cells are grown in 3G10R.

2. Materials and Methods

2.1. Culturing X. fastidiosa Cells. PW and 3G10R liquid media have been prepared essentially as described by Davis et al. [11] and Leite et al. [16], respectively. Cells of *X. fastidiosa* 9a5c have been routinely kept in our laboratory, for over a year, in 20 ml of liquid cultures, which were incubated in an orbital shaker at 28°C and 100 rpm. One-milliliter (1 ml) aliquots were transferred to 19 ml of fresh media every 4-5 days.

To evaluate the behavior of *X. fastidiosa* cells under xylem-based chemistry conditions, bacterial cultures were grown in PW for 3 days, until an OD_{600} = 0.25 (late phase of exponential growth) was reached. A one milliliter-aliquot (1 ml) of this culture was used to inoculate 19 ml of liquid 3G10R and PW media. Bacterial growth in both cultures was monitored on a daily basis, through OD_{600} measurements, providing a direct comparison between *X. fastidiosa* growth patterns observed in 3G10R and standard PW medium.

2.2. Microarray Fabrication. X. fastidiosa microarrays have been constructed as previously described [18, 19]. Representative sequences from approximately 2200 ORFs from the *X. fastidiosa* genome (>90% coverage) were PCR amplified, purified, and spotted onto CMT-GAPS silane-coated slides (Corning), using an Affymetrix 427 arrayer, according to the manufacturer's instructions.

2.3. RNA Extraction, cDNA Labeling, and Hybridization. To evaluate and compare the bacterial transcriptome profiles in these two media, 200-ml bacterial cultures were prepared as described above and cells were harvested for total RNA extraction at day 3 (PW) and day 13 (3G10R), which allowed us to compare bacterial cultures at their maximum growth rates. The RNA samples were extracted and purified with aid of the RNAeasy kit (Qiagen), labeled by incorporation of Cy3- or Cy5-dCTP and hybridized to the microarrays, as previously described [18, 19].

2.4. Image Acquisition and Analysis. Images were analyzed with the TIGR Spotfinder program (v.2.2.4). All spots with median values lower than the median local background plus two Standard Deviations have been flagged and excluded from further analyses. Replicated experiments were performed with two independent RNA preparations from cells cultivated in each medium. For each pair of RNA preparations, two independent hybridizations were performed, with dye swaps within each pair. Since each microarray carries two complete copies of the *X. fastidiosa* genome, replicated hybridizations resulted in a series of 8 independent readings for each probe spotted in the microarrays.

The results from each hybridization were submitted to a series of mathematical transformations with the aid of the software TIGR MIDAS v.2.19. These included filtering out all spots whose integrated intensities were 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. Experiments were then normalized and 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. [20]. The δ factor of the SAM test was adjusted to 0.69, resulting in a Median False Discovery Rate (FDR) = 0.163. For details regarding the use of the TIGR microarray software suite (TM4), see Saeed et al. [21]. Raw and normalized

data from all microarray hybridizations, as well as the microarray complete annotation file have been submitted to NCBI's Gene Expression Omnibus (GEO) and can be accessed through Series number GSE 6619. A Tab-delimited file containing the Significant Genes List and their mean expression ratios can also be accessed through this GEO Series number.

2.5. Real-Time qPCR. All the Real-Time qPCR and RT-PCR reactions were performed using an ABI Prism 7500 Sequence Detection System (Applied Biosystem, USA). Taq-Man EZ RT-PCR kits (Applied Biosystems, USA) were used for RT-PCR reactions, according to the manufacturer's instructions, using 2–5 μg of total *X. fastidiosa* RNA and 1 μl of random nonamers (4 μg/μl). The thermocycling conditions comprised an initial step at 50°C for 2 minutes, followed by 30 minutes at 60°C for reverse transcription. Taq-Man PCR Reagent kits then were used for PCR reactions using 100–200 ng of the resulting cDNA. The thermocycling conditions comprised an initial step at 50°C for 2 minutes, followed by 10 minutes at 95°C, and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. ORF *Xf*1311, which encodes a rod-shaped determining protein (MreD) has been used as an endogenous control for experimental normalization, since the microarray hybridization experiments showed that this ORF is constitutively expressed in both PW and 3G10R. Primers and probes were synthesized through the Applied Biosystems Assay-by-Design service and all reactions were prepared essentially as recommended by the manufacturer.

2.6. Evaluation of Respiratory Rates. *X. fastidiosa* cells were grown into middle exponential phase in PW and subsequently transferred (in a 1 : 20 proportion) into fresh PW and 3G10R cultures. Bacterial growth in both cultures was monitored through OD_{600} measurements until both cultures reached stationary phase. Aliquots were taken from each culture to evaluate O_2 consumption on a daily basis, until day 7 (in PW) and day 13 (in 3G10R). We defined the respiratory rate for each culture as the ratio between O_2 consumption rate ($\Delta O_2/\Delta$min) and the respective OD_{600} value obtained at each time point.

Oxymetric measurements were monitored polarographically by an oxygraph equipped with a Clark-type oxygen electrode (Gilson Medical Electronics, Middleton, WI, USA) in intact cells. After measurement of the optical density, 2.0 ml of PW or 3G10R media containing bacteria were incubated at 30°C and the state 4 respiration was initiated by addition of 10 mM malate plus 10 mM glutamate. Basal respiratory rates were calculated by $\Delta O_2/\Delta$min ratio and the values were normalized by the optical density values.

3. Results

3.1. X. fastidiosa Cells Growing in PW and 3G10R Display Distinct Growth Patterns and Different Transcriptome Profiles. To evaluate the behavior of *X. fastidiosa* cells under xylem-based chemistry conditions, bacterial cultures were monitored in both 3G10R and PW, the complex medium traditionally used to cultivate this bacterium in the laboratory. As observed in Figure 1, PW cultures reached higher cellular densities ($OD_{600} \sim 0.3$) in a shorter period of time (4 days) when compared to cells grown in 3G10R, which had to be cultivated for a period of 14 days in order to reach a similar cellular density ($OD_{600} \sim 0.25$). Moreover, although 3G10R cultures exhibited continuous growth over the course of the experiment, they failed to display the typical profile of a bacterial growth curve, as observed in PW cultures. Such lack of an exponential growth phase in 3G10R cultures is typically observed in bacteria growing in nutrient-restricted environments, a situation that is likely to resemble xylem conditions [22–26]. Recently, Zaini et al. [27] showed that *X. fastidiosa* cells grown in pure xylem sap rapidly reach stationary phase without a detectable exponential growth, probably due to nutrient limitation.

