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 3G10-R-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 colonzation 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 respiratory activity is observed when cells are grown in 3G10R.

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\textsubscript{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\textsubscript{600} measurements, providing a direct comparison between X. fastidiosa growth patterns observed in 3G10R and standard PW medium.

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.

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 prepara- tions, 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 \( \delta \) 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
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 Biosystems, 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 Xf1311, which encodes a rod-shaped determining protein (MreD) has been used as an endogenous control for experimental normalization, as 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 OD600 measurements until both cultures reached stationary phase. Aliquots were taken from each culture, on a daily basis, to monitor bacterial growth through OD600 readings. Measurements were performed in triplicate and graphic shows the average values and their respective standard deviations.

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 (OD600 ∼ 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 (OD600 ∼ 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.

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 Log₂ ratio are overexpressed in 3G10R, while genes with negative Log₂ ratio are overexpressed in PW.

| Functional Group                      | ORF Number | Gene Number | Gene Name | Gene Product                                                                 | Log₂ (3G10R/PW) |
|---------------------------------------|------------|-------------|-----------|-------------------------------------------------------------------------------|-----------------|
| Intermediary Metabolism               |            |             |           |                                                                               |                 |
| Energy metabolism, carbon—Aerobic respiration | Xf0308     | nuoD        |           | NADH-ubiquinone oxidoreductase, NQO4 subunit                                 | −0.93           |
|                                       | Xf0310     | nuoF        |           | NADH-ubiquinone oxidoreductase, NQO1 subunit                                 | −1.08           |
|                                       | Xf0311     | nuoG        |           | NADH-ubiquinone oxidoreductase, NQO3 subunit                                 | −1.14           |
|                                       | Xf0317     | nuoM        |           | NADH-ubiquinone oxidoreductase, NQO13 subunit                                | −1.03           |
|                                       | Xf0347     | dld1        |           | D-Lactate dehydrogenase                                                      | 1.18            |
| Energy metabolism, carbon—Glycolysis  | Xf0303     | tpiA OR tpi |           | Triosephosphate isomerase                                                    | −0.89           |
| Energy metabolism, carbon—TCA cycle   | Xf2548     | sucD        |           | Succinyl-CoA synthetase, alpha subunit                                       | −1.67           |
|                                       | Xf1554     | fumC        |           | Fumarate hydratase                                                           | −1.47           |
|                                       | Xf1554     | fumC        |           | Fumarate hydratase                                                           | −1.36           |
| Energy metabolism, carbon—Electron Transport | Xf1990    | yneN        |           | Thioredoxin                                                                  | −1.14           |
|                                       | Xf0620     | dsbD        |           | c-Type cytochrome biogenesis protein (Copper Tolerance)                      | −0.83           |
| Degradation—Degradation of Small Molecules | Xf1250   | rocF        |           | Arginine deaminase                                                           | −2.00           |
|                                       | Xf1740     | yijl        |           | Glucose dehydrogenase B                                                      | 1.45            |
|                                       | Xf2395     | axeA        |           | Acetylxylan esterase                                                          | 1.75            |
|                                       | Xf2432     | gtaB        |           | UTP-glucose-1-phosphate uridylyl transferase                                  | −1.14           |
|                                       | Xf0610     | galE        |           | UDP-glucose 4-epimerase                                                      | −1.44           |
|                                       | Xf2210     |             |           | Dioxygenase                                                                  | 1.00            |
| Regulatory Functions                  | Xf1354     | ygyA        |           | Transcriptional regulator (MARR Family)                                      | 1.27            |
|                                       | Xf1354     | ygyA        |           | Transcriptional regulator (MARR Family)                                      | 1.55            |
|                                       | Xf1254     | araL        |           | Transcriptional regulator (ARAC Family)                                      | −1.10           |
|                                       | Xf2344     | fur         |           | Transcriptional regulator (FUR Family)                                       | 1.19            |
|                                       | Xf2336     | colR        |           | Two-component system regulatory protein                                      | 1.32            |
|                                       | Xf2534     | colR        |           | Two-component system regulatory protein                                      | −0.95           |
|                                       | Xf1752     |             |           | Transcriptional regulator (LYSR Family)                                      | 1.64            |
|                                       | Xf1733     | AF0343      |           | Tryptophan repressor binding protein                                          | 1.13            |
|                                       | Xf1749     | opdE        |           | Transcriptional regulator                                                    | 1.65            |
|                                       | Xf1730     | yafC        |           | Transcriptional regulator                                                    | 1.97            |
Table 1: Continued.

