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A chitinase is required for *Xylella fastidiosa* colonization of its insect and plant hosts

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Abstract

Xylella fastidiosa colonizes the xylem network of host plant species as well as the foregut of its required insect vectors to ensure efficient propagation. Disease management strategies remain inefficient due to a limited comprehension of the mechanisms governing both insect and plant colonization. It was previously shown that *X. fastidiosa* has a functional chitinase (ChiA), and that chitin likely serves as a carbon source for this bacterium. We expand on that research, showing that a *chiA* mutant strain is unable to grow on chitin as the sole carbon source. Quantitative PCR assays allowed us to detect bacterial cells in the foregut of vectors after pathogen acquisition; populations of the wild-type and complemented mutant strain were both significantly larger than the *chiA* mutant strain 10 days, but not 3 days, post acquisition. These results indicate that adhesion of the *chiA* mutant strain to vectors may not be impaired, but that cell multiplication is limited. The mutant was also affected in its transmission by vectors to plants. In addition, the *chiA* mutant strain was unable to colonize host plants, suggesting that the enzyme has other substrates associated with plant colonization. Lastly, ChiA requires other *X. fastidiosa* protein(s) for its *in vitro* chitinolytic activity. The observation that the *chiA* mutant strain is not able to colonize plants warrants future attention to be paid to the substrates for this enzyme.

INTRODUCTION

Xylella fastidiosa is an economically important vector-borne bacterial plant pathogen [1]. It is the a etiological agent of Pierce's disease of grapevines, as well as diseases in many other crops, including almonds, citruses, coffee, peaches and plums [2]. Vector transmission of *X. fastidiosa*, the only means by which this bacterium naturally spreads, is dependent on xylem sap-sucking insects including sharpshooter leafhoppers (Hemiptera: Cicadellidae). Transmission occurs in a non-circulative yet propagative manner [3]. There is no specific relationship between *X. fastidiosa* genotype and vector species, and vector transmission efficiency is dependent on plant–insect–pathogen interactions [4–6]. Despite the importance of plant-to-plant spread for the epidemiology and management of *X. fastidiosa*, vector transmission and insect colonization remain the most poorly understood aspects of the biology of this bacterium. Once acquired from infected plants, *X. fastidiosa* cells adhere to the cuticular surface of the foregut of vectors [7, 8]. The region in the foregut associated with *X. fastidiosa* colonization and inoculation, the precibarium [7], is a dynamic environment through which ~1000 times the leafhopper's body weight worth of xylem sap is ingested daily, at speeds estimated to reach 8 cm s^{-1} [9]. Therefore, successful cell adhesion to the cuticle has been proposed to be an uncommon event, as suggested by microscopy observations and population abundance estimates by quantative PCR (qPCR) [7, 8].

Xylella fastidiosa was the first bacterial plant pathogen to have its genome fully sequenced [10], offering new tools and important insights into its biology. In particular, studies have shown that interactions between *X. fastidiosa* and its insect host surface are required for vector colonization and transmission [8, 11]. It has been shown that several *X. fastidiosa* proteins, particularly adhesins, play a role in this transmission process [8, 11, 12]. Disruption of those interactions has been demonstrated as a possible approach towards blocking *X. fastidiosa* transmission to plants and thus to control of disease spread [13, 14]. For example, specific *X. fastidiosa*-derived peptides with domains that bind to chitin have been shown to block transmission under greenhouse conditions, possibly by competing with *X. fastidiosa* cells for binding sites on the cuticular foregut of

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Abbreviations: CBM, carbohydrate-binding module; CBP, chitin-binding protein; ChiA, chitinase; NS, neutral site; PWG, periwinkle wilt medium with Gelrite; qPCR, quantitative PCR; XFX, *Xylella fastidiosa* medium.

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One supplementary figure and one supplementary table are available with the online Supplementary Material.

insect vectors [14]. However, little is known about the chemical composition of the cuticle of vectors in the foregut, which is of ectodermic origin and therefore part of the exocuticle (as wings), being predominantly composed of chitin and related carbohydrates. These compounds have been shown to play a central role in *X. fastidiosa* adhesion to vectors [8]. However, subsequent bacterial colonization of insects and the role of chitin in this process remain poorly understood.

