A single genetic locus in the phytopathogen *Pantoea stewartii* enables gut colonization and pathogenicity in an insect host

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**Summary**

Aphids are typically exposed to a variety of epiphytic and phytopathogenic bacteria, many of which have entomopathogenic potential. Here we describe the interaction between *Pantoea stewartii* ssp. *stewartii* DC283 (DC283), an enteric phytopathogen and causal agent of Stewart’s wilt, and the pea aphid, *Acyrthosiphon pisum*. When ingested by aphids, DC283 establishes and aggregates in the crop and gut, preventing honeydew flow and excretion, resulting in aphid death in 72 h. A mutagenesis screen identified a single locus, termed ucp1 (you cannot pass), whose disruption abolishes aphid pathogenicity. Moreover, the expression of ucp1 in *Escherichia coli* is sufficient to mediate the hindgut aggregation phenotype by this normally avirulent species. Ucp1 is related to six other proteins in the DC283 genome, each having a common N-terminal region and a divergent C-terminus, but only ucp1 has a role in pathogenicity. Based on predicted motifs and secondary structure, Ucp1 is a membrane-bound protein that functions in bacterial adhesion and promotes the formation of aggregates that are lethal to the insect host. These results illustrate that the enteric plant pathogenic bacteria have the capacity to exploit alternative non-plant hosts, and retain genetic determinants for colonizing the gut.

**Introduction**

The plant-dependent lifestyle of many insects predisposes them to frequent encounters with epiphytic and plant pathogenic bacteria. For the agriculturally and economically important aphid, many bacterial plant pathogens have been identified as constituents of its gut microbiome (Harada et al., 1996). These associations are of ecological consequence in that several phytopathogens, including *Erwinia aphidicola* (Harada and Ishikawa, 1997; Harada et al., 1997; Santos et al., 2009) *Dickeya dadantii* (Grenier et al., 2006) and *Pseudomonas syringae* pv. *syringae* B728a (Stavrinides et al., 2009), can exploit the aphid as an alternative host, suggesting that these bacteria retain genetic determinants for insect colonization in addition to their regular complement of plant pathogenicity factors.

Little is known about the nature and specificity of the genetic determinants that mediate the interaction between bacterial pathogen and aphid. Exploration of the *D. dadantii*-pea aphid interaction implicated several bacterial Cyt-like toxin genes in aphid pathogenesis (Grenier et al., 2006), and although the Cyt gene homologues of *Bacillus thuringiensis* confer measurable insecticidal activity (Bravo et al., 2007), the *D. dadantii* Cyt homologues are not wholly responsible for its pathogenic potential. In fact, disruption of the cytA-D in *D. dadantii* only slightly attenuates virulence, indicating the requirement of additional virulence factors. In contrast, a *fliL* mutant of the phytopathogen *Pseudomonas syringae* was rendered avirulent towards the pea aphid, suggesting a virulence mechanism linked to bacterial motility (Stavrinides et al., 2009). Other insect-specific virulence factors are likely present in the genome of *P. syringae* (Buell et al., 2003; Feil et al., 2005; Lindeberg et al., 2008), but the identity of these genes is still unknown.

Several studies have implicated members of RHS/YD repeat family in insect–pathogen interactions (Lin et al., 1984; Sadosky et al., 1989; Zhao et al., 1993; Hill et al., 1994; Zhao and Hill, 1995; Minet et al., 1999; Vallet-Gely et al., 2008). The rhs (rearrangement hotspot) genes were first described in *Escherichia coli* and characterized as mosaics whose common core mediates chromosomal rearrangements through homologous exchange (Lin et al., 1984). The RHS family in *E. coli* consists of eight proteins classified into three subfamilies, all having a highly conserved 3.7 kb N-terminal domain but variable C-termini (Wang et al., 1998). There is some suggestion
that these E. coli proteins associate with the bacterial cell surface and bind specific ligands (Wang et al., 1998; Hill, 1999), but this has not been demonstrated experimentally leaving their specific function unknown. RHS/YD repeat proteins of several bacteria have been shown to mediate pathogen–insect interactions, such as the Tcc proteins of Photorhabdus luminescens, which have been implicated in toxicity to insect hosts (Waterfield et al., 2001), and the Sep proteins of Serratia entomophila, which are responsible for the development of amber disease in the grass grub Costelytra zealandica (Hurst et al., 2000). More recently, the RHS-like toxin encoded on the APSE phage in the aphid endosymbiont Candidatus Hamiltonella defensa has been suggested to antagonize development of parasitoid wasp ova and larvae growing within the aphid (Degnan and Moran, 2008). Ultimately, the role and importance of RHS genes in host–bacterial interactions remain unclear.