| Functional Group                                      | ORF Number | Gene Name | Gene Product                                                                 | Log2 (3G10R/PW) |
|-------------------------------------------------------|------------|-----------|-------------------------------------------------------------------------------|-----------------|
| Sugar-Nucleotide Biosynthesis, Conversions            | Xf0260     | xanA      | Phosphoglucomutase/Phosphomannomutase                                         | 0.92            |
| Central Intermediary Metabolism—Pool, Multipurpose Conversions | Xf0880     | yadF      | Carboxy anhydrase                                                            | −1.30           |
| Central Intermediary Metabolism—Amino Sugars          | Xf2255     | acs       | Acetyl coenzyme A synthetase                                                  | −1.37           |
| Central Intermediary Metabolism—Amino Acid Family     | Xf2355     |           | Exo II n-acetyl-beta-glucosaminidase                                           | 1.44            |
| Central Intermediary Metabolism—Pool, Multipurpose Conversions | Xf2223     | thrC      | Threonine synthase                                                           | 1.00            |
| Central Intermediary Metabolism—Pool, Multipurpose Conversions | Xf0863     | met2      | Homoserine O-acetyltransfer                                                   | 1.25            |
| Nucleotides Biosynthesis—Salvage of Nucleosides and Nucleotides | Xf2150 | apaH      | Diadenosine tetraphosphatase                                                  | 1.14            |
| Nucleotides Biosynthesis—Salvage of Nucleosides and Nucleotides | Xf2354 | hpt       | Hypoxanthine-guanine phosphoribosyl transferase                               | 1.08            |
| Nucleotides Biosynthesis—2′-Deoxyribonucleotides       | Xf0580     | PH1695    | Thymidylate kinase                                                           | −0.93           |
| Nucleotides Biosynthesis—2′-Deoxyribonucleotides       | Xf1196     | nrdA OR TP1008 | Ribonucleoside-diphosphate reductase alpha chain                               | 1.10            |
| Nucleotides Biosynthesis—Salvage of Nucleosides and Nucleotides | Xf1503 | gmk OR spoR | Guanylate kinase                                                             | 0.86            |
| Nucleotides Biosynthesis—Purine Ribonucleotides        | Xf1106     | carA      | Carbamoyl-phosphate synthase large chain                                      | −0.96           |
| Cofactors, Prosthetic Groups, Carriers Biosynthesis—Menalphaquinone, Ubiquinone | Xf1487 | ubiE      | Ubiquinone menaquinone transferase                                            | −1.64           |
| Cofactors, Prosthetic Groups, Carriers Biosynthesis—Pantothenate | Xf0229 | panB      | 3-Methyl-2-oxobutanoate hydroxy methyltransferase                             | −1.50           |
| Cofactors, Prosthetic Groups, Carriers Biosynthesis—Biotin | Xf0783 | thiG      | Thiamine biosynthesis protein                                                 | −0.87           |
| Cofactors, Prosthetic Groups, Carriers Biosynthesis—Riboflavin | Xf1748 | MJ0671    | 5-amino-6-(5-phospho ribosylamino) uracil reductase                          | 1.05            |
| Cofactors, Prosthetic Groups, Carriers Biosynthesis—Biotin | Xf2477 | bioD      | Dethiobiotin synthetase                                                      | 1.08            |
| Cofactors, Prosthetic Groups, Carriers Biosynthesis—Biotin | Xf1916 | AF1671    | Coenzyme F390 synthetase                                                      | 1.21            |
| Cofactors, Prosthetic Groups, Carriers Biosynthesis—Others | Xf2269 | DRB0080  | 3-alpha-hydroxysteroid dehydrogenase                                          | −0.93           |
| Fatty Acid and Phosphatidic Acid Biosynthesis          | Xf0572     | fabA      | Beta-hydroxydecanoyl-ACP dehydratase                                         | 1.18            |

Macromolecule Metabolism

DNA metabolism—Replication | Xf0001 | dnaA | Chromosomal replication initiator | −1.02 |
### Table 1: Continued.

| Functional Group                                | ORF Number | Gene Name | Gene Product                                | Log2 (3G10R/PW) |
|-------------------------------------------------|------------|-----------|---------------------------------------------|-----------------|
| DNA metabolism—Recombination                     | Xf0002     | dnaN      | DNA polymerase III, beta chain              | −1.39           |
| DNA metabolism—Repair                            | Xf0002     | dnaN      | DNA polymerase III, beta chain              | −1.15           |
| DNA Metabolism—Restriction, Modification         | Xf0425     | recD      | Exodeoxyribonuclease V alpha chain          | −0.96           |
| DNA Metabolism—Structural DNA Binding Proteins  | Xf0446     | bhh3      | Single-stranded DNA binding protein         | −1.19           |
| RNA Metabolism—Ribosomes—Maturation and Modification | Xf0441   | rim1      | Ribosomal-protein-alanine acetyl transferase | 1.87            |
| RNA Metabolism—Ribosomal Proteins               | Xf1164     | rp/E OR rp5 OR H10790 | 50S ribosomal protein L5 | −0.91           |
| RNA Metabolism—RNA Synthesis, Modification, DNA Transcription | Xf1108 | greA | Transcriptional elongation factor | −1.73           |
| RNA Metabolism—Aminoacyl tRNA Synthetases, tRNA Modification | Xf0428 | proS OR drpA | Tryptophanyl-tRNA synthetase | −1.89           |
| RNA Metabolism—RNA Degradation                  | Xf1505     | rph       | Ribonuclease PH                             | −0.74           |
|                                                  | Xf1041     | rnhB      | Ribonuclease III                            | −1.00           |
## Table 1: Continued.