A previous study identified a functional chitinase (ChiA) in *X. fastidiosa*; it was also suggested that this bacterium could use chitin as a carbon source [15]. Because *X. fastidiosa* multiplies on the chitinous surface of the foregut of vectors [8, 16], and persistently colonizes adult insects [16–18], the cuticle of leafhoppers could potentially serve as a food source, enabling the bacterium to multiply and permanently colonize its vector host. This seems likely given the low nutrient content of xylem sap ingested by the insects. To determine how essential ChiA is to insect colonization, we compared the ability of a *X. fastidiosa chiA* mutant, as well as complemented strains, in order to determine their capacity to grow in the presence or absence of chitin as a carbon source. We also determined the role of ChiA in insect and plant colonization in addition to its transmission between plants. Our results suggest that this enzyme plays important roles not only in insect colonization, but also in dissemination within and between plants.

METHODS

Strains, media and insects

Xylella fastidiosa subspecies *fastidiosa* strain Temecula1 [19] was used in all experiments. *X. fastidiosa* cells were cultured on periwinkle wilt medium with Gelrite (PWG) [16] or on *X. fastidiosa* medium (XFM) [11] at 28 °C. The investigation of the use of chitin as a carbon source was performed by comparing *X. fastidiosa* wild-type, *chiA* mutant or *chiA*-complemented strains for growth on different media supplemented (or not) with chitin (XFM or XFM-chitin) but also depleted (or not) of the additional carbon sources (XFMΔ or XFM) initially present in the medium as previously described [15].

For plant inoculation or vector colonization and transmission experiments, *X. fastidiosa* cells were propagated on XFM solid medium supplemented with galacturonic acid (XFM-Gal; 0.01 % w/v) as an inducer of the transmission process [11]. Adult leafhopper vectors *Grophocephala atropunctata* (Signoret) (Hemiptera: Cicadellidae) were collected from California wild grapes (*Vitis* spp.) in Berkeley, California. Insect colonies were established in a greenhouse on sweet basil (*Ocimum basilicum*) from eggs laid by the field-collected individuals. Because *X. fastidiosa* is not transovarially or transtadially transmitted [20, 21], a colony initiated with eggs and the sequential transfers to new basil plants used to maintain the insects would result in a *X. fastidiosa*-free population. For transmission experiments, adults were used with previously described protocols [11].

Construction of the *X. fastidiosa chiA* mutant strain

Deletion of *chiA* (PD1826) was carried out by transforming the *X. fastidiosa* strain with a construct derived from the suicide vector pFXF*kan* [22], in which two 1000 bp genomic fragments adjacent to PD1826 in the *X. fastidiosa* Temecula1 genome flanked the kanamycin resistance gene. Regions flanking PD1826 were amplified and cloned into pFXF*kan* [23] using the primers listed in Table S1 (available in the online Supplementary Material) to generate pFXF11 (PD1826 :: kan^R). This suicide vector construct was transformed into *X. fastidiosa* Temecula1 by exploiting its natural competence [22, 24]. Transformed cells were cultured on PWG plates supplemented with kanamycin for 2 to 3 weeks at 28 °C. Colonies were screened for loss of PD1826 by PCR using the appropriate primers listed in Table S1. Gene deletion was confirmed by sequencing the PCR product directly amplified from genomic DNA purified from the selected mutant clones.

Complementation of the PD1826-deleted mutant

The gene encoding PD1826 and a 1000 bp region immediately upstream (presumably harboring its promoter) were amplified from the *X. fastidiosa* genome using the primers PD1826-COMP-F2 and PD1826-COMP-R. The PCR product was cloned into the chromosomal-based complementation vector pAX1-Cm^R [24] at the *XhoI* and *XbaI* restriction sites to generate pAX1-CmR/PD1826, which was then transformed into the *X. fastidiosa* PD1826 knockout strain [22, 24]. Recombinant cells were selected on PWG plates supplemented with kanamycin (50 $\mu g\,ml^{-1}$) and chloramphenicol (20 $\mu g\,ml^{-1}$). Integration of the PD1826 allele into a neutral site (NS1) in the chromosome was verified by PCR using primers PD0702-F and PD0703-R [25].

