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Global Gene Expression Analysis of the Heat Shock Response in the Phytopathogen *Xylella fastidiosa*

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***Xylella fastidiosa* is a phytopathogenic bacterium that is responsible for diseases in many economically important crops. Although different strains have been studied, little is known about *X. fastidiosa* stress responses. One of the better characterized stress responses in bacteria is the heat shock response, which induces the expression of specific genes to prevent protein misfolding and aggregation and to promote degradation of the irreversibly denatured polypeptides. To investigate *X. fastidiosa* genes involved in the heat shock response, we performed a whole-genome microarray analysis in a time course experiment. Globally, 261 genes were induced (9.7%) and 222 genes were repressed (8.3%). The expression profiles of the differentially expressed genes were grouped, and their expression patterns were validated by quantitative reverse transcription-PCR experiments. We determined the transcription start sites of six heat shock-inducible genes and analyzed their promoter regions, which allowed us to propose a putative consensus for σ^{32} promoters in *Xylella* and to suggest additional genes as putative members of this regulon. Besides the induction of classical heat shock protein genes, we observed the up-regulation of virulence-associated genes such as *vapD* and of genes for hemagglutinins, hemolysin, and xylan-degrading enzymes, which may indicate the importance of heat stress to bacterial pathogenesis. In addition, we observed the repression of genes related to fimbriae, aerobic respiration, and protein biosynthesis and the induction of genes related to the extracytoplasmic stress response and some phage-related genes, revealing the complex network of genes that work together in response to heat shock.**

When a cell is exposed to a sudden temperature upshift, its gene expression program is altered to counteract this environmental perturbation. A characteristic response of the cells to high-temperature exposure is the increase in the rate of synthesis of a class of proteins designated heat shock proteins (Hsps), which act mainly as molecular chaperones mediating the correct folding and assembly of polypeptides or as proteases degrading the irreversibly unfolded proteins. The Hsps are highly conserved among prokaryotic and eukaryotic organisms (27) and have been classified into families according to their molecular weights: Hsp100, Hsp90, Hsp70, Hsp60, Hsp40, Hsp10, and alpha-Hsps or small heat shock proteins (36). Many of these proteins are also induced under various environmental stress conditions such as nutrient starvation and changes in osmolarity or pH of the medium.

The heat shock response has been extensively studied in both gram-negative and gram-positive bacteria. Among the gram-negative bacteria, the best characterized is *Escherichia coli*, where the induction of Hsps is positively regulated by the alternative sigma factor, σ^{32}, encoded by the *rpoH* gene. Regulation of σ^{32} levels is exerted by a feedback loop where the Hsps (DnaK, DnaJ, and GrpE) direct σ^{32} to FtsH-mediated proteolysis at normal temperatures (18). Since the levels of DnaK and DnaJ are limiting in vivo, the increase of aberrant proteins during heat shock sequesters these chaperones, liberating σ^{32} to bind to the RNA polymerase and directing the polymerase to specific heat shock promoters (63). In addition, temperature upshifts rapidly increase translation of σ^{32} by destabilizing the *rpoH* mRNA secondary structure, thereby increasing the ribosomal accessibility to the translation start site (32). Recently, it was demonstrated that GroEL/S chaperone machinery is also involved in the regulation of σ^{32} activity and stability in *E. coli* (21).

With the availability of whole genome sequences, the global response to temperature increase has been studied at the transcriptional level in diverse bacteria, from model organisms such as *E. coli* (45) and *Bacillus subtilis* (22), through the bioremediation-involved bacterium *Shewanella oneidensis* (16), to diverse mammalian pathogens such as *Campylobacter jejuni* (51), group A *Streptococcus* (50), *Mycoplasma pneumoniae* (59), *Yersinia pestis* (33), and *Neisseria meningitidis* (19). These global analyses revealed not only the induction of the classical Hsps but also changes in the expression levels of genes related to other cellular functions, such as flagellum biogenesis, transcriptional regulation, phage-related proteins, membrane composition, and pathogenesis, to cite a few. Several proteomic studies characterizing the heat shock response at the protein level have also been carried out, revealing extensive global responses of bacteria such as *Bradyrhizobium japonicum* (34), *Agrobacterium tumefaciens* (46), and *Myxococcus xanthus* (38). These approaches represent a step towards the comprehension of these organisms as whole biological systems with interconnected pathways responding to an environmental stimulus.

The present work describes a whole-genome transcriptional analysis of the heat shock response in the phytopathogen *Xylella fastidiosa*, the causative agent of diseases in economically important crops, such as citrus variegated chlorosis (CVC) in

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citrus and Pierce disease in grapevines, among others (41). The diseases are broadly distributed on the American continent, causing heavy economic losses in agriculture. The transmission is mediated by xylem-feeding insects, and it is proposed that *X. fastidiosa* affects the host plants by occluding these water-conductive vessels, causing water stress symptoms (41). *X. fastidiosa* is a gram-negative bacterium, a member of the gamma subdivision of *Proteobacteria*, which inhabits the plant xylem, a nutrient-poor environment. Thus, mechanisms to sense and respond to adverse environmental conditions are certainly of extreme importance for bacterial survival in the plant host. The complete genome sequences of different *Xylella* strains have been determined (10, 49, 56), revealing the presence of genes encoding different Hsps as well as the heat shock sigma factor σ^{32}. However, little is known about stress responses and gene regulation in these organisms.

As a first approach to characterize the response to a temperature upshift in the citrus strain *X. fastidiosa* 9a5c, we have performed microarray time course experiments during heat shock at 40°C. In addition to the induction of classical Hsp genes, we have observed the up-regulation of several genes related to pathogenesis and adaptation, as well as the repression of fimbria-related genes and genes involved in energy metabolism, revealing a complex network of genes that work together in response to heat stress in *Xylella*. To our knowledge, this is the first genome-wide study of the heat shock response in a phytopathogenic bacterium.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *X. fastidiosa* strain 9a5c, isolated from CVC-symptomatic *Citrus* (L.) *osbeck* trees, was grown in Periwinkle wilt (13) broth medium at 29°C with rotary agitation at 100 rpm in the dark. The culture was maintained through weekly serial transfers at a 1/10 dilution in Periwinkle wilt medium. Exponential-phase cells (7-day-old cultures) were subjected to heat shock at 40°C, using a water bath incubator with rotary agitation at 150 rpm. Aliquots (50 ml) of cell cultures were taken at each time point (0, 7, 15, 25, and 45 min), and cells were immediately harvested by centrifugation (5,000 $\times$ *g*, 5 min); the culture medium was discarded, and the cells were immediately frozen on dry ice prior to RNA extraction. *Xylella* growth is impaired at 40°C, but the cells are still viable after incubation for 45 min at high temperature (not shown). At least three independent biological replicates of each experiment were performed.

