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Whole-Genome Expression Profiling of *Xylella fastidiosa* in Response to Growth on Glucose

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ABSTRACT

***Xylella fastidiosa* is the etiologic agent of diseases in a wide range of economically important crops including citrus variegated chlorosis, a major threat to the Brazilian citrus industry. The genomes of several strains of this phytopathogen have been completely sequenced enabling large-scale functional studies. In this work we used whole-genome DNA microarrays to investigate the transcription profile of *X. fastidiosa* grown in defined media with different glucose concentrations. Our analysis revealed that while transcripts related to fastidian gum production were unaffected, colicin-V-like and fimbria precursors were induced in high glucose medium. Based on these results, we suggest a model for colicin-defense mechanism in *X. fastidiosa*.**

INTRODUCTION

XYLELLA FASTIDIOSA IS A GRAM-NEGATIVE XYLEM-LIMITED BACTERIUM which is the etiologic agent of economically important plant diseases, such as Pierce's disease of grapevines (PD) and citrus variegated chlorosis (CVC) (Tyson et al., 1985; Chang et al., 1993; Hopkins and Purcell, 2002). The latter is a major concern to the citrus industry, being responsible for annual losses over $100 million. CVC is considered one of the most devastating citrus diseases and according to Fundecitrus (www.fundecitrus.com.br), of the 180 million productive trees, 70 million will be affected during the next five years.

Upon transmission from infected plants by xylem sap-feeding sharpshooter leafhoppers, *X. fastidiosa* spreads systemically throughout the plant and attaches to xylem vessel walls. Depending on size, these biofilm-like colonies eventually occlude the vascular system, leading to the water-stress symptoms typical of CVC (Roberto et al., 1996; McElrone et al., 2001). Furthermore, it has been proposed that the fastidian exopolysaccharide and fimbriae are involved in the bacterial attachment and survival in the plant as well as in the insect vector (da Silva et al., 2001; Feil et al., 2003; Newman et al., 2004). Thus, the formation

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of biofilm is currently accepted as a crucial factor in the pathogenicity of *X. fastidiosa* (Simpson et al., 2000; Machado et al., 2001; Newman et al., 2003; Osiro et al., 2004). Virulence mechanism might also include toxins, antibiotics and ion sequestration systems (Dow and Daniels, 2000; Simpson et al., 2000), but these remain poorly characterized.

Several *X. fastidiosa* strains have had their genomes completely or partially sequenced and comparative genome analysis of different strains pointed to common virulence determinants as well as strain-specific genomic signatures (Simpson et al., 2000; Bhattacharyya et al., 2002; Nunes et al., 2003; Van Sluys et al., 2003; Koide et al., 2004). These genome sequence data paved the way for high-throughput functional studies such as transcriptome and proteome analyses in an effort to improve our understanding of physiology and pathogenicity of this organism (de Souza et al., 2003; Nunes et al., 2003; Smolka et al., 2003; de Souza et al., 2004; Koide et al., 2004).

In this work we used full-genome DNA microarrays to investigate the transcription profile of *X. fastidiosa* grown in defined media with different glucose concentrations. Our analysis revealed that while transcripts related to fastidian gum production were unaffected, colicin-V-like and fimbria precursors were induced in high glucose medium. Based on these results we suggest a model for colicin-defense mechanism in *X. fastidiosa*.

MATERIALS AND METHODS

Bacterial strain and growth conditions

Triply cloned *X. fastidiosa* strain 9a5c (Li et al., 1999) isolated from citrus variegated chlorosis (CVC) symptomatic *Citrus sinensis* (L.) Osbeck trees (sweet orange) was grown on solid XDM_2 medium (Lemos et al., 2003) at 28°C in the dark. Cells with less than 20 passages were used. After 6 days (late log-phase) of growth, cells were collected and washed in liquid XDM_2 medium without glucose. A 1 mL aliquot of the cell suspension was then plated in XDM_2 with defined glucose concentrations (1, 50, and 250 mM) and grown for 6 more days for total RNA isolation.

To monitor the growth at different concentrations of glucose, the cultures were prepared as described above until the washing step, and then transferred to liquid XDM_2 medium with defined glucose concentrations. Samples were taken every 4 days during 12 days. Growth was monitored by measuring the optical density of resuspended cells and by total protein content. For this, samples were resuspended in 1.0 mL of sonication buffer (10mM Tris-HCl and 5mM $MgCl_2$, pH 7.0) by vortexing. A 50-μL aliquot was removed and diluted in 950 μL of water for measurement of optical density at 600 nm. The remaining cells were sonicated (Sonifier Branson, model 250 at 85W) in an ice water-bath for 5 min, and the extract was clarified by centrifugation at 10,600 $\times$ *g* for 10 min at 4°C and total protein was estimated by the Hartree assay (Hartree, 1972).

Microarray construction, fluorescent labeling, and hybridization

A 6152-element DNA microarray containing unique internal fragments of 2692 CDS spotted at least in duplicate, representing 94.5% of all the 2848 CDS annotated by Simpson et al. (2000) was constructed as previously described (Koide et al., 2004). Expression profiling studies were carried out labeling 20 μg of total RNA with indocarbocyanine or indodicarbocyanine (Cy3 or Cy5) using Cy-Scribe Post Labeling kit (GE Healthcare) according to manufacturer's instructions. Total RNA from 6-day cultures was extracted with TRIzol (Invitrogen), treated with RQ1 DNase I (Promega). Complete removal of genomic DNA was evaluated by PCR.

