Proc. Natl. Acad. Sci. USA
Vol. 96, pp. 9368–9373, August 1999
Microbiology

The *Xanthomonas* Hrp type III system secretes proteins from plant and mammalian bacterial pathogens

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Communicated by Brian J. Staskawicz, University of California, Berkeley, CA, June 10, 1999 (received for review April 2, 1999)

ABSTRACT **Studies of essential pathogenicity determinants in Gram-negative bacteria have revealed the conservation of type III protein secretion systems that allow delivery of virulence factors into host cells from plant and animal pathogens. Ten of 21 Hrp proteins of the plant pathogen *Xanthomonas campestris* pv. *vesicatoria* have been suggested to be part of a type III machinery. Here, we report the *hrp*-dependent secretion of two avirulence proteins, AvrBs3 and AvrRxv, by *X. campestris* pv. *vesicatoria* strains that constitutively express *hrp* genes. Secretion occurred without leakage of a cytoplasmic marker in minimal medium containing BSA, at pH 5.4. Secretion was strictly *hrp*-dependent because a mutant carrying a deletion in *hrcV*, a conserved *hrp* gene, did not secrete AvrBs3 and AvrRxv. Moreover, the Hrp system of *X. campestris* pv. *vesicatoria* was able to secrete proteins from two other plant pathogens: PopA, a protein secreted via the Hrp system in *Ralstonia solanacearum*, and AvrB, an avirulence protein from *Pseudomonas syringae* pv. *glycinea*. Interestingly, *X. campestris* pv. *vesicatoria* also secreted YopE, a type III-secreted cytotoxin of the mammalian pathogen *Yersinia pseudotuberculosis* in a *hrp*-dependent manner. YerA, a YopE-specific chaperone, was required for YopE stability but not for secretion in *X. campestris* pv. *vesicatoria*. Our results demonstrate the functional conservation of the type III system of *X. campestris* for secretion of proteins from both plant and mammalian pathogens and imply recognition of their respective secretion signals.**

Studies of bacterial pathogens of plants and mammals have revealed common strategies to interact with their hosts. A striking example is the conservation of a protein-secretion system essential for pathogenicity in distantly related Gram-negative bacteria. Type III secretion systems serve to deliver virulence factors into host cells and have been identified in 10 pathogenic bacteria, including the mammalian pathogens *Yersinia* spp., *Shigella flexneri*, *Salmonella* spp., enteropathogenic *Escherichia coli*, *Pseudomonas aeruginosa*, and *Chlamydia* spp., and the plant pathogens *Erwinia* spp., *Pseudomonas syringae*, *Ralstonia solanacearum*, and *Xanthomonas campestris* (for a review on type III systems, see ref. 1). Type III secretion systems are encoded by approximately 20 genes clustered in 18- to 40-kb regions, either in the chromosome or on virulence plasmids. Nine genes appear to be homologous in all gene clusters sequenced so far (1). Secretion via the type III pathway is *sec*-independent and occurs without the cleavage of an N-terminal signal sequence. In *Yersinia* spp., for which this secretion system was first characterized, secretion in the culture medium occurs at 37°C in the absence of Ca^{2+}. Type III-secreted proteins in *Yersinia* include YopH, a protein phosphatase, YopE and YopT, two cytotoxins, and YopJ (also called YopP), a protein that induces apoptosis in macrophages (2).

Genes encoding type III secretion systems in plant pathogens were identified by the isolation of mutants that were unable to cause disease in susceptible plants and no longer elicited the hypersensitive reaction (HR), a rapid localized plant cell death, in resistant plant tissue. The corresponding genes were designated *hrp* for "hypersensitive reaction and pathogenicity" (1). In our laboratory, we study *X. campestris* pv. *vesicatoria*, the causal agent of bacterial spot disease in pepper and tomato. The 25-kb *hrp* gene cluster is localized in the chromosome of *X. campestris* pv. *vesicatoria* and contains six transcription units, designated *hrpA* to *hrpF* (3). Based on sequence homologies and protein localization studies, 10 Hrp proteins have been suggested to be part of the core secretion apparatus (refs. 4–7; O.R. and U.B., unpublished data). Two regulators, HrpG and HrpXv, are encoded elsewhere in the genome and govern *hrp* gene expression in *hrp* gene-inducing medium and in plant leaf tissue. HrpG is homologous to response regulators of two-component regulatory systems and activates the expression of *hrpA* and *hrpXv* (8). HrpXv is an AraC-type transcriptional activator of the operons *hrpB* to *hrpF* (9). Although *hrp* gene induction in culture has been obtained (6), no *hrp*-dependent protein secretion was observed so far.

The interaction of *X. campestris* pv. *vesicatoria* with its hosts is determined by gene-for-gene relationships (10). If matching genes for resistance and avirulence are expressed in the plant and in the bacterium, respectively, the bacterium is recognized resulting in the induction of plant-defense responses, such as the HR. In *X. campestris* pv. *vesicatoria*, several avirulence genes have been cloned (10). In some cases, lack of the *avr* (avirulence) gene causes a reduction of bacterial growth in susceptible hosts. However, the function of most *avr* components remains unknown (11). The *avrBs3* gene is one of the best-studied avirulence genes in *X. campestris* pv. *vesicatoria* (12). Interestingly, the recognition of the AvrBs3 protein in pepper plants carrying the corresponding resistance gene, *Bs3*, was shown to occur inside the plant cell (13). Moreover, the function of *avrBs3* is *hrp*-dependent, because *hrp* mutants are no longer able to induce the HR on resistant pepper plants (14). Thus, AvrBs3 is a likely candidate to be delivered via the Hrp apparatus into the plant cell.