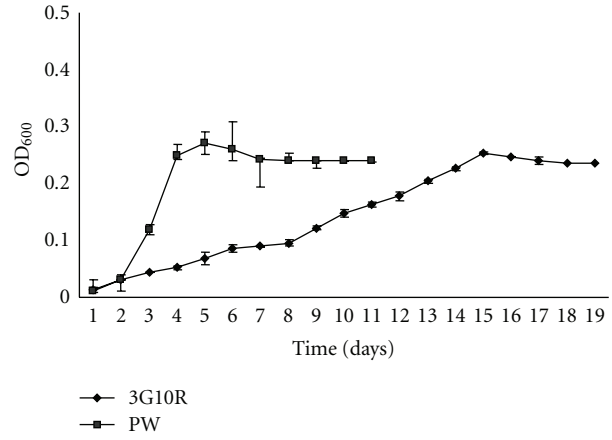

Figure 1: *Xylella fastidiosa* growth patterns in PW and 3G10R media. Both cultures have been made with a 1 : 20 ml inoculum of *X. fastidiosa* 9a5c cells grown into late exponential phase in PW (OD_{600} = 0.25). Cultures were then incubated in an orbital shaker at 28°C and 100 rpm. One milliliter (1 ml) aliquots were taken from each culture, on a daily basis, to monitor bacterial growth through OD_{600} readings. Measurements were performed in triplicate and graphic shows the average values and their respective standard deviations.

To evaluate and compare the bacterial transcriptome profiles in PW and 3G10R, samples from the resulting RNAs were used in competitive hybridizations against *X. fastidiosa* microarrays, as described by Nunes et al. [19]. Replicated experiments were performed with two independent RNA preparations from cells cultivated in each medium, which resulted in a series of 8 independent readings for each probe spotted in the microarrays, as described in the materials and methods. Statistical analysis of such results revealed a total of 132 genes that displayed overexpression in cells grown in 3G10R, while 167 genes were upregulated in cells grown in PW. These genes, as well as their respective changes in expression ratio are shown in Table 1. More detailed information about these genes can be obtained through the Gene expression Omnibus (GEO) webpage, through Series number GSE 6619 (see http://ncbi.nlm.nih.gov/geo). In order to access the overall reliability of these data, we have confirmed gene expression variation of several genes using

Table 1: List of genes that displayed statistically significant variation in gene expression. Genes with positive Log_2 ratio are overexpressed in 3G10R, while genes with negative Log_2 ratio are overexpressed in PW.

Functional Group	ORF Number	Gene Name	Gene Product	Log_2 (3G10R/PW)
Intermediary Metabolism				
Energy metabolism, carbon—Aerobic respiration	*Xf*0308	*nuo*D	NADH-ubiquinone oxidoreductase, NQO4 subunit	−0.93
	*Xf*0310	*nuo*F	NADH-ubiquinone oxidoreductase, NQO1 subunit	−1.08
	*Xf*0311	*nuo*G	NADH-ubiquinone oxidoreductase, NQO3 subunit	−1.14
	*Xf*0317	*nuo*M	NADH-ubiquinone oxidoreductase, NQO13 subunit	−1.03
	*Xf*0347	*dld*1	D-Lactate dehydrogenase	1.18
Energy metabolism, carbon—Glycolysis	*Xf*0303	*tpi*A OR *tpi*	Triosephosphate isomerase	−0.89
Energy metabolism, carbon—TCA cycle	*Xf*2548	*suc*D	Succinyl-CoA synthetase, alpha subunit	−1.67
	*Xf*1554	*fum*C	Fumarate hydratase	−1.47
	*Xf*1554	*fum*C	Fumarate hydratase	−1.36
Energy metabolism, carbon—Electron Transport	*Xf*1990	*yne*N	Thioredoxin	−1.14
	*Xf*0620	*dsb*D	c-Type cytochrome biogenesis protein (Copper Tolerance)	−0.83
Degradation—Degradation of Small Molecules	*Xf*1250	*roc*F	Arginine deaminase	−2.00
	*Xf*1740	*yli*I	Glucose dehydrogenase B	1.45
	*Xf*2395	*axe*A	Acetylxylan esterase	1.75
	*Xf*2432	*gta*B	UTP-glucose-1-phosphate uridylyl transferase	−1.14
	*Xf*0610	*gal*E	UDP-glucose 4-epimerase	−1.44
	*Xf*2210		Dioxygenase	1.00
Regulatory Functions	*Xf*1354	*yyb*A	Transcriptional regulator (MARR Family)	1.27
	*Xf*1354	*yyb*A	Transcriptional regulator (MARR Family)	1.55
	*Xf*1254	*ara*L	Transcriptional regulator (ARAC Family)	−1.10
	*Xf*2344	*fur*	Transcriptional regulator (FUR Family)	1.19
	*Xf*2336	*col*R	Two-component system regulatory protein	1.32
	*Xf*2534	*col*R	Two-component system regulatory protein	−0.95
	*Xf*1752		Transcriptional regulator (LYSR Family)	1.64
	*Xf*1733	AF0343	Tryptophan repressor binding protein	1.13
	*Xf*1749	*opd*E	Transcriptional regulator	1.65
	*Xf*1730	*yaf*C	Transcriptional regulator (LYSR Family)	1.97

Table 1: Continued.