RNA isolation and cDNA labeling. Total RNA was isolated with phenol-chloroform (4), and residual DNA was removed by treatment with 10 U of RQ1 RNase-free DNase I (Promega) and 40 U of the RNase inhibitor RNasin (Promega). The integrity of the RNA was checked through agarose gel electrophoresis, and the lack of residual DNA was checked by PCR. Total RNA (20 μg) was reverse transcribed and labeled using a CyScribe postlabeling kit (GE Healthcare), according to the manufacturer's instructions. Briefly, the RNA was reverse transcribed using the enzyme CyScript in the presence of modified amino allyl-dUTP, an optimized nucleotide mix, buffer, dithiothreitol, and random primers. The resulting amine-modified cDNA was then chemically labeled in the amino allyl group by using CyDye *N*-hydroxysuccinimide esters in 0.1 M sodium bicarbonate (pH 9.0).

Microarray construction and hybridization. Microarray slides containing unique internal PCR amplified fragments of 94.5% of the *Xylella fastidiosa* genome (2,692 genes) spotted at least in duplicate were used for the experiments. Hybridization and washing were performed as previously described (39).

Data acquisition, filtering, and normalization. Microarray slides were scanned with a Generation III DNA scanner (Amersham Biosciences). Fluorescence mean intensity and surrounding median background from each spot were obtained with ArrayVision v6.0 (Imaging Research, Inc). Spots presenting a mean intensity below 2 times the standard deviation of its background in Cy3 and Cy5 simultaneously were eliminated from the subsequent analysis. Saturated signals (intensity of greater than >990 fluorescence units) were also discarded. Normalization was carried out by LOWESS fitting on an *M*-versus-*S* plot, where *M* is the fluorescence log ratio of the heat shock time point relative to the control condition [$M = \log_2(40°C/29°C)$] and *S* is the log mean fluorescence intensity {$S = \log_2[(40°C + 29°C)/2]$} (61). Specifically, for the study of global change in expression levels, dye swap normalization was used. Through dye swap normalization, it is possible to verify if the hypothesis that the majority of the genes do not change holds for the data under analysis instead of assuming this as valid (25). When dealing with dye swap, the normalized *M* is calculated as $M = 1/2\log_2[(cy5/cy3)/(cy5_{swap}/cy3_{swap})] = 1/2\log_2[(k40°C/29°C)/(k29°C/40°C)] = \log_2(40°C/29°C)$, where *cy3* and *cy5* are the fluorescence intensities and *k* is the normalization constant that is canceled out. Since the same quantities of total RNA from cells at 29°C and 40°C were hybridized, global changes in mRNA levels relative to rRNA can be identified, assuming the following conditions: most of total RNA is rRNA, the rRNA remains stable under the experimental conditions used, and the levels of rRNA per cell are roughly the same due to the low growth rate of the bacteria.

Determination of differentially expressed genes. We used intensity-dependent cutoff values for classifying a gene as differentially expressed based on self-self hybridization experiments, as previously described (26, 57). Briefly, the self-self approach consists of hybridizing simultaneously the cDNA from the control sample (29°C) labeled with either Cy3 or Cy5 to estimate the experimental noise. We used credibility intervals of 0.99, a window size of 1.0, and a window step of 0.2. A gene was classified as differentially expressed at a given heat shock time point if at least 80% of its replicates were outside the intensity-dependent cutoff curves, using at least five replicates.

Clustering analysis. Differentially expressed genes presenting the complete time course profile (0, 7, 15, 25, and 45 min) were clustered using the K-means algorithm. To discover the number of groups to be considered, we applied principal-component analysis (PCA) (54). Since the differences between successive PCA components (eingenvalues) go rapidly to near zero after the sixth component (see Fig. S1 at http://blasto.iq.usp.br/~tkoide/Xylella/Heat_shock/), we subdivided the genes in six groups with different expression patterns. A variance-weighted Euclidean distance was used in order to incorporate information for all the replicates (62), using a modified implementation described elsewhere (25). To characterize each group based on functional gene categories, we measured the level of statistical association between "presence in a given group" and "belonging to a functional category," using the Goodman-Kruskal gamma value (*G*) (58). To assess the statistical significance of a given association, we compared it with the association G^* obtained from several randomly simulated lists of genes. We considered that a gene category was overrepresented if the value of the probability $\Pr(G^* > G)$ was smaller than 0.05.

Real-time reverse transcription-PCR (RT-PCR). Primers were designed using the PrimerExpress software (Applied Biosystems). Primer sequences are shown in Table S1 at http://blasto.iq.usp.br/~tkoide/Xylella/Heat_shock/. Five micrograms of total RNA was reverse transcribed using 200 U of SuperScriptII reverse transcriptase (Invitrogen) and 500 ng of random nonamers according to the manufacturer's instructions. A 180-ng amount of the resulting cDNA were used as the template in the PCR, which included 800 nM of the forward and reverse primers and 10 μl of Platinum SYBR Green qPCR SuperMix UDG (Invitrogen). Real-time quantitative PCR was performed in an ABI PRISM 5700 sequence detection system (Applied Biosystems), using default parameters. Assays were performed in triplicate independent time course experiments, using XF2175 (*dnaQ*) to normalize the amount of cDNA per sample (39), since its transcript levels did not change during heat shock in all the microarray experiments performed. The fold change of each gene was calculated using the $2^{-\Delta\Delta CT}$ method (28).

Primer extension assays. Primer extension assays were performed to determine the transcription start site of six genes: XF0381 (*clpB*), XF0616 (*groES*), XF2340 (*dnaK*), XF2341 (*grpE*), XF2234 (*hspA*), and XF2625 (*htpX*). The following oligonucleotides were designed next to the translation start site of each gene and purchased from Invitrogen: XF0381. 5′-GTAAGCTTATCCATCCGC-3′; XF0616, 5′-GATCATGAAGCGGTTTGA-3′; XF2234, 5′-CCAAGGGGTATAACGAAC-3′; XF2340, 5′-AGGTCGATACCAATGATT-3′; XF2341, 5′-CATTCGGGGTGGTCTTGA-3′; and XF2625, 5′-GGCAAACAAGACAATACG-3′. The oligonucleotides were 5′ end labeled with 30μCi of [γ-^{32}P]ATP by using T4 polynucleotide kinase (New England Biolabs) and hybridized to 50 μg of total RNA isolated from cells growing at 29°C or heat shocked at 40°C for 25 min. Annealing was performed at 50°C for 16 h; primer extension and RNA degradation were carried out as previously described (48). Extension products were detected after electrophoresis on denaturing sequencing gels followed by autoradiography, and their sizes were determined by comparison with a DNA sequencing ladder obtained with M13mp18 DNA and the universal primer −40.

