***Pantoea stewartii* subsp. *stewartii* Produces an Endoglucanase That Is Required for Full Virulence in Sweet Corn**

Mojtaba Mohammadi, Lindsey Burbank, and M. Caroline Roper

Department of Plant Pathology and Microbiology, 900 University Avenue, University of California, Riverside 92521, U.S.A.

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***Pantoea stewartii* subsp. *stewartii*, a xylem-dwelling bacterium, is the causal agent of Stewart's wilt and blight of sweet corn. The goal of this study was to characterize the only gene in the *P. stewartii* subsp. *stewartii* genome predicted to encode an endoglucanase (EGase); this gene was designated *engY*. Culture supernatants from *P. stewartii* subsp. *stewartii* and *Escherichia coli* expressing recombinant EngY protein possessed both EGase and xylanase activities. Deletion of *engY* abolished EGase and xylanase activity, demonstrating that EngY appears to be the major EGase or xylanase produced by *P. stewartii* subsp. *stewartii*. Most importantly, our results show that EngY contributes to movement in the xylem and disease severity during the wilting phase of Stewart's wilt but is not required for water-soaked lesion formation.**

Pantoea stewartii subsp. *stewartii* is a xylem-dwelling bacterium that causes Stewart's wilt, a serious disease of sweet corn (*Zea mays*). The pathogen is spread by the corn flea beetle (*Chaetocnema pulicaria*), which transmits the bacteria primarily through scratching wounds created while feeding on the plant (Clafin 2000; Pataky 2004). As *P. stewartii* subsp. *stewartii* initially enters the plant, it colonizes the leaf apoplast and causes localized plant cell lysis, producing a characteristic water-soaked lesion. In later infection stages, the pathogen preferentially colonizes the xylem tissue of the plant, where it multiplies to high cell densities and forms exopolysaccharide (EPS)-encased biofilms that block water flow in the xylem, leading to the subsequent wilting and death of the plant (Beck von Bodman and Farrand 1995; Dolph 1988; Leigh and Coplin 1992). Molecular genetic approaches have identified the type III secretion system and stewartian EPS to be important pathogenicity factors for *P. stewartii* subsp. *stewartii* but, otherwise, the molecular mechanisms that this bacterium utilizes as it infects the sweet corn host are not fully understood (Beck von Bodman and Farrand 1995; Dolph 1988; Leigh and Coplin 1992).

Host cell-wall-degrading enzymes (CWDE) are important virulence factors for many bacterial phytopathogens, including other xylem-dwelling bacteria such as *Xylella fastidiosa*, *Ralstonia solanacearum*, and *Xanthomonas campestris* (Dow et al. 1990, 1998; Gonzalez and Allen 2003; Huang and Allen 1997; Kamoun and Kado 1990; Liu et al. 2004; Ray et al. 2000; Roper et al. 2007). CWDE have specifically been implicated in facilitating systemic movement throughout the xylem tissue (Huang and Allen 2000; Roper et al. 2007). These include

enzymes that break down the pectic, cellulosic, hemicellulosic, and proteinaceous components of the plant cell wall (Carpita and Gibeau 1993; Keegstra et al. 1973). In silico analysis of the *P. stewartii* subsp. *stewartii* genome revealed several open reading frames (ORF) encoding putative host CWDE, including two cellulases, two xylanases, one polygalacturonase, and one endoglucanase (EGase) (ASAP database) (Glasner et al. 2006), indicating that *P. stewartii* subsp. *stewartii* may be capable of host cell wall degradation.

EGases (EC 3.2.1.4) are hydrolytic enzymes that cleave polymers containing β -1,4-linked glucose molecules such as those found in cellulose and the cross-linking glycans (hemicellulose), both constituents of the higher plant cell wall (Buchanan et al. 2000). EGases are implicated in virulence and systemic colonization of the xylem in other bacterial phytopathogens, such as *R. solanacearum* and *X. campestris* pv. *campestris* (Gough et al. 1988; Roberts et al. 1988; Saile et al. 1997). Furthermore, there is also substantial evidence of enzymes classified as EGases, which exhibit xylanase activity in addition to EGase activity (Bauer et al. 1973; Labavitch and Greve 1983).

The most closely related ortholog to the *P. stewartii* subsp. *stewartii* EngY is the minor EGase Y, called CelY, from *P. ananatis* (99% coverage, $E = 0.0$) (De Maayer et al. 2010). CelY has been cloned and characterized from the closely related species *Dickeya dadantii* and is necessary for full virulence of the pathogen (Boccardo et al. 1994; Boyer et al. 1987). Based on the carbohydrate-active enzyme classification, the *P. stewartii* subsp. *stewartii* EngY belongs to the glycoside hydrolase family 8, which are O-glycosyl hydrolases that cleave the glycosidic bond between two or more carbohydrates or between a carbohydrate and a noncarbohydrate moiety. This family includes EGases, endo-1,4- β -xylanase, lichenase, and chitosanase. Enzymes in this family can also hydrolyze β -1,4-linkages in β -D-glucans containing β -1,3 linkages (i.e., the so-called mixed-linkage β -glucans found in cereal plant cell walls) and xylan (Labavitch and Greve 1983). Carboxy methyl cellulose (CMC) is a soluble form of cellulosic glucan composed of β -1,4 carboxymethyl group-substituted glucosyl residues. The mixed-linkage β -D-glucan is a polymer primarily composed of β -1,4-linked glucosyl residues interspersed with β -1,3-linked glucosyl residues. Xylans are polymers of β -1,4-linked xylosyl residues with varying degrees of substitution by arabinosyl or glucuronosyl residues. All represent structural carbohydrates that are found in sweet corn cell walls (Buchanan et al. 2000).