| Functional Group | ORF Number | Gene Name | Gene Product | Log2 (3G10R/PW) |
|------------------|------------|-----------|--------------|-----------------|
| **Protein Metabolism—Translation and Modification** | | | | |
| Protein Metabolism—Protein Degradation | Xf0453 | hflC OR HI0150 | Integral membrane proteinase | 1.65 |
| | Xf2241 | mucD | Periplasmic protease | −0.87 |
| | Xf1479 | ptrB OR tlp | Peptidase | −0.82 |
| | Xf2330 | spfD | Proteinase | −0.85 |
| **Cell Structure** | | | | |
| Murein Sacculus, Peptidoglycan | Xf0799 | ddiB OR ddi | D-Alanine-D-alanine ligase B | −1.69 |
| | Xf0276 | mpl | UDP-N-acetylmuramate-L-alanine ligase | −0.88 |
| **Surface Structures** | | | | |
| | Xf0487 | | Fimbrillin | 1.07 |
| | Xf2539 | | Fimbrial protein | −1.02 |
| | Xf2544 | pilB | Pilus biogenesis protein | −0.79 |
| **Chemotaxis and Mobility—Surface Polysaccharides, Lipopolysaccharides and Antigens** | | | | |
| | Xf1289 | kdsA | 2-dehydro-3-deoxy phosphoocotionate aldolase | −0.90 |
| | Xf1419 | lpxD OR firA OR omisA | Acetyltransferase | 1.05 |
| | Xf1646 | lpxD OR firA | UDP-3-O-(R-3-hydroxy myristoyl)-glucosamine N-acyltransferase | −0.75 |
| | Xf1638 | | Dolichyl-phosphate mannose synthase related protein | −1.02 |
| | Xf0879 | rbpU | Lipopolysaccharide biosynthesis protein | −0.74 |
| | Xf2154 | opsX | Saccharide biosynthesis regulatory protein | −1.00 |
| | Xf0105 | kdsA OR waaA | 3-deoxy-D-manno-octulosonic acid transferase | 1.50 |
| **Membrane Components—Outer Membrane Constituents** | Xf1024 | | Outer membrane protein H.8 precursor | −1.19 |
| **Cellular Processes** | | | | |
| Transport—Cations | | | | |
| | Xf1903 | kup OR trkD | Potassium uptake protein | 1.01 |
| | Xf1903 | kup OR trkD | Potassium uptake protein | 1.40 |
| | Xf0599 | ybiL | TONB-dependent receptor for iron transport | 1.46 |
| | Xf0395 | bfr | Bacterioferritin | −1.22 |
| Transport—Amino Acids, Amines | Xf1937 | gltP | Proton glutamate symport protein | −1.00 |
| Transport—Protein, Peptide Secretion | Xf2685 | sppA | Protease IV | −0.88 |
| | Xf2261 | HI0561 560 | Oligopeptide transporter | −1.12 |
| Transport—Carbohydrates, Organic Acids, Alcohols | Xf0976 | dctA | C4-dicarboxylate transport protein | −1.10 |
| Functional Group                  | ORF Number | Gene Number | Gene Name | Gene Product                                      | Log2 (3G10R/PW) |
|----------------------------------|------------|-------------|-----------|---------------------------------------------------|-----------------|
| Cell Division                    | Xf2281     | DR0012      |           | Chromosome partitioning protein                   | −1.08           |
| Other                            | Xf2251     | ppa         |           | Solute Na+ symporter                              | −1.64           |
|                                  | Xf1728     | F451        |           | Transport protein                                 | 1.11            |
|                                  | Xf1604     | btuE        |           | ABC transporter vitamin B12 uptake permease       | −1.48           |
|                                  | Xf1409     | H1148       |           | ABC transporter ATP-binding protein               | 0.84            |
| Mobile Genetic Elements          |            |             |           |                                                   |                 |
| Transposon- and Intron-Related Functions | Xf1775 | IS629       |           | Reverse transcriptase                             | 1.06            |
|                                  | Xf0535     |             |           | Transposase ORF                                  | −0.80           |
| Phage-Related Functions and Prophages | Xf2522 |           |           | Phage-related protein                             | 1.52            |
|                                  | Xf2522     | trbI        |           | Phage-related protein                             | 1.02            |
|                                  | Xfa0040    |             |           | Conjugal transfer protein                         | −0.98           |
|                                  | Xf2291     |             |           | Phage-related protein                             | 0.95            |
|                                  | Xf0513     | lycV        |           | Phage-related endolysin                           | −1.52           |
|                                  | Xf1786     |             |           | Phage-related protein                             | 1.32            |
|                                  | Xf1706     | GP37        |           | Phage-related tail fiber protein                  | 1.31            |
|                                  | Xf0685     |             |           | Phage-related protein                             | 0.86            |
|                                  | Xf0704     |             |           | Phage-related protein                             | 1.18            |
|                                  | Xf1875     |             |           | Phage-related protein                             | 1.44            |
| Plasmid-Related Functions        | Xfa0006    | traA OR virB3 |           | Conjugal transfer protein                         | −1.13           |
|                                  | Xfa0013    | traAO OR virB9 |           | Conjugal transfer protein                         | −1.37           |
|                                  | Xfa0008    | traAC OR virB5 |           | Conjugal transfer protein                         | −1.54           |
| Pathogenicity, Virulence, and Adaptation |            |             |           |                                                   |                 |
| Toxin production and detoxification | Xf0262 | cvaC        |           | Colicin V precursor                               | 7.29            |
|                                  | Xf0263     | cvaC        |           | Colicin V precursor                               | 1.70            |
|                                  | Xf1011     | frpC        |           | Hemolysin-type calcium binding protein            | −1.45           |
|                                  | Xf1827     | ohr         |           | Organic hydroperoxide resistance protein          | −1.43           |
|                                  | Xf2614     | sodA OR sod |           | Superoxide dismutase (MN)                         | −1.47           |
|                                  | Xf1210     | gst OR H1011 |           | Glutathione S-transferase                         | −1.00           |
|                                  | Xf1890     | gpo         |           | Glutathione peroxidase-like protein               | 0.86            |
|                                  | Xf2135     | frnE        |           | Polyketide synthase (PKS)                         | 1.80            |
|                                  | Xf1897     | tolB        |           | TOLB protein precursor                            | −1.30           |
|                                  | Xf1729     | DR1890      |           | Phenylacetaldehyde dehydrogenase                  | 0.91            |
| Host Cell Wall Degradation       | Xf0818     | engXCA      |           | Endo-1,4-beta-glucanase                           | −0.89           |
| Adaptation Atypical Condition    | Xf2682     | mdoG        |           | Periplasmic glucan biosynthesis protein           | −0.80           |
|                                  | Xf2622     | tapB        |           | Temperature acclimation protein B                 | −1.30           |
| Surface Proteins                 | Xf1516     | uspA1       |           | Surface-exposed outer membrane protein            | −1.28           |
| Exopolysaccharides               | Xf2360     | gumM        |           | Gumm protein                                     | −1.08           |
Table 1: Continued.

