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The Iron Stimulon of *Xylella fastidiosa* Includes Genes for Type IV Pilus and Colicin V-Like Bacteriocins▽†

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Xylella fastidiosa **is the etiologic agent of a wide range of plant diseases, including citrus variegated chlorosis (CVC), a major threat to citrus industry. The genomes of several strains of this phytopathogen were completely sequenced, enabling large-scale functional studies. DNA microarrays representing 2,608 (91.6%) coding sequences (CDS) of *X. fastidiosa* CVC strain 9a5c were used to investigate transcript levels during growth with different iron availabilities. When treated with the iron chelator 2,2′-dipyridyl, 193 CDS were considered up-regulated and 216 were considered down-regulated. Upon incubation with 100 μM ferric pyrophosphate, 218 and 256 CDS were considered up- and down-regulated, respectively. Differential expression for a subset of 44 CDS was further evaluated by reverse transcription–quantitative PCR. Several CDS involved with regulatory functions, pathogenicity, and cell structure were modulated under both conditions assayed, suggesting that major changes in cell architecture and metabolism occur when *X. fastidiosa* cells are exposed to extreme variations in iron concentration. Interestingly, the modulated CDS include those related to colicin V-like bacteriocin synthesis and secretion and to functions of pili/fimbriae. We also investigated the contribution of the ferric uptake regulator Fur to the iron stimulon of *X. fastidiosa*. The promoter regions of the strain 9a5c genome were screened for putative Fur boxes, and candidates were analyzed by electrophoretic mobility shift assays. Taken together, our data support the hypothesis that Fur is not solely responsible for the modulation of the iron stimulon of *X. fastidiosa*, and they present novel evidence for iron regulation of pathogenicity determinants.**

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When challenged with limiting iron concentrations such as those encountered in host tissues, pathogenic bacteria commonly manage iron homeostasis by releasing iron from intracellular reservoirs and increasing the expression of iron acquisition systems (73). This allows survival in an otherwise prohibiting environment, since iron is an essential cofactor for many proteins mediating electron transfer and redox reactions. Besides functioning as a barrier against pathogens, light control of free iron concentration within host tissues reduces the deleterious effects of iron overload, such as the generation of reactive oxygen species (78, 83). Strict control of iron metabolism is also observed for bacteria, and in most cases it is exerted by the ferric uptake regulator, Fur. When bound to Fe^{2+}, this intracellular iron sensor is capable of binding to Fur boxes at operator sequences, blocking the transcription of genes involved in many cellular processes besides iron uptake (1). The description of the iron regulatory circuitry has recently increased in complexity due to the discovery of other transcriptional repressors and of small noncoding regulatory RNAs (23, 51, 82).

Many pathogenic bacteria have also evolved to couple the iron-limiting response to the expression of other virulence determinants, such as exotoxin A in *Pseudomonas aeruginosa* (47), pectinolytic enzymes in *Erwinia chrysanthemi* (76), type IV pili in *Moraxella catarrhalis* (48), and an adhesin in *Staphylococcus aureus* (11). These and other observations support the hypothesis that the iron concentration is sensed as an environmental signal to successfully establish an infection or colonization process (26, 58, 70, 78, 88).

Transcriptome and proteome analyses of microbes subjected to iron-restricted and iron-replete conditions are adding many genes to the iron stimulon (5, 21, 55, 86). These studies have also provided novel information on the dynamic nature of the iron stimulon, such as the growth phase-dependent response (54), the cross talk between regulons (56), and adaptation to particular conditions (24).

Despite the annotation of many genes related to iron metabolism and pathogenicity in the genomic sequences of different strains of the phytopathogen *Xylella fastidiosa* (79, 84), their function and regulation according to iron availability remain to be demonstrated. Here we report the analysis of steady-state levels of transcripts of this bacterium when subjected to iron-restricted and iron-replete conditions in order to describe its iron stimulon and its relation with virulence determinants. We also investigated the contribution of the ferric uptake regulator Fur to the iron stimulon of *X. fastidiosa*.

MATERIALS AND METHODS

Bacterial strain and growth conditions. *X. fastidiosa* strain 9a5c, isolated from symptomatic *Citrus sinensis* (L.) Osbeck trees with citrus variegated chlorosis (CVC) (44), was grown in periwinkle wilt (PW) (16) broth medium at 28°C with rotary agitation at 80 rpm. The total iron concentration in PW medium is in the

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range of 4.5 μM, as determined by inductively coupled plasma atomic emission spectrometry on a Spectro Ciros charge-coupled device (Spectro Analytical Instruments) (data not shown). The culture was maintained through weekly serial transfers at a 1/10 dilution in PW medium, for no longer than 12 weeks. Exponential-phase cells (7-day-old cultures) were subjected to iron limitation by the addition of 200 μM 2,2′-dipyridyl or to iron excess by the addition of 100 μM ferric pyrophosphate to 500-ml cultures. Aliquots (50 ml) of cell cultures were taken at each time point (0, 15, 60, 240, and 960 min), and cells were immediately harvested by centrifugation (3,000 × *g*, 4 min, 4°C); the culture medium was discarded, and the cells were immediately frozen on dry ice prior to RNA extraction.

RNA isolation and cDNA labeling. Total RNA was isolated with Trizol reagent (Invitrogen), and residual DNA was removed by treatment with 10 U of RQ1 RNase-free DNase I (Promega) and 40 U of the RNase inhibitor RNaseOUT (Invitrogen). RNA integrity was checked through denaturing agarose gel electrophoresis, and the lack of residual DNA was checked by PCR. Quantitations were performed on a NanoDrop ND-1000 spectrophotometer. Total RNA (20 μg) was reverse transcribed and labeled using the SuperScript indirect cDNA labeling system (Invitrogen) according to the manufacturer's instructions.

Microarray construction and hybridization. Microarray slides containing unique internal PCR-amplified fragments representing 2,608 *X. fastidiosa* 9a5c coding sequences (CDS) (91.6% of the total) spotted at least in duplicate were constructed as previously described (41) and hybridized by the method of reference 68.

Microarray data acquisition, filtering, normalization, and analysis. Microarray data analysis was performed according to reference 40. For each cell treatment (iron limitation or iron excess), two biological replicates were performed, using dye swap labeling, throughout five-point time series, resulting in at least 20 data points for each CDS before data filtering. We used intensity-dependent cutoff values for classifying a gene as differentially expressed based on self-self hybridization experiments, as previously described (41, 85). Briefly, the self-self approach consists of simultaneously hybridizing the cDNA from the control sample (growth in regular PW medium) labeled with either Alexa Fluor 555 or Alexa Fluor 647 to estimate the experimental noise. We used credibility intervals of 0.98, a window size of 1.0, and a window step of 0.2. A gene was classified as differentially expressed at a given time point if more than 50% of its replicates were outside the intensity-dependent cutoff curves, when at least two replicates were used.

