Identification of a response regulator involved in surface attachment, cell–cell aggregation, exopolysaccharide production and virulence in the plant pathogen *Xylella fastidiosa*

TANJA M. VOEGEL^{1,†}, HARSHAVARDHAN DODDAPANENI², DAVIS W. CHENG², HONG LIN², DRAKE C. STENGER², BRUCE C. KIRKPATRICK¹ AND M. CAROLINE ROPER^{3,*}

¹Department of Plant Pathology, University of California, Davis 95616, CA, USA

²Crop Diseases, Pests & Genetics, USDA-ARS, Parlier 93648, CA, USA

³Department of Plant Pathology and Microbiology, University of California, Riverside 92521, CA, USA

SUMMARY

Xylella fastidiosa, the causal agent of Pierce's disease of grapevine, possesses several two-component signal transduction systems that allow the bacterium to sense and respond to changes in its environment. Signals are perceived by sensor kinases that autophosphorylate and transfer the phosphate to response regulators (RRs), which direct an output response, usually by acting as transcriptional regulators. In the *X. fastidiosa* genome, 19 RRs were found. A site-directed knockout mutant in one unusual RR, designated XhpT, composed of a receiver domain and a histidine phosphotransferase output domain, was constructed. The resulting mutant strain was analysed for changes in phenotypic traits related to biofilm formation and gene expression using microarray analysis. We found that the *xhpT* mutant was altered in surface attachment, cell–cell aggregation, exopolysaccharide (EPS) production and virulence in grapevine. In addition, this mutant had an altered transcriptional profile when compared with wild-type *X. fastidiosa* in genes for several biofilm-related traits, such as EPS production and haemagglutinin adhesins.

INTRODUCTION

The plant pathogenic, Gram-negative bacterium *Xylella fastidiosa* is the causal agent of Pierce's disease (PD) of grapevine and many other economically important diseases, such as almond leaf scorch, citrus variegated chlorosis and oleander leaf scorch (Hopkins, 1989; Hopkins and Purcell, 2002; Purcell and Hopkins, 1996). The bacteria are transmitted in nature by xylem-feeding insects, mainly sharpshooters (Hewitt *et al.*, 1946). Once introduced into the plant xylem, *X. fastidiosa* forms biofilms that are thought to contribute, together with tyloses of host origin, to the occlusion of the xylem vessels, which may result in host plant

death (Hopkins, 1989; Thorne *et al.*, 2006). During *X. fastidiosa* biofilm formation, the cells primarily attach to the surface via large afimbrial adhesins and pili. The cells aggregate via haemagglutinins (HAs) and pili, which leads to the formation of microcolonies that subsequently form a mature matrix-encased biofilm in the xylem vessels (Feil *et al.*, 2007; De La Fuente *et al.*, 2008; Guilhabert and Kirkpatrick, 2005; Li *et al.*, 2007; Meng *et al.*, 2005; Voegel *et al.*, 2010). The *X. fastidiosa* biofilm matrix has been shown to contain exopolysaccharide (EPS), DNA and proteins (Cheng *et al.*, 2010; Roper *et al.*, 2007b).

Similar to other bacteria, *X. fastidiosa* has evolved to cope with changes in its physical and biological environment. The ability to rapidly sense and respond to its environment is particularly pertinent when *X. fastidiosa* makes the abrupt transition from life inside the insect foregut to life inside the plant xylem tissue, and vice versa. Bacteria utilize two-component signal transduction (TCST) systems to sense and respond to their environment (Heeb and Haas, 2001; West and Stock, 2001). The prototypical TCST system is composed of two proteins, a sensor kinase and a cognate response regulator (RR). Diverse abiotic (pH, temperature, osmolarity) and biotic (host-produced or quorum-sensing) signals are typically perceived by the sensor domain of histidine protein kinases (HKs) leading to autophosphorylation of the HK. The phosphoryl group is then transferred to the RR, which modulates the expression of downstream genes, including genes important for biofilm formation and virulence (Heeb and Haas, 2001; West and Stock, 2001). RR proteins typically contain a conserved N-terminal CheY-like receiver domain (REC) that can function either as a stand-alone module (Armitage, 1999; Szurmant and Ordal, 2004) or can be C-terminally associated with a DNA-binding (Stock *et al.*, 2000; West and Stock, 2001), RNA-binding (Shu and Zhulin, 2002), enzymatic output (Galperin, 2006) or protein-binding (Rosario *et al.*, 1994) domain.

In silico analysis of the *X. fastidiosa* genome identified a gene, designated *xhpT*, which encodes an RR containing a prototypical CheY-like REC domain and a histidine phosphotransferase (HPT) protein-binding domain. This is an unusual domain organization for bacterial RRs in general, with an occurrence of less than 0.1% (mostly found in proteobacteria), and is the only one of its type

*Correspondence: Email: mcroper@ucr.edu

†Present address: Department of Biology, University of British Columbia Okanagan, Kelowna, BC, Canada.

found in the *X. fastidiosa* genome (Galperin, 2006, 2010). Interestingly, gene neighbourhood analysis suggested that *xhpT* participates in the regulation of EPS synthesis, because of its location directly upstream of the *gum* operon, which encodes proteins putatively involved in the biosynthesis of the EPS polymer, fastidiosis gum (da Silva *et al.*, 2001). This polymer is a major component of the *X. fastidiosa* biofilm matrix *in vitro* and *in planta* (Roper *et al.*, 2007b). This observation prompted us to investigate the role of XhpT as a regulator of EPS synthesis, as well as of phenotypes associated with other steps involved in biofilm formation. In addition, microarray analyses on an *xhpT* null mutant were conducted. The results further elucidated the contribution of this unusual RR to the biology and pathogenicity of *X. fastidiosa*.

RESULTS

Protein domain analysis of XhpT

The 714-bp gene coding for PD1386, designated XhpT, for *Xylella* histidine-containing phosphotransferase, is located immediately upstream of, but transcribed in the opposite orientation to, the *gum* operon that encodes genes putatively involved in EPS production (da Silva *et al.*, 2001; Simpson *et al.*, 2000; Van Sluys *et al.*, 2003). XhpT is most likely a nonsecretory cytoplasmic protein as analysed by SMART (simple modular architecture research tool). Located at the C-terminus (amino acids 10–118) is a REC superfamily domain, and located at the N-terminus (amino acids 161–235) is an HPt (histidine-containing phosphotransferase) domain.