| Functional Group | ORF Number | Gene Name | Gene Product | Log2 (3G10R/PW) |
|------------------|------------|-----------|--------------|-----------------|
| Other            | Xf1529     | *hsf*     | Surface protein | 1.96          |
|                  | Xf1532     | *oxyR*    | Oxidative stress transcriptional regulator | 0.96          |
|                  | Xf2121     | *vapE*    | Virulence-associated protein E | 1.24          |
|                  | Xf1987     | *vacB*    | VACB protein | −1.35          |
|                  | Xf1114     | *rpfC*    | Regulator of pathogenicity factors | −0.87          |
| ORFs with Undefined Category |          |           |              |                |
|                  | Xf1723     | *yrpG*    | Sugar-phosphate dehydrogenase | 1.30          |
|                  | Xf0088     | *hflX*    | GTP-binding protein | 1.36          |
| Hypothetical Proteins |          |           |              |                |
|                  | Xf1287     |            | Hypothetical protein | 1.40          |
|                  | Xf0493     |            | Hypothetical protein | 0.94          |
|                  | Xf0037     |            | Hypothetical protein | −1.11         |
|                  | Xf1655     |            | Hypothetical protein | 0.82          |
|                  | Xf0726     |            | Hypothetical protein | −1.17         |
|                  | Xf1835     |            | Hypothetical protein | −0.85         |
|                  | Xfa0031    |            | Hypothetical protein | −1.60         |
|                  | Xf2413     |            | Hypothetical protein | 0.96          |
|                  | Xf0871     |            | Hypothetical protein | 1.69          |
|                  | Xf2454     |            | Hypothetical protein | −0.97         |
|                  | Xf1769     |            | Hypothetical protein | −0.80         |
|                  | Xf1803     |            | Hypothetical protein | −2.00         |
|                  | Xf0512     |            | Hypothetical protein | −0.93         |
|                  | Xf0531     |            | Hypothetical protein | −1.72         |
|                  | Xf1868     |            | Hypothetical protein | 1.11          |
|                  | Xf1881     |            | Hypothetical protein | 1.18          |
|                  | Xf0917     |            | Hypothetical protein | 1.25          |
|                  | Xf1738     |            | Hypothetical protein | 1.37          |
|                  | Xf0242     |            | Hypothetical protein | 1.27          |
|                  | Xf1228     |            | Hypothetical protein | 1.01          |
|                  | Xf1279     |            | Hypothetical protein | 1.11          |
|                  | Xf1575     |            | Hypothetical protein | 1.14          |
|                  | Xf2597     |            | Hypothetical protein | −0.94         |
|                  | Xf0516     |            | Hypothetical protein | 1.16          |
|                  | Xf2017     |            | Hypothetical protein | −1.51         |
|                  | Xf1989     |            | Hypothetical protein | −0.94         |
|                  | Xf2410     |            | Hypothetical protein | −1.60         |
|                  | Xf2304     |            | Hypothetical protein | −1.26         |
|                  | Xf0959     |            | Hypothetical protein | 1.24          |
|                  | Xf2115     |            | Hypothetical protein | 1.23          |
|                  | Xf1100     |            | Hypothetical protein | 1.04          |
|                  | Xf1704     |            | Hypothetical protein | 0.95          |
|                  | Xf0974     |            | Hypothetical protein | −1.26         |
|                  | Xf0491     |            | Hypothetical protein | 1.31          |
|                  | Xf1060     |            | Hypothetical protein | 1.77          |
|                  | Xf2151     |            | Hypothetical protein | 1.73          |
| Functional Group | ORF Number | Gene Name      | Gene Product         | Log2 (3G10R/PW) |
|------------------|------------|----------------|----------------------|-----------------|
|                  | Xf2449     | Hypothetical protein | −1.01               |                 |
|                  | Xf2305     | Hypothetical protein | −0.77               |                 |
|                  | Xf1721     | Hypothetical protein | 1.14                |                 |
|                  | Xf0626     | Hypothetical protein | −1.39               |                 |
|                  | Xf2411     | Hypothetical protein | 1.01                |                 |
|                  | Xf1770     | Hypothetical protein | −0.87               |                 |
|                  | Xf1364     | Hypothetical protein | −0.85               |                 |
|                  | Xf1710     | Hypothetical protein | 0.90                |                 |
|                  | Xf1761     | Hypothetical protein | 1.44                |                 |
|                  | Xf1787     | Hypothetical protein | 1.38                |                 |
|                  | Xf0540     | Hypothetical protein | −1.30               |                 |
|                  | Xf1788     | Hypothetical protein | 1.06                |                 |
|                  | Xf0646     | Hypothetical protein | 1.03                |                 |
|                  | Xf2543     | Hypothetical protein | −0.98               |                 |
|                  | Xf0914     | Hypothetical protein | −1.33               |                 |
|                  | Xf2702     | Hypothetical protein | −1.52               |                 |
|                  | Xf0492     | Hypothetical protein | 1.55                |                 |
|                  | Xf1239     | Hypothetical protein | 1.01                |                 |
|                  | Xf0074     | Hypothetical protein | −1.07               |                 |
|                  | Xfa0004    | Hypothetical protein | −1.78               |                 |
|                  | Xf1687     | Hypothetical protein | 1.32                |                 |
|                  | Xf0388     | Hypothetical protein | −0.86               |                 |
|                  | Xf0025     | Hypothetical protein | −1.23               |                 |
|                  | Xf1434     | Hypothetical protein | −1.24               |                 |
|                  | Xf2125     | Hypothetical protein | 0.89                |                 |
|                  | Xf1513     | Hypothetical protein | 1.18                |                 |
|                  | Xf2711     | Hypothetical protein | 1.23                |                 |
|                  | Xf0035     | Hypothetical protein | 1.31                |                 |
|                  | Xf1441     | Hypothetical protein | −1.41               |                 |
|                  | Xf2514     | Hypothetical protein | 1.71                |                 |
|                  | Xf2626     | Hypothetical protein | 1.44                |                 |
|                  | Xf0687     | Hypothetical protein | 1.07                |                 |
|                  | Xf1917     | Hypothetical protein | 1.90                |                 |
|                  | Xf2271     | Hypothetical protein | 1.50                |                 |
|                  | Xf1036     | Hypothetical protein | −0.99               |                 |
|                  | Xfa0017    | Hypothetical protein | −1.98               |                 |
|                  | Xf0529     | Hypothetical protein | 1.09                |                 |
|                  | Xf2103     | Hypothetical protein | −1.05               |                 |
|                  | Xf1986     | Hypothetical protein | −1.05               |                 |
|                  | Xf1700     | Hypothetical protein | 1.12                |                 |
|                  | Xf1719     | Hypothetical protein | 1.08                |                 |
|                  | Xf1753     | Hypothetical protein | 1.44                |                 |
|                  | Xf0019     | Hypothetical protein | 0.85                |                 |
|                  | Xf0293     | Hypothetical protein | −1.15               |                 |
|                  | Xf0300     | Hypothetical protein | 1.67                |                 |
|                  | Xf0279     | Hypothetical protein | 1.79                |                 |
|                  | Xf0735     | Hypothetical protein | −0.94               |                 |
|                  | Xf1010     | Hypothetical protein | −0.97               |                 |
| Functional Group | ORF Number | Gene Name | Gene Product          | Log2 (3G10R/PW) |
|------------------|------------|-----------|-----------------------|-----------------|
|                  | Xf1580     |           | Hypothetical protein   | 0.80            |
|                  | Xf2021     |           | Hypothetical protein   | 1.21            |
|                  | Xf2738     |           | Hypothetical protein   | 1.49            |
|                  | Xf0877     |           | Hypothetical protein   | 1.28            |
|                  | Xf2270     |           | Hypothetical protein   | 1.13            |
|                  | Xf0488     |           | Hypothetical protein   | 1.50            |
|                  | Xf0264     |           | Hypothetical protein   | 4.10            |
|                  | Xf2701     |           | Hypothetical protein   | −1.68           |
|                  | Xf2768     |           | Hypothetical protein   | 1.35            |
|                  | Xf0688     |           | Hypothetical protein   | 0.96            |
|                  | Xf0898     |           | Hypothetical protein   | 1.15            |
|                  | Xf0426     |           | Hypothetical protein   | −1.23           |
|                  | Xf0443     |           | Hypothetical protein   | −1.06           |
|                  | Xf1421     |           | Hypothetical protein   | −1.40           |
|                  | Xf2193     |           | Hypothetical protein   | −2.17           |
|                  | Xf2390     |           | Hypothetical protein   | 1.24            |
|                  | Xf1128     |           | Hypothetical protein   | −1.16           |
|                  | Xf2116     |           | Hypothetical protein   | 1.52            |
|                  | Xf0467     |           | Hypothetical protein   | −1.18           |
|                  | Xf1193     |           | Hypothetical protein   | −0.80           |
|                  | Xf1032     |           | Hypothetical protein   | −1.33           |
|                  | Xf2262     |           | Hypothetical protein   | −1.60           |