RT-qPCR. Reverse transcription (RT) was carried out with 5 μg of *X. fastidiosa* total RNA primed with 500 ng of random hexamers using the SuperScript first-strand synthesis system (Invitrogen). One hundred nanograms of the resulting cDNA was subjected to quantitative PCR (qPCR) with 800 nM (each) of the forward and reverse primers and 10 μl of Platinum Sybr green qPCR SuperMix UDG (Invitrogen) on a GeneAmp 5700 system (Applied Biosystems) using the default thermocycler program for all genes. qPCR assays were performed in triplicate with the gene-specific primer pairs shown in Table S1 in the supplemental material from two biological replicas distinct from those performed for microarray analysis. Threshold values were normalized according to the threshold cycle of CDS XF2175 (*dnaQ*), which is expressed at similar levels under all conditions tested according to our microarray data and was also used previously (15, 40). The change in the expression of each gene was calculated using the $2^{-\Delta\Delta CT}$ method (46).

Preparation of recombinant *X. fastidiosa* Fur. The complete CDS of *fur* (XF2344) was amplified from the genomic DNA of strain 9a5c with primers 5′TGAGAAGCATATGGAATTAAATGATTTACG and 5′CACGGTACCTC CACGACCGCGT. The 400-bp amplicon was purified from the agarose gel with a QIAquick gel extraction kit (Qiagen), digested with KpnI and NdeI, and ligated onto KpnI- and NdeI-linearized pET36b (Novagen). The ligation reaction product was electroporated into *Escherichia coli* BL21(DE3) cells (Invitrogen). The pET36b-Xf-Fur (pXf-Fur) construct was isolated from the kanamycin-resistant bacterial transformants, and the insert was sequenced on both strands. Molecular cloning and recombinant expression procedures were essentially as described elsewhere (2), unless otherwise noted. DNA sequencing was performed with BigDye Terminator mix, version 3.1 (Perkin-Elmer), on an ABI 377 DNA automated sequencer (Applied Biosystems).

E. coli BL21(DE3) cells transformed with pXf-Fur were grown in 50 ml LB medium (2) with 1% glucose and 40 μg/ml kanamycin to an optical density at 600 nm (OD_{600}) of ≈0.3. Cells were then harvested by centrifugation at 3,000 × *g* for 1 min, resuspended in 50 ml LB plus 0.3 mM isopropyl-β-D-thiogalactoside (IPTG), and grown for 3 h at 37°C. Following centrifugation at 3,000 × *g* for 3 min at 4°C, the cell pellet was resuspended in lysis buffer (20 mM Tris [pH 8.0], 2 mM $MnCl_2$, 20 mM imidazole, 10% glycerol, 1 mM β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) and sonicated (Branson Sonifier) in an ice bath for five cycles of 10 s with 30-s intervals. The crude extract was centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatant fraction was subjected to standard Ni^{2+} affinity chromatography using nickel-nitrilotriacetic acid agarose (Qiagen). Recombinant *X. fastidiosa* Fur (Fur_{Xf}) was eluted with lysis buffer supplemented with 25 to 300 mM imidazole. Aliquots of purified recombinant Fur_{Xf} were kept at −20°C and used within 2 weeks.

Protein concentrations were determined with the Bradford reagent (Bio-Rad). Recombinant protein expression and purification were monitored by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (42) followed by staining with Coomassie blue.

EMSA. The experimental procedure for electrophoretic mobility shift assays (EMSA) was modified from reference 18. DNA fragments of ≈150 bp were amplified from the genomic DNA of *X. fastidiosa* strain 9a5c, *E. coli* BL21(DE3), or *P. aeruginosa* PA14 (kindly provided by R. Baldini, University of São Paulo, São Paulo, Brazil) with the following primers: Xf0009F (5′-TCTCATGCTA TGGGAAAAGTTAAA), Xf0009R (5′-TGCCAATGGGCTCTATAACA), Xf0010F (5′-CCGTGTACCGGTCAATTTTT), Xf0010R (5′-CTGTGGCAA CGATGAAAAGA), Xf0599F (5′-CCATTTTGATTACCTGACATCATT), Xf0599R (5′-CAGGGGGATGTAATGTCACC), Xf1712F (5′-AGAAATTGA CGCATTGTTTCC), Xf1712R (5′-CCCAAAAAGCATTGACCTGAT), Xf2344F (5′-CATCGGCTGACTAGGTCGTT), Xf2344R (5′-TAAACAAGAGGTCGC CTTGC), XF16SF (5′-GGTCTTGACATCTGCGGAACTT), XF16SR (5′-CA GCCATGCAGCACCTGTCT), Ec-fhuAF (5′-CCCTTTCTTTTCATCTGGT TG), Ec-fhuAR (5′-ACGCGCCATTGGTATATCTC), Pa-pvdSF (5′-TCTGA AACGCCGAAGAATTT), and Pa-pvdSR (5′-GTGGGGTAAGACCCACA CAT).

The amplicons were purified in GFX columns (GE Healthcare) and then labeled with T4 polynucleotide kinase (Fermentas) and [γ-^{32}P]ATP according to the manufacturer's instructions.

For the DNA-protein interaction assays, 10 nM labeled DNA fragment was mixed with increasing concentrations of recombinant Fur_{Xf} at 37°C for 20 min in ligation buffer (10 mM BisTris [pH 7.5], 5 μg/ml salmon sperm DNA, 10% glycerol, 100 μM $MnCl_2$, 100 μg/ml bovine serum albumin,1 mM $MgCl_2$, 40 mM KCl). Competition with a 60-fold concentration of "cold" DNA was performed for each assay. The reactions were resolved (90 min, 185 V) on native 5% polyacrylamide gels previously run at 185 V for 30 min in 400 mM BisTris (pH 7.5) supplemented with 2 mM $MnCl_2$. Dried gels were exposed for 2 h to a Phosphor Screen (Molecular Dynamics), which was then scanned using Storm (Molecular Dynamics).

Computational search for *X. fastidiosa* Fur boxes. Predicted *X. fastidiosa* promoter regions (−350 to +50 bp of predicted traslation start sites) were retrieved from genomic sequence available at http://www.xylella.lncc.br/. This set was then interrogated with 112 Fur boxes characterized in other bacteria (see Table S2 in the supplemental material), using Cross_match (http://www.phrap.org/phredphrapconsed.html) requiring at least 11/19 hits. The putative Fur_{Xf} boxes mapped to CDS down-regulated at high iron concentrations (HIC) were selected, and base frequencies at each position were determined with WebLogo (13).