In silico search of σ^{32}-dependent promoters. Using experimentally derived information about the σ^{32} putative promoters, we have built probabilistic con-

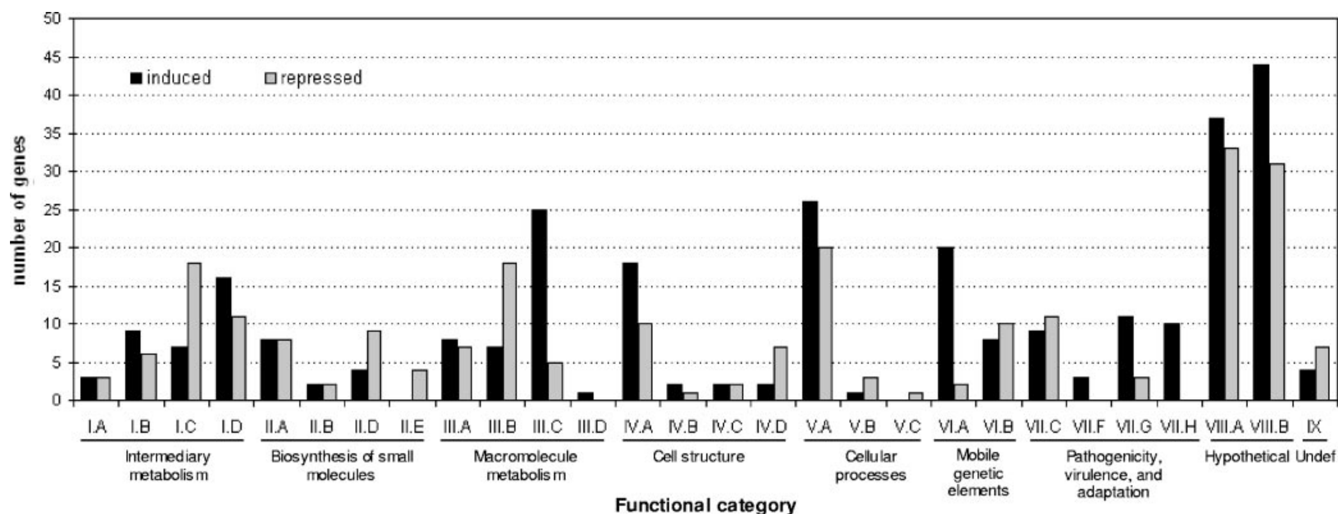


FIG. 1. Differentially expressed genes grouped by functional classification according to the *X. fastidiosa* genome database. Dark bars refer to induced genes, and gray bars refer to repressed genes. Categories: I, intermediary metabolism; I.A, degradation; I.B, central intermediary metabolism; I.C, energy metabolism, carbon; I.D, regulatory functions; II, biosynthesis of small molecules; II.A, amino acid biosynthesis; II.B, nucleotide biosynthesis; II.D, cofactors, prosthetic groups, carrier biosynthesis; II.E, fatty acid and phosphatidic acid biosynthesis; III, macromolecule metabolism; III.A, DNA metabolism; III.B, RNA metabolism; III.C, protein metabolism; III.D, other macromolecule metabolism; IV, cell structure; IV.A, membrane components; IV.B, murein sacculus, peptidoglycan; IV.C, surface polysaccharides, lipopolysaccharides, and antigens; IV.D, surface structures; V, cellular processes; V.A, transport; V.B, cell division; V.C, chemotaxis and mobility; VI, mobile genetic elements; VI.A, phage-related functions and prophages; VI.B, plasmid-related functions; VII, pathogenicity, virulence, and adaptation; VII.C, toxin production and detoxification; VII.F, surface proteins; VII.G, adaptation, atypical conditions; VII.H, other; VIII, hypothetical proteins; VIII.A, conserved hypothetical proteins; III.B, hypothetical proteins; IX, ORFs with undefined category.

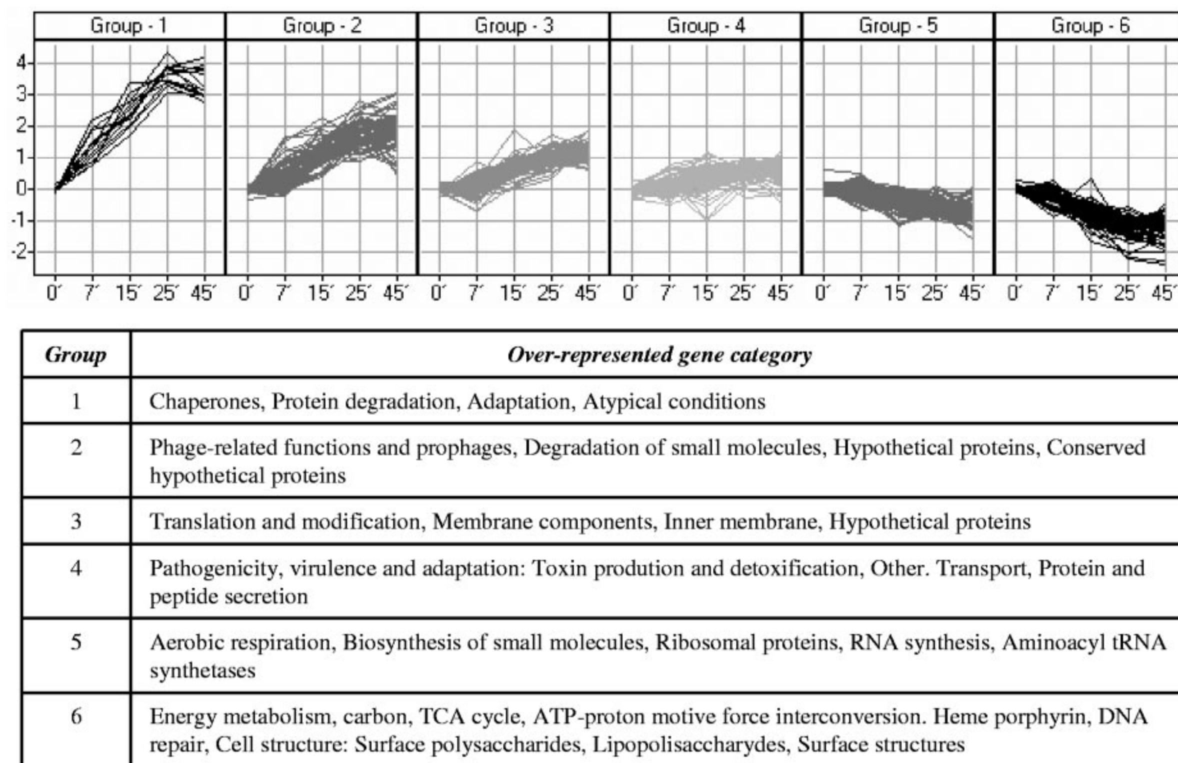
sensus matrices calculating the frequency (percentage) of each nucleotide in the −35 and −10 regions determined in primer extension experiments (see Fig. S2 at http://blasto.iq.usp.br/~tkoide/Xylella/Heat_shock/). The matrices were used separately as input to search for putative σ^{32} promoter sequences in the upstream regions (−200 to −1) of all heat shock-induced genes by using the Patser program with default parameters (55). R scripts were developed to merge Patser output in order to incorporate −35 to −10 spacing (9 to 16 nucleotides), which is freely available at http://blasto.iq.usp.br/~tkoide/Xylella/Heat_shock/. To evaluate the statistical significance of the putative σ^{32} promoter list, we performed the Bayesian analogue of the Fisher's exact test. For that, we calculated the probability *P* that the proportion of putative σ^{32} promoters found among uninduced genes would be higher than the one found among heat shock-induced genes, as a function of the Patser's stringency score. We defined a *P* value of <0.05 as significant, and this *P* value corresponds to a Patser's stringency score cutoff of 0.75 (see Fig. S3 at http://blasto.iq.usp.br/~tkoide/Xylella/Heat_shock/). If more than one promoter sequence was predicted for the same gene, we chose the one presenting the highest Patser mean score.

