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Production of DNA Microarray and Expression Analysis of Genes from *Xylella fastidiosa* in Different Culture Media

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ABSTRACT

DNA Microarray was developed to monitor the expression of many genes from Xylella fastidiosa, allowing the side-by-side comparison of two situations in a single experiment. The experiments were performed using X. fastidiosa cells grown in two culture media: BCYE and XDM_2. The primers were synthesized, spotted onto glass slides and the array was hybridized against fluorescently labeled cDNAs. The emitted signals were quantified, normalized and the data were statistically analyzed to verify the differentially expressed genes. According to the data, 104 genes were differentially expressed in XDM_2 and 30 genes in BCYE media. The present study showed that DNA microarray technique efficiently differentiate the expressed genes under different conditions.

Key words: DNA Microarray, *Xylella fastidiosa*, expression, transcriptome

INTRODUCTION

The genome sequence determination of the phytopathogenic bacterium *X. fastidiosa* strain 9a5c was a world landmark. *X. fastidiosa*-9a5c is associated to citrus variegated chlorosis (CVC) in sweet orange trees (Habermann et al., 2006). It has a main chromosome and two plasmids, composing a total of 2,905 genes. Half of these ORFs present similarity to unknown functions proteins (Simpson et al., 2000). Determination of which genes and under what circumstances are actively expressed represent the next great hurdle in understanding the metabolic and replicative characteristics, as well as in settling the pathogenicity mechanisms approaches.

Various methods are available for gene expression levels detection and quantification, including northern blots (Alwine et al., 1977), S1 nuclease protection (Berks and Sharp, 1977), dotblot analysis (Lennon and Lehrach, 1991), differential display (Liang and Pardee, 1992), sequencing of cDNA libraries (Okubo et al., 1992) and serial analysis of gene expression (SAGE) (Velculescu et al., 1995). However, these methods present some drawbacks, which render them unsuitable when a large number of expression products are analyzed simultaneously (van Hal et al., 2000).

DNA microarray consists of thousands of unique DNA sequences, each of them attached to a known location on a small solid surface. Labeled cDNA or DNA bind to the fixed sequences producing an indicative pattern of nucleic acid sequences, which can be qualitatively and semi-quantitatively analyzed by a computer. Such technology has been used to study many bacterial species, including

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Escherichia coli (Tao et al., 1999), *Mycobacterium tuberculosis* (Wilson et al., 1999), *Caulobacter crescentus* (Laub et al., 2000), *Streptococcus pneumoniae* (De Saizieu et al., 2000), *Helicobacter pylori* (Salama et al., 2000), *Bacillus subtilis* (Yoshida et al., 2001), *Klebsiella peneumoniae* (Dong et al., 2001), *Salmonella enterica* (Detweiler et al., 2001), *Shewanella oneidensis* MR-1 (Murray et al., 2001), *Cortnebacterium glutamicum* (Lange et al., 2003), *Pseudomonas aeruginosa* (Wagner et al., 2003) and *X. fastidiosa* (Koide et al., 2004; Travensolo, 2004; Pashalidis et al., 2005). The DNA microarray technique constitutes the most promising and revolutionary ever-developed technique to study differential gene expression. In this work, the DNA microarrays technique was applied to *X. fastidiosa* gene expression analyses under different conditions (XDM_2 and BCYE media).

MATERIALS AND METHODS

Growth conditions

Genomic DNA extraction was carried out with *X. fastidiosa* strain 9a5c cells grown in BCYE solid medium (Wells et al., 1981) at 28 ºC for six days. RNA extraction was conducted in three Erlenmeyer flasks containing the bacterial cells in 30 ml of XDM_2 (Lemos et al., 2003) or BCYE media (Table 1) at 30 ºC in a rotatory shaker (140 rpm) for four days.

Table 1 - Components in BCYE and XDM_2 media, developed for *X. fastidiosa*.

Components	BCYE	XDM_2
Glucose (10 g/l)	-[a]	+[b]
K_2HPO_4 (2.1 g/l)	-	+
KH_2PO_4 (0.8 g/l)	-	+
$MgSO_4$ $7H_2O$ (0.4 g/l)	-	+
Ferric pyrophosphate (0.125 g/l)[c]	+	+
Aces buffer (10 g/l)	+	-
Activated charcoal (2 g/l)	+	-
Yeast extract	+	-
L-cysteine (0.4 g/l)	+	-
L-serine (0.4 mg/ml)	-	+
L-asparagine (1.0 mg/ml)	-	+
L-methionine (0.4 mg/ml)	-	+
L-glutamine (4.0 mg/ml)	-	+
Vitamin stock solution (10 ml/l)[d]	-	+
Biotin (0.2 ml/l)	-	+
Phenol red (0.1%)	-	+

[a](-) components taken in the media, [b](+) components added to the media, [c]in BCYE media, the ferric pyrophosphate concentration was 0.25 g/l and [d]Vitamin stock solution (10 ml/l) contained 0.2 mg $_D$-biotin, 10 mg thiamine, 10 mg pyridoxine hydrochloride, 5.0 mg nicotinic acid, 0.05 mg vitamin B12 and 350 mg myo-inositol.

