

# Plant-like bacterial expansins play contrasting roles in two tomato vascular pathogens

MATTHEW A. TANCOS<sup>1</sup>†, TIFFANY M. LOWE-POWER<sup>2</sup>, F. CHRISTOPHER PERITORE-GALVE<sup>1</sup>, TUAN M. TRAN<sup>2</sup>‡, CAITILYN ALLEN<sup>2</sup> AND CHRISTINE D. SMART<sup>1,\*</sup>

<sup>1</sup>Plant Pathology and Plant-Microbe Biology Section, School of Integrative Plant Sciences, Cornell University, Geneva, NY 14456, USA

<sup>2</sup>Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706, USA

## SUMMARY

Expansin proteins, which loosen plant cell walls, play critical roles in normal plant growth and development. The horizontal acquisition of functional plant-like expansin genes in numerous xylem-colonizing phytopathogenic bacteria suggests that bacterial expansins may also contribute to virulence. To investigate the role of bacterial expansins in plant diseases, we mutated the non-chimeric expansin genes (*CmEXLX2* and *RsEXLX*) of two xylem-inhabiting bacterial pathogens, the Actinobacterium *Clavibacter michiganensis* ssp. *michiganensis* (*Cmm*) and the  $\beta$ -proteobacterium *Ralstonia solanacearum* (*Rs*), respectively. The *Cmm*  $\Delta$ *CmEXLX2* mutant caused increased symptom development on tomato, which was characterized by more rapid wilting, greater vascular necrosis and abundant atypical lesions on distant petioles. This increased disease severity correlated with larger *in planta* populations of the  $\Delta$ *CmEXLX2* mutant, even though the strains grew as well as the wild-type *in vitro*. Similarly, when inoculated onto tomato fruit,  $\Delta$ *CmEXLX2* caused significantly larger lesions with larger necrotic centres. In contrast, the *Rs*  $\Delta$ *RsEXLX* mutant showed reduced virulence on tomato following root inoculation, but not following direct petiole inoculation, suggesting that the *RsEXLX* expansin contributes to early virulence at the root infection stage. Consistent with this finding,  $\Delta$ *RsEXLX* attached to tomato seedling roots better than the wild-type *Rs*, which may prevent mutants from invading the plant's vasculature. These contrasting results demonstrate the diverse roles of non-chimeric bacterial expansins and highlight their importance in plant–bacterial interactions.

**Keywords:** bacterial plant pathogenesis, *Clavibacter michiganensis*, expansin, horizontal gene transfer, *Ralstonia solanacearum*.

## INTRODUCTION

Plant primary cell walls are highly dynamic structures that fluctuate between rigid and relaxed states, enabling basic biological processes, such as growth, enlargement and cell division (Cosgrove, 1993, 2005). The regulation of cell wall elasticity and plasticity is an intricate process that requires many cell wall-loosening enzymes, including expansins (Cosgrove, 1993; Sampedro and Cosgrove, 2005). Plant expansins loosen the rigid carbohydrate matrix of the cell wall through an uncharacterized non-lytic slippage mechanism (Cosgrove, 2000). This key biological function may explain why expansins are conserved in all vascular plants, with isoforms regulating the cell wall changes required for cellular growth, vascular differentiation, fruit ripening, seed germination, abscission and leaf development (Cho and Cosgrove, 2000; Im *et al.*, 2000; Kende *et al.*, 2004; Rose *et al.*, 1997). Expansins are pH dependent and are activated when plant growth hormones stimulate H<sup>+</sup>-ATPases, which lower the pH of the cell wall matrix by producing a proton differential across the plasma membrane (Cosgrove, 2000).

Recent evidence has suggested that diverse microbes have independently acquired and exploited this plant-derived enzyme for unknown reasons (Nikolaidis *et al.*, 2014). It has been hypothesized that multiple independent horizontal gene transfers have led to the microbial acquisition of plant expansins, with subsequent horizontal gene transfer events within bacterial and fungal phyla (Nikolaidis *et al.*, 2014). Expansins are rare in bacteria; only 3% of sequenced bacteria have expansin xenologues, and these bacterial genera represent diverse ecological niches from free-living saprophytes to plant pathogens (Nikolaidis *et al.*, 2014). Interestingly, systemic xylem pathogens are present in all plant-pathogenic genera with an expansin gene: *Xanthomonas*, *Xylella*, *Ralstonia*, *Dickeya*, *Pectobacterium*, *Acidovorax* and *Clavibacter* (Georgelis *et al.*, 2015; Nikolaidis *et al.*, 2014). The only exception is the genus *Streptomyces* (Nikolaidis *et al.*, 2014). However, no expansin xenologues are present amongst the non-vascular phytopathogenic bacterial genera *Pseudomonas* or *Agrobacterium* (Nikolaidis *et al.*, 2014). Two forms of expansin have been described in bacteria: a chimeric version, which has an endoglucanase domain fused to the expansin domain, and a non-chimeric version (Jahr *et al.*, 2000; Nikolaidis *et al.*, 2014). Most

\*Correspondence: Email: cds14@cornell.edu

†Present address: Foreign Disease-Weed Science Research Unit, USDA-ARS, Fort Detrick, MD 21702, USA

‡Present address: School of Biological Sciences, Nanyang Technological University, 639798, Singapore

bacteria contain only a single expansin (either chimeric or non-chimeric), with the notable exception of the tomato pathogen *Clavibacter michiganensis* ssp. *michiganensis* (*Cmm*), which has both a chimeric (*CmEXLX1*) and non-chimeric (*CmEXLX2*) version (Georgelis *et al.*, 2015; Nikolaidis *et al.*, 2014). Another vascular tomato pathogen, *Ralstonia solanacearum* (*Rs*), only has a non-chimeric expansin, *RsEXLX* (Nikolaidis *et al.*, 2014).

*Cmm*, the internationally quarantined causal agent of tomato bacterial canker, is an economically devastating seed-disseminated pathogen found in all major tomato-producing regions (Bryan, 1930; de León *et al.*, 2011). *Cmm* enters plants epiphytically through natural openings, wounds or infected seed (Bryan, 1930; Carlton *et al.*, 1998; Tancos *et al.*, 2013). Once inside its host, this bacterium systemically spreads throughout the vasculature, plugging and degrading xylem vessels, resulting in significant tissue maceration and impaired water transport, which, in turn, leads to characteristic wilting, marginal necrosis of leaflets, stem cankers and fruit lesions (Bryan, 1930; Tancos *et al.*, 2013; Wallis, 1977). Bacterial canker of tomato is difficult to control because of the lack of resistant cultivars and improper sanitation (Sen *et al.*, 2013; Werner *et al.*, 2002).

*Rs* is a soil-borne  $\beta$ -proteobacterium vascular pathogen that infects a wide host range of economically important crops, including monocots and dicots (Elphinstone, 2005). Root attachment is a critical early step in *Rs* pathogenesis (Yao and Allen, 2006). *Rs* infects roots near the elongation zone, secondary root emergence sites and wounds before the bacterium penetrates the vascular cylinder and colonizes the xylem (Vasse *et al.*, 1995). *Rs* spreads systemically throughout the vasculature, reaching population sizes  $>10^9$  colony-forming units (CFU)/g of stem tissue (Tans-Kersten *et al.*, 2004). These large bacterial populations, which produce abundant extracellular polymeric substances and macerate tissue, reduce xylem sap flow and cause wilting (Vasse *et al.*, 1995). Like *Cmm*, *Rs* is difficult to manage because of the lack of resistant plant cultivars or other effective control strategies (Bae *et al.*, 2015).

Microbial expansins are structurally similar to plant expansins, and the unique cellulose-binding domains are highly conserved (Kerff *et al.*, 2008; Pastor *et al.*, 2015). Purified bacterial expansins from *Bacillus subtilis*, *Xanthomonas campestris*, *Rs* and *Cmm* loosen plant cell walls without lytic activity *in vitro*, but their activity is modest relative to that of plant expansins (Bunterngsook *et al.*, 2015; Georgelis *et al.*, 2014; Kerff *et al.*, 2008). Recent studies have shown that a non-chimeric expansin mutant of the saprophytic biocontrol agent *B. subtilis* exhibits reduced attachment to maize roots (Kerff *et al.*, 2008), but, in contrast, *Xylella fastidiosa* and *Cmm* mutants lacking the endoglucanase-expansin chimeras cause reduced disease symptoms (Ingel *et al.*, 2015; Jahr *et al.*, 2000). However, the function(s) of non-chimeric expansins remains unknown, especially in phytopathogenic species.