Microarray data accession numbers. A detailed description of the microarray can be found in reference 41 and at NCBI's Gene Expression Omnibus (GEO) database (http://www.ncbi.nlm.nih.gov/geo) under accession number GPL2708. The complete data set is publicly available according to MIAME guidelines at the GEO database under accession numbers GSE5886 and GSE5888.

RESULTS AND DISCUSSION

***X. fastidiosa* transcriptome in response to iron availability.** The transcriptional profile of *X. fastidiosa* citrus strain 9a5c in response to iron availability was assessed by incubating cells for different times (15, 60, 240, and 960 min) at HIC or low iron concentrations (LIC). Total RNA was isolated and labeled for hybridization to DNA microarrays comprising 91.6% of the CDS annotated in the genome of strain 9a5c (41, 79). As summarized in Table 1, 1,328 and 1,534 CDS were considered to be expressed at HIC and LIC, respectively. Of these CDS, 218 and 256 were classified as up- or down-regulated at HIC, while 193 and 216 were classified as up- or down-regulated at LIC, respectively. As shown in the Venn diagrams in Fig. 1, among the CDS classified as differentially expressed, 74 and

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TABLE 1. Summary of microarray hybridization results[a]

Condition and time[b]	No. of CDS differentially expressed (% of all CDS expressed under the same conditions)	
	Up-regulated CDS	Down-regulated CDS
HIC		
After 15 min	69 (5.2)	87 (6.6)
After 60 min	136 (10.2)	160 (12.1)
After 240 min	149 (11.2)	158 (11.9)
After 960 min	135 (10.2)	173 (13)
Total	218 (16.4)	256 (19.3)
LIC		
After 15 min	23 (1.5)	55 (3.9)
After 60 min	125 (8.1)	134 (8.7)
After 240 min	74 (4.8)	51 (3.3)
After 960 min	61 (4)	46 (3)
Total	193 (12.6)	216 (14.1)

[a] A total of 2,608 CDS were represented on the microarray. Of these, 1,328 CDS were considered expressed under HIC and 1,534 were considered expressed under LIC.

[b] Total, sum of the nonredundant CDS differentially expressed at all time points.

115 CDS, respectively, were up- or down regulated at both HIC and LIC. The identities of the differentially expressed CDS with their respective changes in expression are shown in Tables S3 to S8 in the supplemental material. In addition, transcriptome maps color coded according to the expression status (induction/repression/no change) of each CDS at HIC or LIC are available at the project website (http://verjo19.iq.usp.br/xylella/microarray/iron/).

It should be pointed out that exposure of *X. fastidiosa* cells to the iron chelator or to an excess of iron for 15, 60, 240, or 960 min did not considerably affect its growth rate in the time frame used in this study (Fig. 2). These treatments also did not alter cell viability, as assessed by immediately transferring cells to solid PW medium after each incubation time and allowing them to grow for 7 days (data not shown).

In order to validate our microarray data, transcript levels of a subset of 44 *X. fastidiosa* CDS were analyzed by RT-qPCR. This subset includes CDS related mainly to iron metabolism and/or pathogenesis whose transcripts appeared to be either up- or down-regulated at HIC or LIC according to microarray data. Figure 3 shows that the transcriptional profiles of CDS XF0371 and XF0290 at HIC and of XF0933 and XF1408 at LIC obtained by microarray experiments are in agreement with the RT-qPCR results. In addition, transcript levels of the 44 selected CDS were evaluated at a single time point after incubation of cells under HIC or LIC conditions (Table 2). Taken together, the microarray and RT-qPCR data for all 44 CDS presented an overall Pearson correlation of 0.77 (Fig. 4), which increased our confidence in the microarray data.

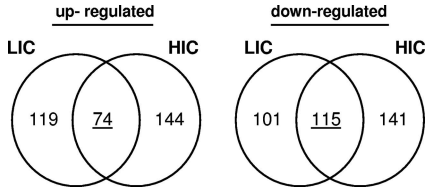

FIG. 1. Summary of differentially expressed CDS according to iron availability. Diagrams show the numbers of CDS up- and down-regulated at either HIC or LIC, as well as the numbers of CDS differentially expressed under both conditions (underlined).

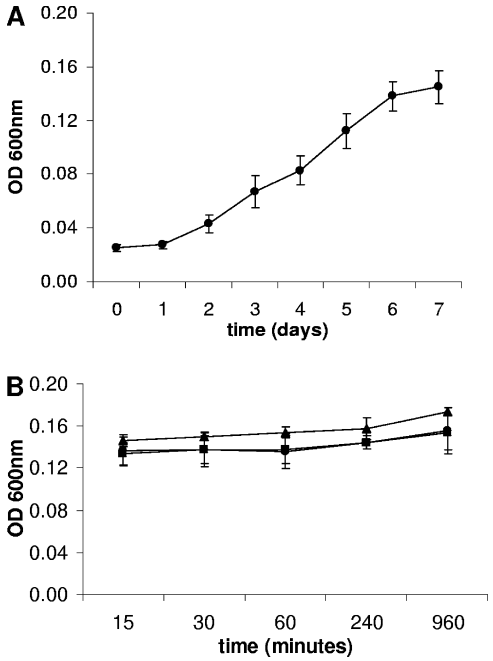

FIG. 2. Growth curves of *X. fastidiosa* 9a5c in PW medium at different iron availabilities. (A) Cells were cultured in PW medium, and OD_{600} was monitored every 24 h for 7 days, after which the culture was divided into three equal portions. (B) One portion received 200 μM 2,2′-dipyridyl (solid squares), the second received 100 μM ferric pyrophosphate (solid triangles), and the third was kept in regular PW medium as a control (solid circles). After 15, 60, 240, or 960 min, the OD for each culture was monitored. Curves show average OD readings from five independent experiments with standard deviations. Samples of cells under the three distinct conditions were taken at each time point shown and plated in solid PW medium in serial dilutions. After 7 days, growth was evaluated, and no difference was observed between samples under control, LIC, or HIC conditions (data not shown).

Functional classification of differentially expressed CDS. From the data presented above, we concluded that 685 CDS exhibited altered expression under at least one of the conditions studied (HIC or LIC). Among these, 285 were originally annotated as hypothetical or conserved hypothetical CDS (79). Using BlastX and Pfam tools, we reannotated and assigned putative functions to 55 of these CDS (see Table S9 in the supplemental material). We believe these observations will be useful for further studies on the biological roles of these previously assigned hypothetical CDS, which indeed are expressed in response to iron availability.

