A Large Repetitive RTX-Like Protein Mediates Water-Soaked Lesion Development, Leakage of Plant Cell Content and Host Colonization in the *Pantoea stewartii* subsp. *stewartii* Pathosystem

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Pantoea stewartii subsp. *stewartii* is the etiological agent of Stewart's wilt and is a serious bacterial pathogen affecting sweet corn. During the leaf blight phase, *P. stewartii* colonizes the leaf apoplast and causes a characteristic water-soaked lesion. The Hrp type III secretion system has been implicated in the water-soaking phenotype, and the goal of this study was to investigate other potential factors that contribute to the plant cellular disruption associated with these lesions. The *P. stewartii* genome contains a gene encoding a large repetitive RTX toxin, designated *rtx2*. RTX toxins comprise a large family of pore-forming proteins, which are widely distributed among gram-negative bacteria. These cytotoxins usually lyse their target host cells and cause significant tissue damage as a consequence. We hypothesized that this RTX-like toxin plays a role in the water-soaking phase of infection due to its predicted cytolytic properties. Based on the data reported here, we conclude that RTX2 contributes significantly to the development of water-soaked lesions and leakage of plant cellular contents and is an important pathogenicity factor for *P. stewartii*.

Pantoea stewartii subsp. *stewartii* (previously *Erwinia stewartii*) is the causal agent of Stewart's wilt of sweet corn, the most serious bacterial disease of sweet corn in the north central and eastern United States. The bacteria are introduced into the plant primarily through the feeding wounds of its insect vector, the corn flea beetle (*Chaetocnema pulicaria*) (Claflin 2000; Pataky 2003). *P. stewartii* colonizes both the leaf apoplast and the xylem, causing leaf blight and wilt symptoms, respectively. During the leaf blight phase, the bacteria cause cellular damage that leads to accumulation of fluids in the surrounding leaf tissue that manifest a water-soaked (WS) lesions. The Hrp type III secretion system has been linked to the water-soaking symptomatology that is observed during the early phase of the *P. stewartii* infection cycle. More specifically, the WtsE protein acts as a type III effector that suppresses host defenses and causes cell death similar to water-soaking when expressed in a heterologous *Escherichia coli* system (Ham et al. 2006, 2008). Subsequently, the bacteria preferentially colonize the xylem

tissue leading to the wilting phase of the disease (Claflin 2000; Dolph et al. 1988; Leigh and Coplin 1992; Pataky 2003). Here, the bacteria grow to the high cell densities that are necessary to produce large amounts of stewartan exopolysaccharide (EPS). Stewartan EPS production is a cell density-dependent phenotype, which is a requisite for *P. stewartii* to produce cell wall-adherent biofilms and effectively colonize the xylem (Beck von Bodman and Farrand 1995; Dolph et al. 1988; Koutsoudis et al. 2006; Leigh and Coplin 1992; von Bodman et al. 1998). The EPS and biofilms produced in the xylem are thought to impede the free flow of xylem fluid, thus causing young plants, in particular, to succumb to wilt. In mature plants, xylem blockage can lead to long wilted areas of the plant with wavy margins.

In this study, we sought to examine a gene predicted to encode a protein belonging to the repeat in toxin (RTX) family of proteins, based on characteristic multidomain protein structure and putative multifunctionality as either cytolytic toxins, adhesins, or both (ASAP database version 8) (Lally et al. 1999; Welch 1991, 2001). RTX proteins are widely distributed, particularly in pathogenic bacteria and are best described for mammalian bacterial pathogens such as *E. coli*, *Bordetella pertussis*, and *Pasteurella haemolytica* (Welch 2001). Notably, little is known about the role of cytolytic RTX toxins in plant pathogenesis. The prototypical RTX toxin is *E. coli* α -hemolysin, but other cytotoxins in this family are lytic against a broad range of target cells other than erythrocytes (Gilbert 2002; Lally et al. 1999). The signature features of RTX toxins are C-terminally located glycine-aspartate rich repeats that participate in Ca^{2+} binding and a C-terminal secretion signal sequence that targets these proteins for export via a type I secretion system (TISS). The list of diverse RTX proteins is rapidly expanding, suggesting that this class of proteins is quite heterogeneous, with individual proteins not always conforming to the classical paradigm of a RTX toxin. Hence, RTX proteins have been further categorized into several subfamilies, including the newly described BAP (biofilm-associated proteins) subfamily of RTX toxins (Satchell 2011). BAP proteins are large repetitive RTX proteins that are cell envelope-associated and function primarily as adhesins rather than toxins (Hinsa et al. 2003; Satchell 2011). Some RTX proteins contain hemagglutinin domains that act as cell-surface localized afimbrial adhesins that mediate cell-cell aggregation, biofilm formation, and virulence for both animal and plant bacterial pathogens

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(Gottig et al. 2009; Guilhabert and Kirkpatrick 2005; Loch et al. 2001; Voegel et al. 2010). The toxigenic component of RTX proteins induces the lysis of target host cells usually by forming pores in the target host membranes (Benz et al. 1994; Gentschev 2003), thus, causing significant tissue damage not unlike that observed in WS lesion formation during the leaf blight phase of Stewart's wilt. These latter attributes, in particular, motivated us to test the *P. stewartii* RTX2 protein for a role in plant tissue damage related to the water-soaking phenotype.

RESULTS

Sequence and domain analysis of RTX2.

RTX2 (ASAP number ACV-0285925 version 8) (Glasner et al. 2006) is a large repetitive protein (2,498 aa) with a predicted molecular mass of 249.8 kDa. The most closely related ortholog for both proteins is the large repetitive protein YeeJ in *Pantoea ananatis*, which has a cryptic role in adhesion (RTX2 shares 75% identity with 93% coverage, E = 0.0) (Genbank accession number WP_029570315.1). Other orthologs include a putative hemagglutinin hemolysin adhesin-related protein from *Erwinia billingiae* (49% identity with 95% coverage, E = 0.0) (accession number YP_03742412.1) (Kube et al. 2010). RTX2 also shares homology (31% identity with 93% coverage, E value = $2e^{-162}$) with a member of the BAP subfamily of RTX proteins (accession number ABX00640.1) found in the human pathogen *Acinetobacter baumannii* (Loehfelm et al. 2008).

ProDom analysis identifies five putative Ca^{2+} -binding domains in RTX2 that are similar to those found in serralyisin, a metalloprotease belonging to the RTX family of exoproteins produced by *Serratia marcescens* (Tao et al. 2006) and a hemolysin-like protein in *Desulfovibrio vulgaris* (Fig. 1) (Heidelberg et al. 2004). These Ca^{2+} -binding domains are necessary for biological activity of RTX toxins (Frey 2006; Gentschev 2003) and suggests that RTX2 requires Ca^{2+} to function (Fig. 1). InterPro scan analysis identified one PKD domain, which is hypothesized to be involved in protein-carbohydrate interactions (Bycroft et al. 1999) (Fig. 1).