Expression and purification of recombinant *X. fastidiosa* ChiA

Purified recombinant His_6-tagged *X. fastidiosa* ChiA was used in this study. Briefly, the *chiA* gene, lacking its initially predicted 19-aa signal peptide [15], was amplified using the primers ChiA-F1 (5′-TCCCGATCGCGTAACACC-3′) and ChiA-R1 (5′-AGTGCCTGTGTTGGGTGG-3′), and then cloned into pGEM-T Easy (Promega). Once the sequence was verified, the fragment was inserted into pET28a(+) (Novagen) and used to transform *E. coli* BL21λDE3 Rosetta. His_6-tagged ChiA was then expressed, purified, and the His_6-tag excised following protocols described previously (Fig. S1) [14]. De-tagged ChiA treated with thrombin for 2 h (lane 4, Fig. S1c) was used in this study. The concentration of recombinant ChiA was assessed using the Bradford procedure, and 500 µl aliquots containing ~1 mg of purified protein were stored at −20 °C until further use.

Assay of ChiA activity *in vitro*

The chitinolytic activity of *X. fastidiosa* and ChiA recombinantly expressed in *E. coli* has been assessed previously [15]. In this study, we followed the protocols used by Killiny *et al.* [15] to measure chitinase activity using 4-methylumbelliferyl *N*-acetyl-β-D-*N,N′,N″*–triacetylchitotriose [4-MU(GlcNAc)$_3$]

as a substrate, following the manufacturer's instructions (Sigma-Aldrich). Whole-protein extracts (wild-type and *chiA* mutant strains) were prepared as described previously [14], with *X. fastidiosa* cells grown on XFM-chitin medium to induce chitinolytic activity. ChiA activity was also tested with recombinant purified ChiA, as well as purified ChiA mixed with whole-protein extracts of the *chiA* mutant for 10 min at 60 r.p.m. using a vertical orbital shaker, before being subjected to native electrophoresis. For each condition, chitinolytic activity was visualized under UV light [26]. Each strain–medium–time point combination was replicated three times.

Insect transmission

Insects (*Graphocephala atropunctata* adults) were fed on a diet solution containing 10^8 cells ml^{-1} of *X. fastidiosa* wild-type, *chiA* mutant or *chiA* mutant complemented strains grown on XFM-Gal medium for 4 h for pathogen acquisition. Insects were then individually transferred to a single leaf on an uninfected grapevine (*Vitis vinifera*) and were provided with an inoculation access period of 24 h. Plants were incubated in a greenhouse for 2 months prior to detection, as described previously [17]. The experiment was fully replicated twice and 12 plants were used for each treatment in each repetition of the experiment.

Insect host colonization

In order to correlate *X. fastidiosa* transmission rates with insect colonization, we measured the *X. fastidiosa* population within insect heads by qPCR, as described previously [11]. Briefly, adult insects were fed on suspensions of *X. fastidiosa* wild-type, *chiA* mutant or *chiA* complemented strains for 4 h. After this acquisition access period, the insects were transferred onto basil plants for 3 and 10 days to enable cell multiplication; 10 individuals (replicates) from each time point were randomly selected for qPCR measurements, which were performed as described previously [27].

