A Temperature-Independent Cold-Shock Protein Homolog Acts as a Virulence Factor in *Xylella fastidiosa*

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Xylella fastidiosa, causal agent of Pierce's disease (PD) of grapevine, is a fastidious organism that requires very specific conditions for replication and plant colonization. Cold temperatures reduce growth and survival of *X. fastidiosa* both in vitro and in planta. However, little is known regarding physiological responses of *X. fastidiosa* to temperature changes. Cold-shock proteins (CSP), a family of nucleic acid-binding proteins, act as chaperones facilitating translation at low temperatures. Bacterial genomes often encode multiple CSP, some of which are strongly induced following exposure to cold. Additionally, CSP contribute to the general stress response through mRNA stabilization and posttranscriptional regulation. A putative CSP homolog (Csp1) with RNA-binding activity was identified in *X. fastidiosa* Stag's Leap. The *csp1* gene lacked the long 5' untranslated region characteristic of cold-inducible genes and was expressed in a temperature-independent manner. As compared with the wild type, a deletion mutant of *csp1* (Δ csp1) had decreased survival rates following cold exposure and salt stress in vitro. The deletion mutant also was significantly less virulent in grapevine, as compared with the wild type, in the absence of cold stress. These results suggest an important function of *X. fastidiosa* Csp1 in response to cellular stress and during plant colonization.

The bacterial pathogen *Xylella fastidiosa* infects multiple hosts, including several important crop and landscape plants. *X. fastidiosa* colonizes xylem tissue, resulting in characteristic leaf scorch symptoms, often leading to decline or death of the plant (Hopkins and Purcell 2002; Purcell et al. 1999). *X. fastidiosa* subsp. *fastidiosa* infection of grapevine, causing Pierce's disease (PD), is mainly prevalent in subtropical climates and, historically, has had a devastating impact on the grape industry in California (Hopkins and Purcell 2002). Bacterial temperature response likely plays a role in pathogenesis of PD, as virulence-associated genes are upregulated in *X. fastidiosa* after exposure to heat and cold conditions are correlated with reduced persistence of PD in grapevine (Feil and Purcell 2001; Koide et al. 2006; Lieth et al. 2011; Meyer 2010). However, little is known regarding temperature acclimation mechanisms of *X. fastidiosa* during plant infection.

Optimal replication of *X. fastidiosa* occurs at 28°C, under laboratory conditions. Restricted growth and loss of viability have been observed for *X. fastidiosa* cultivated at lower temperatures in vitro (Feil and Purcell 2001; Meyer 2010). A crucial and highly

conserved aspect of bacterial adaptation to cold temperatures is the production of cold-shock proteins (CSP), a large number of which belong to the CspA family of nucleic acid-binding proteins and are strongly induced by temperature change (Etchegaray and Inouye 1999; Graumann and Marahiel 1998; Graumann et al. 1997; Lee et al. 1994; Yamanaka et al. 1998). In addition to being highly conserved in prokaryotes, bacterial CSP also are remarkably similar to eukaryotic cold-shock domain proteins, many of which are involved in cold-shock response and drought tolerance in plants (Kim et al. 2013; Nakaminami et al. 2006; Schröder et al. 1995). CspA family proteins facilitate transcription and translation at low temperatures by acting as chaperones to prevent the formation of RNA secondary structure as well as in a regulatory capacity to stabilize specific mRNA molecules (Bae et al. 2000; Cohen-Or et al. 2010; Jiang et al. 1997; Nakaminami et al. 2006; Schindelin et al. 1993). Cold-inducible gene expression is often the result of posttranscriptional regulation, involving an unusually long 5' untranslated region (UTR) (Janiyani and Ray 2002; Jiang et al. 1996; Kaan et al. 1999). In *Escherichia coli* at 37°C, the 160-nt *cspA* 5' UTR interacts with a portion of the coding region, obstructing the ribosomal binding site (Breaker 2010; Giuliadori et al. 2010). At lower temperatures, a series of stem loop structures are formed within the 5' UTR, allowing uninhibited access to the ribosomal binding site and coding region (Giuliadori et al. 2010). This mechanism facilitates cold-inducible expression of CSP and allows for adaptation of bacterial cells to cold temperatures. A number of cold-inducible genes are characterized by similar 5' UTR sequences of 150 to 200 nt in length (Jiang et al. 1996; Mitta et al. 1997; Nakashima et al. 1996; Wang et al. 1999). However, other mechanisms of cold-dependent regulation, such as *E. coli* CspE and *Salmonella enterica* CspH, which have shorter 5' UTR sequences, are attributed to differential stability of mRNA at different temperatures (Kim et al. 2001; Uppal et al. 2008).

In addition to cold adaptation, there also is evidence that CSP are involved in other aspects of bacterial survival and virulence. Induction of CSP can increase survival under conditions of osmotic or oxidative stress as well as during stationary phase (Balhasteros et al. 2010; Kandrór et al. 2002; Loepfe et al. 2010; Michaux et al. 2012; Schmid et al. 2009). Plant pathogens often encounter reactive oxygen species (ROS) during infection of the host, as a result of the immune response or cell lysis (Burbank and Roper 2014; Flores-Cruz and Allen 2011; Fones and Preston 2012). Production of ROS increases when plants are subjected to pathogen invasion or abiotic stress such as temperature extremes (Lamb and Dixon 1997; Wise 1995). Additionally, numerous changes in the host plant following cold stress, including elevated levels of abscisic acid and phenolic compounds in xylem sap (Król et al. 2015; Meyer and Kirkpatrick

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2011; Wilhelm et al. 2011), may be another challenge to successful infection of *X. fastidiosa* under these conditions. A demonstrated role of CSP as virulence factors has been limited to animal pathogens and has been linked to stress adaptation during intracellular colonization (Loepfe et al. 2010; Michaux et al. 2012; Wang et al. 2014). We chose to investigate the role of a representative *X. fastidiosa* CSP homolog in response to cold, cellular stress conditions, and during plant infection to elucidate the role of this protein in *X. fastidiosa* survival, replication, and development of PD.