Isolation of the genomic DNA and total RNA

The genomic DNA was extracted according to the methodology described by Ausubel et al. (1992) with some modifications, which included the addition of 100 µl of RNAse solution (200 µg/ml), previously treated with proteinase K, and incubation at 37 ºC for 1 h. The RNA extraction methodology (Chomczynski and Sacchi, 1987) involved a monophasic solution of phenol and guanidine isothiocyanate - Trizol (Invitrogen, Carlsbad, CA, USA). The RNAs samples were obtained from *X. fastidiosa* cells grown in two different media (XDM_2 and BCYE). The cells were treated with DNAse I, purified with the NucleoSpin® RNA II BD Bioscience kit (Clontech, Mountain View, CA, USA), resuspended in DEPC treated H_2O and stored at – 80 ºC. The DNA integrity was analyzed by electrophoresis in 1.0% (w/v) agarose gel in 1 x TBE buffer (89 mM Tris, 89 mM Boric acid; 2.5 mM EDTA, pH 8.3) containing 0.5 µg/ml ethidium bromide. RNA integrity was verified in a 1.2% (w/v) agarose gel in 10 x buffer (200 mM MOPS; 50 mM sodium acetate; 10 mM EDTA), DEPC-treated H_2O and 6.7% w/v formaldehyde.

Synthesis of the fluorescent cDNA from total RNA

Synthesis of fluorescent cDNA was carried out with a CyScribe cDNA Post Labeling kit (GE Healthcare, Piscataway, NJ, USA) using 30 µg of total RNA, 15 µg of random primers (GE Healthcare, Piscataway, NJ, USA), 1 µl of control synthetic RNA in a final volume of 11 µl. Synthetic RNA from Lucidea Universal ScoreCard kit (GE Healthcare, Piscataway, NJ, USA) was used as the transcriptase reaction control. The mixture was incubated at 70 ºC for 5 min and cooled to room temperature for 10 min, to allow the primers and mRNA template annealing. An aliquot of 9 µl of the following mix was added to each tube: 4 µl 5 x CyScript buffer, 2 µl 0.1 M DTT, 1 µl nucleotide mix, 1 µl AA-dUTP and 1 µl CyScript reverse transcriptase. The reverse transcriptase reaction occurred at 37 ºC for 3 h in a programmable thermocycler (PTC-100 Programmable Thermal Controller - MJ Research, Inc.). The reaction was neutralized with 20 µl of 2 M HEPES. The cDNAs were purified by precipitation with a solution of 3 M sodium acetate and 75 µl of 100% ethanol, followed by storage at – 20 ºC overnight. After centrifuging and washing with 70% (v/v) ethanol, the cDNA was resuspended in 30 µl of CyDye, which was previously diluted in 0.1 M sodium bicarbonate buffer (pH 9.0).

The sample was kept at 25 ºC in the dark for 1 h and the labeling reaction was interrupted by adding 15 µl of 4 M hydroxylamine for 15 min at 25 ºC. The sample was then resuspended in 400 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) and concentrated using a YM 30 Microcon-like column (Millipore, Billerica, MA, USA). The CyDye incorporation efficiency was monitored by absorbance measurements at different wavelengths: 260 nm (for DNA concentration), 550 nm (for Cy3) and 650 nm (for Cy5).

Microarray construction

Specific primer pairs were designed for the whole *X. fastidiosa* genome using the PRIMER3 software. These primer pairs were used to amplify the 2,600 ORFs of *X. fastidiosa*-9a5c genome. The primer pairs were composed by 16 to 19 nucleotides and presented a Tm (melting temperature) ranged from 48 to 57 ºC. PCR reactions were carried out in 1 x PCR buffer (50 mM KCl, 200 mM Tris-HCl, pH 8.4), 2 mM $MgCl_2$, 10 mM dNTP, 2 U Taq DNA polimerase, 5 pMol of each primer, 60 ng of genomic DNA and pure sterile water to complete the volume to 100 µl. The following reaction conditions were used: 94 ºC for 2 min, 35 cycles (94 ºC for 1 min, 58 ºC for 1 min and 72 ºC for 1 min and 30 s) and a final step of 72 ºC for 5 min. All products were analyzed by electrophoresis in 1.5% (w/v) agarose gel in 1 x TBE buffer containing 0.5 µg/ml ethidium bromide.