Because of the presence of putative CWDE genes in the genome of *P. stewartii* subsp. *stewartii* and its lifestyle as a xylem-dwelling bacterium, we hypothesized that CWDE allow *P. stewartii*

artii subsp. *stewartii* to colonize the xylem by digesting xylem pit membranes that separate xylem vessels. This would, presumably, facilitate movement within the xylem and potentially enable the bacteria to utilize the cell wall structural carbon-rich nutrients released by this enzymatic activity. In this study, we confirmed that the *engY* ORF encodes a functional EGase that is capable of degrading substrates containing β -1,4-linked glucosyl and xylosyl residues similar to those that *P. stewartii* subsp. *stewartii* would encounter in sweet corn cell walls, and that a deletion in the *engY* gene abolishes this enzymatic activity. We also demonstrate that EngY is an important virulence factor that contributes to the systemic infection of sweet corn.

RESULTS

Culture supernatants of *P. stewartii* subsp. *stewartii* possess EGase and xylanase activity.

We tested precipitated proteins from *P. stewartii* subsp. *stewartii* DC283 culture supernatants for the ability to degrade three polysaccharide substrates: CMC, a mixed-linkage β -D-glucan, and xylan. EGase and xylanase activities were confirmed by the production of reducing-end groups (micrograms of glucose released per milligram of protein) when assayed in the presence of CMC, mixed-linkage β -D-glucan, and xylan (Fig. 1A to C). The

results represent the means of enzyme activities expressed as micrograms of glucose equivalent released per milligram of protein. We also tested enzymatic activity associated with the cell pellet (data not shown) but found that the majority of the EGase and xylanase activity was found in the culture supernatant, indicating that the enzyme capable of degrading these three substrates is primarily secreted from the cell.

Enzymatic activity of the EngY protein expressed in *Escherichia coli*.

The precipitated culture supernatants from *Escherichia coli* BL21 (pEXP2::engY) expressing the *P. stewartii* subsp. *stewartii* EngY protein were capable of degrading CMC, mixed-linkage β -D-glucan, and xylan, as indicated by the production of reducing ends when incubated with these substrates (Fig. 2A to C). The β -1,4-linked glucan and xylan backbones look alike in terms of sugar ring size (both have six-membered rings) and the hydroxyl group orientations of the glucose and xylose residues in the polymer backbone are the same. Accordingly, it is not uncommon that EGases also possess xylanase activity (Bauer et al. 1973; Labavitch and Greve 1983). The pEXP2-GW/lacZ vector supplied with the pDEST expression kit (Invitrogen, Grand Island, NY, U.S.A.) served as the negative control in these experiments, and precipitated proteins from *E. coli* harboring

Fig. 1. Enzymatic activity of proteins precipitated from *Pantoea stewartii* subsp. *stewartii* culture supernatants. Degradation of either **A**, carboxymethyl cellulose sodium salt (CMC); **B**, mixed-linkage β -D-glucan (β -glucan) from barley bran; or **C**, beech wood xylan as indicated by the production of reducing ends in reaction mixtures of proteins precipitated from the culture supernatants of either *P. stewartii* subsp. *stewartii* DC283, $\Delta engY$, or the complemented strain, $\Delta engY$ (pBBR1::engY) as a function of time. Reaction mixtures were incubated at 37°C overnight. Similar results were obtained from at least three independent experiments and the results represent the mean of three replicates from one representative experiment.

Fig. 2. Enzymatic activity of the EngY protein expressed in *Escherichia coli*. Degradation of either **A**, carboxymethyl cellulose sodium salt (CMC); **B**, mixed-linkage β -D-glucan (β -glucan) from barley bran; or **C**, beech wood xylan as indicated by the production of reducing ends in reaction mixtures of proteins precipitated from the culture supernatants of either *E. coli* BL21(DE3) pLysS carrying pEXP2::engY, or BL21(DE3) pLysS carrying the negative control vector pEXP2-DEST::lacZ as a function of time. Reactions mixtures were incubated at 37°C overnight. Similar results were obtained from at least three independent experiments and the results represent the mean of three replicates from one representative experiment.

this plasmid did not break down the substrates tested in the reducing sugar assay. These results confirm that the *engY* ORF encodes an enzyme that is capable of degrading polymers contain β -1,4-linked glucosyl and xylosyl residues molecules.

A deletion mutation in *P. stewartii* subsp. *stewartii* *engY* abolishes EGase and xylanase activity.