Functional Group	ORF Number	Gene Name	Gene Product	Log_2 (3G10R/PW)
Sugar-Nucleotide Biosynthesis, Conversions	*Xf*0260	*xan*A	Phosphoglucomutase/ Phosphomannomutase	0.92
Central Intermediary Metabolism—Pool, Multipourpose Conversions	*Xf*0880	*yad*F	Carbonic anhydrase	−1.30
	*Xf*2255	*acs*	Acetyl coenzyme A synthetase	−1.37
Central Intermediary Metabolism—Amino Sugars	*Xf*2355		Exo II n-acetyl-beta-glucosaminidase	1.44
Biosynthesis of Small Molecules				
Amino Acids Biosynthesis—Aspartate family, pyruvate family	*Xf*2272	*met*E	5-methyltetrahydro pteroyltriglutamate–homocysteine methyltransferase	1.44
	*Xf*1121	*met*F OR *AQ*_1429	5,10-methylene tetrahydrofolate reductase	0.92
	*Xf*2223	*thr*C	Threonine synthase	1.00
	*Xf*0863	*met*2	Homoserine O-acetyltransferase	1.25
Amino Acids Biosynthesis—Aromatic Amino Acid Family	*Xf*0624	*aro*E	Shikimate 5-dehydrogenase	1.64
Nucleotides Biosynthesis—Salvage of Nucleosides and Nucleotides	*Xf*2150	*apa*H	Diadenosine tetraphosphatase	1.14
	*Xf*2354	*hpt*	Hypoxanthine-guanine phosphoribosyl transferase	1.08
Nucleotides Biosynthesis – 2′ Deoxyribonucleotides	*Xf*0580	PH1695	Thymidylate kinase	−0.93
	*Xf*1196	*nrd*A OR TP1008	Ribonucleoside-diphosphate reductase alpha chain	1.10
Nucleotides Biosynthesis—Purine Ribonucleotides	*Xf*1503	*gmk* OR *spo*R	Guanylate kinase	0.86
Nucleotides Biosynthesis—Pyrimidine Ribonucleotides	*Xf*1107	*car*B OR *pyr*A	Carbamoyl-phosphate synthase large chain	−0.99
	*Xf*1106	*car*A	Carbamoyl-phosphate synthase small chain	−0.96
Cofactors, Prosthetic Groups, Carriers Biosynthesis—Menaquinone, Ubiquinone	*Xf*1487	*ubi*E	Ubiquinone menaquinone transferase	−1.64
Cofactors, Prosthetic Groups, Carriers Biosynthesis—Pantothenate	*Xf*0229	*pan*B	3-Methyl-2-oxobutanoate hydroxy methyltransferase	−1.50
Cofactors, Prosthetic Groups, Carriers Biosynthesis—Thiamin	*Xf*0783	*thi*G	Thiamine biosynthesis protein	−0.87
Cofactors, Prosthetic Groups, Carriers Biosynthesis—Riboflavin	*Xf*1748	MJ0671	5-amino-6-(5-phospho ribosylamino) uracil reductase	1.05
Cofactors, Prosthetic Groups, Carriers Biosynthesis—Biotin	*Xf*2477	*bio*D	Dethiobiotin synthetase	1.08
Cofactors, Prosthetic Groups, Carriers Biosynthesis—Others	*Xf*1916	AF1671	Coenzime F390 synthetase	1.21
Fatty Acid and Phosphatidic Acid Biosynthesis	*Xf*2269	DRB0080	3-alpha-hydroxysteroid dehydrogenase	−0.93
	*Xf*0572	*fab*A	Beta-hydroxydecanoyl-ACP dehydratase	1.18
Macromolecule Metabolism				
DNA metabolism—Replication	*Xf*0001	*dna*A	Chromosomal replication initiator	−1.02

Table 1: Continued.

Functional Group	ORF Number	Gene Name	Gene Product	Log_2 (3G10R/PW)
	*Xf*0002	*dna*N	DNA polymerase III, beta chain	−1.39
	*Xf*0002	*dna*N	DNA polymerase III, beta chain	−1.15
	*Xf*a0003	*top*A OR *sup*X	Topoisomerase I	−1.60
	*Xf*1353	*par*C	Topoisomerase subunit	0.98
DNA metabolism—Recombination	*Xf*0425	*rec*D	Exodeoxyribonuclease V alpha chain	−0.96
	*Xf*0425	*rec*D	Exodeoxyribonuclease V alpha chain	−1.02
	*Xf*0423	*ecb* OR *ror*A	Exodeoxyribonuclease V beta chain	1.30
DNA metabolism—Repair	*Xf*1902	*ruv*B OR HL0312	Holliday junction binding protein, DNA helicase	−1.20
	*Xf*2692	*ung*	Uracil-DNA glycosylase	−1.18
DNA Metabolism—Restriction, Modification	*Xf*0935	LLAIIA	Methyltransferase	−0.83
	*Xf*1804	SPHIM	Site-specific DNA-methyltransferase	1.12
	*Xf*1774	*hpa*IIM	DNA methyltransferase	−0.81
DNA Metabolism—Structural DNA Binding Proteins	*Xf*0446	*bbh*3	DNA-binding protein	−1.19
	*Xf*1644	*ssb*	Single-stranded DNA binding protein	1.05
RNA Metabolism—Ribosomes—Maturation and Modification	*Xf*0441	*rim*I	Ribosomal-protein-alanine acetyl transferase	1.87
	*Xf*0939	*rlu*D OR *sfh*B	Ridosomal large subunit pseudoeridine synthase D	−1.02
RNA Metabolism—Ribosomal Proteins	*Xf*1164	*rpl*E OR rpl5 OR HI0790	50S ribosomal protein L5	−0.91
	*Xf*0238	*rps*O OR *sec*C	30S ribosomal protein S15	−1.23
	*Xf*1166	*rps*H OR *rps*8 OR HI0792	30S ribosomal protein S8	−1.4
	*Xf*1169	*rps*E OR *spc*	30S ribosomal protein S5	−1.14
RNA Metabolism—RNA Synthesis, Modification, DNA Transcription	*Xf*1108	*gre*A	Transcriptional elongation factor	−1.73
	*Xf*0227	*pcn*B	Polynucleotide adenyltransferase	−1.31
	*Xf*2632	*rpo*C OR *tab*B	RNA polymerase beta subunit	1.09
	*Xf*2606	*rlu*C	Pseudourylate synthase	−1.08
RNA Metabolism—Aminoacyl tRNA Synthetases, tRNA Modification	*Xf*0428	TM0492	Tryptophanyl-tRNA synthetase	−1.89
	*Xf*0445	*pro*S OR *drp*A	Prolyl-tRNA synthetase	−1.08
	*Xf*0134	*val*S OR HI1391	Valyl-tRNA synthetase	−0.96
	*Xf*0169	*tyr*S OR HI1610	Tyrosyl-tRNA synthetase	1.93
	*Xf*1314	*que*A	S-Adenosylmethionine tRNA ribosyltransferase-isomerase	−1.00
	*Xf*0736	*thr*S	Threonyl-tRNA synthetase	−1.08
RNA Metabolism—RNA Degradation	*Xf*1505	*rph*	Ribonuclease PH	−0.74
	*Xf*1041	*rnh*B	Ribonuclease HII	−1.00

Table 1: Continued.