The amplified products were suspended in 50% (v/v) dimethyl sulfoxide (DMSO) to a final concentration of 100 to 300 ng/µl. The suspension was arranged in duplicate, at 250 µm of distance, on CMT-GAPS™ silane-coated slides (Corning, NY, USA) using an Affimetrix 417 Arrayer (Affymetrix, Santa Clara, CA, USA), according to the manufacturer's instructions. The printed microarrays were re-hydrated (42 ºC for 10 s), dried (70 ºC for 1 min) and fixed in a cross-linked UV camera (1300 x 100 µJ cm^2). The arrays were kept at 70 ºC during 2 h and then stored at room temperature under vacuum. Genetically distant negative controls were also included in this array with human (pHUM1 and pHUM7) and plant genes (707050B11 - Rubisco), as well as synthetic controls from various species, such as: human, mouse, leaven, *Arabidopsis* spp., *Archaeabacteria* and *E. coli*, which were obtained from Lucidea Universal ScoreCard kit (GE Healthcare, Piscataway, NJ, USA).

Hybridization and Washing

Arrays were hybridized and washed in a GeneTac Hybridization Station (Genomic Solutions, Ann Arbor, MI, USA). Initially, the arrays were denatured at 65 ºC for 5 min. A solution containing 8 µl of blocking liquid (GE Healthcare, Piscataway, NJ, USA RPN 3601), 19 µl of SSC 20 x, 5.5 µl of 2% w/v SDS, 100 pMol of each cDNA labeled with the fluorescent dyes Cy3 and Cy5, in a final volume of 110 µl, was heated at 95 °C for 2 min. This solution was injected in the hybridization chamber to cover the arrays. Arrays were hybridized for 12 h at 42 ºC. Afterwards, the slides were washed at 25 ºC with the following solutions: 2 x SSC plus 0.5% (w/v) SDS (high stringency), 0.5 x SSC (medium stringency) and 0.05 x SSC (low stringency). All the washing steps consisted of 10 cycles of solution flow (10 s) and incubation (20 s). The slides were dried for 15 min and submitted to fluorescence detection.

Image acquisition and data analyses

Hybridized arrays were scanned with an Affymetrix 418 Array Scanner (Affymetrix, Santa Clara, CA, USA) at different wavelengths: 550 nm (Cy3) and 650 nm (Cy5). The localization and identity of each spot in the slide were defined in a text file, which was created with the CloneTracker2 program (BioDiscovery, El Segundo, CA, USA). The signal of each spot was quantified with the ImaGene software (v. 4.1, BioDiscovery, El Segundo, CA, USA) where the Cy3 and Cy5 fluorescent dyes images were overlapped and the spots were classified, according to morphology and intensity. Data transformation was carried out by background signal subtraction from each spot signal using the local background, which was obtained by the GeneSight-Lite program (BioDiscovery, El Segundo, CA, USA). The Cy5/Cy3 signals intensities ratio, obtained in BCYE and XDM_2 media, respectively, were normalized according to the total signal intensity average. The replicas within each slide were combined to the average of their values and the statistical analysis was performed using the Significance Analysis of Microarrays (SAM) program (http://www-stat.stanford.edu/~tibs/SAM) (Tusher et al., 2001).

RESULTS AND DISCUSSION

Using DNA microarrays for genomic analysis of *Xylella fastidiosa*

Construction of DNA microarrays

X. fastidiosa-9a5c was used as the model organism for these investigations. Therefore each of the *X. fastidiosa* genome ORFs was represented by 2,600 amplicons, which were synthesized by pairs of primers. The amplifications were considered successful when only one product was visualized, within a size range of 0.3 to 1.0 kb. The final concentration of the amplified ORFs ranged from 100 to 300 ng/µl. The minimum required DNA concentration depends on some factors, such as probe length, base composition and binding capacity of the arrays substrate (Deyholos and Galbraith, 2001). The effect of probe length (0.2 – 1.2 kb) spotted in different concentrations was shown by Heller et al. (1997). The authors concluded that a probe concentration of 125 ng/µl, or higher, affected the signal intensities of probes shorter than 200 bp. If the DNA concentration was lower than 100 ng/µl, loss of sensitivity and underestimation of differential expression degree between samples were observed (Yue et al., 2001). Human (pHUM1 and pHUM7) and plant genes (707050B11-Rubisco), as well as synthetic genes from several species (human, mouse, rat, yeast, plant and bacteria) were also included in this array as negative controls. These controls play an important role in microarray data analysis because they allow signal levels evaluation from nonspecific hybridization. Any spot on the array, which presents a signal not significantly stronger than the one from the negative control, should be scored as absent from the fluorescently labeled target (Holloway et al., 2002a).

