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 σ32 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 fimbrae, 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 number of 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 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. 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, σ32, encoded by the rpoH gene. Regulation of σ32 levels is exerted by a feedback loop where the Hsps (DnaK, DnaJ, and GrpE) direct σ32 to FtsH-mediated proteolysis at normal temperatures. Since the levels of DnaK and DnaJ are limiting in vivo, the increase of aberrant proteins during heat shock sequesters these chaperones, liberating σ32 to bind to the RNA polymerase and directing the polymerase to specific heat shock promoters. In addition, temperature upshifts rapidly increase translation of σ32 by destabilizing the rpoH mRNA secondary structure, thereby increasing the ribosomal accessibility to the translation start site. Recently, it was demonstrated that GroEL/S chaperone machinery is also involved in the regulation of σ32 activity and stability in E. coli.

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 and Bacillus subtilis, through the bioremediation-involved bacterium Shewanella oneidensis, to diverse mammalian pathogens such as Campylobacter jejuni, group A Streptococcus, Mycoplasma pneumoniae, Yersinia pestis, and Neisseria meningitidis. 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 proteome level have also been carried out, revealing extensive global responses of bacteria such as Bradyrhizobium japonicum, Agrobacterium tumefaciens, and Myxococcus xanthus. 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
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 σ52. 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 × 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. The RNA was treated using the enzyme CyScript in the presence of modified amino allyl-dUDP, an optimized nucleotide mix, buffer, dithiothreitol, and random primers. The resulting amine-modified cDNA was then chemically labeled using Cy5 Cy3 S-ddUTP (Perkin-Elmer) and hybridized to 50,000 cDNA spots. The oligonucleotides were 5′-end labeled with 32P-γATP 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. Minced was performed at 50°C for 16 h; primer extension and RNA degradation were carried out as previously described (40). Extension products were analyzed by gel 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 stress-responsive promoters.** Using experimentally derived information about the σ52 putative promoters, we have built probabilistic con...
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-experiment experiments, using at least five replicates, as described in Materials and Methods.

Globally, about 20% of the genes were significantly upregulated 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/.

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; IA, degradation; IB, central intermediary metabolism; IC, energy metabolism, carbon; ID, regulatory functions; II, biosynthesis of small molecules; IA, amino acid biosynthesis; IB, nucleotide biosynthesis; ID, cofactors, prosthetic groups, carrier biosynthesis; IIE, fatty acids and phosphatidic acid biosynthesis; III, macromolecule metabolism; IIIA, DNA metabolism; IIIB, RNA metabolism; IIC, protein metabolism; III, other macromolecule metabolism; IV, cell structure;IVA, membrane components; IVB, murein sacculus, peptidoglycan; IVC, surface polysaccharides, lipopolysaccharides, and antigens; IVD, surface structures; V, cellular processes; VA, transport; VB, cell division; VC, chemotaxis and mobility; VI, mobile genetic elements; VLA, plasmid-related functions and prophages; VLB, plasmid-related functions; VII, pathogenicity, virulence, and adaptation; VILC, toxin production and detoxification; VII, surface proteins; VIIIA, adaptation, atypical conditions; VIIIB, other; VIIIC, hypothetical proteins; VIIID, hypothetical proteins; IX, ORFs with undefined category.

sense 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 σ²⁷ promoter sequences in the up-stream 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 σ²⁷ promoters found among unin-duced 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.
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 downregulated 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...
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 $\sigma^{70}$ 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 [$M = \log_{2}(40^\circ C/29^\circ C)$] 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.