Microarray data accession numbers. A detailed description of the array can be found in reference 26 and at NCBI's Gene Expression Omnibus (GEO) database (8; http://www.ncbi.nlm.nih.gov/geo) under accession number GPL2708. The complete data set is publicly available according to MIAME guidelines (7) at the GEO database under accession number GSE4161.

RESULTS AND DISCUSSION

Global gene expression analysis. To determine gene expression changes in *X. fastidiosa* citrus strain 9a5c in response to heat shock, we performed time course experiments by transferring the bacterial cells from 29°C to 40°C and extracting samples after different time points to investigate global transcript levels by using DNA microarrays. A particular gene was classified as differentially expressed if at least 80% of the replicates were outside the credibility intervals defined by self-self experiments, using at least five replicates, as described in Materials and Methods.

Globally, about 20% of the genes were significantly up-regulated or down-regulated over a period of 45 min after the temperature upshift: 261 genes were induced (9.7% of the genes), and 222 genes were repressed (8.3% of the genes). The numbers of differentially expressed genes in each of the four heat shock time points (7 min, 15 min, 25 min, and 45 min) were 28, 90, 182, and 166 for the induced genes and 3, 49, 128, and 156 for the repressed ones, respectively. Among differentially expressed genes, 110 originally classified as encoding hypothetical or conserved hypothetical proteins were reannotated using BlastP and Pfam tools (2, 9); 67 genes were assigned a putative function based on electronic annotation, and 43 genes encoding hypothetical proteins were classified as encoding conserved hypothetical proteins. A complete list of the differentially expressed genes is available in Tables S2 and S3 at http://blasto.iq.usp.br/~tkoide/Xylella/Heat_shock/. In addition, an *X. fastidiosa* genome and KEGG metabolic maps, color coded according to the expression status (induction/repression/no change) of each gene during the heat shock time course study, is available at http://blasto.iq.usp.br/~tkoide/Xylella/Heat_shock/. The functional classification of differentially expressed genes according to *X. fastidiosa* genome database is shown in Fig. 1. Many hypothetical and conserved hypothetical genes presented altered expression levels upon heat shock. Among the induced genes, 37 (14.2%) encode conserved hypothetical proteins and 44 (16.8%) encode hypothetical proteins. The repressed genes included 33 encoding conserved hypothetical proteins (14.8%) and 31 encoding hypothetical proteins (13.9%). Although these numbers correspond to approximately 31% of the induced genes and 29% of the repressed ones, they are not higher than those observed in the whole *Xylella* genome, since 51% of the annotated genes are hypothetical.



Group	Over-represented gene category
1	Chaperones, Protein degradation, Adaptation, Atypical conditions
2	Phage-related functions and prophages, Degradation of small molecules, Hypothetical proteins, Conserved hypothetical proteins
3	Translation and modification, Membrane components, Inner membrane, Hypothetical proteins
4	Pathogenicity, virulence and adaptation: Toxin prodution and detoxification, Other. Transport, Protein and peptide secretion
5	Aerobic respiration, Biosynthesis of small molecules, Ribosomal proteins, RNA synthesis, Aminoacyl tRNA synthetases
6	Energy metabolism, carbon, TCA cycle, ATP-proton motive force interconversion. Heme porphyrin, DNA repair, Cell structure: Surface polysaccharides, Lipopolisaccharydes, Surface structures

FIG. 2. K-means clustering with six groups, using the complete profiles of differentially expressed genes. The *y* axis shows the *M* values [$M = \log_2(40°C/29°C)$], and the *x* axis shows the heat shock time points. The table shows the overrepresented gene categories for each cluster. A functional category was considered overrepresented if its statistical association with its presence in the cluster is significant ($P < 0.05$).

Time course view of the heat shock response. To have a global structured view of the expression patterns of *X. fastidiosa* genes that are differentially expressed during heat shock, we performed a clustering analysis using the K-means algorithm with six groups, as indicated by the PCA analysis. With the aim of characterizing each cluster based on functional categories, we searched for overrepresented gene categories in each group, as described in Materials and Methods. In Fig. 2 we show the K-means clustering expression profiles and the respective overrepresented gene categories. The complete list of genes in each group is provided in Table S4 at http://blasto.iq.usp.br/~tkoide/Xylella/Heat_shock/.

We have observed two main patterns of transcriptional induction during heat shock: genes with a rapid increase in transcript levels, presenting a maximum after 25 min of exposure to high temperature followed by a reduction in expression levels (group 1), and those with a smooth and constant increase in expression (groups 3 and 4). Group 2 seems to be a hybrid between these two main patterns. Group 1 mainly contains genes encoding chaperones and proteases, which showed the highest induction levels. In addition, genes from group 1 show a transient increase in expression, which is a known characteristic of the heat shock response, as we can observe a decrease in transcript levels for these genes by 45 min of exposure to high temperature. Group 2 is characterized by the presence of genes associated with phage-related functions, prophages, and hypothetical proteins. Almost half of the genes in this group (33 genes) present expression patterns similar to those of group 1, i.e., with the characteristic kinetics of heat shock genes but with lower expression ratios. Among these, four are classical Hsp genes: *htpX*, *dnaJ*, *clpA*, and *hslU*. The remaining 42 genes present expression patterns similar to those of group 3, with slightly higher expression ratios. Group 3 contains genes related to protein synthesis and modification and to membrane components. Group 4 presents the lowest induction ratios and has clustered together 15 genes related to pathogenicity, virulence, and adaptation. Among them, eight are related to toxin production and detoxification, and five are related to other functions. The category of protein and peptide secretion was also overrepresented in this group.

Group 5 is composed of genes that were slightly down-regulated during heat shock and contains genes related to aerobic respiration, biosynthesis of small molecules, ribosomal proteins, RNA synthesis, and aminoacyl-tRNA synthetases. Group 6 clusters genes with the highest repression levels and which are related to cellular structures such as surface structures and polysaccharides. These down-regulated surface structure genes are related to those encoding type IV pili. Moreover, this group also contains genes related to energy metabolism involved in the tricarboxylic acid cycle and ATP-proton motive force interconversion. The repression of genes related to metabolism and protein synthesis might indicate a diminished metabolic activity during heat shock.