The *X. fastidiosa* DNA arrays were made with non-purified amplicons. Studies on microarray gene expression analysis, using non purified amplified products, have shown no significant differences between the purified and non purified PCR products. However low alteration on the hybridization signals (6%) have been observed, when compared to the purified products (Diehl et al., 2001).

The amplicons were printed on silane-coated glass microscope slides by an Affymetrix 417 Arrayer. A fraction of the ring fluid is transferred onto the microscope slide when the pins pass through the sample and push a tiny drop to the surface. This robot is designed to collect samples from 96 or 384-well microtitre plates, with four pins and rings simultaneously. Each ring collects 0.5 - 1.0 µl of probe solution and the pin deposits about 50 pl of material on each slide, making 100 - 150 µm diameter spots. However, the size of the resulting spot is a function of the pin diameter, the pin material, the fluid viscosity, and the dynamics of interaction between the fluid and the surface. For this reason, aqueous solutions deposited on hydrophobic or hydrophilic surfaces spread differently before evaporation (Mace et al., 2000).

The main advance of DNA microarray technology arises from the array small size, which allows higher sensitivity, enables the screening of a larger number of genes and provides the opportunity to use smaller amounts of starting material, compared to conventional techniques (van Hal et al., 2000). Southern et al. (1999) described that in addition to the glass rigidity, its desirable optical properties, low porosity and low inherent fluorescence, help to minimize the hybridization volumes and background signals. However, some of the common problems found in glass slides are due to

the spot morphology and the high background, which may be related to the batch variability (Ye et al., 2001).
In the present experiments, problems of spots shape variation were observed, such as deviations in circle uniformity of nearly 50% of the spots as well as absence of some spots (data not shown). Other problems associated to the irregular spot morphology were pin defects, low humidity throughout the printing and low DNA concentration. As quoted elsewhere, the problem of DNA concentration was corrected by gel analysis in order to quantify the amplified DNA, immediately prior to the printing.
The ideal relative humidity during the printing process should be 50 – 60% since it minimizes the formation of "doughnut" - shaped spots and improves the spot morphology homogeneity (Holloway et al., 2002b). Finally, the change of the pins for new ones had to be solved. The pins were always cleaned at the end of each cycle, allowing the same set of pins to be used in different array without cross-contamination. The hypothesis of pins damage in contact with the glass slide is strongly supported by variations in height between the slides. For this reason, both the pin strength and speed must be corrected in order to avoid its impact on deformation and the spot morphology modification.
Before robotic printing, some geometric tests were performed to determine the best spot distribution on the slides (data not shown). The linear geometry is an excellent standard for the printing of many samples (plates) and their replicas. However, the array geometry is used to control the localization of these replicates and the spaces in the slides, so that a small volume is necessary to the hybridization. In this experiment, the spots were printed in arrays of 4 x 2.
After spotting, the arrays were submitted to a heat treatment for both DNA sequences attaching to the glass surface and denaturation. The spotted amplicons were re-hydrated, dried and fixed in a cross-linked UV camera. The arrays were kept at 70 °C for 2 h and then stored at room temperature under vacuum.
The used slides were coated with gamma-amino propyl silane because it limited the spotted DNA droplet dispersion and enhanced the slides hydrophobicity, also increasing the adherence of the deposited DNA (Duggan et al., 1999). DNA is bound to the substrate through the electrostatic interaction between the silane amine groups. The positively charged primary amines attract the DNA negatively charged phosphodiester backbone (Stillman and Tonkinson, 2001). UV-irradiation enhances and stabilizes such interaction by generating free-radical-mediated coupling of thymidine residues and carbon atoms of the alkyl amine (Holloway et al., 2002a).

Synthesis of fluorescent labeled cDNA

Preparation of fluorescent labeled cDNAs was carried out by total RNA extraction, and its concentration was determined by absorbance measurement at 260 nm (A_{260}). One unit of absorbance at 260 nm corresponds to 40 µg of RNA per ml. The A_{260}/A_{280} ratio provides an approximate purity of RNA, but this approximation is influenced by pH. If RNA is diluted in water, the A_{260}/A_{280} ratio falls between 1.5 – 1.9. On the other hand, if RNA is diluted in 10 mM Tris-HCl buffer (pH 7.5), a value within the range 1.9 – 2.1 is expected (Wilfinger et al., 1997). The quality of RNA is critical factor for microarray analysis. The extraction and purification steps are particularly critical since they can cause RNA contamination, which can mediate significant non-specific binding of fluorescently labeled cDNAs to the slide surface (Duggan et al., 1999). The RNA integrity was checked by formaldehyde agarose gel electrophoresis, where the occurrence of the two ribosomal subunit bands (23S and 16S containing of 2.9 and 1.5 kb, respectively) were examined.
The fluorescent labeled cDNAs were prepared from total *X. fastidiosa* RNA by reverse transcription. Total RNA was used because most of the mRNAs produced by bacteria do not have a poly (A) tail and are difficultly separated. Lucchini et al. (2001) stated that cDNAs, generated by specific primer (genome-directed primers), showed higher sensitivity and specificity than the probes obtained by random priming. However, no satisfactory results was obtained with the synthesized primers for *X. fastidiosa*.
A limitation of this technology is the large amount of RNA required for hybridization. According to Ye et al. (2001), 7 - 15 µg of total RNA in combination with 6 µg of random hexamers generally yielded good labeling efficiency and reasonable signal intensity with Cy5 or Cy3 dyes, on glass slides arrays. Labeling efficiency by reverse transcription depends on the incorporation efficiency and on the amount of specific nucleotides present in a particular mRNA species.