Fluorescent cDNAs were combined and denatured by heating to 95°C for 2 min and quickly chilled on ice. The targets were then applied to the microarray slide and covered with a 24 $\times$ 60 mm coverslip (Corning). The hybridization proceeded for 16 h at 42°C. The slides were then washed in 1 $\times$ SSC + 0.2% SDS for 10 min at 55°C, twice in 0.1 $\times$ SSC + 0.2% SDS also for 10 min at 55°C, 0.1 $\times$ SSC for 1 min at room temperature and finally in deionized water for 1 min at room temperature and dried with N_2 gas.

Data acquisition, ratio normalization, and statistical analysis

Microarray slides were scanned using a Generation III DNA scanner (GE Healthcare) and fluorescence intensity values from each spot were extracted using ArrayVision 7.0 software (Imaging Research Inc.). We used the usual "reference design", with the 50 mM glucose growth condition as the reference, and prepared three biological replicates for each growth condition (Churchill, 2002). Each microarray slide has at least two technical replicates measured for both channels and, therefore, each CDS has at least $3 \times 2 \times 2 = 12$ intensity measures. A CDS is defined as "detectable" if the majority of its intensity measurements are brighter than the 90% percentile of its surrounding background intensity. In microarray context, a given CDS intensity measurement cannot be detected significantly above its local background due to a series of reasons ranging from limitations on dynamic range or high level of local background hybridization, to real lack of transcriptional activity. On the other hand, a given CDS successfully detected can have its intensity value due to unspecific hybridization or artifacts. We deemed detected CDS as being transcriptionally active but we do not call the not detected CDS as not transcribed acknowledging the limitations above. Since our hybridization stringency is relatively high and the array was constructed with specific primers (Koide et al., 2004), we believe that our "detectable thus expressed" approximation is reasonable.

The $\log_2$-ratio results are calculated only for those CDS that could be detected in both co-hybridized conditions. CDS that could be observed only in one condition relative to other are treated separately since are differentially expressed but do not have measurable expression ratio. Data normalization was carried out essentially as previously described by Koide et al. (2004) using LOWESS fitting on M versus S plot, where M is the $\log_2$-ratio of background subtracted fluorescence intensities (I_{Cy3} and I_{Cy5}), defined as $M = \log_2(I_{Cy5}/I_{Cy3})$, and S is the logarithm of the average intensity, defined as $S = \log_2(I_{Cy5}/2 + I_{Cy3}/2)$ (see supplemental Figs. S1.1–S1.23).

To determine hybridization noise and to estimate dynamic cutoff values for classifying a CDS according to its relative expression levels we used the hybridization data collected from two duplicated biological replicates of self-self hybridization (independent harvest of *X. fastidiosa* grown at 50 mM glucose). The intensity-dependent noise-threshold cutoff values were obtained according to the rationale introduced by Koide et al. (2004), with the improvements described in http://blasto.iq.usp.br/~rvencio/HTself . We used a sliding window of size 1.0, step 0.2 and defined 0.995 credibility intervals. A CDS was considered as consistently differentially expressed if the majority of its M replicated measurements were outside of the 0.995 curves defined by the self-self hybridizations. Using these criteria, up- or down-regulated categories were defined for samples grown at 1 mM and at 250 mM glucose (see supplemental Fig. S2). The complete data set is publicly available under the MIAME guidelines (Brazma et al., 2001) at supplemental web site (http://verjo19.iq.usp.br/xylella/microarray/glucose).

Real-time quantitative RT-PCR

cDNA was generated from 5 μg of total RNA using 200 U of SuperscriptII reverse transcriptase (Invitrogen) and 500 ng of random nonamer primers, according to the manufacture's instructions. cDNA was diluted with nuclease free water to 35 ng/μL and stored at -20°C before use. Real-time quantitative PCR was performed using an ABI PRISM 5700 Sequence Detection System (Applied Biosystems) using default parameters. The PCR mixture included 10 μL of Platinum® SYBR® Green qPCR SuperMix UDG (Invitrogen), 800 nM forward and reverse primers and 180ng template cDNA. In order to confirm the generation of specific PCR products, the PCR was immediately followed by melting curve analysis of the RT-PCR product according to the manufacturer's recommendations (Applied Biosystems). Primers were purchased from Bio-Synthesis, Inc. Primers for amplification of XF0305 or XF2157 were used as endogenous controls to normalize the amount of total RNA per sample. The fold-change of each gene was calculated by using the $2^{-\Delta\Delta C}$T method, as described by Livak and Schmittgen (2001) from three independent experiments. We considered a CDS differentially expressed relative to 50mM glucose standard condition if the mean minus one standard deviation on obtained fold-change exceeded the 1.5-fold value cutoff. PCR primers were designed with PRIMER EXPRESS 2.0 software (Applied Biosystems), and their sequences are given in Table 1.

Table 1. Genes and Primers Used in Real-Time Quantitative PCR

Gene ID[a]	*Gene name*	*Primers*
XF0262	*cvaC*	F 5′GGCGGTATTGCTGGTGCTAT
		R 5′AGCGAAGGTGCCGTTAAAGA
XF0287	*rpfB*	F 5′CGGGCTTCGACAAACTTGA
		R 5′GACGGAGCGCTGGATCAC
XF0305	*nuoA*	F 5′TTCATCGTGCCTTGGACTCA
		R 5′CAGCGCTCCCTTCTTCCATA
XF1115	*rpfF*	F 5′GCTTGGAGGAGGCTTCGAA
		R 5′CACCCCTTCCTCAGCTACGA
XF2361	*gumK*	F 5′GCATGCATCTTTCGGTATTGC
		R 5′CCAAAGCGTGTCGGATAAGAG
XF2157	*dnaQ*	F 5′GGTGCCGAACTGATTATTCACA
		R 5′CAACCGCGATAACTCGTAATCAA

[a]Simpson et al. (2000).