The genes described here as being related to temperature stress were selected based on their expression change in relation to the majority of the genes, a usual procedure adopted in

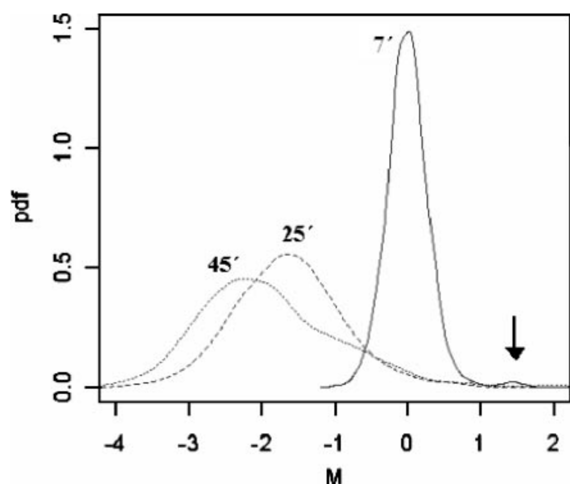


FIG. 3. Global gene expression changes in response to heat shock. The graph shows smoothed histograms of expression log ratios [$M = \log_2(40°C/29°C)$] for 7, 25, and 45 min of heat shock, as indicated. The arrow highlights the early induced heat shock genes (7 min). The *x* axis shows the expression log ratios, (*M*), and the *y* axis shows the probability density function (pdf).

microarray data analysis. Since we applied the LOWESS normalization procedure, our microarray analysis was carried out assuming that expression of most genes does not change upon heat shock. However, it has been recently reported that heat shock elicits a significant global reduction in gene expression levels in human endothelial cells (53). This effect could be detected only by using normalization procedures based on well-calibrated external controls or costly dye swap schemes. Since we had a set of experiments that were amenable to dye swap normalization, which does not assume that the majority of the genes do not change, we investigated whether such a global change in gene expression levels occurs during heat shock in *Xylella*. The dye swap normalization was applied to two microarray slides from each of the following heat shock time points: 7, 25, and 45 min. Figure 3 shows the distribution of log ratio values (*M*) from dye swap-normalized experiments for the heat shock time points considered, indicating a global reduction in expression levels as *Xylella* cells are exposed to high temperature for longer periods.

At the first heat shock time point (7 min), we can observe that most genes are distributed around $M = 0$, indicating that there was no net change in expression levels. There is a small peak that corresponds to the early-induced heat shock genes (Fig. 3). At subsequent time points, there is a global shift in expression ratios to negative *M* values, indicating a possible global reduction in mRNA levels during heat shock. The longer the time of exposure to heat stress, the greater the observed shift to negative *M* values. We could speculate that this global reduced transcription during heat shock could be the consequence of the reduced stability of the major sigma factor σ^{70} at elevated temperatures, as has been demonstrated for *E. coli* and *Caulobacter crescentus* (11, 48).

Real-time RT-PCR validation of expression profiles. To validate the profiles of gene expression, we have performed real-time RT-PCR experiments for 10 selected genes, using triplicate biological experiments for the entire heat shock time course. The overall Spearman correlation between the log ratio values [$M = \log_2(40°C/29°C)$] from microarray experiments and real-time RT-PCR was 0.89, indicating a good overall correlation and therefore validating the microarray expression profiles. Figure 4 shows the data comparing the results from microarray and RT-PCR experiments for the selected genes.

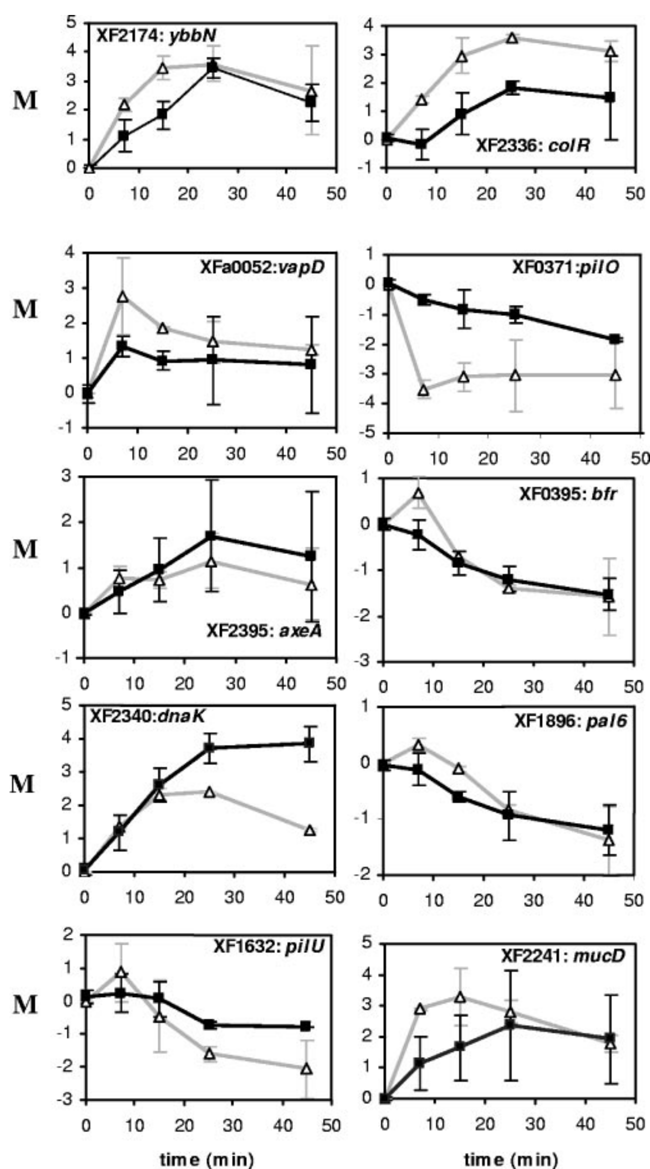


FIG. 4. Time course expression levels for 10 selected genes evaluated by real-time RT-PCR (open triangles) and microarray experiments (black squares), where $M = \log_2(40°C/29°C)$. The results are the median values from three independent biological samples.

Functional classification of differentially expressed genes. (i) Heat shock genes. We observed the induction of genes encoding proteins from different Hsp families that are known to be involved in the heat shock response in different organisms: the chaperones GrpE (Hsp20), DnaK (Hsp70), DnaJ (Hsp40), GroES (Hsp10), GroEL (Hsp60), and ClpB (Hsp100), clustered in group 1; ATP-dependent proteases such as Lon and ClpP (both in group 4), ClpA (group 2), HslU

TABLE 1. Most-highly-induced genes in microarray experiments (group 1 in the K-means cluster) and respective fold induction at each time point

Gene	Function	Gene name	Fold induction at min:			
			7	15	25	45
XF0381	Chaperone	*clpB*	2	5	14	14
XF0615	Chaperone	*groEL*	4	5	15	7
XF0616	Chaperone	*groES*	4	7	20	9
XF1484	Protease	*hslV*	2	4	9	7
XF2174	Thioredoxin	*ybbN*	2	4	11	9
XF2233	Chaperone	*dnaJ*	2	6	15	18
XF2234	Chaperone	*hspA*	2	10	11	8
XF2340	Chaperone	*dnaK*	2	6	12	14
XF2341	Cochaperone	*grpE*	3	5	14	8
XF2594	Protease	*b2494*	2	8	13	15
XF2625	Protease	*htpX*	3	5	14	12
XFa0048	Putative mobilization protein	*mobC*	5	7	10	8

(group 2), and HslV (group 1); and other heat shock proteins such as HtpX (group 1), HlsO (Hsp33, group 4), HtpG (Hsp90, group 2), and HspA (α-Hsp, group 1). Among the induced genes, we identified three that were originally annotated as encoding hypothetical proteins (XF0862 [group 3], XF0882 [group 3], and XF2594 [group 1]) and which were reannotated here as peptidases.