The *xhpT* mutant is altered in cell–cell aggregation and surface attachment

The *xhpT* null mutant formed fewer bacterial cell aggregates (Fig. 1A) and was compromised in attachment to a solid surface (Fig. 1B) after 9 days of incubation in liquid culture when compared with wild-type (WT) cells. Both strains reached stationary phase by 9 days of incubation, which was the time point at which all phenotypes and gene expression profiles were measured.

The *xhpT* mutant produces more EPS matrix

Protein A double-antibody-sandwich enzyme-linked immunosorbent assay (ELISA) using *X. fastidiosa*-specific EPS antibodies was employed to quantify EPS associated with *X. fastidiosa* cells grown on PD3 plates (Roper *et al.*, 2007b). The *xhpT* mutant produced significantly more EPS relative to WT *X. fastidiosa* when cells were assayed at a concentration of 10^8 colony-forming units (cfu)/mL (Fig. 2).

Virulence and host colonization studies

PD symptom onset began at 8 weeks post-inoculation in grapevines inoculated with either *xhpT* or WT *X. fastidiosa*. We scored

Fig. 1 (A) Cell–cell aggregation assay of *Xylella fastidiosa* wild-type (WT) Fetzter and *xhpT* mutant. The mutant forms fewer cell aggregates relative to WT cells. (B) Surface attachment of *X. fastidiosa* WT Fetzter and *xhpT*. The mutant attaches less well to polystyrene surfaces relative to WT *X. fastidiosa*. Error bars indicate standard deviation. Letters indicate groups assigned by Tukey's honestly significant difference test. OD, optical density.

plants at 18 weeks post-inoculation on the arbitrary disease scale and observed that more WT-inoculated plants reached a rating of '5' (dead or dying) relative to plants inoculated with the *xhpT* mutant strain, meaning that more plants inoculated with the *xhpT* mutant strain exhibited fewer PD symptoms at this time point and were still alive. Specifically, the survival rate of plants inoculated with WT was only $60\% \pm 8.94\%$ at 18 weeks post-inoculation, whereas the survival rate was $90\% \pm 5.48\%$ for plants inoculated with the *xhpT* mutant (Fig. 3), indicating that a mutation in *xhpT* resulted in a reduction in overall virulence.

Twelve weeks after inoculation, bacteria were quantified from infected petioles. There was no significant difference in the

Fig. 2 Quantification of exopolysaccharide (EPS) production in *Xylella fastidiosa* wild-type (WT) Fetzer and *xhpT* mutant using protein A double-antibody-sandwich enzyme-linked immunosorbent assay (ELISA). The mutant produces more EPS relative to WT cells. Error bars indicate standard deviation. Letters indicate groups assigned by Tukey's honestly significant difference test.

Fig. 3 Survival rates of *Vitis vinifera* var. Thompson seedless grapevines inoculated with *Xylella fastidiosa* wild-type (WT) Fetzer and *xhpT* mutant strain, 18 weeks after inoculation. Plants inoculated with the *xhpT* mutant showed an increased survival rate relative to plants inoculated with WT.

bacterial population between the two strains at the point of inoculation ($P = 0.5939$) or 25 cm above the point of inoculation ($P = 0.4725$), indicating that the mutant was capable of colonizing the plants to WT levels. Therefore, the decrease in virulence cannot be attributed to a colonization defect, indicating that XhpT may be involved in the regulation of virulence factors that specifically affect PD symptom development. No bacteria were recovered from plants inoculated with buffer alone.

Microarray and quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analyses

To test the effects of a mutation in *xhpT* at the transcriptional level, microarray analysis was performed. Overall, 520 genes were at least two-fold up-regulated and 115 genes were at least two-fold down-regulated in the *xhpT* mutant relative to WT, as shown in Tables S1 and S2 (see Supporting Information). Select genes with differences in expression levels were organized into functional groups and are listed in Table 1 (genes that were up-regulated) and Table 2 (genes that were down-regulated).

qRT-PCR was used to validate differences in gene regulation found by microarray analysis for PD0146, PD0215 (*cvaC*), PD0956, PD1299, PD1367 (*colR*), PD1391 (*gumH*), PD1395 (*gumC*), PD1396 (*gumB*), PD1702, PD1703, PD1711 (*tonB*) and PD3039, and the down-regulation of PD1792 (*hxfB*) and PD2118 (*hxfA*). The qRT-PCR data for all genes listed above followed the same trend as the microarray data, thus confirming the robustness of the microarray data.

DISCUSSION

RRs are part of TCST systems and act as phosphorylation-regulated switches coupling microbial responses to environmental cues. With an increasing number of annotated bacterial genomes, new RRs that possess atypical domain architectures have emerged (for an overview of domain architectures, see Galperin, 2006, 2010). Among the different possibilities for output domains, less than 0.1% of RRs from a nonredundant set of completely sequenced bacterial genomes possess an Hpt domain (Galperin, 2010) and, in *X. fastidiosa*, XhpT is the only protein that has been identified with a REC-Hpt domain architecture. In this study, we sought to characterize the role of the XhpT RR based on genetic linkage to the *gum* operon and its unique domain structure within the *X. fastidiosa* genome.

The simplest and most common example of a signal transduction pathway is a TCST system composed of a sensor HK and its cognate RR. Because XhpT does not possess an obvious DNA-binding domain, direct action as a transcriptional regulator is unlikely. Instead, XhpT may serve as a 'signal integration locus' within a multistep signal transduction network (e.g. a phosphorelay). This more complex type of two-component phosphotransfer scheme involves several proteins, rather than just a sensor kinase and its cognate RR. The phosphoryl group is commonly transferred from a hybrid kinase via a histidine-containing phosphotransfer protein (Hpt) to the DNA-binding RR (West and Stock, 2001). In *X. fastidiosa*, we speculate that XhpT transfers phosphate to the receiver domain of an unidentified downstream RR that acts as a transcription factor, initiating a change in gene expression (Goulian, 2010). Twelve genes listed in Tables 1 and 2 that

Table 1 Genes that are at least two-fold up-regulated in *xhpT*, organized into functional groups. A complete list can be found in Table S1 (see Supporting Information).