RESULTS

X. fastidiosa encodes a CspA homolog.

In *X. fastidiosa* subsp. *fastidiosa*, a homolog of CspA was identified (Csp1, encoded by *csp1* [PD1380]) that shares 53% amino acid sequence identity with the major CSP (CspA) from *E. coli* K-12 (ECDH10B_3735) and 52% identity with a CspA homolog (XC_1828) from *Xanthomonas campestris* pv. *campestris* (Fig. 1A) (Dehal et al. 2010; Marchler-Bauer et al. 2013). Among different subspecies of *X. fastidiosa*, the Csp1 sequence is highly conserved. The Csp1 protein of the *X. fastidiosa* strain Stag's Leap shares 100% amino acid sequence identity with CSP homologs in strains Temecula-1 and M23 (subsp. *fastidiosa*), M12 (subsp. *multiplex*), and Ann-1 (subsp. *sandyi*) and shares 97% amino acid identity with a CSP homolog from strain 9a5c (subsp. *pauca*) (Dehal et al. 2010; Marchler-Bauer et al. 2013) (Fig. 1B). Conserved nucleic acid-binding domains characteristic of CspA superfamily proteins (Marchler-Bauer et al. 2013; Weber et al. 2002) are retained in Csp1 homologs of all four *X. fastidiosa* subspecies (Fig. 1B). A second CspA homolog also is present in the *X. fastidiosa* genome (Csp2, encoded by *csp2* [PD1993]). However, as a viable knockout mutant could not be obtained, characterization of *csp2* is reserved for further study.

X. fastidiosa Csp1 is important for in vitro cold tolerance.

A knockout mutant of *X. fastidiosa csp1* ($\Delta csp1$) was constructed to study the function of Csp1 in *X. fastidiosa* Stag's Leap. Wild-type Stag's Leap and $\Delta csp1$ were tested for survival after exposure cold (4°C) in vitro. Percent survival was determined based on number of CFU before and after cold treatment of cell suspensions in 1× phosphate buffered saline (PBS) for 24 h at 4°C (Fig. 2). The deletion mutant had significantly reduced survival (7%) as compared with the wild type (66%) (Fig. 2A). Complementation of $\Delta csp1$ by chromosomal insertion of a wild-type copy of *X. fastidiosa csp1* at a

neutral location, using the method of Matsumoto et al. (2009), restored mean survival rate to 74% (Fig. 2A), showing that *X. fastidiosa* Csp1 contributes to survival at low temperature. In addition, complementation with CspA homologs from *E. coli* and *Xanthomonas campestris* pv. *campestris* also restored cold survival in $\Delta csp1$ to 54% and 60%, respectively (Fig. 2A). These results suggest that the function of *X. fastidiosa* Csp1 is conserved with CspA homologs of other bacterial species. Insertion of *X. fastidiosa csp1*, *E. coli cspA*, or *Xanthomonas campestris* pv. *campestris cspA* into a neutral chromosomal region of wild-type Stag's Leap did not significantly increase cold tolerance, as quantified by percent survival after 24 h at 4°C (Fig. 2B), confirming that differences in CSP activity between different species do not account for the cold sensitivity of *X. fastidiosa*. It appears that cold response in *X. fastidiosa* is at least partially dependent on Csp1 in vitro.

X. fastidiosa Csp1 contributes to salt stress tolerance but not oxidative stress tolerance in vitro.

It was relevant to determine if *X. fastidiosa* Csp1 was involved in survival under stress conditions other than cold. As compared with wild-type *X. fastidiosa* Stag's Leap, which had a survival rate of 67%, $\Delta csp1$ had decreased survival (9%) following incubation in a concentrated salt solution (0.3M NaCl) for 1 h (Fig. 3). Survival of the complemented strain $\Delta csp1/csp1^+$ (46%) did not significantly differ from the wild type (Fig. 3). Incubation in either 0.1 M NaCl or 0.05 M NaCl after 48 or 72 h, respectively, also resulted in decreased survival of $\Delta csp1$ as compared with wild type (data not shown). Because CSP can, in some cases, contribute to bacterial resistance to oxidative stress (Loepfe et al. 2010; Wang et al. 2014), hydrogen peroxide sensitivity was quantified, using a disc inhibition assay (Matsumoto et al. 2009). However, the inhibition zone radius was not significantly different for $\Delta csp1$ as compared with wild-type Stag's Leap, indicating that Csp1 is most likely not part of the response to peroxide stress (data not shown).

X. fastidiosa Csp1 binds single-stranded RNA.

X. fastidiosa Csp1 was expressed in and purified from *E. coli* for characterization of protein function. RNA binding activity was determined by electrophoretic mobility shift assay, using labeled single-stranded RNA probes of 100 bases in length (Fig. 4). The observed band shift that occurred with the addition of higher concentrations of Csp1 demonstrated binding activity. No band shift was observed using bovine serum albumin as a non-RNA binding protein control (Fig. 4). This result is

Fig. 1. Csp1 is a cold shock protein homolog encoded by *Xylella fastidiosa*. **A**, Multiple sequence alignment of protein sequences comparing homologs from different bacterial species and **B**, different *X. fastidiosa* strains. Protein sequences were obtained from the MicrobesOnline database (Dehal et al. 2010) and alignment was performed using Clustal Omega (Sievers et al. 2011). Nucleic acid binding domains were identified using the Conserved Domain Database (Marchler-Bauer et al. 2013).

consistent with a potential role of *X. fastidiosa* Csp1 in stabilizing translation at low temperatures by altering secondary structure in single-stranded RNA.

Short 5' UTR of *X. fastidiosa* *csp1* does not facilitate cold-inducible expression.