The used labeling kit was developed as a two-step procedure. The first step involves the incorporation of amino allyl-dUTP (AA-dUTP) during cDNA synthesis using an optimized nucleotide mixture. The second step involves the chemically labeled amino allyl-modified cDNA using CyDye NHS-esters. Coupling reactions of amino allyl-modified cDNA were performed separately with Cy3 and Cy5 and both targets were combined in the hybridization solution. The amount of target used for hybridization depends on the array format and the labeling method. Targets containing 100 pMol of incorporated fluorescent dye were employed. Such amount was calculated from the formulas: (OD_{550} x dilution factor x total volume)/0.15 for Cy3 and (OD_{650} x dilution factor x total volume)/0.25 for Cy5, where the obtained values were in pMol (Table 2).

Hybridization conditions and Image Analysis

Hybridization conditions (sample concentration, ionic strength, temperature and time) depend on the fragments size present on the array. Thus, the hybridization and washing protocols used in this study were optimized for *X. fastidiosa*. After hybridization and washing, the arrays were submitted to fluorescence reading in an Affymetrix 418 Array Scanner at different wavelengths: 550 nm and 650 nm. The GMS 418 Array Scanner is a digital confocal laser scanning epifluorescent microscope. Either Cy3 or Cy5 fluorescent-dye tagged to sample on slides can be observed in such microscope. The Affymetrix 418 Array Scanner is capable of exciting and detecting two-channel different wavelengths. Images were obtained optically and then reported in grayscale or as false-colored data to quantify the signal intensity of each spot.

Table 2 - Expected Cy3 and Cy5 measurements using the CyScribe cDNA Post Labeling kit (GE Healthcare).

Target	A_{550}	A_{650}	Expected absorbance[a]	Total yield (pmol) [b]
Cy3 (1) [c]	0.087	-		2,175
Cy3 (2)	0.093	-	0.04 – 0.15	2,325
Cy3 (3)	0.081	-		2,025
Cy5 (1)[d]	-	0.135		2,025
Cy5 (2)	-	0.119	0.04 – 0.15	1,785
Cy5 (3)	-	0.071		1,065

[a]The expected absorbance range is indicated for the probe analysis in the Atlas™ Glass Fluorescent Labeling Kit User Manual (Clontech, Mountain View, CA, USA – www.Clontech.com); [b]The yield was calculated by the values of the measured absorbance (A_{260}) x absorbance of 1 unit at 260 nm x dilution factor. For example, the RNA concentration in XDM_2 (1) was calculated as 1,932 x 40 x 50 = 3,864 µg/µL; [c]CyScribe Cy3 reactive dye incorporated in XDM_2 media cDNA and [d]CyScribe Cy5 reactive dye incorporated in BCYE media cDNA.

Data acquisition and Normalization

There is a variety of software tools developed for array images processing. The main goal is to reduce a spot image of varied intensities into a table with intensity measurements of each spot. The localization and identity of each gene in the slide was defined in a text file created with the CloneTracker2 program. The signal was quantified with the ImaGene software, where two images from the Cy3 and Cy5 fluorescent dyes were overlapped and the spots classified according to morphology and intensity. The computer displays an electronic symbol as a false-color image where a red or green spot corresponds to an expression of a gene in sample 1 or 2, correspondingly, while a yellow-orange spot indicates that the gene has been expressed at similar levels in both samples. Aiming the data transformation, the background signal was subtracted from the signal of each spot using the local background obtained by the GeneSight-Lite program. Data obtained from Cy5/Cy3 measured signal intensities ratio (from BCYE and XDM_2 media, respectively) were normalized according to the average intensity of the total signal.

At least two sources of error are involved in the microarray experiments, such as biological (culture-to-culture variation) and operational ones (variation in the measurements themselves) which affect the measured gene expression levels (Liao and Sabatti, 2002).