By examining the distribution of differentially expressed CDS into the functional categories according to the *X. fastidiosa* genome database, we observed that CDS involved with cell division, protein, and RNA and energy metabolism are enriched among the down-regulated CDS either at HIC or at LIC (Fig. 5). Repression of genes belonging to these categories has been detected in other bacteria subjected to similar con-

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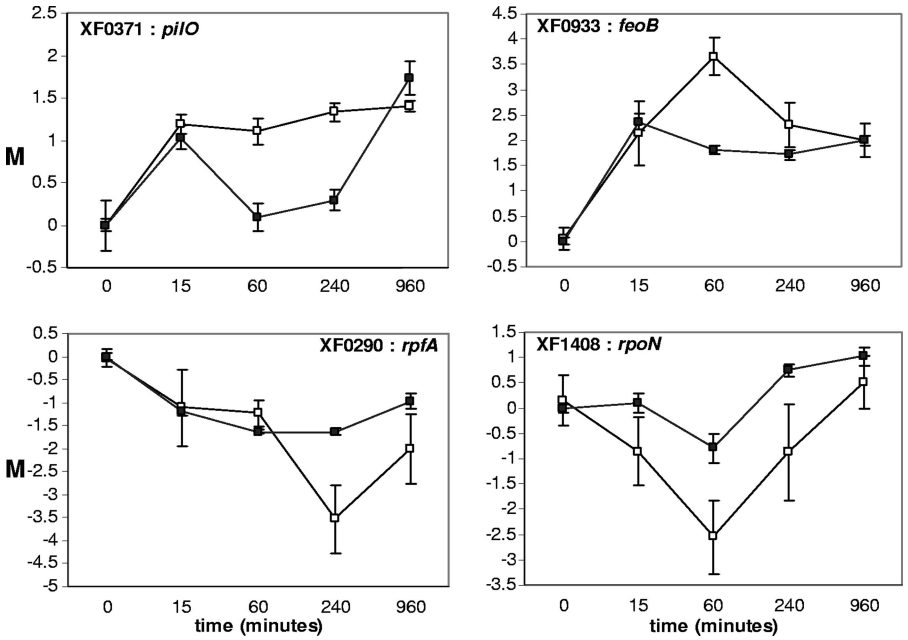

FIG. 3. Microarray data validation. Shown are expression profiles of selected CDS by microarray (open squares) and RT-qPCR (solid squares) from cells treated with 100 μM ferric pyrophosphate (left) or with 200 μM 2,2′-dipyridyl (right). In all plots, M stands for the $\log_2$ of normalized expression ratios (treatment/control).

ditions (3, 23, 63). In *Bacillus subtilis*, iron starvation repressed the expression of two cytochrome systems (*cydABCD* and *qcrAB*) and a number of genes encoding ribosomal proteins (3). Iron depletion also repressed genes encoding ribosomal proteins in *Neisseria gonorrhoeae* (23). Moreover, iron overload repressed cytochrome genes *cyoABCDE* in *P. aeruginosa* (63). These observations suggest that extreme changes in iron levels lead to a general decrease in bacterial metabolism. Despite this, we observed that exposure of *X. fastidiosa* cells to the iron chelator or to an excess of iron under our experimental conditions did not affect the growth rate or cell viability (Fig. 2).

Among the 685 CDS differentially expressed when cells were incubated under HIC and/or LIC conditions (see Tables S3 to S8 in the supplemental material), we highlight those related to iron homeostasis, pathogenicity, and regulatory functions (Table 2). These groups of CDS are discussed in detail below.

Iron uptake, use, and storage. The *X. fastidiosa* genome encodes at least 67 CDS that are related to iron metabolism according to the previous annotation (79). As expected, many of these CDS presented changes in their transcript levels under HIC or LIC conditions (Table 2; see also Tables S3 to S8 in the supplemental material). After 15 min under conditions of iron shortage, increases in transcript levels for the receptors of free iron (*feoAB*) and of iron-complexed compounds (*fiu*) were detected by microarray hybridization (see Table S5 in the supplemental material). Up-regulation of these CDS at LIC was further confirmed by RT-qPCR (Table 2). On the other hand, transcripts encoding intracellular iron reservoirs were less abundant when cells were incubated under low-iron conditions, including the major iron storage protein, bacterioferritin, and other proteins containing iron-sulfur clusters such as RpfA, SdhB, ferredoxins, electron transfer proteins, and flavoproteins. A similar effect has been reported for *Helicobacter pylori*, in which transcripts for ferritin were down-regulated under iron limitation (6). Indeed, studies with several bacteria support the view that modulation of intracellular levels of iron-containing proteins in addition to the control of iron uptake maintains iron homeostasis (83).

Under the iron-replete condition, CDS XF0599 (similar to the Fiu receptor) and XF0550, which encodes a hypothetical protein similar to TonB-dependent receptors, were down-regulated (Table 2). In contrast, *feoAB* transcript levels were not affected. The expression pattern of *fiu* displays a typical Fur-regulated profile, that is, repression under iron-rich conditions and derepression in iron starvation. As will be discussed further, CDS XF0599 is a good example of a Fur-regulated gene in *Xylella*. Moreover, no differential expression was observed for any member of the TonB-ExbBD energy-transducing system (XF0009, XF0010, and XF0011) either at HIC or at LIC (data not shown), although Fur-mediated control of these CDS has been reported for other bacteria (8, 65, 71).

Our data showed that other CDS that contain iron or a Fe-S cluster were also down-regulated under iron overload, including *qcrA* (a component of cytochrome *c*) and *cyoDCA* (components of cytochrome *o*). As discussed above, repression of cytochrome components upon iron overload has also been described for *P. aeruginosa* (63). *hemB* was also repressed under this condition. HemB performs the second step in porphyrin and heme biosynthesis and is essential to soy symbiosis with *Bradyrhizobium japonicum* (10). These authors also propose that δ-aminolevulinic acid is the only heme-containing intermediate capable of being translocated from the plant to the endosymbiont.

In addition, *rpfAB* and *mexB* were down-regulated both at HIC and at LIC (see Table S8 in the supplemental material).