The 5' UTR region of *X. fastidiosa* *csp1* mRNA was amplified and sequenced by 5' RACE (rapid amplification of cDNA ends) to determine its potential role as a cold-inducible regulatory element (Fig. 5). The 5' UTR of *X. fastidiosa* *csp1* was 54 nt in length, shorter than expected for cold-inducible regulation. To quantify expression relative to temperature, a reporter

construct in pCR8/GW/TOPO was created by fusing the luciferase open reading frame (ORF) 3' proximal to the *X. fastidiosa* *csp1* ORF. The resulting construct (pCR8-Xfcsp1-luc) retained the *X. fastidiosa* *csp1* 5' UTR. A similar luciferase reporter construct (pCR8-ECcspA-luc) was made using *E. coli* *cspA* (including the 5' UTR) as a cold-inducible control. CSP ORF sequences were included in reporter constructs, as sequences downstream of the start codon, known as the downstream box, are important for cold-inducible expression (Mittal et al. 1997). *E. coli* recombinants bearing reporter plasmids pCR8-Xfcsp1-luc and pCR8-ECcspA-luc were transferred from 37 to 15°C

Fig. 2. Cold tolerance of $\Delta csp1$. Percent cell survival of *Xylella fastidiosa* was quantified based on counts of CFU before and after 24 h at 4°C. **A**, Mean percent cell survival of wild type (WT), a deletion mutant of *csp1* ($\Delta csp1$), and $\Delta csp1$ complemented with *csp1* from *X. fastidiosa* ($\Delta csp1/csp1^+$) or *cspA* of *Escherichia coli* ($\Delta csp1/ECcspA$). **B**, Mean percent cell survival of WT and *X. fastidiosa* strains with a second copy of the cold shock protein homolog from *X. fastidiosa* (*Xf csp1*⁺), *E. coli* (*Ec cspA*), or *Xanthomonas campestris* pv. *campestris* (*Xcc cspA*⁺). Bars represent means ($\pm$ standard error) combined from three independent experiments, each consisting of three replicates plated in duplicate. Means with different letters are significantly different based on analysis of variance followed by a Tukey-Kramer multiple comparison test ($\alpha = 0.05$).

Fig. 3. Osmotic stress tolerance of $\Delta csp1$. Percent survival of *X. fastidiosa* was quantified based on counts of CFU before and after incubation in 0.3M NaCl for 1 h, and the mean percent survival of wild type (WT), a deletion mutant of *csp1* ($\Delta csp1$), and $\Delta csp1$ complemented with *csp1* from *X. fastidiosa* ($\Delta csp1/csp1^+$) is shown. Bars represent means ($\pm$ standard error) combined from three independent experiments, each consisting of three replicates plated in duplicate. Means with different letters are significantly different based on analysis of variance followed by a Tukey-Kramer multiple comparison test ($\alpha = 0.05$).

Fig. 4. RNA-binding activity of Csp1. Purified *X. fastidiosa* Csp1 was used to determine RNA-binding activity, using an electrophoretic mobility shift assay. Reduced electrophoretic mobility of single-stranded RNA probes with the addition of Csp1 indicates binding activity. Binding reactions contained 20 ng of biotin-labeled RNA and increasing concentrations of purified Csp1-His (1, 3, and 5 μ g). Bovine serum albumin (5 or 10 μ g) was used as a nonbinding protein control.

and luciferase protein expression was quantified by Western blot (Fig. 6). Expression of luciferase from pCR8-ECcspA-luc was induced after temperature drop and showed increasing levels over the course of 2 h at 15°C (Fig. 6). In contrast, luciferase expression from pCR8-Xfcsp1-luc was constitutive in relation to temperature (Fig. 6). Based on this evidence, it appears that *X. fastidiosa* Csp1 is not cold-inducible through the function of its 5' leader RNA. To address the possibility of *X. fastidiosa*-specific regulatory elements, the Xfcsp1-luc reporter construct also was inserted into the broad-host range plasmid pBBR1-MCS5 (replication competent in *X. fastidiosa*) and was transformed into wild-type Stag's Leap. Reporter expression in *X. fastidiosa* was not increased by temperature shift to 15°C (data not shown), confirming that *X. fastidiosa* Csp1 expression is regulated in a temperature-independent manner.

Csp1 is required for full virulence in grapevine.

There is little or no evidence for a role of CSP in virulence of plant pathogens. To evaluate the role of *X. fastidiosa* Csp1 in the infection process, susceptible *Vitis vinifera* (Chardonnay) plants were inoculated with wild-type *X. fastidiosa* Stag's Leap, $\Delta csp1$, and $\Delta csp/csp1^+$ and were observed for symptom development at 12 weeks postinoculation (Fig. 7A and B). Severity of disease symptoms was rated based on the 0 to 5 scoring system by Guilhabert and Kirkpatrick (2005) (Fig. 7B). The mean disease score of $\Delta csp1$ -inoculated vines was significantly reduced in comparison with wild type-inoculated plants (Fig. 7B). Plants inoculated with $\Delta csp/csp1^+$ showed symptoms similar to wild type-inoculated plants. As inoculated plants were grown in a temperature-controlled greenhouse during the summer, this reduction in virulence was not due to cold stress. In addition to visible disease symptoms, bacterial titer in planta was quantified by quantitative polymerase chain reaction (qPCR), using total DNA extracted from petioles taken from 15 cm above the point of inoculation (Fig. 8). *X. fastidiosa* DNA quantity was significantly lower in $\Delta csp1$ -inoculated plants compared with wild type-infected vines. Partial complementation of bacterial DNA quantity was

observed in plants inoculated with $\Delta csp/csp1^+$ (Fig. 8). These results indicate that *X. fastidiosa* Csp1 is a virulence factor important for colonization of the plant host in the absence of cold stress.

To address the question of whether Csp1 also is important for cold survival in planta, inoculated grapevines were separated

Fig. 6. Temperature-independent expression of *Xylella fastidiosa* Csp1. Luciferase reporter expression of constructs in *Escherichia coli* bearing the *luc* open reading frame (ORF) fused 3'-proximal to *E. coli* *cspA* (*Ec cspA-luc*) or *X. fastidiosa* *csp1* (*Xf csp1-luc*) ORF. Each construct included the native promoter and 5' intranuclear region of the respective bacterial species. Cultures were grown at 37°C and were shifted to 15°C to evaluate induction upon exposure to cold. Total protein samples (1 µg) were extracted before or after (30 min, 1 h, and 2 h) temperature shift; luciferase was detected following sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with a firefly luciferase monoclonal antibody. A construct bearing only the *luc* ORF with no promoter served as a negative control (–control).

