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Type III secretion-dependent host defence elicitation and type III secretion-independent growth within leaves by *Xanthomonas campestris* pv. *campestris*

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SUMMARY

In many plant–bacterial interactions, loss of the type III secretion system (T3SS) severely reduces bacterial growth, symptom causation and suppression of defences in host plants. In the present study of *Xanthomonas campestris* pv. *campestris* (Xcc), Xcc strain B305 grew better than strain B186 in *Arabidopsis thaliana* after hydathode inoculation, and B305 strains mutated to the loss of T3SS (Δ*hrcC* and/or Δ*hrpE*; also Δ*hrcC*Δ*flgBC*) grew similarly to wild-type B305 in Arabidopsis leaves. Unlike Xcc strain B186, wild-type B305 was relatively inefficient in secreting the exogenous T3S effector AvrBsT, but Δ*hrcC* and/or Δ*hrpE* attenuated the disease symptoms caused by Xcc B305, showing that the partially compromised T3SS of this strain still promotes necrotic leaf symptoms. In contrast with the T3SS-dependent defence suppression that has been observed for some other plant pathogenic bacteria, the Xcc B186 and B305 wild-type strains (which are virulent on Arabidopsis) caused greater elicitation of host *PR-1* and *PR-5* expression and callose deposition in comparison with their respective T3SS mutants. A defence-suppressing/virulence-enhancing activity of the Xcc T3SS effector suite was detectable when co-inoculation with wild-type Xcc B186 increased the growth of Δ*hrcC* Xcc, but this activity did not prevent the above defence elicitation. Experiments using T3SS mutants and Arabidopsis *fls2* mutants suggested that FLS2 does not play a prominent role in restriction of the examined Xcc strains. However, ectopic overexpression of the *Pseudomonas syringae* effector AvrPto promoted *in planta* growth of wild-type and Δ*hrcC* Xcc. In summary, the T3SS components or effector suite from virulent Xcc strains elicit some host defence responses, but suppress other defences and stimulate more severe disease symptoms, AvrPto-disruptable elements other than FLS2 apparently contribute to the host restriction of Xcc, and in some virulent Xcc strains the T3SS is not absolutely required for wild-type levels of bacterial growth within the plant.

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INTRODUCTION

Xanthomonas campestris pv. *campestris* (Xcc) causes black rot disease in cruciferous plants, and is considered to be one of the most economically significant diseases of cruciferous crops worldwide (Alvarez, 2000; Williams, 1980). *Arabidopsis thaliana* is a natural host for pathogenic Xcc (Simpson and Johnson, 1990), making the Xcc–*Arabidopsis* system an attractive model for study. Unlike many other well-studied *Pseudomonas* and *Xanthomonas* plant pathogens, Xcc is a vascular pathogen that typically enters the plant via the hydathodes or wounds (Alvarez, 2000; Hugouvieux *et al*., 1998; Lu *et al*., 2008; Schroth *et al*., 1991). Hence, differences can be anticipated in the modes of virulence employed by Xcc as opposed to these other bacterial pathogens.

Bacterial type III secretion systems (T3SSs) mediate elaborate interactions with hosts by translocating effector proteins into the cytosol of eukaryotic host cells (Buttner and He, 2009; da Cunha *et al*., 2007; Galan and Wolf-Watz, 2006; McCann and Guttman, 2008). The primary known contribution of these effectors to the virulence of plant pathogens is host defence suppression. In *Pseudomonas* and *Xanthomonas* plant pathogens, effector proteins delivered by the T3SS suppress host defence responses by manipulating a variety of host pathways (Block *et al*., 2008; da Cunha *et al*., 2007; He *et al*., 2007a; Kay and Bonas, 2009; Kim *et al*., 2008, 2009b; Zhou and Chai, 2008). In Xcc, it is known that the T3SS plays an essential role in pathogenicity, and the gene cluster that encodes the core components of the Xcc T3S apparatus has been described (Arlat *et al*., 1991; Cornelis and Van Gijsegem, 2000; Kamoun and Kado, 1990; Kay and Bonas, 2009; Qian *et al*., 2005; da Silva *et al*., 2002; White *et al*., 2009). However, some plant pathogenic *Xylella* and *Pectobacterium* bacteria lack any detectable T3SS, yet are successful pathogens (Kim HS *et al*., 2009a; Lambais *et al*., 2000; Van Sluys *et al*., 2002).

Relevant to the present study, the *hrcC* gene encodes the core outer membrane component of the T3SS in diverse bacteria, and *hrcC* mutants in *Pseudomonas* and *Xanthomonas* have been demonstrated to be deficient in T3S (Bonas, 1994; Keshavarzi *et al*., 2004; Roine *et al*., 1997). The HrpE pilin is a major Hrp pilus component that is unique to xanthomonads (Cornelis and

Van Gijsegem, 2000; Weber and Koebnik, 2005). This T3SS pilus serves as a conduit for the transfer of bacterial effector proteins into the plant cell cytosol, and is required, for example, for productive interaction of *Xanthomonas campestris* pv. *vesicatoria* (Xcv) with pepper host plants (Casper-Lindley *et al*., 2002; Weber *et al*., 2005).

One of the first indications that a primary role of bacterial T3SS effectors is host defence suppression came from microscopy studies with T3SS mutants of Xcv, which caused much greater plant cell wall thickening and papilla formation than did wild-type bacteria (Brown *et al*., 1995, 1998). In subsequent studies, when wild-type and Δ*hrcC* mutants of Xcv were mix-inoculated into pepper host plants, the bacterial population of the Δ*hrcC* mutant was significantly larger than after inoculation with the Δ*hrcC* mutant only (Keshavarzi *et al*., 2004). T3S-dependent suppression of basal defence responses triggered by bacterial lipopolysaccharides was demonstrated (Keshavarzi *et al*., 2004). Individual *Xanthomonas* T3S effector proteins have also been shown to suppress defence (Kim *et al*., 2008, 2009b; Metz *et al*., 2005). *Xanthomonas* bacteria can also suppress plant defences independent of the T3SS, such as via xanthan gum or cyclic β-(1,2) glucans (Rigano *et al*., 2007; Yun *et al*., 2006).

Xanthomonas T3S effectors can play other roles in pathogenicity (for example, AvrBs3 acts as a plant transcription factor that provokes the developmental reprogramming of host cells; Kay and Bonas, 2009; White *et al*., 2009). Recently, it has been demonstrated that several Xcc T3SS effectors, including XopXccN and AvrXccC, are required for full virulence (Jiang *et al*., 2008, 2009; Wang *et al*., 2007). By contrast, deletion of eight known *avr* genes in Xcc had no detectable effect on pathogenicity (Castaneda *et al*., 2005). Many additional *hrp* gene- or T3SS-associated regulators of Xcc virulence systems have been defined recently (Chao *et al*., 2008; Jiang *et al*., 2006; Wang *et al*., 2008; Wei *et al*., 2007). Two avr proteins, AvrXccFM and AvrACXcc8004, can induce an atypical hypersensitive response (HR), i.e. HR in vascular tissues (Castaneda *et al*., 2005; Xu *et al*., 2008; see, for comparison, (Godard *et al*., 2000). Hence, the *Xanthomonas* T3SS and effectors are starting to be understood. However, these studies did not focus on the defence-activating activity of the T3SS effector suite in virulent bacteria.

In addition to the primary T3SS, the present study tested the flagellum secretion system. The flagellum spans the bacterial membrane, and distal components are secreted through the base of the structure via a specialized T3SS (Macnab, 1999). There are examples in which the flagellum-associated secretion system is used as a virulence protein export machine (Journet *et al*., 2005). FlgB and FlgC are among five proteins that make up the rod structure of the flagellum; hence, the deletion of *flgB* and *flgC* genes should block the secretion of any distal components and flagellum-secreted virulence effectors (Aldridge and Hughes, 2002).

Microbe-associated molecular patterns (MAMPs, also called PAMPs), such as flagellin, have been characterized as broadly conserved molecular patterns that are used by host innate immune systems to recognize microbes (Ausubel, 2005; Jones and Dangl, 2006; Nurnberger *et al*., 2004). Our group has demonstrated previously that some Xcc strains (including B305) express a flagellin that is detectable by the FLS2 flagellin receptor, whereas other Xcc strains (including B186) express a flagellin that is not detectable by Arabidopsis FLS2 (Sun *et al*., 2006). However, we found that isogenic Xcc flagellin (*fliC*) gene replacement strains expressing eliciting or noneliciting flagellins grew similarly in Arabidopsis leaves that carried a functional FLS2. Resistance against Xcc in Arabidopsis was enhanced if purified eliciting flagellin was used to trigger *FLS2*-dependent responses 1 day prior to Xcc infection, but the FLS2 system had no detectable effect on disease outcome when naive plants were infected by Xcc that expressed a detectable flagellin. We hypothesized that Xcc bacteria might actively suppress flagellin-elicited host defences (Sun *et al*., 2006); this has been shown for *Pseudomonas syringae* bacteria (Gohre *et al*., 2008; Shan *et al*., 2008; de Torres *et al*., 2006; Xiang *et al*., 2008).

