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Mol Plant Pathol. Author manuscript; available in PMC 2019 May 01.

Published in final edited form as:
Mol Plant Pathol. 2018 May ; 19(5): 1210–1221. doi:10.1111/mpp.12611.

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

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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* subsp. *michiganensis* (*Cmm*) and the β-proteobacterium *Ralstonia solanacearum* (*Rs*), respectively. The *Cmm* *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 *CmEXLX2* mutant even though the strains grew equally well as wildtype *in vitro*. Similarly, when inoculated onto tomato fruit, *CmEXLX2* caused significantly larger lesions with larger necrotic centers. In contrast, the *Rs* *RsEXLX* mutant had 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, *RsEXLX* attached to tomato seedling roots better than wildtype *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

horizontal gene transfer; expansin; bacterial plant pathogenesis; *Clavibacter michiganensis*; *Ralstonia solanacearum*

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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; Cosgrove, 2005). Regulating 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 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^+-ATPases, which lower the pH of the cell wall matrix by producing a proton differential across the plasma membrane (Cosgrove, 2000).

Recent evidence suggests that diverse microbes independently acquired and exploited this plant-derived enzyme for unknown reasons (Nikolaidis *et al.*, 2014). It is 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 xenologs, 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 xenologs are present among the non-vascular phytopathogenic bacterial genera *Pseudomonas* or *Agrobacterium* (Nikolaidis *et al.*, 2014). Two forms of expansins 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 bacteria contain only a single expansin (either chimeric or non-chimeric), with the notable exception of the tomato pathogen *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), which has both a chimeric (*CmEXLX1*) and non-chimeric version (*CmEXLX2*) (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).

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Bacterial canker of tomato is difficult to control due to the lack of resistant cultivars and improper sanitation (Sen *et al.*, 2013; Werner *et al.*, 2002).

Rs is a soil-borne β-proteobacteria 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 $>10^9$ 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 due to 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* had reduced attachment to maize roots (Kerff *et al.*, 2008), but, in contrast, *Xylella fastidiosa* and *Cmm* mutants lacking the endoglucanase-expansin chimeras caused reduced disease symptoms (Ingel *et al.*, 2015; Jahr *et al.*, 2000). However, the function(s) of non-chimeric expansins remain unknown, especially in phytopathogenic species.

Here we investigate the effect of non-chimeric bacterial expansins on pathogenesis and colonization of tomato by two vascular pathogens: the Gram-positive *Cmm* and the Gram-negative *Rs.* Because of the pathogens' niche overlap, 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. While the *Cmm CmEXLX2* mutant was hypervirulent and grew faster *in planta*, an *Rs RsEXLX* mutant hyper-attached 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 CmEXLX2* and *Rs RsEXLX* mutants did not express *CmEXLX2* or *RsEXLX,* while the wildtype and complemented mutants both did (Figure S1, Figure S2). *In vitro* growth of the *Cmm* strains was not significantly different from wildtype in nutrient-rich LB medium (P=0.994) and nutrient-poor tomato xylem sap (P=0.729) (Figure S3). Similarly, *RsEXLX* grew at the same rate as wildtype in both rich (P=0.493) and minimal medium (P=0.301) (Figure S3). The *CmEXLX2* mutation did not

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influence host recognition in *Mirabilis jalapa* (four o'clock plants). *CmEXLX2* induced a strong hypersensitive response (HR) indistinguishable from the HR induced by the wildtype and complemented mutant strain (Table 1).

Plant expansins facilitate cell wall loosening as plant cells develop and grow. While the bacterial BsEXLX1 expansin contributed to the autolysis of *B. subtilis*, BsEXLX1 lacked peptidoglycan-lytic activity (Kerff *et al.*, 2008). Because BsEXLX1 influenced *B. subtilis* cell wall integrity, we measured the autolysis rates of *CmEXLX2* and *RsEXLX* in *Cmm* and *Rs*, respectively. Autolysis of *CmEXLX2* and *RsEXLX* did not differ from the corresponding wildtype or complemented mutant strains (P=0.141 for *Cmm* and P=0.086 for *Rs*) (Figure S4).