Fig. 5. *Xylella fastidiosa* *csp1* has a short 5' untranslated region (UTR). **A**, Sequence of the 5' UTR of *X. fastidiosa* *csp1* and of *Escherichia coli* *cspA*. **B**, Schematic representation of 5' UTR of *X. fastidiosa* and of *E. coli* cold-shock homolog genes. The mRNA 5' terminus of each was determined by sequencing cloned 5' rapid amplification of cDNA end products.

into two separate treatments, a control group that remained in a greenhouse at or above 25°C and a cold-treated group that was placed in a cold chamber at 4°C. Both treatments were under reduced light to promote dormancy. Each treatment contained at least 20 plants per strain inoculated. After 6 weeks of cold treatment, all the grapevines were returned to the greenhouse and were placed under a 10-h daylight cycle to grow out, for evaluation of *X. fastidiosa* persistence. After 6 weeks of regrowth, bacterial titer was determined by qPCR. In wild type-inoculated plants, the titer of *X. fastidiosa* was reduced 36% in the cold-treated samples compared with the untreated controls. In $\Delta csp1$ -inoculated plants, there was a 40% reduction in bacterial titer in cold-treated plants, which was not significantly different from the wild type. These results suggest that the contribution of Csp1 to *X. fastidiosa* cold tolerance in planta is minimal.

DISCUSSION

X. fastidiosa is a fastidious bacterium, requiring very specific conditions for optimal growth and replication. *X. fastidiosa* does not replicate at or below 12°C and, in many cases, shows

rapid loss of viability at 5°C (Feil and Purcell 2001). In contrast, many plant-pathogenic bacteria are able to consistently withstand a much greater range of temperatures both in vitro and in planta (Feil and Purcell 2001; Smirnova et al. 2001). The narrow range of temperatures conducive to growth and survival of *X. fastidiosa* in vitro suggests a limited ability to adapt to temperature fluctuations.

Cold exposure in mesophilic bacteria leads to disruption of transcription and translation, loss of membrane integrity, and disruption of cell metabolism (Cao-Hoang et al. 2010; Phadtare 2011; Phadtare and Severinov 2010). The severe impact of cold stress on bacterial cells has led to an evolved cold response, often including CSP with similar or overlapping function. In *E. coli*, nine different CSP (CspA to CspI) are expressed in response to cold stress, environmental changes, or the transition to stationary phase (Goldstein et al. 1990). Cold shock-induced expression of CSP is often substantial; CspA alone becomes up to 10% of the total cellular protein of *E. coli* at 15°C (Goldstein et al. 1990). Three CspA homologs in *Bacillus subtilis* are induced transiently after a temperature shift from 37 to 15°C and are important for optimal protein synthesis (Graumann et al. 1996, 1997). Similarly, *Listeria monocytogenes* CspA and CspD are dispensable for growth at 37°C but necessary for replication at lower temperatures (Schmid et al. 2009). In species with several CSP homologs, mutagenesis studies have demonstrated that multiple gene knockouts are necessary to see a reduction in cold-shock survival but that loss of all CSP at once is often lethal (Graumann et al. 1997; Schmid et al. 2009; Xia et al. 2001).

Certain members of the CSP family are temperature-independent, such as *E. coli* CspC and CspE, which are expressed constitutively at 37°C and are involved in regulation of the general stress response (Shenhar et al. 2012), or CspD, which is growth-phase dependent (Yamanaka and Inouye 1997). Expression of *Listeria monocytogenes* CspA and CspD are induced by high salt concentrations and at least one CSP is necessary for

Fig. 7. *Xylella fastidiosa* $\Delta csp1$ has reduced virulence in grapevine. Susceptible grapevines (*Vitis vinifera* cv. Chardonnay) were needle-inoculated with wild-type Stag's Leap (WT), a *csp1* deletion mutant ($\Delta csp1$), or the complemented deletion mutant ($\Delta csp1/csp1^+$). Plants inoculated with phosphate buffered saline served as negative controls. **A**, Symptom development 12 weeks postinoculation. **B**, Disease rating based on the 0 to 5 rating system by Roper et al. (2007). Bars represent means ($\pm$ standard error); means with different letters are significantly different based on analysis of variance followed by a Tukey-Kramer multiple comparison test ($\alpha = 0.05$).

Fig. 8. *Xylella fastidiosa* $\Delta csp1$ has lower bacterial titer in planta. Susceptible grapevines (*Vitis vinifera* cv. Chardonnay) were needle-inoculated with wild-type Stag's Leap (WT), a *csp1* deletion mutant ($\Delta csp1$) or the complemented deletion mutant ($\Delta csp1/csp1^+$). Plants inoculated with phosphate buffered saline served as negative controls. Bacterial titer was determined by quantitative polymerase chain reaction 12 weeks postinoculation. Quantity of *X. fastidiosa* DNA was determined based on a standard curve of wild-type Stag's Leap genomic DNA and was normalized to total DNA concentration. Bars represent means ($\pm$ standard error); means with different letters are significantly different based on analysis of variance followed by a Tukey-Kramer multiple comparison test ($\alpha = 0.05$).

osmotic-stress tolerance (Schmid et al. 2009). *Clostridium botulinum* also requires a set of CSP for survival and growth when exposed to high NaCl concentrations, low pH, or ethanol, in addition to cold stress (Derman et al. 2015; Söderholm et al. 2011). We found that *X. fastidiosa* Csp1 was important for survival of osmotic stress by exposure to 0.3 M NaCl, suggesting a role in certain aspects of environmental adaptation beyond cold-shock response. Although this salt concentration is higher than what would be encountered during plant colonization, experiments conducted with lower salt concentrations produced similar results, albeit over a longer timeframe (data not shown).

Accumulation of ROS is another component of temperature acclimation in plants as part of the stress-response signaling pathway (Chinnusamy et al. 2010); and in bacterial cells, CSP can be important for oxidative-stress defense (Chattopadhyay et al. 2011; Loepfe et al. 2010). However, sensitivity of *X. fastidiosa* to hydrogen peroxide was not affected by loss of Csp1. This may be due to predominance of the inducible oxidative-stress response, which includes production of detoxifying enzymes (Matsumoto et al. 2009), or the presence of other protective proteins, possibly a role of a second CSP, Csp2. Although some level of ROS are produced in xylem tissue (Barceló 1998), it is also possible that *X. fastidiosa* does not normally encounter the same magnitude of ROS, due to its specific habitat, and does not necessarily require the same level of oxidative-stress defense as other plant pathogens. However, it is demonstrated overall that Csp1 contributes to survival of *X. fastidiosa* under certain stress conditions in vitro.