Many RTX toxins are secreted through a cognate TISS usually cotranscribed with the gene encoding the RTX toxin. Notably, RTX2 does not possess a TISS signal peptide, nor is it found in an operon with a cognate TISS. Thus, this protein is unlikely to be secreted via this mechanism. However, ProDom analysis identified two domains in RTX2 that are indicative of autotransported proteins, suggesting that RTX2 is exported across the cell wall via an autotransport mechanism (Fig. 1). In addition, RTX2 contains five C-terminal transmembrane domains, which suggests that the protein may be tethered to the bacterial cell surface following autotransport (Fig. 1).

The $\Delta rtx2$ mutant is compromised in virulence and WS lesion development.

Because of the homology to cytolytic toxins, we predicted that the RTX protein has a role in virulence and, specifically, in the early water-soaking phenotype characteristic of *P. stewartii*

infections. We confirmed this prediction, with a deletion mutant in *rtx2* (Table 1, CR54). Wild-type (WT) *P. stewartii*, $\Delta rtx2$, and its complemented counterpart were inoculated separately into 7-day-old sweet corn seedlings, using the whorl inoculation method that tests specifically for WS lesion development (Coplin et al. 1986). The WT parental strain, *P. stewartii*, was fully virulent and caused the formation of WS lesions, which enlarged rapidly to eventually give rise to large confluent lesions that spanned the entire leaf, giving a disease rating of 4, based on a disease index rating scale of 0 to 4 (Figs. 2A and 3). In contrast, plants inoculated with the $\Delta rtx2$ mutant failed to develop typical leaf blight symptoms and retained a healthy appearance for the duration of the experiment. The symptoms of these plants gave a disease rating that was statistically similar to the negative control, i.e., sterile phosphate-buffered saline, pH 7.4, with 0.2% Tween 20 buffer (1× PBST)-inoculated control plants (Fig. 2A). In terms of lesion development, the $\Delta rtx2$ mutant strain was conspicuously free of visible WS lesions (Fig. 3). WS lesion formation and leaf collapse were fully restored by the complemented mutant strain (CR64) that carries a functional copy of *rtx2* on plasmid pMCR29 (Table 1) (Figs. 2A and 3). When inoculated using the scratch-inoculation method, which mimics the natural inoculation process and allows the bacteria to colonize the apoplast and the xylem, the $\Delta rtx2$ mutant did not cause WS lesions or wilt (Fig. 2B).

A mutation in *rtx2* limits growth in planta.

To evaluate the role of the *rtx2* locus in bacterial growth in the host, populations of the $\Delta rtx2$ mutant strain and the complemented counterpart strain were inoculated into 7-day-old sweet corn seedlings and were compared with WT DC283 bacteria, using the whorl inoculation technique. We assessed bacterial growth in planta 6 days after inoculation (Fig. 4) and found that the number of bacteria was 2.3×10^7 CFU/g (CFU per gram of leaf tissue) for the $\Delta rtx2$ mutant, which was significantly lower than the growth of the WT strain, which grew to an average titer of 1.2×10^{10} CFU/g. These data indicate that the $\Delta rtx2$ mutant strain was still able to colonize the plant tissue, although at lower numbers than the WT, but did not induce visible disease symptoms. In trans expression of a functional *rtx2* gene in strain CR64 (*rtx2/rtx2+*) fully reversed the $\Delta rtx2$ mutant phenotype by restoring wild-type in planta growth and colonization (Fig. 4).

The apoplastic fluid of symptomatic leaves exhibiting WS lesions is enriched in protein.

We reason that *P. stewartii* creates WS lesions as a mechanism to release nutrients from the plant cells, and we quantified total protein content as a marker for nutrient levels into the apoplastic space. Indeed, the apoplastic fluid of leaves harvested from plants inoculated with WT *P. stewartii* contained significantly more protein (1,647.78 μ g/ml) than the apoplastic fluid extracted from leaves harvested from seedlings inoculated with $\Delta rtx2$, which contained 1,019.81 μ g of protein per milliliter (Fig. 5). The apoplastic fluid extracted from plants inoculated with the $\Delta rtx2/rtx2+$ complemented strain had

Fig. 1. Predicted domain structure of RTX1 and RTX2. The predicted amino acid sequences of RTX2 were analyzed using ProDom and InterPro Scan software. The Ca^{2+} domains are those commonly found in proteins belonging to the RTX exoprotein family. RTX2 possesses two domains found in autotransported proteins and a PKD domain involved in adhesion to carbohydrate surfaces. The presence of five transmembrane domains in the C-terminus suggests that RTX2 is localized to the bacterial membrane via the C-terminus.

similar levels (1,604.98 µg/ml) of protein as apoplastic fluid from seedlings inoculated with WT *P. stewartii* (Fig. 5). Apoplastic fluid extracted from 1× PBST-inoculated negative control plants contained 517.38 µg of protein per milliliter, which was less than all of the other treatments and belonged to its own statistical group (Fig. 5).

The apoplastic fluid of symptomatic leaves contains elevated levels of dissolved solutes.

We quantified solute concentration (reported as osmotic potential [Ψ_s]) as an indicator of leakage of plant cellular contents into the leaf apoplast. We extracted leaf apoplastic fluid from plants inoculated with either WT *P. stewartii*, $\Delta rtx2$, $\Delta rtx2/rtx2+$, or 1× PBST and found that, indeed, the apoplastic fluid extracted from seedlings inoculated with WT had significantly higher Ψ_s , as compared with apoplastic fluid extracted from seedlings inoculated with $\Delta rtx2$ or 1× PBST, indicating leakage of solutes into the apoplast. Specifically, the solute concentration was 5.6-fold higher in seedlings inoculated with WT, as compared with those inoculated with $\Delta rtx2$ (Fig. 6). The differences in dissolved solutes (Ψ_w) were statistically significant, based on a Student's *t* test using $P \leq 0.05$. Dissolved solute levels were restored to near WT levels in leaf apoplastic fluid of plants inoculated with the $\Delta rtx2/rtx2+$ complemented strain.

Coinoculation of $\Delta rtx2$ and $\Delta wtsE$ restores water-soaking.