DNA and protein sequence analysis

DNA and protein sequence similarity searches were done with BLAST tools (Altschul et al., 1997) at the NCBI databases and at the *X. fastidiosa* 9a5c genome sequencing consortium database (http://aeg.lbi.ic.unicamp.br/xf/). Sequence alignment was performed with CLUSTAL W software (Thompson et al., 1994). Protein secondary structure prediction was done with NNPREDICT software (Kneller et al., 1990).

RESULTS AND DISCUSSION

Analysis of X. fastidiosa *growth at different concentrations of glucose*

The recent development of defined media for *X. fastidiosa* (Campanharo et al., 2003; Lemos et al., 2003; Almeida et al., 2004; Leite et al., 2004) allowed us to examine the growth kinetics and global gene expression under different glucose concentrations. As shown in Figure 1, growth in XDM_2 containing 1 mM and 250 mM glucose is strikingly different compared to standard XDM_2, which has 50 mM glucose. After 8 days of growth, cell density reached OD_{600} ~1.2 at the standard conditions and was 3–4-fold lower at 1 mM and 250 mM glucose. It can also be observed that cultures grown at low glucose reach the stationary phase within 4 days of growth while this stage is only reached after 8 days at the standard conditions. Interestingly, even after 12 days the stationary phase was not reached for cultures grown at 250 mM glucose. Their log phase was delayed for ~8 days, possibly when glucose declined to optimal levels. In all of the conditions the cellular density correlated well with total protein content. This correlation suggests that *X. fastidiosa* does not alter synthesis of fastidian gum (exopolysaccharide) in response to glucose variations.

Global analysis of X. fastidiosa *gene expression in response to glucose concentration*

We next monitored global transcription profiles of *X. fastidiosa* grown in XDM_2 with 1, 50, and 250 mM glucose. For this we used DNA microarrays representing 94.5% of the 2848 previously annotated coding sequences (CDS) (Simpson et al., 2000; Koide et al., 2004). The microarray slides were competitively hybridized with fluorescently labeled cDNA prepared from RNA samples of 6 day-old cultures. As detailed in Materials and Methods, we considered a CDS as expressed if its intensity measurements were consistently and significantly above the local background. As represented in the diagrams of Figure 2, the analysis of hybridization data show that 2143 CDS were considered expressed at 50mM glucose. On the other hand, only 554 and 1711 CDS were expressed by *X. fastidiosa* cells grown at 1 mM and 250 mM, respectively. These results show that when *X. fastidiosa* was grown in 1 mM glucose XDM_2 an enormous set of

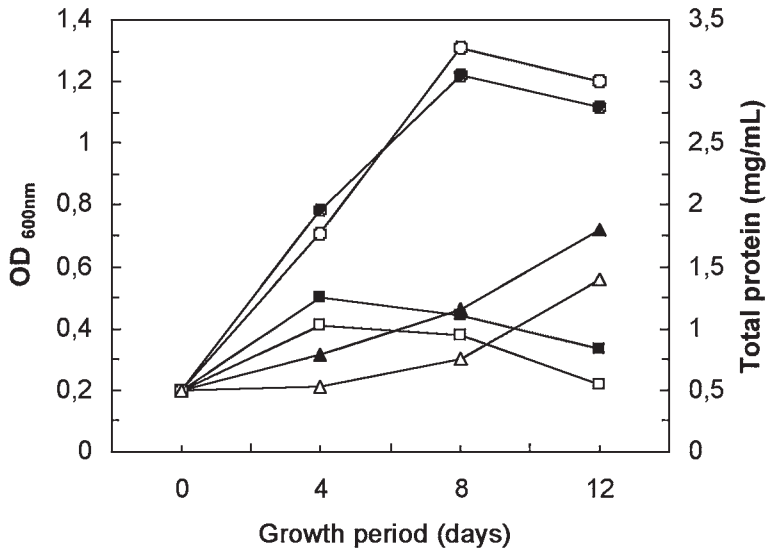


FIG. 1. Growth curves of *Xylella fastidiosa* 9a5c in XDM_2 medium with different concentrations of glucose. Closed symbols represent optical density readings at 600 nm. Open symbols represent total protein content. Squares, circles and triangles represent cultures grown at 1, 50, and 250 mM of glucose, respectively.

transcripts were down-regulated compared to the standard medium. This effect was also observed in cells growing in 250 mM glucose, though to a lesser extent. CDS expressed in each condition are listed in supplemental Table S1 and represented on interactive transcriptome maps that summarize all our hybridization results at the project site (http://verjo19.iq.usp.br/xylella/microarray/glucose). The transcriptome maps provide an overview of genome activity in each hybridization condition, enriched with additional information on each CDS. The project site also offers raw and normalized data and additional information on data collection, processing and statistical analysis.