In this study, we have established conditions for *hrp*-dependent secretion of proteins by *X. campestris* pv. *vesicatoria* in culture. We show that the avirulence proteins AvrBs3 and AvrRxv from *X. campestris* pv. *vesicatoria* are secreted via the Hrp machinery. Furthermore, we demonstrate that the Hrp

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Abbreviations: HR, hypersensitive reaction; avr, avirulence; GUS, β-glucuronidase; hrp, hypersensitive reaction and pathogenicity; HA, hemagglutinin.

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secretion system from *Xanthomonas* is promiscuous for the secretion of heterologous proteins from bacterial pathogens of both plants and mammals.

MATERIALS AND METHODS

Bacterial Strains, Growth Conditions, and Plasmids. Bacterial strains used in this study were *E. coli* strains DH5α (Bethesda Research Laboratories), DH5α λpir (15), and HB101 (16) and *P. syringae* pv. *maculicola* M4 (17). *X. campestris* pv. *vesicatoria* strains 82–8 and 56 were described previously (10, 18). Strain 82* is a derivative of 82–8 that expresses a mutated version of *hrpG.* The amino acid substitution E44K leads to constitutive *hrp* gene expression in noninducing medium (K.W., O.R., and U.B., unpublished data). *E. coli* cells were cultivated at 37°C in LB and *Xanthomonas* strains at 30°C in NYG broth (19), in *hrp*-inducing medium XVM2 (6), or in minimal medium A (see ref. 20) (MA) and M9 (20) supplemented with sucrose (10 mM) and casamino acids (0.3%). Antibiotics were added to the media at the following final concentrations: 100 μg/ml ampicillin, 25 μg/ml kanamycin, 10 μg/ml tetracycline, 100 μg/ml rifampicin, and 100 μg/ml spectinomycin. Plasmids were introduced into *E. coli* by electroporation and into *Xanthomonas* by conjugation, by using pRK2013 as a helper plasmid in triparental matings as described previously (7).

Generation of a Mutation in the Secretion Apparatus. The nonpolar deletion mutant 82*Δ*hrcV* was constructed as follows. First, a suicide plasmid, pRO1, was constructed by replacing the gene encoding the antibiotic resistance marker of pSB360 (21). The *Sma*I 6.2-kb fragment was ligated to the *Hin*dIII filled-in Ω fragment that confers spectinomycin resistance (22), resulting in pRO1. Plasmid pUCB11o, which carries a 5,900-bp insert encompassing *hrcV*, was linearized with *Sfi*I, rendered blunt, and digested with *Eco*RV. Religation created a 562-bp deletion in *hrcV* (from position 752 to 1314 with respect to putative translation start; pUCBΔ*hrcV*). The insert of this plasmid was cloned into the *Bam*HI site of the suicide vector pRO1, giving pROΔ*hrcV*, which was introduced into 82* as described (7). For complementation analysis of the resulting mutant 82*Δ*hrcV*, the Ω fragment was introduced into the *Sna*BI site of pUCB11o, thereby disrupting the *hrpC3* gene, which is downstream of *hrcV* in the same operon. The insert of the resulting plasmid was cloned into the broad host-range vector pLAFR6 (12), giving pSCOB, which complemented strain 82*Δ*hrcV*.

Plant Material and Plant Inoculations. Inoculation of tomato cultivar Hawaii 7998, which recognizes *avrRxv* (23), and the near-isogenic pepper cultivars Early Cal Wonder (ECW) and ECW-30R (which carries the resistance gene *Bs3*) were performed as described (12).

Secretion Experiments. Bacteria were cultivated in MA, pH 7.0, at 30°C overnight and resuspended to an optical density of 0.1 (600 nm) in 4 ml of MA at pH 5.4 (acidified by addition of HCl) containing BSA (50 μg/ml, New England Biolabs). After 4–5 h, 0.5 ml of total culture (total protein) was precipitated for 30 min on ice with 10% trichloroacetic acid (TCA). After centrifugation at 14,000 $\times$ *g* for 10 min at 4°C, protein precipitates were washed with ethanol and resuspended in Laemmli buffer (1/10 vol) (24). The remaining culture was filtered with a low protein-binding filter (HT Tuffryn, 0.45 μm; Gelman). Filtrates (supernatants) were precipitated with TCA and resuspended in Laemmli buffer (1/50 to 1/200 vol). Aliquots of 5 μl were analyzed by immunoblotting as described previously (6). Antibodies used were polyclonal anti-AvrBs3 antibody (14), polyclonal anti-HrcN antiserum (U.B., unpublished data), anti-Flag M2 mAb (IBI/Kodak), monoclonal antihemagglutinin (HA) antibody (Eurogentec, Brussels), polyclonal anti-PopA antiserum (provided by C. Boucher, Toulouse, France), and specific anti-YopE and anti-YerA antisera (provided by H. Wolf-Watz, Umea, Sweden). Horseradish peroxidase-labeled goat anti-mouse or goat anti-rabbit antibodies were used as secondary antibodies. Reactions were visualized by enhanced chemiluminescence (Amersham Pharmacia).