WtsE, a type III effector implicated in WS lesion formation (Ham et al. 2006), is thought to suppress host defenses immediately after infection as well as serving a secondary role as a cell death factor, which contributes to lesion formation. Because a mutation in *rtx2* had such a profound effect on WS lesion formation, we sought to investigate whether the $\Delta wtsE$ and $\Delta rtx2$ mutants could complement one another, which might be indicative that they serve separate, complementary roles in the infection process that leads to WS lesion formation. Indeed, WS lesion formation was restored when the two strains were coinoculated together into the whorls of sweet corn plants (Fig. 7).

DISCUSSION

Our prediction that RTX2 mediates cellular damage and lesion formation was substantiated by the protein structure and function predictions of the RTX2 protein and was confirmed by

the mutant analysis. Specifically, we show that the $\Delta rtx2$ mutant strain is completely deficient in inducing the water-soaking phenotype. We infer that this cellular damage function correlates with the presence of five Ca^{2+} -binding hemolysin domains predicted from the amino acid sequence of RTX2. Further, RTX2 is predicted to assume a β -barrel protein tertiary structure, based on analysis performed using the SCRATCH protein prediction software. Other known RTX lytic toxins also adopt a β -barrel conformation, which is thought to be essential for their interaction with host cell membranes and formation of pores in the target cell, leading to cell rupture and collapse (Gilbert 2002). Another interesting feature of RTX2 is an extended C-terminus in RTX2, which contains five transmembrane domains. These transmembrane domains indicate that RTX2 may be associated with the bacterial envelope, similar to the ShlA hemolysin in *S. marcescens* (Hertle 2000).

Based on the type of inoculation method used, it is possible to separate the water-soaked lesion phase from the wilting phase of Stewart's wilt. When whorls of seedlings are inoculated, the plants are not wounded and the pathogen does not enter the xylem. This method specifically tests the ability of the bacterium to cause WS lesions in the leaf apoplastic space. A second method, the scratch-inoculation technique, creates wounds using a syringe needle. The inoculum is then deposited in the wound, which allows bacteria to enter the plant and colonize both the xylem and the leaf apoplast. This method mimics the natural infection process facilitated by the corn flea beetle feeding behavior and allows assessment of a strain's ability to cause the wilting that follows WS lesion formation in young seedlings. The $\Delta rtx2$ mutant did not incite WS lesions when inoculated using either the whorl or scratch inoculation methods. Interestingly, when introduced directly into the xylem using the scratch inoculation method, the $\Delta rtx2$ mutant did not cause wilting either. This suggests that the $\Delta rtx2$ mutant is essentially locked into the apoplastic phase of infection and does not effectively colonize the xylem, which is required to cause the wilting symptom. We know that *P. stewartii* produces stewartan EPS, which is a critical factor for robust biofilm formation that insures successful xylem colonization, only in the presence of fermentable sugars and at cell densities greater than 3×10^8 cells per milliliter (von Bodman et al. 1998). We reason that the inability to cause WS lesions limits nutritional availability for the bacteria, thus preventing the bacteria from

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotypes ^a	Reference or source
Strains		
<i>P. stewartii</i> subsp. <i>stewartii</i>		
DC283	SS104 wild type, Nal ^R	Coplin et al. 1986
CR54	$\Delta rtx2$, Nal ^R , parent strain DC283	This study
CR64	$\Delta rtx2$, pMCR29, parent strain DC283	This study
AA005	DC283 <i>wtsE::aphT,Km^r</i>	Ham et al. 2006
<i>Escherichia coli</i>		
S17-1λ <i>pir⁺</i>	RP4 Mob+	Simon et al. 1982
EC100	<i>pir⁺</i>	Epicentre
DH10B	$\Delta lacX74$ <i>araΔ139 Δ(ara-leu)</i>	Invitrogen
Plasmids		
pMCR29	<i>rtx2</i> cloned into pBBR1	This study
pMCR31	<i>rtx2::kan</i> cloned into pDONR/Zeo	This study
pMCR32	<i>rtx2::kan</i> cloned into pKNG101	This study
pKD4	Km ^R , source of kan cassette	Datsenko and Wanner 2000
pDONR-Zeo	Cloning vector, Zeo ^R	Invitrogen
pAUC40	Suicide vector, Gateway <i>attR-Cm^R</i> cassette cloned in pKNG101, Sm ^R , Cm ^R	Carlier et al. 2009
pBBR1MCS-4	Broad host range vector, Ap ^R	Kovach et al. 1995
pFLP2	Ap ^R , source of Flp recombinase	Hoang et al. 1998

^a Nal^R, Ap^R, Km^R, Gm^R, Sm^R, Cm^R, and Zeo^R = nalidixic acid, ampicillin, kanamycin, gentamycin, streptomycin, chloramphenicol, and zeocin resistance, respectively.

achieving the cell densities high enough to effectively colonize the xylem as well as limiting the amount of available plant-associated sugars necessary for the production of EPS that is needed to systemically colonize the xylem (Mohammadi et al. 2012).

Indeed, RTX proteins contribute to colonization in the host for several bacterial species, including *Pasteurella pneumotropica*, *Vibrio vulnificus*, *Haemophilus ducreyi*, and *S. marcescens* (Lo et al. 2011; Marre et al. 1989; Sasaki et al. 2011; Wood et al. 1999). These cytolytic toxins can facilitate the spread of bacteria through host tissues, allowing the bacteria to acquire essential nutrients from the damaged host cells (Alouf 2001). Specific examples include a *S. marcescens* hemolysin that contributes to colonization of the rat urinary tract and a cell-associated hemolysin that facilitates the invasiveness of *H. ducreyi* into human epithelial cells during genital ulcer formation (Hertle 2000; Marre et al. 1989; Wood et al. 1999). Our leaf colonization demonstrated that the $\Delta rtx2$ mutation limits in planta growth compared with the parental strain. Thus, we envision a scenario in which RTX2 contributes to the growth of *P. stewartii* in the plant by inducing plant cell-wall collapse and release of nutrients and water from the damaged plant cells that allow the bacteria to proliferate to the cell numbers that will achieve the physiological conditions necessary to continue the infection process and effectively transition from the apoplast to the xylem

environment. Collectively, the results from the extracted leaf apoplastic fluid support our hypothesis that RTX2 mediates plant cell lysis. It is important to note that several *P. stewartii* mutants that are deficient in colonization can still effectively incite WS lesions (Coplin and Cook 1990); therefore, the lack of WS lesion development is primarily due to the absence of the RTX2 cytolysin and is not simply a consequence of a reduction in plant colonization.