We constructed a targeted deletion in the *P. stewartii* subsp. *stewartii* *engY* gene. To determine whether EngY was solely or partially responsible for the EGase or xylanase activity measured in *P. stewartii* subsp. *stewartii* DC283 supernatants, we determined the ability of the Δ *engY* mutant to degrade CMC, a mixed-linkage β -D-glucan, and xylan using a reducing sugar assay. Proteins precipitated from the culture supernatants of strain Δ *engY* lacked the ability to degrade all three substrates (Fig. 1A to C). Furthermore, complementation with a wild-type (WT) copy of *engY* in strain Δ *engY* (pBBR1::*engY*) restored CMCase, xylanase, and mixed-linkage β -glucanase activities to levels that exceeded that of the WT. This is likely due to the fact that the *engY* gene was cloned into the multi-copy pBBR1-MCS4 complementation vector and expression was driven off of the strong *lacZ* promoter in addition to the native promoter. These results indicate that the enzyme encoded by the *engY* gene is the primary EGase or xylanase produced by *P. stewartii* subsp. *stewartii* in vitro.

EngY is required for full virulence and movement in planta.

There are two phases of Stewart's wilt: leaf blight and wilt. In the leaf blight phase, the bacteria primarily colonize the leaf

apoplast, causing the formation of water-soaked lesions to appear on the leaves. In the wilting phase, the bacteria systemically colonize the xylem, where they reach high population numbers leading to the blockage of water flow in the xylem tissue. When *P. stewartii* subsp. *stewartii* is inoculated into the whorl of a sweet corn seedling, the bacteria only colonize the leaf apoplast and do not enter the xylem. Therefore, this method specifically tests the ability to form water-soaked lesions (Coplin et al. 1986). We saw no difference in virulence on sweet corn seedlings when the Δ *engY* and *P. stewartii* subsp. *stewartii* DC283 strains were individually whorl inoculated (data not shown), indicating that EngY has no obvious role in water-soaked lesion formation.

In contrast, when the bacteria were inoculated using the stem-scratch technique, we saw a decrease in the ability of the Δ *engY* strain to cause wilting. The stem inoculation technique mimics wounding created by the corn flea beetle vector during feeding, deposits the bacteria into the apoplast and the xylem, and tests the ability to incite both leaf blight and wilt symptoms. Scratch-inoculated plants challenged with *P. stewartii* subsp. *stewartii* DC283 exhibited the typical water-soaked lesions, ooze, and wilt symptoms associated with Stewart's wilt. These inoculated plants averaged 3.25 on the disease index scale and 75% were wilted (Fig. 3). However, the plants inoculated with the Δ *engY* strain developed fewer wilt symptoms than those inoculated with *P. stewartii* subsp. *stewartii* DC283, rated an average of 1.67 on the disease index scale, and only 33% developed wilting symptoms (Fig. 3). The reduced wilting phenotype for the Δ *engY* strain was partially complemented with a WT copy of *engY* supplied on plasmid pBBR1::*engY* (Fig. 3). Contingency table analysis showed that the disease ratings for plants inoculated with Δ *engY* were statistically different from those of the plants inoculated with WT *P. stewartii*

Fig. 3. Contribution of EngY to virulence. Fourteen-day-old sweet corn seedlings ('Jubilee') were inoculated with 5 μ l of either *Pantoea stewartii* subsp. *stewartii* DC283; Δ *engY*; the complemented strain, Δ *engY* (pBBR1::*engY*); or 1 $\times$ phosphate-buffered saline (PBS) Tween 20 buffer using the scratch-inoculation technique. Virulence was assessed 8 days post inoculation on a 0-to-5 rating scale. Data represent the mean of at least 12 replicates. Similar results were obtained from at least three independent experiments. Disease severity data was analyzed using a six-by-two χ^2 contingency table analysis and letters indicate treatments that are significantly different from one another at $P \leq 0.05$. Plants inoculated with 1 $\times$ PBS Tween 20 buffer did not develop any Stewart's wilt symptoms. The table summarizes the percentage of plants displaying wilt symptoms (rating 3 or more on the disease index scale).

Fig. 4. EngY plays a role in acropetal dissemination within the xylem. Dissemination was measured by inoculating approximately 10^7 green fluorescent protein (GFP)-tagged bacteria into the midrib of 14-day-old sweet corn ('Jubilee') seedling leaves and evaluated after 72 h of incubation. Active bacterial dissemination within the xylem of sweet corn (Jubilee) seedlings was assessed by epifluorescence microscopy measured against the passive infiltration of fluorescent microspheres. The dashed line indicates the distance the fluospheres traveled. The GFP-labeled wild-type strain DC283 showed significant dissemination in the acropetal direction (toward base of the leaves) direction, whereas the GFP-labeled Δ *engY* mutant strain did not move significantly beyond the passive infiltration distance of the fluorescent microspheres. Data were analyzed using a one-way analysis of variance test and each statistical mean was square-root transformed to meet test assumptions. The graph represents the means from at least three biological replicates each with at least 10 technical replicates.

subsp. *stewartii* DC283 or the complemented strain, Δ engY carrying pBBR1::engY ($P = 0.001$). The Δ engY scratch-inoculated plants developed a similar level of leaf blight symptoms (water-soaking) when compared with *P. stewartii* subsp. *stewartii* DC283 inoculated plants (data not shown). These results demonstrate that EngY is required for wilting but not water-soaked lesion formation.