The normalization procedure is a suitable approach for minimizing the variations so that a common base for comparison is established. There are a number of reasons that justify data normalization, including unequal quantities of starting RNA, differences of labeling or detection efficiencies between the used fluorescent dyes, and

systematic biases in the measured expression levels (Quackenbush, 2002). However current normalization methods are not applicable to all conditions.
Normalization can be carried out in different ways: within the slide, in order to adjust the dye incorporation efficiency; between two slides, for dye swap experiments; and across slides, for repetition of the same experiments (Yang et al., 2001). In this case, it would be employed to the entire data set (an overall normalization), instead of applying it to particular physical data subset or sub grid (a local normalization). Quackenbush (2002) suggested that the local normalization has the advantage of enabling corrections for systematic spatial variation in the array, including inconsistencies among the spotting pens used to make the array, variability in the slide surface and slight local differences in hybridization conditions across the array.

In this work, the identification of expressed genes in three cDNA microarrays independent experiments was focused. The replicas within and between the slides were combined by the average of their values and the statistical analysis was done using the SAM program. This method assigns a score (*d*) to each gene (*i*) based on the change in gene expression relative to the standard deviation of repeated measurements for that gene. For this purpose, 100 repeated permutations of the data were used in order to determine if the genes expression was significantly related to the response and if the significance cutoff was determined by a delta parameter ($\Delta = 0.49$), which was based on the false significant number (FSN = 0.42). The genes with scores higher than a threshold or fold change ($\geq$ 1.5 x) were deemed potentially significant and the percentage of such genes, occasionally identified, was the false discovery rate (FDR) (Fig. 1).

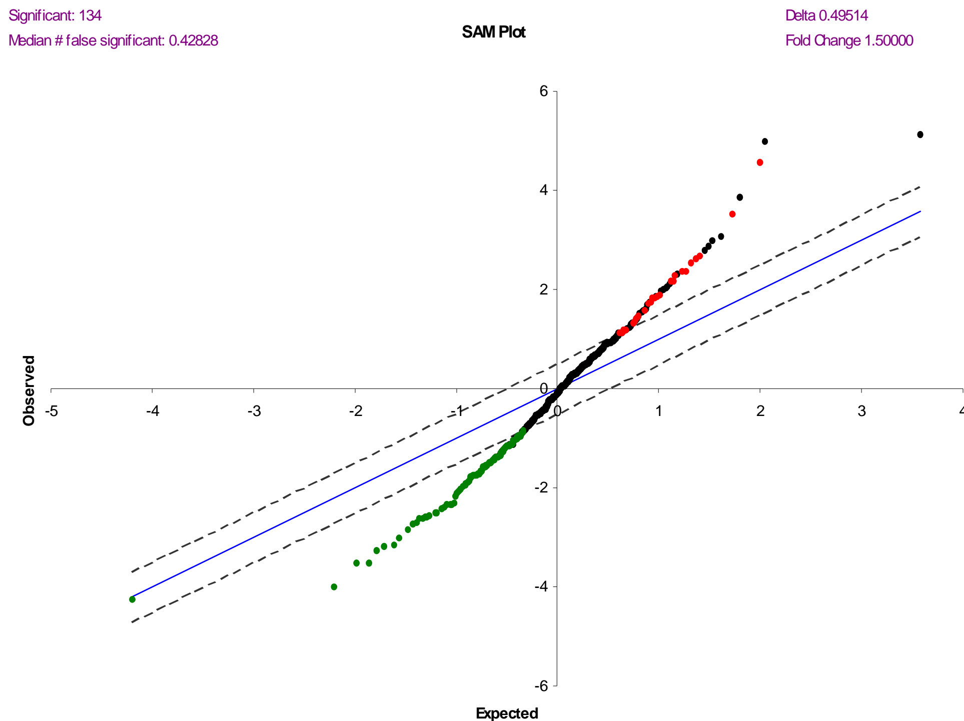

Figure 1 - Identification of genes with significant changes in expression measured by the SAM program. Each gene in the microarray is represented by a point. (A) green points – genes differentially expressed in the XDM_2 medium and (B) red points – genes differentially expressed in the BCYE medium. Total significant genes=134, FSN=0.42, Δ=0.49 and 1.5 x.

Among the analyzed genes, approximately 5.15% (134 genes) were detected as differentially expressed under the studied media conditions. From these, 30 genes (22.4%) showed higher expression in the BCYE medium and 104 (77.6%) in the XDM_2 medium. The differentially expressed genes were distributed among the respective functional categories (http://unicamp.lbi.ic.unicamp.br/xf) (Fig. 2).
According to the results obtained by the microarray analysis, bacteria cultivated in XDM_2 medium expressed a higher number of significant genes than those cultivated in BCYE medium. This was expected since XDM_2 defined medium contained a smaller variety of nutrients than BCYE complex medium. Analysis of the 14 categories revealed that eight of them (57.1 %) showed expressed genes in both the media (Table 3 and 4).