Functional Group	ORF Number	Gene Name	Gene Product	Log_2 (3G10R/PW)
	*Xf*2615	*rna*SA3	Ribonuclease	1.00
Protein Metabolism—Translation and Modification	*Xf*0644	*mip*	Peptidyl-propyl cis-trans isomerase	−1.11
	*Xf*2629	*fus*A	Elongation factor G	−0.90
Protein Metabolism—Protein Degradation	*Xf*0220	*pep*Q	Proline dipeptidase	−1.13
	*Xf*0453	*hfl*C OR HI0150	Integral membrane proteinase	1.65
	*Xf*2241	*muc*D	Periplasmic protease	−0.87
	*Xf*1479	*ptr*B OR *tlp*	Peptidase	−0.82
	*Xf*2330	*slp*D	Proteinase	−0.85
Cell Structure				
Murein Sacculus, Peptidoglycan	*Xf*0416	*vac*J	Lipoprotein precursor	−0.78
	*Xf*0799	*ddl*B OR *ddl*	D-Alanine-D-alanine ligase B	−1.69
	*Xf*0276	*mpl*	UDP-N-acetylmuramate-L-alanine ligase	−0.88
Surface Structures	*Xf*0487		Fimbrillin	1.07
	*Xf*2539		Fimbrial protein	−1.02
	*Xf*2544	*pil*B	Pilus biogenesis protein	−0.79
Chemotaxis and Mobility—Surface Polysaccharides, Lipopolysaccharides and Antigens	*Xf*1289	*kds*A	2-dehydro-3-deoxy phosphooctonate aldolase	−0.90
	*Xf*1419	*lpx*D OR *fir*A OR *oms*A	Acetyltransferase	1.05
	*Xf*1646	*lpx*D OR *fir*A	UDP-3-O-(R-3-hydroxy myristoyl)-glucosamine N-acyltransferase	−0.75
	*Xf*1638		Dolichyl-phosphate mannose synthase related protein	−1.02
	*Xf*0879	*rfb*U	Lipopolysaccharide biosynthesis protein	−0.74
	*Xf*2154	*ops*X	Saccharide biosynthesis regulatory protein	−1.00
	*Xf*0105	*kdt*A OR *waa*A	3-deoxy-D-manno-octulosonic acid trasnferase	1.50
Membrane Components—Outer Membrane Constituents	*Xf*1024		Outer membrane protein H.8 precursor	−1.19
Cellular Processes				
Transport—Cations	*Xf*1903	*kup* OR *trk*D	Potassium uptake protein	1.01
	*Xf*1903	*kup* OR *trk*D	Potassium uptake protein	1.40
	*Xf*0599	*ybi*L	TONB-dependent receptor for iron transport	1.46
	*Xf*0395	*bfr*	Bacterioferritin	−1.22
Transport—Amino Acids, Amines	*Xf*1937	*glt*P	Proton glutamate symport protein	−1.00
Transport—Protein, Peptide Secretion	*Xf*2685	*spp*A	Protease IV	−0.88
	*Xf*2261	HI0561 560	Oligopeptide transporter	−1.12
Transport—Carbohydrates, Organic Acids, Alchohols	*Xf*0976	*dct*A	C4-dicarboxylate transport protein	−1.10

Table 1: Continued.

Functional Group	ORF Number	Gene Name	Gene Product	Log_2 (3G10R/PW)
Cell Division	*Xf*2281	DR0012	Chromosomepartitioning protein	−1.08
Other	*Xf*2251	*ppa*	Solute Na+ symporter	−1.64
	*Xf*1728	F451	Transport protein	1.11
	*Xf*1604	*btu*E	ABC transporter vitamin B12 uptake permease	−1.48
	*Xf*1409	HI1148	ABC transporter ATP-binding protein	0.84
Mobile Genetic Elements				
Transposon- and Intron-Related Functions	*Xf*1775	IS629	Reverse transcriptase	1.06
	*Xf*0535		Transposase ORFA	−0.80
Phage-Related Functions and Prophages	*Xf*2522		Phage-related protein	1.52
	*Xf*2522		Phage-related protein	1.02
	*Xf*a0040	*trb*I	Conjugal transfer protein	−0.98
	*Xf*2291		Phage-related protein	0.95
	*Xf*0513	*lyc*V	Phage-related endolysin	−1.52
	*Xf*1786		Phage-related protein	1.32
	*Xf*1706	GP37	Phage-related tail fiber protein	1.31
	*Xf*0685		Phage-related protein	0.86
	*Xf*0704		Phage-related protein	1.18
	*Xf*1875		Phage-related protein	1.44
Plasmid-Related Functions	*Xf*a0006	*tra*A OR *vir*B3	Conjugal transfer protein	−1.13
	*Xf*a0013	*tra*AO OR *vir*B9	Conjugal transfer protein	−1.37
	*Xf*a0008	*tra*AC OR *vir*B5	Conjugal transfer protein	−1.54
Pathogenicity, Virulence, and Adaptation				
Toxin production and detoxification	*Xf*0262	*cva*C	Colicin V precursor	7.29
	*Xf*0263	*cva*C	Colicin V precursor	1.70
	*Xf*1011	*frp*C	Hemolysin-type calcium binding protein	−1.45
	*Xf*1827	*ohr*	Organic hydroperoxide resistance protein	−1.43
	*Xf*2614	*sod*A OR sod	Superoxide dismutase (MN)	−1.47
	*Xf*1210	*gst* OR HI0111	Glutathione S-transferase	−1.00
	*Xf*1890	*gpo*	Glutathione peroxidase-like protein	0.86
	*Xf*2135	*frn*E	Polyketide synthase (PKS)	1.80
	*Xf*1897	*tol*B	TOLB protein precursor	−1.30
	*Xf*1729	DR1890	Phenylacetaldehyde dehydrogenase	0.91
Host Cell Wall Degradation	*Xf*0818	*eng*XCA	Endo-1,4-beta-glucanase	−0.89
Adaptation Atypical Condition	*Xf*2682	*mdo*G	Periplasmic glucan biosynthesis protein	−0.80
	*Xf*2622	*tap*B	Temperature acclimation protein B	−1.30
Surface Proteins	*Xf*1516	*usp*A1	Surface-exposed outer membrane protein	−1.28
Exopolysaccharydes	*Xf*2360	*gum*M	Gumm protein	−1.08

Table 1: Continued.

Functional Group	ORF Number	Gene Name	Gene Product	Log_2 (3G10R/PW)
Other	*Xf*1529	*hsf*	Surface protein	1.96
	*Xf*1532	*oxy*R	Oxidative stress transcriptional regulator	0.96
	*Xf*2121	*vap*E	Virulence-associated protein E	1.24
	*Xf*1987	*vac*B	VACB protein	−1.35
	*Xf*1114	*rpf*C	Regulator of pathogenicity factors	−0.87
ORFs with Undefined Category				
	*Xf*1723	*yrp*G	Sugar-phosphate dehydrogenase	1.30
	*Xf*0088	*hfl*X	GTP-binding protein	1.36
Hypothetical Proteins				
	*Xf*1287		Hypothetical protein	1.40
	*Xf*0493		Hypothetical protein	0.94
	*Xf*0037		Hypothetical protein	−1.11
	*Xf*1655		Hypothetical protein	0.82
	*Xf*0726		Hypothetical protein	−1.17
	*Xf*1835		Hypothetical protein	−0.85
	*Xfa*0031		Hypothetical protein	−1.60
	*Xf*2413		Hypothetical protein	0.96
	*Xf*0871		Hypothetical protein	1.69
	*Xf*2454		Hypothetical protein	−0.97
	*Xf*1769		Hypothetical protein	−0.80
	*Xf*1803		Hypothetical protein	−2.00
	*Xf*0512		Hypothetical protein	−0.93
	*Xf*0531		Hypothetical protein	−1.72
	*Xf*1868		Hypothetical protein	1.11
	*Xf*1881		Hypothetical protein	1.18
	*Xf*0917		Hypothetical protein	1.25
	*Xf*1738		Hypothetical protein	1.37
	*Xf*0242		Hypothetical protein	1.27
	*Xf*1228		Hypothetical protein	1.01
	*Xf*1279		Hypothetical protein	1.11
	*Xf*1575		Hypothetical protein	1.14
	*Xf*2597		Hypothetical protein	−0.94
	*Xf*0516		Hypothetical protein	1.16
	*Xf*2017		Hypothetical protein	−1.51
	*Xf*1989		Hypothetical protein	−0.94
	*Xf*2410		Hypothetical protein	−1.60
	*Xf*2304		Hypothetical protein	−1.26
	*Xf*0959		Hypothetical protein	1.24
	*Xf*2115		Hypothetical protein	1.23
	*Xf*1100		Hypothetical protein	1.04
	*Xf*1704		Hypothetical protein	0.95
	*Xf*0974		Hypothetical protein	−1.26
	*Xf*0491		Hypothetical protein	1.31
	*Xf*1060		Hypothetical protein	1.77
	*Xf*2151		Hypothetical protein	1.73