Initially hypothesizing that the secretion systems of Xcc help to suppress flagellin-triggered plant basal defences, the present study disrupted type III Hrp and Flg secretion systems in Xcc strains B94, B186 and/or B305. We observed that Xcc strain B305 is much less efficient than strain B186 in secreting the Xcv T3S effector AvrBsT. Intriguingly, after hydathode inoculation, Xcc B305 mutants disrupted for T3SS grew to population sizes in plants similar to those of the wild-type strain. Loss of T3SS delayed and reduced symptom formation on plants. Moreover, for multiple Xcc strains, the induction of host basal defence was reduced rather than increased when the T3SS was knocked out. Wild-type Xcc strains, such as B186, are virulent on Arabidopsis Col-0 and grow with no detectable elicitation of an HR, yet produce T3SS components and/or effectors that induce as well as suppress host defence responses.

RESULTS

Loss of T3SS imposes different effects on bacterial growth in Arabidopsis leaves among three different Xcc strains

Previously studied *P. syringae* and *X. campestris* pathovars carrying a mutated *hrcC* are deficient in T3S, exhibit loss of pathogenicity on host plants and fail to induce an HR on resistant hosts (Alfano and Collmer, 1996, 2004; Keshavarzi *et al*., 2004). We used homologous recombination to construct unmarked *hrcC* gene deletion mutants (Δ*hrcC*) of Xcc strains B94,

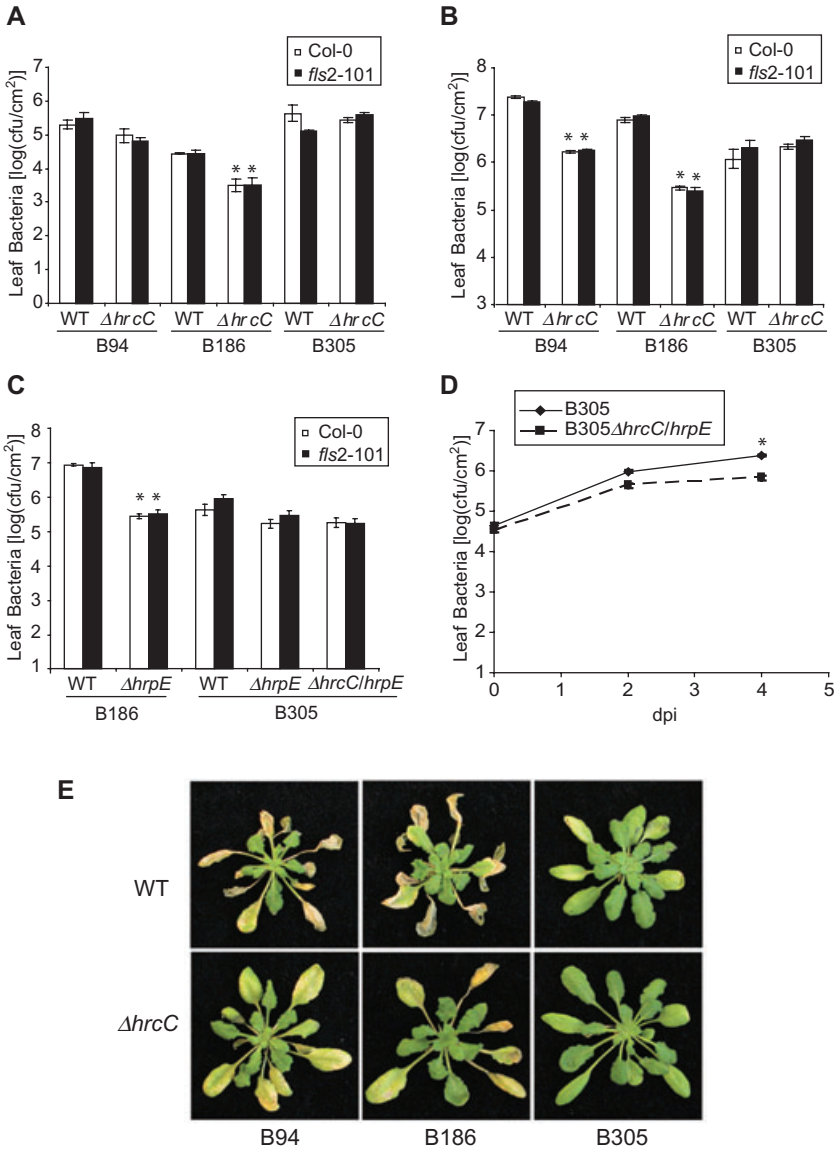

Fig. 1 Deletion of *hrcC* or *hrpE* causes different *in planta* bacterial growth phenotypes of *Xanthomonas campestris* pv. *campestris* (Xcc) B94, B186 and B305. (A) Xcc population sizes within Arabidopsis leaves 4 days after hydathode inoculation (inoculation by spraying bacteria onto rosette leaves of guttating plants). Bars are means ± standard error. *Significant difference from the wild-type version of the same strain on the same host genotype (*t*-test, $P < 0.05$). WT, wild-type strain; Δ*hrcC*, isogenic strain with unmarked deletion of *hrcC* gene. This and all other experiments in all figures were repeated with similar results on at least two separate dates unless specifically noted. (B, C) Xcc leaf populations 3 days after inoculation of Arabidopsis by vacuum infiltration. (D) *In planta* growth curve of B305 Δ*hrcC/hrpE* and wild-type B305 after inoculation by vacuum infiltration into Arabidopsis Col-0 leaves (dpi, days post-inoculation). (A–D) Mean ± standard error of mean; bar fill in (A), (B) and (C) indicates plant genotype. cfu, colony-forming units. (E) Representative leaf disease symptoms 7 days after vacuum infiltration. Xcc strain genotype noted below and to the left of the photographs.

B186 and B305, precisely deleting from the start to the stop codon of the open reading frame. To corroborate the essential role of T3SS in Xcc virulence (Arlat *et al*., 1991; Kamoun and Kado, 1990), these Δ*hrcC* and wild-type strains were spray inoculated onto Arabidopsis leaves after the induction of guttation droplets. The B186 Δ*hrcC* strain exhibited reduced population levels as expected, but the B305 Δ*hrcC* and B94 Δ*hrcC* strains grew to population levels similar to those of the isogenic wild-type parent strains (Fig. 1A). When bacteria were introduced directly into the leaf interior by vacuum infiltration, the Δ*hrcC* mutation caused reduced bacterial populations for the B94 and B186 strains, but the B305 strain once again grew to similar levels with or without *hrcC* (Fig. 1B). Deletion of *hrcC* was confirmed by Southern blot

(Fig. S1, see Supporting Information). Loss of *hrcC* function of all three Xcc strains was also confirmed by complementation with *hrcC* (data not shown) and by other means (see following sections). Hydathode invasion after spray inoculation was indicated by the classic Xcc V-shaped lesions formed at leaf margins (Fig. S2, see Supporting Information). Based on the results of Figs 1A,B, strains B186 and B305 were chosen for more in-depth study.

To further confirm the finding of T3SS-independent growth, a separate T3SS gene (*hrpE*) was deleted from Xcc strains B186 and B305, and a double mutant of B305 was also constructed. On plant inoculation by vacuum infiltration, the Δ*hrpE* mutation greatly reduced the virulence of B186, but caused significantly less effect on the growth of B305 (Fig. 1C). Similar growth of Xcc B305 with or without *hrcC* was observed in three different Arabidopsis ecotypes (Fig. 1A and Fig. S3, see Supporting Information). As noted in a previous study, wild-type B305 grew less well than other wild-type Xcc after vacuum infiltration into Arabidopsis (Sun *et al*., 2006; and compare Fig. 1A,B). The experiment reported in Fig. 1D examined this and, although a 10-fold or greater increase in bacterial populations occurred for B305 strains even after vacuum infiltration, a difference was observed between B305 and the B305 Δ*hrcC*Δ*hrpE* mutant. The results after spray inoculation of guttating plants (as in Fig. 1A) may be of more interest, where wild-type B305 reproducibly established larger populations than B186 in Arabidopsis leaves. By spray inoculation, loss of the TTSS reproducibly caused no significant reduction in bacterial population growth of B305 within leaves.