Gene	Gene product	Fold change
EPS production		
PD1388/ <i>gumK</i>	GumK protein	3.79
PD1391/ <i>gumH</i>	GumH protein	2.66
PD1395/ <i>gumC</i>	GumC protein	3.4
PD1396/ <i>gumB</i>	GumB protein	3.59
Attachment/aggregation/motility		
PD0060/ <i>fimD</i>	Outer membrane usher protein precursor	2.13
PD0731/ <i>xadA</i>	Outer membrane protein-XadA	2.9
PD1928/ <i>pilR</i>	TCST, regulatory protein	3.1
PD1924/ <i>pilA</i>	Fimbrial protein	2.45
PD1148/ <i>pilU</i>	Twitching motility protein	2.43
PD1691/ <i>pilQ</i>	Fimbrial assembly protein	2.41
PD1147/ <i>pilT</i>	Twitching motility protein	2.91
Gene regulation		
PD0092/ <i>lexA</i>	LexA repressor	2.16
PD0595/ <i>yybA</i>	Transcriptional regulator/MarR family	5.07
PD0708	Virulence regulator	3.62
PD0945/ <i>exsB</i>	Transcriptional regulator	2.73
PD1153/ <i>algR</i>	TCST, regulatory protein	2.16
PD1276/ <i>algH</i>	Transcriptional regulator	2.11
PD1367/ <i>colR</i>	TCST, regulatory protein	9.55
PD1905/ <i>xrvA</i>	Virulence regulator	2.7
PD1909	Transcriptional regulator	3.1
PD1919/ <i>colR</i>	TCST, regulatory protein	2.49
PD2050	TCST, hybrid sensor and regulatory protein	2.35
Potential virulence factors		
PD0215/ <i>cvaC</i>	Colicin V precursor	8.72
PD0499/ <i>cvaB</i>	Colicin V secretion	2.25
PD0529/ <i>guxA</i>	Cellulose, 1,4- β -cellobiosidase	2.34
PD0708	Virulence regulator	3.6
PD0855/ <i>virK</i>	VirK protein	7.05
PD1341/ <i>vapI</i>	Virulence-associated protein	3.73
PD1640/ <i>bglX</i>	β -Glucosidase	2.24
PD1702	Hyp protein w/lipase domain	8.41
PD1703	Hyp protein w/lipase domain	33.47
PD1905/ <i>xrvA</i>	Virulence regulator	2.7
PD1964/ <i>tolC</i>	Outer membrane export factor	6.01
PD2061/ <i>egl</i>	Endo-1,4- β -glucanase	3.47

EPS, exopolysaccharide; TCST, two-component signal transduction.

Table 2 Genes that are at least two-fold down-regulated in *xhpT*, organized into functional groups. A complete list can be found in Table S2 (see Supporting Information).

Gene	Gene product	Fold change
Cell-cell aggregation		
PD1792/ <i>hxfB</i>	Haemagglutinin-like protein	0.35
PD2118/ <i>hxfA</i>	Haemagglutinin-like protein	0.3
Attachment/aggregation/motility		
PD0024/ <i>pilE</i>	PilE protein	0.42
PD00845/ <i>pilG</i>	Pilus protein	0.43
PD1926	Fimbrial assembly protein	0.25
PD1929/ <i>pilS</i>	TCST, sensor protein	0.49
Gene regulation		
PD0279	GGDEF family protein	0.47
Potential virulence factors		
PD0310	Pathogenicity-related protein	0.44
PD1412/ <i>hlyB</i>	Toxin secretion	0.42
PD1439/ <i>mviN</i>	Virulence factor	0.46

TCST, two-component signal transduction.

putatively encode proteins involved in various regulatory pathways were differentially expressed in the *xhpT* mutant, suggesting that there may be branched regulatory pathways in *X. fastidiosa*. Two general scenarios of branched pathways have been described

in other bacteria (Laub and Goulian, 2007). The first is a 'many-to-one' pathway in which more than one HK is responsible for the phosphorylation of the same RR. The second is a 'one-to-many' pathway in which one HK phosphorylates several RRs, as exem-

plified in bacterial chemotaxis systems, where the HK CheA phosphorylates the two RRs CheY and CheB (Goulian, 2010; Kirby, 2009). The existence of 19 RRs and only nine HKs in the *X. fastidiosa* genome further suggests considerable branching among the regulatory networks in *X. fastidiosa*. This strategy could be advantageous for *X. fastidiosa* by allowing greater signalling precision within the pathways that control complex phenotypes, such as biofilm formation. Biofilm formation is a process that generally follows a developmental sequence beginning with initial attachment to a surface and microcolony formation, followed by EPS matrix production and biofilm maturation (O'Toole *et al.*, 2000), and appropriate timing of the steps in biofilm formation is often critical for successful host colonization. The integration of XhpT in a phosphorelay controlling biofilm formation may also allow *X. fastidiosa* to integrate multiple environmental signals into this pathway, thereby providing robust regulation over the phenotypes involved in this complex microbial behaviour.

Both surface attachment and cell–cell aggregation are early steps in the process of biofilm formation. A visible reduction was observed in the ability of the *xhpT* mutant to attach to the side-wall of a polystyrene tube and to form cell–cell aggregates when the mutant was grown in liquid medium. These observations, together with the microarray analysis results, prompted further study of the phenotypes involved in the different stages of biofilm formation, including surface attachment, cell–cell aggregation and EPS production. Indeed, there was a quantitative difference in the ability of the *xhpT* mutant to initially attach to a polystyrene surface and to form cell aggregates. A gene encoding a cell surface adhesin, XadA, was up-regulated in the *xhpT* mutant (Table 1). In WT *X. fastidiosa* biofilms, very little XadA protein is evident in the early phase of biofilm formation, but, as the biofilm matures, XadA accumulates and displays a distinct temporally regulated spatial 'island-like' distribution, suggesting that it plays a role in the maintenance of biofilm structure (Caserta *et al.*, 2010). We speculate that the deregulated/increased expression of *xadA* probably leads to an improperly skewed distribution of the XadA protein within the biofilm, causing an overall defect in biofilm stability/formation. Furthermore, an increased EPS layer surrounding the cells might prevent secretion of the extracellular XadA protein, thereby rendering the cells unable to form a stable biofilm. HxfA and HxfB function as cell–cell adhesins in *X. fastidiosa* (Guilhabert and Kirkpatrick, 2005) and the decrease in expression of HxfA and HxfB in the *xhpT* mutant probably contributes to the marked decrease in cell–cell aggregation observed for the *xhpT* mutant.