Plant host colonization

Certified pathogen-free dormant cuttings of grape cultivar Cabernet Sauvignon were rooted in a mix of perlite and vermiculite (1 : 1) on a misting bench in a greenhouse. Certified cuttings were kindly provided by Foundation Plant Services, University of California, Davis. Following root development, cuttings were transplanted into 5 cm pots filled with Supersoil potting soil (Rod McLellan Company, San Mateo, CA) and, after roots appeared, plants were transferred into 1-gallon pots containing a mix of Supersoil (50 %), sand (25 %) and vermiculite (25 %), and maintained in an insect-free glasshouse in the same facility. Pathogen inoculum was prepared by growing *X. fastidiosa* wild-type, *chiA* mutant or *chiA* complemented cells on XFM-Gal medium. Twenty microlitres of a suspension (10^9 cells ml^{-1}) of each strain were used to mechanically inoculate 6-month-old grapevines at the stem base using standard procedures [16]. Inoculations were performed twice (experimental blocks), 15 plants were inoculated in each block and the plants were maintained as described previously [28]. Eight weeks after inoculation, petioles were removed from immediately adjacent to the inoculation point as well as 15 cm above the point of inoculation. Sampling close to the inoculation point was done to test *X. fastidiosa* persistence within the plant, whereas the more distant samples enable detection of movement and colonization of vines. The petioles collected were macerated and cells cultured on PWG plates to confirm bacterial presence [16]. qPCR, as described above, was used on a subset of samples (five per treatment) to estimate bacterial populations in the petioles of leaves 1 and 15 cm above the inoculation site. Foliar symptoms of Pierce's disease (leaf scorching) were evaluated 2 months after inoculation.

Statistical analyses

Differences in the role of ChiA activity on *X. fastidiosa* growth in various media were assessed using repeated-measure two-way ANOVA, considering day as a repeated-measures random effect and strain and media as fixed effects. Optical density was natural log transformed to meet linear model assumptions. We tested for differences in insect transmission using a logistic mixed-effects regression model, with media as a fixed effect and each experimental repetition was treated as a block random effect. For the plant colonization data, a logistic regression with penalized maximum likelihood estimation was used due to quasi-separation (i.e. some factor levels were zero). Differences among the strains on insect colonization were assessed using ANOVA; *X. fastidiosa* population data (excluding insects below the qPCR detection threshold) were natural log transformed to meet linear model assumptions, and Tukey's honest significant difference test was used for pair-wise comparisons among treatments. R 3.2.1 software was used for all analyses [29], with the lme4 package for repeated-measures and logistic-mixed-effects regression [30], the brglm package for logistic regression with penalized maximum likelihood [31], and the multcomp package for pair-wise comparisons [32].

RESULTS

Xylella fastidiosa requires ChiA to exploit chitin as a carbon source

Although Killiny *et al.* [15] demonstrated that chitin promoted *X. fastidiosa* growth in culture, they did not demonstrate that *X. fastidiosa* could grow on chitin as a sole carbon source. We thus generated a *chiA* mutant and a complemented *chiA* mutant strain to address this question. These strains, as well as the wild-type, were each grown in a basal medium supplemented with two organic acids (succinate and citrate) as carbon sources (XFM), XFM supplemented with colloidal chitin (XFM-chitin), XFM devoid of the organic acids but supplemented with colloidal chitin (XFMΔ-chitin), and XFM devoid of any carbon source (XFMΔ). No strain grew in XFMΔ, the medium without carbon sources (XFMΔ main effect: df=253, $t= -9.86$, $P<0.0001$) (Fig. 1). The *chiA* mutant strain was unable to grow in the XFMΔ-chitin medium (*chiA* mutant x XFMΔ-chitin interaction: df=253, $t= -5.492$, $P <0.0001$). Both the