Table 1: Continued.

Functional Group	ORF Number	Gene Name	Gene Product	Log_2 (3G10R/PW)
	*Xf*2449		Hypothetical protein	−1.01
	*Xf*2305		Hypothetical protein	−0.77
	*Xf*1721		Hypothetical protein	1.14
	*Xf*0626		Hypothetical protein	−1.39
	*Xf*2411		Hypothetical protein	1.01
	*Xf*1770		Hypothetical protein	−0.87
	*Xf*1364		Hypothetical protein	−0.85
	*Xf*1710		Hypothetical protein	0.90
	*Xf*1761		Hypothetical protein	1.44
	*Xf*1787		Hypothetical protein	1.38
	*Xf*0540		Hypothetical protein	−1.30
	*Xf*1788		Hypothetical protein	1.06
	*Xf*0646		Hypothetical protein	1.03
	*Xf*2543		Hypothetical protein	−0.98
	*Xf*0914		Hypothetical protein	−1.33
	*Xf*2702		Hypothetical protein	−1.52
	*Xf*0492		Hypothetical protein	1.55
	*Xf*1239		Hypothetical protein	1.01
	*Xf*0074		Hypothetical protein	−1.07
	*Xf*a0004		Hypothetical protein	−1.78
	*Xf*1687		Hypothetical protein	1.32
	*Xf*0388		Hypothetical protein	−0.86
	*Xf*0025		Hypothetical protein	−1.23
	*Xf*1434		Hypothetical protein	−1.24
	*Xf*2125		Hypothetical protein	0.89
	*Xf*1513		Hypothetical protein	1.18
	*Xf*2711		Hypothetical protein	1.23
	*Xf*0035		Hypothetical protein	1.31
	*Xf*1441		Hypothetical protein	−1.41
	*Xf*2514		Hypothetical protein	1.71
	*Xf*2626		Hypothetical protein	1.44
	*Xf*0687		Hypothetical protein	1.07
	*Xf*1917		Hypothetical protein	1.90
	*Xf*2271		Hypothetical protein	1.50
	*Xf*1036		Hypothetical protein	−0.99
	*Xf*a0017		Hypothetical protein	−1.98
	*Xf*0529		Hypothetical protein	1.09
	*Xf*2103		Hypothetical protein	−1.05
	*Xf*1986		Hypothetical protein	−1.05
	*Xf*1700		Hypothetical protein	1.12
	*Xf*1719		Hypothetical protein	1.08
	*Xf*1753		Hypothetical protein	1.44
	*Xf*0019		Hypothetical protein	0.85
	*Xf*0293		Hypothetical protein	−1.15
	*Xf*0300		Hypothetical protein	1.67
	*Xf*0279		Hypothetical protein	1.79
	*Xf*0735		Hypothetical protein	−0.94
	*Xf*1010		Hypothetical protein	−0.97

Table 1: Continued.

Functional Group	ORF Number	Gene Name	Gene Product	Log_2 (3G10R/PW)
	*Xf*1580		Hypothetical protein	0.80
	*Xf*2021		Hypothetical protein	1.21
	*Xf*2738		Hypothetical protein	1.49
	*Xf*0877		Hypothetical protein	1.28
	*Xf*2270		Hypothetical protein	1.13
	*Xf*0488		Hypothetical protein	1.50
	*Xf*0264		Hypothetical protein	4.10
	*Xf*2701		Hypothetical protein	−1.68
	*Xf*2768		Hypothetical protein	1.35
	*Xf*0688		Hypothetical protein	0.96
	*Xf*0898		Hypothetical protein	1.15
	*Xf*0426		Hypothetical protein	−1.23
	*Xf*0443		Hypothetical protein	−1.06
	*Xf*1421		Hypothetical protein	−1.40
	*Xf*2193		Hypothetical protein	−2.17
	*Xf*2390		Hypothetical protein	1.24
	*Xf*1128		Hypothetical protein	−1.16
	*Xf*2116		Hypothetical protein	1.52
	*Xf*0467		Hypothetical protein	−1.18
	*Xf*1193		Hypothetical protein	−0.80
	*Xf*1032		Hypothetical protein	−1.33
	*Xf*2262		Hypothetical protein	−1.60
Conserved Hypothetical Proteins				
	*Xf*a0045		Conserved hypothetical protein	−2.22
	*Xf*2450		Conserved hypothetical protein	−1.22
	*Xf*2609		Conserved hypothetical protein	−0.87
	*Xf*1754		Conserved hypothetical protein	1.83
	*Xf*0805		Conserved hypothetical protein	−0.81
	*Xf*2493		Conserved hypothetical protein	1.13
	*Xf*2088		Conserved hypothetical protein	1.26
	*Xf*0196		Conserved hypothetical protein	−1.95
	*Xf*1750		Conserved hypothetical protein	1.36
	*Xf*1745		Conserved hypothetical protein	1.24
	*Xf*2647		Conserved hypothetical protein	1.13
	*Xf*2252		Conserved hypothetical protein	−2.81
	*Xf*2010		Conserved hypothetical protein	−1.06
	*Xf*2237		Conserved hypothetical protein	−0.85

Table 1: Continued.