The *Cmm CmEXLX2* mutant causes increased symptoms on tomato seedlings and fruit

Tomato seedlings inoculated with *CmEXLX2* consistently developed earlier and more severe wilt symptoms than the wildtype or complemented strains (P<0.0001) (Table 1). While 60% of plants inoculated with the wildtype and complement developed symptoms by 15 and 13 days post inoculation (DPI), respectively, *CmEXLX2* reached this disease incidence at 10 DPI. All three *Cmm* strains produced typical stem cankers at the site of inoculation, but *CmEXLX2* also produced multiple atypical cankers in the vascular bundles of distant petioles that appeared approximately 11–14 DPI (Table 1, Figure 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 later stages of disease development (Figure 1). Lesions on petioles were never observed on plants inoculated with the wildtype strain and were rarely formed by the complemented mutant strain.

The *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 if systemic movement influenced symptom development and *in planta* population sizes, 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). Consistent with our findings at 1 cm above the inoculation site, the *CmEXLX2* population (2.41×10^9 CFU/g) at 5 cm was larger (P=0.024) than wildtype (6.85×10^8 CFU/g). However, 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 if CmEXLX2 influences movement of *Cmm* between xylem vessels or vascular bundles, we inoculated tomato plants with EGFP-expressing *Cmm* strains and visualized intra- or intervascular colonization. EGFP-expressing *Cmm* wildtype ($CmEXLX2^+$) and *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). This qualitative approach revealed no detectable differences in patterns of xylem colonization, lateral movement, or parenchyma cell colonization between the strains at any time point or distance from the inoculation site.

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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, Figure S5).

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

Symptom development on tomato seedlings infected with the *Rs RsEXLX* mutant

Since 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, while the cut-petiole technique bypasses root infection by introducing *Rs* directly into the xylem. In contrast to *Cmm*, *RsEXLX* had wildtype virulence when directly introduced into tomato stems (P=0.106), but was reduced in virulence following soil-drench inoculation (P=0.0465) (Figure 3A–B, Figure S6). By 14 DPI, only 56% of plants inoculated with *RsEXLX* displayed symptoms, compared to 87% and 89% of plants inoculated with the wildtype and complemented mutant strains, respectively.

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

The virulence defect of *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 *RsEXLX* mutant hyper-attached to roots in comparison to wildtype (P<0.0001). Following a 2-hour incubation of tomato seedlings in a bacterial suspension, 6% of the *RsEXLX* population attached to roots compared to only 3.3% and 3.8% of the wildtype and complemented strain populations, respectively (Figure 3C). In contrast, there were no differences in root attachment between the three *Cmm* strains (P=0.4647) (Table S1B).

To determine if the attachment phenotype was root-specific, we measured 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* wildtype and expansin mutant strains (P=0.111), or *Cmm* strains (P=0.117), but the *Cmm* complemented strain produced less biofilm than wildtype (Figure S7).

Heterologous expression of *Rs* expansin in *Cmm*

Because the *CmEXLX2* and *RsEXLX* mutants had contrasting virulence phenotypes, we tested whether the heterologous expression of *RsEXLX* could complement the hypervirulence of *CmEXLX2*. *RsEXLX* was fused with the *Cmm* expansin secretion

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sequence and promoter so that the *Rs* gene was expressed and secreted like the *Cmm* expansin; this hybrid fusion strain was named C *CmP:RsEXLX*$^{+}$ (Figure S8). Similar to previous *Cmm* experiments, no differences in root attachment were observed between strains (*P*=0.4647). The expansin-hybrid also behaved similarly to *CmEXLX2* and the complemented mutant, producing less biofilm than the wildtype strain (*P*=0.0094) when grown in pure tomato xylem sap. The expansin-hybrid did not restore the *Cmm*-wildtype-level virulence and canker severity when inoculated into tomato, but had a trend of less disease than *CmEXLX2* (*P*=0.5230) (Figure S9). Although both *CmEXLX2* and *RsEXLX* are non-chimeric expansins, they only share 27% amino acid identity, which could explain why *RsEXLX* did not restore wildtype-level virulence (Table 2, Figure S10).