EPS production is a primary component of the matrix during late biofilm formation and maturation in *X. fastidiosa* (Roper *et al.*, 2007b). Interestingly, the *xhpT* gene is directly upstream from, but transcribed in the opposite direction to, the *gum* operon encoding genes that probably synthesize EPS (da Silva *et al.*, 2001; Simpson *et al.*, 2000; Van Sluys *et al.*, 2003), suggesting that XhpT plays an important role in the regulation of EPS production. The

up-regulation of genes involved in EPS biosynthesis and export probably accounts for the increased EPS production phenotype observed for the *xhpT* mutant. Indeed, a marked increase in total EPS matrix production was observed in the *xhpT* mutant, indicating that XhpT acts as part of a regulatory pathway that represses EPS synthesis. The overproduction of EPS could further explain why the *xhpT* mutant was compromised in attachment to a solid surface, as well as cell–cell aggregation. In other bacterial systems, the constitutive or early production of EPS can prevent initial surface adhesion and disrupt overall biofilm formation (Koutsoudis *et al.*, 2006). Deregulated expression of the EPS layer that coats the bacterial cell surface could prevent physical access of other *X. fastidiosa* adhesins and surface proteins, thereby disrupting surface attachment and aggregation.

Genes PD1702 and PD1703 were highly up-regulated in the *xhpT* mutant with fold changes of 8.41 and 33.47, respectively. Both genes encode for conserved hypothetical proteins that possess a LIP domain potentially functioning as a lipase. In *Xanthomonas oryzae* pv. *oryzae*, the causal agent of bacterial leaf blight on rice, lipase proteins are potent inducers of host defence responses. This may also be the case for *X. fastidiosa*, which could account for the decreased virulence observed for the *xhpT* mutant (Jha *et al.*, 2007). In addition, the increased layer of EPS could trap secreted lipases, preventing access to the plant cell wall.

Although iron is an essential nutrient and necessary for cell growth, an excess of free iron can cause the production of damaging oxygen radicals via the Fenton reaction, in which iron catalyses the breakdown of hydrogen peroxide to hydroxyl radicals which are damaging to cell components (Imlay, 2008; Zheng *et al.*, 1999). A fine balance is established by various genetic regulatory systems to ensure that a sufficient amount of essential iron is available to the cell without having an excess (Zheng *et al.*, 1999). Fur, a well-studied repressor of iron uptake (Hantke, 2001), was down-regulated in the *xhpT* mutant, but, at the same time, several genes encoding proteins involved in the detoxification of reactive oxygen species (ROS) were up-regulated. We speculate that the *xhpT* mutant responds to this deregulated increase in iron uptake by overproducing ROS scavenging enzymes to compensate for the uncontrolled uptake of iron and subsequent formation of damaging ROS.

Interestingly, 12 genes that encode putative virulence factors were up-regulated in the *xhpT* mutant, yet a decrease in virulence was observed. This strongly suggests that appropriate temporal expression of certain virulence factors is critical for normal progression of PD.

Although we did not elucidate the exact mechanism by which XhpT regulates gene expression in *X. fastidiosa*, we demonstrated that this protein plays a central role in controlling a variety of phenotypes affecting biofilm formation and virulence. These findings suggest that XhpT is a critical component of the regulatory hierarchy governing host invasion for this plant pathogen.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions

Xylella fastidiosa was grown on solid PD3 medium (Davis *et al.*, 1981) with appropriate antibiotics when needed (kanamycin at 5 µg/mL) at 28 °C for 9 days. Liquid cultures were grown by the inoculation of a bacterial suspension of 10⁸ cfu/mL in a 1:100 dilution into liquid PD3 medium. Cultures were grown for 9 days at 28 °C with agitation at 100 rpm.

For the microarray gene expression study, *X. fastidiosa* WT Fetzter strain and the *xhpT* null mutant were first grown from –80 °C glycerol stock on PD3 agar medium at 28 °C for 14 days. Cells were harvested from plates and transferred into PD3 liquid medium (Davis *et al.*, 1981). Both strains were adjusted to a cell concentration equal to 10⁵ cfu/mL and plated onto solid PD3 agar medium and grown for another 8 days. Cells were then harvested from plates and immediately stored in liquid nitrogen. Each strain was cultured on nine 15-cm plates from which three replicates were obtained by pooling three plates per replicate. All strains used in this study are described in Table 3.

Generation of the *xhpT* mutant strain

PD1386 (*xhpT*) was PCR amplified from WT *X. fastidiosa* (Fetzter) genomic DNA with the primer pair 1386for/1386rev (Table 3). The resulting PCR product was cloned into pCR2.1-TOPO (Life Technologies, Carlsbad, CA, USA) to generate pCR2.1-*xhpT*. The *xhpT* insert was subcloned into pUC18, a suicide plasmid in *X. fastidiosa*, to generate pUC18-*xhpT*. A kanamycin resistance gene was randomly inserted into pUC18-*xhpT* using the EZ-Tn5™ <KAN-2> Insertion Kit (Epicentre, Madison, WI, USA) according to the manufacturer's protocol, generating pUC18-*xhpT*::kan. The location of the kanamycin cassette insertion was confirmed by sequencing using the primers kan-2RP-1/kan-2FP-1 (Life Technologies). The pUC18-*xhpT*::kan mutagenesis construct was electroporated into *X. fastidiosa* WT Fetzter as described previously (Guilhabert *et al.*, 2001), creating strain *xhpT*. Primer pair *xhpT*for/*xhpT*rev was used to confirm double cross-over

events in the transformants (Fig. 4A,B). In addition, Southern blot analysis was used to confirm single insertion of the kanamycin resistance gene cassette into the WT Fetzter genome as described previously (Guilhabert and Kirkpatrick, 2005) (data not shown).