Functional Group	ORF Number	Gene Name	Gene Product	Log_2 (3G10R/PW)
	*Xf*a0032	SCJ21.16	Conserved hypothetical protein	−1.06
	*Xf*0758	*yje*E	Conserved hypothetical protein	−1.40
	*Xf*0407	*ycc*W	Conserved hypothetical protein	0.98
	*Xf*0552	*yra*L	Conserved hypothetical protein	−0.92
	*Xf*2651	*ycb*Y	Conserved hypothetical protein	−1.19
	*Xf*2575	DR0386	Conserved hypothetical protein	−0.86
	*Xf*0363	*yia*D	Conserved hypothetical protein	−1.78
	*Xf*0066	*ylb*K	Conserved hypothetical protein	1.10
	*Xf*2179	*ybe*N	Conserved hypothetical protein	1.29
	*Xf*2153	HI0260.1	Conserved hypothetical protein	−1.14
	*Xf*0553	HI1655	Conserved hypothetical protein	−1.29
	*Xf*2014	DR0566	Conserved hypothetical protein	1.14
	*Xf*0139	*yjg*P	Conserved hypothetical protein	1.15
	*Xf*2474	*yje*K	Conserved hypothetical protein	−0.79
	*Xf*2096	MTH1196	Conserved hypothetical protein	−1.93
	*Xf*1054	TM1087	Conserved hypothetical protein	−0.91
	*Xf*0554	*yra*N	Conserved hypothetical protein	−0.85
	*Xf*0339	*btu*B OR *bfe* OR *cer*	Conserved hypothetical protein	−0.91
	*Xf*1272	RV1827 OR MTCY1A11.16C	Conserved hypothetical protein	−1.02
	*Xf*1405	*yhb*J	Conserved hypothetical protein	−0.88
	*Xf*1808	*yba*B	Conserved hypothetical protein	−1.19
	*Xf*1829	RP471	Conserved hypothetical protein	−1.05
	*Xf*0941	*yux*K	Conserved hypothetical protein	−0.80

an alternative approach. Thus, we performed Real-Time qPCR experiments with the same RNA samples used in the microarray hybridizations, aiming at double-checking the changes in expression of 16 genes present in Table 1 (~5% of all modulated genes). These genes have been randomly chosen from different functional categories and all displayed average expression ratios that correlate with the microarray results (see Figure 2).

Interestingly, we were able to verify that several genes directly associated with pathogenicity, virulence and adaptation had their transcription modulated in response to growth in xylem-based chemical conditions. This group includes

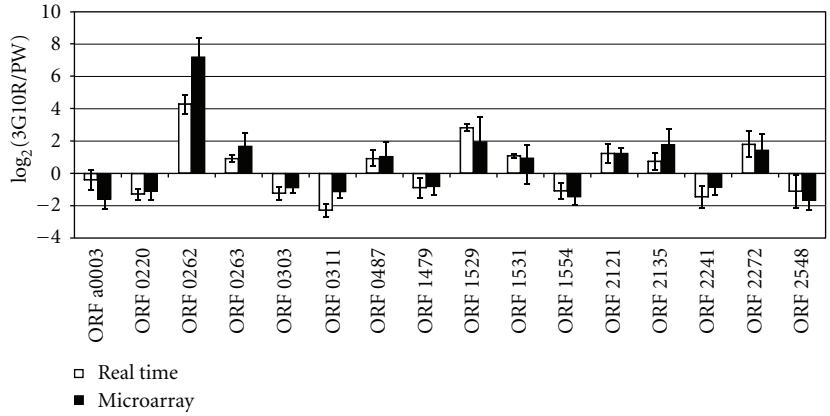

Figure 2: Evaluation of transcriptional modulation of selected genes by Real-Time qPCR. In order to confirm the reliability of the microarray experiments, 16 genes have been randomly selected and their transcription modulation was verified by Real-Time qPCR. The same RNA samples used in the microarray hybridizations were converted to cDNA and the relative expression ratios (RQ) of these genes have been measured with the aid of specific Taq-Man probes. ORF *Xf*1311, which encodes a rod-shaped determining protein (MreD), has been used as an endogenous control for experimental normalization, since the microarray hybridization experiments showed that this ORF is constitutively expressed in both PW and 3G10R. Variations in transcriptional modulation were calculated having the expression levels in PW as a reference and are represented by the $\log_2$ ratio of the relative quantifications (RQ). Experiments were performed in triplicate and graphic shows the average values and their respective standard deviations.

genes associated with adaptation to atypical conditions (such as the temperature acclimatation protein TAPB (ORF *Xf*2622) and the oxidative stress transcriptional regulator OxyR (ORF *Xf*1532)); surface proteins (including adhesion factors, such as the outer membrane protein Hsf (ORF *Xf*1529)), and genes involved in toxin production and/or detoxification (such as the colicin precursors encoded by ORFs *Xf*0262 and *Xf*0263), among others (see Table 1 for details).

The lack of aminoacids in 3G10R also seems to lead to overexpression of at least four genes directly involved in the biosynthesis of such molecules (represented by ORFs *Xf*0624, *Xf*0863, *Xf*1121, *Xf*2223 and *Xf*2272). On the other hand, cells that are grown on the peptide-based diet provided by PW display an increased production of proteolytic enzymes, such as MucD (ORF *Xf*2241), PtrB (ORF *Xf*1479) and PepQ peptidase (ORF *Xf*0220), which has been shown to play a major role in lactic acid bacteria, providing the cells with amino acids derived from extracellular protein sources during milk fermentation [28].

The transcriptome results also show that the elevated growth rate of *X. fastidiosa* cells kept in PW is associated with the upregulation of several genes involved in a series of metabolic pathways and processes that are important to sustain continued bacterial growth [29]. These include ORFs associated with DNA replication, recombination and repair, such as *dna*A (the chromosomal replication initiator, encoded by ORF *Xf*0001), *dna*N (the β chain of DNA polymerase III, encoded by ORF *Xf*0002), *rec*D (the alpha chain of exodeoxyribonuclease V, encoded by ORF *Xf*0425), *ruv*B (a Holiday junction-associated helicase, encoded by ORF *Xf*1902) and *ung* (an uracil-DNA glycosilase, encoded by ORF *Xf*2692).

However, since elevated growth rates establish a higher demand for energy consumption, they can only be maintained if ATP production is increased. Thus, it is interesting to verify that growth in PW is associated with overexpression of several genes involved in all major steps of the central metabolic pathway, such as triose phosphate isomerase (*Xf*0303) (glycolytic pathway); succinyl-coA synthase (*Xf*2548) and fumarate hydratase C (*Xf*1554) (Krebs cycle), as well as genes from the *nuo* operon (represented by ORFs *Xf*0308, *Xf*0310, *Xf*0311 and *Xf*0317, resp.). Genes from this operon encode subunits of the NADH Dehydrogenase I complex, the first component of the respiratory electron transport chain. Interestingly, coordinated overexpression of such genes has already been shown to occur in *E. coli* cells submitted to differing culture conditions [30, 31].