In addition, the gene expression profiles revealed that 211 out of 551 sequences (see supplemental Table S2), that had been considered invalid CDS in the re-annotation of *X. fastidiosa* strain 9a5c genome (Van Sluys et al., 2002), were transcriptionally active in the standard XDM_2 medium. The fact that we have detected transcriptional activity of these predicted CDS demonstrates the usefulness of DNA microarrays as a complementary tool to validate genome annotations. Given 187 of these expressed CDS are conserved in *X. fastidiosa* strain Temecula, responsible for Pierce's disease in grapevines, we intend to reannotate these CDS as conserved hypotheticals.

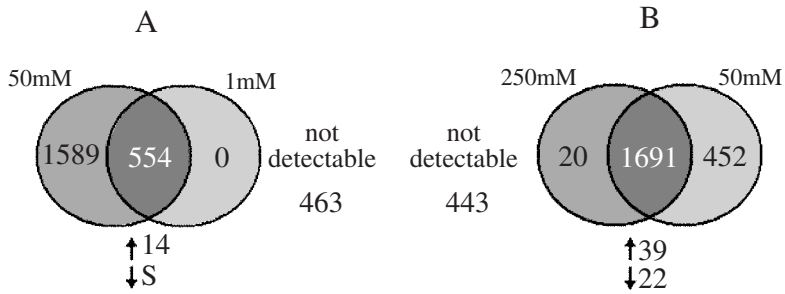


FIG. 2. Gene expression changes in three glucose concentrations. The number of CDSs expressed in cells grown at 50 versus 1 mM (**A**), and 50 versus 250 mM glucose (**B**). White numbers correspond to CDS detected in both conditions, for which differential expression analysis was performed. The number of up- and down-regulated CDS in each condition are indicated below the circles. Undetectable CDS were excluded from further analysis.

Functional characteristics of differentially expressed genes in response to growth on glucose

As mentioned above, transcriptional activity was drastically reduced in *X. fastidiosa* cells grown at 1 mM glucose given that a total of 1589 CDS were not expressed. Moreover, cells grown at 250 mM glucose also reduced the overall number of expressed genes considering 452 CDS were not expressed. Even though these large sets of CDS (supplemental Table S1) should be assigned as differentially expressed relative to growth at 50mM, their precise hybridization ratios were not be calculated. Therefore, the biological implication of these observations will not be addressed in this work. We chose to analyze differentially expressed genes exclusively with genes expressed in both conditions and for these precise ratios were calculated (Fig. 2). When comparing 50 mM versus 1 mM, 14 and 8 out of 554 CDS were found to be up- and down-regulated, respectively (Tables 2 and S3). When 50 mM versus 250 mM were compared, 39 and 22 out of 1691 CDS were up- and down-regulated, respectively (Tables 3 and S3).

Among the 14 CDS up-regulated in 1 mM glucose listed in Table 2, we highlight one that encodes a sugar ABC transporter ATP-binding protein (XF1067) and another that encodes an ATP-dependent Clp protease proteolytic subunit (XF1187). This protease assembles into a disk-like structure with a central cavity, resembling the structure of eukaryotic proteasomes (Kessel et al., 1995). Interestingly, Clp-dependent proteolysis has been recently linked to general bulk protein breakdown at the transition from growing to non-growing phases in *B. subtilis* (Kock et al., 2004).

Table 3 presents the 39 CDS found to be up-regulated upon growth in 250 mM glucose. Twenty-six of them were annotated as hypothetical or conserved hypothetical. Eleven of these reside between CDS XF1648 and XF1693 that comprises a region denoted as XfP4 (for phage number 4). Although the region harboring CDS XF1648-XF1657 is currently not annotated as belonging to this phage, we suspect this phage region starts at CDS XF1642 and not at XF1658 as currently proposed (Simpson et al., 2000). XF1642 encodes a phage integrase whose genomic location is indicative of an island or horizontally transferred region in *X. fastidiosa* 9a5c (Moreira et al., 2004). Expression of phage sequences have been shown to be under the control of nutrient availability in a microarray comparison of *X. fastidiosa* grown in rich and nutrient-limited media (Nunes et al., 2003).

Other up-regulated genes in this condition include XF0395, XF1189, XF2234 and XF2625. The first encodes bacterioferritin, an intracellular reservoir of ferric iron, the second an ATP-dependent serine endopeptidase (Lon), and the other two encode heat shock proteins. This observation suggests *X. fastidiosa* might be undergoing some kind of stress due to growth on high glucose concentration. In *Pseudomonas aeruginosa*, bacterioferritin has been proposed to be involved indirectly in the resistance to redox stress

Table 2. Up-Regulated Genes at 1 mM Glucose

Gene ID[a]	*Product*	*Gene name*	*Ratio*
XF0094	Cell division protein	*ftsJ*	1.6
XF0388	Hypothetical protein	—	2.0
XF0561	Hypothetical protein	—	2.0
XF1067	Sugar ABC transporter ATP-binding protein	DR2153	1.9
XF1187	ATP-dependent Clp protease proteolytic subunit	*clpP*	2.1
XF1189	ATP-dependent serine proteinase La	*lopA*	1.8
XF1530	Subunit C of alkyl hydroperoxide reductqase	*ahpC*	1.9
XF1554	Fumarate hydratase	*fumC*	1.9
XF1693	Hypothetcal protein	—	2.1
XF1796	Bifunctional transcriptional repressor of the biotin operon/ biotin acetyl-CoA-carboxylase synthetase	*birA*	1.4
XF1810	Conserved hypothetical protein	HI0961	2.0
XF2377	Hypothetical protein	—	2.0
XFa0034	Conserved hypothetical protein	STMD1.84	1.8
XFb0001	Replication protein	—	2.1

[a]Simpson et al. (2000).