Determination of β-Glucuronidase Activity. To express β-glucuronidase (GUS) in *X. campestris* pv. *vesicatoria*, the *uidA* gene from pKEx4tr-G (25) was cloned into the *Eco*RI/*Hin*dIII sites of pDSK602, giving pDGUS. This plasmid was introduced into strains 82* and 82*Δ*hrcV*. To determine GUS activities, aliquots of supernatants and total bacterial culture (cells and supernatants) were taken before TCA precipitation. 4-Methylumbelliferyl β-D-glucuronide was used as a substrate in a modified procedure from ref. 26 in which sodium phosphate concentration of the extraction buffer was increased to 100 mM. One unit is defined as 1 nmol of 4-methylumbelliferone released per minute and per bacterium.

Epitope Tagging of the AvrRxv Protein. The Flag epitope (IBI/Kodak) was engineered at the C terminus of AvrRxv by PCR, resulting in the replacement of the stop codon by a threonine and a serine codon, followed by the Flag-coding sequence (DYKDDDDK), a stop codon, and a *Hin*dIII restriction site. The construct was expressed under the control of the *lac* promoter present in the broad host-range vector pLAFR3 (27). For PCR amplification, plasmid pXV9009 (28) was used as a template by using primers 5′-GGAATTCTATGTGCGACTCC-3′ and 5′-CCCAAGCTTCACTTGTCATCGTCGTCCTTGTAGTCACTAGTGGATTCTAAGGCG-3′. After digestion of the PCR products by *Eco*RI and *Hin*dIII (specific sites are underlined in primer sequence), the 1,163-bp fragment was cloned into pUC118 (29). Clones were checked by sequence analysis. Subsequently, the *Eco*RI/*Hin*dIII insert was cloned into pLAFR3, giving pLRF.

Expression of *popA*, *avrB*, *yopE*, and *yerA* in *X. campestris* pv. *vesicatoria*. To express *popA* in *X. campestris* pv. *vesicatoria*, plasmid pAZ13 (provided by C. Boucher) was digested with *Xba*I and *Sac*I. The 1.6-kb insert containing *popA* first was cloned into the corresponding sites of pUC118 and then as an *Eco*RI-*Pst*I fragment into pLAFR3, resulting in pLAZ13. This construct contains 387 bp upstream of the translation start of *popA* and was shown to be functional, because supernatants of *R. solanacearum popA* mutant strain GM1551 (pLAZ13) induced the HR on petunia and tobacco. To express *yopE* and *yerA* of *Y. pseudotuberculosis* in *X. campestris* pv. *vesicatoria*, both genes were cloned in pLAFR3 under the control of the *lac* promoter, giving pLKW1. A 1,050-bp *Eco*RI-*Hin*dIII fragment of pAF19 (30) containing *yopE* was cloned into pBluescript-KS II (Stratagene) and pLAFR3, resulting in pBKW2 and pLKW2, respectively. The *yerA* gene was isolated on a *Dpn*I fragment from pAF19 and ligated into *Sma*I-digested pBKW2, resulting in pBKW1. The untranslated region between *yerA* and *yopE* was 375 bp long. The insert from pBKW1 was cloned as a *Bam*HI/*Hin*dIII fragment into pLAFR3.

RESULTS

The Avirulence Protein AvrBs3 from *X. campestris* pv. *vesicatoria* Is Secreted in a *hrp*-Dependent Manner. Previous studies have shown that recognition of the avirulence protein AvrBs3 from *X. campestris* pv. *vesicatoria* by *Bs3*-resistant pepper plants requires functional *hrp* genes and occurs inside the plant cell (13). We, therefore, assumed that AvrBs3 is secreted via the Hrp type III secretion system and we used it as a reporter to establish secretion conditions. Because bacteria grown in *hrp* gene-inducing medium do not secrete proteins in a *hrp*-dependent manner, we aimed for conditions that would disconnect *hrp* gene induction from secretion. We used strain 82*, a derivative of 82–8 (10) that contains *avrBs3* and constitutively expresses *hrp* genes because of a mutation in the regulatory gene *hrpG* (K.W., O.R., and U.B., unpublished

data). As a control for a cytoplasmic protein, the *uidA* gene, encoding GUS, was expressed in strain 82* by using plasmid pDGUS. Total protein extracts and culture supernatants were analyzed for the presence of AvrBs3 by immunoblotting. 82*(pDGUS) did not secrete AvrBs3 in solid or liquid NYG medium, in *hrp*-inducing medium XVM2, or in minimal medium at pH 7.0. Secretion in XVM2 and minimal medium could not be induced by changing growth temperature (in a range from 20 to 30°C), by addition of various concentrations of sodium, fructose, and sucrose, or by addition of cellulose acetate (mimicking plant cell walls) at logarithmic and stationary growth phase, respectively. Secretion was not observed in a minimal medium with a pH range of from 7.8 to 9.0. Bacterial growth in a minimal medium at pH 5.6 with different ion concentrations (Na^{2+}, Ca^{2+}, Fe^{2+}, and Mg^{2+}) or the addition of oxidized or reduced glutathione also failed to induce secretion.