RT-PCR was carried out to confirm the absence of *xhpT* RNA transcript in the mutant strain (Fig. 4C). RNA was isolated and cDNA generated as described below (microarray analysis) from WT Fetzter and the *xhpT* mutant. The cDNA was employed as a template for a subsequent PCR using the primer pair *xhpT*cDNAfor/*xhpT*cDNArev. All plasmids and primer sequences used in this study are shown in Table 3.

Fig. 4 (A) Primer binding and EZ-Tn5™ <KAN-2> cassette insertion sites in *xhpT*. (B) Confirmation of the integration of the EZ-Tn5™ <KAN-2> cassette into the wild-type (WT) *Xylella fastidiosa* Fetzter chromosome by polymerase chain reaction (PCR). Lane 1, 1-kb plus ladder; lanes 2 and 3, PCR products obtained using the primer pair *xhpT*for and *xhpT*rev and DNA from WT Fetzter (lane 2) and *xhpT* (lane 3). (C) Reverse transcriptase-PCR. Lane 1, 1-kb plus ladder; lanes 2 and 3, PCR products obtained using the primer pair *xhpT*cDNAfor and *xhpT*cDNArev and cDNA from WT Fetzter (lane 2) and *xhpT* (lane 3).

Table 3 Primers, plasmids and strains used in this study.

Primers, plasmids and strains	Sequences (5'–3') and characteristics	Source
Primer		
1386for	TCGAGATTGCGTTCAT	This study
1386Srev	TGGTGCTGATACTCTGTGTACT	This study
kan-2FP-1	ACCTACAACAAAGCTCTCATCAACC	Life Technologies
kan-2RP-1	GCAATGTAACATCAGAGATTTTGAG	Life Technologies
<i>xhpT</i> for	GACCTGCGGAACCACTGTAT	This study
<i>xhpT</i> rev	TGTTGGTGCGTTGTTTGATT	This study
<i>xhpT</i> cDNAfor	ACACCGGCGCTGGCACATAC	This study
<i>xhpT</i> cDNArev	GCAGCTGGCTGTAGCGCGT	This study
Plasmids		
pCR2.1-TOPO	TA-cloning vector, Ap ^R Km ^R lacZ, T7	Life Technologies
pCR2.1- <i>xhpT</i>	pCR2.1-TOPO with <i>xhpT</i> PCR product, Km ^R	This study
pUC18	pMB1 derivative, rep (pMB1), Ap ^R , lacZ	Roche
pUC18- <i>xhpT</i>	pUC18 with <i>xhpT</i> PCR product, Ap ^R	This study
pUC18- <i>xhpT</i> ::kan	pUC18- <i>xhpT</i> with kanamycin cassette, Ap ^R , Km ^R	This study
Strains		
<i>X. fastidiosa</i> Fetzter	Wild-type (WT)	Hendson <i>et al.</i> , 2001
<i>xhpT</i>	<i>X. fastidiosa</i> Fetzter PD1386:: (EZ::TN<Kan-2>Tnp)	This study

Growth curve, cell–cell aggregation and surface attachment assays

For growth curve analysis, the strains were grown in 40 mL of PD3 medium. Cell growth was monitored daily for 9 days by dispersing the cells and measuring the turbidity as an optical density at 600 nm (OD_{600nm}).

For cell–cell aggregation assays, *X. fastidiosa* cultures were incubated for 9 days in liquid PD3 medium in 15-mL polypropylene tubes without shaking. The turbidity of the upper culture medium (OD_3), composed mostly of dispersed cells, was measured using a spectrophotometer at 600 nm. The culture medium was returned to the original tube, the settled aggregate masses were dispersed by pipetting and the total cell culture was measured (OD_t). The relative percentage of aggregated cells was estimated as follows: percentage of aggregated cells = $(OD_t - OD_3)/OD_t \times 100$ (Burdman *et al.*, 2000).

For surface attachment assays, *X. fastidiosa* cultures were incubated for 9 days in liquid PD3 medium in 15-mL polystyrene tubes in a vertical position without shaking. Attachment to the surface walls of the tubes was assessed by a crystal violet staining method (Espinosa-Urgel *et al.*, 2000; Leite *et al.*, 2004). After the incubation period, the PD3 medium was discarded and a 0.1% (w/v) aqueous solution of crystal violet was added to each tube, allowed to incubate for 15 min and rinsed with deionized H_2O . The remaining stain was eluted from the bacterial ring using ethanol. The absorbance of the ethanol–crystal violet solution was measured at 540 nm. All assays were performed in triplicate and each experiment was repeated twice.

Quantification of *X. fastidiosa* EPS *in vitro*

Protein A double-antibody-sandwich ELISA was used to detect EPS produced by *X. fastidiosa* cells grown on PD3 plates for 9 days as described previously (Roper *et al.*, 2007b). Anti-EPS F(ab)₂ fragments were prepared from anti-Xf EPS immunoglobulin G (IgG) (Roper *et al.*, 2007b) and EPS was quantified using protein A–alkaline phosphatase conjugate. Each sample was replicated four times and the experiment was repeated twice.

Virulence assay and quantification of *in planta* bacterial populations

Pathogenicity assays using *Vitis vinifera* var. Thompson seedless grapevines grown in a glasshouse were performed as described previously (Guilhabert and Kirkpatrick, 2005; Hill and Purcell, 1997; Purcell *et al.*, 1999; Roper *et al.*, 2007a). Fifteen plants were used for inoculation of each strain. In brief, 20 μ L of bacterial inoculum was mechanically inoculated into the stem at the base of the grapevine using a 20-gauge syringe needle. Eight weeks post-inoculation, symptoms were rated biweekly using a disease rating scale of 0–5, with '0' being healthy and '5' being dead or dying (Guilhabert and Kirkpatrick, 2005). *In planta* bacterial populations were quantified from petiole tissue. Petioles were harvested, surface sterilized, ground in 1 $\times$ phosphate-buffered saline (PBS) and 20 μ L of the ground tissue was plated onto PD3 medium. The plates were incubated at 28 °C for 9 days and the colonies were enumerated.