Table 3. Up-Regulated Genes at 250 mM Glucose

Gene ID[a]	*Product*	*Gene name*	*Ratio*
XF0032	PilY1 gene product	*pilY1*	2.1
XF0261	Colicin V immunity protein[b]	*cvi*	2.3
XF0262	Colicin V precursor	*cvaC*	2.5
XF0264	Colicin V precursor[b]	*cvaC*	2.4
XF0395	Bacterioferritin	*bfr*	2.3
XF0667	Hypothetical protein	—	1.9
XF0700	Hypothetical protein	—	2.3
XF0703	Hypothetical protein	—	2.0
XF1189	ATP-dependent serine proteinase La	*lopA*	1.7
XF1217	Hypothetical protein	—	3.0
XF1224	PilY1 gene product	*pilY1*	2.2
XF1287	Hypothetical protein	—	2.0
XF1550	Oxoglutarate dehydrogenase	*odhA*	1.5
XF1556	Hypothetical protein	—	1.8
XF1648	Hypothetical protein	—	3.6
XF1649	Conserved hypothetical protein	b2360	3.1
XF1650	Hypothetical protein	—	2.3
XF1655	Hypothetical protein	—	2.6
XF1659	Hypothetical protein	—	3.9
XF1661	Hypothetical protein	—	2.8
XF1662	Hypothetical protein	—	2.0
XF1663	Phage-related protein	—	1.9
XF1673	Hypothetical protein	—	2.4
XF1683	Hypothetical protein	—	2.0
XF1693	Hypothetical protein	—	2.4
XF1754	Conserved hypothetical protein	*orf1*	2.1
XF1948	Colicin V production protein	*cvpA*	2.0
XF2005	Hypothetical protein	—	1.8
XF2173	Hypothetical protein	—	2.0
XF2234	Low molecular weight heat shock protein	*hspA*	2.7
XF2377	Hypothetical protein	—	2.0
XF2380	Hypothetical protein	—	2.0
XF2510	Hypothetical protein	—	2.8
XF2550	Outer membrane hemolysin activator protein	*hecB*	1.6
XF2625	Heat shock protein	*htpX*	2.5
XFa0024	Hypothetical protein	—	1.8
XFa0033	Hypothetical protein	—	1.6
XFa0045	Conserved hypothetical protein	—	1.6
XFb0001	Replication protein	—	2.1

[a]Simpson et al. (2000).

[b]Currently annotated as hypothetical proteins.

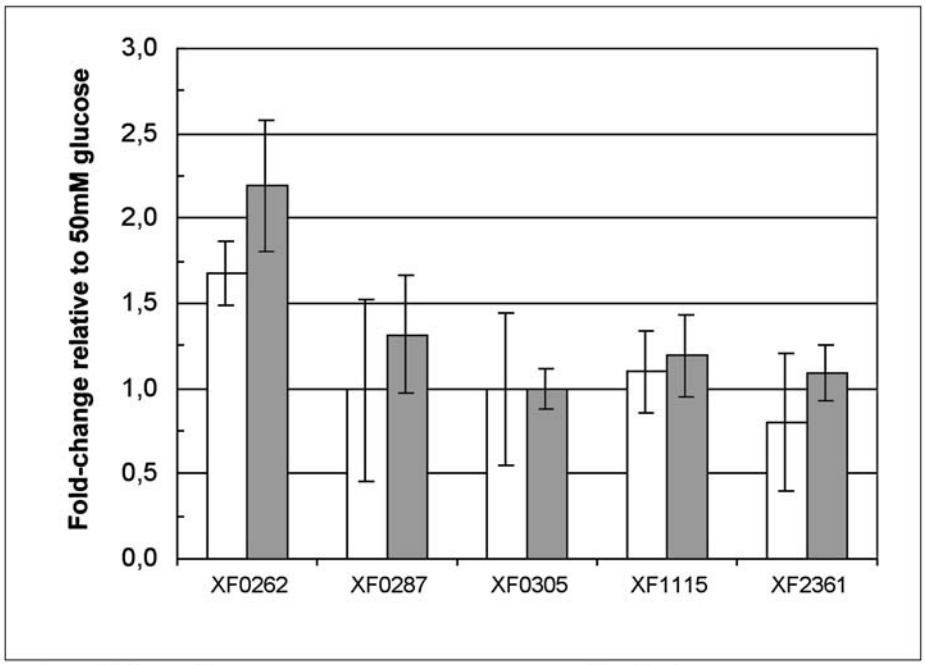
(Ma et al., 1999) and the Lon protease homologue of *Brucella abortus* is essential for survival in a variety of stresses (Robertson et al., 2000).

When cultivated at 250 mM glucose *X. fastidiosa* also up-regulates two CDS (XF0032 and XF1224) that encode PilY1, a transmembrane protein involved in export and correct assembly of the Type-IV pili (Alm et al., 1996). Type IV pili are multifunctional devices at the bacterial surface that can act as virulence factors because of pilus-based motility or formation of biofilms (Nudleman and Kaiser, 2004). Fimbriae- and pili-mediated attachment of *X. fastidiosa* to xylem or cibarium has been suggested as essential to successful colonization (Purcell and Hopkins, 1996; Simpson et al., 2000). Other virulence determinants induced on high glucose include colicin-V relates genes, which will be further discussed below.