Here, we investigate the effect of non-chimeric bacterial expansins on the pathogenesis and colonization of tomato by two vascular pathogens: the Gram-positive *Cmm* and the Gram-negative *Rs*. Because of the niche overlap of the pathogens, we hypothesized that non-chimeric expansins from *Cmm* and *Rs* would contribute similarly to vascular colonization. However, we found contrasting roles for expansins in these two systems. Although the *Cmm*  $\Delta$ *CmEXLX2* mutant was hypervirulent and grew faster *in planta*, an *Rs*  $\Delta$ *RsEXLX* mutant hyperattached to tomato roots and was reduced in virulence after root inoculation.

## RESULTS

We used a genetic approach to investigate the roles of non-chimeric plant-like bacterial expansins in the pathogenesis of two vascular plant-pathogenic bacteria. Gene nomenclature for bacterial expansins was based on the current standard for microbial expansins (Georgelis *et al.*, 2015; Kende *et al.*, 2004). *CmEXLX2* was disrupted in a virulent *Cmm* New York strain (Cmm0317) that naturally lacked the *CmEXLX1* plasmid-borne chimeric expansin (Tancos *et al.*, 2015). As expected, the *Cmm*  $\Delta$ *CmEXLX2* and *Rs*  $\Delta$ *RsEXLX* mutants did not express *CmEXLX2* or *RsEXLX*, whereas the wild-type and complemented mutants both did (Figs S1 and S2, see Supporting Information). *In vitro* growth of the *Cmm* strains was not significantly different from that of the wild-type in nutrient-rich Luria–Bertani (LB) medium ( $P = 0.994$ ) and nutrient-poor tomato xylem sap ( $P = 0.729$ ) (Fig. S3, see Supporting Information). Similarly,  $\Delta$ *RsEXLX* grew at the same rate as the wild-type in both rich ( $P = 0.493$ ) and minimal ( $P = 0.301$ ) medium (Fig. S3). The *CmEXLX2* mutation did not influence host recognition in *Mirabilis jalapa* (four o'clock plants).  $\Delta$ *CmEXLX2* induced a strong hypersensitive response (HR) indistinguishable from the HR induced by the wild-type and complemented mutant strain (Table 1).

Plant expansins facilitate cell wall loosening as plant cells develop and grow. Although the bacterial BsEXLX1 expansin contributes to the autolysis of *B. subtilis*, BsEXLX1 lacks peptidoglycan-lytic activity (Kerff *et al.*, 2008). Because BsEXLX1 influenced *B. subtilis* cell wall integrity, we measured the autolysis rates of  $\Delta$ *CmEXLX2* and  $\Delta$ *RsEXLX* in *Cmm* and *Rs*, respectively. Autolysis of  $\Delta$ *CmEXLX2* and  $\Delta$ *RsEXLX* did not differ from the corresponding wild-type or complemented mutant strains ( $P = 0.141$  for *Cmm* and  $P = 0.086$  for *Rs*) (Fig. S4, see Supporting Information).

### The *Cmm* $\Delta$ *CmEXLX2* mutant causes increased symptoms on tomato seedlings and fruit

Tomato seedlings inoculated with  $\Delta$ *CmEXLX2* consistently developed earlier and more severe wilt symptoms than the wild-type or complemented strains ( $P < 0.0001$ ) (Table 1). Although 60% of plants inoculated with the wild-type and complemented strains

**Table 1** Virulence of *Clavibacter michiganensis* ssp. *michiganensis* strains on tomato plants.

Strain	HR*	AUDPC <sup>†</sup>	Disease incidence <sup>‡</sup>	Petiole lesion length (cm) <sup>§</sup>	CFU/g <i>in planta</i> <sup>¶</sup>	
					9 DPI	21 DPI
Cmm0317	+	139 ( $\pm 36$ ) B	15/15	0	$2.78 \times 10^8$ B	$5.36 \times 10^8$ C
$\Delta$ CmEXLX2	+	430 ( $\pm 64$ ) A	15/15	2.16 ( $\pm 0.70$ )	$3.72 \times 10^9$ A	$4.82 \times 10^9$ A
C $\Delta$ CmEXLX2 <sup>+</sup>	+	157 ( $\pm 21$ ) B	15/15	0.59 ( $\pm 0.21$ )	$2.25 \times 10^8$ B	$2.30 \times 10^9$ B
Water control	–	0.0	0/15	0	0.0	0.0

\*Induction of a hypersensitive response (HR) in *Mirabilis jalapa*: +, positive for HR reaction; –, negative for HR reaction.

<sup>†</sup>The mean area under the disease progress curve (AUDPC) for disease severity for three independent experiments is given. Differences among strains were determined with PROC GLIMMIX ( $P < 0.05$ ) followed by Tukey–Kramer post-test ( $P < 0.05$ ). AUDPC values followed by the same letters are not significantly different. Numbers in parentheses indicate the standard error associated with the AUDPC values.

<sup>‡</sup>Expressed as the number of wilting plants/number of inoculated plants characterized at 21 days post-inoculation (DPI).

<sup>§</sup>The mean length of lesions located on the inoculated petiole at 21 DPI. Numbers in parentheses indicate the standard error associated with the lesion lengths.

<sup>¶</sup>The mean *in planta* population [colony-forming units (CFU)/g] recovered from a 0.5-cm section of tomato stem tissue located 1 cm above the inoculation site at both 9 and 21 DPI. Significant differences amongst strains were tested by PROC GLIMMIX ( $P < 0.05$ ) followed by Tukey–Kramer post-test ( $P < 0.05$ ). *In planta* populations followed by the same letters are not different.

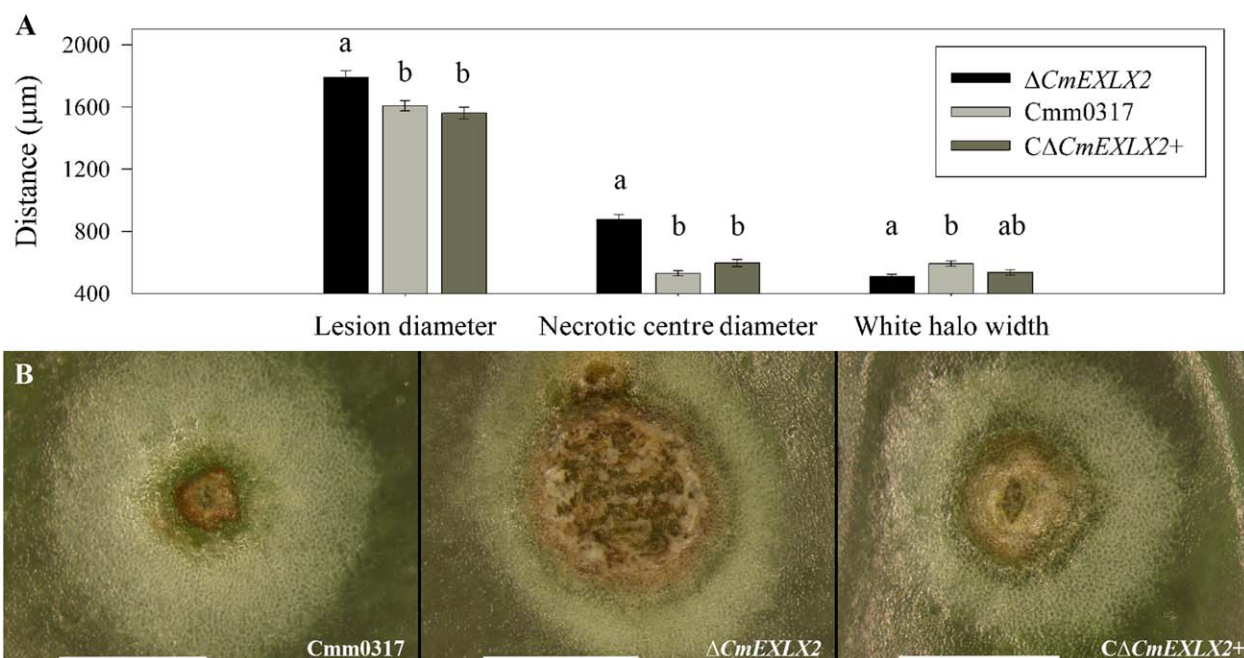
developed symptoms by 15 and 13 days post-inoculation (DPI), respectively,  $\Delta$ CmEXLX2 reached this disease incidence at 10 DPI. All three *Cmm* strains produced typical stem cankers at the site of inoculation, but  $\Delta$ CmEXLX2 also produced multiple atypical cankers in the vascular bundles of distant petioles that appeared at approximately 11–14 DPI (Table 1, Fig. 1). These atypical petiole lesions extended along the tracks of the vascular bundles; necrosis was confined to the vasculature, with healthy tissue surrounding the lesions until the later stages of disease development (Fig. 1). Lesions on petioles were never observed on plants inoculated with the wild-type strain and were rarely formed by the complemented mutant strain.