Microarray analysis

RNA isolation and cDNA synthesis

The RiboPure-Bacteria Kit (Life Technologies) was used to isolate total RNA. RNA was purified and concentrated using an RNeasy Mini Kit (Qiagen, Germantown, MD, USA) with on-column DNase treatment. Samples were processed to yield ~20 μ g of bacterial RNA. RNA samples were then dissolved in water and quantified on a fluorometer using RiboGreen reagent according to the manufacturer's instructions (Life Technologies). Double-stranded cDNA was synthesized using a cDNA Synthesis Kit (Life Technologies), and cDNA samples were shipped to the Nimblegen hybridization service facility (Roche Nimblegen, Madison, WI, USA). Both RNA and cDNA integrity were evaluated on a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA).

Probe design

Probe design was carried out in collaboration with Nimblegen, Inc. based on the available genomes for four *Xylella* strains (9a5c, Ann1, Dixon, Temecula). To design 60-mer oligos for all the chromosomal annotated genes, three levels of stringency were used.

Group I oligos. In this category, oligos were selected with a GC value of 45%–59%, with no more than five homopolymer stretches of As, Ts, Gs or Cs. The GC cut-off was determined based on the analysis of the GC distribution of the 60-mer probes on a scale of 0–100. For this, two strains of *X. fastidiosa* (Ann1 and 9a5c) were used and the GC range for the 60-mers was plotted at 5% intervals to determine the number of probes per interval. GC averaged around 50%–60%. To ensure optimal hybridization specificity and to eliminate cross-hybridization, these oligos were BLAST screened so that none matched more than 15 bp consecutively to any other location in the genome. This set had the largest number of oligos representative of the four strains (82.4%–89.7%).

Group II oligos. For this category, the BLAST screening was omitted (8.5%–12.5%).

Group III oligos. For this category, both BLAST and GC screening were omitted (1.6%–5.7%).

Next, for genes which had more than 10 oligos that passed the set criteria, the 10 oligos from the 3-end of the gene were selected. For *Xylella* plasmids and NPT2 gene oligos, no BLAST or GC restrictions were placed.

Microarray analysis

To reduce experimentally and hybridization dye introduced biases, three biological replicates per experimental stage using single colour hybridization were carried out. Slides were scanned using a GenePix 4.0 scanner and associated software (Molecular Devices Corp., Sunnyvale, CA, USA), and data representing raw spot intensities were subjected to robust multi-array average (RMA) (Irizarry *et al.*, 2003). We used the RMA model of probe-specific background correction to the perfect match probes. Next, these corrected probe values were subjected to quantile normalization, and a median polish method was applied to compute a single expression value for all the probes of a particular gene. Differentially expressed genes were identified using SAM software to generate fold differences and *q* values with a delta value of 1.2. Only genes with at least a two-fold change in expression value were selected for further analyses (Tusher *et al.*, 2001).

Validation of microarray data

RNA was isolated as described above and cDNA was generated using the High Capacity RNA-to-cDNA Kit (Life Technologies). Primers were generated using Primerexpress software (Applied Biosystems). *DnaQ* (PD1217), coding for the epsilon chain of DNA-polymerase III, was used as an endogenous control. PCRs were performed using SYBR Green PCR Master Mix (Applied Biosystems) employing an ABI PRISM 7000 Sequence Detector System (Applied Biosystems). The PCR thermal cycling conditions were as follows: an initial step at 50 °C for 2 min; 10 min at 95 °C; and 40 cycles, with one cycle consisting of 15 s at 95 °C and 1 min at 60 °C. Serial dilutions of WT Fetzter cDNA were used to calculate the standard curve for all primer pairs. The primer efficiency is $E = 10^{(-1/\text{slope})}$, with the slope derived from the standard curve. Each sample was processed in duplicate on each plate, with two plates in total. The Pfaffl equation was used for relative quantification (Pfaffl, 2001): $\text{ratio} = (E_{\text{target}})^{\Delta\text{Ct, target}(\text{control-treated})} / (E_{\text{ref}})^{\Delta\text{Ct, ref}(\text{control-treated})}$.

ACKNOWLEDGEMENTS

This research was supported by the California Department of Food and Agriculture Pierce's Disease/Glassy Winged Sharpshooter Research Program. The authors would like to thank Jerome Braun (Department of Statistics, University of California, Davis, CA, USA) for assistance with the statistical analysis.