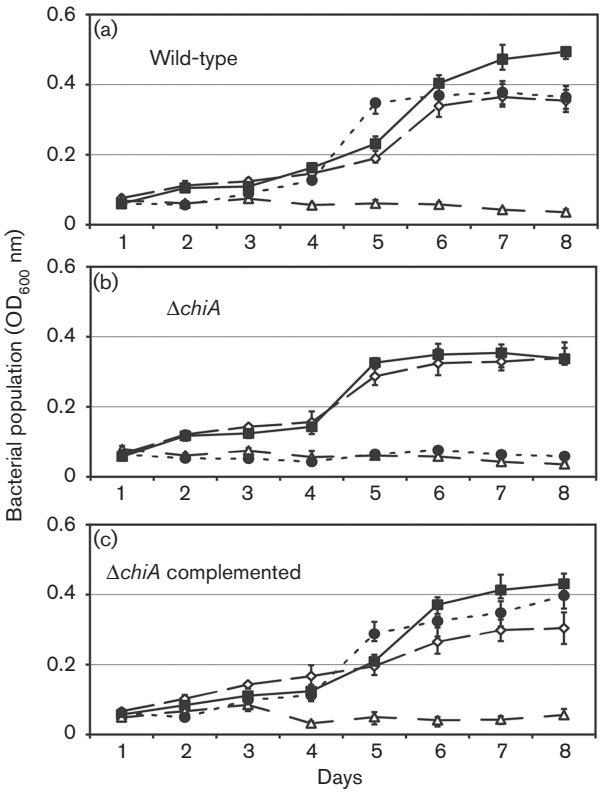

Fig. 1. *In vitro* growth of *X. fastidiosa* wild-type (a), *chiA* mutant (b) and its complemented strain (c) in a basal medium without a carbon source (XFMΔ, triangles), basal medium supplemented with chitin (XFM-chitin, squares), medium with chitin as the sole carbon source (XFMΔ-chitin, circles) and on media with organic acids as carbon sources (XFM, empty diamonds). Differences among media, strains and dates were modelled using a repeated-measures two-way ANOVA. Sample size: three biological replicates for each strain–media–date combination. Error bars represent ±SE.

wild-type and the complemented *chiA* mutant strain exhibited equivalent growth in all four media (df=253, t= −0.393, P=0.695) (Fig. 1). Together, these results demonstrate that ChiA is required for *X. fastidiosa* to utilize chitin as a carbon source, and that chitin is sufficient to support growth of this bacterium *in vitro*.

Xylella fastidiosa proteins are required for ChiA chitinase activity

We hypothesized that ChiA requires carbohydrate-binding protein partners to mediate interactions with its substrate (s). Substrate (4-MU(GlcNAc)$_3$) cleavage, releasing the fluorescent product (4-MU), was observed for whole-cell protein extracts of wild-type cells as well as protein extracts from the *chiA* mutant strain supplemented with recombinant ChiA (Fig. 2, lanes 1 and 4), but not the *chiA* mutant strain or ChiA alone. These data support the hypothesis that ChiA interacts with other *X. fastidiosa* protein(s) to function as a chitinase, presumably to facilitate chitin binding.

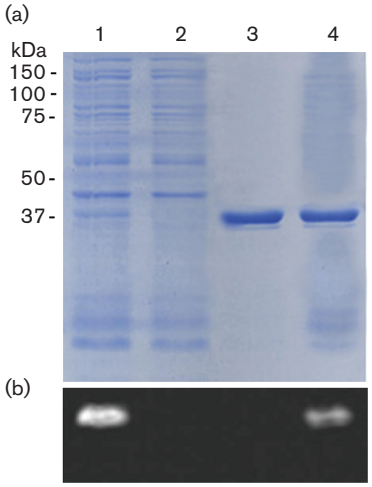

Fig. 2. Impact of the presence or absence of whole *X. fastidiosa* protein extract on the chitinolytic activity of ChiA. (a) Colloidal blue-stained SDS gel containing: lane 1, total proteins of *X. fastidiosa* wild-type Temecula cells; lane 2, total proteins of *chiA* mutant; lane 3, recombinant purified ChiA only; and lane 4, recombinant purified ChiA incubated with total proteins from the *chiA* mutant. (b) Detection of enzymatic activity after incubation of the same samples with the substrate [4-MU(GlcNAc)$_3$; Sigma-Aldrich], which releases a fluorescent product (4-MU) that can be visualized under UV light if subjected to chitinolytic activity.

Vector transmission of the *chiA* mutant strain is reduced

Because chitin and chitin-related carbohydrates are targets for insect colonization by *X. fastidiosa*, we tested whether ChiA was necessary for its vector transmission (Fig. 3a). Transmission of the wild-type strain was efficient (61 % efficiency; 14 out of 23 plants tested), while the *chiA* mutant strain exhibited reduced vector transmission with only six out of 23 plants tested becoming infected (25.8 % efficiency). The transmission efficiency of the complemented *chiA* mutant strain was restored to a level equivalent to the wild-type strain (12 out of the 23 plants tested, 52.3 % efficiency). The transmission of the wild-type strain was significantly higher than the *chiA* mutant strain (n=23, Z= −2.32, P=0.02), but was not significantly different from the complemented strain (n=23, Z= −0.59, P=0.55).