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 the loss of non-chimeric bacterial expansins divergently affected virulence of two vascular pathogens of tomato, *Cmm* and *Rs* (Nikolaidis *et al.*, 2014).

The *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 *CmEXLX2* caused atypical necrotic lesions on the vasculature of distant petioles. The quick onset of symptoms by *CmEXLX2* was not correlated with increased intra- or intervascular spread, because EGFP-expressing strains had similar rates of vascular infection both above and below the site of inoculation. In parallel with the increased stem tissue necrosis, *CmEXLX2* caused larger necrotic tomato fruit lesions. Fruit infected with *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.

The *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 acropetal regions of the stem were colonized; at 10 cm above the inoculation site, 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 *CmEXLX2* growth was not significantly different from wildtype *in vitro,* the mutant's increased *in planta* population size could have been due to the increased necrosis induced by the mutant. The older, more established *CmEXLX2* populations near the inoculation site may degrade vascular tissue more quickly, which likely released 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

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increased availability of plant-derived nutrients and subsequent influx of bacterial growth as established populations macerate plant tissue.

The molecular mechanism underlying the increased necrosis of *CmEXLX2* is unknown. We favor 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 likely due to the similarity between the D2 domains of BsEXLX1 and other type-A cellulose-binding modules (Georgelis *et al.*, 2012). While canonical CWDEs like pectinases, cellulases, and hemicellulases cleave plant cell wall polysaccharides, plant α-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 *CmEXLX2* likely 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 we 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, Figure 4, and Figure S10). Previous studies have shown that disrupting 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 of 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 *RsEXLX* mutant had wildtype virulence when directly introduced into the tomato vasculature. However when inoculated via the soil to the natural root infection court, *RsEXLX* caused significantly less disease than its wildtype 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; Huang and Allen, 2000; Liu *et al.*, 2005). We infer that the *RsEXLX* mutant's hyper-attachment to tomato roots delays symptom development in the soil-drench assay because the attached *RsEXLX* cannot efficiently enter and colonize the root vasculature.

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Plant and bacterial expansins have diverse functions. Within the plant kingdom, distinct cell-specific expansins modulate different aspects of plant cellular growth (Cosgrove, 2000; Cosgrove, 2015). *Arabidopsis thaliana* maintains at least 24 α-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, while the *B. subtilis* non-chimeric expansin increased *B. subtilis* attachment to maize roots (Kerff *et al*., 2008). Moreover, we demonstrated that RsEXLX is functionally different from CmEXLX2; unlike CmEXLX2, RsEXLX could not complement the hypervirulence phenotype of the *Cmm* *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 likely influence substrate specificity and activity (Pastor *et al*., 2015).

Only a subset of plant-associated bacteria possess 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; Lambert and Loria, 1989b). 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 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 highlights the dynamic nature of plant-bacterial interactions. 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

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attachment. It would be of interest to further characterize the role of RsEXLX in *Rs* root infections, and elucidate the targets and molecular mechanisms of these diverse bacterial plant-like expansins.

EXPERIMENTAL PROCEDURES

Bacterial strains/plasmids and primers used in this study are listed in Table 3 and S3, respectively. For further experimental procedural details, see Supporting Information Text S1.

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 pGCMEβGM plasmid as described (Stork *et al.*, 2008; Tancos *et al.*, 2013). Depending on the assay, *Cmm* isolates were incubated for 3–4 days at 27°C in Luria-Bertani (LB) (Miller, 1972), SB (Kirchner *et al.*, 2001; Stork *et al.*, 2008), or D2ANX media (Hadas *et al.*, 2005). When required, LB medium was supplemented with the antibiotics gentamicin (40 μg/ml), neomycin (100 μg/ml), or ampicillin (100 μg/ml) (Fisher Scientific; Pittsburgh, PA).