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TABLE 2. Transcript levels of selected CDS analyzed by RT-qPCR

Gene ID	Predicted product	Gene name	Expression[a] with:	
			HIC	LIC
Iron uptake, use, and storage				
XF0932	Ferrous iron transport protein	*feoA*	⇆	↑
XF0933	Ferrous iron transport protein	*feoB*	⇆	↑
XF0599	TonB-dependent receptor for iron	*fiu*	↓	↑
XF0395	Bacterioferritin	*bfr*	↑	↑
XF0550	TonB-dependent receptor for iron[b]		↓	⇆
XF1073	Succinate dehydrogenase iron-sulfur protein	*sdhB*	⇆	↓
XF1889	Ferredoxin-NADP reductase	*fpr*	↓	↓
XF0557	Electron transfer protein azurin I	*az1*	↓	↓
XF0253	Electron transfer flavoprotein alpha subunit	*etfA*	↓	↓
XF0287	Regulator of pathogenicity factors	*rpfB*	↓	↓
XF2094	Multidrug-efflux transporter	*mexB*	↓	↓
XF0908	Cytochrome *c* oxidoreductase	*petA*	↓	⇆
XF1387	Cytochrome *o* ubiquinol oxidase, subunit IV	*cyoD*	↓	⇆
XF1388	Cytochrome *o* ubiquinol oxidase, subunit III	*cyoC*	↓	⇆
XF1390	Cytochrome *o* ubiquinol oxidase, subunit II	*cyoA*	↓	⇆
XF2306	δ-Aminolevulinic acid dehydratase	*hemB*	↓	⇆
XF2344	Ferric uptake regulator	*fur*	⇆	⇆
XF1382	Conserved hypothetical protein (DPS domain)		↓	⇆
Cell division				
XF1320	Cell division topological specificity factor	*minC*	↓	↓
XF1321	Septum site-determining protein	*minD*	↓	↓
XF1322	Cell division inhibitor	*minE*	↓	↓
XF0800	Cell division protein	*ftsZ*	↓	⇆
XF0801	Cell division protein	*ftsA*	↓	⇆
XF0802	Cell division protein	*ftsQ*	↓	⇆
Energy metabolism				
XF1535	Citrate synthase	*gltA*	↓	↓
XF2596	Isocitrate dehydrogenase	*icd*	↓	↓
XF0942	Malate:quinone oxidoreductase	*yojH*	↓	↓
XF2547	Succinyl-CoA synthetase, beta subunit	*sucC*	↓	↓
XF2548	Succinyl-CoA synthetase, alpha subunit	*sucD*	↓	⇆
XF0290	Aconitase	*rpfA*	↓	↓
Pilus and fimbriae				
XF0029	Pre-pilin leader sequence	*pilV*	⇆	↓
XF0033	PilE protein	*pilE*	⇆	↓
XF1632	Twitching motility protein	*pilU*	↓	↓
XF1633	Twitching motility protein	*pilT*	↓	⇆
XF1953	Pilus biogenesis protein	*pilJ*	↑	↓
XF1954	Pilus biogenesis protein	*pilI*	↑	⇆
XF1955	Pilus protein	*pilG*	↑	⇆
XF2544	Pilus biogenesis protein	*pilB*	↑	↓
XF2545	Two-component system, regulatory protein	*pilR*	⇆	↓
XF0370	Fimbrial assembly membrane protein	*pilN*	↑	⇆
XF0371	Fimbrial assembly membrane protein	*pilO*	↑	⇆
XF0372	Fimbrial assembly protein	*pilP*	↑	⇆
XF0082	Chaperone protein precursor	*fimC*	↓	↓
XF0083	Fimbrial subunit precursor	*fimA*	↓	↓
XF1407	Sigma-54 modulation protein	*rpoX*	↓	↓
XF1408	RNA polymerase sigma-54 factor	*rpoN*	↓	↓
XF1843	Nitrogen regulatory protein P-II	*glnB*	↑	↑
XF1848	Two-component system, regulatory protein	*ntrC*	⇆	↑
XF1849	Two-component system, sensor protein	*ntrB*	⇆	↑
Colicin V-like bacteriocins				
XF0261	Colicin immunity protein[b]	*cvi*	↑	↑
XF0262	Colicin V precursor	*cvaC*	↑	↑
XF0263	Colicin V precursor	*cvaC*	↑	⇆
XF0264	Colicin V precursor[b]	*cvaC*	⇆	↑
XF1216	Colicin V secretion protein	*cvaA*	↑	⇆
XF1220	Colicin V secretion ABC transporter	*cvaB*	↑	⇆
Transcriptional regulators				
XF0216	MarR family	*prsX*	↑	↑
XF0972	LuxR/UhpA family	*glpR*	⇆	↑
XF1354	MarR family	*yybA*	⇆	↑
XF1490	MarR/EmrR family		↑	⇆

[a] Arrows indicate up-regulation (↑), down-regulation (↓), or no change (⇆) in transcript levels.
[b] Currently annotated as a conserved hypothetical protein in the *Xylella* genome databank. CDS categorization does not necessarily follow the genome categories assigned by Simpson et al. (79).

rpfB (XF0287) encodes a long-chain fatty acyl coenzyme A (CoA) ligase that is involved in the synthesis of a diffusible signal factor, a molecule important for the pathogenicity of *Xanthomonas campestris* (4). *rpfB* expression is regulated by cell density in both *Xanthomonas* and *Xylella*, for which the existence of a diffusible signal factor has also been proposed (4, 12, 59, 77). MexB (XF2094) is a translocator inserted into the inner membrane that transports multiple drugs and cations

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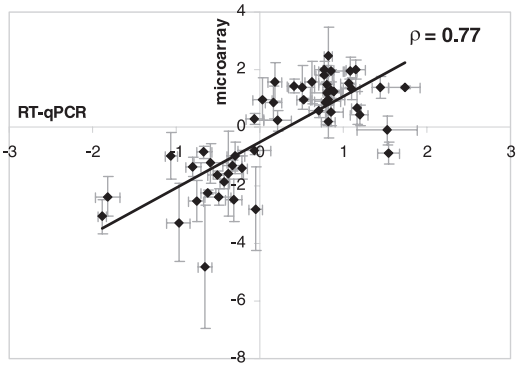

FIG. 4. Overall correlation of the microarray and RT-qPCR data. Both axes show the $\log_2$ of normalized expression ratios (treatment/control). The methodologies showed a Pearson correlation of 0.77.