In our clustering analysis, group 1 genes presented the highest induction levels (Table 1). Except for XF2174 and XFa0048, which encode, respectively, a thioredoxin (YbbN) and a putative mobilization protein C from plasmid pXF51, all the remaining genes encode classical Hsps, which are regulated by the heat shock sigma factor σ^{32} in other gram-negative bacteria (63). Therefore, to obtain a consensus sequence for putative σ^{32}-dependent promoters in *Xylella*, we performed primer extension assays to determine the transcription start sites for six genes from this group: *dnaK*, *grpE*, *clpB*, *groES*, *htpX*, and *hspA*.

The transcription start sites were mapped by using as the template total RNA from cells grown at normal temperature (29°C) and from cells isolated after 25 min of heat shock at 40°C, where we observed the peak of induction of these genes. Figure 5 shows the sizes of the extension products obtained for each gene and the alignment of the putative −35 and −10 promoter sequences inferred from the transcription start sites determined. It can be observed that, for all six genes tested, the amount of extension product is larger at 40°C than at 29°C, confirming the increase in mRNA levels during heat shock determined in the microarray experiments.

In *Xylella*, *groES* is organized in a probable operon with *groEL*, and its putative promoter presents −35 and −10 regions very similar to the consensus for *E. coli* σ^{32}-dependent promoters (Fig. 5A). The *X. fastidiosa groESL* operon also possesses a CIRCE element (*c*ontrolling *i*nverted *r*epeat of *c*haperone *e*xpression; TTAGCACTC-N9-GAGTGCTAA) located 48 nucleotides upstream of the *groES* translation start site. This regulatory sequence is the binding site of the repressor protein HrcA, constituting the CIRCE-HrcA system, which is known to regulate the expression of heat shock genes in several bacterial species. In gram-positive bacteria, CIRCE-HrcA controls induction at high temperature of several heat shock genes, whereas in gram-negative bacteria such as *C. crescentus* and *Agrobacterium tumefaciens*, CIRCE is found only in front of the *groESL* operon and its role is restricted to repressing *groESL* expression under nonstress conditions (5, 6, 35). In *X. fastidiosa*, the CIRCE element is also found only in front of *groESL*, but whether or not CIRCE-HrcA controls heat shock induction of this operon remains to be established.

Concerning the *dnaK* gene, although its transcription start site could be mapped and a putative σ^{32} promoter could be found, the signal corresponding to the extension product was very weak, even at 40°C (Fig. 5B). This result is not compatible with the high expression levels observed for *dnaK* in the microarray experiments and confirmed by real-time RT-PCR. The *dnaK* gene (XF2340) is organized in a putative operon, *hrcA-grpE-dnaK-dnaJ*. The extension product of 61 nucleotides for the *grpE* gene revealed a higher mRNA level, which may indicate that *dnaK* is transcribed mainly from the promoter located upstream of *grpE* (Fig. 5C). The transcription start site of the *grpE* gene was mapped near the translation start site proposed by Weng and collaborators (60). It is interesting to note that the *hrcA* gene was not classified as differentially expressed according to microarray data, since it did not pass the established statistical criteria. Thus, our data indicate that *hrcA* is probably transcribed independently from *grpE*.

(ii) Determination of a consensus sequence for σ^{32}-dependent promoters in *Xylella*. A putative consensus sequence for *X. fastidiosa* σ^{32}-dependent promoters was obtained by comparing the −35 and −10 regions determined during mapping of the transcription start sites of several heat shock genes. Using this experimentally derived information, we have built probabilistic consensus matrices and used an in silico approach to suggest putative σ^{32}-dependent promoters. As shown in Fig. 5G, the consensus for *Xylella* σ^{32} promoters is similar to those proposed for other gram-negative bacteria, mainly in the −35 region. Additional searches for this consensus sequence were carried out in the 5′ regions of other 261 heat shock-induced genes in *Xylella*, as described in Materials and Methods. The search revealed 42 putative members of the σ^{32} regulon that presented increased transcription levels upon heat shock. This number is statistically significant ($P < 0.05$) compared to the proportion of putative σ^{32} promoters found in uninduced genes. Among the 42 genes, besides the six genes from which the consensus sequence was determined, we found heat shock-related genes such as *htrA*, encoding a protease; *hslV*, which is in an operon with *hslU*, encoding a probable proteasome-like system; and *hslO*, from the Hsp33 family. Moreover, five phage-related genes and nine genes encoding hypothetical or conserved hypothetical proteins all displayed matching σ^{32} promoter sequences in their 5′ noncoding regions. Putative promoters with highest scores were more abundant in genes from group 1. It is important to note that this analysis should be carefully considered, since the search for the consensus sequence was performed within the 200 nucleotides upstream of each translation start site, without knowledge of the real transcription start site of the gene. A complete list of the genes and their putative promoter sequences is in Table S5 at http://blasto.iq.usp.br/~tkoide/Xylella/Heat_shock/.

(iii) Extracytoplasmic stress response. In our microarray analysis, we also observed changes in transcript levels of genes related to the bacterial cell envelope. For instance, among the