The phylotype I sequevar 18 *Rs* strain GMI1000 was used in this study. *Rs* was routinely grown in casamino acid, peptone, glucose media (CPG) or Boucher's minimal media (BMM) with 20 mM glucose at 28°C (Boucher *et al.*, 1985). When required, media were supplemented with the antibiotic gentamicin (25 μg/ml) (Fisher Scientific; Pittsburgh, PA). The unmarked *Rs* *RsEXLX* mutant was generated by natural transformation with pUFR80-RSc0818KO as described in (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 loss of the sacB-containing pUFR80 vector backbone by homologous recombination. Sucrose resistant clones were restruck on unamended media. PCR screening with RSc0818intF/R primers and GoTaq Green PCR mastermix (Promega, Madison, WI) 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 the MasterPure Gram-Positive DNA purification kit (Epicentre, Madison, WI) according to the manufacturer's instructions. Amplification was performed in a C1000 Touch Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA) 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 Lab. Inc., Mountain View, CA), according to the manufacturer's instructions. PCR products were purified with a DNA Clean & Concentrator – 25 kit (Zymo Research Inc., Irvine, CA) according to the manufacturer's instructions.

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RNA was extracted from 6 ml of *Cmm* suspension grown in LB medium to an OD_{600nm}= 1.4. Total RNA was extracted using a ZR Fungal/Bacterial RNA MiniPrep kit (Zymo Research Corporation, Irvine, CA). Extraction was followed by an additional DNase treatment using TURBO DNA-free DNase (Fisher Scientific; Pittsburgh, PA). Quantity and quality (260/280 ratio) of DNase-treated RNA was determined with a NanoDrop ND 1000 (NanoDrop Technologies, Wilmington, DE) and a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA). Reverse transcription was performed with RNA to cDNA EcoDry Premix with random hexamers (Clontech Laboratories, Inc. Mountain View, CA) using 300 ng of RNA per reaction. To ensure no DNA remained in the RNA, controls were performed with pure RNA (100 ng/reaction).

Assessing *in vitro* growth and hypersensitive response 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 (Chalupowicz *et al.*, 2012; Hiery *et al.*, 2013). Pooled collected sap was filter-sterilized through a 0.22 μm PES membrane 500-ml filter system (Corning Inc. Corning NY) and frozen in aliquots at −20°C.

Bacteria for *in vitro* growth assays were initially grown in LB medium for 28 hours and then adjusted to OD_{600nm}= 0.6 in water and inoculated (10 μl) into individual wells (n=9/strain) of a 96-well Falcon tissue culture plate containing LB medium or tomato sap (190 μl) (Corning Inc., Corning, NY). The plate was incubated at 28°C with shaking in a BioTek microplate reader (Winooski, VT). 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 ability to induce a hypersensitive response (HR) in *Mirabilis jalapa* (four o'clock plants). *Cmm* strains were grown for 24–32 h in LB medium (gentamicin and/or neomycin were added for *CmEXLX2* or the complemented strain) while shaking at 160 rpm in 125 ml flasks at 27°C, and the OD_{600nm} was adjusted to 0.8 (10^8 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 an $OD_{600\ nm}$=0.1 in CPG or BMM and 200 μl of the cell suspensions were added to individual wells (n=3/strain) of a 96-well Falcon tissue culture plate (Corning Inc., Corning, NY). The plate was incubated at 28°C with shaking in a BioTek microplate reader (Winooski, VT). 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× PBS with 0.02% SDS (pH=7.0), and 200 μl suspensions were seeded into a 96-well microtiter plate. Absorbance at 590 nm (*Cmm*) or 600 nm (*Rs*) of bacterial suspensions was monitored every 30 min for 10–12 h at 28°C without agitation, using a

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BioTek microplate reader (BioTek, Winooski, VT). The experiment was performed three times.