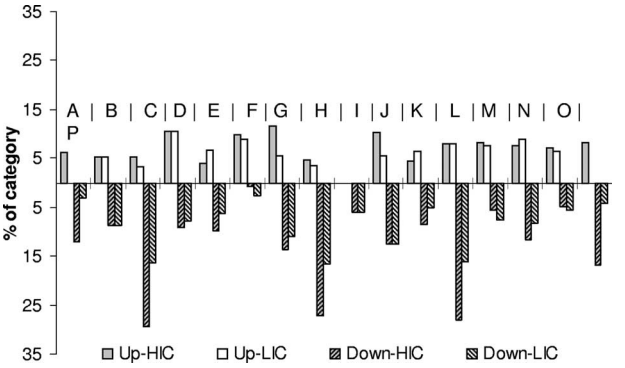

FIG. 5. Functional categorization of differentially expressed CDS. Shaded and open bars show the percentage of up-regulated CDS in each functional category when *X. fastidiosa* cells were exposed to either HIC or LIC, respectively. Hatched bars indicate the percentages of down-regulated CDS. Letters above bars represent functional categories as follows: A, degradation of small molecules (33 CDS); B, central intermediary metabolism (58 CDS); C, energy metabolism (92 CDS); D, regulatory functions (77 CDS); E, biosynthesis of small molecules (226 CDS); F, DNA metabolism (111 CDS); G, RNA metabolism (183 CDS); H, protein metabolism (85 CDS); I, metabolism of other macromolecules (17 CDS); J, cell structure (145 CDS); K, transport (141 CDS); L, cell division (25 CDS); M, mobile genetic elements (144 CDS); N, pathogenicity, virulence, and adaptation (147 CDS); O, hypothetical (1,534 CDS); P, undefined (24 CDS). The 55 reannotated CDS are included in category O in order to follow the original classification by Simpson et al. (79).

through a complex also formed by MexA and OprM (61). In *P. aeruginosa* this gene is induced under iron-limiting conditions, and its product is possibly involved in pyoverdine secretion (69).

The hypothetical CDS XF1382 was also down-regulated under these conditions, and further investigations revealed the presence of a DPS domain (cd01043 [49]) also present in ferritin. Some DPS proteins bind to DNA nonspecifically, conferring protection from oxidative damage, and induction under iron-restricted conditions has also been reported (37).

Transcripts for bacterioferritin, encoded by XF0395, showed opposing results for microarray versus RT-qPCR experiments. The high expression levels observed under both conditions generated signals close to saturation, and this might have generated the discrepancy between the methodologies. RT-qPCR data suggest a positive regulation, in accordance with results observed for *E. coli* and *P. aeruginosa* (51, 89). In these bacteria, the small noncoding regulatory RNAs RyhB and PrrF anneal with different mRNAs including *bfr* and are themselves regulated by Fur, thus causing the observed "up-regulation" of transcripts under abundant iron conditions. In addition, the RNA chaperone Hfq (encoded by XF0089) was up-regulated at LIC (see Table S5 in the supplemental material). This chaperone is essential for the stability of RyhB in *E. coli* (50). In silico searches indicate that no RyhB or PrrF orthologs are encoded in the *Xylella* genome. However, we do not exclude the possibility of the existence of a functional analog, since RyhB and PrrF show no resemblance at the DNA sequence level.

Our data suggest that *X. fastidiosa* does not suffer oxidative stress under the iron-replete condition, since no induction of *sodA* was observed. In *H. pylori*, Fur-mediated control of *sodB* expression is used against oxidative stress (25). However, no *sodB* (Fe superoxide dismutase) ortholog has been identified in the *Xylella* genome (79). Curiously, members of the peroxiredoxin family of antioxidant peroxidases encoded in the *Xylella* genome, *bcp* (XF0961) and *ahpCF* (XF1530 to -31), were repressed under this condition, as were transcripts for thioredoxin, thioredoxin reductase (XF1990 and -1448), ferredoxin (XF1889), and organic hydroperoxide resistance protein (XF1827). In *Staphylococcus aureus*, *bcp*, *ahcCF*, and *trxB* (thioredoxin reductase) are controlled by PerR, a member of the Fur family also involved in sensing oxidative stress (36), but no PerR ortholog has been described for *Xylella*.

Pilus and fimbriae. Type IV pili are polymeric structures present on the cell surfaces of many bacteria. They exert diverse functions, such as adhesion to host surfaces and formation of cellular aggregates, and assist in cell movement (52). *X. fastidiosa* has functional type I and IV pili that participate in biofilm formation; the former promotes cell adhesion, while the latter is essential for cell motility (45, 53). Our data indicate that at LIC, CDS related to these apparatuses, *pilVE*, *pilU*, *pilJ*, and *pilBR*, were down-regulated (Table 2). At HIC, transcripts for *pilUT* and *pilB* were less abundant while transcripts for *pilNOP* and *pilJIG* were more abundant. In addition, under both conditions tested, the type I pilus components *fimC* and *fimA* were down-regulated. A *fimA* mutant of a Pearce's disease-causing strain exhibited a reduced size and number of fimbriae as well as reduced cell aggregation and size. However, this mutant still remained pathogenic to grapevines, corroborating the hypothesis that *Xylella* pathogenesis is multifactorial (28). It is not clear how and to what extent the alterations in transcript levels presented here modify the morphology and function of pili and fimbriae. The precise mechanism behind the transcriptional control of these CDS also remains unclear. Sigma-54 (RpoN) and its modulator RpoX (encoded by XF1408 and XF1407, respectively) might be involved in this response, since these two CDS were down-regulated under both HIC and LIC conditions (Table 2). Additionally, CDS XF1843, XF1848, and XF1849 were up-regulated. The latter two form a two-component system involved in the response to nitrogen, regulated by protein P-II (encoded by XF1843). When phosphorylated, NtrC (XF1848) activates sigma-54 (74). When inactive, sigma-54 is still capable of forming a closed

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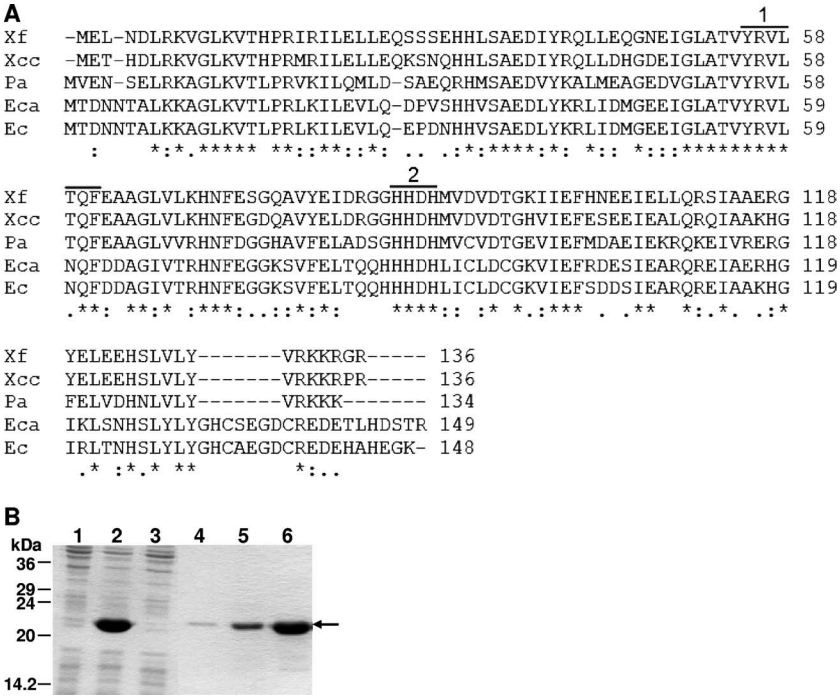