Secretion of the AvrBs3 protein, however, was achieved by incubation of bacteria for 4–5 hr in a minimal medium that was acidified by addition of HCl to pH 5.4 and supplemented with BSA (50 μg/ml) (Fig. 1, lane 3). To test whether secretion was *hrp*-dependent, a nonpolar mutation in *hrcV*, a conserved *hrp* gene (4, 31), was introduced into 82*. The resulting strain, 82*Δ*hrcV*(pDGUS), expressed AvrBs3 (Fig. 1, lane 2); however, AvrBs3 was not detected in bacterial supernatants (Fig. 1, lane 4). To rule out bacterial lysis as a reason for AvrBs3 detection in the supernatant of 82*(pDGUS), protein samples were tested for the presence of HrcN, a conserved type III system-ATPase (4, 31). Localization studies by using a polyclonal antiserum specific for HrcN indicated that approximately 60% of this intracellular protein is in the soluble fraction, most likely in the cytoplasm (O.R. and U.B., unpublished data). As shown in Fig. 1, HrcN was present in total protein extracts of both 82*(pDGUS) and 82*Δ*hrcV*(pDGUS) (lanes 1 and 2), but it was not detected in supernatants (lanes 3 and 4). In addition, the activity of GUS, a normally cytoplasmic protein, was determined. The GUS activity in supernatants was equally low for both strains [0.61 ± 0.13 unit $\times 10^{-10}$ for strain 82*(pDGUS) and 1.18 ± 0.15 unit $\times 10^{-10}$ for strain 82*Δ*hrcV*(pDGUS)]. GUS activity in total protein extracts (lysed cells plus supernatant) reached 277.41 ± 24.87 units $\times 10^{-10}$ for 82*(pDGUS) and 280.11 ± 47.93 units $\times 10^{-10}$ for 82*Δ*hrcV*(pDGUS). These data confirm that the presence of AvrBs3 in the supernatant of 82*(pDGUS) was not a result of bacterial lysis but of AvrBs3 secretion in a *hrp*-dependent manner.

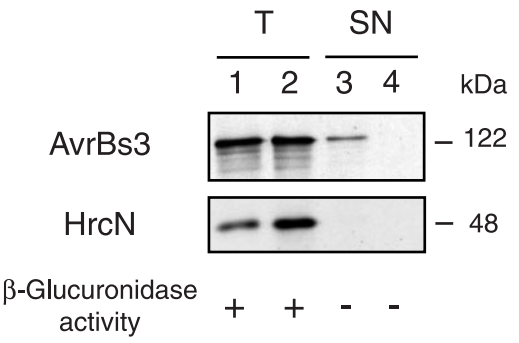

FIG. 1. *hrp*-dependent secretion of AvrBs3. Bacteria were incubated for 4 hr in minimal medium MA at pH 5.4 with BSA (50 μg/ml). Total protein extracts (T, lanes 1 and 2) and filtered supernatants (SN, lanes 3 and 4) were precipitated with TCA and concentrated 10- and 50-fold, respectively. Five-microliter protein samples of strains 82*(pDGUS) (lanes 1 and 3) and 82*Δ*hrcV*(pDGUS) (lanes 2 and 4) were separated by SDS/PAGE (8% polyacrylamide) and analyzed by immunoblotting by using polyclonal antibodies directed against AvrBs3 (upper blot) and HrcN (lower blot). The molecular mass (kDa) of the proteins is indicated. For β-glucuronidase activities, see text.

Effect of pH and BSA on Secretion of AvrBs3. The effect of the pH on secretion of AvrBs3 by 82*(pDGUS) was tested in the presence of BSA (50 μg/ml). At pH 7.0 or 5.8, AvrBs3 was not detectable in the supernatant (Fig. 2*A*), but the amount of AvrBs3 in the supernatant increased as the pH of the medium was lowered from 5.6 to 5.0 (Fig. 2*A*). The effect of the BSA concentration on AvrBs3 secretion was determined in MA medium at pH 5.4. Amounts of AvrBs3 in the supernatant decreased as the BSA concentration was lowered from 100 to 0 μg/ml (Fig. 2*B*). Throughout the remainder of this study, MA medium at pH 5.4 containing 50 μg/ml BSA was used as secretion medium. Under these conditions we estimated that 15–20% of the total amount of AvrBs3 was secreted. This was done by comparing signal intensities of dilutions of total protein extracts and supernatants in immunoblotting analyses (data not shown).