Interestingly, when inoculated separately, neither the $\Delta rtx2$ nor the $\Delta wtsE$ -inoculated plants developed WS lesions, but coinoculation with the two strains restored WS lesion formation. WtsE is the only known type III effector involved in *P. stewartii* virulence and has an apparent dual role in suppressing the host immune response as well as causing a host cell death associated with WS lesions (HR) (Ham et al. 2006, 2008, 2009). We propose a model in which, within minutes of infection, WtsE suppresses host defenses, including the hypersensitive response, which provides protection for the infecting bacteria. Following this, RTX2 serves as the primary cell lysis factor, which releases nutrients and water that allow the bacteria to become established in the plant. In addition to suppressing the host defense response, WtsE also has a secondary role as a cell death factor. Ham et al. (2006) report that this is a slow sustained cell death, and we propose that WtsE likely acts temporally downstream of RTX2 in this secondary role as an

Fig. 2. Virulence assays. **A**, The whorls of 7-day-old sweet corn seedlings (var. Jubilee) were inoculated with 100 μ l of a 10^5 CFU/ml inoculum of either wild-type (WT) DC283, $\Delta rtx2$, $\Delta rtx2/rtx2+$, or sterile phosphate-buffered saline, pH 7.4, with 0.2% Tween 20 buffer (1 $\times$ PBST). Overall disease severity ratings were taken 6 days postinoculation. Plants inoculated with $\Delta rtx2$ appeared healthy and showed no water-soaked (WS) lesions, whereas plants inoculated with WT DC283 developed confluent WS lesions. Pathogenicity was restored with a copy of *rtx2* supplied on plasmid pMCR29 in strain *rtx2/rtx2+*. Plants inoculated with 1 $\times$ PBST buffer did not develop any symptoms. **B**, The stems of 10-day-old sweet corn seedlings (var. Jubilee) were wounded with a syringe needle and 5 μ l of a 10^5 CFU/ml inoculum of either WT DC283, $\Delta rtx2$, $\Delta rtx2/rtx2+$, or 1 $\times$ PBST was placed in the wound. Overall disease severity ratings were assessed 7 days postinoculation. Similar to the whorl-inoculated plants, seedlings inoculated with $\Delta rtx2$ appeared healthy and showed no WS lesions or wilt, whereas plants inoculated with WT DC283 developed confluent WS lesions and subsequent wilting. WS lesion development and wilt was restored with a copy of *rtx2* supplied on plasmid pMCR29 in strain *rtx2/rtx2+*. Plants that were wound-inoculated with 1 $\times$ PBST buffer did not develop any symptoms. Disease severity data were analyzed using a Dunnett multiple comparison test and letters indicate treatments that are significantly different from one another $P \leq 0.05$. The graphs represent the means of data collected from at least 15 replicates (three biological replicates with five technical replicates each).

Fig. 3. Lesion length assay. The whorls of 7-day-old sweet corn seedlings (var. Jubilee) were inoculated with 100 μ l of a 10^5 CFU/ml inoculum of either wild-type (WT) DC283, $\Delta rtx2$, $\Delta rtx2/rtx2+$, or sterile phosphate-buffered saline, pH 7.4, with 0.2% Tween 20 buffer. The longest lesion on the first fully expanded leaf was measured 6 days postinoculation. Plants inoculated with $\Delta rtx2$ had no water-soaked (WS) lesions. WS lesion formation was fully restored with a wild-type copy of *rtx2* carried on plasmid pMCR29 in strain *rtx2/rtx2+*. Lesion length data were analyzed using a Dunnett multiple comparison test and letters indicate treatments that are significantly different from one another $P \leq 0.05$. The graph represents the means of data collected from at least 15 replicates (three biological replicates with five technical replicates each).

elicitor of cell death and that it is not the primary contributor to WS lesion formation. This indicates that *P. stewartii* relies on finely tuned, spatio-temporally defined and sequential processes that are necessary for successful infection of the susceptible sweet corn host, in which initial deployment of WtsE dampens host defense responses to allow the infection process to proceed such that RTX2 can then serve as a cell lysis factor.

We also tested a role of RTX2 as an adhesin, based on the presence of large repetitive adhesin motifs homologous to hemagglutinins as well as to the BAP subfamily of RTX proteins that have been implicated in promoting bacterial-host adhesion during biofilm formation (Gottig et al. 2009; Guilhabert and Kirkpatrick 2005; Loch et al. 2001; Voegel et al. 2010). However, adhesion assays conducted on an abiotic PVC surface or an artificial cellulose substrate (cellophane) showed no difference in adhesion between $\Delta rtx2$ and WT *P. stewartii* (data not shown), indicating that RTX2 does not play an obvious role in adhesion to these surfaces. However, this finding does not rule out a potential adhesin function to biologically relevant substrates, such as other carbohydrates found in the

plant cell wall or chitin in the insect vector. In fact, the presence of an adhesion-related domain (PKD) suggests that these proteins may be involved in adhesion to carbohydrate surfaces, which the pathogen would encounter, particularly in the xylem of the host, in which the bacteria develop robust biofilms. RTX2 could play a cryptic role in adhesion, much like the close ortholog YeeJ in *E. coli* (Roux et al. 2005), that would not be apparent in the crystal violet adhesion experiment.

In this study, we have identified and characterized RTX2, a large repetitive protein that shares similarities in domain structure and sequence homology to proteins belonging to the family of RTX proteins. Most importantly, we showed that the RTX2 protein is a major virulence factor for *P. stewartii* during the WS lesion phase of infection. While the full-length RTX2 protein shares similarities with previously described RTX toxins, it cannot be categorized into a specific subfamily of RTX proteins. The RTX family of proteins is an expanding class of proteins and *P. stewartii* RTX2 may represent a new type of RTX-like toxin important in plant pathogenesis.

MATERIALS AND METHODS

Strains and growth conditions.

All relevant strains are listed in Table 1. The *P. stewartii* strains were grown on nutrient agar (Difco Laboratories, Detroit) and Luria-Bertani (LB) broth (Difco Laboratories) at 28°C in the presence of nalidixic acid (30 µg/ml), kanamycin (30 µg/ml), or ampicillin (100 µg/ml), when needed. *E. coli* strains were grown at 37°C in LB in the presence of kanamycin (30 µg/ml) or ampicillin (100 µg/ml), as needed. *E. coli* DH10B was used as a cloning host and S17-1 served as a donor for conjugal transfer of R6K-based plasmid constructs into *P. stewartii*.

Standard DNA manipulations.