Pathogenicity and fruit infection assays

Cultures for *Cmm* strains were prepared and adjusted to an OD_{600nm}= 0.8 as described above, and inoculated into 3 week old tomato seedlings by the cotyledon clipping method (n=5/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) with a 14-hr light/10-hr dark photoperiod in the greenhouse. Tomato plants were screened daily for characteristic wilting and chlorosis associated with bacterial canker as previously described (Balaji *et al.*, 2008; Tancos *et al.*, 2015). Disease severity was quantified by counting the number of individual leaflets wilting relative to the total number of individual leaflets present on the three oldest leaves. Observations continued until all plants were wilting or until 21 days post inoculation (DPI). The mean area under the disease progress curve (AUDPC) was calculated from 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/treatment) and at 21 DPI (n=5/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) using a TissueLyser (Retsch, Newtown, PA) as described (Balaji and Smart, 2012; Tancos *et al.*, 2013). Subsequent dilutions and population counts were performed as described (Tancos *et al.*, 2013). The experiment was performed twice.

Immature green tomato fruit (8–12 mm in diameter) were inoculated with wildtype *Cmm*, *CmEXLX2*, and the complemented mutant strain at a density of 10^8 cells/ml using a #2 horse-hair paintbrush as previously described (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/strain) approximately 16 days post inoculation 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 Corp., Shinjuku, Tokyo, Japan) connected to a Nikon Digital Sight-Qi1Mc camera (Nikon Corp., Chiyoda, Tokyo, Japan). Nikon's NIS-Elements V. 4.1 software (Nikon Corp., Chiyoda, Tokyo, Japan) was used to measure the total lesion diameter, necrotic center 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.

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 °C climate controlled chamber with a 12 hr day/12 hr 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 $OD_{600\ nm}$=0.200. Bacterial suspensions (50 ml per plant) were poured into the soil of 17-day old unwounded plants

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(n=15 plants/strain), which corresponds to an inoculum of ~5×10^8 CFU/g soil. For cut-petiole inoculations, strains were grown overnight in 5 ml CPG in test tubes. Cultures were washed once, and cell density was adjusted to ~250 CFU/μl. The oldest petiole of 21-day old plants was delicately removed with a sharp razor blade. A 2 μl drop containing 500 CFU was placed on the cut petiole. For both *Rs* virulence assays, wilting symptoms of each plant were rated daily on a 0–4 disease index scale: 0, no symptoms; 1, 25% leaves wilted; 2, 50% leaves wilted; 75% leaves wilted; 100% leaves wilted.

Cmm movement and its influence on *in planta* growth

To assess differences in bacterial movement *in planta*, OD_{600nm}= 0.8 suspensions of *Cmm* wildtype, *CmEXLX2*, and the complemented mutant strain were prepared as described above. To measure acropetal movement, three week old tomato seedlings with 3 true leaves were inoculated by the cotyledon clipping method (n= 3/treatment). To quantify i*n 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 cm and 10 cm above the inoculation site and processed to determine bacterial populations as described above. Negative control plants were cotyledon clipped with sterile water. The experiment was performed three times with a total of 9 plants/strain.

Assessing differences in the lateral movement of *Cmm* strains

To visualize *in planta* movement of bacteria, wildtype and *CmEXLX2* strains were transformed with the eGFP transient expression vector pK2-22 (Chalupowicz *et al.*, 2012). EGFP-expressing strains adjusted to an OD_{600nm}= 0.8 were inoculated into 3 week old tomato seedlings by the cotyledon clipping method and plants (n=6/treatment/time point) were harvested and screened at 5 DPI, 7 DPI, 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). An argon laser (488 nm excitation) and a green helium neon laser (543 nm excitation) was used to excite the eGFP bacteria and 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 were added when necessary) as described above. Bacterial suspensions (125 μl) were added to the individual wells of a 24-well Falcon tissue culture plate (Corning Inc., Corning, NY) containing 375 μl of pure tomato xylem sap. The plates were briefly agitated and incubated statically at 27°C for five days, stained with 0.1% crystal violet, and solubilized with 30% acetic acid as described in (Davey and O'Toole, 2000; Kwasny and Opperman, 2010). Attached bacteria were quantified with an absorbance of 590 nm using the BioTek micoplate 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.