In this study, we examined the interaction between the enteric Stewart’s wilt pathogen Pantoea stewartii ssp. stewartii DC283 (DC283) (Coplin et al., 1986) and the pea aphid, Acyrthosiphon pismum. Previous work identified two Pantoea species that are aggressive pathogens of aphids (Harada and Ishikawa, 1997; Grenier et al., 2006), and due to the association of P. stewartii with an insect vector, the corn flea beetle (Chaetocnema pulicaria), and its ability to establish in the plant vasculature during plant pathogenesis (Leigh and Coplin, 1992; von Bodman et al., 2003), we hypothesized that our pathosystem may have potentially relevant ecological implications. A genetic screen in aphids designed to identify hypovirulent DC283 mutants recovered an rhs-like gene that enables bacteria to form aggregates in the gut of the pea aphid, ultimately reducing honeydew excretion and causing death. Disruption of this gene in DC283 results in complete abolition of aphid pathogenicity, and transfer of the gene to E. coli is sufficient to convert this normally avirulent strain into a pathogen.

Results

The ucp1 mutant of DC283 is avirulent

Virulence assays that tested the oral toxicity of DC283 towards the aphid revealed that DC283 is an aggressive pathogen, reaching titres of 5 × 10⁶ cfu and killing the aphid within 72 h (Fig. 1). Transposon mutagenesis of DC283 yielded a hypovirulent mutant that was disrupted in a sequence resembling an rhs gene, which we called ucp1 (you cannot pass). Growth of the mutant line, DC283 (ucp1::Tn5), in the aphid was substantially lower than wild-type, reaching titres of only 1 × 10³ cfu/aphid by 96 h. Survival of aphids fed the mutant line DC283 (ucp1::Tn5) was the same as that for the no-bacteria control.

Functional features of Ucp

Ucp contains a predicted kinase domain, similar to those in the phosphofructokinase B family (PS00583), as well as three transmembrane (TM) domains. Despite its current annotation as a member of the RHS family of proteins (COG3209), it does not possess the 22-residue repeat containing the YD motif that characterizes this family, but can instead be placed in the DUF808 protein family (domain of unknown function). Similarity searches of ucp1-like genes in the DC283 genome yielded six potential homologues, ranging from 1200 to 1300 bp in length, which we have named ulg1–6 (ucp1-like gene). The seven genes are similar at their 5’ ends (i.e. the initial 663 bp) but differ from one another in that they have unique 3’ regions. A particularly striking feature was the variation in GC contents across each of the seven genes: the conserved N-terminus has a GC content of 64% while the 3’ end averages only 42% GC (Fig. 2). A similar chimeric pattern of compositional variation is apparent in all members of this gene family. Like Ucp, each of the Ulg proteins contains a positionally conserved predicted kinase domain, but they are variable in the number and location of their predicted TM domains. Most have TM regions between amino acids 137–159 (TM1), 165–185 (TM2) and 206–225 (TM3), except for Ulg1, which has an additional predicted TM domain (TM0) between amino acids 98–118, and whose TM2 lies between amino acids 174–194. Ulg3 lacks TM3, and Ulg4 has a predicted F-box in its C-terminus, a domain with potential role in protein–protein interactions.
The ucp1-like genes are clustered in the DC283 genome, with ucp1 and ulg1 being arranged convergently and separated by two genes commonly associated with mobile elements (Fig. 3). Disruption of ucp1 is therefore less likely to have polar effects. ulg2, ulg3 and ulg4 are organized co-linearly, with the latter two ORFs overlapping and possibly co-transcribed from a single regulatory promoter. Upstream of these three genes is a putative nematicidal gene belonging to the RHS/YD repeat family. ulg5 and ulg6 are adjacent but separated by 625 bp, suggesting that they are controlled by distinct promoters.