The  $\Delta$ CmEXLX2 mutant reached higher populations *in planta* at 1 cm above the inoculation site at both 9 DPI ( $P = 0.0048$ ) and 21 DPI ( $P < 0.0001$ ) (Table 1). To determine whether systemic movement influenced symptom development and *in planta* population size, we also assessed *Cmm* populations 5 and 10 cm above the inoculation site at 21 DPI. All three *Cmm* strains were detectable 5 and 10 cm above the site of inoculation (Table S1A, see Supporting Information). Consistent with our findings at 1 cm above the inoculation site, the  $\Delta$ CmEXLX2 population ( $2.41 \times 10^9$  CFU/g) at 5 cm was larger ( $P = 0.024$ ) than the wild-type population ( $6.85 \times 10^8$  CFU/g). However, the population sizes of the two strains were not different 10 cm above the inoculation site ( $P = 0.46$ ) (Table S1A).

Plant vasculature enables water and nutrient transport; it is composed of a network of vascular bundles that contain aggregated xylem vessels and phloem tissue. To determine whether CmEXLX2 influences the movement of *Cmm* between xylem vessels or vascular bundles, we inoculated tomato plants with enhanced green fluorescent protein (eGFP)-expressing *Cmm* strains and visualized intra- or intervascular colonization.



**Fig. 1** Atypical tomato petiole lesions associated with the *Clavibacter michiganensis* ssp. *michiganensis* expansin mutant  $\Delta$ CmEXLX2. (A) An individual petiole lesion rupturing a vascular bundle. Necrosis appeared to be confined to the vasculature with healthy plant tissue surrounding the petiole lesions. (B) Multiple lesions localized to the vasculature of an individual petiole. Black arrows highlight the large canker lesions present along multiple vascular tracks.



**Fig. 2** Deletion of *Clavibacter michiganensis* ssp. *michiganensis* (*Cmm*) expansin gene *CmEXLX2* accelerates tomato fruit lesion development. (A) Lesion and halo sizes on tomato fruit ( $n = 100$ ) inoculated with the three *Cmm* strains. Fruit lesion distances followed by the same letter are not significantly different. Differences amongst treatments for mean lesion size were determined with PROC GLIMMIX ( $P < 0.05$ ) followed by Tukey–Kramer post-test ( $P < 0.01$ ). (B) Representative images of tomato fruit lesions: Cmm0317 (wild-type),  $\Delta CmEXLX2$  (expansin mutant) and C $\Delta CmEXLX2^+$  (complement). Scale bar, 1 mm. Error bars correspond to the standard error.

eGFP-expressing *Cmm* wild-type ( $CmEXLX2^+$ ) and  $\Delta CmEXLX2$  strains were both visible in xylem vessels at 3 cm above and below the inoculation site at 5, 7 and 9 DPI (Table S2A,B, see Supporting Information). This qualitative approach revealed no detectable differences in the patterns of xylem colonization, lateral movement or parenchyma cell colonization between the strains at any time point or distance from the inoculation site. By 9 DPI, all *Cmm* strains were present at high concentrations, and bacteria were visible in macerated vessel elements and in the surrounding parenchyma cells (Table S2B and Fig. S5, see Supporting Information).

When inoculated onto immature green fruit, all three *Cmm* strains produced characteristic bacterial canker lesions, which have a necrotic centre surrounded by a white halo. Fruit lesions appeared at 3–4 DPI, but phenotypic differences between strains became evident at 7–10 DPI. The  $\Delta CmEXLX2$  lesions were larger and exhibited more necrosis than lesions caused by either the wild-type or complemented mutant strain ( $P < 0.0001$ ), but  $\Delta CmEXLX2$  lesions were surrounded by thinner white halos compared with the wild-type lesions ( $P = 0.0008$ ) (Fig. 2).

#### Symptom development on tomato seedlings infected with the *Rs* $\Delta RsEXLX$ mutant

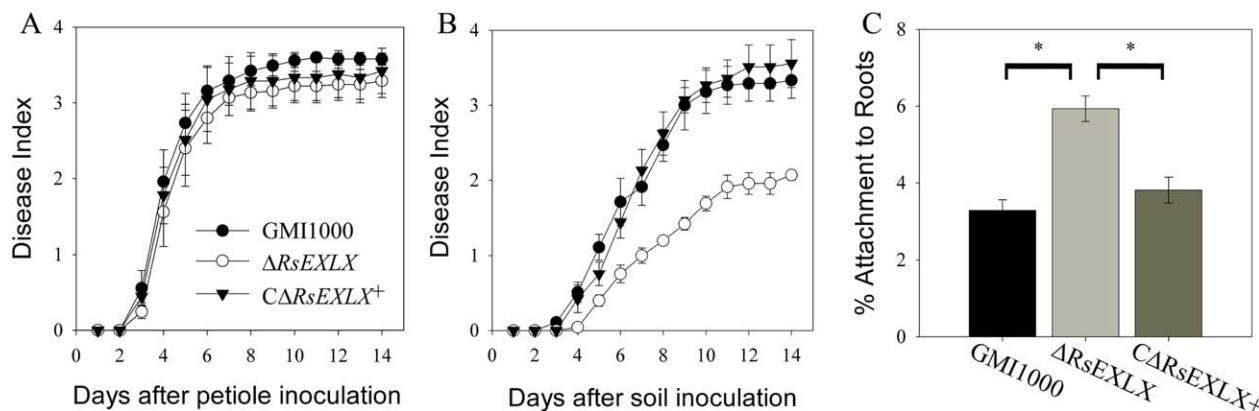
As plant-like expansins have been acquired by many xylem-dwelling plant-pathogenic bacteria, we hypothesized that

expansins would also affect the virulence of another xylem pathogen, *Rs*. Because *Rs* infects host plants via the roots before systemically colonizing the xylem, two inoculation methods were used to distinguish possible roles of the *Rs* expansin. A soil drench inoculation of unwounded plants mimics the natural route of *Rs* infection, whereas the cut petiole technique bypasses root infection by introducing *Rs* directly into the xylem. In contrast with *Cmm*,  $\Delta RsEXLX$  showed wild-type virulence when directly introduced into tomato stems ( $P = 0.106$ ), but was reduced in virulence following soil drench inoculation ( $P = 0.0465$ ) (Figs 3A,B and S6, see Supporting Information). By 14 DPI, only 56% of plants inoculated with  $\Delta RsEXLX$  displayed symptoms, compared with 87% and 89% of plants inoculated with the wild-type and complemented mutant strains, respectively.

#### Expansins influence *Rs*, but not *Cmm*, attachment to host roots

The virulence defect of  $\Delta RsEXLX$  following soil drenching, but not direct xylem inoculation, suggested that *RsEXLX* contributes to early infection. To investigate the initial step of the infection process, bacterial attachment of *Cmm* and *Rs* to tomato seedling roots was measured. The  $\Delta RsEXLX$  mutant hyperattached to roots in comparison with the wild-type ( $P < 0.0001$ ). Following a 2-h incubation of tomato seedlings in a bacterial suspension, 6% of





**Fig. 3** Virulence and root attachment of *Ralstonia solanacearum* (*Rs*) expansin mutant  $\Delta RsEXLX$ . (A) Bacteria were directly inoculated into tomato stem vasculature by placing 500 colony-forming units (CFUs) onto the surface of a freshly cut leaf petiole [ $P = 0.1056$ , repeated-measures analysis of variance (ANOVA)]. (B) Plants were naturalistically inoculated by drenching the soil around the plants with approximately  $5 \times 10^8$  CFU bacteria/g soil ( $P = 0.0465$ , repeated-measures ANOVA). Experiments were repeated three times with total  $n = 45$  plants per strain. (C)  $10^4$  CFU *Rs* were incubated for 2 h with sterile tomato seedling roots. The percentage of bacteria attached to roots after washing was measured by serial dilution plating of ground roots. \* $P < 0.0001$  by ANOVA. Error bars correspond to the standard error.

the  $\Delta RsEXLX$  population attached to roots compared with only 3.3% and 3.8% of the wild-type and complemented strain populations, respectively (Fig. 3C). In contrast, there were no differences in root attachment between the three *Cmm* strains ( $P = 0.4647$ ) (Table S1B).