Secretion of AvrRxv. After having established conditions that allow AvrBs3 secretion in culture, we subsequently analyzed whether another avirulence protein, isolated from a different strain of *X. campestris* pv. *vesicatoria*, was secreted. AvrRxv is an avirulence protein present in strain 75–3 (23), and like AvrBs3, its recognition by resistant tomato lines depends on *hrp* gene function (32). AvrRxv was chosen because it is the only avirulence protein in a plant pathogen, besides its homolog AvrBsT (32), that shares similarity with virulence proteins of mammalian pathogens, i.e., YopJ from *Yersinia pseudotuberculosis* (33), YopP from *Y. enterocolitica* (34), and AvrA from *Salmonella typhimurium* (35) (47.6 and 45.9% similarity to AvrRxv, respectively). To allow immunodetection of AvrRxv, the Flag epitope was fused to the C terminus of the protein in plasmid pLRF. Avirulence activity of this construct was tested after conjugation into *X. campestris* pv. *vesicatoria* 56, a strain virulent on tomato cultivar Hawaii (18). Transconjugants carrying pLRF induced the HR on Hawaii. Total protein extracts of 82* and 82*Δ*hrcV* strains containing pLRF or the empty vector (pLAFR3) were analyzed by immunoblotting (Fig. 3). The Flag-specific antibody reacted with two proteins of 44 and 47 kDa in size. The presence of a 47-kDa protein in bacterial extracts probably is a result of an alterna-

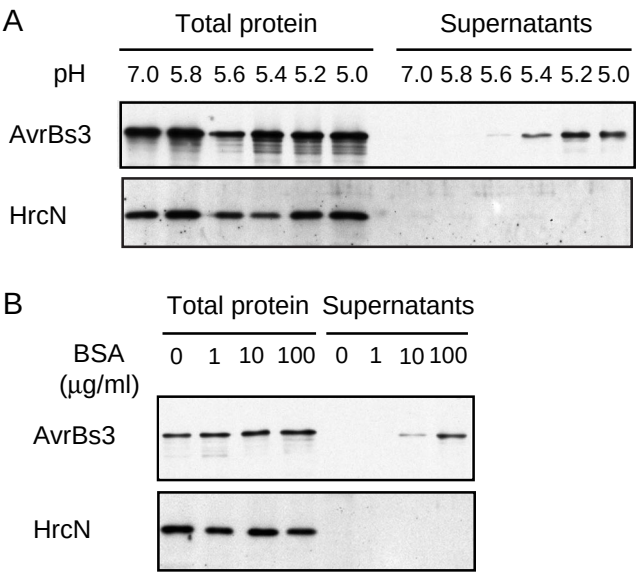

FIG. 2. Effect of pH and BSA on AvrBs3 secretion. Total protein extracts and supernatants of strain 82*(pDGUS) were separated by SDS/PAGE (8% polyacrylamide) and analyzed by immunoblotting by using AvrBs3- and HrcN-specific antibodies. (*A*) Effect of pH on AvrBs3 secretion. pH of the MA medium was varied from 7.0 to 5.0 in the presence of BSA (50 μg/ml). (*B*) Effect of BSA on AvrBs3 secretion. BSA concentration was varied from 0 to 100 μg/ml in the MA medium at pH 5.4.

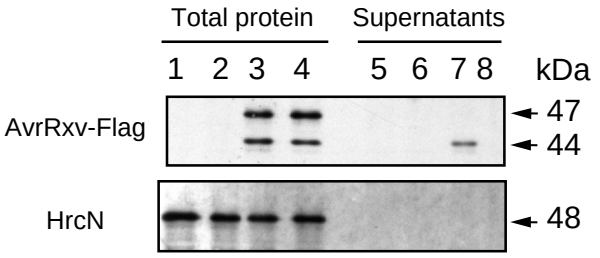

FIG. 3. *hrp*-dependent secretion of AvrRxv. Total protein (lanes 1–4) and supernatants (lanes 5–8) of 82*(pLAFR3) (lanes 1 and 5), 82*Δ*hrcV*(pLAFR3) (lanes 2 and 6), 82*(pLRF) (lanes 3 and 7), and 82*Δ*hrcV*(pLRF) (lanes 4 and 8) were separated by SDS/PAGE (10% polyacrylamide) and analyzed by immunoblotting by using anti-Flag antibody (*Upper*) or anti-HrcN antibody (*Lower*). The molecular mass of the proteins is indicated by an arrow.

tive translational start in the upstream region of *avrRxv* resulting in the addition of 25 aa. Only the 44-kDa protein was present in the supernatant of strain 82*(pLRF), and it was absent from the supernatant of the mutant 82*Δ*hrcV*(pLRF) (Fig. 3, lanes 7 and 8). No bacterial lysis was detectable by using the HrcN-specific antibody (Fig. 3), indicating that the 44-kDa protein was secreted in a *hrp*-dependent manner. Approximately 10–15% of the total amount of the 44-kDa protein was secreted.