The pores in the pit membranes that separate one xylem vessel from another in corn plants are too small to allow passage of bacteria (Shane et al. 2000). In order to breach the pit membrane barrier, *P. stewartii* subsp. *stewartii* must actively digest them, likely through the action of glycosyl hydrolases, such as EngY, that are capable of degrading the polysaccharide constituents of that compose the xylem pit membranes. We tracked the movement of a green fluorescent protein (GFP)-labeled Δ engY mutant in the xylem of sweet corn by placing the inoculum in a leaf wound and compared it with the movement of GFP-labeled WT *P. stewartii* subsp. *stewartii* DC283. Movement was assessed 72 h later by fluorescence microscopy. We compared acropetal and basipetal movement of the pathogen away from the point of inoculation using a one-way analysis of variance to test differences among means, and the movement values were square-root transformed to meet test assumptions. Statistical significance was tested at the 5% confidence level. Our results show that Δ engY (pHC60::gfp) was compromised in acropetal (Fig. 4) but not basipetal (data not shown) movement within the xylem. Acropetal movement was partially restored in strain Δ engY (pBBR1::engY) (pHC60::gfp) that carries a WT copy of engY.

DISCUSSION

In this study, we identified and characterized an EGase or xylanase for the phytopathogen *P. stewartii* subsp. *stewartii* and demonstrated that this EGase can degrade complex polysaccharide substrates containing β -1,4-linked glucosyl or β -1,4-linked xylosyl backbones similar to those found in corn plant cell walls. Most importantly, our data demonstrate that this enzyme contributes to virulence and dissemination within the xylem.

P. stewartii subsp. *stewartii* systemically colonizes the xylem, which results in the wilting of young seedlings. Xylem vessels are separated by diaphragm-like structures that function as filters that block the passage of particulates, microbes, and air embolisms from one xylem vessel to another. These filters, while typically referred to as pit membranes, are actually made of primary plant cell wall tissue (Esau 1965). Primary plant cell walls are composed of cellulose microfibrils embedded in a polysaccharide matrix of pectin and cross-linking glycans (hemicellulose) (Buchanan et al. 2000; Gibeaut and Carpita 1994). Sweet corn is a commelinoid monocot and the major cross-linking glycan in this group of plants is glucuronoarabinoxylan (GAX). GAX consists of a backbone of β -1,4-linked xylosyl residues with side chains of glucuronic acid and arabinose (Buchanan et al. 2000; Gibeaut and Carpita 1994). In addition, members of the plant order Poales, which includes sweet corn, contain a unique mixed-linkage β -glucan. This polymer is primarily composed of cellotriose and cellotetraose (three and four β -1,4-linked glucosyl residues, respectively) connected by β -1,3 linkages (Buchanan et al. 2000; Carpita 1996).

The small pores in the pit membrane have been estimated to be between 5 and 20 nm in other plant species (Choat et al. 2003; Perez-Donoso et al. 2010; Zimmerman 1983) and the pores in the *Z. mays* pit membranes are estimated to be 4.9 ± 0.7 nm in size (Shane et al. 2000), which is much too small to allow passive passage of a *P. stewartii* subsp. *stewartii* cell,

which is 400 to 700 by 900 to 2,000 nm in size (Bradbury 1967). How *P. stewartii* subsp. *stewartii* breaches this barrier is poorly understood. We speculated that the bacterium utilizes plant CWDE to digest xylem pit membranes because it is a strategy used by other xylem-inhabiting phytopathogenic bacteria to facilitate movement through the xylem tissue (Huang and Allen 2000; Roper et al. 2007). *P. stewartii* subsp. *stewartii* may also potentially metabolize the cell wall structural carbon-rich nutrients released by this enzymatic activity. We chose the commercially available model substrates CMC, mixed-linkage β -glucan, and xylan because they most closely represent the structures of cellulosic glucans and hemicellulosic glycans (GAX), and hemicellulosic glucans (mixed-linkage β -D-glucan) that *P. stewartii* subsp. *stewartii* would presumably encounter in the pit membranes found in sweet corn xylem conduits, and demonstrated that the *P. stewartii* subsp. *stewartii* EngY degraded all three of these substrates. The enzymatic action of EngY may aid in passage through xylem pit membranes or contribute to release of nutrients from the host cell wall by digesting the cross-linking glycans and, possibly, cellulose found in the primary walls of sweet corn.

When tested in planta, we confirmed that the Δ engY mutant was significantly compromised in the ability to move acropetally within the xylem, indicating that loss of the ability to degrade β -1,4-linked glucans or xylans results in decreased movement in the xylem. We also found that EngY contributes to disease severity during the wilting phase of Stewart's wilt but not during the water-soaking (leaf blight) phase. This suggests that the impairment in dissemination throughout the xylem leads to a reduction in wilt disease development in sweet corn. This was similarly demonstrated in *R. solanacearum*, where inactivation of a single EGase caused a partial reduction of wilting in tomato when wound inoculated into tomato stems and also hindered systemic colonization of the xylem (Roberts et al. 1988; Saile et al. 1997).