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Root attachment assays

To assess root attachment by *Cmm* and *Rs* strains, surface disinfested tomato seeds were germinated on wet sterile filter paper until roots were approximately 2 cm in length. Emergent seedlings were individually collected, placed on 1% water agar, inoculated along the root axis with 10 μl of bacterial suspension, and incubated at room temperature for 2 hours. Following incubation, the roots were aseptically removed and pooled in 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

M. Tancos was supported by grant no. DGE-1144153 from the NSF-Graduate Research Fellowships Program. Additional support was provided by federal formula funds administered through the New York State Agricultural Experiment Station and the University of Wisconsin-Madison College of Agricultural and Life Sciences. T. Lowe-Power was funded by NIH National Research Service Award T32 GM07215 and by Agriculture and Food Research Initiative Competitive Grant # 2015-67011-22799 from the USDA NIFA. T. Tran was supported by NSF Grant # IOS1456636. Special thanks to Shaun Stice, Chase Crowell, Greg Vogel, and members of the Smart lab for assisting with experiments. We would also like to thank Dr. Tom Burr for critical reading of the manuscript. The authors declare no conflicts of interest.

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FIGURE 1.

Atypical tomato petiole lesions associated with the *Clavibacter michiganensis* subsp. *michiganensis* expansin mutant *CmEXLX2*. (A) An individual petiole lesion rupturing a vascular bundle. Necrosis appeared 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.

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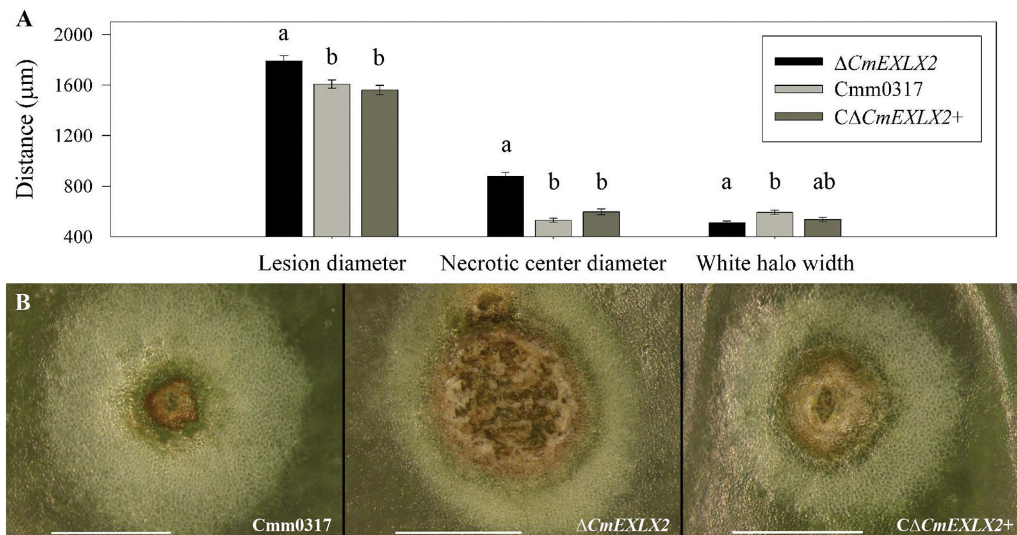

FIGURE 2.

Deleting *Clavibacter michiganensis* subsp. *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 among treatments for mean lesion size were determined with PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.01$). (B) Representative images of tomato fruit lesions are shown below: Cmm0317 (wildtype), *CmEXLX2* (expansin mutant), and C *CmEXLX2*$^{+}$ (complement). Scale bar: 1 mm. Error bars correspond to the standard error.