Reporter expression indicated that *X. fastidiosa* Csp1 is not induced by a drop in temperature in vitro. It is likely that Csp1 is regulated in a manner similar to temperature-independent CSP, *E. coli* CspC and CspE (Phadtare and Inouye 2001; Shenhar et al. 2012). *X. fastidiosa* does not appear to have the highly redundant inducible cold-shock response found in many

cold-adapted bacterial species, perhaps due to its reduced genome and the specific niche colonized. *X. fastidiosa* encodes only two CspA family homologs in its genome. These two proteins alone may be responsible for both an inducible cold response and the other observed stress survival functions of CSP studied in other systems. Multiple attempts to create a knockout mutant in the second *X. fastidiosa* CSP (Csp2) were unsuccessful, suggesting lethality or a significant loss of fitness. In light of our findings that *X. fastidiosa* Csp1 is expressed independent of temperature, it is possible that Csp2 is more important for an inducible cold-shock response and for cold survival in planta. Furthermore, less redundancy in the cold-shock response may contribute to inherent sensitivity of *X. fastidiosa* to low temperatures.

In a number of animal pathogens, CSP are important for survival during host invasion as well as regulation of specific virulence-associated proteins (Erova et al. 2008; Loepfe et al. 2010; Michaux et al. 2012; Wang et al. 2014). Previously, no evidence for a specific role of CSP in plant infection was available, in spite of the fact that several plant-pathogenic bacterial species possess cold-induced virulence factors (Budde et al. 1998; Wei et al. 1992). Notably, $\Delta csp1$ was less virulent in grapevine under summer growing conditions without being subjected to cold stress. Reduced bacterial populations observed in $\Delta csp1$ -inoculated grapevines support the hypothetical role of Csp1 in stress survival during plant infection. However, this does not rule out a potential role of *X. fastidiosa* Csp1 in virulence-associated gene regulation through RNA binding activity.

MATERIALS AND METHODS

Bacterial strains and culture conditions.

All bacterial strains used are listed in Table 1. *E. coli* strains Top10 and BL21 Star (Life Technologies, San Francisco, CA,

Table 1. Strains and plasmids used in this study

Strains/plasmids	Characteristics ^a	Source
<i>Xylella fastidiosa</i> subsp. <i>fastidiosa</i> Stag's Leap	Wild-type <i>X. fastidiosa</i> , parent strain for mutants	
$\Delta csp1$	Knockout in PD1380, Cm ^R	This study
$\Delta csp1/csp1^+$	$\Delta csp1$ complemented by chromosomal insertion of <i>csp1</i> Cm ^R , Gm ^R	This study
$\Delta csp1/EccspA^+$	$\Delta csp1$ complemented by chromosomal insertion of <i>Escherichia coli cspA</i> Cm ^R , Gm ^R	This study
$\Delta csp1/Xccsp^+$	$\Delta csp1$ complemented by chromosomal insertion of <i>Xanthomonas campestris</i> pv. <i>campestris cspA</i> Cm ^R , Gm ^R	This study
<i>X. fastidiosa/csp1^+</i>	Wild-type Stag's Leap with chromosomal insertion of <i>X. fastidiosa csp1</i> , Gm ^R	
<i>X. fastidiosa/EccspA^+</i>	Wild-type Stag's Leap with chromosomal insertion of <i>E. coli cspA</i> , Gm ^R	This study
<i>X. fastidiosa/Xccsp^+</i>	Wild-type Stag's Leap with chromosomal insertion of <i>X. campestris cspA</i> Gm ^R	This study
<i>Escherichia coli</i> Top10	Cloning host, F [−] <i>mcrA</i> $\Delta(mrr-hsdRMS-mcrBC)$, $\Phi80lacZ\Delta M15$, $\Delta lacX74$, <i>recA1</i> , <i>araD139</i> , $\Delta(araleu)7697$, <i>galU</i> , <i>galK</i> , <i>rpsL</i> , (Str ^R), <i>endA1</i> , <i>nupG</i>	Life Technologies
<i>Escherichia coli</i> BL21 Star (DE3)	Expression host, F [−] <i>ompT</i> <i>hsdS_B</i> (<i>r_B−</i> , <i>m_B</i>) <i>galcdmre131</i> (DE3)	Life Technologies
<i>Xanthomonas campestris</i> 8004	Spontaneous rifampicin-resistant strain derived from <i>Xanthomonas campestris</i> pv. <i>campestris</i> NCPPB No. 1145	
Plasmids		
pCR8/GW/TOPO	TA cloning vector, Sp ^R , <i>att</i> sites for Gateway cloning	Life Technologies
pET101/D-TOPO	Expression vector, Ap ^R , C-terminal 6xHis tag	Life Technologies
pCR8- <i>csp1::chl</i>	<i>csp1::cat</i> cloned into pCR8/GW/TOPO, Sp ^R , Cm ^R	This study
pCR8-Xf <i>csp1</i> ORF	<i>X. fastidiosa csp1</i> cloned into pCR8/GW/TOPO, Sp ^R	This study
pCR8-EccspAORF	<i>E. coli cspA</i> cloned into pCR8/GW/TOPO, Sp ^R	This study
pCR8-XccspORF	<i>Xanthomonas campestris</i> pv. <i>campestris cspA</i> cloned into pCR8/GW/TOPO, Sp ^R	This study
pAX1-Gm	Suicide vector for recombination into neutral site 1 on <i>X. fastidiosa</i> Temecula chromosome, Gm ^R	Matsumoto et al. 2009
pAX1-Gm/GW	pAX1-Gm with Gateway cassette	This study
pAX1-Xf <i>csp1</i>	<i>X. fastidiosa csp1</i> cloned into pAX1-Gm, Gm ^R	This study
pAX1-EccspA	<i>E. coli cspA</i> cloned into pAX1-Gm, Gm ^R	This study
pAX1-Xccsp	<i>Xanthomonas campestris</i> pv. <i>campestris cspA</i> cloned into pAX1-Gm, Gm ^R	This study
pET101- <i>csp1</i>	<i>X. fastidiosa csp1</i> cloned into pET101/D-TOPO for inducible expression, Ap ^R	This study
pGEM-luc	Promoterless firefly luciferase gene (<i>luc</i>) in pGEM vector, Ap ^R	Promega
pCR8-luc	<i>luc</i> cloned into pCR8/GW/TOPO	This study
pCR8-EC <i>cspA-luc</i>	Reporter construct with <i>E. coli cspA-luc</i> , Sp ^R	This study
pCR8-Xf <i>csp1-luc</i>	Reporter construct with <i>X. fastidiosa csp1-luc</i> , Sp ^R	This study