We also made and tested B305 strains with deletion of the flagellar basal body *flgB/C* genes that have an impact on the secretion of flagellar and other proteins (Journet *et al*., 2005), as well as a B305 Δ*hrcC*Δ*flgB/C* double mutant to disrupt both predicted T3SSs of Xcc. These strains also established leaf populations similar to those of wild-type B305 in Arabidopsis Col-0 leaves (Fig. S4, see Supporting Information). The phenotypes of the bacterial strains used in this study are summarized briefly in Table S1 (see Supporting Information).

Loss of T3SS attenuates disease symptoms caused by Xcc

Although the loss of T3SS function had less effect on the *in planta* growth of Xcc B305 than B186, lesion formation by B305 T3SS mutants was attenuated. Under one type of standard experimental conditions, disease symptoms became visible on Arabidopsis leaves 4 days after vacuum infiltration of wild-type B305, whereas disease symptoms caused by B305 Δ*hrcC* were not evident until 7 days after inoculation (Fig. 1E, and data not shown). The behaviour of Xcc wild-type and Δ*hrcC* strains was also tested for compatible interaction with *Nicotiana benthamiana* plants and, 5 days after syringe infiltration into leaf mesophyll, the B305 wild-type and Δ*hrcC* strains again caused markedly different disease symptoms (Fig. 2A). Xcc B305 caused complete chlorosis/necrosis in the infiltrated leaf area, whereas

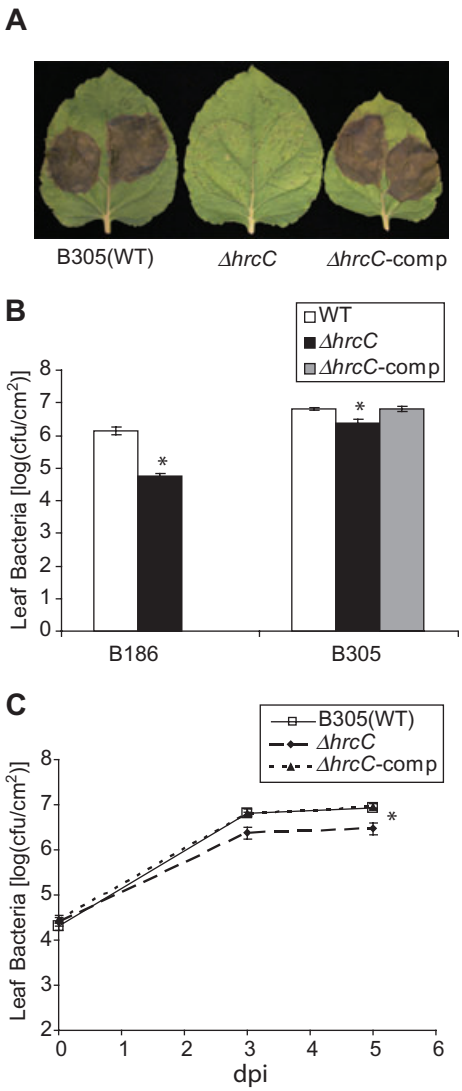

Fig. 2 Virulence of (leaf symptoms caused by) *Xanthomonas campestris* pv. *campestris* (Xcc) B305 on *Nicotiana benthamiana* is greatly attenuated by the loss of the *hrcC* gene despite sustained growth *in planta*. (A) Necrosis caused by Xcc B305 strains 5 days after syringe infiltration of the same strain onto *N. benthamiana* leaf. No tissue collapse was evident until at least 3 days after inoculation. WT, wild-type B305; Δ*hrcC*, B305Δ*hrcC*; Δ*hrcC*-comp, B305Δ*hrcC* carrying a plasmid with wild-type *hrcC* locus under the control of the native promoter. (B) Xcc populations in syringe-infiltrated *N. benthamiana* leaves. (C) Xcc B305 populations in syringe-infiltrated *N. benthamiana* leaves; dpi, days post-inoculation. (B, C) Data are mean ± standard error; bar fill and *x*-axis, or line and symbol type, denote bacterial genotypes as described in (A). *Significant difference from the wild-type version of the same strain (*t*-test, $P < 0.05$). cfu, colony-forming units.

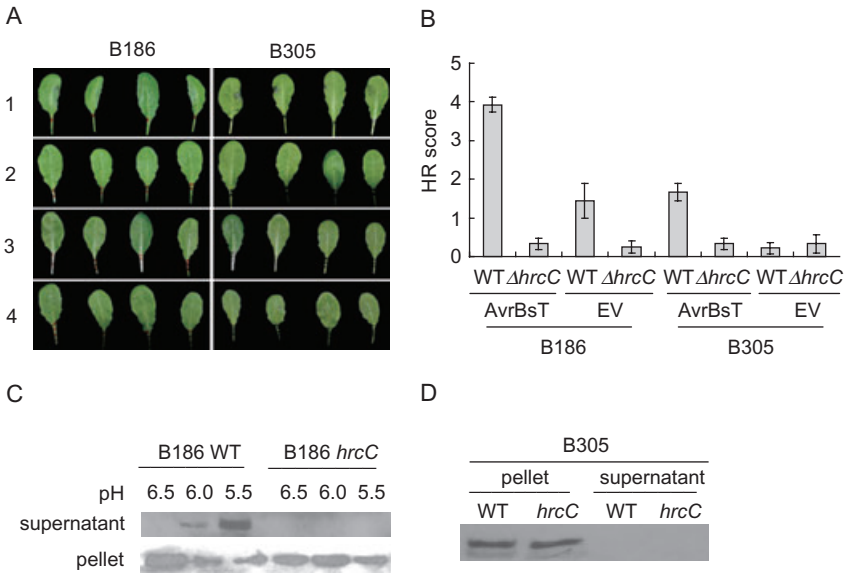

Fig. 3 Type III secretion (T3S) effector AvrBsT is secreted efficiently by *Xanthomonas campestris* pv. *campestris* (Xcc) B186, but inefficiently by Xcc B305, and is not detectably secreted in Δ*hrcC* mutants. (A) Hypersensitive response (HR) tissue collapse 24 h after inoculation with Xcc strains as noted [four leaves are shown for each Xcc strain; inoculation at optical density at 600 nm (OD_{600}) = 0.3]. 1, wild-type strains with the *AvrBsT-HA* construct; 2, Δ*hrcC* strains with the *AvrBsT-HA* construct; 3, wild-type strains with the pDD62 empty vector (EV); 4, Δ*hrcC* strains with EV. (B) HR scores for leaves inoculated as in (A). Scores were recorded for at least eight leaves per strain; means ± standard error are shown. (C, D) Western blotting for the presence of AvrBsT-HA detected using anti-haemagglutinin (HA) antibody after growth of Xcc B186 with the *AvrBsT-HA* construct (C) in *hrp*-inducing culture medium (SMMXC, pH 6.5–5.5 as noted) or B305 with the *AvrBsT-HA* construct (D) in SMMXC medium, pH 5.5.

B305 Δ*hrcC* produced only a few small water-soaked spots in the inoculated leaf area. The virulence of the B305 Δ*hrcC* mutant was restored when a plasmid-borne wild-type *hrcC* gene was introduced into the Δ*hrcC* strain (Fig. 2A); B186 Δ*hrcC* was also complemented for growth in plants when a plasmid carrying wild-type *hrcC* was added (data not shown). The severe chlorosis/necrosis caused by B305 was unlikely to be the consequence of an HR, because these symptoms only appeared 3–4 days after inoculation. Similarly, no rapid HR was observed after inoculation of Arabidopsis with Xcc B305 (Figs 1 and 3). B186 Δ*hrcC* grew to much lower levels than wild-type B186 in *N. benthamiana*, whereas a much smaller reduction in the growth of B305 Δ*hrcC* was observed relative to wild-type B305 (Fig. 2B). In these *N. benthamiana* mesophyll inoculation experiments, B305 grew relatively better than B186 (Fig. 2B), in contrast with the Arabidopsis mesophyll inoculation results of Fig. 1B,C. The population size of B305 Δ*hrcC* increased more than 100-fold in *N. benthamiana* plant leaves 3 days after inoculation (Fig. 2C). Overall, the above results suggest that the Xcc B305 T3SS effector suite or components promote leaf necrosis during compatible interactions and speed the development of disease symptoms, but, in contrast with most previously studied plant pathogenic proteobacteria, *in planta* growth of Xcc B305 is substantially less dependent on the T3SS.