REFERENCES

- Armitage, J.P. (1999) Bacterial tactic responses. *Adv. Microb. Physiol.* **41**, 229–289.
- Burdman, S., Jurkevitch, E., Soria-Diaz, M.E., Serrano, A.M. and Okon, Y. (2000) Extracellular polysaccharide composition of *Azospirillum brasilense* and its relation with cell aggregation. *FEMS Microbiol. Lett.* **189**, 259–264.
- Caserta, R., Takita, M.A., Targon, M.L., Rosselli-Murai, L.K., de Souza, A.P., Peroni, L., Stach-Machado, D.R., Andrade, A., Labate, C.A., Kitajima, E.W., Machado, M.A. and de Souza, A.A. (2010) Expression of *Xylella fastidiosa* fimbrial and afimbrial proteins during biofilm formation. *Appl. Environ. Microbiol.* **76**, 4250–4259.
- Cheng, D.W., Lin, H. and Civerolo, E.L. (2010) Extracellular genomic DNA mediates enhancement of *Xylella fastidiosa* biofilm formation *in vitro*. *J. Plant Pathol.* **92**, 415–420.
- Davis, M.J., French, W.J. and Schaad, N.W. (1981) Axenic culture of the bacteria associated with phony disease of peach and plum leaf scald. *Curr. Microbiol.* **6**, 309–314.
- De La Fuente, L., Burr, T.J. and Hoch, H.C. (2008) Autoaggregation of *Xylella fastidiosa* cells is influenced by type I and type IV pili. *Appl. Environ. Microbiol.* **74**, 5579–5582.
- Espinosa-Urgel, M., Salido, A. and Ramos, J.L. (2000) Genetic analysis of functions involved in adhesion of *Pseudomonas putida* to seeds. *J. Bacteriol.* **182**, 2363–2369.
- Feil, H., Feil, W.S. and Lindow, S.E. (2007) Contribution of fimbrial and afimbrial adhesins of *Xylella fastidiosa* to attachment to surfaces and virulence to grape. *Phytopathology*, **97**, 318–324.
- Galperin, M.Y. (2006) Structural classification of bacterial response regulators: diversity of output domains and domain combinations. *J. Bacteriol.* **188**, 4169–4182.
- Galperin, M.Y. (2010) Diversity of structure and function of response regulator output domains. *Curr. Opin. Microbiol.* **13**, 150–159.
- Goulian, M. (2010) Two-component signaling circuit structure and properties. *Curr. Opin. Microbiol.* **13**, 184–189.
- Guilhabert, M.R. and Kirkpatrick, B.C. (2005) Identification of *Xylella fastidiosa* antivirulence genes: hemagglutinin adhesins contribute a biofilm maturation to *X. fastidiosa* and colonization and attenuate virulence. *Mol. Plant–Microbe Interact.* **18**, 856–868.
- Guilhabert, M.R., Hoffman, L.M., Mills, D.A. and Kirkpatrick, B.C. (2001) Transposon mutagenesis of *Xylella fastidiosa* by electroporation of Tn5 synaptic complexes. *Mol. Plant–Microbe Interact.* **14**, 701–706.
- Hantke, K. (2001) Iron and metal regulation in bacteria. *Curr. Opin. Microbiol.* **4**, 172–177.
- Heeb, S. and Haas, D. (2001) Regulatory roles of the GacS/GacA two-component system in plant-associated and other Gram-negative bacteria. *Mol. Plant–Microbe Interact.* **14**, 1351–1363.
- Hendson, M., Purcell, A.H., Chen, D., Smart, C., Guilhabert, M. and Kirkpatrick, B. (2001) Genetic diversity of Pierce's disease strains and other pathovars of *Xylella fastidiosa*. *Appl. Environ. Microbiol.* **67**, 895–903.
- Hewitt, W.B., Houston, B.R., Frazier, N.W. and Freitag, J.H. (1946) Leafhopper transmission of the virus causing Pierce's disease of grape and dwarf of alfalfa. *Phytopathology*, **36**, 117–128.
- Hill, B.L. and Purcell, A.H. (1997) Populations of *Xylella fastidiosa* in plants required for transmission by an efficient vector. *Phytopathology*, **87**, 1197–1201.
- Hopkins, D.L. (1989) *Xylella fastidiosa*—xylem-limited bacterial pathogen of plants. *Annu. Rev. Phytopathol.* **27**, 271–290.
- Hopkins, D.L. and Purcell, A.H. (2002) *Xylella fastidiosa*: cause of Pierce's disease of grapevine and other emergent diseases. *Plant Dis.* **86**, 1056–1066.
- Imlay, J. (2008) Cellular defenses against superoxide and hydrogen peroxide. *Annu. Rev. Biochem.* **77**, 755–776.
- Irizarry, R.A., Hobbs, B., Collin, F., Beazer-Barclay, Y.D., Antonellis, K.J., Scherf, U. and Speed, T.P. (2003) Exploration, normalization, and summaries of high density oligonucleotide array probe level data. *Biostatistics*, **4**, 249–264.
- Jha, G., Rajeshwari, R. and Sonti, R.V. (2007) Functional interplay between two *Xanthomonas oryzae* pv. *oryzae* secretion systems in modulating virulence on rice. *Mol. Plant–Microbe Interact.* **20**, 31–40.
- Kirby, J.R. (2009) Chemotaxis-like regulatory systems: unique roles in diverse bacteria. *Annu. Rev. Microbiol.* **63**, 45–59.
- Koutsoudis, M.D., Tsalts, D., Minogue, T.D. and von Bodman, S.B. (2006) Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. *Proc. Natl. Acad. Sci. USA*, **103**, 5983–5988.
- Laub, M.T. and Goulian, M. (2007) Specificity in two-component signal transduction pathways. *Annu. Rev. Genet.* **41**, 121–145.
- Leite, B., Andersen, P.C. and Ishida, M.L. (2004) Colony aggregation and biofilm formation in xylem chemistry-based media for *Xylella fastidiosa*. *FEMS Microbiol. Lett.* **230**, 283–290.
- Li, Y.X., Hao, G.X., Galvani, C.D., Meng, Y.Z., De la Fuente, L. and Hoch, H.C. (2007) Type I and type IV pili of *Xylella fastidiosa* affect twitching motility, biofilm formation and cell–cell aggregation. *Microbiology*, **153**, 719–726.
- Meng, Y.Z., Li, Y.X., Galvani, C.D., Hao, G.X., Turner, J.N., Burr, T.J. and Hoch, H.C. (2005) Upstream migration of *Xylella fastidiosa* is pilus-driven twitching motility. *J. Bacteriol.* **187**, 5560–5567.
- O'Toole, G., Kaplan, H.B. and Kolter, R. (2000) Biofilm formation as microbial development. *Annu. Rev. Microbiol.* **54**, 49–79.
- Pfaffl, M.W. (2001) A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45.
- Purcell, A.H. and Hopkins, D.L. (1996) Fastidious xylem-limited bacterial plant pathogens. *Annu. Rev. Phytopathol.* **34**, 131–151.
- Purcell, A.H., Saunders, S.R., Hendson, M., Grebus, M.E. and Henry, M.J. (1999) Causal role of *Xylella fastidiosa* in Oleander leaf scorch disease. *Phytopathology*, **89**, 53–58.
- Roper, M.C., Greve, L.C., Warren, J.G., Labavitch, J.M. and Kirkpatrick, B.C. (2007a) *Xylella fastidiosa* requires polygalacturonase for colonization and pathogenicity in Vitis vinifera grapevines. *Mol. Plant–Microbe Interact.* **20**, 411–419.
- Roper, M.C., Greve, L.C., Labavitch, J.A. and Kirkpatrick, B.C. (2007b) Detection and visualization of an exopolysaccharide produced by *Xylella fastidiosa* *in vitro* and *in planta*. *Appl. Environ. Microbiol.* **73**, 7252–7258.
- Rosario, M.M.L., Fredrick, K.L., Ordal, G.W. and Hermann, J.D. (1994) Chemotaxis in *Bacillus subtilis* requires either of 2 functionally redundant CheW homologs. *J. Bacteriol.* **176**, 2736–2739.
- Shu, C.Y.J. and Zhulin, I.B. (2002) ANTAR: an RNA-binding domain in transcription antitermination regulatory proteins. *Trends Biochem. Sci.* **27**, 3–5.
- da Silva, F.R., Vettore, A.L., Kemper, E.L., Leite, A. and Arruda, P. (2001) Fastidious gum: the *Xylella fastidiosa* exopolysaccharide possibly involved in bacterial pathogenicity. *FEMS Microbiol. Lett.* **203**, 165–171.
- Simpson, A.J.G., Reinach, F.C., Arruda, P., Abreu, F.A., Acencio, M., Alvarenga, R., Alves, L.M., Araya, J.E., Baia, G.S., Baptista, C.S., Barros, M.H., Bonaccorsi, E.D., Bordin, S., Bové, J.M., Briones, M.R., Bueno, M.R., Camargo, A.A., Camargo, L.E., Carraro, D.M., Carrer, H., Colauto, N.B., Colombo, C., Costa, F.F., Costa, M.C., Costa-Neto, C.M., Coutinho, L.L., Cristofani, M., Dias-Neto, E., Docena,