PopA from *R. solanacearum* and AvrB from *P. syringae* Are Secreted by *X. campestris* pv. *vesicatoria*. Subsequently, the ability of the Hrp secretion system from *X. campestris* pv. *vesicatoria* to export proteins from other plant pathogenic bacteria was examined. The similarity between the *X. campestris* pv. *vesicatoria* and *R. solanacearum hrp* gene clusters (36) prompted us to test PopA, a secreted protein from *R. solanacearum* (37). *popA* was expressed in pLAFR3 under the control of the *lac* promoter (pLAZ13) in strains 82* and 82*Δ*hrcV*. Immunoblotting analyses showed that both strains expressed PopA1 (Fig. 4*A*, lanes 3 and 4). However, PopA1

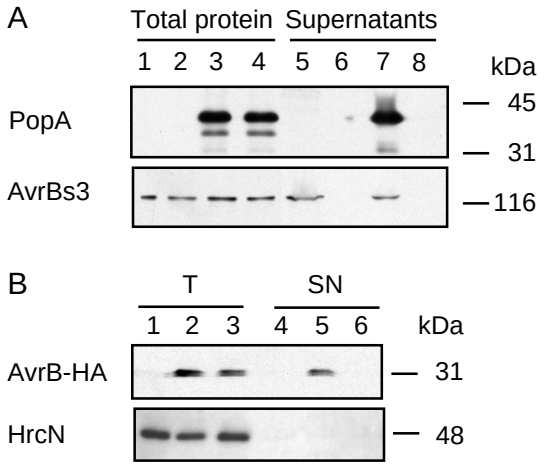

FIG. 4. PopA from *R. solanacearum* and AvrB from *P. syringae* are secreted by *X. campestris* pv. *vesicatoria*. (*A*) Immunoblotting analysis of total protein (lanes 1–4) and supernatants (lanes 5–8) of strains 82*(pLAFR3) (lanes 1 and 5), 82*Δ*hrcV*(pLAFR3) (lanes 2 and 6), 82*(pLAZ13) (lanes 3 and 7), and 82*Δ*hrcV*(pLAZ13) (lanes 4 and 8) by using an antibody directed against PopA (*Upper*) or against AvrBs3 (*Lower*). Total protein samples and supernatants were concentrated 10- and 100-fold, respectively. Molecular mass markers are indicated on the right. (*B*) Immunoblotting analysis of total protein (T, lanes 1–3) and supernatants (SN, lanes 4–6) of strains 82*(pVSP61) (lanes 1 and 4), 82*(pEM240) (lanes 2 and 5), and 82*Δ*hrcV*(pEM240) (lanes 3 and 6) by using anti-HA antibody (*Upper*) and anti-HrcN antibody (*Lower*). Total protein samples and supernatants were concentrated 50- and 200-fold, respectively.

was detectable only in the supernatant of 82*(pLAZ13) (Fig. 4*A*, lane 7). This was a result of specific secretion, because HrcN was not detected in the bacterial supernatants (data not shown). Approximately 10–15% of PopA1 was secreted in these conditions. Secretion of AvrBs3 by 82*(pLAZ13) was similar to that of 82*(pLAFR3), which harbors the vector alone (Fig. 4*A*, lanes 5 and 7), showing that expression of *popA* in *Xanthomonas* has no negative effect on AvrBs3 secretion.

In a similar experiment, we tested for secretion of AvrB, an avirulence protein from *P. syringae* pv. *glycinea*, a more distantly related plant pathogen. AvrB recognition in resistant plants depends on *hrp* genes (38) and has been shown to occur in the plant cell (39). A HA epitope-tagged version of AvrB was expressed in *X. campestris* pv. *vesicatoria* by using plasmid pEM240 (E. Marois and J. Dangl, unpublished data). In this construct, *avrB* is under the control of the promoter of *avrRpm1* (17), an avirulence gene from *P. syringae* pv. *maculicola*. Although *avr* and *hrp* gene regulation in *X. campestris* pv. *vesicatoria* differs from that in *P. syringae*, the construct used allowed low expression of the AvrB-HA protein in *Xanthomonas* (Fig. 4*B*, lanes 2 and 3). The AvrB-HA fusion protein was detected only in the supernatant of 82*(pEM240) (lane 5) and not in the supernatant of 82*Δ*hrcV*(pEM240) (lane 6). Again, the intracellular marker, HrcN, was not detectable in the supernatants (Fig. 4*B*). *X. campestris* pv. *vesicatoria*, therefore, is able to secrete PopA and AvrB, proteins from two different plant pathogenic bacteria via the Hrp-secretion pathway.

***X. campestris* pv. *vesicatoria* Secretes YopE of *Y. pseudotuberculosis*.** The ability of *Xanthomonas* to secrete PopA and AvrB prompted us to examine the secretion of a protein from a mammalian pathogen. The *Y. pseudotuberculosis* cytotoxin YopE, which is secreted via the Ysc/Lcr type III secretion system (40), was chosen for this study. Because secretion of YopE in *Yersinia* requires a specific chaperone, YerA (41), both *yopE* and *yerA* (on pLKW1) were expressed in *Xanthomonas* strains 82* and 82*Δ*hrcV*. Immunoblotting analyses showed that the bacteria expressed YopE, YerA, and the endogenous protein AvrBs3 (Fig. 5, lanes 3 and 4). Most interestingly, YopE was detected in the supernatant of 82*(pLKW1) but not in the supernatant of the mutant 82*Δ*hrcV*(pLKW1) (Fig. 5, lanes 9 and 10). The presence of YopE in the culture medium resulted from specific secretion, because the cytosolic YerA protein (42) was detected only in whole protein extracts and not in culture supernatants (Fig. 5). We estimated that 10–15% of the total amount of YopE was