Disruption of Xcc T3SS does not reveal a detectable impact of host FLS2-mediated basal immunity

Previous work has shown that Xcc strains B305 and B94 express a flagellin that elicits defence responses in Arabidopsis Col-0, whereas the flagellin of Xcc B186 is noneliciting (Sun *et al*., 2006). In that study, an Xcc B186 gene replacement strain expressing a defence-eliciting flagellin infected Arabidopsis similarly to wild-type B186 that produces noneliciting flagellin (Sun *et al*., 2006). In the present study, the hypothesis that one or more T3SS effectors of Xcc suppress FLS2-mediated defences was examined. Each of the tested Xcc strains grew similarly in Arabidopsis Col (wild-type *FLS2*$^{+}$) and *fls2-101* mutant plants (Fig. 1A–C). In particular, no contribution of FLS2 to the restriction of Xcc growth became evident after disruption of the T3SS in Δ*hrcC* or Δ*hrpE* strains.

Xcc B305 is less efficient than B186 in secreting T3S effectors

The *avrBsT*-dependent HR elicited in Arabidopsis ecotype Pi-0 by Xcc strains can be used to confirm the presence of a functioning T3SS (Cunnac *et al*., 2007). To further test the T3SS functionality of wild-type and mutant strains of Xcc B186 and B305, an *avrBsT*

construct or empty vector pDD62 was electroporated into the wild-type and Δ*hrcC* mutant strains. Successful transformation was confirmed by Western blot analyses (Fig. 3C,D). Four- to six-week-old Pi-0 plants were used for macroscopic HR analyses. The severity of HR was photographed and scored at 24 h after the leaves had been syringe inoculated with different Xcc strains at high cell density [optical density at 600 nm (OD_{600}) = 0.3] (Fig. 3A,B). Xcc B186 expressing AvrBsT-haemagglutinin (AvrBsT-HA) elicited severe HRs, whereas B186 with pDD62 did not. Xcc B186 Δ*hrcC* expressing AvrBsT-HA lost the ability to cause an HR. Compared to Xcc B186 with AvrBsT-HA, Xcc B305 with AvrBsT-HA caused an evident, but less severe, HR with subtle tissue collapse on most inoculated leaves (Fig. 3A,B), suggesting that the T3SS of Xcc B305 is functional but possibly less efficient in delivering AvrBsT-HA into Arabidopsis cells than that of B186. *In vitro* secretion assays were performed for these Xcc strains to confirm the secretion of AvrBsT-HA. In these independent experiments, the hrp-inducing SMMXC medium (MMXC plus 100 μg/mL bovine serum albumin) at different pH values (Wang *et al*., 2007) was used to culture Xcc and to trigger the secretion of T3S effectors. For Xcc B186 expressing AvrBsT-HA, Western blot analyses demonstrated that the AvrBsT effector was not detectably secreted into SMMXC medium at pH 6.5, but the amount of AvrBsT-HA in the supernatant increased as the pH of the medium was lowered from 6.0 to 5.5 (Fig. 3C). AvrBsT-HA was not detectable in the supernatant of the Xcc B186 Δ*hrcC* mutant at any pH, indicating that the *hrcC* deletion completely disrupted the T3SS of B186 (Fig. 3C). In contrast, for Xcc B305, AvrBsT-HA was detected only in cell pellets and, in repeated experiments, was not detectable in the supernatant, even when the wild-type strain with AvrBsT-HA was cultured in SMMXC medium at pH 5.5 (Fig. 3D).

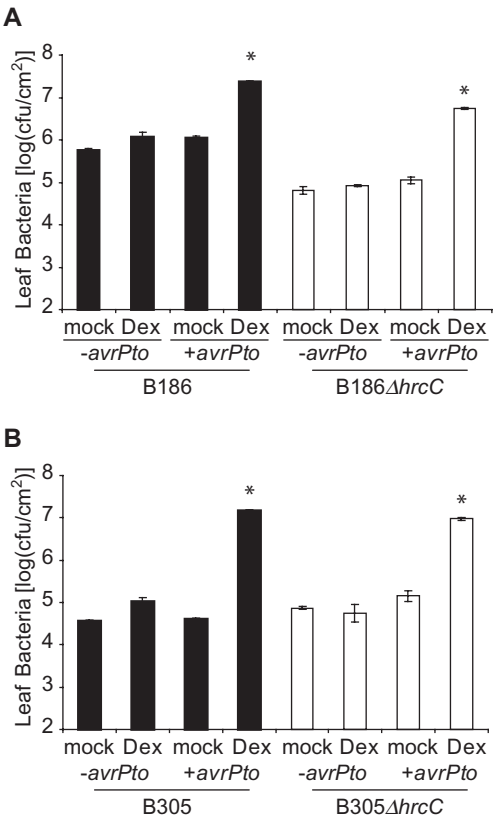

Fig. 4 Dexamethasone-inducible expression of *avrPto* in Arabidopsis promotes the *in planta* growth of *Xanthomonas campestris* pv. *campestris* (Xcc) wild-type and Δ*hrcC* strains after vacuum infiltration. (A) Population sizes of Xcc B186 wild-type and Δ*hrcC* strains in transgenic plants carrying *avrPto* under the control of a dexamethasone-inducible promoter (+*avrPto*), or nontransgenic Col *gl-1* plants (-*avrPto*), after spraying plants with dexamethasone (Dex) or with buffer (mock) 24 h prior to inoculation. (B) Population sizes of Xcc B305 wild-type and Δ*hrcC* strains in an experiment similar to that in (A). All data are means ± standard error. *Significant difference from the same strain on -*avrPto* plants (*t*-test, $P < 0.05$). cfu, colony-forming units.

The set of host defences disrupted by *Pseudomonas* effector AvrPto contribute to the restriction of Xcc growth

AvrPto is an effector protein from *P. syringae* pv. *tomato* that disrupts host defences by disrupting the activity of at least one and probably more MAMP receptors (FLS2, EFR, possibly others including the co-receptor BAK1; Shan *et al*., 2008; Xiang *et al*., 2008, 2011). In the absence of a detectable effect of FLS2 on Xcc (see previous section), ectopic AvrPto expression was used to determine whether the broader suite of AvrPto-sensitive host defence components had an impact on Xcc. Transgenic plants were used in which AvrPto was conditionally overexpressed 1 day prior to inoculation under the control of a dexamethasone (Dex)-inducible promoter (Aoyama and Chua, 1997; Hauck *et al*., 2003). Leaf populations of the Xcc B186 wild-type and Δ*hrcC* strains grew to approximately 20-fold and 50-fold higher levels, respectively, when AvrPto was overexpressed, in comparison with the same strains in Dex-sprayed plants lacking the P_{Dex}-*avrPto* construct or in mock-sprayed P_{Dex}-*avrPto* transgenic plants (Fig. 4A). Induced AvrPto expression also increased significantly the leaf population sizes of B305 wild-type and Δ*hrcC* strains (Fig. 4B). Hence, the AvrPto effector assisted the growth of Xcc B186 and B305, even though mutational removal of only FLS2 did not.

The T3SS components or effector suite in Xcc induces host basal defence responses while promoting virulence

For compatible interactions of plants with virulent *P. syringae* and Xcv bacteria, the T3S effector suite has been shown to