Genomic DNA was extracted with a MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, WI, U.S.A.). Plasmid DNA was purified with a QIAprep spin miniprep kit

Fig. 4. Leaf colonization assay. The whorls of 7-day-old sweet corn seedlings (var. Jubilee) were inoculated with 100 µl of a 10^5 CFU/ml inoculum of either wild-type (WT) DC283, $\Delta rtx2$, or sterile phosphate-buffered saline, pH 7.4, with 0.2% Tween 20 buffer (1× PBST). At 6 days post-inoculation, leaf tissue was harvested, surface sterilized, and ground in sterile 1× PBS. The tissue grindate was serially diluted and plated onto nutrient agar containing 30 µg/ml nalidixic acid. Colonies were enumerated and normalized to tissue wet weight. The data indicate that, despite its nonpathogenic phenotype, the $\Delta rtx2$ mutant colonizes the plant tissue to high levels, albeit at a significantly lower level than WT DC283. The decrease in colonization for strain $\Delta rtx2$ was fully restored with a WT copy of *rtx2* carried on plasmid pMCR29. No bacteria were recovered from plants inoculated with the 1× PBST buffer control. The data here represent the mean of at least 15 replicates for each strain (three biological replicates with five technical replicates each) and were analyzed using a Dunnett multiple comparison test and letters indicate treatments that are significantly different from one another $P \leq 0.05$.

Fig. 5. RTX2 mediates release of proteins into the leaf apoplast. The Bradford assay was used to determine total protein concentration in the filtered apoplastic fluid extracted from sweet corn seedlings inoculated with either wild-type (WT) DC283, $\Delta rtx2$, $\Delta rtx2/rtx2+$, or sterile phosphate-buffered saline, pH 7.4, with 0.2% Tween 20 buffer (1× PBST), using the whorl inoculation method that allows for robust colonization of the leaf apoplastic spaces and not the xylem. Seedlings inoculated with WT DC283 or the $\Delta rtx2/rtx2+$ complemented strain had significantly elevated levels of normalized total protein concentration when compared with $\Delta rtx2$ or 1× PBST-inoculated plants. Error bars represent the standard error from the mean. Letters indicate significant differences in the total protein concentration among the different treatments. The differences were statistically significant based on a Mann-Whitney test at the 0.05 level.

(Qiagen, Valencia, CA, U.S.A.). DNA fragments were amplified using Takara Ex Taq DNA polymerase (Takara Bio USA, Madison, WI, U.S.A.). Synthetic oligonucleotide primers were ordered from Integrated DNA Technologies (Coralville, IN, U.S.A.). All restriction enzymes were purchased from New England Biolabs (Ipswich, MA, U.S.A.) or Invitrogen, Inc. (Carlsbad, CA, U.S.A.).

Construction of the $\Delta rtx2$ mutant.

The $\Delta rtx2$ mutant was constructed in the *P. stewartii* strain (DC283) using a modified Gateway (Invitrogen)-based technique adapted from Choi and Schweizer (2005). In three separate reactions, polymerase chain reaction (PCR) primer pairs *rtx2B*-UpF-GW and *rtx2B*-UpR-Kan2, *rtx2B*-DnF-Kan2 and *rtx2revDn*-GW, and *pKD4 fwd2* and *pKD4rev2* were designed to amplify the 5' and 3' flanking regions of the target gene and the kanamycin cassette (from *pKD4*), respectively. The entire open reading frame of *rtx2* was deleted. All relevant primer sequences can be found in Table 2. The three PCR products were assembled by PCR overlap extension. The assembled PCR product was recombined into the pDONR/Zeo vector (Invitrogen) to create plasmid pMCR31, which was also linearized with *AatII* and was recombined with pAUC40 to create plasmid pMCR32. Plasmid pMCR32 was introduced into *P. stewartii* (DC283) by conjugation, and transconjugants were selected on LB medium containing kanamycin at 30 $\mu\text{g}/\text{ml}$. The merodiploid state was resolved by sucrose counterselection and antibiotic selection. Double crossover events were confirmed using PCR with primers designed to anneal to the flanking regions and the kanamycin resistance (Kan^R) marker. The Kan^R cassette was subsequently excised by FLP recombinase carried on plasmid pFLP2, leaving an 85-bp *frr* scar to create the unmarked mutant strain CR54 ($\Delta rtx2$). The pFLP2 plasmid was cured by sucrose selection to create an unmarked, nonpolar deletion mutant. The native promoter region was left intact.

Fig. 6. RTX2 mediates leakage of solutes into the leaf apoplastic space. Water potential (ψ_w) was used to determine the solute concentration in the filtered apoplastic fluid from sweet corn seedlings inoculated with wild-type (WT) DC283, $\Delta rtx2$, $\Delta rtx2/rtx2+$, or sterile phosphate-buffered saline, pH 7.4, with 0.2% Tween 20 buffer (1x PBST), following the whorl inoculation method. Seedlings inoculated with WT DC283 or $\Delta rtx2/rtx2+$ had significantly lower ψ_w , indicating an increase in dissolved solutes compared with $\Delta rtx2$ or the 1x PBST. Error bars represent the standard error from the mean. Letters indicate significant differences in the ψ_w among treatments. The differences were statistically significant based on a Student's *t* test using $P \leq 0.05$.

For the complementation strain, the entire coding region of *rtx2* (including the ribosomal binding site) was amplified by PCR, using the primer pairs listed in Table 2. The resulting PCR products were digested with the appropriate restriction enzymes and were ligated into the pBBR1-MCS4 vector (Kovach et al. 1995) to create plasmid pMCR29 (pBBR1::rtx2). This plasmid was electroporated into *E. coli* S17-1 and was introduced by conjugal transfer into the $\Delta rtx2$ mutant to create strain CR64. We took care to design the mutagenesis scheme to create a nonpolar mutation. Similarly, all phenotypes tested in this study were fully restored by the corresponding wild-type *rtx2* gene, confirming the transcriptional or translational integrity of this mutation. Growth curves for all mutants and complemented strains did not show a significant growth defect in minimal AB medium supplemented with 0.2% glucose as compared with WT DC283, based on analysis using a Student's *t* test (P value = 0.05) (data not shown). All primer sequences are listed in Table 2.

Virulence assays on sweet corn seedlings.

Whorl inoculation. Sweet corn (var. Jubilee) seedlings were grown in a controlled environmental growth chamber at 28°C day and 25°C night temperatures, 80% humidity, a 12-h light and 12-h dark cycle. The whorls of 7-day-old sweet corn (var. Jubilee) seedlings were inoculated with 100 μl of a 10^5 CFU/ml suspension of either the wild type *P. stewartii*, $\Delta rtx2$, the corresponding ($\Delta rtx2/rtx2+$) complemented strain, or 1x PBS buffer to assess water-soaking ability (Coplin and Cook 1990). For the coinoculation of $\Delta wtsE$ and $\Delta rtx2$, a cell suspension was made by mixing the two strains (1:1 ratio) to a final concentration of 10^5 CFU/ml, and 100 μl of the mixed suspension was used for inoculation. At 6 days postinoculation, seedlings were rated for disease severity on a scale of 0 to 4: 0 = no symptoms, 1 = a few lesions but no ooze, 2 = many lesions and some ooze, 3 = confluent lesions and ooze, and 4 = leaf necrosis and collapse. Each strain was inoculated into five individual plants and each experiment was repeated at least three times.