FIG. 6. Characterization of recombinant Fur_{Xf}. (A) Alignment of Fur proteins from different bacteria (all from GenBank): Xf, *Xylella fastidiosa*; Xcc, *Xanthomonas campestris* pv. *campestris*; Pa, *Pseudomonas aeruginosa* PAO1; Eca, *Erwinia carotovora* subsp. *atroseptica* SCRI1043; Ec, *Escherichia coli* K-12. Numbers 1 and 2 above the sequences indicate domains important for DNA and Fe^{2+} binding, respectively. (B) Purification of recombinant Fur_{Xf} by Ni^{2+} affinity chromatography. Lane 1, 10 μg of the soluble fraction of a noninduced culture; lane 2, 10 μg of the soluble fraction of a culture induced with IPTG; lane 3, 10 μg of flowthrough; lanes 4 to 6, fractions eluted with 75, 100, and 150 mM imidazole, respectively. Arrow indicates recombinant Fur_{Xf}.

complex with its target DNA sequences, blocking transcription by steric hindrance (20). In *Vibrio fischeri* and *Dichelobacter nodosus*, type IV pilus biogenesis requires sigma-54 (67, 90), and *Pseudomonas rpoN* mutants were significantly less virulent in multiple hosts (34). Type IV pilus-mediated twitching motility has been shown to be crucial for *Xylella* colonization of upstream vascular regions in planta (53), and here we show that iron is an environmental signal capable of modulating the transcriptional control of these virulence determinants.

Colicin V-like bacteriocins. Colicin V is a pore-forming toxin produced by *E. coli* that acts against closely related sensitive bacteria. Along with the toxin, encoded by *cvaC*, the system involves a production protein encoded by *cvpA*, activation and secretion proteins (CvaB and CvaA) that function assisted by TolC, and an immunity protein, Cvi (27, 33, 87, 91). In *E. coli*, *cvaA*, *cvaB*, *cvaC*, and *cvi* are carried by plasmid pColV-K30 (29), whereas in *X. fastidiosa*, these CDS are dispersed in two regions of the main chromosome (68, 79). According to our data, *cvi* (XF0261), *cvaC* (XF0262, XF0263, and XF0264), *cvaA* (XF1216), and *cvaB* (XF1220) transcripts are more abundant at both LIC and HIC than under control conditions (Table 2). Previous reports have shown the influence of intracellular iron levels on the expression of this toxin in *E. coli* (9). The CDS related to colicin V-like production are among the CDS most frequently expressed by *Xylella*, even under standard growth conditions in PW medium, suggesting that this toxin is a common weapon employed by this pathogen, possibly to counterbalance its slow growth and to allow efficient competition with other microorganisms within the xylem and insect foregut. In fact, *cvaC* was among the differentially expressed CDS identified in a study comparing *X. fastidiosa* freshly isolated from citrus plants with *X. fastidiosa* after several passages in axenic culture, highlighting the importance of *cvaC* in planta (22).

Transcriptional regulators. Our expression data indicate that transcriptional regulators of the MarR and LuxR families are modulated by iron in *X. fastidiosa* (Table 2; see also Tables S3 to S6 in the supplemental material). At LIC, transcript levels for CDS XF0216 and XF1354 were increased. XF0216 and XF1490 transcript levels also increased at HIC. All these CDS are members of the MarR family. Members of this family are widely distributed and regulate functions such as the synthesis of antibiotics and exoenzymes in *Erwinia carotovora*, antimicrobial factors in *Serratia marcescens*, and adhesins and invasion factors in *Yersinia pseudotuberculosis* (57, 81).

Another CDS up-regulated at LIC was XF0972, which encodes a LuxR-type regulator. The LuxR domain is present in many regulators that become phosphorylated by sensor members of two-component systems and are involved in a series of biological functions such as nitrogen fixation in *Rhizobium meliloti*, bioluminescence in *V. fischeri*, and sporulation in *B. subtilis* (39, 75, 80). Regulators with LuxR domains are also involved in quorum sensing by derivatives of homoserine lactones, and recently it was demonstrated that *Erwinia carotovora* ExpR activates the transcription of a global RNA regulator (14). Iron deprivation also caused reductions in transcript

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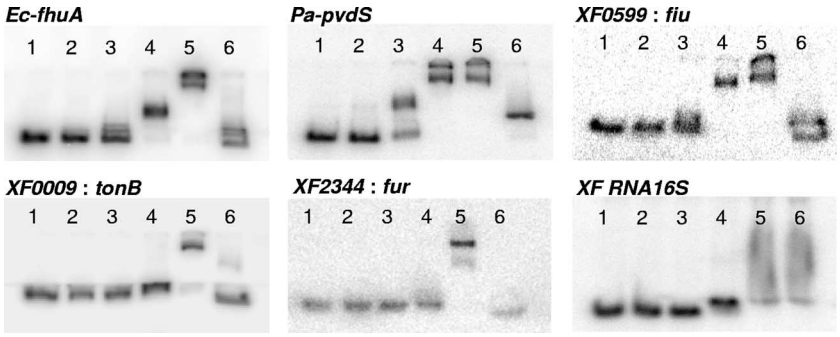

FIG. 7. EMSA of promoter regions and Fur_{Xf}. In all assays, 10 nM labeled DNA was incubated with recombinant Fur_{Xf} (0, 10 nM, 100 nM, 1 μM, 10 μM, and 10 μM in the presence of unlabeled DNA at a 60-fold excess [lanes 1 to 6, respectively]). The mobility shift seen with 10 μM Fur_{Xf} (lane 5) was considered nonspecific, given that this amount caused some retardation of the DNA fragment from the 16S rRNA gene and of fragments from the promoter sequences of *tonB* (XF0009) and *fur* (XF2344), which show no evidence of Fur regulation based on microarray and RT-qPCR data plus in silico Fur box searches.

levels of the regulator *pilR* (XF2545). Orthologs of *pilR* are directly involved with type IV pilus biosynthesis (67), and its negative regulation may account for the lower transcript levels observed for other *pil* CDS observed under iron limitation.