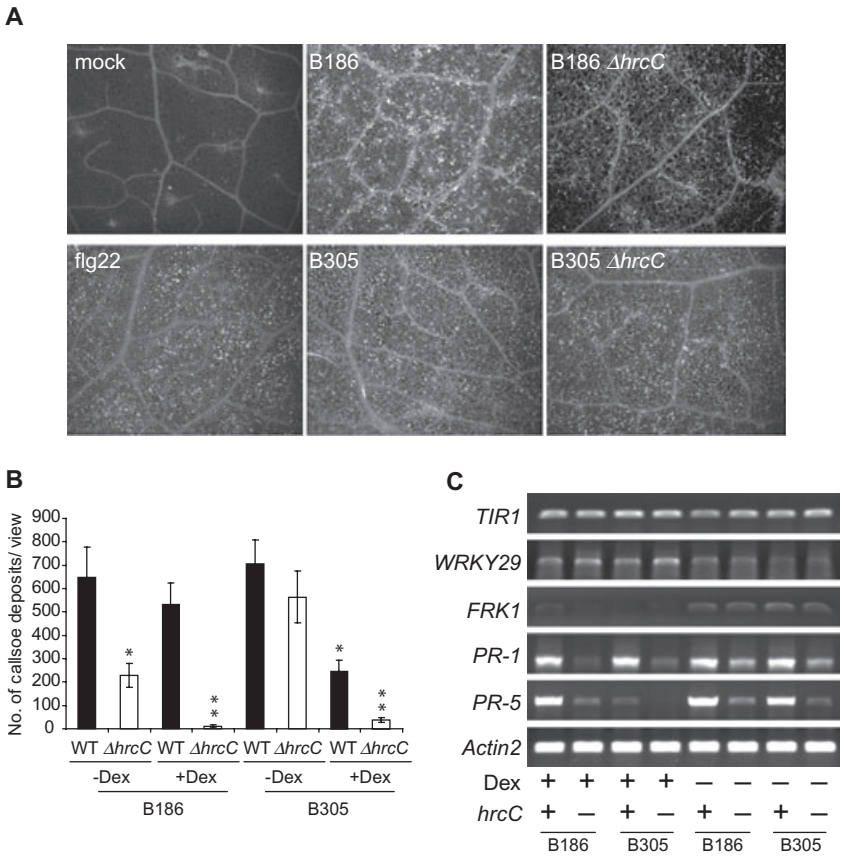

Fig. 5 Elicitation of Arabidopsis basal defence responses by *Xanthomonas campestris* pv. *campestris* (Xcc) is impaired, not elevated, when the *hrcC* gene is deleted. (A) Representative images of callose deposition in leaves 24 h after infiltration of Arabidopsis Col-0 leaves with flg22 peptide or with Xcc strains of the indicated genotype. (B) Quantification of callose deposits per field of view 24 h after infiltration of Xcc strains into Arabidopsis Col-0 plants carrying *avrPto* under the control of a dexamethasone-inducible promoter, activated in some samples by dexamethasone application 24 h prior to bacterial inoculation. Top and bottom lines of the *x*-axis annotation indicate the bacterial genotype, middle line indicates plant treatment (–Dex, mock treatment; +Dex, with dexamethasone treatment). Mean ± standard error and statistical significance from other plants treated with the same bacterial strain (*t*-test, $P < 0.05$) are shown; asterisk above Δ*hrcC*—Dex is for difference from wild-type (WT)—Dex; asterisk above WT +Dex is for difference from WT—Dex; double asterisk above Δ*hrcC* +Dex is for difference from both Δ*hrcC*—Dex and WT +Dex. (C) Abundance of the indicated mRNAs 24 h after the treatments described in (B). For *hrcC* genotypes, + indicates wild-type *hrcC*$^{+}$ and—indicates Δ*hrcC*. Equivalent tissue quantities were extracted and assayed using semiquantitative reverse transcription-polymerase chain reaction (RT-PCR); *Actin2* served as a control for mRNA equivalence.

suppress plant basal defences, such as callose deposition and pathogenesis-related (PR) gene expression, that are triggered by MAMP detection (Alfano and Collmer, 2004; Block *et al*., 2008; Speth *et al*., 2007). We examined callose deposition in plant leaves after Xcc inoculation to determine whether the Xcc T3S effector suite successfully suppresses plant basal defences. Infiltration of flg22 peptide as a positive control induced callose deposition in Arabidopsis Col-0 leaves, as expected (Fig. 5A). Wild-type Xcc B186 and B305 strains also induced callose deposition (Fig. 5A,B). In contrast with T3SS-deficient mutants of *P. syringae* pv. *tomato* and Xcv, which elicit more callose deposition than do isogenic wild-type strains (e.g. Brown *et al*., 1995; Hauck *et al*., 2003), Xcc B186 Δ*hrcC* partially lost the ability to induce callose deposition (Fig. 5A,B). Equally interesting, the B305 wild-type and Δ*hrcC* strains that grow to similar levels within leaves had a similar ability to trigger callose deposition (Fig. 5A,B). Similar callose deposition was observed on wild-type and *fls2-101* plants for Xcc B186 and B305 strains and their derivative Δ*hrcC* strains (Fig. S5, see Supporting Information). This latter result suggests that Xcc elicitors other than flagellin are primarily responsible for the triggering of callose deposition in Arabidopsis.

Callose deposition assays were also performed after Xcc infiltration into Arabidopsis leaves that overexpressed the *P. syringae* effector AvrPto. Consistent with published findings for *P. syringae* bacteria, AvrPto reduced callose deposition in plants infiltrated with Xcc B305, B30 5Δ*hrcC* and B186 Δ*hrcC* strains.

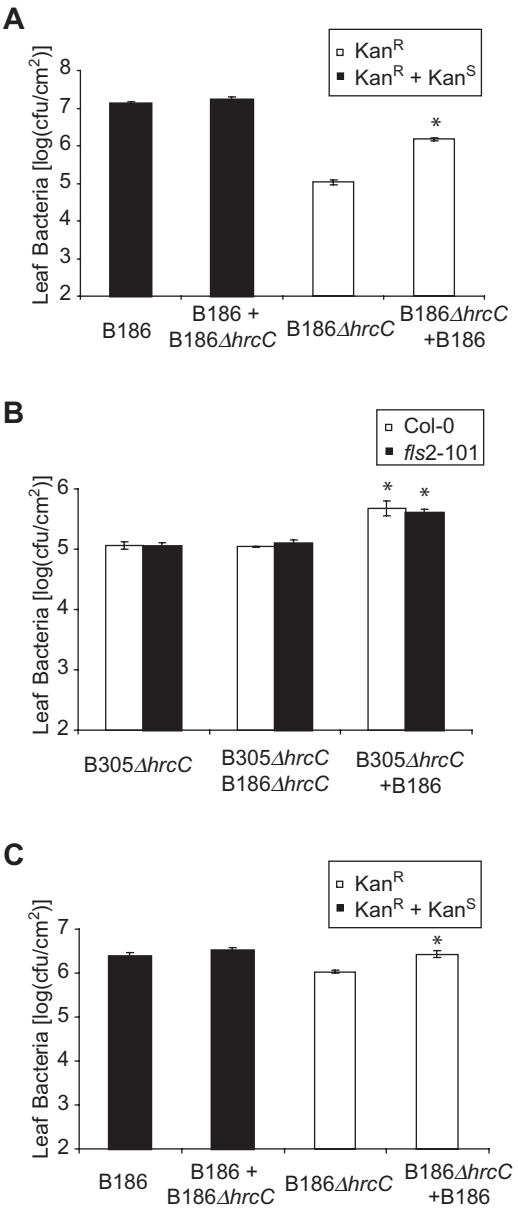

Fig. 6 Co-inoculation of Arabidopsis with wild-type (WT) *Xanthomonas campestris* pv. *campestris* (Xcc) B186 promotes the *in planta* growth of Xcc Δ*hrcC* strains. *x*-axis labels denote bacteria that were applied to the plant; data are means ± standard error. *Significant difference between population sizes of the first stain listed in the presence/absence of the second strain listed (*t*-test, $P < 0.05$). In (A) and (C), open bars show the main result (leaf population of Xcc Δ*hrcC* strain carrying a Kan^R plasmid in the absence or presence of co-inoculated WT Xcc B186), and filled bars show control data (leaf population of all Kan^S and/or Kan^R Xcc bacteria). (A) Leaf population of B186Δ*hrcC* 3 days after mix-inoculation [at an optical density at 600 nm (OD_{600}) = 0.0005, low cell density] with WT B186 (at OD_{600} = 0.005, high cell density). (B) Leaf population of B305Δ*hrcC* 3 days after mix-inoculation (at OD_{600} = 0.0005, low cell density) with WT B186 or B186Δ*hrcC* (inoculated at OD_{600} = 0.005, high cell density). In (B), open bars show data for growth in WT Arabidopsis Col-0 and filled bars show data for a Col-0 *fls2*$^-$ mutant. (C) Leaf population of B186Δ*hrcC* 3 days after mix-inoculation (at OD_{600} = 0.005, high cell density) with WT B186 (at OD_{600} = 0.0005, low cell density). cfu, colony-forming units.

The callose deposition caused by Xcc B186 remained prominent in plants that expressed AvrPto (Fig. 5B).

The expression of PR and MAMP-induced genes (van Loon *et al*., 2006; Navarro *et al*., 2004) was monitored using semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR). Once again, unlike *P. syringae* or Xcv T3SS mutants, Xcc Δ*hrcC* mutants exhibited distinctly less, rather than more, elicitation of *PR-1* and *PR-5* gene expression in comparison with that elicited by wild-type Xcc (Fig. 5C). The presence/absence of *hrcC* did not alter significantly the expression of the MAMP-inducible *WRKY29* and *FRK1* genes. Consistent with callose deposition assays, AvrPto expression suppressed *PR-5* gene expression induced by Xcc B305, but did not detectably suppress Xcc B186-induced PR gene expression. Returning to the primary observation, during compatible interactions with virulent Xcc strain B186, Xcc T3SS components or the T3S effector suite caused more, rather than less, induction of benchmark basal defence responses, such as callose deposition and *PR-1* or *PR-5* gene expression.