Fig. 7. Coinoculation of $\Delta rtx2$ and $\Delta wtsE$ restores full virulence. Sweet corn seedlings inoculated via the whorl inoculation method with either $\Delta rtx2$ or $\Delta wtsE$ did not develop significant water-soaked (WS) lesions and were statistically similar to the sterile phosphate-buffered saline, pH 7.4, with 0.2% Tween 20 buffer (1x PBST) treated plants. However, when the two strains were coinoculated, these plants developed lesions similar to wild-type DC283-inoculated plants. Plants inoculated with 1x PBST did not develop WS lesions. Letters indicate significant differences among treatments.

Lesion length. Seven-day-old sweet corn plants were inoculated as described above. Measurements of the longest lesion on the first fully expanded leaf were taken 6 days postinoculation. Each strain was inoculated into five individual plants and each experiment was repeated at least three times.

Scratch Inoculation. Sweet corn seedlings were wound inoculated as previously described (Herrera et al. 2008). Cells were grown overnight in AB minimal medium, 0.2% glucose (Clark and Maaloe 1967), with shaking at 180 rpm at 28°C and were subcultured and allowed to grow to an optical density at 600 nm (OD_{600}) = 0.4. Bacteria was then harvested by centrifugation, and the pellet was washed twice with 1× PBST and was then resuspended to OD_{600} = 0.1. This suspension (5 µl), containing approximately 5×10^7 cells per milliliter in 1× PBST, was then placed over a wound created by scratching the stem of a 10-day-old sweet corn seedling with a sterile 20-gauge syringe needle. Plants were observed for development of wilting symptoms after 7 days postinoculation, as previously described (Burbank and Roper 2014). In brief, the plants were rated on an arbitrary scale of 0 = no symptoms, 1 = a few lesions, 2 = spreading lesions that coalesce, 3 = severe lesions plus some wilt, and 4 = severe wilting and death of the plant. The results are shown as the mean disease score of all replicates. Each strain or control was inoculated into at least five individual plants. The complete experiment was repeated independently at least three times. Positive control plants were infected with WT DC283, while seedlings inoculated with sterile 1× PBST buffer only served as negative controls.

Plant colonization.

The whorls of 7-day-old sweet corn plants were inoculated using the whorl inoculation technique. Each strain was inoculated into five individual plants and each experiment was repeated at least three times. The oldest leaf was harvested 6 days postinoculation and was surface-sterilized by submerging in 20% bleach for 30 s and 95% ethanol for 30 s, and was washed twice for 30 s each in sterile distilled H₂O. The sterile excised leaves were then placed in a mesh grinding bag (Agdia, Inc., Elkhart, IN, U.S.A.) with 2 ml of sterile 1× PBS buffer and were ground with a hammer. Grindate (1 ml) was removed and serially diluted, and 100 µl of each dilution was plated onto nutrient agar containing 30 µg of nalidixic acid per

milliliter or nutrient agar containing nalidixic acid (30 µg/ml) and ampicillin (100 µg/ml).

Apoplastic fluid extraction.

Plants were inoculated as described above. The first fully expanded leaf was harvested from each inoculated plant 4 days postinoculation. The wet weight for each leaf tissue was obtained. Following this, the leaves were surface-sterilized using the following procedure. Leaves were soaked in 70% ethanol followed by 10% bleach and were rinsed twice in sterile distilled H₂O (30 s per solution). The apoplastic fluid was then extracted from the leaf by centrifugation, using a modified protocol from Joosten (2012). Briefly, a 1-ml filter tip (USA Scientific, Orlando, FL, U.S.A.) was cut and the leaf was rolled gently and inserted into the top portion of the tip and was placed into a microcentrifuge tube. Samples were centrifuged at $5,510 \times g$ for 1 to 2 min to collect apoplastic fluid. The leaf was removed from the tube and the apoplastic fluid was filtered using Ultrafree- MC-VV centrifugal 0.1-µm filters (EMD Millipore, Darmstadt, Germany) to remove bacterial cells.

Quantification of total protein in leaf apoplastic fluid.

The total protein concentration was determined by using the Quick start Bradford assay (Bio-Rad, Hercules, CA, U.S.A.). Protein content was quantified in 20 µl of filtered apoplastic fluid, using a bovine serum albumin standard curve. This value was then converted to micrograms per milliliter of apoplastic fluid and was normalized by an A-B scale. The A-B normalization equation used was $y = 1 + (x - A)(b - a)/(B - A)$, where A is the minimum value of the samples, B is the maximum of the samples, a is 1, b is 10, and x is the data point.

Measurements of water potential in leaf apoplastic fluid.

Water potential (ψ_w) was used to provide a measure of the concentration of dissolved solutes in the filtered leaf apoplastic fluid. ψ_w was quantified using a Psypro water-potential data-logger fitted with the PCT-55/PST-55 soil hygrometer/psychrometer (Eli Tech Group WESCOR, Logan, UT, U.S.A.) probe. A small paper filter was placed in the collection chamber and 10 µl of the apoplastic fluid was applied to the paper filter. The probe was then placed in the chamber and water potential values were collected every hour for 20 h. The water potential values were calculated based on a sodium chloride standard curve.

Table 2. List of primer sequences used in this study

Primer	Primer sequence ^a	Source
pKD4 fwd2	TAGGCTGGAGCTGCTTCGAAGTTC	Amplifies kanamycin cassette from pKD4
pKD4rev2	CATATGAATATCCTCCTTAGTTCCTATTCCG	Amplifies kanamycin cassette from pKD4
rtx2B-UpF-GW	TACAAAAAGCAGGCTAATGCGCTGGAGGCCAGCCAGG	Mutagenesis construct
rtx2B-UpR-Kan2	GAAGCTTCAAGCAGCTCCAGCCTATTGCCAGCCTGATCAACCACA	Mutagenesis construct
rtx2B-DnF-kan2	CGGAATAGGAAGTAAGGAGGATATTCATATGCGCAACGTTGCGCGATAAATC	Mutagenesis construct
pKD13fwd	CGATTGTGTAGGCTGGAGCTGCTTC	Amplifies kanamycin cassette from pKD13
pKD13rev	AATTAATTCGGGGATCCGTCGACC	Amplifies kanamycin cassette from pKD13
GW-attB1	GGGGACAAGTTTGTACAAAAAGCAGGCT	Amplifies polymerase chain reaction (PCR) overlap extension products, contains attB sites
GW-attB2	GGGGACCACTTTGTACAAGAAAGCTGGGT	Amplifies PCR overlap extension products, contains attB sites
rtx2fwdchk	ATTGCAACGTCCAGCATCCCTAATC	Verification of gene replacement
rtx2revchk	CGTACACCAACAGGCCACAGGTAA	Verification of gene replacement
rtx2BXhoI fwd	TCGGTACTCGAGGCCAGCGATCAGGTAC	Complementation primer
rtx2XbaI rev	ATGCGATCTAGATTATCGCGCAACGTTG	Complementation primer

^a All primer sequences are shown 5' to 3'.