3.2. Increased Growth Rate in PW Is Associated with Upregulation of Genes from the Electron Transport Chain and Consequent Enhancement of Respiratory Activity. As mentioned before, PW is the most commonly used medium to cultivate *Xylella fastidiosa* under laboratory conditions, since this formulation has been shown to sustain efficient growth of all isolates of this phytobacterium [11]. Thus, the positive modulation of genes directly involved in oxidative phosphorylation, might lead to increased aerobic respiratory activity and consequent ATP production, which seems to greatly improve on the fastidious nature of this bacterium. Thus, we decided to verify O_2 consumption in PW-grown cells as a way to indirectly estimate the activation of aerobic respiration in *X. fastidiosa*. This experiment allowed us to verify not only the activation of the aerobic respiration, but also to obtain biological confirmation of a major metabolic change originally predicted solely on the transcriptome data.

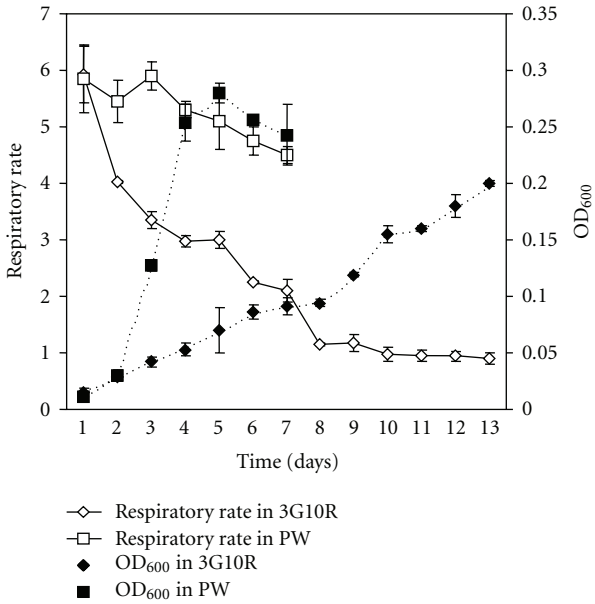

Figure 3: Evaluation of respiratory rates in *Xylella fastidiosa* cells growing in PW and 3G10R. *X. fastidiosa* cells were grown into middle exponential phase in PW and subsequently transferred (in a 1 : 20 proportion) into fresh PW and 3G10R cultures. Bacterial growth in both cultures was monitored through OD_{600} measurements and aliquots were taken from each culture to evaluate O_2 consumption with the aid of an oxygraph in intact cells. Respiratory rate for each culture was calculated as the ratio between O_2 consumption and the respective OD_{600} value obtained at each time point. Measurements were taken until day 7 (in PW) and day 13 (in 3G10R). Experiments were performed in triplicate and graphic shows the average values and their respective standard deviations.

As shown in Figure 3, *X. fastidiosa* cells transferred from PW to 3G10R displayed a continued decrease in the respiratory rate, which is unaffected in cells transferred to fresh PW medium. A direct comparison between the results observed for the PW culture, at day 3, and the 3G10R culture, at day 13, (the same time points used for transcriptome comparisons) shows that cells grown in PW display overexpression of several genes involved in all major steps of the central metabolic pathway, as well as a respiratory rate that is about five times greater than that observed with cells grown in 3G10R. Thus, the results from this experiment confirmed that there is a significant increase in oxidative phosphorylation when *X. fastidiosa* cells are grown in PW (as previously inferred from the analysis of transcriptome data), which helps to explain the effectiveness of this culture medium in sustaining continued and vigorous growth of *X. fastidiosa* strains.

4. Discussion

The recent development of xylem-based chemistry media, such as 3G10R, has provided an interesting instrument to study several aspects of *X. fastidiosa* behavior under laboratory conditions, where this phytopathogen is typically grown in complex media, such as PW [11]. Interestingly, both PW and 3G10R are capable of sustaining growth of *X. fastidiosa* cells *in vitro*, although significant differences have been observed in the bacterial growth rates.

Nonetheless, when growing in PW, where *X. fastidiosa* cells have been shown to display an increased respiratory rate, as well as an enhanced growth profile, we can observe coordinated upregulation of enzymes from the central metabolic pathway, particularly of the NADH Dehydrogenase I complex, a phenomenon also observed to occur in *E. coli* grown in different media [30, 31]. This results in strong activation of the aerobic respiratory metabolism, providing the cells with the necessary energy for increased bacterial replication. However, at this point, we do not know the exact mechanism(s) that might be responsible to trigger such a respiratory activation, nor if it plays any role during plant colonization or onset of disease, when the endophytic population of *X. fastidiosa* seems to increase dramatically inside xylem vessels [32, 33]. It seems unlikely, however, that this metabolic switch occurs only on the account of oxygen concentration, since both cultures were kept under the same aeration conditions during all experimental steps described throughout this work.

Incidentally, this situation seems to resemble the fermentative-to-respiratory shift observed in *Lactococus lactis*, a gram-positive, microaerophilic bacterium, with a fermentative metabolism that produces mainly L-lactate from carbohydrates [34, 35]. *L. lactis*, as well as other members of the *Streptococcaceae* family, such as *Streptococcus agalactiae* and *Enterococcus fecalis*, multiply mainly via a fermentative metabolism, even in the presence of oxygen. Curiously, in spite of the fact that these bacteria carry all genes and enzymes necessary to undergo aerobic respiration, prolonged aeration of *L. lactis* cultures can lead to growth inhibition, DNA degradation and cell death, probably due to the formation of hydrogen peroxide and hydroxyl radicals during aerobic respiration, associated with an incomplete set of oxidative stress-resistance enzymes [36]. However, if exogenous haem is provided during aerated growth, *L. lactis* cells can undergo a metabolic shunt towards respiratory metabolism, leading to increased ATP production, improved growth and a dramatic increase in long-term survival, when compared to growth in standard fermentation conditions [35]. Further details regarding the fermentation-respiration shift in *L. lactis* are not completely understood, but it has been documented that the process depends on cytochrome BD (encoded by the *cya*BD genes) and is controled by the Catabolite Control Protein (CcpA) [37]. Although more direct evidence is still needed to further clarify this issue, it is tempting to speculate if the presence of hemin chloride in PW might be acting as an exogenous source of haem and activating an analogous mechanism in *X. fastidiosa* cells that would lead to an increase in aerobic respiration.

The observed modulation of triose phosphate isomerase (*Xf*0303) is also noteworthy, since preliminary studies failed to detect specific activity of several genes from the Glycolytic pathway in bacterial crude extracts, such as aldolase, glyceraldehyde 3-phosphate dehydrogenase and enolase [38]. On the other hand, the activity of glucose 6-phosphate

dehydrogenase was detected in these same extracts, leading the authors to suggest that *X. fastidiosa* cells do not use the glycolytic pathway to oxidize glucose, which would be preferably metabolized by the Entner-Dudoroff pathway [38]. In *X. fastidiosa*, all genes of the Entner-Dudoroff pathway are encoded by a single operon, which encompasses ORFs *Xf*1061 to *Xf*1065, but we did not observe overexpression of any such genes in either of the media, even in 3G10R, which has glucose as the sole carbon source.