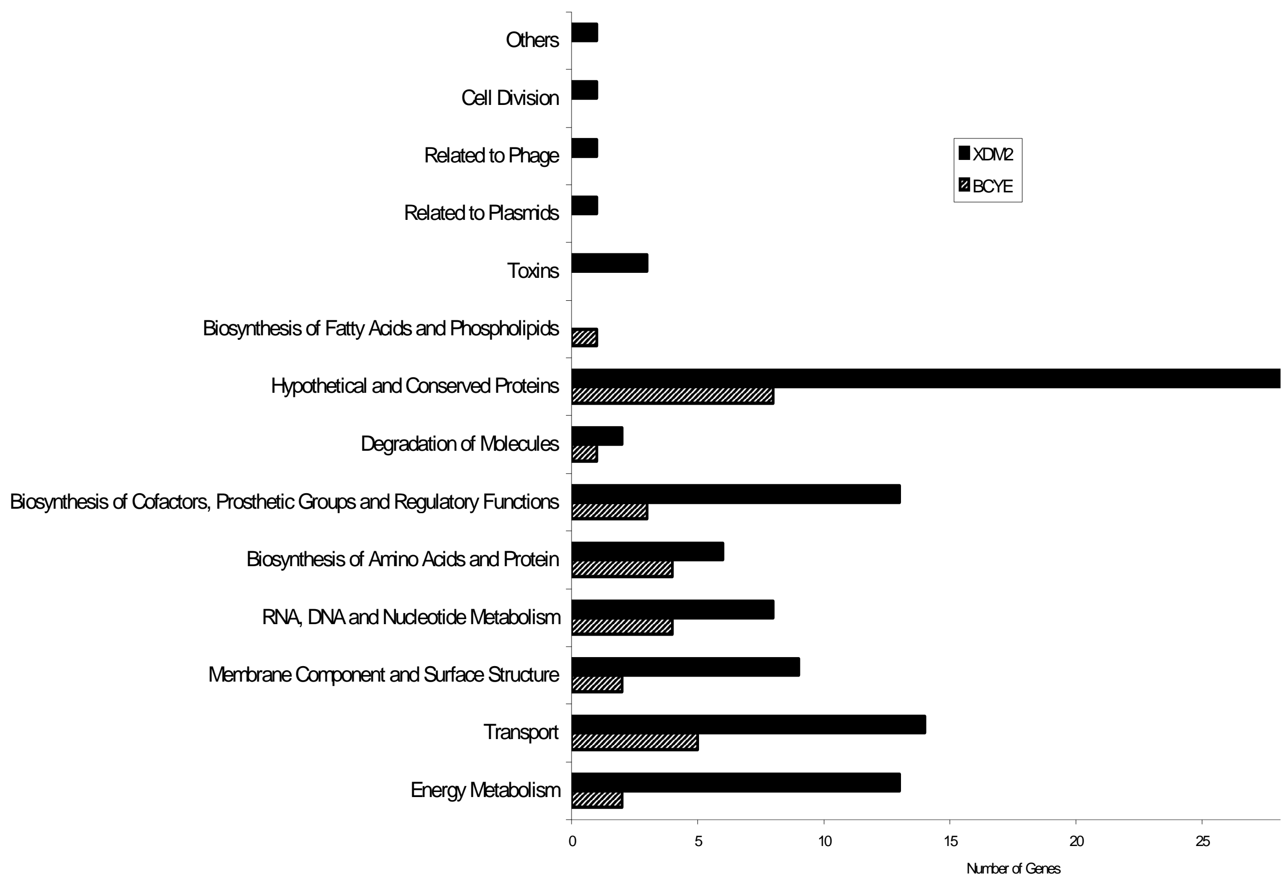

Figure 2 - Functional classes of *Xylella fastidiosa* 9a5c regulated genes. The differentially expressed genes were distributed in the respective functional categories and the number of genes for each category is shown.

Activities such as energy metabolism, transport, RNA, DNA and nucleotide metabolism, biosynthesis of amino acids, proteins and cofactors are considered essential for *X. fastidiosa* survival under any condition. The expression of genes related to the glycolytic pathway (6-phosphofrutokinase, glyceraldehydes-3-phosphate dehydrogenase, triosephosphate isomerase and pyruvate kinase type II) suggest that this pathway is active and that glucose is degraded into pyruvate in *X. fastidiosa* cells.
Others functional categories were observed under particular growth condition. Activities related to toxin production (*frpC*, *gaa* and *cvaB*), plasmids (*taxC*), phage (*int*), cell division (*ftsW*) and others (*bcp*) were only observed in XDM_2 medium. Among these, the depicted toxins belong to the RTX family (gene *frpC*) and the production of