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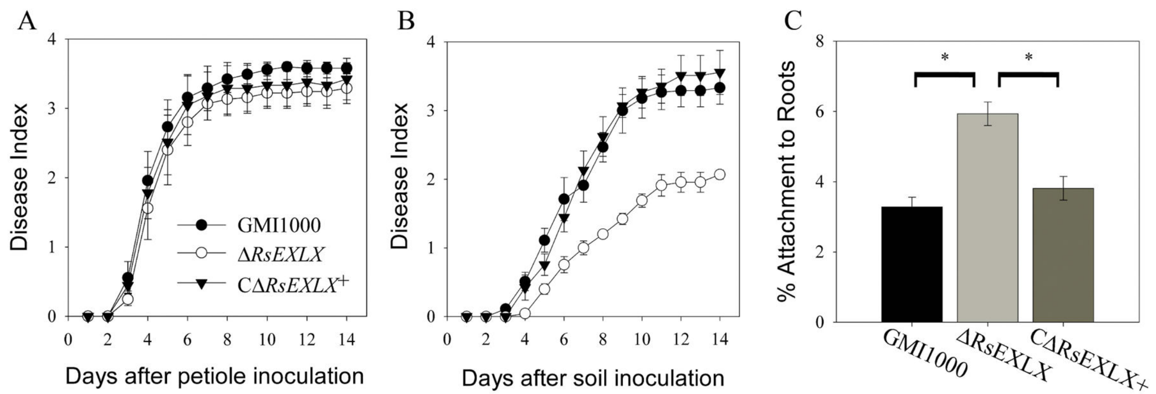

FIGURE 3.

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

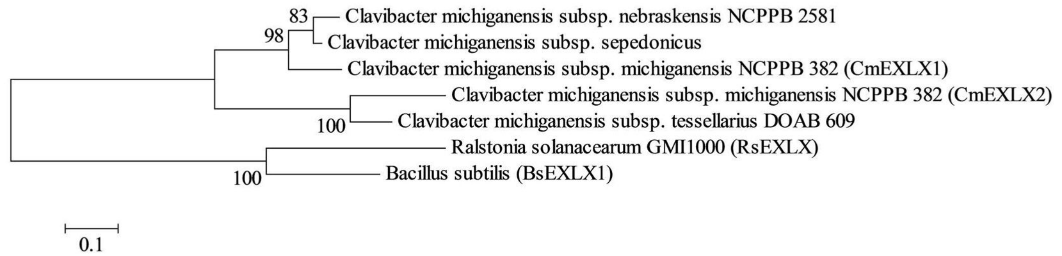

FIGURE 4.

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 in the number of substitutions per site (Tamura *et al.*, 2011).

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TABLE 1

Virulence of *Clavibacter michiganensis* subsp. *michiganensis* strains on tomato plants.

Strain	HR[a]	AUDPC[b]	Disease incidence[c]	Petiole lesion length (cm)[d]	CFU/g *in planta*[e]	
					9 DPI	21 DPI
Cmm0317	+	139 (± 36) [B]	15/15	0	2.78×10^{8} [B]	5.36×10^{8} [C]
CmEXLX2	+	430 (± 64) [A]	15/15	2.16 (±0.70)	3.72×10^{9} [A]	4.82×10^{9} [A]
C *CmEXLX2*^{+}	+	157 (± 21) [B]	15/15	0.59 (±0.21)	2.25×10^{8} [B]	2.30×10^{9} [B]
Water control	–	0.0	0/15	0	0.0	0.0

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

[b]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 posttest ($P<0.05$). AUDPC values followed by the same superscript are not significantly different. Numbers in parentheses indicate the standard error associated with the AUDPC values.

[c]Expressed as the number of wilting plants/number of inoculated plants characterized at 21 days post inoculation.

[d]The mean length of lesions located on the inoculated petiole at 21 days post inoculation. Numbers in parentheses indicate the standard error associated with the lesion lengths.