ChiA is required for *X. fastidiosa* colonization of the insect host

Xylella fastidiosa attaches to the cuticular foregut of vectors, forming a persistent biofilm that increases in population size with time. To determine the role of ChiA in insect colonization, we estimated bacterial population size in insects 3 and 10 days after cell acquisition. When assessed at 3 days after acquistion, all tested strains had equivalent *X. fastidiosa* populations (Fig. 3b; n=6, $F_{2,14}$=0.33, P=0.73). However, by day 10, population sizes of the different *X. fastidiosa* strains differed significantly (n=8, $F_{2,20}$=103.20,

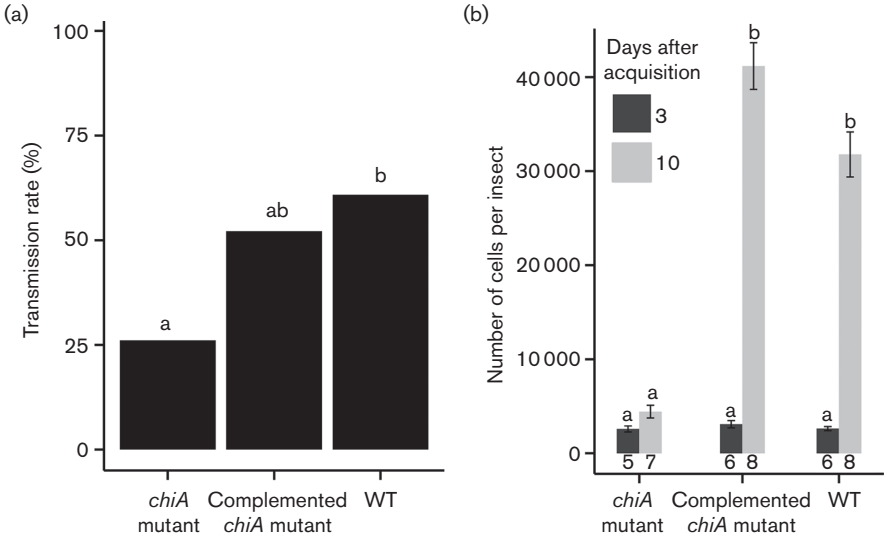

Fig. 3. Role of ChiA in *X. fastidiosa* vector transmission and colonization. (a) Transmission rate of *X. fastidiosa* wild-type, *chiA* mutant and the complemented *chiA* mutant strains; treatments with a given letter do not differ (α=0.05) according to a mixed-effects logistic regression model. (b) Number of *X. fastidiosa* cells detected by qPCR after 3 days (black bars) and 10 days (grey bars) post acquisition; means with the same letter are not significantly different according to Tukey's honest significant difference test (α=0.05) according to an ANOVA model. Numbers under bars indicate the number of *X. fastidiosa*-positive individuals included in the analyses (i.e. negative insects were excluded). Error bars represent ±SE.

P<0.0001). Populations of the *chiA* mutant strain remained low, suggesting a lack of multiplication within vectors, while those of the complemented *chiA* mutant strain reached the high populations found in the wild-type strain (Fig. 3b).

ChiA activity is required for *X. fastidiosa* colonization of plants

Despite the fact that plants do not contain chitin and that ChiA was shown to be a functional chitinase, we tested whether plant colonization was affected in the *chiA* mutant strain. Following mechanical inoculation of plants, all strains were detected close to the inoculation site (<1 cm) and no significant difference was observed among treatments 2 months after infection (Table 1; wild-type versus *chiA* mutant: *Z*=0.56, *P*=0.56; wild-type versus complemented mutant: *Z*=−1.0, *P*=0.32). Importantly, while the wild-type and complemented *chiA* mutant strains were detected at equal frequencies at 15 cm above the inoculation site (Table 1; *Z*=0.22, *P*=0.83), the *chiA* mutant was not (Table 1; *Z*= −3.16, *P*=0.002). Using qPCR, bacterial populations were assessed for each strain at 1 and 15 cm above the inoculation site, 12 weeks after infection; there were no differences at 1 cm, but the *chiA* mutant strain was not detected at 15 cm, while the wild-type and complemented strains were detected with similar population sizes (Table 1). Furthermore, the *chiA* mutant strain did not cause disease symptoms, while plants inoculated with either the wild-type or the complemented strain were symptomatic (Table 1).