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LITERATURE CITED

- Alouf, J. E. 2001. Pore-forming bacterial protein toxins: An overview. *Curr. Top. Microbiol. Immunol.* 257:1-14.
- Beck von Bodman, S., and Farrand, S. K. 1995. Capsular polysaccharide biosynthesis and pathogenicity in *Erwinia stewartii* require induction by an N-acylhomoserine lactone autoinducer. *J. Bacteriol.* 177:5000-5008.
- Benz, R., Hardie, K. R., and Hughes, C. 1994. Pore formation in artificial membranes by the secreted hemolysins of *Proteus vulgaris* and *Morganella morganii*. *Eur. J. Biochem.* 220:339-347.
- Burbank, L., and Roper, M. C. 2014. OxyR and SoxR modulate the inducible oxidative stress response and are implicated during different stages of infection for the bacterial phytopathogen *Pantoea stewartii* subsp. *stewartii*. *Mol. Plant-Microbe Interact.* 27:479-490.
- Bycroft, M., Bateman, A., Clarke, J., Hamill, S. J., Sandford, R., Thomas, R. L., and Chothia, C. 1999. The structure of a PKD domain from polycystin-1: Implications for polycystic kidney disease. *EMBO J.* 18:297-305.
- Carlier, A., Burbank, L., and von Bodman, S. B. 2009. Identification and characterization of three novel EsaI/EsaR quorum-sensing controlled stewartan exopolysaccharide biosynthetic genes in *Pantoea stewartii* ssp. *stewartii*. *Mol. Microbiol.* 74:903-913.
- Choi, K. H., and Schweizer, H. P. 2005. An improved method for rapid generation of unmarked *Pseudomonas aeruginosa* deletion mutants. *BMC Microbiol.* 5:30.
- Clafflin, L. E. 2000. Stewart's bacterial wilt, 3rd ed. The American Phytopathological Society, St. Paul, MN, U.S.A.
- Clark, J. D., and Maaloe, O. 1967. DNA replication and the cell division cycle in *Escherichia coli*. *J. Mol. Biol.* 23:99-112.
- Coplin, D. L., and Cook, D. 1990. Molecular genetics of extracellular polysaccharide biosynthesis in vascular phytopathogenic bacteria. *Mol. Plant-Microbe Interact.* 3:271-279.
- Coplin, D. L., Frederick, R. D., Majerczak, D. R., and Haas, E. S. 1986. Molecular cloning of virulence genes from *Erwinia stewartii*. *J. Bacteriol.* 168:619-623.
- Datsenko, K. A., and Wanner, B. L. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc. Natl. Acad. Sci. U.S.A.* 97:6640-6645.
- Dolph, P. J., Majerczak, D. R., and Coplin, D. L. 1988. Characterization of a gene cluster for exopolysaccharide biosynthesis and virulence in *Erwinia stewartii*. *J. Bacteriol.* 170:865-871.
- Frey, J. 2006. Genetics and phylogeny of RTX cytotoxins. Pages 570-577 in: *The Comprehensive Sourcebook of Bacterial Protein Toxins*, 3rd Ed. Elsevier Press. Amsterdam.
- Gentschev, I. G. W. 2003. Type I secretion protein secretion systems in gram-negative bacteria: *Escherichia coli* α -hemolysin secretion. Pages 121-139 in: *Protein Secretion Pathways in Bacteria*. B. Oudega, ed. Kluwer Academic Publishers, Boston.
- Gilbert, R. J. C. 2002. Pore-forming toxins. *Cell. Mol. Life Sci.* 59:832-844.
- Glasner, J. D., Rusch, M., Liss, P., Plunkett, G., 3rd, Cabot, E. L., Darling, A., Anderson, B. D., Infield-Harm, P., Gilson, M. C., and Perna, N. T. 2006. ASAP: A resource for annotating, curating, comparing, and disseminating genomic data. *Nucleic Acids Res.* 34:D41-D45.
- Gottig, N., Garavaglia, B. S., Garofalo, C. G., Orellano, E. G., and Ottado, J. 2009. A filamentous hemagglutinin-like protein of *Xanthomonas axonopodis* pv. *citri*, the phytopathogen responsible for citrus canker, is involved in bacterial virulence. *PLoS One* 4:e4358.
- Guilhabert, M. R., and Kirkpatrick, B. C. 2005. Identification of *Xylella fastidiosa* antivirulence genes: Hemagglutinin adhesins contribute a biofilm maturation to *X. fastidiosa* and colonization and attenuate virulence. *Mol. Plant-Microbe Interact.* 18:856-868.
- Ham, J. H., Majerczak, D., Ewert, S., Sreerekha, M. V., Mackey, D., and Coplin, D. 2008. WtsE, an AvrE-family type III effector protein of *Pantoea stewartii* subsp. *stewartii*, causes cell death in non-host plants. *Mol. Plant Pathol.* 9:633-643.
- Ham, J. H., Majerczak, D. R., Arroyo-Rodriguez, A. S., Mackey, D. M., and Coplin, D. L. 2006. WtsE, an AvrE-family effector protein from *Pantoea stewartii* subsp. *stewartii*, causes disease-associated cell death in corn and requires a chaperone protein for stability. *Mol. Plant-Microbe Interact.* 19:1092-1102.
- Ham, J. H., Majerczak, D. R., Nomura, K., Mecey, C., Uribe, F., He, S. Y., Mackey, D., and Coplin, D. L. 2009. Multiple activities of the plant pathogen type III effector proteins WtsE and AvrE require WxxxE motifs. *Mol. Plant-Microbe Interact.* 22:703-712.
- Heidelberg, J. F., Seshadri, R., Haveman, S. A., Hemme, C. L., Paulsen, I. T., Kolonay, J. F., Eisen, J. A., Ward, N., Methe, B., Brinkac, L. M., Daugherty, S. C., Deboy, R. T., Dodson, R. J., Durkin, A. S., Madupu, R., Nelson, W. C., Sullivan, S. A., Fouts, D., Haft, D. H., Selengut, J., Peterson, J. D., Davidsen, T. M., Zafar, N., Zhou, L., Radune, D., Dimitrov, G., Hance, M., Tran, K., Khouri, H., Gill, J., Utterback, T. R., Feldblyum, T. V., Wall, J. D., Voordouw, G., and Fraser, C. M. 2004. The genome sequence of the anaerobic, sulfate-reducing bacterium *Desulfovibrio vulgaris* Hildenborough. *Nat. Biotechnol.* 22:554-559.
- Herrera, C. M., Koutsoudis, M. D., Wang, X., and von Bodman, S. B. 2008. *Pantoea stewartii* subsp. *stewartii* exhibits surface motility, which is a critical aspect of Stewart's wilt disease development on maize. *Mol. Plant-Microbe Interact.* 21:1359-1370.
- Hertle, R. 2000. Serratia type pore forming toxins. *Curr. Protein Pept. Sci.* 1:75-89.
- Hinsa, S. M., Espinosa-Urgel, M., Ramos, J. L., and O'Toole, G. A. 2003. Transition from reversible to irreversible attachment during biofilm formation by *Pseudomonas fluorescens* WCS365 requires an ABC transporter and a large secreted protein. *Mol. Microbiol.* 49:905-918.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J., and Schweizer, H. P. 1998. A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally located DNA sequences: Application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* 212:77-86.
- Joosten, M. H. 2012. Isolation of apoplastic fluid from leaf tissue by the vacuum infiltration-centrifugation technique. *Method. Microbiol.* 835: 603-610.
- Koutsoudis, M. D., D.Tsaltas, T. D.Minogue, and S. B.von Bodman. 2006. Quorum-sensing regulation governs bacterial adhesion, biofilm development, and host colonization in *Pantoea stewartii* subspecies *stewartii*. *Proc. Natl. Acad. Sci. U.S.A.* 103:5983-5988.
- Kovach, M. E., Elzer, P. H., Hill, D. S., Robertson, G. T., Farris, M. A., Roop, R. M., 2nd, and Peterson, K. M. 1995. Four new derivatives of the broad-host-range cloning vector pBBR1MCS, carrying different antibiotic-resistance cassettes. *Gene* 166:175-176.
- Kube, M., Migdoll, A. M., Gehring, I., Heitmann, K., Mayer, Y., Kuhl, H., Knaust, F., Geider, K., and Reinhardt, R. 2010. Genome comparison of the epiphytic bacteria *Erwinia billingiae* and *E. tasmaniensis* with the pear pathogen *E. pyrifoliae*. *BMC Genomics* 11:393.
- Lally, E. T., Hill, R. B., Kieba, I. R., and Korostoff, J. 1999. The interaction between RTX toxins and target cells. *Trends Microbiol.* 7:356-361.
- Leigh, J. A., and Coplin, D. L. 1992. Exopolysaccharides in plant-bacterial interactions. *Annu. Rev. Microbiol.* 46:307-346.
- Lo, H. R., Lin, J. H., Chen, Y. H., Chen, C. L., Shao, C. P., Lai, Y. C., and Hor, L. I. 2011. RTX toxin enhances the survival of *Vibrio vulnificus* during infection by protecting the organism from phagocytosis. *J. Infect. Dis.* 203:1866-1874.
- Locht, C., Antoine, R., and Jacob-Dubuisson, F. 2001. *Bordetella pertussis*, molecular pathogenesis under multiple aspects. *Curr. Opin. Microbiol.* 4:82-89.
- Loehfelm, T. W., Luke, N. R., and Campagnari, A. A. 2008. Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. *J. Bacteriol.* 190:1036-1044.
- Marre, R., Hacker, J., and Braun, V. 1989. The cell-bound hemolysin of *Serratia marcescens* contributes to uropathogenicity. *Microb. Pathog.* 7:153-156.
- Mohammadi, M., Burbank, L., and Roper, M. C. 2012. *Pantoea stewartii* subsp. *stewartii* produces an endoglucanase that is required for full virulence in sweet corn. *Mol. Plant-Microbe Interact.* 25:463-470.
- Pataky, J. K. 2003. Stewart's wilt of corn. *APSnet Features*. Online. doi: 10.1094/APSnetFeature-2003-0703
- Roux, A., Beloin, C., and Ghigo, J. M. 2005. Combined inactivation and expression strategy to study gene function under physiological conditions: Application to identification of new *Escherichia coli* adhesins. *J. Bacteriol.* 187:1001-1013.