Surprisingly, based on our microarray data, the *rpf* and *gum* genes were not induced by the glucose increment used in this study. This was further confirmed by real-time quantitative PCR performed with selected genes (Fig. 3). Our initial premise was that fastidian gum production would be increased as a function of glucose concentration, as observed for *Xanthomonas campestris* pv. *campestris* (Vojnov et al., 2001) and *Xanthomonas axonopodis* pv. *citri* (our unpublished results). These results are specially intriguing since RpfB and RpfF are 70% and 44% identical, respectively, between *X. fastidiosa* and *X. axonopodis* pv. *citri*. These genes are essential to the synthesis of a diffusible signal factor (DSF) (Barber et al., 1997) which in turn activates a transduction pathway that leads to transcriptional regulation of genes required for pathogenic traits, such as EPS and exoenzyme synthesis (Slater et al., 2000). The expression of *rpf* and *gum* genes in *X. fastidiosa* has been recently shown to be affected by cell density *in vitro*, being significantly induced at high density conditions (Scarpari et al., 2003). Taken together, these observations lead us to conclude that cell density but not nutrient availability triggers fastidian gum production.

X. fastidiosa *expresses colicin-V–like toxins and putative anti-toxin*

X. fastidiosa 9a5c genome encodes two colicin-like precursor proteins encoded by *cva*C genes XF0262 and XF0263 (Simpson et al., 2000). Colicin-V is an antibacterial polypeptide toxin produced by *E. coli*,



Gene ID[a]	Gene name	Product
XF0262	*cvaC*	colicin V precursor
XF0287	*rpfB*	regulator of pathogenicity factors
XF0305	*nuoA*	NADH-ubiquinone oxidoreductase, NQO7 subunit
XF1115	*rpfF*	regulator of pathogenicity factors
XF2361	*gumK*	Glycosyltransferase IV

[a] according to Simpson *et al.*, 2000.

FIG. 3. Gene expression of selected CDS in response to glucose variations. Real-time quantitative polymerase chain reactions using templates derived from total RNA samples of *X. fastidiosa* cultures grown at 1 mM glucose (white bars) and 250 mM glucose (gray bars) compared to the standard growth condition (50 mM glucose). Standard error bars from three independent experiments are shown. XF0305 (NADH-ubiquinone oxidoreductase, NQO7 subunit) was used as the normalizer of total RNA input.

which acts against closely related sensitive bacteria (Havarstein et al., 1994). Its synthesis is regulated by CvpA and activation and secretion depend on two other proteins, CvaB and CvaA. The first is an ATP-dependent ABC transporter inserted in the inner membrane of the cell, which transports colicin from the citosol to the periplasm after the cleavage of a signal peptide, and the second is a transport protein inserted in the outer membrane that transports colicin from the periplasm to the external medium, usually with assistance of TolC (Waters and Crosa, 1991; Skvirsky et al., 1995; Zhang et al., 1995). In *X. fastidiosa* 9a5c *cvaA* and *cvaB* are dispersed in the chromosome (Simpson et al., 2000), different from *E. coli*, where these genes are organized in operon present in plasmid pColV (Gilson et al., 1987). Processed colicin-V is lethal against bacteria that have a specific receptor (Cir) but do not express a protein (Civ) that confers immunity against it (Zhang et al., 1995).

Our microarray results indicated that *cva*C XF0262 and the neighboring XF0264 are up-regulated in cells grown at 250 mM glucose (Table 3). Induction of XF0262 was also verified by real-time quantitative PCR (Fig. 3). XF0264 is currently annotated as hypothetical, despite its strong aminoacid similarity to the other two cvaC found in *X. fastidiosa* 9a5c genome (Fig. 4A). The tandem organization of the *cva*C genes in this strain suggests a genomic expansion as this gene is found as single-copy in other strains of *X. fastidiosa* with genomes completely (*X. fastidiosa*-Temecula) or partially sequenced (*X. fastidiosa*-Ann1 and *X. fastidiosa*-Dixon) (Bhattacharyya et al., 2002; Van Sluys et al., 2003). *cvpA* (XF1948) was also up-regulated in this condition, while *cvaA*, *cvaB* and *tolC* were not.

A

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XF0263 MRELTSIEMN NVSGGDLATR IEASIVFGVS AFFAGSIWGG TRGGDGGGIL GVGSIAQGVG
XF0264 MRELTSIEMN NVSGGDFATR LEASIVGGIA AFFAGSIWGG TRGGDGGGVL GIGSIGQGVG
XF0262 MRELTLTEID NVSGADLGSR LSAAIVGGVA AFFAGSIWGG TRGGDGGGIL GVGSIGQGVG
       *****  *:: ****.*:.:* :.*:** *:: ********** ********:* *:***.****

XF0263 MVYGGIVGGI GGLIAGFVLD KNVTYNYAVG FYNSLFNGTFTK (283nt)
XF0264 MVYGGIAGAI GGAIGCFVLD KDVTYSYTTG FMNSLFSGNFAK (227nt)
XF0262 MVYGGIAGAI GGAIAGFVLD KDVIYSYTNG FMSSIFNGTFAK (205nt)
       ******.*.* ** *. **** *:* *.*: * * .*:*.*.*:*
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B

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       ------HHHHHHHHHH---HHHEEHHHHHH-H-------HHHHEHHHHHHHEEEEE----
Q841V5 MDRKRTKLELLFAFIINATAIYIALAIYDCVFREKDFLSMHTFCFSALMSAICYFVGDNY
XF0261        MRSLFGQVFNTNPSDVNEDTVSASLAG-FWFKLSSRIERVLGSLGRQFAGQGY
              -------E-----------HHH-HHH.HHHHH--HHHHHHHHH-HHHH----