DISCUSSION

The results support our hypothesis that a *Xylella fastidiosa chiA* mutant strain would be deficient in chitin utilization and, consequently, would have reduced population size and be affected in its vector transmission. The results show that the *chiA* mutant strain is not able to grow *in vitro* on a medium with chitin as a sole carbon source. In addition,

Table 1. Grapevine host plant colonization by *Xylella fastidiosa* strains

Results indicate that cells of all strains were viable at the site of inoculation, but the *chiA* mutant strain did not move within plants and did not cause disease symptoms, as observed for the two other strains.

Strain	Proportion of culture-positive plants at two points above inoculation site*, and bacterial populations (mean ±SEM)†		Disease symptoms	N‡
	1 cm	15 cm		
Wild-type	17/19 | 4.8±1.7	15/19 | 4.7±1.8	26/27	30
chiA mutant	19/20 | 4.8±1.7	0/20 | 0±0	0/27	30
Complemented *chiA* mutant	9/12 | 4.8±1.5	10/12 | 4.8±1.7	12/15	15

*Not all plants inoculated were tested for the presence of live *X. fastidiosa* cells with the culturing assay.

†Bacterial population estimates are based on five randomly selected plant replicates (log10 transformed, number of cells μg^{-1} of total DNA).

‡Total number of plants inoculated.

the *chiA* mutant strain was deficient in insect colonization, presumably because cells cannot use chitin as a nutrient to establish a normal population within the vector foregut. Affected insect colonization would likely lead to reduced vector transmission, as observed here. However, it has been previously shown that the presence of chitin also results in the differential expression of a variety of genes involved in vector and plant colonization [11]. In particular, fimbrial and afimbrial adhesins, associated with cell adhesion within insects, were upregulated. It is likely that such gene expression is modulated by the breakdown products of chitin, and thus the *chiA* mutant may not have been able to properly respond to chitin signals since chitin breakdown was inhibited. In the *chiA* mutant strain, the potential regulatory function with which ChiA activity may be associated could be altered resulting in impaired colonization. Future work is necessary to disentangle these interconnected factors.

It is intriguing that bacteria in the genus *Xanthomonas*, a sister group to *Xylella*, have a ChiA homologue [15]. However, *Xanthomonas* spp. are not vector-transmitted. We thus hypothesized that ChiA has other functions besides those associated with vector colonization and transmission. ChiA has been identified as a virulence factor of the bacterial pathogens of vertebrate hosts, which also lack endogenous chitin [33]. In those cases it has been suggested that GlcNAc-containing glycans may be the targets of ChiA, such as *N*-acetyllactosamine, which is common in vertebrate glycoconjugates [34]. When the *X. fastidiosa chiA* mutant was inoculated into grapevines, none of the plants infected with the *chiA* mutant developed symptoms, while most plants infected with the wild-type or the *chiA* mutant complemented strain developed disease symptoms. The lack of virulence was not due to the death of cells inoculated into plants, since live cells of the *chiA* mutant strain were recovered from the inoculation site. However, 2 months after inoculation, only the wild-type and the complemented strain were recovered as little as 15 cm above the inoculation site. These results suggest that ChiA contributes to vessel-to-vessel movement of *X. fastidiosa* within plants, a process needed for dispersal through the plant and for disease symptom development [35]. To date, only *pglA* (encoding polygalacturonase A) has been shown to be important for *X. fastidiosa* movement among vessels, and consequently, symptom development [36]. While a *pglA* mutant was less virulent to grapevines than the wild-type control, it was recovered with high frequency and at populations only 10-fold lower than the wild-type strain at 37 cm above the inoculation site [36]. This indicates that the *pglA* mutant was only moderately impaired in host colonization relative to that of the *chiA* mutant strain. Our results suggest different *in planta* roles for PglA and ChiA given that no within-plant movement was observed with the *chiA* mutant strain. Surprisingly, the carbon sources used by this bacterium within plants remain unknown. The *pglA* mutant strain is deficient in pectin degradation *in vitro*, but evidence remains lacking for pectin or its major subunit, galacturonic acid, as carbon sources for *X. fastidiosa* [11]. Preliminary *in vitro* work with the *chiA* mutant and wild-type strains suggested that ChiA was not involved in pectin, glucan or cellulose degradation by *X. fastidiosa* (A. Sicard and R. Almeida, unpublished data). We propose that ChiA is involved in the processing of GlcNAc-containing glycans in plants, which is similar to that found recently for vertebrate hosts [34].