- Sasaki, H., Ishikawa, H., Sato, T., Sekiguchi, S., Amao, H., Kawamoto, E., Matsumoto, T., and Shirama, K. 2011. Molecular and virulence characteristics of an outer membrane-associated RTX exoprotein in *Pasteurella pneumotropica*. *BMC Microbiol.* 11:55.
- Satchell, K. J. 2011. Structure and function of MARTX toxins and other large repetitive RTX proteins. *Annu. Rev. Microbiol.* 65:71-90.
- Simon, R., Priefer, U., and Pühler, A. 1982. A broad host range mobilization system for in vivo genetics engineering: Transposon mutagenesis in gram-negative bacteria. *Biotechnology* 1:784-769.
- Tao, K., Long, Z., Liu, K., Tao, Y., and Liu, S. 2006. Purification and properties of a novel insecticidal protein from the locust pathogen *Serratia marcescens* HR-3. *Curr. Microbiol.* 52:45-49.
- Voegel, T. M., Warren, J. G., Matsumoto, A., Igo, M. M., and Kirkpatrick, B. C. 2010. Localization and characterization of *Xylella fastidiosa* haemagglutinin adhesins. *Microbiology* 156: 2172-2179.
- von Bodman, S. B., Majerczak, D. R., and Coplin, D. L. 1998. A negative regulator mediates quorum-sensing control of exopolysaccharide production in *Pantoea stewartii* subsp. *stewartii*. *Proc. Natl. Acad. Sci. U.S.A.* 95:7687-7692.
- Welch, R. A. 1991. Pore-forming cytolysins of gram-negative bacteria. *Mol. Microbiol.* 5:521-528.
- Welch, R. A. 2001. RTX toxin structure and function: A story of numerous anomalies and few analogies in toxin biology. Pates 85 to 107 in: *Pore Forming Toxins*. F. G. Van der Gout, ed. Springer Press, New York.
- Wood, G. E., Dutro, S. M., and Totten, P. A. 1999. Target cell range of *Haemophilus ducreyi* hemolysin and its involvement in invasion of human epithelial cells. *Infect. Immun.* 67:3740-3749.