The difference in carbon source also seems to be important in determining the expression of genes associated with other aspects of the cellular metabolism, such as aminoacid biosynthesis (in 3G10R), as opposed to proteolytic enzymes (in PW). Interestingly, the coordinated upregulation of proteolytic enzymes is indicative that *X. fastidiosa* cells, like lactic acid bacteria, have developed an efficient mechanism dedicated to process extra cellular proteins as a major way to obtain amino acids from exogenous sources [39]. This idea is also consistent with the elevated growth rates observed with cells grown in PW, a significantly rich medium, which is based on relatively high concentrations of protein hydrolisates, such as tryptone and peptone [11].

In spite of providing more adequate nutritional conditions to sustain continued growth of fastidious microorganisms, complex media are not likely to resemble the harsh nutritional conditions found in xylem sap. Since 3G10R does not receive nutrients from any complex source, it is likely to be much more restricted in nutrient availability [16]. Moreover, this formulation incorporates a few important chemical characteristics that resemble xylem composition of plants known to be infected by *X. fastidiosa*, such as the use of glucose as a major carbon source [22–24] and the presence of L-glutamine, which is the most abundant amino acid detected in the sap of grapevines [25, 26] and seems to be essential for *in vitro* growth of *X. fastidiosa* cells [11, 40]. The antioxidant tripeptide glutathione (GSH) has also been detected in the composition of xylem fluid of poplar and spruce trees [41, 42] and is present in the composition of 3G10R at a similar concentration [16].

The presence of glucose seems to be an important characteristic of 3G10R in resembling xylem, since this metabolite has already been identified in the chemistry composition of xylem fluid from many plant species, such as grapevine [22], maize [43], cabbage [44], poplar [24] and oak [23], among others [45]. However, the exact glucose concentration found in the xylem sap of different plants has been shown to vary significantly, depending on the species, genotype, season, time of day, age of plants and nutritional status. In poplar trees, such concentration has been shown to range from 0.2 to 15 mM [24], although there have been reports of this nutrient at $<50\,\mu$M concentration in the xylem of grapevines (a typical *X. fastidiosa* host) [16]. Thus, the $\sim$10 mM glucose concentration present in 3G10R might be higher than the concentration typically encountered by *X. fastidiosa* cells during the process of plant infection and colonization.

Although glucose is generally viewed as an energy source for growing microorganisms, this substance has also been shown to act as a precursor for the biosynthesis of several bacterial cell wall components and exopolysaccharides (EPSs), which have been proposed to act as virulence factors in *X. fastidiosa* and many other pathogenic bacteria ([46–48], reviewed in [4]). Moreover, increased production of EPS is one major characteristic of *X. fastidiosa* cells freshly isolated from infected plants and such primarily isolated cells have been shown to be more effective in the process of plant colonization, when compared to cells submitted to continued growth in PW [49]. Coincidentally, while growing in 3G10R, *X. fastidiosa* cells have also been shown to synthesize increased amounts of EPS, leading to more intense biofilm formation [16]. It has even been proposed that the preferential use of glucose to drive EPS synthesis could be an explanation to the fastidious growth of *X. fastidiosa* cells, especially in 3G10R, where these molecules are expected to act as the major source of energy as well [16]. Interestingly, when *X. fastidiosa* cells are grown in this medium, we observed increased expression of *xan*A (ORF *Xf*0260), which encodes a phosphoglucomutase that converts glucose 6-P into glucose 1-P, which in turn, acts as a precursor of UDP-Glucose and UDP-Galactose, which are involved in the biosynthesis of different types of EPS [50]. Moreover, it has already been shown that increased expression of phosphoglucomutase can lead to an increase in the production of EPS in *Lactococus lactis* [51].

EPS production is one of the most important aspects of biofilm formation, which is believed to be an important pathogenicity factor in *X. fastidiosa* cells [52]. Other adhesion factors have been detected as preferentially expressed in 3G10R, which might be directly correlated with the more intense cellular aggregation and biofilm formation observed in this medium [16]. One of these putative adhesion factors is represented by ORF *Xf*0487, which encodes a 20 kDa fimbrillin subunit of bacterial fimbreae, and may be involved in bacterial adherence and invasion [53]. Pili and fimbreae have been implicated in plant infection and migration via a twitching motility mechanism that seems to be of paramount importance to the colonization process of *X. fastidiosa* [54]. Another important component of the cellular outer membrane structure that has been shown to be upregulated in 3G10R is the *hsf* gene (ORF *Xf*1529), which encodes a surface fibril that belongs to a family of high molecular weight autotransporter adhesins [55]. This protein has been originally characterized as an important virulence factor from *Haemophilus influenzae* type b, which causes meningitis and other serious invasive human diseases. In this bacterium, the Hsf protein has been shown to form trimeric fiber-like structures on the bacterial surface that mediate adhesion to epithelial cells [56]. Hsf is also suspected to act as a virulence factor in *X. fastidiosa*, since overexpression of this protein occurs in *X. fastidiosa* cells that display higher infective capacity, as well as in bacteria directly isolated from infected plants [17, 49].

Three bacteriocin genes (*Xf*0262, *Xf*0263 and *Xf*0264) have been found to be overexpressed in 3G10R-cultivated cells, suggesting that increased production of such molecules might be important to *X. fastidiosa* cells in competing with other endophytic bacteria within the xylem [57]. These molecules belong to a class of structurally related proteins

that kill target cells by membrane permeabilization. Some of them have been known to kill different types of bacteria, constituting a strategic advantage for microorganisms that colonize highly competitive environments [58]. Although little is known about the *X. fastidiosa* bacteriocins so far, it is interesting to verify that the bacteriocin encoded by *Xf*0263 has also been identified as overexpressed in *X. fastidiosa* cells that display higher infective capacity, as well as in bacteria directly isolated from infected plants [17, 49], while the proteins encoded by *Xf*0262 and *Xf*0264 are induced in response to glucose [59].

Although we are aware that defined media, like 3G10R, do not constitute a perfect simulation of the environment inhabited by xylem-dwelling endophytes, this formulation has clearly incorporated some important chemical aspects of xylem fluid composition, which induce transcriptional activation of some putative pathogenicity-associated genes in *X. fastidiosa* cells. Moreover, some of these genes have also been shown to be specifically upregulated in cells directly isolated from infected plants, as well as in freshly isolated *X. fastidiosa* cultures, which are known to display a higher infective capacity. The dependence of aggregation and biofilm formation on the nutrient composition of xylem fluid suggests that xylem chemistry is important in resistance/susceptibility to disease [27, 60, 61]. Thus, the transcriptome profile of *X. fastidiosa* cells grown in xylem-based chemistry media is more likely to represent the metabolome of *X. fastidiosa* cells *in planta*, reinforcing the idea that such media formulations should be preferred for metabolic studies of this phytopathogen.

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