To determine whether the attachment phenotype was root specific, we measured the attachment of *Rs* and *Cmm* strains with an *in vitro* biofilm formation assay (Kwasny and Opperman, 2010). No differences in attachment were detected between the *Rs* wild-type and expansin mutant strains ( $P = 0.111$ ), or *Cmm* strains ( $P = 0.117$ ), but the *Cmm* complemented strain produced less biofilm than the wild-type (Fig. S7, see Supporting Information).

#### Heterologous expression of *Rs* expansin in *Cmm*

Because the  $\Delta CmEXLX2$  and  $\Delta RsEXLX$  mutants showed contrasting virulence phenotypes, we tested whether the heterologous expression of *RsEXLX* could complement the hypervirulence of  $\Delta CmEXLX2$ . *RsEXLX* was fused with the *Cmm* expansin secretion sequence and promoter, so that the *Rs* gene was expressed and secreted in a similar manner to the *Cmm* expansin; this hybrid fusion strain was named  $C\Delta CmP:RsEXLX^+$  (Fig. S8, see Supporting Information). Similar to previous *Cmm* experiments, no differences in root attachment were observed between strains ( $P = 0.4647$ ). The expansin-hybrid also behaved similarly to  $\Delta CmEXLX2$  and the complemented mutant, producing less biofilm than the wild-type strain ( $P = 0.0094$ ) when grown in pure tomato xylem sap. The expansin-hybrid did not restore the *Cmm* wild-type-level virulence and canker severity when inoculated into tomato, but showed a tendency to induce less severe disease than  $\Delta CmEXLX2$  ( $P = 0.5230$ ) (Fig. S9, see Supporting

Information). Although both *CmEXLX2* and *RsEXLX* are non-chimeric expansins, they only share 27% amino acid identity, which could explain why *RsEXLX* did not restore wild-type-level virulence (Table 2 and Fig. S10, see Supporting Information).

#### DISCUSSION

Functional plant-like bacterial expansins were first studied in *B. subtilis*, and then identified in a wide array of plant-associated bacteria (Kerff *et al.*, 2008; Nikolaidis *et al.*, 2014). However, the biological function of non-chimeric bacterial expansins, which lack endoglucanase domains, has not been investigated in phytopathogenic bacteria. We found that the loss of non-chimeric bacterial expansins divergently affected the virulence of two vascular pathogens of tomato, *Cmm* and *Rs* (Nikolaidis *et al.*, 2014).

The  $\Delta CmEXLX2$  mutant caused more severe disease on tomato, characterized by a faster onset of unilateral wilting, increased necrosis and larger pathogen population sizes. Although all three *Cmm* strains produced typical stem lesions at the site of inoculation, only  $\Delta CmEXLX2$  caused atypical necrotic lesions on the vasculature of distant petioles. The quick onset of symptoms by  $\Delta CmEXLX2$  was not correlated with increased intra- or inter-vascular spread, because eGFP-expressing strains showed similar rates of vascular infection both above and below the site of inoculation. In parallel with the increased stem tissue necrosis,  $\Delta CmEXLX2$  caused larger necrotic tomato fruit lesions. Fruit infected with  $\Delta CmEXLX2$  had larger, more blistered lesions with more necrosis and less noticeable 'white halos'. Unfortunately, fruit lesions are a relatively unexplored disease symptom associated with bacterial canker, and the significance of the 'white halo' that surrounds the lesion remains unknown.

**Table 2** Overview of bacterial phenotypes associated with the mutation of bacterial expansins.

Bacterium	Lifestyle	Primary route of colonization	Protein structure	Gene	Percentage identity*	Expansin mutant phenotype		Reference
						Disease symptoms	Root attachment	
<i>Clavibacter michiganensis</i> ssp. <i>michiganensis</i>	Vascular phytopathogen	Foliar	Non-chimeric	<i>CmEXLX2</i>	100 (100)	Increase	No difference	This study
<i>Clavibacter michiganensis</i> ssp. <i>michiganensis</i>	Vascular phytopathogen	Foliar	Chimeric	<i>CmEXLX1</i>	17 (54)	Decrease	n/a	Jahr <i>et al.</i> (2000)
<i>Ralstonia solanacearum</i>	Vascular phytopathogen	Root	Non-chimeric	<i>RsEXLX</i>	27 (34)	Decrease	Hyperattachment	This study
<i>Bacillus subtilis</i>	Saprophyte	Root	Non-chimeric	<i>BsEXLX1</i>	28 (36)	n/a	Decrease	Kerff <i>et al.</i> (2008)

\*Percentage amino acid identity of the full-length protein relative to the non-chimeric *Clavibacter michiganensis* ssp. *michiganensis* expansin *CmEXLX2*. The number in parentheses denotes the amino acid identity of the most similar regions via the pairwise sequence alignment tool EMBOSS Matcher (Goujon *et al.*, 2010).

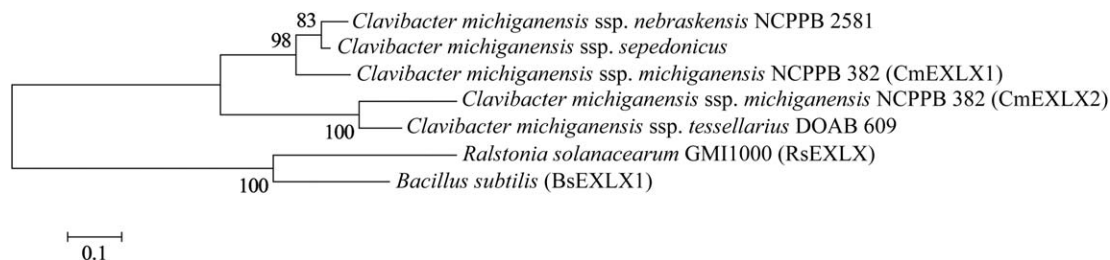
The  $\Delta$ *CmEXLX2* mutant strain reached larger *in planta* populations at 1 and 5 cm above the site of inoculation. However, the differences in population size between *Cmm* strains diminished as the acropetal regions of the stem were colonized; at 10 cm above the inoculation site, the population sizes of the three strains were not significantly different. Therefore, acropetal *in planta* populations and the vascular GFP movement study both demonstrate that systemic movement alone does not appear to have caused the increased *in planta* populations. Because  $\Delta$ *CmEXLX2* growth was not significantly different from that of the wild-type *in vitro*, the mutant's increased *in planta* population size could have been a result of the increased necrosis induced by the mutant. The older, more established  $\Delta$ *CmEXLX2* populations near the inoculation site may degrade vascular tissue more quickly, which probably releases plant-derived nutrients into the nutrient-poor xylem (Fatima and Senthil-Kumar, 2015). The greater rates of necrosis observed on the stems and fruit may be directly associated with the increased availability of plant-derived nutrients and the subsequent influx of bacterial growth as established populations macerate plant tissue.