^a Cm^R, Sp^R, Ap^R, Gm^R, and Str^R indicate resistance to chloramphenicol, spectinomycin, ampicillin, gentamycin, and streptomycin, respectively.

U.S.A.) were used as cloning and protein expression hosts, respectively. *E. coli* strains were grown in Luria-Bertani medium (LB) at 37°C, unless otherwise indicated, with the addition of antibiotics when necessary. For *E. coli*, antibiotics were used at the following concentrations (µg/ml): ampicillin, 100; spectinomycin, 100; kanamycin, 30; chloramphenicol, 35; and gentamycin, 10. *X. fastidiosa* Stag's Leap wild type and mutant derivatives were grown in PD3 (Davis et al. 1981) medium at 28°C. For *X. fastidiosa* strains, PD3 was supplemented, when necessary, with kanamycin, gentamycin, or chloramphenicol, at a concentration of 5 µg/ml. *Xanthomonas campestris* pv. *campestris* 8004 was grown in LB medium at 28°C.

ΔcspI mutant construction.

The genome sequence of *X. fastidiosa* Temecula-1 (Van Sluys et al. 2003) was used for reference in the absence of the full genome sequence of the Stag's Leap strain. All primer sequences used for PCR amplification are listed in Table 2. The ORF in Stag's Leap corresponding to Temecula 1 locus tag PD1380 is referred to as *cspI*. This region (including 1 kb upstream and downstream) was PCR-amplified, using primers csp1LFfwd and csp1RFrev, and was sequenced prior to mutagenesis. Nucleotide sequence of *cspI* was 100% identical to PD1380 of Temecula-1. One-kilobase DNA fragments upstream and downstream of *cspI* were PCR-amplified, using Stag's Leap genomic DNA as template and primer pairs csp1LFfwd and csp1LFrevChl and csp1RFfwdChl and csp1RFrev. A chloramphenicol resistance cassette from the Tn5 HyperMu transposon (Epicentre, Madison, WI, U.S.A.) was PCR-amplified separately, using primers Chlfwd and Chlrev. Primers csp1LFrevChl and csp1RFfwdChl contain 15 base pairs of sequence homologous to the chloramphenicol resistance marker. Upstream and downstream flanking regions, and the chloramphenicol resistance marker, were annealed together using overlap extension PCR (Heckman and Pease 2007). The combined PCR

product of approximately 3kb was gel-purified and cloned into vector pCR8/GW/TOPO (Life Technologies), following the manufacturer's instructions, to create pCR8-csp1::chl. This plasmid was transformed into *X. fastidiosa* by electroporation, following the protocol of Guilhabert et al. (2001). Homologous recombination and gene replacement were selected for by plating transformants on PD3 with chloramphenicol. Chloramphenicol-resistant colonies were screened by colony PCR, using primer sets RST31 and RST33 (*X. fastidiosa*-specific primers) and csp1outfwd and csp1outrev (gene-specific primers). Correct mutants (Δ*cspI*) were further confirmed by cloning and sequencing the site of recombination.

Mutant complementation.

For complementation of mutant Δ*cspI*, the *cspI* ORF and flanking sequences were inserted into a neutral region of the chromosome, as described by Matsumoto et al. (2009). Briefly, *cspI* with an additional 250bp upstream and downstream (to include all native promoter and terminator sequences) was PCR-amplified, using primers csp1ORFfwd and csp1ORFrev and was cloned into vector pCR8 and GW/TOPO, following the manufacturer's instructions, creating the pCR8-csp1ORF. Chromosomal insertion vector pAX1-Gm (Matsumoto et al. 2009) was modified using the Gateway vector conversion kit (Life Technologies) to create vector pAX1-Gm/GW. The resulting construct (pCR8-csp1ORF) was recombined with pAX1-Gm/GW, using LR clonase enzyme mix (Life Technologies), according to the manufacturer's protocol, to create insertion construct pAX1-Xfcsp1. This plasmid was transformed into Δ*cspI* by electroporation; recombination into the neutral site was confirmed by cloning and sequencing the site of recombination. This complemented strain was called Δ*cspI/cspI*⁺.

Strains Δ*csp/EccspA*⁺ and Δ*csp/Xccsp*⁺ were created in a similar manner, except with CSP genes derived from *E. coli* (ECDH10B_3735) or *X. campestris* pv. *campestris* (XC_1828).