The majority of the ucp1 coding sequence is contained within genes annotated as RHS-like, nematicidal, or hypothetical in the enteric bacteria Yersinia intermedia (ZP_00832839), Erwinia tasmaniensis (YP_001906495, YP_001908938), Hamiltonella defensa (ACJ10121), Providencia stuartii (ZP_02960661) and Xenorhabdus bovienii (CAC19493). These genes are usually much longer than ucp1, and their products often bear characteristics of the RHS/YD repeat family of proteins. Similarly, ulg1 is similar over its entire length to the C-terminus of the Erwinia tasmaniensis nematicidal gene 1 (E = 1e – 79, YP_001908938), whereas both ulg3 and ulg6 show full-length similarity to the C-terminus of the Xenorhabdus bovienii nematicidal gene 2 (E < 8e – 54, CAC19493).

ucp1 is sufficient for aphid pathogenicity

To determine whether ucp1 was sufficient for entomopathogenicity, aphids were allowed to feed on a recombinant strain of E. coli that constitutively expressed ucp1. Identical to the observed effect of DC283 on aphids, the E. coli expressing ucp1 was rendered toxic to aphids, reaching bacterial titres of 10⁶ cfu/aphid and resulting in death by 72 h (Fig. 1). In contrast, E. coli vector control (pET-21b) surpassed 10⁴ cfu, with aphids surviving beyond 96 h. To determine the pathogenic potential of each of the ulg alleles, we tested the toxicity of E. coli expressing each of these genes in similar lethality assays. In each case, these lines behaved like the vector control and were avirulent to aphid hosts. We also assayed the pathogenicity of these constructs in the monocot aphids Sitobion avenae and Schizaphis graminum, and as with the pea aphid, E. coli-expressing ucp1 are lethal to both species, with death occurring in 3 days.

Given the chimeric nature of ucp1 and the ulg family, and the fact that expression of only ucp1 is lethal to aphids, we attempted to determine if the C-terminus alone was responsible for Ucp1 functionality. Aphids feeding on E. coli expressing different portions of ucp1 (Fig. 2) survived beyond those aphids feeding on E. coli (ucp1), indicating that none of the regions tested was sufficient for entomopathogenicity.

Pathology and Ucp1 mode of action

By observing aphids during the pathogenicity assays, it became apparent that honeydew production was abated drastically in aphids feeding on DC283 (Fig. S1). Although originally attributed to the cessation of feeding due to infection, aphids were immobile and tapped into their food source continually over the 72 h period, as if feeding actively during this time. Microscopic examination of the aphids after 48 h revealed a non-turgid body and a sunken abdomen characteristic of starved aphids. A similar pathology was observed for aphids feeding on E. coli expressing ucp1 (although honeydew production was not affected as dramatically). In contrast, DC283 (ucp1::Tn5)-fed aphids produced substantially more honeydew and remained fully engorged over the same 72 h period.

Microscopic examination of the gut from aphids feeding on DC283 revealed the formation of solid aggregates in the crop and hindgut (Fig. 4, 1A and 1B). Bacterial aggre...
gates began to form in the crop by 24 h, and by 48 h, our dissections revealed large spherical aggregates in the hindgut that were often suspended in a cloudy haze of bacteria. During dissection, aggregates often flowed out of the crop, moving through the foregut and midgut, and settling in the hindgut, indicating that they were not permanently bound to either the crop or gut epithelium. Such aggregates were also observed in the crop of aphids feeding on DC283 (ucp1::Tn5) (Fig. 2A) but were never present in the hindgut (Fig. 2B). In some preparations, the crop was completely filled with bacteria, which could be released by puncturing the crop (Fig. 5).

Because E. coli (ucp1) was also lethal to aphids, we looked for commonalities between the gut of aphids fed on E. coli (ucp1) with those fed DC283. Both produced bacterial aggregates in the crop and the hindgut, with many preparations being filled from crop to hindgut with an opaque tubular formation of bacteria (Fig. 4, 3A and 3B). Much like that of DC283 (ucp1::Tn5)-fed aphids, the gut of aphids fed the E. coli vector control contained few aggregates in the crop but none in the hindgut (Fig. 4, 4A and 4B). Aphids fed on diet without bacteria lacked aggregates in their crop and gut (data not shown).