The molecular mechanism underlying the increased necrosis of  $\Delta$ *CmEXLX2* is unknown. We favour the hypothesis that *CmEXLX2* competes with the numerous *Cmm* cell wall-degrading enzymes (CWDEs) for unique binding sites on the plant cell wall. In support of this idea, Georgelis *et al.* (2012) demonstrated that the *B. subtilis* *BsEXLX1* expansin competes with type-A cellulose-binding modules for binding sites in crystalline cellulose, which is probably a result of the similarity between the D2 domains of *BsEXLX1* and other type-A cellulose-binding modules (Georgelis *et al.*, 2012). Although canonical CWDEs, such as pectinases, cellulases and hemicellulases, cleave plant cell wall polysaccharides, plant  $\alpha$ -expansins induce rapid cell wall extension with no lasting structural changes (McQueen-Mason *et al.*, 1992; Yuan *et al.*, 2001). Without substrate competition from the *Cmm* expansin, *Cmm* CWDEs, which include a polygalacturonase, and several pectate lyases, xylanases, cellulases and other endoglucanases (Gartemann *et al.*, 2008), may have increased efficiency, resulting

in major structural changes and the breakdown of the plant cell wall (i.e. necrosis). Under infection-mimicking conditions, *CmEXLX2* and the aforementioned *Cmm* CWDE proteins were measured at similar levels (Savidor *et al.*, 2012).

The increased necrosis caused by  $\Delta$ *CmEXLX2* probably poses a fitness cost at another life cycle stage, such as during seed or leaf infections. For example, *X. fastidiosa* *rpff* quorum sensing mutants are hypervirulent in grape, but are poorly transmitted by their insect vectors because they attach too strongly to host surfaces (Newman *et al.*, 2004). We investigated the role of the expansin in root attachment by *Cmm*, but did not find any differences between strains. The non-chimeric *CmEXLX2* investigated in this study shares only 54% identity (amongst expansin domain regions) with the chimeric *CmEXLX1* present in other *Cmm* strains (Table 2 and Figs 4, S10). Previous studies have shown that disruption of the endoglucanase-expansin chimeras in *Cmm* and *X. fastidiosa* reduces disease symptoms (Ingel *et al.*, 2015; Jahr *et al.*, 2000; Laine *et al.*, 2000). This reduction in disease symptoms might be attributed to the adjacent endoglucanase domain present in chimeric expansins (Ingel *et al.*, 2015; Jahr *et al.*, 2000; Nikolaidis *et al.*, 2014).

We hypothesized that non-chimeric bacterial expansins of other vascular plant pathogens may attenuate virulence similar to *CmEXLX2*. The Gram-negative vascular pathogen *Rs* was selected for comparative studies because it shares tomato as a host, and purified *RsEXLX* has validated expansin activity *in vitro* (Georgelis *et al.*, 2014). Unlike *Cmm*, the  $\Delta$ *RsEXLX* mutant showed wild-type virulence when directly introduced into the tomato vasculature. However, when inoculated via the soil to the natural root infection court,  $\Delta$ *RsEXLX* caused significantly less disease than its wild-type parent. In contrast, *Rs* mutants lacking cellulases or pectinases typically have reduced virulence following both soil drench and cut petiole inoculations (Huang and Allen, 1997, 2000; Liu *et al.*, 2005). We infer that the hyperattachment of the  $\Delta$ *RsEXLX* mutant to tomato roots delays symptom development in the soil drench assay because the attached  $\Delta$ *RsEXLX* cannot efficiently enter and colonize the root vasculature.



**Fig. 4** Maximum-likelihood phylogenetic tree for bacterial expansins represented by *Clavibacter michiganensis*, *Ralstonia solanacearum* and *Bacillus subtilis*. Alignment gaps were excluded, and the total number of sites used was 188 with 1000 repetitions. Bootstrap values are shown at the nodes if greater than 50%. The tree is drawn to scale, with branch lengths measured as the number of substitutions per site (Tamura *et al.*, 2011).

Plant and bacterial expansins have diverse functions. Within the plant kingdom, distinct cell-specific expansins modulate different aspects of plant cellular growth (Cosgrove, 2000, 2015). *Arabidopsis thaliana* maintains at least 24  $\alpha$ -expansins with several putative roles in cell wall modification, and *Zinnia elegans* has a subset of three xylem-specific expansins that vary in temporal and spatial expression (Cosgrove, 2000; Im *et al.*, 2000). Similarly, bacterial non-chimeric expansins appear to have undergone intense selective pressures to adapt to their hosts. *Cmm* is primarily a foliar pathogen that accesses xylem vessels through wounds or natural openings, consistent with our finding that CmEXLX2 acts in the xylem and plays no detectable role in root attachment. In contrast, RsEXLX reduced *Rs* attachment to tomato roots, whereas the *B. subtilis* non-chimeric expansin increased *B. subtilis* attachment to maize roots (Kerff *et al.*, 2008). Moreover, we have demonstrated that RsEXLX is functionally different from CmEXLX2; unlike CmEXLX2, RsEXLX could not complement the hypervirulence phenotype of the *Cmm*  $\Delta$ CmEXLX2 mutant. These results indicate that non-chimeric expansins can play at least three distinct and contrasting functions adapted to the biology of the microbe that wields them (Table 2). The structural targets of plant and microbial expansins remain unknown, but differences in expansin structure and isoelectric points probably influence substrate specificity and activity (Pastor *et al.*, 2015).

Only a subset of plant-associated bacteria possesses expansin genes. Although some saprophytes and plant pathogens have expansins, other notorious plant-associated microbes, such as *Pseudomonas* or *Rhizobium*, lack expansins (Nikolaidis *et al.*, 2014). Expansins are pH dependent, with an optimal pH range of 4.5–6.0, which overlaps the pH range of xylem fluid (pH 5.5) and tomato fruit tissue (pH 4.4) (Bollard, 1960; Cosgrove, 2005; Jones, 1999; Urrestarazu *et al.*, 1996). Perhaps an acidic microenvironment, like that of xylem or fruit tissue, provides the optimal pH environment for expansin activity, unlike the relatively neutral pH of apoplastic fluid. We hypothesize that vascular-inhabiting bacterial pathogens horizontally acquired and maintained the expansin gene to exploit this acidic microenvironment. However, putative endoglucanase-expansin chimeras are present in several non-

vascular plant-associated microbes, such as the non-vascular *Xanthomonas* species *X. oryzae* pv. *oryzicola*, *X. translucens* and *X. campestris* pv. *raphani* (Nikolaidis *et al.*, 2014; Ryan *et al.*, 2011). *Streptomyces* also contains non-vascular phytopathogenic bacteria possessing bacterial expansins; however, some phytopathogenic *Streptomyces* sp., such as *S. acidiscabies*, can tolerate acidic soils with pH values ranging between 4 and 5.5, depending on the strain (Lambert and Loria, 1989a,b). Furthermore, *S. acidiscabies* is likely to be the most recent microbe to acquire a plant-like expansin because the sequence is more similar to plant expansins than is any known microbial expansin (Nikolaidis *et al.*, 2014). Regardless of the bacterial-colonized microenvironment, whether xylem or soil, acidity appears to be important.

The independent horizontal acquisition of a plant gene and its subsequent prokaryotic-specific adaptations highlight the dynamic nature of plant–bacterial interactions. The appropriation of plant expansins may have allowed microbes to manipulate or possibly mimic the biological processes of their hosts. In *Cmm*, CmEXLX2 influences vascular and fruit necrosis, leading to larger *in planta* populations and increased symptom progression. In contrast, RsEXLX appears to contribute to root infection, possibly by modulating root attachment. It would be of interest to further characterize the role of RsEXLX in *Rs* root infections, and to elucidate the targets and molecular mechanisms of these diverse bacterial plant-like expansins.

## EXPERIMENTAL PROCEDURES

The bacterial strains/plasmids and primers used in this study are listed in Tables 3 and S3 (see Supporting Information), respectively. Further experimental procedural details are given in Text S1 (see Supporting Information).