Conserved Hypothetical Proteins

|                  | Xfa0045    |           | Conserved hypothetical protein | −2.22 |
|                  | Xf2450     |           | Conserved hypothetical protein | −1.22 |
|                  | Xf2609     |           | Conserved hypothetical protein | −0.87 |
|                  | Xf1754     |           | Conserved hypothetical protein | 1.83  |
|                  | Xf0805     |           | Conserved hypothetical protein | −0.81 |
|                  | Xf2493     |           | Conserved hypothetical protein | 1.13  |
|                  | Xf2088     |           | Conserved hypothetical protein | 1.26  |
|                  | Xf0196     |           | Conserved hypothetical protein | −1.95 |
|                  | Xf1750     |           | Conserved hypothetical protein | 1.36  |
|                  | Xf1745     |           | Conserved hypothetical protein | 1.24  |
|                  | Xf2647     |           | Conserved hypothetical protein | 1.13  |
|                  | Xf2252     |           | Conserved hypothetical protein | −2.81 |
|                  | Xf2010     |           | Conserved hypothetical protein | −1.06 |
|                  | Xf2237     |           | Conserved hypothetical protein | −0.85 |
Table 1: Continued.

| Functional Group | ORF Number | Gene Name | Gene Product | Log2 (3G10R/PW) |
|------------------|------------|-----------|--------------|-----------------|
|                  |            |           | Conserved hypothetical protein | −1.06 |
|                  | Xfa0032    | SCJ21.16  | Conserved hypothetical protein | −1.40 |
|                  | Xf0758     | yjeE      | Conserved hypothetical protein | 0.98  |
|                  | Xf0407     | yceW      | Conserved hypothetical protein | −0.92 |
|                  | Xf0552     | yraL      | Conserved hypothetical protein | −1.19 |
|                  | Xf2651     | ycbY      | Conserved hypothetical protein | −0.86 |
|                  | Xf2575     | DR0386    | Conserved hypothetical protein | −1.78 |
|                  | Xf0363     | yiaD      | Conserved hypothetical protein | 1.10  |
|                  | Xf0066     | yibK      | Conserved hypothetical protein | 1.29  |
|                  | Xf2179     | ybeN      | Conserved hypothetical protein | −1.14 |
|                  | Xf2153     | HI0260.1  | Conserved hypothetical protein | −1.29 |
|                  | Xf0553     | HI1655    | Conserved hypothetical protein | 1.14  |
|                  | Xf2014     | DR0566    | Conserved hypothetical protein | 1.15  |
|                  | Xf0139     | yjeP      | Conserved hypothetical protein | −0.79 |
|                  | Xf2474     | yjeK      | Conserved hypothetical protein | −1.93 |
|                  | Xf0339     | btuB OR yhe OR cer | Conserved hypothetical protein | −0.91 |
|                  | Xf1272     | RV1827 OR MTCY1A11.16C | Conserved hypothetical protein | −1.02 |
|                  | Xf1405     | yhbJ      | Conserved hypothetical protein | −0.88 |
|                  | Xf1808     | ybaB      | Conserved hypothetical protein | −1.19 |
|                  | Xf1829     | RP471     | Conserved hypothetical protein | −1.05 |
|                  | Xf0941     | yuxK      | 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
genes associated with adaptation to atypical conditions (such as the temperature acclimatation protein TAPB (ORF Xf2622) and the oxidative stress transcriptional regulator OxyR (ORF Xf1532)); surface proteins (including adhesion factors, such as the outer membrane protein Hsf (ORF Xf1529)), and genes involved in toxin production and/or detoxification (such as the colicin precursors encoded by ORFs Xf0262 and Xf0263), among others (see Table 1 for details).