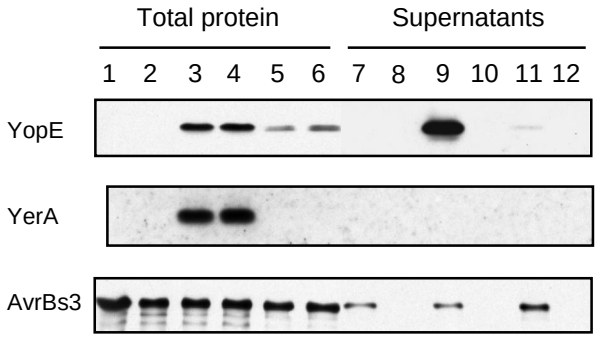

FIG. 5. YopE from *Y. pseudotuberculosis* is secreted by *X. campestris* pv. *vesicatoria*. Immunoblotting analysis of total protein (lanes 1–6) and supernatants (lanes 7–12) of strains 82*(pLAFR3) (lanes 1 and 7), 82*Δ*hrcV*(pLAFR3) (lanes 2 and 8), 82*(pLKW1) (lanes 3 and 9), 82*Δ*hrcV*(pLKW1) (lanes 4 and 10), 82*(pLKW2) (lanes 5 and 11), and 82*Δ*hrcV*(pLKW2) (lanes 6 and 12) by using antibodies specific for YopE (23 kDa) (*Top*), YerA (14 kDa) (*Middle*), and AvrBs3 (122 kDa) (*Bottom*). Total protein samples and supernatants were concentrated 10- and 100-fold, respectively. To allow YopE detection in lane 11, lanes 7–12 were overexposed.

secreted under these conditions. Secretion of AvrBs3 by 82*(pLKW1) was similar to that of 82* harboring the vector alone (Fig. 5, lanes 7 and 9), showing that expression of *yopE* in *Xanthomonas* has no negative effect on AvrBs3 secretion.

When *yopE* was expressed without its chaperone *yerA* (with plasmid pLKW2), the amount of YopE detectable in total protein extracts was reduced greatly in comparison with strains expressing *yopE* and *yerA* (Fig. 5, compare lanes 5 and 6 with lanes 3 and 4). This also was observed for whole-cell extracts of *E. coli* DH5α (data not shown). Nevertheless, small amounts of YopE were detected in the supernatant of 82*(pLKW2) (Fig. 5, lane 11). These results show that *X. campestris* pv. *vesicatoria* indeed does secrete YopE of *Y. pseudotuberculosis* via the Hrp secretion system.

Expression of *popA*, *avrB*, and *yopE* in *X. campestris* pv. *vesicatoria* Has No Effect on Pathogenicity. Bacterial strains expressing heterologous proteins were tested for their interaction with the plant. *Xanthomonas* strains 82* carrying an empty vector or expressing *popA*, *avrB*, and *yopE* [82*(pLAFR3), 82*(pLAZ13), 82*(pEM240), and 82*(pLKW1), respectively] were inoculated into pepper. None of the strains displayed a difference in disease-symptom formation or in HR induction in susceptible ECW and resistant ECW-30R pepper plants. We also tested whether *X. campestris* pv. *vesicatoria* expressing *avrB* induces an HR on *Arabidopsis* ecotype Columbia, which carries the corresponding resistance gene *RPM1*. In contrast to *P. syringae* pv. *maculicola* M4 strain containing *avrB* on plasmid pEM240, 82*(pEM240) did not induce an HR on *Arabidopsis* ecotype Columbia (data not shown). Thus, *X. campestris* pv. *vesicatoria* was not able to deliver the AvrB signal in *Arabidopsis*.

DISCUSSION

For plant pathogenic bacteria, *hrp* gene function in type III protein secretion has been difficult to study either *in planta* or in plant tissue culture. Therefore, we established *in vitro* conditions for *hrp*-dependent secretion by *X. campestris* pv. *vesicatoria*. Two avirulence proteins of *Xanthomonas*, AvrRxv and AvrBs3, were found to be secreted in a Hrp-dependent manner. These endogenous avirulence proteins were good candidates for type III secretion because their function depends on *hrp* genes. In other plant pathogenic bacteria, secreted proteins include nonspecific elicitors of the HR such as harpins and PopA (37, 43–46), HrpA, the subunit of the *P. s.* pv. *tomato* Hrp pilus (47), and DspA (also designated DspE), an essential pathogenicity factor of *Erwinia amylovora* (48). However, although harpin was secreted by *P. syringae* in *hrp*-inducing medium, the avirulence protein AvrB was not detected in culture supernatants (39), indicating that secretion of some proteins in this system may be gated differentially. In contrast to other plant bacterial pathogens, *Xanthomonas hrp* gene expression and protein secretion appear to be even more tightly controlled. Although all known *hrp* loci are expressed in a synthetic medium, XVM2 (6), and some Hrp proteins apparently are localized properly in the bacterial membranes (ref. 6; O.R. and U.B., unpublished data), it has been impossible to detect any specifically secreted proteins in culture supernatants. This suggests that *Xanthomonas* requires an additional trigger for secretion. The breakthrough became possible by disconnecting *hrp* gene induction from secretion conditions by constitutive gene expression and growth of the bacteria in a minimal medium at an acidic pH. In *P. syringae* (38) and *E. amylovora* (49), pH values between 5.5 and 5.7 were shown to be important for *hrp* gene induction. However, for *X. campestris* pv. *vesicatoria*, *hrp*-gene induction in XVM1 medium is almost abolished at acidic pH and optimal at pH 6.5–7.5 (50). The acidic pH that triggered secretion *in vitro* might reflect the situation in natural infections, i.e., in the plant leaf apoplast. However, because our experiments were done in a synthetic medium and in the absence of plant cells, we cannot rule out that *in planta* the trigger for secretion is a specific plant molecule. In some experiments, in which BSA was omitted, AvrBs3 was detected occasionally in bacterial supernatants, along with a large number of degradation products. Because BSA does not induce secretion at pH 7.0 (Fig. 2) and can be replaced by a different protein, e. g., triosephosphate isomerase (data not shown), BSA most probably plays a role in stabilizing secreted proteins rather than triggering secretion.