Table 2. Primer sequences used for polymerase chain reaction amplification

Primer	Sequence	Source or reference
RST31	GCGTTAATTTTCGAAGTGATTTCGATTGC	Minsavage et al. 1994
RST33	CACCATTCGTATCCCGGTG	Minsavage et al. 1994
csp1LFfwd	GTCGCTAGTGGTGTGATGATTAA	This study
csp1LFrevchl	CGATAGGATCCGTGGTGATGCGAGA	This study
csp1RFfwdchl	GAAAAGGATCCGTGAAATGTCGCGT	This study
csp1RFrev	CACGCTCCCATTCGCATATACA	This study
Chlfwd	AACGGATCCTATCGTCAATTATTACCTCCA	This study
Chlrev	ACGGATCCTTTTCGACCGAATAAATACC	This study
Csp1 ORF fwd	TCTCCAACCTGGGCGGC	This study
Csp1 ORF rev	TTTTAATGAACACTAGGATGGTTGA	This study
ECcspAfw	GTATCAGTCGCCATGGTAAAGGAA	This study
ECcspArev	AAGCAGAGATTACAGGCTGTTTACGTTACC	This study
<i>Xanthomonas campestris</i> pv. <i>campestris</i> cspA fwd	CAAGACCTGGCGGGAGTG	This study
<i>Xanthomonas campestris</i> pv. <i>campestris</i> cspA rev	TTAGACCGCCTGCACCTG	This study
Csp1expfwd	CACCATGCAGAGCGGTACAGTTAAG	This study
Csp1exprev	TGCTGGCGTGATATTCGATGCTTG	This study
csp1outfwd	GTTGTTTGGCCAGCGTGTGG	This study
csp1outrev	GAGCAAGCACTCTTATTACCGACCC	This study
Xf145-60F	TACATCGGAATCTACCTTATCGTG	Ledbetter and Rogers 2009
Xf145-60R	ATGCGGTATTTAGCGTAAGTTTC	Ledbetter and Rogers 2009
<i>Xylella fastidiosa</i> RACE 1	CCCTGCGTCACGTATAAGT	This study
EC RACE 1	CTTTAGCGCCGCTTTTCGATG	This study
<i>X. fastidiosa</i> RACE 2	GGGAGTCCCATCATCCGGTA	This study
<i>Escherichia coli</i> RACE 2	AGAGCCATCGTCAGGAGTGA	This study
LucFwd	ATGGAAGACGCCAAAAACATAAAG	This study
LucRev	AATTTGGACTTTCCGCCCTTCTTGG	This study
Xf5UTRfwd	GTTGCGCGAGAGTGATTGTC	This study
XfORFlucrev	ATGTTTTTGGCGTCTTCCATTATTGGCGTGATATTCGATG	This study
EC5UTRfwd	CCTGATGACAGGACCGTTTTCACAC	This study
ECORFlucrev	TGTTTTTGGCGTCTTCCATCAGGCTGGTTACGTTACCAGC	This study

Both genes were PCR-amplified (including an additional 250bp upstream and downstream) from genomic DNA of *E. coli* Top10 or *X. campestris* pv. *campestris* 8004, using primer sets ECcspAfw/ECcspArev or XCcspAfw/XCcspArev, respectively. The amplification products were cloned into vector pCR8/GW/TOPO to create plasmids pCR8-EccspA and pCR8-Xccsp. These plasmids were recombined with insertion vector pAX1-Gm/GW to create pAX1-EccspA and pAX1-Xccsp. These two plasmid constructs were then transformed into *X. fastidiosa* $\Delta csp1$ to create strains $\Delta csp1/EccspA^+$ and $\Delta csp1/Xccsp^+$. Strains *X. fastidiosa* *csp1*⁺, *X. fastidiosa*/EccspA⁺, and *X. fastidiosa*/Xccsp⁺ were created by transforming wild-type *X. fastidiosa* Stag's Leap with insertion vectors pAX1-Xfcspl, pAX1-EccspA, and pAX1-Xccsp.

Cold tolerance assay.

X. fastidiosa strains were grown on PD3 plates for 7 days at 28°C. Cells were harvested by scraping off plates and resuspended in sterile 1× PBS. Starting cell density was normalized by measuring optical density at 600nm (OD₆₀₀) and adjusting the concentration to OD₆₀₀ = 0.1 with 1× PBS. A 1-ml aliquot of cells from each strain was removed and serial dilutions were plated on PD3 to serve as an untreated control. Cold-treated samples were kept at 4°C for 24 h and, then, serial dilutions were plated on PD3. CFU were counted for quantification after 7 days of incubation at 28°C. Percent survival indicates number of colonies in the cold-treated sample divided by number of colonies for the corresponding untreated control. Experiments were repeated at least three times, including a total of at least nine independent biological replicates.

Salt tolerance.

Cells were grown on PD3 plates for 1 week and were harvested in 1× PBS. Cell concentration was normalized to OD₆₀₀ = 0.25 and was divided into 1-ml aliquots. Cells were concentrated by centrifugation, were resuspended in either 1× PBS (control) or NaCl at a concentration of 0.3, 0.1, or 0.05 M, and were placed on a shaker (180 rpm) for 1 h (0.3 M treatment), 48 h (0.1 M treatment), or 72 h (0.05 M treatment) at 28°C. After incubation, cells were centrifuged and were washed twice in 1× PBS to remove salt, and serial dilutions were plated on PD3. CFU were quantified after 10 days of growth at 28°C. Percent survival was based on number of colonies from salt-treated samples divided by number of colonies for the corresponding untreated control. Experiments were repeated at least three times, including a total of at least nine independent biological replicates.

Hydrogen peroxide sensitivity.

Hydrogen peroxide sensitivity was quantified using a disc inhibition assay as described by Matsumoto et al. (2009). Cells were grown on PD3 for 1 week at 28°C, were harvested with PD3 liquid medium, and were normalized to OD₆₀₀ = 0.1. Cell suspensions (500 µl) were mixed with 5 ml of PD3 top agar (0.8% agar) and were spread on PD3 plates. A small paper disc was placed in the center of each plate and was soaked with 10 µl of 0.5% hydrogen peroxide. Zone of inhibition radii were measured after 7 days of growth at 28°C. Experiments were performed three separate times to include a total of nine biological replicates.

Expression and purification of *X. fastidiosa* Csp1.

The ORF of *csp1*, sans stop codon, was PCR-amplified, using primers csp1expfwd and csp1exprev, and were cloned into the inducible expression vector pET101/D-TOPO (Life Technologies). Expression clones of pET101-csp1 were screened by sequencing to determine correct orientation and reading frame in relation to the C-terminal 6xHis tag and to confirm correct sequence. For induced expression, pET101-csp1 was

transformed into BL21 Star Competent cells (Life Technologies). Protein expression was induced by addition of 400 µM isopropyl-β-D-thiogalactoside to midlog phase cultures, followed by 4 h of growth at 37°C. Cells were harvested by centrifugation and were frozen at -20°C. Frozen cell pellets were lysed with B-PER bacterial protein extraction reagent (Life Technologies) with 100 µg of lysozyme per milliliter (Sigma-Aldrich, St. Louis), protease inhibitor cocktail (Sigma-Aldrich), and 4U of Turbo Dnase per milliliter (Life Technologies) was added. Recombinant Csp1-His was purified from cell lysates using HisPur Ni-NTA columns (Life Technologies) and was eluted with 250 µM imidazole (Sigma-Aldrich). Purified protein samples were concentrated by filtration, using Ambion Ultra 0.5 centrifugal filter devices (EMD Millipore, Darmstadt, Germany).