- C., El-Dorri, H., Facincani, A.P., Ferreira, A.J., Ferreira, V.C., Ferro, J.A., Fraga, J.S., França, S.C., Franco, M.C., Frohme, M., Furlan, L.R., Garnier, M., Goldman, G.H., Goldman, M.H., Gomes, S.L., Gruber, A., Ho, P.L., Hoheisel, J.D., Junqueira, M.L., Kemper, E.L., Kitajima, J.P., Krieger, J.E., Kuramae, E.E., Laigret, F., Lambais, M.R., Leite, L.C., Lemos, E.G., Lemos, M.V., Lopes, S.A., Lopes, C.R., Machado, J.A., Machado, M.A., Madeira, A.M., Madeira, H.M., Marino, C.L., Marques, M.V., Martins, E.A., Martins, E.M., Matsukuma, A.Y., Menck, C.F., Miracca, E.C., Miyaki, C.Y., Montero-Vitorello, C.B., Moon, D.H., Nagai, M.A., Nascimento, A.L., Netto, L.E., Nhani, A. Jr, Nobrega, F.G., Nunes, L.R., Oliveira, M.A., de Oliveira, M.C., de Oliveira, R.C., Palmieri, D.A., Paris, A., Peixoto, B.R., Pereira, G.A., Pereira, H.A. Jr, Pesquero, J.B., Quaggio, R.B., Roberto, P.G., Rodrigues, V., de M Rosa, A.J., de Rosa, V.E. Jr, de Sá, R.G., Santelli, R.V., Sawasaki, H.E., da Silva, A.C., da Silva, A.M., da Silva, F.R., da Silva, W.A. Jr, da Silveira, J.F., Silvestri, M.L., Siqueira, W.J., de Souza, A.A., de Souza, A.P., Terenzi, M.F., Truffi, D., Tsai, S.M., Tuhako, M.H., Vallada, H., Van Sluys, M.A., Verjovski-Almeida, S., Vettore, A.L., Zago, M.A., Zatz, M., Meidanis, J. and Setubal, J.C. (2000) The genome sequence of the plant pathogen *Xylella fastidiosa*. *Nature*, **406**, 151–157.
- Stock, A.M., Robinson, V.L. and Goudreau, P.N. (2000) Two-component signal transduction. *Annu. Rev. Biochem.* **69**, 183–215.
- Szurmant, L. and Ordal, G.W. (2004) Diversity in chemotaxis mechanisms among the bacteria and archaea. *Microbiol. Mol. Biol. Rev.* **68**, 301–319.
- Thorne, E.T., Stevenson, J.F., Rost, T.L., Labavitch, J.M. and Matthews, M.A. (2006) Pierce's disease symptoms: comparison with symptoms of water deficit and the impact of water deficits. *Am. J. Enol. Vitic.* **57**, 1–11.
- Tusher, V.G., Tibshirani, R. and Chu, G. (2001) Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. USA*, **98**, 5116–5121.
- Van Sluys, M.A., de Oliveira, M.C., Monteiro-Vitorello, C.B., Miyaki, C.Y., Furlan, L.R., Camargo, L.E.A., da Silva, A.C., Moon, D.H., Takita, M.A., Lemos, E.G., Machado, M.A., Ferro, M.I., da Silva, F.R., Goldman, M.H., Goldman, G.H., Lemos, M.V., El-Dorri, H., Tsai, S.M., Carrer, H., Carraro, D.M., de Oliveira, R.C., Nunes, L.R., Siqueira, W.J., Coutinho, L.L., Kimura, E.T., Ferro, E.S., Harakava, R., Kuramae, E.E., Marino, C.L., Giglioti, E., Abreu, I.L., Alves, L.M., do Amaral, A.M., Baia, G.S., Blanco, S.R., Brito, M.S., Cannavan, F.S., Celestino, A.V., da Cunha, A.F., Fenille, R.C., Ferro, J.A., Formighieri, E.F., Kishi, L.T., Leoni, S.G., Oliveira, A.R., Rosa, V.E. Jr, Sasaki, F.T., Sena, J.A., de Souza, A.A., Truffi, D., Tsukumo, F., Yanai, G.M., Zaros, L.G., Civerolo, E.L., Simpson, A.J., Almeida, N.F. Jr, Setubal, J.C. and Kitajima, J.P. (2003) Comparative analyses of the complete genome sequences of Pierce's disease and citrus variegated chlorosis strains of *Xylella fastidiosa*. *J. Bacteriol.* **185**, 1018–1026.
- Voegel, T.M., Warren, J.G., Matsumoto, A., Igo, M.M. and Kirkpatrick, B.C. (2010) Localization and characterization of *Xylella fastidiosa* haemagglutinin adhesins. *Microbiology*, **156**, 2172–2179.
- West, A.H. and Stock, A.M. (2001) Histidine kinases and response regulator proteins in two-component signaling systems. *Trends Biochem. Sci.* **26**, 369–376.
- Zheng, M., Doan, B., Schneider, T.D. and Storz, G. (1999) OxyR and SoxRS regulation of Fur. *J. Bacteriol.* **181**, 4639–4643.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1 Genes that are at least two-fold up-regulated in *xhpT*.

Table S2 Genes that are at least two-fold down-regulated in *xhpT*.