Characterization of the Fur protein of *X. fastidiosa*. The ferric uptake regulator protein (Fur) is known to play a central role in iron homeostasis in the organisms for which large-scale expression data are available (3, 19, 63, 66). As more organisms are studied, however, other transcriptional regulators are proving to take part in the iron stimulon (23, 32, 38, 82).

To investigate how much of the iron stimulon is controlled by Fur in *Xylella*, we performed functional studies with the Fur protein and putative Fur boxes. A *fur* ortholog (Fur_{Xf}) is present in the genome of strain 9a5c, and its predicted amino acid sequence shows 61% identity to that of *P. aeruginosa* Fur. Sequence alignment of Fur_{Xf} (Fig. 6A) with other members of the *Proteobacteria* revealed the highly conserved motifs YRVLNQF and HHDH, which are important for targeting the DNA sequence (Fur box) and for binding to an Fe^{2+}, respectively (30). Initial sequence alignment revealed an extra 28-residue N-terminal segment on Fur_{Xf} that is not present in any of the Fur proteins in COG0735 (data not shown). Therefore, we concluded this to be a wrong assignment of the translational start and excluded it from further analysis. Hence, the sequence coding for the remaining 136 amino acids was used for preparation of the recombinant protein that was purified by His-tagged affinity chromatography (Fig. 6B).

Recombinant Fur_{Xf} was then used in EMSA to check its binding to Fur box-like DNA sequences. Purified Fur_{Xf} was initially assayed with the promoter regions of *E. coli fhuA* ($fhuA_{Ec}$) and *P. aeruginosa pvdS* ($pvdS_{Pa}$) (Fig. 7). Both CDS have been shown to be Fur regulated, and the corresponding Fur boxes have been determined (7, 62). The recognition of both Fur boxes by Fur_{Xf} encouraged us to search for conserved Fur boxes in the strain 9a5c genome. A similarity search in the promoter regions of strain 9a5c using a collection of 112 Fur boxes characterized in other bacteria (see Table S2 in the supplemental material) retrieved 653 putative *Xylella* Fur boxes. Sixty-one candidates mapped within the promoter regions of 49 CDS negatively regulated at HIC. Among these CDS is XF0599, which encodes a putative TonB-dependent outer membrane receptor similar to *E. coli* Fiu. XF0599 is indeed a good example of Fur-mediated transcriptional regulation in *X. fastidiosa*, as we mentioned above. Along with the antagonism observed between its transcript levels and the iron concentration, its promoter region contains two Fur boxes with 13/19 and 14/19 perfect matches with the *E. coli* consensus 5′-GATAATGATAATCATTATC, which are also recognized by recombinant Fur_{Xf} according to the EMSA for which results are shown in Fig. 7. Based on these results, a consensus was

FIG. 8. Sequence logo of the Fur box of *X. fastidiosa* 9a5c. Searches for Fur boxes in the *Xylella* genome were performed using 112 previously characterized Fur boxes listed in Table S2 in the supplemental material. The putative Fur_{Xf} boxes that mapped to CDS down-regulated at HIC were selected, and a consensus was built from 36 putative *Xylella* Fur boxes similar to those found in the operator sequences of XF0599, $fhuA_{Ec}$, and $pvdS_{Pa}$. Base frequencies at each position were determined with WebLogo (13).

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built from 36 putative Fur boxes similar to those found in the operator sequences of XF0599, $fhuA_{Ec}$, and $pvdS_{Pa}$ (Fig. 8).

Our microarray data along with the Fur box searches suggest that only 20% of iron-regulated CDS are also Fur regulated in *X. fastidiosa*. Alternatively, other nonconserved Fur boxes might exist in this bacterium. Although other regulators may take part in the iron stimulon, the recognition of unknown target sequences by Fur cannot be excluded. On the other hand, several promoters containing consensus Fur boxes identified by bioinformatics searches appeared not to be regulated by iron or Fur in *Campylobacter jejuni*, indicating that the Fur box consensus needs experimental refinement in each case (35).

Despite the fact that *tonB* (XF0009) was not considered differentially expressed in our array experiments, its promoter region was also assayed for Fur_{Xf} interaction, although no conserved Fur box was detected in it. As expected, no gel retardation was observed (Fig. 7). We also analyzed the promoter region of *fur*, since autoregulation has been reported in different bacterial species (17). No conserved Fur box was detected, and neither gel retardation nor differential expression was observed for *X. fastidiosa fur* (Fig. 7). Moreover, the fur_{Xf} promoter is bidirectional. As previously pointed out (72), none of the bacteria where the *omlA-fur* context is conserved are capable of the otherwise frequently observed autoregulation of Fur.

Concluding remarks. Whole-genome transcriptome analysis has provided a wealth of information for organisms such as the citrus-infecting strains of *X. fastidiosa*, for which genetic tools are still in their infancy. By investigating the response of this bacterium to extreme variations in iron concentrations, we found that many CDS expected to be iron regulated were not significantly affected by the iron shift, such as hemolysin-like RTX toxins or the hemagglutinin-like secreted proteins previously shown to be involved in biofilm maturation within the xylem vessels (31). Interestingly, the iron stimulon of *Xylella* goes beyond uptake and storage systems, encompassing type IV pilus and colicin V production. This suggests that iron sensing might be important in the early stages of plant colonization, to activate systems that allow efficient translocation throughout xylem vessels and competition against endophytes, and not for biofilm formation. According to current models of biofilm formation by *Xylella*, cells initially multiply and adhere to each other and host surfaces; later, they synthesize fastidian gum and hemagglutinin-like adhesins for biofilm maturation, when vessel clogging occurs and symptoms become evident (43, 60, 64, 77). These findings warrant further studies in order to better understand the regulation of *X. fastidiosa* pathogenicity determinants as well as to evaluate their importance for its survival within both host and vector. Our results also indicate that although iron concentration influences the abundance of many transcripts, only a fraction of these transcripts appear to be directly regulated by Fur. Other transcriptional regulators are possibly involved, particularly those regulated by the iron shift. Combined transcriptome and proteome approaches (21, 86) are needed to verify whether Fur-independent regulation extends to the post-transcriptional level.

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