Co-inoculation with wild-type Xcc B186 increases the growth of Δ*hrcC* Xcc

As noted previously, wild-type strains of Xcv have not only been shown to inhibit basal defences in pepper, but also to partially restore the multiplication of isogenic nonpathogenic Δ*hrcC* bacteria on co-inoculation into plant leaves (Keshavarzi *et al*., 2004). We traced the *in planta* growth of kanamycin-resistant Xcc B186 Δ*hrcC* and B305 Δ*hrcC* strains in Arabidopsis on co-inoculation with wild-type and kanamycin-sensitive Xcc B186. Bacterial growth assays indicated that wild-type B186 assisted the multiplication of B186 Δ*hrcC* and B305 Δ*hrcC* (Fig. 6A,B). Co-inoculation with B186 Δ*hrcC* did not increase the growth of B305 Δ*hrcC* (Fig. 6B). A greater growth increase was observed when the inoculum contained a 10:1 rather than a 1:10 ratio of wild-type to Δ*hrcC* bacteria (Fig. 6A vs. Fig. 6C). Hence, the Xcc T3SS or its effector suite causes a greater induction of defence responses (such as callose and PR genes; Fig. 5) and more host leaf necrosis (Figs 1 and 2). The Xcc T3SS effector suite also, in at least some instances, contributes demonstrably to bacterial growth (Figs 1 and 6).

DISCUSSION

Pseudomonas, *Ralstonia* and *Xanthomonas* mutants deficient in *hrcC* or other core components of the T3SS consistently exhibit a

near-complete loss of pathogenicity on host plants (Alfano and Collmer, 1996, 2004; Kamoun and Kado, 1990; Lindgren *et al*., 1986; Rahme *et al*., 1991; see also Coburn *et al*., 2007). In contrast, although Xcc strain B186 was strongly dependent on the T3SS for *in planta* growth, we found that the loss of T3SS core structural genes (*hrcC* or *hrpE*) had a distinctly minor impact on the *in planta* growth of Xcc B305. However, Xcc B305 Δ*hrcC* or Δ*hrpE* strains exhibited a delay and overall reduction in disease symptom development in Arabidopsis and *N. benthamiana*. The Δ*hrcC* disease phenotype was restored to wild-type levels if a full-length *hrcC* gene was introduced into the mutant strain. B305 Δ*hrcC*Δ*flgBC* double mutants also grew like the wild-type strain when vacuum infiltrated into the leaf, suggesting that the *in planta* growth of Xcc B305 Δ*hrcC* could not be attributed to the alternative secretion of T3S effectors via the flagellar biosynthetic apparatus, as has been observed in some systems (Journet *et al*., 2005). Some plant pathogenic bacteria have multiple T3S systems. It would be interesting to determine whether Xcc B305 carries other secretion systems that substitute for the canonical T3SS in contributing to *in planta* growth.

The differences in symptom development between wild-type B305 and B305 Δ*hrcC* indicate that the T3SS of wild-type B305 is sufficiently functional to secrete effectors that promote disease causation in mesophyll cells. However, direct assays for AvrBsT protein secretion and tests for AvrBsT-dependent HR (Fig. 3) indicated that, in contrast with Xcc B186, wild-type B305 carries a defective or differently regulated T3SS, which is consistent with the relative independence of B305 from T3SS noted in the preceding paragraph. We also observed that wild-type B305 and B305 Δ*hrcC* triggered a similar amount of callose deposition (Fig. 5), and that the populations of B305 and B305 Δ*hrcC* were similar in AvrPto-expressing Arabidopsis plants (Fig. 4B), whereas this was not the case for Xcc B186 (Figs 4A and 5). Previous studies have shown that some T3SS-independent factors, such as the major exopolysaccharide xanthan gum or cyclic β-(1,2) glucans in Xcc, suppress host defences and induce plant susceptibility (Rigano *et al*., 2007; Yun *et al*., 2006). These findings are consistent with our observations in suggesting that, at least in some strains, factors other than T3S effectors can contribute significantly to Xcc virulence.

The *in planta* growth of B305 was comparable with that of B186 Δ*hrcC* after vacuum infiltration, and B305 was less efficient in secreting the Xcv AvrBsT effector. These observations might suggest that B305 is a universally less virulent strain. However, given the fact that the population size of B305 was equal to or larger than that of B186 after spray inoculation onto Arabidopsis leaves (Fig. 1A; see also Sun *et al*., 2006), other explanations cannot be ruled out. It is known that some vascular pathogens, such as *Xylella fastidiosa* and certain strains of *Pectobacterium carotovorum* and *Pectobacterium wasabiae*, have no T3SS (Kim *et al*., 2009a; Lambais *et al*., 2000; Van Sluys *et al*., 2002). For vascular pathogens, a T3SS may be less essential in the vessel-colonizing phase of a vascular lifestyle, where a smaller proportion of the bacterial population is in contact with 'living' plant cells. This hypothesis is consistent with our observations. In some cases, the *in planta* bacterial population of B305 was significantly higher than that of Xcc B305 T3SS mutants after pressure infiltration into leaf mesophyll, despite no differences after hydathode inoculation into Arabidopsis. In addition, Xcc B305 was more virulent than Xcc B186 after hydathode inoculation, but this was reversed when bacteria were introduced into the entire mesophyll by vacuum infiltration. Pathogens such as Xcc are likely to exhibit only partial overlap between the mechanisms required for vascular and mesophyll colonization, with strain-to-strain differences apparent. Significant strain-to-strain differences in virulence mechanisms may be an increasingly common observation in pathogenesis research given that, for example, 15%–20% of the genes in Xcc pathovars are absent or highly divergent among strains (He *et al*., 2007b; Lu *et al*., 2008). Hence, the Xcc B305/B186 contrast may be useful for future studies examining the specific structures, secreted molecules, regulatory pathways and other features that allow bacteria to successfully invade through hydathodes and colonize vascular tissues.

In the present study, co-inoculation with wild-type Xcc B186 increased the *in planta* growth of Xcc Δ*hrcC* mutants, revealing some defence suppression activity by the wild-type strain. This observation is consistent with many previous studies (Alfano and Collmer, 2004; Block *et al*., 2008; Brown *et al*., 1995; Jakobek *et al*., 1993; Speth *et al*., 2007). However, in contrast with many of these previous studies with other bacteria, wild-type Xcc B305 and B186 elicited more host callose deposition and PR gene expression than did their T3SS-defective mutants, on a compatible host. Similarly, in a compatible interaction between turnip and Xcc strain 8004, wild-type bacteria induced a host β-1,3-glucanase gene more strongly than did an *hrp* mutant (Newman *et al*., 1994). This phenomenon was also observed in two recent studies published after the present report was submitted (Oh *et al*., 2010; Rong *et al*., 2010).

Given the reduced macroscopic damage caused by T3SS-defective mutants, even in cases in which these mutants achieved similar leaf population levels, the simplest explanation for the greater elicitation of host defences by the T3SS-defective mutants is that the T3SS components or effectors of Xcc contribute directly to the elicitation of host responses as well as to the suppression of host defences. However, other alternative explanations for the data obtained cannot be ruled out. In many cases, defences are more evident in plants in which bacteria are multiplying at a higher rate, causing more damage and/or producing more T3SS-independent elicitors. This explanation may be more relevant to the concept of the host recognition of 'patterns of pathogenesis'—that the immune system responds to PAMPs in

the context of additional signals (Vance *et al*., 2009). A recent study on Xcc 8004 has suggested that effector-triggered immunity contributes to the induction of defence responses in Arabidopsis, such as callose deposition and PR gene expression—a third explanation of this phenomenon (Rong *et al*., 2010). Other studies have also demonstrated that several T3S effectors have quantitative (weak) avirulence activities on their susceptible hosts (Vinatzer *et al*., 2006). Our findings and those of others apparently demonstrate that, as bacterial pathogens and their hosts co-evolve, strains can be present whose T3SS effector suite plays a greater or lesser role in facilitating growth within hosts, and in eliciting rather than suppressing defences. Individual effectors are well known to serve as virulence factors or as strong avirulence factors depending on the host genotype, but there are also effectors that are only weak avirulence factors or trigger an atypical HR. It is interesting to consider that a mild defence-eliciting activity of effectors may provide adaptive utility to the pathogen.