**Discussion**

We identified a single gene, ucp1, responsible for DC283 pathogenicity in aphids. Disruption of ucp1 completely abolishes the virulence attributes of DC283, and the heterologous expression of this one gene is sufficient to render a non-pathogenic strain of E. coli virulent. The formation of prominent aggregates in the crop and hindgut of aphids fed ucp1-containing bacteria suggests a primary role for Ucp1 in bacterial adhesion and aggregation. This
activity is supported by the structural and functional features of \textit{ucp1} predicted from its sequence: Ucp1 contains several putative TM domains, indicating membrane localization and a possible role in substrate or matrix binding.

Based on its sequence similarity to portions of full-length RHS family members, \textit{ucp1} was originally believed to encode a putative RHS family protein; however, it is substantially smaller than most genes encoding RHS/YD repeat proteins and does not possess the ligand-binding YD repeat motif characteristic of this family (Lin \textit{et al.}, 1984; Sadosky \textit{et al.}, 1989; Minet \textit{et al.}, 1999). Still, Ucp1 and the Ulg relatives retain several structural and organizational features ascribed to the original RHS family of proteins from \textit{E. coli}, including mosaicism marked by conserved 5′-cores with divergent and seemingly non-homologous 3′ ends, and drastically different base compositions at their 5′ and 3′ ends (Lin \textit{et al.}, 1984; Sadosky \textit{et al.}, 1989; Zhao \textit{et al.}, 1993; Hill \textit{et al.}, 1994; Zhao and Hill, 1995; Hill, 1999). Although the functional implications of the disparate GC composition between 5′ and 3′ ends of \textit{ucp1} are unclear, the evolution of seven related proteins with non-homologous 3′ ends may be significant from the perspective of host–pathogen interactions.

Based on its predicted secondary structure, we propose that Ucp1 and its relatives are membrane bound, with the hypervariable C-terminus being exposed to the extracellular environment (Fig. 6). Our proposed model is complicated by the predicted N-terminal kinase domain, which would be localized to the periplasm instead of the cytoplasm (Fig. 6). Nonetheless, if our model is correct, \textit{ucp1} could function in manner analogous to the Microbial Surface Component Recognizing Adhesive Matrix Molecules (MSCRAMM) family of adhesin proteins used by animal pathogens, which bind to key proteinaceous components of the eukaryotic host cell to facilitate pathogenesis (Patti \textit{et al.}, 1994). The unique Ucp C-terminus may bind components of the aphid gut epithelial cells, and because only \textit{ucp1} can confer pathogenicity towards the aphid, the related Ulg proteins could function to bind alternative matrix molecules in different hosts. Under this scenario, the variability of the exposed C-terminus may be the result of selection imposed by host immunity (Korotkova \textit{et al.}, 2007). Similar patterns are seen in type III effectors of pathogenic bacteria, which are shuffled into new variants by the process of terminal re-assortment (Stavrinides \textit{et al.}, 2006; 2008; McCann and Guttman, 2008), and by the \textit{Neisseria gonorrhoeae} pilin gene, which is shuffled into new variants via recombination with inactive pilin gene variants in the genome (Seifert \textit{et al.}, 1988). Alternatively, the C-terminus of Ucp1 does not bind eukaryotic proteins, but rather other exposed Ucp C-termini of nearby cells, linking them together into bacterial matrices.

Fig. 6. Proposed model of Ucp structure and localization. Three transmembrane (TM) domains loop through the outer membrane of the Gram-negative cell, with the C-terminal hypervariable region exposed to the extracellular environment and the putative N-terminal kinase domain localized to the periplasm.
Because the RHS members share highly similar N-terminal regions, the distinct phenotype conferred by Ucp1 is specified by its C-terminus; however, the C-terminus alone does not advance aphid lethality and the aggregation phenotype, as it was insufficient for protein functionality (Fig. 2). Even fragments that incorporated all three TM domains and the predicted kinase domain did not retain functionality (Fig. 2), suggesting the N-terminus possesses key regions for protein function or conformational folding. Nonetheless, it is clear that Ucp promotes the formation of spherical bacterial aggregates in the aphid alimentary tract, as well as the accumulation of free bacteria, obstructing the normal flow through the aphid gut and reducing honeydew expulsion.