The lack of amino acids in 3G10R also seems to lead to overexpression of at least four genes directly involved in the biosynthesis of such molecules (represented by ORFs Xf0220, Xf0262, Xf1121, Xf2223 and Xf2272). 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 Xf2241), PtpB (ORF Xf1479) and PepQ peptidase (ORF Xf0220), 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 dnaA (the chromosomal replication initiator, encoded by ORF Xf0001), dnaN (the β chain of DNA polymerase III, encoded by ORF Xf0002), recD (the alpha chain of exodeoxyribonuclease V, encoded by ORF Xf0425), ruvB (a Holiday junction-associated helicase, encoded by ORF Xf1902) and ung (an uracil-DNA glycosilase, encoded by ORF Xf2692).

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 (Xf0303) (glycolytic pathway); succinyl-coA synthase (Xf2548) and fumurate hydratase C (Xf1554) (Krebs cycle), as well as genes from the nus operon (represented by ORFs Xf0308, Xf0310, Xf0311 and Xf0317, 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 O2 consumption in PW-grown 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.
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 Lactococcus 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 cyaBD 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 (Xf0303) 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, glycaldehyde 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 Xf1061 to Xf1065, 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 μM concentration in the xylem of grapevines (a typical X. fastidiosa host) [16]. Thus, the ~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 xanA (ORF Xf0260), 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 Lactococcus 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 Xf0487, which encodes a 20 kDa fimbrillin subunit of bacterial fimbrae, and may be involved in bacterial adherence and invasion [53]. Pili and fimbrae 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 Xf1529), 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 (Xf0262, Xf0263 and Xf0264) 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 Xf0263 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 Xf0262 and Xf0264 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.

References

[1] J. M. Wells, B. C. Raju, H. Y. Hung, W. G. Weisburg, L. Mandelco-Paul, and D. J. Brenner, “Xylella fastidiosa gen nov sp nov: gram-negative, xylem-limited, fastidious plant bacteria related to Xanthomonas spp.,” International Journal of Systematic Bacteriology, vol. 37, pp. 136–143, 1987.
[2] D. L. Hopkins, “Xylella fastidiosa: xylem-limited bacterial pathogen of plants,” Annual Review of Phytopathology, pp. 271–290, 1989.
[3] A. H. Purcell and D. L. Hopkins, “Fastidious xylem-limited bacterial plant pathogens,” Annual Review of Phytopathology, vol. 34, pp. 131–151, 1996.
[4] S. Chatterjee, R. P. P. Almeida, and S. Lindow, “Living in two worlds: the plant and insect lifestyles of Xylella fastidiosa,” Annual Review of Phytopathology, vol. 46, pp. 243–271, 2008.
[5] V. S. da Silva, C. S. Shida, F. B. Rodrigues, et al., “Comparative genomic characterization of citrus-associated Xylella fastidiosa strains,” BMC Genomics, vol. 8, article 474, 2007.
[6] L. M. Moreira, R. F. de Souza, N. F. Almeida Jr., et al., “Comparative genomics analyses of citrus-associated bacteria,” Annual Review of Phytopathology, vol. 42, pp. 163–184, 2004.
[7] A. J. Simpson, F. C. Reinach, P. Arruda, et al., “The genome sequence of the plant pathogen Xylella fastidiosa. The Xylella fastidiosa Consortium of the Organization for Nucleotide Sequencing and Analysis,” Nature, vol. 406, no. 6792, pp. 151–159, 2000.
[8] A. Bhattacharyya, S. Stilwagen, N. Ivanova, et al., “Whole-genome comparative analysis of three phytopathogenic Xylella fastidiosa strains,” Proceedings of the National Academy of Sciences of the United States of America, vol. 99, no. 19, pp. 12403–12408, 2002.
[9] M. A. Van Sluys, M. C. de Oliveira, C. B. Monteiro-Vitorello, et al., “Comparative analyses of the complete genome sequences of Pierce’s disease and citrus variegated chlorosis strains of Xylella fastidiosa,” Journal of Bacteriology, vol. 185, no. 3, pp. 1018–1026, 2003.
[10] M. J. Davis, A. H. Purcell, and S. V. Thomson, “Isolation media for the Pierce’s disease bacterium,” Phytopathology, vol. 70, pp. 425–429, 1980.
[11] M. J. Davis, W. J. French, and N. W. Schaad, “Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald,” Current Microbiology, vol. 6, no. 5, pp. 309–314, 1981.
[12] J. S. Hartung, J. Beretta, R. H. Bralnsky, J. Spisso, and R. F. Lee, “Citrus variegated chlorosis bacterium: axenic culture, pathogenicity, and serological relationships with other strains of Xylella fastidiosa,” Phytopathology, vol. 84, no. 6, pp. 591–597, 1994.
[13] J. C. Campanharo, M. V. F. Lemos, and E. G. de Macedo Lemos, “Growth optimization procedures for the phytopathogen Xylella fastidiosa,” Current Microbiology, vol. 46, no. 2, pp. 99–102, 2003.
[14] J. M. Wells, B. C. Raju, G. Nyland, and S. K. Lowe, “Medium for the isolation and growth of the bacteria associated with plum leaf scald and phony peach disease,” Applied and Environmental Microbiology, vol. 42, pp. 357–363, 1981.
[15] M.-A. Selosse, E. Baudoin, and P. Vandenoonhuys, “Symbiotic microorganisms, a key for ecological success and protection of plants,” Comptes Rendus Biologies, vol. 327, no. 7, pp. 639–648, 2004.
[16] B. Leite, P. C. Andersen, and M. L. Ishida, “Colonization and biofilm formation in xylem chemistry-based media for Xylella fastidiosa,” FEMS Microbiology Letters, vol. 230, no. 2, pp. 283–290, 2004.
[17] A. A. de Souza, M. A. Takita, E. O. Pereira, H. D. Coletta-Filho, and M. A. Machado, “Expression of pathogenicity-related genes of Xylella fastidiosa in vitro and in planta,” Current Microbiology, vol. 50, no. 4, pp. 223–228, 2005.
[18] R. Costa de Oliveira, G. M. Yanai, N. H. Muto, et al., “Competitive hybridization on spotted microarrays as a tool to conduct comparative genomic analyses of Xylella fastidiosa strains,” FEMS Microbiology Letters, vol. 216, no. 1, pp. 15–21, 2002.
[19] L. R. Nunes, Y. B. Rosato, N. H. Muto, et al., “Microarray analyses of Xylella fastidiosa provide evidence of coordinated transcription control of laterally transferred elements,” Genome Research, vol. 13, no. 4, pp. 570–578, 2003.
[20] V. G. Tusher, R. Tibshirani, and G. Chu, “Significance analysis of microarrays applied to the ionizing radiation response,” Proceedings of the National Academy of Sciences of the United States of America, vol. 98, no. 9, pp. 5116–5121, 2001.
[21] A. I. Saeed, N. K. Bhagabati, J. C. Braisted, et al., “TM4 microarray software suite,” Methods in Enzymology, vol. 411, pp. 134–193, 2006.
[22] P. C. Andersen, B. V. Brodbeck, and R. F. Mizell, “Water stress and nutrient solution-mediated changes in water relations and amino acids, organic acids, and sugars in xylem fluid of Prunus salicina and Lagerstroemia indica,” Journal of the American Society for Horticultural Science, vol. 120, no. 1, pp. 36–42, 1995.
[23] J. Kreuzwieser, M. Graus, A. Wisthaler, A. Hansel, H. Rennenberg, and J.-P. Schnitzler, “Xylem-transported glucose as
an additional carbon source for leaf isoprene formation in *Quercus robur*, New Phytologist, vol. 156, no. 2, pp. 171–178, 2002.