### Bacterial strains and growth conditions

The *Cmm* strain (Cmm0317) used in the present study was a virulent New York field strain, which naturally lacked the *celA* gene (chimeric expansin CmEXLX1) (Tancos *et al.*, 2015). The CmEXLX2 mutant was transformed by insertional mutagenesis of *Cmm* (strain Cmm0317) with the pGCME $\beta$ GM plasmid, as described previously (Stork *et al.*, 2008; Tancos *et al.*, 2013). Depending on the assay, *Cmm* isolates were incubated for 3–4 days at 27 °C in LB (Miller, 1972), sorbitol broth (SB) (Kirchner *et al.*,

**Table 3** Bacterial strains and plasmids used in this study.

Strain or plasmid	Description*	Reference/source
<b>Strains</b>		
<i>Clavibacter michiganensis</i> ssp. <i>michiganensis</i>		
Cmm0317	Wild-type virulent New York strain	Tancos <i>et al.</i> (2015)
$\Delta$ CmEXLX2	Cmm0317 mutant strain with disrupted <i>CmEXLX2</i>	This study
C $\Delta$ CmEXLX2 <sup>+</sup>	$\Delta$ CmEXLX2 transformed with pHNExpA (complement)	This study
C $\Delta$ CmP:RsEXLX <sup>+</sup>	$\Delta$ CmEXLX2 transformed with pHNRsExpA ( <i>C. michiganensis</i> ssp. <i>michiganensis</i> : <i>Ralstonia solanacearum</i> expansin hybrid)	This study
<i>Escherichia coli</i>		
Zymo 5 $\alpha$ (DH5 $\alpha$ )	Cloning strain	Zymo Research, Irvine, CA, USA
<i>E. coli</i> ER2925	<i>dam</i> and <i>dcm</i> methylation-negative strain	New England Biolabs, Ipswich, MA, USA
<i>Ralstonia solanacearum</i>		
GMI1000	Wild-type strain isolated from tomato in French Guyana; phylotype I sequevar 18	Salanoubat <i>et al.</i> (2002)
$\Delta$ RsEXLX	GMI1000 mutant with unmarked <i>RsEXLX</i> deletion	This study
C $\Delta$ RsEXLX <sup>+</sup>	$\Delta$ RsEXLX with a wild-type copy of RSc0818comp in the chromosome	This study
<b>Plasmids</b>		
<i>C. michiganensis</i> ssp. <i>michiganensis</i>		
pGEM-T Easy	Cloning vector; Amp <sup>r</sup> ; 3 kb	Promega, Madison, WI, USA
pHN216	<i>E. coli</i> – <i>Clavibacter</i> shuttle vector; Gm <sup>r</sup> Nm <sup>r</sup> ; 13.8 kb	Laine <i>et al.</i> (1996)
pGnR-BsiWI	pGEM-T Easy:Gm <sup>r</sup> cassette (from pHN216); Amp <sup>r</sup> Gm <sup>r</sup> ; 3.8 kb	This study
pGCME $\beta$	pGEM-T Easy: <i>CmEXLX2</i> ; 3.6 kb	This study
pGCME $\beta$ GM	pGEM-T Easy: <i>CmEXLX2</i> ::Gm <sup>r</sup> cassette; Amp <sup>r</sup> Gm <sup>r</sup> ; 4.3 kb	This study
pIDT-FLExpA	IDT vector:full length <i>CmEXLX2</i> and promoter; Amp <sup>r</sup> ; 3 kb	IDT, Coralville, IA, USA
pHNExpA	Full-length <i>CmEXLX2</i> cloned into pHN216; Nm <sup>r</sup> ; 13.1 kb	This study
pHNRsExpA	<i>CmEXLX2</i> promoter and signal peptide fused to <i>RsEXLX</i> cloned into pHN216; Nm <sup>r</sup> ; 13.1 kb	This study
pK2–22	eGFP-expressing plasmid; Nm <sup>r</sup> ; 13.5 kb	Chalupowicz <i>et al.</i> (2012)
<i>R. solanacearum</i>		
pUFR80	Cloning vector; Kan <sup>r</sup> Suc <sup>s</sup> ; 7.8 kb	Castañeda <i>et al.</i> (2005)
pUFR80-RSc0818KO	pUFR80:unmarked $\Delta$ RsEXLX cassette; Kan <sup>r</sup> Suc <sup>s</sup> ; 9.4 kb	This study
pUC18t-MiniTn7t(Gm)	Wide host range complementation vector; Gm <sup>r</sup> ; 4.6 kb	Choi <i>et al.</i> (2005)
pMiniTn7-RSc0818comp	pUC18t-MiniTn7t(Gm):full length <i>RsEXLX</i> ; Gm <sup>r</sup> ; 6.1 kb	This study

\*Amp<sup>r</sup>,  $\beta$ -lactamase; Gm<sup>r</sup>, gentamicin acetyltransferase; Kan<sup>r</sup>, aminoglycoside 3'-phosphotransferase; Nm<sup>r</sup>, neomycin phosphotransferase; Suc<sup>s</sup>, levansucrase.

2001; Stork *et al.*, 2008) or D2ANX (Hadas *et al.*, 2005) media. When required, LB medium was supplemented with the antibiotics gentamicin (40  $\mu$ g/mL), neomycin (100  $\mu$ g/mL) or ampicillin (100  $\mu$ g/mL) (Fisher Scientific, Pittsburgh, PA, USA).

The phylotype I sequevar 18 *Rs* strain GMI1000 was used in this study. *Rs* was routinely grown in casamino acid, peptone, glucose (CPG) medium or Boucher's minimal medium (BMM) with 20 mM glucose at 28 °C (Boucher *et al.*, 1985). When required, media were supplemented with the antibiotic gentamicin (25  $\mu$ g/mL) (Fisher Scientific). The unmarked *Rs*  $\Delta$ RsEXLX mutant was generated by natural transformation with pUFR80-RSc0818KO, as described previously (Boucher *et al.*, 1985; Lowe *et al.*, 2015). Briefly, transformants were plated on kanamycin to select for vector integration at the *RsEXLX* locus. Then, kanamycin-resistant clones were counter-selected on 5% w/v sucrose to select for the loss of the *sacB*-containing pUFR80 vector backbone by homologous recombination. Sucrose-resistant clones were restructured on unamended medium. Polymerase chain reaction (PCR) screening with RSc0818intF/R primers and GoTaq Green PCR mastermix (Promega, Madison, WI, USA) was used to confirm the loss of *RsEXLX*.

## PCR analysis and RNA expression

Plasmid constructs and chromosome integrations were confirmed with gene-specific PCR and sequencing (Table S3). Genomic *Cmm* DNA was extracted with a MasterPure Gram-Positive DNA purification kit (Epicentre, Madison, WI, USA), according to the manufacturer's instructions. Amplification was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) using EmeraldAmp GT PCR Master Mix (Takara Bio Inc., Otsu, Shiga, Japan) or Advantage-GC 2 Polymerase Mix with a final GC-Melt concentration of 1.0 M for high GC sequences (Clontech Laboratories Inc., Mountain View, CA, USA), according to the manufacturer's instructions. PCR products were purified with a DNA Clean & Concentrator-25 kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions.

RNA was extracted from 6 mL of *Cmm* suspension grown in LB medium to an optical density at 600 nm (OD<sub>600 nm</sub>) of 1.4. Total RNA was extracted using a ZR Fungal/Bacterial RNA MiniPrep kit (Zymo Research). Extraction was followed by an additional DNase treatment using TURBO DNA-free DNase (Fisher Scientific). The quantity and quality (260/280 ratio)



of DNase-treated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE, USA) and a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). Reverse transcription was performed with RNA to cDNA EcoDry Premix with random hexamers (Clontech Laboratories Inc.) using 300 ng of RNA per reaction. To ensure that no DNA remained in the RNA, controls were performed with pure RNA (100 ng per reaction).

### Assessment of *in vitro* growth and HR assays

*In vitro* bacterial growth rates were compared in LB medium and pure tomato xylem sap over 48 h. Xylem sap was harvested from tomato seedlings (*Solanum lycopersicum*), cultivar Mountain Fresh Plus, as described previously (Chalupowicz *et al.*, 2012; Hiery *et al.*, 2013). Pooled collected sap was filter sterilized through a 0.22- $\mu$ m PES membrane 500-mL filter system (Corning Inc., Corning, NY, USA) and frozen in aliquots at  $-20^{\circ}\text{C}$ .

Bacteria for *in vitro* growth assays were initially grown in LB medium for 28 h and then adjusted to  $\text{OD}_{600\text{ nm}} = 0.6$  in water and inoculated (10  $\mu$ L) into individual wells ( $n = 9/\text{strain}$ ) of a 96-well Falcon tissue culture plate containing LB medium or tomato sap (190  $\mu$ L) (Corning Inc.). The plate was incubated at  $28^{\circ}\text{C}$  with shaking in a BioTek microplate reader (Winooski, VT, USA). The bacterial density was measured at 590 nm every 2 h for 48 h. The mean area under the growth curve was calculated for both LB medium and pure tomato xylem sap. The experiment was performed three times.