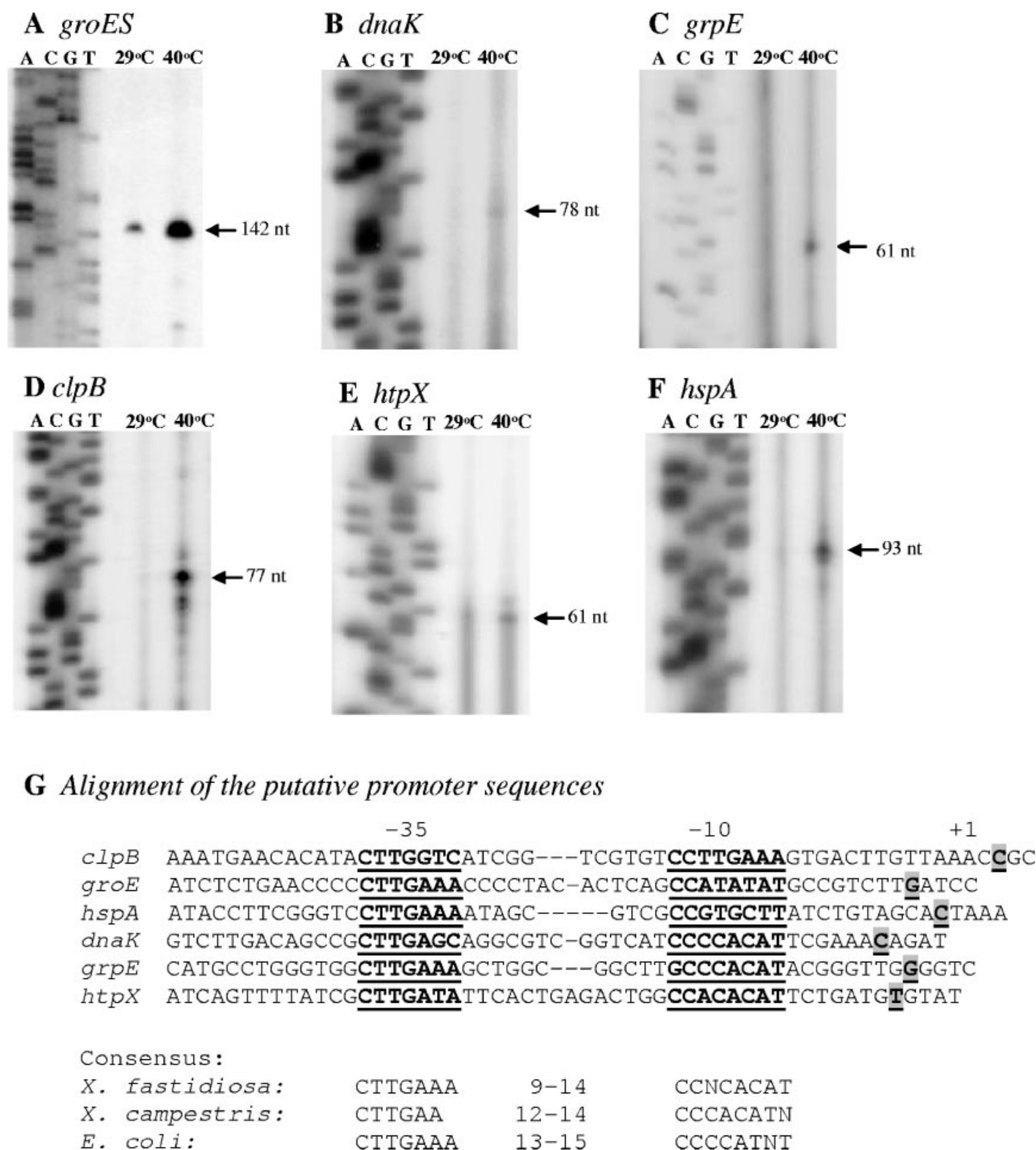


FIG. 5. Primer extension assays for the genes *groES* (A), *dnaK* (B), *grpE* (C), *clpB* (D), *htpX* (E), and *hspA* (F). The reference sequence is from phage M13mp18. The sizes of the extension products are indicated. nt, nucleotides. Panel G shows the alignment of the putative −35 and −10 promoter regions inferred from the transcription start sites, which are boxed. The consensus sequences for σ^{32} promoters in *Xanthomonas campestris* (60) and *E. coli* (18) are shown for comparison.

induced genes, we found *rfbA* (XF0256, group 4) and *glmU* (XF1140, group 3), involved in lipopolysaccharide biosynthesis, as well as *rfbU* (XF0879, group 3), *mltB* (XF2184, group 3), *rlpA* (XF2185, group 2), and *murA* (XF1415, group 4), involved in peptidoglycan biosynthesis. Since alterations in lipopolysaccharide structure affect the ratio of outer membrane proteins, differential transcription of these envelope-related genes is probably involved in the extracytoplasmic stress response. Studies characterizing the σ^E regulon in *E. coli* indicate that this sigma factor controls the expression of genes involved in periplasmic folding catalysis, genes related to lipid A biosynthesis, and genes encoding lipoproteins (1). We observed the induction of many genes that have been characterized as members of the σ^E regulon in *E. coli*, such as *mucD* (XF2241, group 2) and *degP* (XF0285, group 3), which encode periplasmic proteases that have been implicated in virulence in *Pseudomonas aeruginosa*, *Shigella flexneri*, and *Klebsiella pneumoniae* (42); *rseA* (XF2240, group 2), a negative regulator of σ^E activity in *E. coli*; *bacA* (XF1841, group 2), which is involved in lipopolysaccharide assembly and peptidoglycan biosynthesis (43, 44); and *dsbA* (XF1436, group 4), encoding a disulfide oxidoreductase that assists in the formation of disulfide bonds in extracytoplasmic proteins. It is interesting to note that *dsbA* and *degP* have been characterized in many bacterial pathogens

as important virulence factors (42). In *Xylella*, *dsbA* was only slightly induced (group 4) upon heat stress, whereas the *degP* homologues (*mucD* [group 2] and *htrA* [group 3]) presented higher induction ratios.

(iv) Genes involved in protein biosynthesis. During heat shock, we observed the down-regulation of a set of nine genes encoding ribosomal proteins (*rplV*, *rplP*, *rpsQ*, *rplN*, *rplX*, *rpsE*, *rplO*, and *rplJ* [all in group 5] and *rpsD* [group 6]) and four genes encoding aminoacyl-tRNA synthetases (*tyrS*, *proS*, *thrS*, and *hisS* [group 5]), indicating a general shutdown of protein synthesis. In *E. coli*, most of the regulation of rRNA synthesis results from changes in the intracellular concentration of ppGpp, with high ppGpp levels leading to reduced transcription from the rRNA promoters (40). Interestingly, the genes encoding RelA and SpoT, involved in ppGpp metabolism, were up-regulated during heat shock in *Xylella*, with both genes being clustered in group 3. In agreement, it has been reported that the ability of σ^{32} to compete with σ^{70} is diminished in cells lacking ppGpp *in E. coli* (23).

(v) Mobile genetic elements. During the entire heat shock time course, we observed the induction of a cluster of genes from plasmid pXF51 (XFa0047 and XFa0049 to XFa0052 [group 2] and XFa0048 [group 1]) encoding a nickase TaxC, a mobilization protein MobC, a hypothetical protein, the stability-determining protein StbB, a hypothetical protein, and virulence-associated protein D (VapD), respectively. The VapD protein is associated with virulence in *Dichelobacter nodosus* (24) and orthologues have been identified in other virulent bacteria; however, its function in *Xylella* is still unknown (29). In addition to plasmid-related genes, genes from four phages integrated in the *X. fastidiosa* 9a5c genome were also induced: 11 genes from XfP1, 9 from XfP2, 1 from XfP3, and 8 from XfP4. These genes encode phage-related proteins and conserved hypothetical or hypothetical proteins.