[24] S. Mayrhofer, U. Heizmann, E. Magel, et al., "Carbon balance in leaves of young poplar trees," Plant Biology, vol. 6, no. 6, pp. 730–739, 2004.

[25] P. C. Andersen and B. V. Brodbeck, "Diurnal and temporal changes in the chemical profile of xylem exudates from *Vitis rotundifolia*," Physiologia Plantarum, vol. 75, pp. 63–70, 1989.

[26] P. C. Andersen and B. V. Brodbeck, "Influence of fertilization on xylem fluid chemistry of *Vitis rotundifolia* Noble and *Vitis* hybrid Suwannee," American Journal of Enology and Viticulture, vol. 42, pp. 245–251, 1991.

[27] P. A. Zaini, L. De La Fuente, H. C. Hoch, and T. J. Burr, "Grapevine xylem sap enhances biofilm development by *Xylella fastidiosa*," FEMS Microbiology Letters, vol. 295, no. 1, pp. 129–134, 2009.

[28] F. Morel, J. Frot-Coutaz, D. Aubel, R. Portalier, and D. Atlan, "Characterization of a prolidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 with an unusual regulation of biosynthesis," Microbiology, vol. 145, no. 2, pp. 437–446, 1999.

[29] E. Harry, L. Monahan, and L. Thompson, "Bacterial cell Q. Hua, C. Yang, T. Oshima, H. Mori, and K. Shimizu, "Analysis of gene expression in *Escherichia coli* in response to changes of growth-limiting nutrient in chemostat cultures," Applied and Environmental Microbiology, vol. 70, no. 4, pp. 2354–2366, 2004.

[30] H. T. Tao, C. Bausch, C. Richmond, F. R. Blattner, and T. Conway, "Functional genomics: expression analysis of *Escherichia coli* growing on minimal and rich media," Journal of Bacteriology, vol. 181, no. 20, pp. 6425–6440, 1999.

[31] K. L. Newman, R. P. P. Almeida, A. H. Purcell, and S. E. Lindow, "Use of a green fluorescent stain for the analysis of *Xylella fastidiosa* colonization of *Vitis vinifera*," Applied and Environmental Microbiology, vol. 69, no. 12, pp. 7319–7327, 2003.

[32] E. Alves, C. R. Marucci, J. R. S. Lopes, and B. Leite, "Leaf symptoms on plum, coffee and citrus and the relationship with the extent of xylem vessels colonized by *Xylella fastidiosa*," Journal of Phytopathology, vol. 152, no. 5, pp. 291–297, 2004.

[33] A. K. Sijpesteijn, "Induction of cytochrome formation and stimulation of oxidative dissimilation by hemin in *Streptococcus lactis* and *Leuconostoc mesenteroides*," Antonie van Leeuwenhoek, vol. 36, no. 1, pp. 335–348, 1970.

[34] P. Duwat, S. Sourice, B. Cesselin, et al., "Respiration capacity of the fermenting bacterium *Lactococcus lactis* and its positive effects on growth and survival," Journal of Bacteriology, vol. 183, no. 15, pp. 4509–4516, 2001.

[35] P. Duwat, R. de Oliveira, D. Ehrlich, and S. Boiteux, "Repair of oxidative DNA damage in grampositive bacteria: the *Lactococcus lactis* Fpg protein," Microbiology, vol. 141, no. 2, pp. 411–417, 1995.

[36] P. Gaud, G. Lambertet, S. Poncet, and A. Gruss, "CcpA regulation of aerobic and respiration growth in *Lactococcus lactis*," Molecular Microbiology, vol. 50, no. 1, pp. 183–192, 2003.

[37] A. P. Facicnani, J. A. Ferro, J. M. Piazza Jr., et al., "Carbohydrate metabolism of *Xylella fastidiosa*: detection of glycolytic and pentose phosphate pathway enzymes and cloning and expression of the enolase gene," Genetics and Molecular Biology, vol. 26, no. 2, pp. 203–211, 2003.

[38] E. Guédon, P. Renault, S. D. Ehrlich, and C. Delorme, "Transcriptional pattern of genes coding for the proteolytic system of *Lactococcus lactis* and evidence for coordinated regulation of key enzymes by peptide supply," Journal of Bacteriology, vol. 183, no. 12, pp. 3614–3622, 2001.