The loss of disease symptomatology despite relatively unaltered bacterial titres, observed for Xcc B305, provides a unique form of evidence for the contribution of a portion of the Xcc T3SS effector suite to disease symptom development. More typically, loss of symptom-inducing effectors also causes a major decrease in bacterial growth within plants (White *et al*., 2009). The T3S effector suite of Xcc has been partially predicted and studied (Castaneda *et al*., 2005; Jiang *et al*., 2008, 2009; Qian *et al*., 2005; Wang *et al*., 2007; Xu *et al*., 2008). It would be interesting to discover the Xcc T3S effectors that act as lesion-promoting factors, and to determine whether these are the same as or different from the Xcc effectors that elicit defence responses.

The plant FLS2 receptor activates biologically significant defences in response to the flagellins of many bacteria, yet some Xcc strains cause similar disease whether or not they have a flagellin that is detectable by Arabidopsis FLS2 (Chinchilla *et al*., 2006; Forsyth *et al*., 2010; Gomez-Gomez and Boller, 2000, 2002; Pfund *et al*., 2004; Sun *et al*., 2006). In the present study, we observed that FLS2-mediated bacterial growth restriction was not unmasked when the T3SS was knocked out, suggesting that T3S effectors are not the primary reason why FLS2 plays a less detectable role in defence against Xcc than it does, for example, with *P. syringae* pv. *tomato* (Zipfel *et al*., 2004). Previous work has shown that Arabidopsis FLS2-mediated defences can restrict Xcc growth if they are activated with flagellin 1 day prior to Xcc infection, but, on naive Arabidopsis, the presence or absence of FLS2 does not have a detectable impact on the outcome of Xcc infections (Sun *et al*., 2006). With no evidence for strong FLS2 suppression via these Xcc secretion systems, other hypotheses for the lack of FLS2 impact on Xcc merit testing. Xcc strains may not make or expose sufficient flagellin within host leaves to elicit an adequate defence response; non-T3S compounds [such as cyclic β-(1,2) glucans] may suppress FLS2-mediated defences; or the plant vasculature (the most typical route for Xcc infection) may be less sensitive to flagellin than is the leaf mesophyll.

AvrPto is a widely conserved T3S effector of *Pseudomonas* species, and can suppress multiple host defences during compatible interactions (Hauck *et al*., 2003; He *et al*., 2006). AvrPto binds directly FLS2, EFR and possibly other MAMP receptors or co-receptor proteins for defence suppression (Shan *et al*., 2008; Xiang *et al*., 2008, 2011). Induced expression of *avrPto* in transgenic Arabidopsis plants has been reported to enhance the growth of a *P. syringae* pv. *tomato* strain DC3000 Δ*hrcC* mutant, but to have no significant growth-promoting effect on wild-type *P. syringae* pv. *tomato* strain DC3000 that expresses an endogenous *avrPto* (Hauck *et al*., 2003). In the present study, we found that the expression of *avrPto* greatly benefited the growth of both wild-type and Δ*hrcC* mutant strains of Xcc. This suggests that FLS2-independent defence pathways targeted by AvrPto play a significant role in the Xcc–host interaction and restrict Xcc growth in the host mesophyll, as also indicated recently for Xcv (Kim *et al*., 2009b).

AvrPto allowed greater growth of wild-type B186, but did not suppress significantly the callose or PR gene mRNA levels elicited by wild-type B186. This indicates that callose deposition and expression of *PR-1* and *PR-5* are not the major defence responses that restrict the *in planta* growth of Xcc B186. Hence, caution may be advisable in the use of callose deposition as a marker for PAMP-triggered immunity in the study of Xcc–Arabidopsis interactions. Because the induction of these responses was reduced by Δ*hrcC* mutation of B186, B186 apparently carries T3S effectors or structural components that elicit defences in Arabidopsis via mechanisms that are not inhibited by AvrPto. AvrPto has been predicted to inhibit MAMP (PAMP) perception rather than T3S effector-triggered immunity (Jones and Dangl, 2006). It is probable that MAMPs from the T3SS mutants elicit basal defences that can be inhibited by AvrPto (callose accumulation, expression of FRK1 and weak expression of PR genes), whereas T3S effectors from Xcc B186 apparently elicit defences (more callose deposition and stronger PR gene expression) via additional pathways that are not inhibited by AvrPto.

To summarize, the contributions of the T3SS were found to differ in certain respects for Xcc relative to other plant pathogenic bacteria, and to differ between wild-type Xcc strains. The T3SS is required for the *in planta* bacterial growth of some, but not all, Xcc strains, whereas its loss significantly attenuates disease symptom causation in all examined cases. The impacts of FLS2 on defence against wild-type Xcc have not been detected previously and, in the present study, disruption of the T3SS did not reveal any latent effects of FLS2-mediated defences that are suppressed by the Xcc T3SS. The contribution of ectopic AvrPto expression to Xcc growth within plants suggests that one or more host factors targeted by AvrPto, other than FLS2, contribute

significantly to the restriction of Xcc growth. Lastly, the T3SS components or effector suite in virulent Xcc induces host basal defence responses while promoting virulence.

EXPERIMENTAL PROCEDURES

Xcc strains

Xanthomonas campestris pv. *campestris* wild-type strains, originally from the collection of Dr Norman Schaad, were the kind gift of Dr Wesley Chun and Dr Al Poplawsky at the University of Idaho, Moscow, ID, USA. Xcc B94 was collected from field mustard, Xcc B186 from cauliflower and the source of Xcc B305 was not recorded.

Molecular biology methods

Standard molecular biology methods were used (Ausubel *et al*., 1997) unless noted. For DNA blot analyses, genomic DNA (500 ng) from Xcc strains was digested with *Sac*II or *Xho*I and probed with a ^{32}P-labelled PCR product generated with the primer set hrcC-probe-F/hrcC-probe-R, with final washing of the blot in 0.1 × standard saline citrate (SSC), 0.1% sodium dodecylsulphate (SDS) at 65 °C.

Construction of Xcc mutant strains with *hrcC*, *hrpE* and *flgB/C* single, double and triple gene deletions using nonmarker homologous recombination

DNA was isolated from different Xcc strains using a genomic DNA isolation kit (Promega, Madison, WI, USA). Two fragments, approximately 1 kb in length, upstream and ending at the start codon of the *hrcC* gene, or downstream and beginning at the stop codon of the *hrcC* gene, were independently PCR amplified from Xcc genomic DNA using *Pfu* polymerase with the respective primer sets hrcC-5′-F/hrcC-del-R and hrcC-del-F/hrcC-3′-R with underlined *Bam*HI and *Xho*I restriction sites, respectively (Table S2, see Supporting Information). These two PCR products were gel purified and added together into a splice overlap PCR (the pairs of 'del' primers carry complementary sequences and will anneal). The resultant PCR fragment carrying flanking regions of the *hrcC* gene, but lacking the *hrcC* open reading frame, was cloned into the pUFR80 suicide vector (Castaneda *et al*., 2005), a *sacB* suicide vector that allows the generation of precise unmarked chromosomal gene replacements/deletions in Gram-negative bacteria (Ried and Collmer, 1987). Construction and screening of Xcc Δ*hrcC* mutants were performed following the procedures described by Sun *et al*. (2006). Briefly, pUFR80-Δ*hrcC* plasmids were electroporated into Xcc and subjected to kanamycin selection. Xcc transformant single colonies with kanamycin resistance were picked and cultured in nutrient yeast glycerol (NYG) medium (Daniels *et al*., 1984) overnight with no kanamycin, and then spread onto nutrient yeast glycerol agar (NYGA) plates with 5% sucrose to select sucrose-insensitive clones. The gene deletion genotype of kanamycin-sensitive/sucrose-insensitive Xcc colonies was confirmed by colony PCR and by sequencing PCR products, as well as by Southern blot analyses. The same strategy was applied to construct precise open reading frame deletion strains for *hrpE* and *flgB/C*, except that *Bam*HI and *Hin*dIII restriction sites were created by PCR for deletion fragments (Table S2). For double mutants, the Δ*hrcC* single mutant strain was used to construct the second deletion. For complementation experiments, the full-length *hrcC* gene, including the 5′ and 3′ regulatory sequences (687 and 225 bp, respectively, for *hrcC*), was amplified by PCR using the respective primer sets hrcC-comp-F/hrcC-comp-R (Table S2). Hence, the *hrcC* complementation construct carried short segments of adjacent open reading frames from separate operons, but no other full-length genes. The *hrcC* complementation PCR fragments were cloned into the wide-host-range vector pVSP61 (Loper and Lindow, 1987) and electroporated into the specified Xcc strains.