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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Gene name</th>
<th>Fold induction at 7 min</th>
<th>15 min</th>
<th>25 min</th>
<th>45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>XFO0381</td>
<td>Chaperone</td>
<td>clpB</td>
<td>2</td>
<td>5</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>XFO0165</td>
<td>Chaperone</td>
<td>groEL</td>
<td>4</td>
<td>5</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>XFO0166</td>
<td>Chaperone</td>
<td>groES</td>
<td>4</td>
<td>7</td>
<td>20</td>
<td>9</td>
</tr>
<tr>
<td>XFI1484</td>
<td>Protease</td>
<td>hslV</td>
<td>2</td>
<td>4</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>XFI2174</td>
<td>Thioredoxin</td>
<td>ybbN</td>
<td>2</td>
<td>4</td>
<td>11</td>
<td>9</td>
</tr>
<tr>
<td>XFI2223</td>
<td>Chaperone</td>
<td>dnaA</td>
<td>2</td>
<td>6</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>XFI2224</td>
<td>Chaperone</td>
<td>dnaK</td>
<td>2</td>
<td>10</td>
<td>11</td>
<td>8</td>
</tr>
<tr>
<td>XFI2340</td>
<td>Chaperone</td>
<td>dnaK</td>
<td>2</td>
<td>6</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td>XFI2341</td>
<td>Chaperone</td>
<td>gprE</td>
<td>3</td>
<td>5</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>XFI2439</td>
<td>Protease</td>
<td>h2494</td>
<td>2</td>
<td>8</td>
<td>13</td>
<td>15</td>
</tr>
<tr>
<td>XFI2625</td>
<td>Protease</td>
<td>htpX</td>
<td>3</td>
<td>5</td>
<td>14</td>
<td>12</td>
</tr>
<tr>
<td>XFI0048</td>
<td>Putative mobilization protein</td>
<td>mobC</td>
<td>5</td>
<td>7</td>
<td>10</td>
<td>8</td>
</tr>
</tbody>
</table>

In our clustering analysis, group 1 genes presented the highest induction levels (Table 1). Except for XFI2174 and XFI0048, 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 σ32 in other gram-negative bacteria (63). Therefore, to obtain a consensus sequence for putative σ32-dependent promoters in Xylella, we performed primer extension assays to determine the transcription start sites for six genes from this group: dnaK, gprE, clpB, groES, htpX, and dnaA.

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 σ32-dependent promoters (Fig. 5A). The X. fastidiosa groESL operon also possesses a CIRCE element (controlling inverted repeat of chaperone expression; 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 σ32 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 (XFI2340) is organized in a putative operon, hrcA-gprE-dnaK-dnaI. The extension product of 61 nucleotides for the gprE gene revealed a higher mRNA level, which may indicate that dnaK is transcribed mainly from the promoter located upstream of gprE (Fig. 5C). The transcription start site of the gprE 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 gprE.

(ii) Determination of a consensus sequence for σ32-dependent promoters in Xylella. A putative consensus sequence for X. fastidiosa σ32-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 σ32-dependent promoters. As shown in Fig. 5G, the consensus for Xylella σ32 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 σ32 regulon that presented increased transcription levels upon heat shock. This number is statistically significant (P < 0.05) compared to the proportion of putative σ32 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 hsoO, from the Hsp33 family. Moreover, five phage-related genes and nine genes encoding hypothetical or conserved hypothetical proteins all displayed matching σ32 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://blastoq.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
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 mucA (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 \( \sigma^{32} \) 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 \( \sigma^{32} \) 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 \( \sigma^{25} \) 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.

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, 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 \( \alpha^{32} \) promoters in \( Xanthomonas campestris \) (60) and \( E. coli \) (18) are shown for comparison.

G Alignment of the putative promoter sequences

<table>
<thead>
<tr>
<th>Gene</th>
<th>Consensus:</th>
</tr>
</thead>
<tbody>
<tr>
<td>clpB</td>
<td>CTTGAAA 9–14</td>
</tr>
<tr>
<td>groES</td>
<td>CTTGAAA 13–15</td>
</tr>
<tr>
<td>hspA</td>
<td>CTTGAAA 13–15</td>
</tr>
<tr>
<td>htpX</td>
<td>CTTGAAA 13–15</td>
</tr>
</tbody>
</table>

\( X. fastidiosa \): CTTGAAA 9–14  CCCCACAT
\( X. campestris \): CTTGAAA 12–14  CCCCACATN
\( E. coli \): CTTGAAA 13–15  CCCCATNT
as important virulence factors (42). In *Xylella*, *dshA* was only slightly induced (group 4) upon heat stress, whereas the *degP* homologues (*mucD* [group 2] and *hrtA* [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 *rplD* [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^70* to compete with *σ^52* 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* 9a1 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* (group 5), which encode subunits of the NADH dehydrogenase, and genes related to the tricarboxylic acid cycle (*succB*, *succC*, and *succD* [group 6]), to glycolysis (*gapA5* 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 *cydC* and *ccmB* (group 3), 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 aero tolerance 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), *xpsD* (*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 bacterial 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 cytoplastic stress. Six of these genes had their transcription start sites determined, allowing the proposal of a consensus sequence for σ32-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 σ21. 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 σ21 in Xylella gene expression and its relation to the σ32 regulon will be investigated in a transcriptome study which is being carried out with a 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 a 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 σ32 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 XFP1020; 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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