Although we had originally predicted that no aggregates would form in ucp1-deficient bacteria, some aggregates were observed in aphids fed either E. coli empty-vector control or DC283 (ucp1::Tn5). In these cases, however, the aggregates were confined to the crop, and the hindgut was always entirely clear, unlike the situation in aphids fed on bacteria expressing ucp1. This suggests that the aggregates of ucp1-deficient bacteria may erode in the crop such that they do not impede the flow of honeydew. Aggregates of ucp1-expressing bacteria may therefore be more resilient, accumulating until they begin to block the natural flow of honeydew. It appears that the aggregation phenotype of ucp1-proficient strains is not simply due to ucp1 increasing bacterial growth, since bacteria lacking the gene can grow to higher densities in the aphid (Fig. 1).

The ecological significance of the DC283–aphid interaction remains uncertain since ingested DC283 are trapped within a dead-end host and are not readily transmitted to new hosts. In contrast, the interaction between P. syringae and the pea aphid described previously appears ecologically relevant, since bacteria acquired from plant surfaces by the aphid can replicate within the aphid host, and eventually escape to the plant surface via the aphid honeydew prior to host death (Stavrinides et al., 2009). In the DC283–aphid interaction, the abatement of honeydew production coupled with aphid lethality effectively prevents escape into the environment, hindering bacterial dissemination and reducing overall fitness. Still, the recurrent isolation of enteric phytopathogens from aphids suggests that colonization of this insect host is frequent. Recently, the aphid pathogen E. aphidicola was identified as a plant pathogen that can infect both snap bean (Phaseolus vulgaris) and fava bean (Vicia faba) (Santos et al., 2009), plants that commonly serve as hosts to pea aphids (Sohi and Swenson, 1964; Blackman and Eastop, 2000; Nault et al., 2004). In addition, E. aphidicola, was shown originally to group phylogenetically with phytopathogenic Erwinia ananas and Erwinia herbicola (Harada et al., 1997), both of which have since been re-assigned to the genus Pantoea (Mergaert et al., 1993), suggesting species of Pantoea are commonly associated with aphids.

The ability of P. stewartii to colonize and aggregate in the aphid gut so effectively lends additional support to the idea that enteric plant pathogens were originally associated with insect hosts, but may have evolved the potential to colonize plants after their frequent deposition into the phyllosphere by insects (Stavrinides, 2009). The results of this study illustrate that the enteric phytopathogens, although typically considered restricted to plant hosts, may retain the genetic means for establishing and colonizing the intestinal environment of alternative non-plant hosts with which they share a common niche and are likely to encounter frequently.

**Experimental procedures**

**Aphid lines, bacterial strains and growth conditions**

Cultures of DC283r, a spontaneous rifampicin-resistant mutant of DC283 that was used for all experiments in this study, were grown in Luria–Bertani (LB) medium at 25°C, and Escherichia coli BL21 (DE3) was grown in LB medium at 37°C (Table S1). Where applicable, antibiotics were supplemented at the following concentrations: ampicillin (150 μg ml⁻¹), chloramphenicol (64 μg ml⁻¹), rifampicin (50 μg ml⁻¹) and kanamycin (50 μg ml⁻¹). The pea aphid, Acyrthosiphon pisum strain 5 A, was maintained at 20°C on fava bean plants (Vicia faba L.). For screening and growth assays, aphids were maintained on an artificial diet containing essential amino acids, vitamins and sucrose (Febvay et al., 1988). Fava bean plants were grown by sowing fava bean seeds in 1:1 ratio of Sunshine Mix to vermiculite.

**Transposon mutagenesis and screening**

Transposon mutagenesis of DC283 was performed using the mini-Tn5 vector pBSL118 in E. coli VPE42 (carrying kanamycin and ampicillin resistance), which was mobilized into the recipient DC283r using the helper plasmid RK600 (chloramphenicol resistance) (Table S1). To facilitate this triparental mating, the three parental strains were combined in a 1:1:1 ratio, spotted on LB agar plates (without antibiotics) and incubated overnight at 28–30°C. Traces of bacterial lawns were resuspended in 10 mM MgSO₄, and serial dilutions plated on LB medium containing rifampicin and kanamycin. Screening of single transconjugants was performed in 96-well microtitre plates as described previously (Stavrinides et al., 2009). Plates were kept at ambient temperature and scored after 3–4 days. Transconjugants recovered from wells of surviving aphids were confirmed to be DC283 via PCR and re-assayed eight times against aphids. A total of over 1100 mutants were screened.