*Cmm* strains were tested for their ability to induce an HR in *Mirabilis jalapa* (four o'clock plants). *Cmm* strains were grown for 24–32 h in LB medium (gentamicin and/or neomycin was added for  $\Delta\text{CmEXLX2}$  or the complemented strain) with shaking at 160 rpm in 125-mL flasks at  $27^{\circ}\text{C}$ , and  $\text{OD}_{600\text{ nm}}$  was adjusted to  $0.8 (10^8 \text{ CFU/mL})$  with sterile water. Bacterial suspensions were syringe injected into the abaxial surface of the expanded *M. jalapa* leaf using a needleless 10-mL syringe.

*In vitro* growth of *Rs* strains was measured in CPG and BMM broth. Strains were grown overnight in CPG broth, pelleted and washed twice in water. Bacterial suspensions were adjusted to  $\text{OD}_{600\text{ nm}} = 0.1$  in CPG or BMM, and 200  $\mu$ L of the cell suspensions were added to individual wells ( $n = 3/\text{strain}$ ) of a 96-well Falcon tissue culture plate (Corning Inc.). The plate was incubated at  $28^{\circ}\text{C}$  with shaking in a BioTek microplate reader. The bacterial density was measured at 600 nm every 15 min for 24 h. The experiment was performed three times.

### Cell autolysis assays

*Cmm* and *Rs* strains were tested for differences in autolytic rates. Bacterial strains were suspended in  $1 \times$  phosphate-buffered saline (PBS) with 0.02% sodium dodecylsulfate (SDS) (pH 7.0), and 200- $\mu$ L suspensions were seeded into a 96-well microtitre plate. The absorbance at 590 nm (*Cmm*) or 600 nm (*Rs*) of bacterial suspensions was monitored every 30 min for 10–12 h at  $28^{\circ}\text{C}$  without agitation using a BioTek microplate reader. The experiment was performed three times.

### Pathogenicity and fruit infection assays

Cultures for *Cmm* strains were prepared and adjusted to  $\text{OD}_{600\text{ nm}} = 0.8$  as described above, and inoculated into 3-week-old tomato seedlings by the cotyledon clipping method ( $n = 5/\text{treatment}$ ) (Xu *et al.*, 2010). Tomato seedlings (cv. Mountain Fresh Plus) were grown in a Fafard

professional formula growing mix (Sun Gro Horticulture, Agawam, MA, USA) with a 14-h light/10-h dark photoperiod in the glasshouse. Tomato plants were screened daily for characteristic wilting and chlorosis associated with bacterial canker, as described previously (Balaji *et al.*, 2008; Tancos *et al.*, 2015). Disease severity was quantified by counting the number of individual wilted leaflets relative to the total number of individual leaflets present on the three oldest leaves. Observations continued until all plants had wilted or until 21 DPI. The mean area under the disease progress curve (AUDPC) was calculated from the disease severity (Madden *et al.*, 2007). Each of the three treatments had five plants per replicate, and the entire experiment was performed three times.

To quantify *Cmm* populations *in planta*, tomato seedlings were harvested at 9 DPI ( $n = 3/\text{treatment}$ ) and at 21 DPI ( $n = 5/\text{treatment}$ ). A 0.5-cm section of tomato stem tissue was harvested 1 cm above the inoculation site and homogenized with a sterile 5-mm stainless steel grinding bead (Qiagen, Valencia, CA, USA) using a TissueLyser (Retsch, Newtown, PA, USA), as described previously (Balaji and Smart, 2012; Tancos *et al.*, 2013). Subsequent dilutions and population counts were performed as described previously (Tancos *et al.*, 2013). The experiment was performed twice.

Immature green tomato fruit (8–12 mm in diameter) were inoculated with wild-type *Cmm*,  $\Delta\text{CmEXLX2}$  and the complemented mutant strain at a density of  $10^8$  cells/mL using a #2 horse-hair paintbrush, as described previously (Medina-Mora *et al.*, 2001; Tancos *et al.*, 2013). Tomato fruit were brushed with sterile distilled water as a negative control. Fruit were harvested ( $n = 4/\text{strain}$ ) at approximately 16 DPI and divided into five or six equal vertical cross-sections. All of the lesions represented in two randomly selected sections were visualized using an Olympus SZX2 dissecting microscope (Olympus Corporation, Shinjuku, Tokyo, Japan) connected to a Nikon Digital Sight-Qi1Mc camera (Nikon Corporation, Chiyoda, Tokyo, Japan). Nikon's NIS-Elements V.4.1 software (Nikon Corporation) was used to measure the total lesion diameter, necrotic centre diameter and the width of the lesion's 'white halo', at the widest point of the respective variables, for 100 individual lesions/strain. The experiment was performed twice.

The virulence of *Rs* isolates was determined on tomato plants following soil soaking and cut petiole inoculations. For *Rs* assays, tomato plants (cv. Bonny Best) were grown in ReadyMix Potting Soil in a  $28^{\circ}\text{C}$  climate-controlled chamber with a 12-h day/12-h night cycle. At 14 days post-sowing, seedlings were transplanted into individual 5-inch pots. For soil soaking inoculations, strains were grown overnight in 100 mL CPG in 250-mL flasks. Cultures were washed once in water and resuspended in water to  $\text{OD}_{600\text{ nm}} = 0.200$ . Bacterial suspensions (50 mL per plant) were poured into the soil of 17-day-old unwounded plants ( $n = 15$  plants/strain), which corresponds to an inoculum of approximately  $5 \times 10^8$  CFU/g soil. For cut petiole inoculations, strains were grown overnight in 5 mL CPG in test tubes. Cultures were washed once, and the cell density was adjusted to  $\sim 250$  CFU/ $\mu$ L. The oldest petiole of 21-day old plants was delicately removed with a sharp razor blade. A 2- $\mu$ L drop containing 500 CFU was placed on the cut petiole. For both *Rs* virulence assays, the wilting symptoms of each plant were rated daily on a 0–4 disease index scale: 0, no symptoms; 1,  $\leq 25\%$  leaves wilted; 2,  $\leq 50\%$  leaves wilted; 3,  $\leq 75\%$  leaves wilted; 4,  $\leq 100\%$  leaves wilted.

### *Cmm* movement and its influence on *in planta* growth

To assess differences in bacterial movement *in planta*,  $\text{OD}_{600\text{ nm}} = 0.8$  suspensions of *Cmm* wild-type,  $\Delta\text{CmEXLX2}$  and the complemented

mutant strain were prepared as described above. To measure acropetal movement, 3-week-old tomato seedlings with three true leaves were inoculated by the cotyledon clipping method ( $n = 3/\text{treatment}$ ). To quantify *in planta* bacterial populations at disparate distances, tomato seedlings were harvested at 21 DPI. A 0.5-cm section of tomato stem tissue was harvested 5 and 10 cm above the inoculation site and processed to determine the bacterial populations as described above. Negative control plants were cotyledon clipped with sterile water. The experiment was performed three times with a total of nine plants/strain.

### Assessment of differences in the lateral movement of *Cmm* strains

To visualize the *in planta* movement of bacteria, wild-type and  $\Delta\text{CmEXLX2}$  strains were transformed with the eGFP transient expression vector pK2–22 (Chalupowicz *et al.*, 2012). eGFP-expressing strains adjusted to an  $\text{OD}_{600\text{ nm}}$  of 0.8 were inoculated into 3-week-old tomato seedlings by the cotyledon clipping method and plants ( $n = 6/\text{treatment}/\text{time point}$ ) were harvested and screened at 5, 7 and 9 DPI. At each time point, microscopic analysis was performed on tomato stem cross-sections taken at 1, 2 and 3 cm above the inoculation site and  $-1$ ,  $-2$  and  $-3$  cm below the inoculation site. We measured the number of protoxylem, vascular bundles and xylem parenchyma cells infected with the eGFP-expressing strains. Plant sections were visualized using an Olympus BX61 microscope connected to a confocal laser scanning microscope (CLSM) system (Olympus Fluoview FV-300, Melville, NY, USA). An argon laser (488 nm excitation) and a green helium neon laser (543 nm excitation) were used to excite the eGFP bacteria and to induce plant autofluorescence, respectively (Tancos *et al.*, 2013).