Because of the variety of other polysaccharide types expected to be present in sweet corn cell walls, it is likely that *P. stewartii* subsp. *stewartii* also utilizes other CWDE to facilitate its growth in the apoplast and xylem vessels. Several ORF encoding putative β -1,4 cellulases and xylanases and one polygalacturonase (PG) have been identified in the *P. stewartii* subsp. *stewartii* genome and these may also be involved in degradation of the hemicellulosic and pectic portions of the plant cell wall. However, these are apparently not required for degradation of CMC, mixed-linkage β -D-glucan, or xylan because a mutation in engY completely abolished enzymatic activity against all three of these substrates. This does not completely rule out the role of the other putative cellulases or xylanases that may be contributing to hemicellulose degradation in planta because the enzymatic assays conducted in this study were performed with *P. stewartii* subsp. *stewartii* cells grown in rich culture medium. These enzymes could be expressed during the infection process while remaining undetectable in culture supernatants. Degradation of crystalline cellulose is a complex process and usually involves the coordinated activities of multiple enzymes, such as EGases and cellobiohydrolases, which often exist in complexes called cellulosomes (Bayer et al. 2004). *P. stewartii* subsp. *stewartii* cannot be considered truly cellulolytic because it does not appear to possess the full suite of enzymes necessary for complete hydrolysis of crystalline cellulose and likely cannot completely digest the cellulose that is found in the microfibrils of the cell wall. Thus, we speculate that EngY is primarily involved in breaking down the cross-linking glycans rather than cellulose microfibrils.

In other systems, the pectic matrix plays a critical role establishing the pore size of the pit membranes (Baron-Epel 1988; Buchanan et al. 2000; Perez-Donoso et al. 2010; Zwieniecki et

al. 2001). PG hydrolyze pectin polymers and are involved in the systemic colonization of the xylem for some xylem-dwelling bacterial pathogens (Huang and Allen 2000; Perez-Donoso et al. 2010; Roper et al. 2007). One ortholog to a PG was identified in the *P. stewartii* subsp. *stewartii* genome. Although pectinase (PG or pectate lyase) activity was not detected in *P. stewartii* subsp. *stewartii* culture supernatants or cell pellets (M. C. Roper, unpublished data), this does not preclude the possibility of pectinase production in planta. Pectins are in relatively low amounts in cereal cell walls but their abundance in pit membranes is unknown and they could play a key role in dictating pit membrane porosity as in other plant systems. Until immunohistochemical assays as performed with grapevine pit membranes (Sun et al. 2011) are executed in sweet corn, this question will remain unanswered.

The xylem sap is considered to have a dilute nutrient composition with a relatively limited availability of a carbon source. Despite this assumption, *P. stewartii* subsp. *stewartii* can reach high cell densities in the xylem (approximately 10^{10} CFU/g of plant tissue) and, while doing so, produces copious amounts of a carbohydrate-rich stewartan EPS (Nimtz et al. 1996), which suggests that the bacteria must have access to an ample carbon source in the xylem. As plant cell walls are being synthesized, the cell wall polymers remain soluble until they are cross-linked at the cell's exterior surface. It is reasonable to speculate that *P. stewartii* subsp. *stewartii* takes advantage of the transient availability of these soluble cell wall precursors as they are being synthesized and deposited in the cell wall in developing xylem tissue (Buchanan et al. 2000). Mixed-linkage β -D-glucans are one of the few known developmental-stage-related polysaccharides in plants, and they accumulate to approximately 30% of the noncellulosic corn coleoptile cell wall polysaccharide material during cell elongation and enlargement (Hoson et al. 1992; Inouhe and Nevins 1991a,b). Synthesis and hydrolysis of these β -D-glucans occurs simultaneously during growth and they are presumably relatively abundant in young, rapidly growing sweet corn seedlings.

Young sweet corn seedlings are highly susceptible to Stewart's wilt compared with mature corn plants and it is perhaps the metabolism of the mixed-linkage β -D-glucan that *P. stewartii* subsp. *stewartii* exploits as it colonizes rapidly growing seedlings.

A possible role for the EGase in virulence is implied by its potential to degrade plant cell wall components (i.e., glucans or xylans). EngY plays a small role in dissemination of the pathogen throughout the xylem tissue and may facilitate degradation of cell wall material by other enzymes (i.e., pectinase and protease). Furthermore, EngY could possibly participate in the release of carbohydrates found in the plant cell wall that could be utilized as nutrients. This study is the first account of host CWDE being produced by *P. stewartii* subsp. *stewartii* and offers insight into the mechanisms of virulence this pathogen uses as it invades and colonizes sweet corn plants.

MATERIALS AND METHODS

Strains and growth conditions.

P. stewartii subsp. *stewartii* (DC283) and *E. coli* strains were cultured and maintained on nutrient agar and Luria-Bertani (LB) agar (Becton Dickinson, Franklin Lakes, NJ, U.S.A.). For enzyme, protein, and nucleic acid extractions, bacterial strains were grown in LB liquid medium. Nalidixic acid (30 μ g/ml), kanamycin (Kan) (25 μ g/ml), streptomycin (100 μ g/ml), spectinomycin (100 μ g/ml), ampicillin (100 μ g/ml), and tetracycline (10 μ g/ml) were added to the medium when appropriate. Bacterial strains and plasmids are presented in Table 1.