Mutated genes were identified by inverse PCR (Ochman et al., 1988) as follows: genomic DNA from each mutant was extracted using the Gentra Puregene Kit (Qiagen, CA, USA). –1 μg of gDNA was digested using either HincII and EcoRI in
Cloning and transformations

The ucp1 and ulg genes, along with all ucp1 deletion derivatives (Δ685-1221), Δ1-357, Δ1-495 and Δ1-609, were amplified with Pfu-Turbo (Stratagene, California, USA) using gene-specific primers (Table S2) and purified with the Qiagen PCR purification kit. PCR products and the pET-21b(+) expression vector (Novagen, New Jersey, USA) (Table S1) were each subjected to 16 h restriction digests with EcoRI and HindIII using 5–10 units of enzyme in a 40 μl reaction volume, and purified with the Qiagen PCR purification kit. PCR products were ligated into the pET-21b(+) vector, and the ligation mix transformed directly into competent E. coli BL21 (DE3) (Chung et al., 1989). Transformants were selected on LB containing ampicillin, and positives were confirmed via PCR in a set of reactions employing two vector primers, one vector and one insert-specific primer, and two insert-specific primers.

Bacterial growth and virulence assays

Second and third instar aphids were allowed to feed for 17–18 h on artificial diet containing 107 cfu ml⁻¹ of bacteria, after which, aphids were transferred to fresh diet that lacked bacteria. Three aphids were sampled at 24 h intervals, macerated together in 100 μl of 10 mM MgSO₄ and dilutions plated on LB containing rifampin. Three independent growth assays were performed.

Qualitative virulence assays were conducted on second and third instar aphids, which were allowed to feed on artificial diet containing 10⁷ cfu ml⁻¹ of bacteria. For assays examining the contribution of ulg1–6 to pathogenicity, and determining the functional domain of ucp1, IPTG was supplemented in all treatments (including the no-bacteria control) to a final concentration of 0.5 mM. Aphids were maintained on assay plates at ambient temperature for the duration of the assay. Five independent assays were performed.

Genomics and sequence analysis

Identification of ucp1 and related sequences within the DC283 genome was achieved by BLASTn and tBLASTx against a customized database constructed from sequence data obtained from Baylor College of Medicine Sequencing Center (http://www.hgsc.bcm.tmc.edu). The ucp1 and ucp1-like homologues in other species were recovered by BLASTn and tBLASTx searches against the nr database. The DNA sequence of Pantoea stewartii ssp. stewartii DC283 was supported by 2004-35600-14174 from the USDA to George Weinstock (BCM-HGSC), Susanne von Bodman (University of Connecticut, CT, USA), Nicole Perna (University of Wisconsin, Madison, WI, USA) and David L. Coplin (The Ohio State University, Columbus, OH, USA). Motif and domain architecture of all proteins were analysed using both InterproScan (Zdobnov and Apweiler, 2001) and PredictProtein (Rost et al., 2004).

Microscopy

Aphid dissections were conducted under a Leica dissecting microscope retrofitted with a Nikon camera. Aphid guts were removed and dissected using two fine-tipped tweezers by pinching and pulling either the head or the last segment on the abdomen directly in phosphate buffered saline. Higher magnification images were taken on a Nikon light/fluorescence compound microscope.

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References


Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Honeydew excretion by aphids ingesting *ucp1*-proficient and *ucp1*-deficient DC283. Aphids feeding on wild-type DC283 excrete less honeydew than those feeding on DC283 (*ucp1::Tn5*). Aphids feeding on DC283 (*ucp1::Tn5*) produce equivalent amounts of honeydew to those feeding on the no-bacteria control. Error bars show standard deviation.

**Table S1.** Strains and constructs.

**Table S2.** Primers.

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