       ------------------
Q841V5 YSISDKIKRRSYENSDSK
XF0261 LWERTDSAQNFELN
       EEE-----------
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FIG. 4. (**A**) Comparison of *X. fastidiosa* 9a5c CvaC proteins. The deduced aminoacid sequences of XF0262, XF0263 and XF0264 were aligned using CLUSTALW. Identical and conserved aminoacids are denoted with (*) and (:), respectively. Sequences encoded by spotted amplicons are underlined. (**B**) Pairwise alignment of candidate *X. fastidiosa* Cvi with *E. coli* Cvi. The deduced amino acid sequences of XF0261 and *E. coli cvi* (UniProt accession number Q841V5) were aligned. Bold and underlined letters represent identical and conserved aminoacids, respectively. Secondary structures were predicted using NNPREDICT. H and E represent helices and β-strands, respectively.

Although the colicin-V secretory machinery is complete in *X. fastidiosa* 9a5c, paradoxically no gene encoding a putative immunity protein (*cvi*) was annotated based on Blast searches (Simpson et al., 2000). We then reasoned if a *cvi* analog could be among hypothetical CDS co-regulated with *cvaC*. Among these, the gene that fulfilled the requirements for a *X. fastidiosa cvi* was the hypothetical CDS XF0261, exactly upstream of the *cvaC* genes. XF0261 encodes a protein with similar size to *E. coli cvi* (66 and 74 aminoacids, respectively) and despite their low sequence similarity shown by pair-wise alignment, their predicted secondary structure turned out to be quite similar (Fig. 4B). Both are rich in helixes in the central region and have a small fraction of β-sheets at the carboxi terminus. It has been proposed that the helix-rich structure allows *cvi* insertion in the inner membrane in *E. coli* (Waters and Crosa, 1991). Further experimentation to demonstrate the functionality of this putative *X. fastidiosa* Cvi is warranted. If indeed XF0261 is a *cvi* analog, we can postulate that *X. fastidiosa* is capable of synthesizing, secreting and defending itself against colicin-V. A model for this response is presented in Figure 5. Colicin secretion would allow efficient competition within the xylem and/or insect foregut. In fact, it has been demonstrated that *X. fastidiosa* lives in an endophytic environment in citrus plants (Araujo et al., 2001; Lacava et al., 2004).

X. axonopodis pv *citri*, a member of the same family as *X. fastidiosa*, also infects orange trees but seems incapable of producing colicin-V since *cvaA* and *cvaC* are absent. In *X. campestris* pv *campestris*, besides *cvaA* and *cvaC*, *cvaB* was not found (da Silva et al., 2002). A possible explanation for this is the different life style and habitat of *Xanthomonas*. By living in the mesophyl, an environment rich in nutrients and carbon sources, and by being able to intensively degrade plant tissues, they might not need to antagonize other bacteria in order to reduce local competition, differently from *Xylella*.

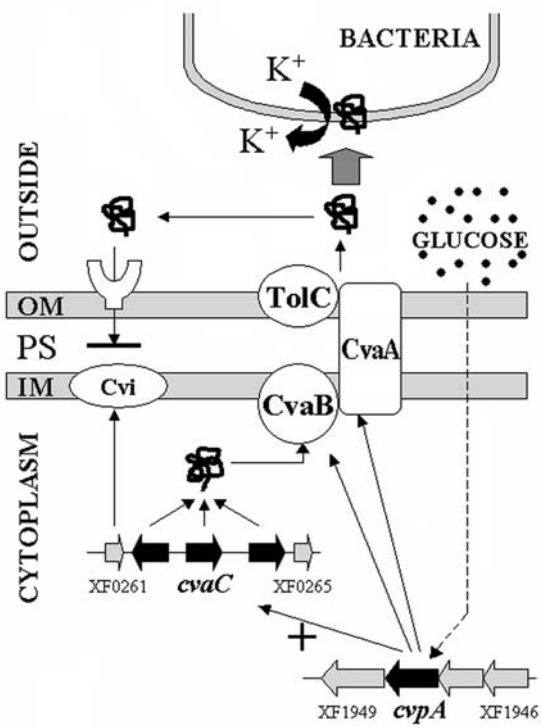

FIG. 5. Model of regulation, synthesis and immunity against colicin-V produced by *X. fastidiosa* 9a5c. Presence of the XF0261 protein inhibits colicin-V toxicity against *X. fastidiosa* 9a5c but allows K^+ efflux, cell membrane imbalance and death of susceptible bacteria. IM, PS, and OM indicate inner membrane, periplasm and outer membrane, respectively.

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SUPPLEMENTAL METHODS

DNA microarray construction

According to Koide et al. (2004). See: http://verjo19.iq.usp.br/xylella/microarray/glucose. PCR primers were designed to amplify unique internal fragments of 200-1000 bp of each predicted CDS described in the annotated genome sequence of *X. fastidiosa* strain 9a5c (http://aeg.lbi.ic.unicamp.br/xf). Primers (18–23mers) with equivalent predicted melting temperature were designed with the use of a perl program that ran PRIMER3 (www-genome.wi.mit.edu/genome_software/ other/primer3.html) for the complete CDS list, automatically testing many parameter settings and also guaranteeing that primers hybridized only to a single genome location. Oligonucleotides were synthesized by MWG and Operon Technologies. Genomic or cosmid DNA, obtained in the *X. fastidiosa* genome sequencing project (Simpson et al., 2000), were used as template in the first round of PCR amplification, and 200-fold-diluted PCR products were used as templates for PCR reamplification to increase product concentration when necessary.