Construction of the *P. stewartii* subsp. *stewartii* Δ engY mutant.

The Δ engY mutant was constructed based on a previously described method (Choi and Schweizer 2005) that utilizes Gateway cloning technology (Invitrogen). The flanking regions of the *engY* gene (500 bp upstream of the start codon and 500 bp downstream of the stop codon) were amplified separately from

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotypes ^a	Reference or source
Strains		
<i>Pantoea stewartii</i> subsp. <i>stewartii</i>		
DC283	SS104 WT, Nal ^r	Coplin et al. 1986
Δ engY	Deletion of <i>engY</i> , Nal ^r	This study
<i>Escherichia coli</i>		
BL21	BL21 (DE3) pLysS, F ⁻ , <i>ompT dcm lon hsdS_B (r_B⁻ m_B⁻) galλ</i> (DE3) pLysS, Cm ^r	Promega Corp.
DH10 β	F ⁻ <i>endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 Φ80lac ZΔM15 araD139 Δ(ara, leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ⁻</i>	Invitrogen
EC100pir ⁺	F <i>mcrA (mrr-hsdRMS-mcrBC) 80dlacZ M15 ΔlacX74 recA1 endA1 araD139 (ara, leu)7697 galU galK λ⁻ rpsL nupG pir⁺(DHFR)</i>	Episentre
S17-1 λ pir ⁺	RP4, Mob ⁺ , Sm ^r	Simon et al. 1982
Plasmids		
pKD4	Kanamycin cassette, Ap ^r , Km ^r	Datsenko and Wanner 2000
pDONR-Zeo	Cloning vector, Zeo ^r	Invitrogen
pAUC40	Suicide vector, Gateway <i>attR</i> -Cm ^r cassette cloned in pKNG101 Sm ^r , Cm ^r	Carlier et al. 2009
pBBR1MCS-4GW	pBBR1MCS-4 modified as a Gateway destination vector	Carlier et al. 2009; Kovach et al. 1995
pKNG101	Suicide vector, Sm ^r	Kaniga et al. 1991
pFLP2	<i>sacB⁺</i> , source of FLP recombinase, Ap ^r	Hoang et al. 1998
pHC60::gfp	Broad host range vector carrying <i>gfp</i> , Tc ^r	Cheng and Walker 1998
pMOJ1	<i>engY::kan</i> cloned into pDONR-Zeo, Km ^r	This study
pMOJ3	<i>engY::kan</i> cloned into pKNG101, Km ^r , Sm ^r	This study
pBBR1::engY	<i>engY</i> cloned into pBBR1MCS-4GW, Ap ^r	This study
pMOJ9	pCR8/GW/TOPO::engY ORF, Sp ^r	This study
pEXP2	Gateway compatible expression vector pEXP2-DEST::lacZ	Invitrogen
pEXP2::engY	<i>engY</i> cloned into pEXP2, Ap ^r	This study
pEXP2-GW/lacZ	pEXP2-DEST control plasmid, Ap ^r Zeo ^r	Invitrogen

^a Nal^r, Cm^r, Sm^r, Ap^r, Km^r, Zeo^r, Tc^r, and Sp^r indicate resistance to nalidixic acid, chloramphenicol, streptomycin, ampicillin, kanamycin, zeocin, tetracycline, and spectinomycin, respectively; *gfp* = green fluorescent protein gene.

P. stewartii subsp. *stewartii* DC283 genomic DNA using primer pairs GW-engF/engR-Kan for the upstream flanking region and engF-Kan/engR for the downstream flanking region (Table 2) using optimized polymerase chain reaction (PCR) conditions. The Kan resistance marker (1.4 kb) was amplified from the pKD4 plasmid (Datsenko and Wanner 2000) using pKD4fwd and pKD4rev primers designed to overlap the 5' and 3' flanking regions of the *engY* amplicons and Kan cassette, respectively. In a second round of PCR, the three purified DNA fragments generated from the first round were combined into one tube (without primers) and assembled by PCR overlap extension: primer pair GWattB1/GWattB2 was added after 10 rounds of PCR and then the rest of the PCR reaction was carried out. The assembled PCR product containing *attP* recombination sites was then recombined into the pDONR/Zeo vector (Invitrogen) using the Gateway BP clonase II mix (Invitrogen) following the manufacturer's instruction to create pMOJ1. pMOJ1 was recombined into pKNG101-attR using the LR clonase II kit according to manufacturer's instruction (Invitrogen) to create pMOJ3. pMOJ3 was introduced into *E. coli* S17-1 λ by electroporation and was then conjugally transferred into *P. stewartii* subsp. *stewartii* DC283. The merodiploid state was resolved by sucrose counter-selection. Sucrose-resistant colonies were selected for Kan resistance and streptomycin susceptibility and double-crossover events were confirmed by PCR using primers endoglucfwd/endoglucchkrev. Following marker-exchange mutagenesis, the Kan cassette used for selection was excised by FLP recombinase carried on pFLP2 (Hoang et al. 1998), leaving an 85-bp *frr* scar to create the unmarked strain Δ engY.