### Crystal violet staining assay for *Cmm* attachment

Differences in bacterial attachment were assessed with pure tomato xylem sap. Bacterial isolates were grown in LB broth (gentamicin and/or neomycin was added when necessary), as described above. Bacterial suspensions (125  $\mu\text{L}$ ) were added to the individual wells of a 24-well Falcon tissue culture plate (Corning Inc.) containing 375  $\mu\text{L}$  of pure tomato xylem sap. The plates were briefly agitated and incubated statically at 27 °C for 5 days, stained with 0.1% crystal violet and solubilized with 30% acetic acid, as described previously (Davey and O'Toole, 2000; Kwasny and Opperman, 2010). Attached bacteria were quantified with an absorbance of 590 nm using a BioTek microplate reader (Davey and O'Toole, 2000; Kwasny and Opperman, 2010). The experiment was performed three times with independently derived media for a total of 27 absorbance readings/strain/medium.

### Root attachment assays

To assess root attachment by *Cmm* and *Rs* strains, surface disinfested tomato seeds were germinated on wet sterile filter paper until the roots were approximately 2 cm in length. Emergent seedlings were individually collected, placed on 1% water agar, inoculated along the root axis with 10  $\mu\text{L}$  of bacterial suspension and incubated at room temperature for 2 h. Following incubation, the roots were aseptically removed and pooled into groups of four for each respective strain, and gently washed to remove any non-adherent bacteria. The pooled roots ( $n = 4$ ) were homogenized and dilution plated. The experiment was performed three times.

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## SUPPORTING INFORMATION

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

**Fig. S1** Ethidium bromide-stained agarose gel showing the analysis of the expression of *CmEXLX2* *in vitro*. Products of reverse transcription-polymerase chain reaction (RT-PCR) of *CmEXLX2* with RNA obtained from wild-type (Cmm0317), *CmEXLX2* mutant ( $\Delta$ *CmEXLX2*) and complemented ( $C\Delta$ *CmEXLX2*<sup>+</sup>) strains (top gel). As a constitutively expressed control, the housekeeping gene *gyrB* was amplified using the same RT-PCR conditions (bottom gel).

**Fig. S2** Deletion of *RsEXLX* in *Ralstonia solanacearum*. (A) Schematic diagram of *RsEXLX* deletion. (B) Ethidium bromide-stained agarose gel (1.5% w/v) showing the genotype of GMI1000,  $\Delta$ *RsEXLX* and  $C\Delta$ *RsEXLX*<sup>+</sup>. Polymerase chain reaction (PCR) was conducted on genomic DNA using *RsEXLX*-specific primers (left) or *R. solanacearum* universal primers (right) as a control.

**Fig. S3** *In vitro* growth of *Clavibacter michiganensis* ssp. *michiganensis* (*Cmm*) and *Ralstonia solanacearum* (*Rs*) strains. Top: growth of Cmm0317 (wild-type),  $\Delta$ *CmEXLX2* (*Cmm* expansin mutant),  $C\Delta$ *CmEXLX2*<sup>+</sup> (*Cmm* complement) and  $C\Delta$ *CmP:RsEXLX*<sup>+</sup> (*R. solanacearum* expansin-hybrid mutant) in nutrient-rich Luria–Bertani medium and nutrient-poor tomato xylem sap medium. Bottom: growth of *Rs* GMI1000 (wild-type),  $\Delta$ *RsEXLX* (*Rs* expansin mutant) and  $C\Delta$ *RsEXLX*<sup>+</sup> (*Rs* complement) in Boucher's minimal medium or casamino acid, peptone, glucose (CPG)-rich medium. OD, optical density.

**Fig. S4** Autolysis of *Ralstonia solanacearum* (*Rs*) and *Clavibacter michiganensis* ssp. *michiganensis* (*Cmm*) strains. (A) *Rs* strains incubated in phosphate-buffered saline (PBS) or PBS with 0.02% w/v sodium dodecylsulfate (SDS). (B) *Cmm* strains incubated in PBS with 0.02% w/v SDS. WT, wild-type.

**Fig. S5** Inter- and intravascular colonization of tomato xylem vessels infected with enhanced green fluorescent protein (eGFP)-*Clavibacter michiganensis* ssp. *michiganensis* Cmm0317 (wild-type) and  $\Delta$ *CmEXLX2* (expansin mutant) at the specified days post-inoculation (DPI). All confocal microscopy images were generated by merging two channels (488-nm and transmitted light). Scale bar, 200  $\mu$ m.

**Fig. S6** The mean area under the disease progress curve (AUDPC) for the disease severity of *Ralstonia solanacearum* GMI1000 (wild-type),  $\Delta$ *RsEXLX* (*Rs* expansin mutant) and  $C\Delta$ *RsEXLX*<sup>+</sup> (*Rs* complement) of tomato. Differences amongst strains were determined by one-way analysis of variance (ANOVA) ( $P < 0.05$ ) using Dunnett's multiple comparison test

( $P < 0.05$ ). AUDPC values with the same letter are not significantly different. Three independent experiments are presented. Error bars correspond to the standard error.

**Fig. S7** *In vitro* biofilm attachment of *Clavibacter michiganensis* ssp. *michiganensis* Cmm0317 (wild-type),  $\Delta$ *CmEXLX2* (expansin mutant),  $C\Delta$ *CmEXLX2*<sup>+</sup> (complement) and  $C\Delta$ *CmP:RsEXLX*<sup>+</sup> (*Ralstonia solanacearum* expansin-hybrid mutant) in tomato sap. Error bars correspond to the standard error.

**Fig. S8** GelRed-stained agarose gel showing the analysis of expression of  $C\Delta$ *CmP:RsEXLX*<sup>+</sup> *in vitro*. Products of reverse transcription-polymerase chain reaction (RT-PCR) of *RsEXLX* with RNA obtained from wild-type (Cmm0317) and  $C\Delta$ *CmP:RsEXLX*<sup>+</sup> strains (top gel). As a constitutively expressed control, the housekeeping gene *gyrB* was amplified using the same RT-PCR conditions (bottom gel).

**Fig. S9** The mean area under the disease progress curve (AUDPC) for the disease severity of *Clavibacter michiganensis* ssp. *michiganensis* Cmm0317 (wild-type),  $\Delta$ *CmEXLX2* (*Cmm* expansin mutant),  $C\Delta$ *CmEXLX2*<sup>+</sup> (*Cmm* complement) and  $C\Delta$ *CmP:RsEXLX*<sup>+</sup> (*Ralstonia solanacearum* expansin-hybrid mutant) on tomato. Differences amongst strains were determined using the lme4 package in R v. 3.3.2 to create a linear mixed effects model ( $P < 0.05$ ) followed by Tukey–Kramer post-test ( $P < 0.05$ ). AUDPC values with the same letter are not significantly different. Three independent experiments are presented. Error bars correspond to the standard error.

**Fig. S10** Protein alignment for the chimeric *Clavibacter michiganensis* ssp. *michiganensis* expansin (CmEXLX1), *C. michiganensis* ssp. *michiganensis* non-chimeric expansin (CmEXLX2), *Ralstonia solanacearum* non-chimeric expansin (RsEXLX) and *Bacillus subtilis* non-chimeric expansin (BsEXLX1). Protein alignment was performed with the default settings of Clustal Omega (Goujon *et al.*, 2010), and the image was generated with BoxShade in RTF\_new format (<http://www.ch.embnet.org>). The shading correlates to amino acid similarity (grey boxes) and conservation (black boxes).

**Table S1** Phenotypic traits of *Clavibacter michiganensis* ssp. *michiganensis* (*Cmm*) strains on tomato tissue. (A) *In planta* populations of *Cmm* strains colonizing tomato stems, 5 and 10 cm above the site of inoculation, at 21 days post-inoculation. (B) *In planta* attachment of *Cmm* strains colonizing tomato roots following a 2-h incubation.

**Table S2** *Clavibacter michiganensis* ssp. *michiganensis* colonization and vascular movement within tomato stem tissue with enhanced green fluorescent protein (eGFP)-expressing wild-type and *CmEXLX2* mutant strains. (A) Amount of colonization and movement present at 5 and 7 days post-inoculation. (B) Amount of colonization and movement present at 9 days post-inoculation.

**Table S3** Oligonucleotides used in this study.

**Text S1** Materials and methods.