The reactions were done in 96-well plates. The mixture in each well contained 100 ng of DNA, 0.5 U of Biolase Taq polymerase (Bioline), 0.2 mM of each dNTP (Invitrogen), 1.5 mM MgCl2 and the primers at 0.5 μM, in a total volume of 100 μL. A 5-min denaturing step at 95°C was applied, followed by 40 cycles of 95°C for 45 sec, 50°C for 30 sec, 72°C for 1 min and a final step at 72°C for 10 min. 4 μL of each PCR reaction were checked for product size and concentration by electrophoresis in 1.2% agarose gels. The amplicons were then purified with 96-well MultiScreen purification plates (Millipore), and an equal volume of dimethyl sulfoxide was added to the purified products ($\sim$100 ng/μL final concentration). Generation III DNA spotter (Amersham Biosciences) was used to array the samples onto coated type-7 glass slides (Amersham Biosciences). This spotter arrays two technical replicas of each sample, one in each longitudinal half of the slides. Thus, a 6152-element array was printed, representing 2692 CDS spotted at least in duplicate. After deposition, the spotted DNA samples were crosslinked to the coated slides by applying 50 mJ of UV light and the slides were stored desiccated at $\sim$10% relative humidity at room temperature until use.

Hybridization conditions

Labeled DNA or cDNA fragments from both strains were combined in the hybridization mixture containing 50% formamide and hybridization buffer (Amersham Biosciences) in a final volume of 54 μL. The mixture was heated to 92°C for 2 min, cooled on ice, and applied to the microarray. A cover slip was used to spread the solution throughout the microarray and the slide was then placed in a 50-mL Falcon tube that was sealed and horizontally positioned for hybridization in a 42°C water bath for 16 h. After hybridization, slides were washed at 55°C for 10 min in 1 $\times$ SSC buffer containing 0.2% SDS and twice for 10 min in 0.1 $\times$ SSC buffer containing 0.2% SDS, followed by 1 min at room temperature in 0.1 $\times$ SSC and a quick rinse in ddH2O. After drying with N_2 in a clean room, the slides were ready for scanning.

Data acquisition and analysis

A Generation III DNA scanner (Amersham Biosciences) was used to acquire monochromatic images of 10 μm/pixel from the microarray slides, corresponding to channels Cy3 (532-nm laser and PMT at 700 V) and Cy5 (633-nm laser and PMT at 750 V). Images were analyzed with ArrayVision 7.0 software (Imaging Research Inc.). The median-trimed mean density (MTMdens) of signal intensity was the measure chosen for signal quantification. This measure removes pixels with signals below or above 4 MADs (median

of absolute deviation) of the mean signal intensity of all pixels within a spot, such as those representing dust particles. The median of the background intensity was calculated for a frame of 24,000 μm^2 around each spot of 180 μm in diameter, and the value was subtracted from the spot's MTMdens value. This represents the raw data used in the normalization procedure.

Normalization procedure

Several imbalance errors affect the true ratio measure. We have assumed that all imbalance due to enzyme efficiency, wavelength detection, dye and brightness can be approximated by multiplicative factors that are contained into just one normalization constant that depends non-linearly on signal intensities. In order to normalize the Cy3 and Cy5 background-subtracted signal intensities (I_{Cy3} and I_{Cy5}), we have used the hypothesis that the great majority of genes are not differentially expressed and therefore the predominant ratio must be one. This is a reasonable hypothesis given that our microarray contains fragments of all CDS of the 9a5c strain genome. We have performed the LOWESS fitting on M vs S plot in order to obtain, locally and non-parametrically, the normalization constant and thus normalized ratios, following Yang et al. (2002). As defined in our work, $S = \log_2((I_{Cy5} + I_{Cy3})/2)$ and $M = \log2(I_{Cy5}/I_{Cy3})$. Lowess normalization eliminated the dye bias of the ratios.

Intensity-dependent ratio cutoff level

We have performed homotypical (or self-self) hybridizations (9a5c strain independently grown in 50 mM of glucose labeled with either Cy3-dCTP or Cy5-dCTP and hybridized simultaneously to the same microarray) in order to derive intrinsic experimental variability of the 1:1 ratio (noise) and to set an upper and lower limit for this noise. With this approach, we have detected a clear dependence between ratio, estimated by M, and average foreground intensity of each CDS, estimated by S. Fold change of CDS with intensities above but close to background strongly varied inside $[0;\infty]$, or in logarithm scale, inside $[-(;\infty]$ when the expected result is ratio $= 1$ or $\log_2(\text{ratio}) = 0$. We used three independent experiments (three slides, six images, two biological replicas) as samplings from experimental error around 1:1 bi-dimensional probability density distribution. We conditioned this distribution in arbitrary S intervals to make it one-dimensional and estimated the density distribution using Kernel Density Estimators (Silverman, 1986). Finally, we integrated this density around mode peak until 0.995 probability was reached, to determine an intensity-dependent ratio cutoff level. These cutoffs levels were subsequently used in the analysis of replicas of hybridization experiments; spots outside these credibility intervals present strong evidence against 1:1 ratio. The method used is an improvement of the rationale introduced by Koide et al. (2004) and is available online at http://blasto.iq.usp.br/~rvencio/HTself.

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