[e]The mean *in planta* population recovered from a 0.5 cm section of tomato stem tissue located 1-cm above the inoculation site at both 9 and 21 days post inoculation. Significant differences among strains were tested by PROC GLIMMIX ($P<0.05$) followed by Tukey-Kramer posttest ($P<0.05$). *In planta* populations followed by the same superscript are not different.

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TABLE 2

Overview of bacterial phenotypes associated with the mutation of bacterial expansins.

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

[a]Percent amino acid identify of the full-length protein, relative to the non-chimeric *Clavibacter michiganensis* subsp. *michiganensis* expansin *CmEXLX2*. The number in parenthesis denotes the amino acid identity of the most similar regions via the pairwise sequence alignment tool EMBOSS Matcher (Goujon *et al.*, 2010).

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TABLE 3

Bacterial strains and plasmids used in this study.

Strain or Plasmid	Description[a]	Reference/Source
Strains		
C. michiganensis subsp. *michiganensis*		
Cmm0317	Wildtype virulent New York strain	(Tancos *et al.*, 2015)
CmEXLX2	Cmm0317 mutant strain with disrupted *CmEXLX2*	This study
C *CmEXLX2*⁺	*CmEXLX2* transformed with pHNExpA (complement)	This study
C *CmP:RsEXLX*⁺	*CmEXLX2* transformed with pHNRsExpA (*C.m.* subsp. *michiganensis: R. solanacearum* expansin hybrid)	This study
Escherichia coli		
Zymo 5α (DH5α)	Cloning strain	Zymo Research
E.coli ER2925	*dam* and *dcm* methylation-negative strain	New England Biolabs
R. solanacearum		
GMI1000	Wildtype strain isolated from tomato in French Guyana ; phylotype I sequevar 18	(Salanoubat *et al.*, 2002)
RsEXLX	GMI1000 mutant with unmarked *RsEXLX* deletion	This study
C *RsEXLX*⁺	*RsEXLX* with a wildtype copy of RSc0818comp in the chromosome	This study
Plasmids		
C. michiganensis subsp. *michiganensis*		
pGEM-T Easy	Cloning vector; Amp^r; 3 kb	Promega
pHN216	*E. coli–Clavibacter* shuttle vector; Gm^r Nm^r; 13.8 kb	(Laine *et al.*, 1996)
pGnR-BsiWI	pGEM-T Easy:Gm^r cassette (from pHN216); Amp^r Gm^r; 3.8 kb	This study
pGCMEβ	pGEM-T Easy:*CmEXLX2*; 3.6 kb	This study
pGCMEβGM	pGEM-T Easy:*CmEXLX2*::Gm^r cassette; Amp^r Gm^r; 4.3 kb	This study
pIDT-FLExpA	IDT vector:Full length *CmEXLX2* and promoter; Amp^r; 3 kb	IDT
pHNExpA	Full-length *CmEXLX2* cloned into pHN216; Nm^r; 13.1 kb	This study
pHNRsExpA	*CmEXLX2* promoter and signal peptide fused to *RsEXLX* cloned into pHN216; Nm^r; 13.1 kb	This study
pK2-22	eGFP-expressing plasmid; Nm^r; 13.5 kb	(Chalupowicz *et al.*, 2012)
R. solanacearum		
pUFR80	Cloning vector; Kan^r Suc^s; 7.8 kb	(Castañeda *et al.*, 2005)
pUFR80-RSc0818KO	pUFR80:Unmarked *RsEXLX* cassette; Kan^r Suc^s; 9.4 kb	This study

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Strain or Plasmid	Description[a]	Reference/Source
pUC18t-MiniTn7t(Gm)	Wide host range complementation vector; Gm^r; 4.6 kb	(Choi *et al.*, 2005)
pMiniTn7-RSc0818comp	pUC18t-MiniTn7t(Gm):full length *RsEXLX*; Gm^r; 6.1 kb	This study

[a] Gm^r, Gentamicin acetyltransferase; Nm^r, neomycin phosphotransferase; Amp^r, β-lactamase; Kan^r, aminoglycoside 3′-phosphotransferase; Suc^S, levansucrase