Plant inoculation and pathogen growth assays

All strains were grown at 28 °C on NYGA medium. For hydathode inoculation, 3-week-old *Arabidopsis* Col-0 and *fls2-101* mutant plants were placed in a dew chamber (wall, 10 °C; water, 28 °C; air, 22 °C) for 4–5 h until guttation drops formed at the edges of the leaves. Bacteria at approximately 1×10^9 colony-forming units (cfu)/mL in 10 mM $MgCl_2$/0.04% Silwet L-77 were then gently misted onto the leaves, and the plants were returned to their normal growth chamber and covered with transparent domes to maintain increased humidity for 2 days. To assess bacterial populations in hydathode-infected leaves, four entire leaves of similar size were removed 4 days after inoculation, surface sterilized in 70% ethanol for 45 s and rinsed three times with sterile distilled water. Leaf discs were then punched from surface-sterilized leaves before grinding in 10 mM $MgCl_2$, and extracts were serially diluted on NYGA plates as described above. Triplicate samples were tested (12 leaves per treatment). For vacuum infiltration, a fresh culture of bacteria was scraped off the plate, resuspended in 10 mM $MgCl_2$ with 0.005% Silwet L-77 (Lehle Seeds, Round Rock, TX, USA) to 10^6 cfu/mL and vacuum infiltrated into leaves. The same concentration of Silwet L-77 was included in all mock treatments. Three days after infection, leaf discs from four different leaves were combined and ground in 10 mM $MgCl_2$ with a microcentrifuge tube plastic pestle, with triplicate replication (12 leaves in total). The samples were vortex mixed, diluted 1:10 serially, plated on NYGA solid medium and colonies were counted after 2 days of growth at 28 °C. Colonies with a non-Xcc morphology were only rarely observed. For syringe inoculation in callose deposition assays, a fresh culture of bacteria was scraped

off the plate, resuspended in 10 mM $MgCl_2$ to 10^8 cfu/mL and infiltrated into leaves by syringe infiltration using a 1-cm^3 plastic syringe with no needle. Unless otherwise noted, bacteria were applied to plant leaves by vacuum infiltration and populations were sampled 3 days after inoculation. For the experiments in Fig. 6, the unmarked Xcc Δ*hrcC* strains were electroporated with pVSP61 plasmid (no insert) with a kanamycin resistance gene to allow the differentiation of wild-type (Kan^S) and Δ*hrcC* mutant (Kan^R) strains. One-way analysis of variance was performed using Minitab (release 14).

Macroscopic HR assays

Arabidopsis thaliana Pi-0 plants were grown for 4–6 weeks at 22 °C with a 12-h light/12-h dark cycle, and fully expanded leaves were used for macroscopic HR analyses. Wild-type and mutant strains of Xcc B186 and B305 with either pDD62(empty vector) or pDD62-avrBsT-HA (Cunnac *et al*., 2007) were cultured in NYG medium overnight and resuspended in 1 mM $MgCl_2$ at $OD_{600} = 0.3$ for inoculation. Leaf mesophyll tissue was infiltrated using a syringe with no needle. The severity of macroscopic HR tissue collapse was scored at 24 h after inoculation according to the following scale: 0, no collapse; 1, hints of collapse, leaf looks normal but inoculated region is slightly less turgid when touched on the edge; 2, obvious but subtle tissue collapse, inoculated areas retaining original green colour and somewhat turgid; 3, intermediate or patchy, some areas grey–green and collapsed; 4, strong response, entire inoculated area grey–green and collapsed; 5, severe, entire inoculated area thin and drying out.

Secretion assays and Western blot analysis

Secretion assays were performed as described previously with minor modifications (Rossier *et al*., 1999; Wang *et al*., 2007). The wild-type and *hrcC* mutant Xcc with AvrBsT-HA were cultured in NYG medium for 24 h. After washing twice with 1 mM $MgCl_2$, the cells were diluted in 10 mL of the hrp-inducing secretion medium SMMXC to $OD_{600} = 0.4$, and cultured at 28 °C for another 5 h. The total culture (0.2 mL) was precipitated for 1 h on ice with 10% trichloroacetic acid (TCA). After centrifugation at 15 000 ***g*** for 40 min at 4 °C, protein precipitates were washed with ethanol and resuspended in 40 μL of Laemmli buffer. The supernatant was collected after centrifugation at 4000 ***g*** for 10 min at 4 °C, and then filtered through 0.45-μm filters. The supernatant was precipitated with TCA and resuspended in Laemmli buffer (1/200 v/v). Proteins were separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and detected by immunoblot using anti-HA antibody.

RNA isolation and RT-PCR

Three leaves of Col *gl-1* and *avrPto* transgenic plants (Hauck *et al*., 2003) were collected 1 day after pressure inoculation of Xcc B186, B305 wild-type and mutant strains for RNA isolation. Total RNA was isolated and subjected to on-column DNase digestion using an RNeasy mini kit according to the protocol provided (Qiagen, Valencia, CA, USA). cDNA was synthesized by reverse transcriptase Superscript III (Invitrogen, Carlsbad, CA, USA) using 2 μg of total RNA as template. One microlitre of cDNA product from RT reactions was used as template for PCR performed with GoTaq polymerase (Promega, Madison, WI, USA). The primer sets used to track the expression of different genes are listed in Table S2. PCR products were separated by gel electrophoresis and stained with ethidium bromide before viewing.

Callose deposition assay

For callose studies, bacteria were applied by syringe inoculation at 10^8 cfu/mL in 10 mM $MgCl_2$, and flg22 peptide (Gomez-Gomez *et al*., 1999) was applied as a 10 μM solution in water. Callose staining was performed 24 h after bacterial inoculation, as described previously (Gomez-Gomez *et al*., 1999). Briefly, the bacteria-infiltrated leaves were detached from the plants and fixed overnight in 1% glutaraldehyde, cleared and dehydrated with 100% ethanol. The leaves were sequentially rehydrated with 50% ethanol and 67 mM K_2HPO_4, and stained with 0.01% aniline blue for callose observation. Callose deposits were examined with an Olympus BX60 photomicroscope (Tokyo, Japan) with a 4′,6-diamidino-2-phenylindole (DAPI) fluorescence filter. The number of callose deposits was quantified (typically six replicates per treatment) with IMAGE PRO PLUS software (Media Cybernetics, Bethesda, MD, USA).

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SUPPORTING INFORMATION

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

Fig. S1 Southern blot analysis indicates that *Xanthomonas campestris* pv. *campestris* (Xcc) B94, B186 and B305 contain one copy of *hrcC*. M, marker; WT, wild-type.
Fig. S2 Symptom differences on Arabidopsis leaves after spray inoculation with *Pseudomonas syringae* pv. *tomato* (Pst) DC3000 (A) and *Xanthomonas campestris* pv. *campestris* (Xcc) strains (B). Note that DC3000 causes disease symptoms in the midst of leaves primarily through stomates, whereas Xcc causes disease symptoms at the margins of leaves primarily through hydathodes.
Fig. S3 Similar growth of *Xanthomonas campestris* pv. *campestris* (Xcc) strain B305 with or without a functional hrcC in the leaves of Arabidopsis ecotype Ws and Ler plants. All data are means ± standard error.
Fig. S4 In vacuum-infiltrated Arabidopsis leaves, growth of *Xanthomonas campestris* pv. *campestris* (Xcc) B305 was slightly impaired by Δ*hrcC*, but not detectably impaired by Δ*hrpE*, Δ*flgB/C*, Δ*tatC* single, double or triple gene deletions. All data are means ± standard error. Asterisks above the bars indicate significantly different from wild-type (WT) B305 on same plant genotype (analysis of variance, $P < 0.05$). (A) Population sizes of B305 and mutants, as noted, in vacuum-infiltrated Arabidopsis Col-0 and Col-0 *fls*2-101 leaves. (B) Growth of B305 and mutants in Arabidopsis leaves (typically at least 10-fold within 2 days after inoculation) after vacuum infiltration.
Fig. S5 Elicitation of Arabidopsis basal defence responses by *Xanthomonas campestris* pv. *campestris* (Xcc) was impaired when the *hrcC* gene was deleted. Xcc infiltration elicits callose deposits in *fls2*-101 leaves, whereas flg22 treatment does not.
Table S1 Mutant strains used in this study, with phenotypes noted.
Table S2 Primers for gene knockout, complementation and reverse transcription-polymerase chain reaction (RT-PCR) experiments.

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