colicin V (gene *cvaB*). The *cvaB* gene from *E. coli*, together with two other genes, *cvaA* and *tolC* mediates the elimination of colicin V (ColV) to the external environment, in order to destroy the target cell by membrane rupture (Zhong and Tai, 1998). On the other hand, the expression of the *fadG* gene can be assigned to the fatty acid biosynthesis. Such expression occurred at elevated levels in BCYE medium. The *E. coli fab* genes presented higher expression levels in the rich media, suggesting that the regulation of the phospholipids biosynthesis genes might be dependent on the growth speed, since they required a higher amount of membrane compounds (Tao et al., 1999). However, it has already been demonstrated that *X. fastidiosa* growth was higher in XDM_2 than in BCYE medium in a 14 days period (Lemos et al., 2003). Therefore *fab* gene expression is possibly mediated by one or more signal molecules found in BCYE medium.

This technology has been used to study the behavior of *X. fastidiosa* genes under different conditions. The study presented here illustrated the best conditions to analyze the expressed genes of this important bacterium with the microarrays technology.

Table 3 - *Xylella fastidiosa* genes induced in XDM_2 media.

Category	Gene name (ORF)
Energy metabolism	*fumB*, *pfkA*, *gapA*, *acnB*, *petB*, *atpG*, *az*1, *tpiA*, *yahK*, *gcvT*, *mdh*, *rfbC*, *odhA*
Transport	f451, *sac*1, *algS*, *citN*, *ccmA*, *malG*, *yecS*, *feoB*, *afuA*, *sppA*, *secY*, *ynhE*, *xpsH*, *yheS*
Membrane components and surface structure	dc14, *murD*, XF0881, *pilY*1, *pilP*, *mreB*, XF2542, *mrkD*, *pilU*
RNA, DNA and nucleotide metabolism	*holA*, *tgt/vacC*, *mutY*, *purE*, *recG*, *holB*, *metG*, SCF 11.04
Biosynthesis of amino acids and proteins	*lysA*, *pspB*, *hisD*, *aroE*, *aroE*, *trpG*
Biosynthesis of cofactors, prosthetic groups and regulatory functions	*bioB*, *panC*, *tctD*, *bioA*, *sspB*, *algR*, *sspA*, *colR*, *ribD*, *folK*, *hemB*, *pilR*, *ribA*
Degradation of molecules	*est*, *lipP*
Toxins	*gaa*, *frpC*, *cvaB*
Related to plasmid	*taxC*
Related to phage	*int*
Cell division	*ftsW*
Others	*bcp*
Hypothetical and conserved proteins	hi0457, dr0620, tm1181, XF1240, XF0172, XF1753, XF2688, XF0358, XF2687, XFa0028, XF0272, XF0201, XF2428, XF1117, XF1086, XF1798, XF2510, XF2400, XF0601, XF2449, XF0638, XF2074, XFa0018, XF2023, XF1881, XF0357, XF1323, XF2647, XF2363, XF2427, XF1854, XF0766

Table 4 - *Xylella fastidiosa* genes induced in BCYE media.

Category	Gene name (ORF)
Energy metabolism	*pdhB*, *pykA*
Transport	*xpsD*, *secA*, *gltP*, *tatD*, dr0830
Membrane components and surface structure	*mopB*, *pilQ*
RNA, DNA and nucleotide metabolism	*ilaIIA*, *gltX*, *purM*, *tyrS*
Biosynthesis of amino acids and proteins	*metA*, *argM*, *dcp*, *lon*
Biosynthesis of cofactors, prosthetic groups and regulatory functions	*thiL*, *phoR*, *hemF*
Degradation of molecules	*dhaA*
Biosynthesis of fatty acids and phospholipids	*fabG*
Hypothetical and conserved proteins	rv2514c, b2520, dr1792, XF0473, XF1620, XF0374, XF2041, XF2734

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RESUMO

DNA Microarray foi desenvolvida para monitorar a expressão de muitos genes de *Xylella fastidiosa*, permitindo a comparação de duas situações distintas em um único experimento. Os experimentos foram feitos utilizando células de *X. fastidiosa* cultivada em dois meios de cultura: BCYE e XDM_2. Pares de oligonucleotídeos iniciadores foram sintetizados, depositados em lâminas de vidro e o arranjo foi hibridizado contra cDNAs marcados fluorescentemente. Os sinais emitidos foram quantificados, normalizados e os dados foram estatisticamente analisados para verificar os genes diferencialmente expressos. De acordo com nossos dados, 104 genes foram diferencialmente expressos para o meio de cultura XDM_2 e 30 genes para o BCYE. No presente estudo, nós demonstramos que a técnica de DNA microarrays eficientemente diferencia genes expressos sob diferentes condições de cultivo.

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