(vi) Intermediary metabolism. Many genes related to aerobic respiration were repressed during heat shock in *Xylella*, mainly after prolonged exposure to high temperature. For instance, genes *nuoA*, *nuoD*, *nuoH*, and *nuoL* (group5), which encode subunits of the NADH dehydrogenase, and genes related to the tricarboxylic acid cycle (*sucB*, *sucC*, and *sucD* [group 6]), to glycolysis (*pfk*6 and *gapA5* [group 5]), and to ATP proton motive force (*atpH*, *atpF*, and *atpE* [group 6]) were shown to be repressed. Concerning the electron transport system, genes encoding the cytochrome *o* ubiquinol oxidase (*cyoD*, *cyoC*, and *cyoB* [group 6]) were also down-regulated. In addition, we observed the induction of *cycJ* and *ccmB* (group3), genes involved in cytochrome *c* biogenesis, which are induced under low-oxygen conditions (52).

(vii) Iron transport-related genes. Further support for the hypothesis of low oxygen pressure during heat shock came from the observation that gene XF0932 (group 4), related to ferrous ion transport, was induced during temperature upshift. Since ferrous ion is more stable under low-oxygen conditions and is readily used by bacteria (3), *Xylella* could favor this transport system during heat shock. In addition, the *bfr* gene (group 6), encoding a bacterioferritin, was repressed. This iron storage protein helps to increase aerotolerance by sequestering iron and, consequently, limiting the oxidative damage that may result from a Fenton reaction (3, 50).

(viii) Pathogenicity, virulence, and adaptation. Besides the induction of *degP* and *vapD*, other genes related to pathogenicity, virulence, and adaptation presented differential expression during heat shock. We observed the induction of two genes encoding pore-forming exotoxins from the RTX family (31): a hemolysin (XF0668, group 4) and a bacteriocin (XF2407, group 4). In agreement, two genes involved in hemolysin secretion (XF2397 and XF2398, group 4) were also up-regulated. Interestingly, the expression of genes related to colicin production was reduced during heat shock: the colicin V precursor CvaC and colicin secretion protein CvaA were down-regulated after 45 min of heat shock.

Two genes encoding enzymes putatively involved in xylan degradation were induced during heat stress in *Xylella*: XF0878 (polysaccharide deacetylase, group 2) and XF2395 (acetyl xylan esterase, group 2). Xylan is the major hemicellulose component of plant cell walls (12), which can be degraded by these enzymes either to provide carbon sources or to facilitate the migration of the bacteria within xylem vessels.

A set of six genes related to the type II secretion system was also induced upon temperature stress: *xpsE* (XF1517, group 2), *xpsF* (XF1518, group 2), *xpsH* (XF1520, group 4), *xpsJ* (XF1522, group 3), *pefL* (XF1524, group 4), and *xpsM* (XF1525, group 4). Type II secretion systems have been implicated in the export of different virulence factors, such as toxins and hydrolytic enzymes (47). In agreement, some genes of the Sec system (*secA*, *secF*, and *secG*) acting in protein translocation from the cytoplasm to the periplasm were also induced.

Genes related to nonfimbrial adhesins also presented increased transcript levels upon heat stress: genes encoding hemagglutinin-like secreted proteins (XF2196 [group 2] and XF2775 [group 3]) and the adhesin gene *uspA1* (XF1516 [group 3]). It is worth noting that *uspA1* was shown to be expressed at higher levels in *X. fastidiosa* freshly isolated from the plant host than in bacterial cultures that had become less virulent after several passages in nutrient medium (15), indicating a possible role in bacterial pathogenesis. Concerning hemagglutinin-like genes, a recent study with *X. fastidiosa* strain Temecula showed that mutants with mutations in these genes induced more severe symptoms in grapevines than the wild-type strain, in contrast to studies with other pathogenic bacteria, where the mutants showed decreased virulence or colonization (20). It was suggested that in *Xylella*, the hemagglutinins attenuate the colonization capacity, although they might play a role in mediating cell-cell aggregation to form colonies and contribute to biofilm maturation.

In addition, *phoQ* (XF0390 [group 4]) and *colR* (XF2336 [group 2] and XF2354 [group 3]), which are genes related to two-component signal transduction systems, were induced upon heat shock in *Xylella*. It is worth noting that the PhoPQ system is required for virulence in several bacterial species, such as *Salmonella*, *Yersinia*, and the plant pathogen *Erwinia carotovora* (17). The *colR* gene has also been characterized as being related to pathogenicity in *Pseudomonas fluorescens*, since mutations in this gene impair root colonization by the bacteria (14). Gene XF1020 (group 4), encoding a protein related to pathogenesis, also presented increased transcript levels during heat shock. Mutation of its orthologue in *Xan-*

thomonas campestris resulted in reduction of virulence, although its function is still unknown (37).

Final remarks. Using whole-genome transcriptional profiling, we obtained important information about the effect of temperature upshift in *Xylella* gene expression. We observed an early and dramatic transient increase in the expression of genes encoding heat shock proteins that act in response to cytoplasmic stress. Six of these genes had their transcription start sites determined, allowing the proposal of a consensus sequence for σ^{32}-dependent promoters in *Xylella*.

We have also observed the induction of genes known to be involved in the periplasmic stress response, which is mediated in other bacteria by the alternative sigma factor σ^E. Several genes related to the outer membrane, lipopolysaccharide biosynthesis, and periplasmic protein folding showed altered expression levels in our microarray assays. Further characterization of the role of σ^E in *Xylella* gene expression and its relation to the σ^{32} regulon will be investigated in a transcriptome study which is being carried out with an *rpoE* null mutant of *X. fastidiosa* strain J1a12 (J. F. da Silva Neto, T. Koide, S. L. Gomes, and M. V. Marques, unpublished data). Attempts to obtain an *rpoH* null mutant strain of *X. fastidiosa* strain J1a12 were unsuccessful (J. F. da Silva Neto and M. V. Marques, personal communication), which might indicate that σ^{32} plays an essential role in *Xylella* even at normal temperatures.

Interestingly, several genes related to pathogenicity, virulence, and adaptation were induced upon heat shock. Some of them are uncharacterized genes that were linked to reduced virulence in other pathogens, such as *vapD* and XF1020; however, their precise role in *Xylella* pathogenesis remains to be unraveled. The induction of the bacteriocin gene and the repression of the colicin V gene might indicate a differential mechanism for competition in the environment under different stress conditions. Moreover, the higher expression of nonfimbrial adhesins indicates an important role of bacterial adhesion in response to stress, which might be linked to bacterial pathogenesis.

It is worth noting that the progress of CVC disease caused by *Xylella* is more accentuated during spring and summer (30), when the disease symptoms are also more severe. We might speculate that expression of some of the genes related to virulence and adaptation described here as induced upon heat exposure could contribute, at least in part, to the success of the bacterial infection in the host plant. Since the response to environmental insults is of primordial importance in bacterial pathogens, we believe that our study will contribute to understanding of the mechanisms of virulence and adaptation in *Xylella fastidiosa.*

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