RNA binding assay.

Plasmid pCR8/GW/TOPO, linearized with *EcoRI* (New England Biolabs, Ipswich, MA, U.S.A.), was used as a template for in vitro transcription of biotin labeled RNA probes (150 nt), using AmpliScribe T7 Biotin-RNA kit (Epicentre). Transcribed RNA was purified by phenol-chloroform extraction and ethanol precipitation. Binding assays were performed in 10 µl of binding buffer (Jiang et al. 1997) containing 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50 mM KCl, and 7.4% glycerol. Binding reactions contained 20 ng RNA and increasing concentrations of purified Csp1-His (1, 3, and 5 µg). Bovine serum albumin (Sigma-Aldrich) was used as a nonbinding protein control (5 or 10 µg). After 10 min of incubation at room temperature, samples were electrophoresed on a 5% acrylamide gel in 0.5× tris-borate-EDTA at 200 V for 35 min and were transferred to a Biotodyne B positively charged nylon membrane (Life Technologies). Following transfer, blots were placed face down on a 302 nm UV transilluminator for 2 min for cross-linking. RNA detection was carried out using the Chemiluminescent detection module (Life Technologies), with imaging on a FluorChem E CCD imager (ProteinSimple, San Jose, CA, U.S.A.).

Sequencing of 5' UTR.

Total RNA was extracted from *X. fastidiosa* cultures grown on PD3 plates for 6 days at 28°C and from *E. coli* cultures grown on LB overnight at 37°C. RNA extraction was done following the protocol by Jahn et al. (2008). RNA samples were treated with Turbo Dnase (Life Technologies), following the manufacturer's protocol to remove trace genomic DNA. Total RNA (1 µg) was used as template for 5' RACE (Scotto-Lavino et al. 2007), using the 5' RACE system kit (Life Technologies), with primers specific for *csp1* of *X. fastidiosa* (XfRACE1) or *cspA* of *E. coli* (ECRACE1), for the respective RNA samples. Products were PCR-amplified using primers XfRACE2 or ECRACE2 along with Abridged Anchor Primer, provided as part of the kit. PCR products were gel-purified and were cloned into pCR8/GW/TOPO for sequencing.

Luciferase reporter assay.

Reporter constructs were made with a promoterless firefly luciferase gene cloned from pGem-luc (Promega, Madison, WI, U.S.A.). The *luc* ORF was PCR-amplified using primers Lucfwd and Lucrev. This DNA fragment was cloned into pCR8/GW/TOPO to serve as a promoterless control for the reporter assays (pCR8-luc). DNA fragments consisting of the promoter, 5' UTR, and ORF (without a stop codon) of *X. fastidiosa* *csp1* and *E. coli* *cspA* were PCR-amplified using primer sets Xf5UTRfwd and XfORFlucrev and EC5UTRfwd and ECORFlucrev, respectively. These fragments were then attached to the *luc* PCR product using overlap extension PCR and were cloned into pCR8/GW/TOPO, creating reporter fusion constructs pCR8-Xfcspl-luc and pCR8-EccspA-luc.

All three plasmids were transformed into *E. coli* Top10 for propagation. Reporter strains were grown at 37°C until reaching a cell density of OD₆₀₀ = 0.5; 1-ml aliquots were removed for untreated controls. Cold-treated samples were incubated at 15°C on a shaker for up to 2 h. All samples were harvested by centrifugation and were lysed in 300 µl of B-Per bacterial protein extraction reagent, as described for Csp1-His purification. Total protein (1 µg) from each sample was used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with firefly luciferase monoclonal antibody (mouse-Luc17) (Life Technologies). Blots were developed with Pierce SuperSignal West Pico chemiluminescent substrate (Life Technologies) and were imaged with FluorChem E CCD imager (ProteinSimple).

Virulence assays.

Grapevines (*Vitis vinifera* cv. Chardonnay) were inoculated with wild-type Stag's Leap, $\Delta cspI$, and $\Delta csp/csp^+$, using the method by Roper et al. (2007). Plants (20 plants per treatment) were observed for symptom development weekly and were assigned a disease rating (0 to 5) based on the system by Roper et al. (2007). Bacterial titer was determined by qPCR at 12 weeks postinoculation. Total DNA was extracted from grapevine petioles 15 cm above the point of inoculation. qPCR was performed using AB SYBR Green master mix (Life Technologies) following the manufacturer's instructions, with 5 µl of total DNA as template and primer set XF145-60fwd and XF145-60rev (Ledbetter and Rogers 2009). Titer of *X. fastidiosa* was determined based on a standard curve of wild-type Stag's Leap genomic DNA, normalized to total DNA concentration. For cold treatment of inoculated vines, plants after 12 weeks postinoculation were pruned back to a single cane about 2 feet long and were separated into two treatment groups (20 plants per strain of each treatment). Plants in the control group remained in the greenhouse at or above 25°C and cold-treated vines were placed in a cold chamber at 4°C. Both treatments were under reduced light conditions to promote dormancy. After 6 weeks of cold treatment, all vines were returned to the greenhouse and put under a 10-h daylight cycle to grow out. After 6 weeks of regrowth, bacterial titer was determined by qPCR as described above.

Tests for statistical significance.

Significance of quantitative differences among treatments (Figs. 2, 3, 7, and 8) was determined using one way analysis of variance followed by a Tukey-Kramer multiple comparison test ($\alpha = 0.05$). All statistical analyses were performed using Origin 2015 graphing and analysis software (OriginLab Inc., Northampton, MA, U.S.A.).

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