For complementation analysis, the entire coding region of *engY*, including the native promoter and ribosomal binding site, was amplified by PCR using primers endoglucfwd/endoglucchkrev. The resulting PCR products were cloned into pCR8/GW/TOPO (Invitrogen) to create pMOJ9. The *engY* ORF in pMOJ9 was recombined into the Gateway compatible pBBR1MCS4-GW vector (Carlier et al. 2009; Kovach et al. 1995), which contains *attR* sites, using the Gateway LR clonase II reaction mix (Invitrogen) to create pBBR1::engY. The pBBR1::engY construct was checked by sequencing to ensure that there were no introduced mutations and then electroporated into Δ engY to create strain Δ engY (pBBR1::engY).

Extraction and precipitation of proteins from culture supernatants.

Single colonies of *P. stewartii* subsp. *stewartii* DC283, Δ engY, and Δ engY (pBBR1::engY) were grown overnight in 2 ml of LB broth. A 125-ml Bellco flask containing 25 ml of LB

broth was inoculated with 250 μ l of the overnight culture and grown at 28°C, 200 rpm. Cells were grown to an optical density at 600 nm (OD_{600}) of 1.0 and collected by centrifugation. Total proteins in the culture supernatant were precipitated by slowly adding ammonium sulfate to 85% saturation on ice. The precipitated proteins were centrifuged and resuspended in 2 ml of 100 mM sodium acetate buffer (pH 6.0) and dialyzed overnight against 3 liters of the same buffer at 4°C with stirring in regenerated cellulose dialysis tubing (MWCO = 12,000 to 14,000) (Fisherbrand, Fisher Scientific, Pittsburgh). The dialysate was used for downstream enzymatic assays.

Enzymatic activity assays.

The ability of the *P. stewartii* subsp. *stewartii* strains to digest three different substrates—CMC sodium salt (Fisher Scientific), mixed-linkage β -D-glucan from barley bran (Sigma-Aldrich, St. Louis), and xylan from beech wood (Sigma-Aldrich)—was tested. These substrates were chosen because of their structural similarities to carbohydrate polymers typically found in sweet corn cell walls. A reducing sugar assay was used to demonstrate enzymatic activity based on a protocol described by Dygert and associates (1965). Substrates were dissolved in 100 mM sodium acetate buffer (pH 6.0) to a final concentration of 1.0 mg/ml by heating briefly and vortexing. A 0.5-ml sample of the dialysate from the ammonium-sulfate-precipitated culture supernatants was incubated with 0.5 ml of each substrate individually. Protein extract incubated without substrate and substrate incubated without protein extract served as enzyme and substrate blanks, respectively. The reaction mixtures and controls were incubated in a 37°C water bath for 48 h. Reaction mixture (50 μ l) was transferred into a screw-cap tube and mixed with 1 ml of reagent A (40 g of Na_2CO_3 , 16 g of glycine, and 450 mg of $CuSO_4 \cdot 5H_2O$ per liter in dH_2O) and 1 ml of reagent B (neocuproine-HCl at 1.2 g/liter in dH_2O). Each sample was vortexed and boiled for 12 min and cooled down to room temperature. The OD was read at 454 nm using a Biomate 3 UV/visible spectrophotometer (Thermo Scientific, Waltham, MA, U.S.A.). D-glucose at final concentrations of 5, 10, 15, and 20 μ g was used to obtain a calibration curve. Absorbance values for buffer blank, substrate blank, and enzyme blank were subtracted from the corresponding enzyme or substrate reaction samples and then converted to nanograms of reducing sugar. Total soluble protein concentration in culture supernatant was measured following the Bradford method using the Quick Start Bradford dye Reagent 1 $\times$ (Bio-Rad Laboratories, Hercules, CA, U.S.A.) (Bradford 1976). Bovine serum albumin fraction IV at 5, 10, 25, 50, and 100 μ g in 2 ml of

Table 2. List of primer sequences used in this study

Primer	Primer sequence ^a	Source
pKD4fwd	GAGAATAGGAACCTTCGGAATAGGAACCTCAA	This study
pKD4rev	CTATTCTCTAGAAAAGTATAGGAACCTCAGAGCG	This study
GW-attB1 ^b	GGGGACAAGTTTGTACAAAAAAGCAGGCT	Choi and Schweizer 2005
GW-attB2 ^b	GGGGACCACTTTGTACAAAGAAAGCTGGGT	Choi and Schweizer 2005
GW-endogluc upstream flank fwd ^c	TACAAAAAAGCAGGCTCAGCTCTATTCCGCTACCTGTT	This study
Endogluc upstream flank rev-Kan ^d	gaacttcgaagcagctccagcta CAGAGCACCTTATTGATATCGGAAATGC	This study
Endogluc downstream flank fwd-Kan ^d	cggaaataggaactaaggaggatattcatatg TGATGCCCGCTCTGCGGCATGCTGTT	This study
GW-endogluc downstream flank rev ^c	TACAAGAAAGCTGGGTTTTTCGAAGTTCGCATGCAGCGCCTGG	This study
Endoglucchkfwd	ATAAAGATGTCACGATTGGCGGGC	This study
Endoglucchkrev	AAAGCGGTAAGGTGATTTCACCG	This study
Endoglucfwd (–150 upstream ORF) ^e	AGACGGCATTGCTGCCATTCT	This study
Endoglucrev	ATCAGGCGCGTGCTTCTCCT	This study

^a Primer sequences are oriented from 5' to 3'.