[39] C. J. Chang and R. C. Donaldson, "*Xylella fastidiosa*: cultivation in chemically defined medium," Phytopathology, vol. 83, no. 2, pp. 192–194, 1993.

[40] A. Schneider, J. Kreuzwieser, R. Schupp, J. J. Sauter, and H. Rennenberg, "Thiol and amino acid composition of the xylem sap of poplar trees (*Populus canadensis* ‘robusta’)," Canadian Journal of Botany, vol. 72, no. 3, pp. 347–351, 1994.

[41] B. Köstner, R. Schupp, E.-D. Schulze, and H. Rennenberg, "Organic and inorganic sulfur transport in the xylem sap and the sulfur budget of *Picea abies* trees," Tree Physiology, vol. 18, no. 1, pp. 1–9, 1998.

[42] P. Mäkelä, J. E. McLaughlin, and J. S. Boyer, "Imaging and quantifying carbohydrate transport to the developing ovaries of *maize*," Annals of Botany, vol. 96, no. 5, pp. 939–949, 2005.

[43] Y. Onda and K. Ito, "Changes in the composition of xylem sap during development of the spadix of skunk cabbage (*Symlocarpus foetidus*)," Bioscience, Biotechnology and Biochemistry, vol. 69, no. 6, pp. 1156–1161, 2005.

[44] U. Heizmann, J. Kreuzwieser, J.-P. Schnitzler, N. Brüggemann, and H. Rennenberg, "Assimilate transport in the xylem sap of pedunculate oak (*Quercus robur*) saplings," Plant Biology, vol. 3, no. 2, pp. 132–138, 2001.

[45] Y.-W. He, C. Wang, L. Zhou, H. Song, J. M. Dow, and L.-H. Zhang, "Dual signaling functions of the hybrid sensor kinase RpfC of *Xanthomonas campestris* involve either phosphorelay or receiver domain-protein interaction," Journal of Biological Chemistry, vol. 281, no. 44, pp. 33414–33421, 2006.

[46] S.-J. Liaw, H.-C. Lai, and W.-B. Wang, "Modulation of swarming and virulence by fatty acids through the RsbA protein in *Proteus mirabilis*," Infection and Immunity, vol. 72, no. 12, pp. 6836–6845, 2004.

[47] D. Osirio, I. A. Colnago, A. M. M. B. Otoboni, et al., "A kinetic model for *Xylella fastidiosa* adhesion, biofilm formation, and virulence," FEMS Microbiology Letters, vol. 236, no. 2, pp. 313–318, 2004.

[48] A. de Souza, M. A. Coletta-Filho, and T. J. Burr, "Analysis of gene expression in two growth states of *Xylella fastidiosa* and its relationship with pathogenicity," Molecular Plant-Microbe Interactions, vol. 16, no. 10, pp. 867–875, 2003.

[49] J. C. Boels, M. Kleerebezem, and W. M. de Vos, "Engineering of carbon distribution between glycolysis and sugar nucleotide biosynthesis in *Lactococcus lactis*," Applied and Environmental Microbiology, vol. 69, no. 2, pp. 1129–1135, 2003.

[50] W. M. de Vos, "Metabolic engineering of sugar catabolism in lactic acid bacteria," Antonie van Leeuwenhoek, vol. 70, no. 2–4, pp. 223–242, 1996.

[51] A. A. de Souza, M. A. Coletta-Filho, et al., "Gene expression profile of the plant pathogen *Xylella fastidiosa* during biofilm formation in vitro," FEMS Microbiology Letters, vol. 237, no. 2, pp. 341–353, 2004.

[52] E. Nudleman and D. Kaiser, "Pulling together with type IV pili," Journal of Molecular Microbiology and Biotechnology, vol. 7, no. 1–2, pp. 52–62, 2004.

[53] Y. Meng, Y. Li, C. D. Galvani, et al., "Upstream migration of *Escherichia coli* and citrus and the relationship between *Haemophilus III*, "Architecture and adhesive activity of the *Haemophilus pedunculate oak (*Quercus robur*)," New Phytologist, vol. 96, no. 5, pp. 939–949, 2005.

[54] J. Kreuzwieser, "Pulling together with type IV pili," Journal of Molecular Microbiology and Biotechnology, vol. 7, no. 1–2, pp. 52–62, 2004.
...influenzae Hsf adhesin," Journal of Bacteriology, vol. 187, no. 13, pp. 4656–4664, 2005.

[56] J. W. St. Geme III, D. Cutter, and S. J. Barenkamp, “Characterization of the genetic locus encoding Haemophilus influenzae type b surface fibrils,” Journal of Bacteriology, vol. 178, no. 21, pp. 6281–6287, 1996.

[57] J. Muñoz-Rojas, L. E. Fuentes-Ramírez, and J. Caballero-Mellado, “Antagonism among Gluconacetobacter diazotrophicus strains in culture media and in endophytic association,” FEMS Microbiology Ecology, vol. 54, no. 1, pp. 57–66, 2005.

[58] D. Drider, G. Fimland, Y. Héchard, L. M. McMullen, and H. Prévost, “The continuing story of class IIa bacteriocins,” Microbiology and Molecular Biology Reviews, vol. 70, no. 2, pp. 564–582, 2006.

[59] S. Pashalidis, L. M. Moreira, P. A. Zaini, et al., “Whole-genome expression profiling of Xylella fastidiosa in response to growth on glucose,” OMICS, vol. 9, no. 1, pp. 77–90, 2005.

[60] P. C. Andersen, B. V. Brodbeck, S. Oden, A. Shriner, and B. Leite, “Influence of xylem fluid chemistry on planktonic growth, biofilm formation and aggregation of Xylella fastidiosa,” FEMS Microbiology Letters, vol. 274, no. 2, pp. 210–217, 2007.

[61] J. L. Bi, C. K. Dumenyo, R. Hernandez-Martinez, D. A. Cooksey, and N. C. Toscano, “Effect of host plant xylem fluid on growth, aggregation, and attachment of Xylella fastidiosa,” Journal of Chemical Ecology, vol. 33, no. 3, pp. 493–500, 2007.