Most bacterial chitinases exhibit a complex, multi-domain architecture, including catalytic domains conferring their glycoside hydrolase activity (GH18), as well as additional domains with no obvious catalytic role. Those domains, called carbohydrate-binding modules (CBMs), are responsible for ligand recognition and binding and are considered non-catalytic accessory modules that often accompany the catalytic domain [37]. Functional studies have shown that some CBMs can enhance substrate affinity and increase catalytic efficiency [38]. No known conserved motifs of CBMs were found in the ChiA sequence of *X. fastidiosa* [15]. Alternatively, CBMs can also be found on chitin-binding proteins (CBPs). CBPs are independent proteins, not structurally linked to their associated chitinases, that mediate interactions with the targeted substrates [39, 40]. Our *in vitro* experimental design aimed to determine whether there were CBPs associated with the chitinolytic activity of ChiA. Chitin degradation products were observed only when ChiA was mixed with other *X. fastidiosa* proteins. It is possible that the lack of ChiA-alone activity may have been due to protein-folding problems during recombinant expression in *E. coli*, or protein purification, and that the remaining chitinolytic function may not be detectable using the assay developed in the study. However, a chitin-binding domain has not been identified in the *X. fastidiosa* ChiA [15], and chitinolytic activity was observed when the recombinant ChiA was mixed with the protein extract of the *chiA* mutant strain (which also lacked chitinolytic activity). Based on these results, it is likely that the *X. fastidiosa* ChiA requires at least one other *X. fastidiosa* protein in order to cleave chitin. Such proteins could play a role in chitin degradation by linking ChiA to its substrate, or by somehow 'activating' ChiA following post-translational modifications (e.g. phosphorylation or methylation) in the presence of chitinous substrates. Because an *E. coli* strain expressing *X. fastidiosa* ChiA was also capable of degrading the same substrate tested here [15], CBMs potentially implicated in chitin degradation in *X. fastidiosa* may be structurally conserved proteins. *E. coli* has been shown to have a functional yet suppressed chitinase, which was also not extracellularly secreted [41]. The assay used with *X. fastidiosa* did not demonstrate chitinolytic activity by the *E. coli* strain used unless it was transformed with *chiA* from *X. fastidiosa* [15], in which case it was assumed that *E. coli* had a CBM partner required for ChiA activity. It is possible that this unknown CBM partner is also used by the *E. coli* ChiA, when expressed. In addition, CBMs can increase the range of substrates degraded by associated enzymes [42]. The search for ChiA partners could be of interest to better understand the details of how this enzyme interacts with its substrates in

both insect and plant hosts of *X. fastidiosa*. The intervention of alternate CBPs, different from those interacting with ChiA during insect colonization, could explain the inability of the *chiA* mutant to colonize its other host plants. Differential regulation of *X. fastidiosa* proteins within its two hosts is an additional argument in favour of such a dual catalytic role for ChiA.

These results show that ChiA apparently plays multiple roles in *X. fastidiosa* colonization of plants and insects. However, the mechanisms involved in ChiA activity remain unknown. For example, we expect that the partners associated with ChiA activity in plants and insects are different, as the substrates are most likely not the same. An in-depth characterization of ChiA should lead to further insights into the biology of this pathogen, since it is involved in the colonization of both hosts essential for its lifestyle. Strategies aimed at blocking ChiA activity should also be investigated, as those could lead to reduced movement of the pathogen within plants and, consequently, reduced disease symptoms.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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