^b Gateway sequences are in bold

^c Gateway overlapping sequences in target gene are shown in bold.

^d Kanamycin (Kan) overlapping sequences in target genes are presented in lowercase bold.

^e ORF = open reading frame.

reagent was used as a standard. After 20 min of incubation, absorbance was read at 595 nm. Enzyme activities were reported as nanograms of glucose equivalent released per milligram of protein.

Cloning and expression

P. stewartii subsp. *stewartii* EngY in *E. coli*.

The *engY* ORF was subcloned from pMOJ9 into the expression vector pEXP2-DEST (Invitrogen) using the Gateway LR clonase II reaction mix (Invitrogen) to create pEXP2::*engY*. This expression vector fuses a 6-histidine tag to the C-terminal end of the EngY protein. pEXP2::*engY* was transformed into chemically competent *E. coli* DH10 β by heat shock. The plasmid was extracted and the construct was confirmed by PCR, sequencing, and restriction analysis to confirm that the insertion had been cloned in-frame with no introduced mutations. pEXP2::*engY* was introduced into the expression strain, *E. coli* BL21 (DE3) pLysS, by electroporation. Single colonies were chosen and grown to OD₆₀₀ = 0.5. Protein expression was induced once the cell density reached OD₆₀₀ = 0.5 by adding isopropyl β -D-1-thiogalactopyranoside to a final concentration of 1 mM. Cultures were grown at 37°C to OD₆₀₀ = 1.0. *E. coli* strain BL21 (DE3) pLysS transformed with the control vector pEXP2-DEST::lacZ supplied with the pEXP2-DEST cloning kit was used as negative control. Protein extraction, purification, and enzymatic assays were performed as described above for *P. stewartii* subsp. *stewartii*.

Virulence assays.

Seed of sweet corn ('Jubilee') (Syngenta Seeds, Boise, ID, U.S.A.) were propagated in a Marathon-treated mixture of peat and vermiculite under greenhouse conditions. Single colonies of either *P. stewartii* subsp. *stewartii* DC283, Δ *engY*, or Δ *engY* (pBBR1::*engY*) were grown overnight, washed twice in 1 $\times$ phosphate-buffered saline buffer (PBS), and resuspended in the same buffer to OD₆₀₀ = 1.0. For the stab-inoculation assay, 14-day-old corn seedlings were stab inoculated six times in the stem slightly below the primary leaf using a sterile 20-gauge syringe needle (Becton Dickinson) and 5 μ l (approximately 10⁷ cells) of bacterial inoculum was placed over the wound. For the whorl-inoculation assay, the whorls of 7-day-old sweet corn (Jubilee) seedlings were inoculated with 150 μ l of a 10⁷ CFU/ml suspension of WT *P. stewartii* subsp. *stewartii* DC283, Δ *engY*, or Δ *engY* (pBBR1::*engY*) or 1 $\times$ PBS buffer to assess water-soaking ability (Coplin et al. 1986). Seedlings were assessed daily for symptom development and rated using an arbitrary disease index scale where 0 = no symptoms; 1 = small water-soaking lesions; 2 = larger water-soaking lesions; 3 = water-soaking lesions, ooze, and slight wilting; 4 = severe wilting; and 5 = dead. Seedlings inoculated with 1 $\times$ PBS buffer were used as negative controls. Both experiments were repeated three times with at least 10 technical replicates for each experiment.

In planta movement assay.

Plasmid pHC60 harboring the *gfp*_{S65t} gene (Cheng and Walker 1998) was conjugally transferred into the *P. stewartii* subsp. *stewartii* DC283, Δ *engY*, and Δ *engY* (pBBR1::*engY*) strains to create strains DC283 (pHC60::*gfp*), Δ *engY* (pHC60::*gfp*), and Δ *engY* (pBBR1::*engY*, pHC60::*gfp*), respectively. Inoculum was prepared by growing the GFP-tagged strains overnight in 2 ml of LB broth containing the appropriate antibiotics, washing twice in 1 $\times$ PBS, and resuspending in 1 $\times$ PBS buffer. Cell density was adjusted to OD₆₀₀ = 1.0. Fourteen-day-old sweet corn seedlings were stab inoculated in the leaf midrib using a sterile syringe needle and 5 μ l of inoculum was placed over the stab wound. Bacterial movement within the leaf tissue

was monitored 72 h after inoculation using a Leica fluorescence stereoscope Fluo Tm equipped with a Hg Arc lamp as described by Herrera and associates (2008). The distances traveled acropetally and basipetally from the inoculation site were measured.

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asap.ahabs.wisc.edu/asap/home.php