Sequence similarities of Hrp proteins from *X. campestris* pv. *vesicatoria* with components of type III secretion systems from plant and mammalian pathogens is suggestive of functional conservation. Recently, AvrB and AvrPto from *P. syringae* were found to be secreted by *E. coli* via the cloned Hrp system from *Erwinia chrysanthemi* (51), showing that this heterologous system is promiscuous for secretion of proteins from other plant pathogens. The idea of promiscuity also is supported by findings in this study. The *hrp* gene cluster from *R. solanacearum* is highly similar to that of *X. campestris* pv. *vesicatoria* in terms of overall sequence similarity, operon structure, and the *hrp* gene regulatory system (36). It, therefore, is not surprising that *Xanthomonas* secretes PopA from *R. solanacearum*. By contrast, the *hrp* gene cluster of *P. syringae* is quite different (36), and, yet, we found AvrB secretion by *Xanthomonas*. Even more interesting was the finding of YopE secretion by *X. campestris* pv. *vesicatoria*, an example of type III secretion of a virulence factor from a mammalian pathogen in a plant pathogenic bacterium. YopE is a cytotoxin in *Yersinia* spp. that is translocated into eukaryotic cells via the *ysc/lcr* type III secretion pathway (2). Functional conservation of the secretion and translocation machinery has been shown previously for virulence proteins of *Yersinia*, *Salmonella*, and *Shigella* (30). Our study shows that the conservation of type III secretion systems can be extended to plant pathogens. The chaperone YerA appears to stabilize YopE but is dispensable for YopE secretion in *Xanthomonas*. Similar observations have been described for YopE and YerA in *Y. pseudotuberculosis* (42). There is no evidence for the existence of specific chaperones for any of the secreted proteins presented in this study. The only chaperone described in plant pathogenic bacteria is the *E. amylovora* DspB protein (also called DspF), which is necessary for the secretion of DspA *in vitro* (48).

That the type III system of *X. campestris* is promiscuous for secreted proteins from different origins implies recognition of their respective secretion signals. No sequence homology was found in the N terminus of the proteins secreted in this study. Type III secretion signals have been best studied for *Yersinia* Yop proteins and were found to reside in the N-terminal domain. Minimal regions from the N-terminal domains of Yops (as short as 15 aa for YopE) were shown to be sufficient to allow secretion of a reporter protein (2). The secretion of the 44- but not the 47-kDa AvrRxv protein might indicate that, for *X. campestris* pv. *vesicatoria* also, the signal is present in the N-terminal region of the protein. Intriguingly, experiments by Anderson and Schneewind (52) point to a secretion signal in the 5′ end of the mRNA rather than in the peptide sequence of Yop proteins. Future work on type III secretion signals of proteins from plant pathogenic bacteria is needed to fully understand the underlying mechanism.

Data presented here establish the presence of a functional type III protein secretion system in *X. campestris* pv. *vesicatoria*, encoded by the *hrp* genes. In mammalian pathogens, type III secretion systems have been shown to translocate specific proteins into the host cell (1). In support of communality between plant and mammalian pathogenic bacteria, previous studies have demonstrated that certain avirulence proteins induced genotype-specific cell death (HR) when they were expressed in the plant cell (11). However, transport of bacterial avirulence proteins from the pathogen into the plant cell remains to be demonstrated. It also is not known whether

heterologously secreted proteins are translocated into the plant cell. Expression of AvrB or YopE in *Xanthomonas* did not alter pathogenicity or HR induction in plants. Our assay might not have been sensitive enough to observe some subtle effects on plant cells. Inoculation of *avrB*-expressing *X. campestris* pv. *vesicatoria* in *Arabidopsis* ecotype Columbia did not result in an HR. One possible explanation could be the low expression of AvrB in *X. campestris* pv. *vesicatoria*. Alternatively, AvrB might carry two signals, one for secretion, which is recognized by *X. campestris* pv. *vesicatoria*, and another signal for transfer into plant cells, which is not functional in *Xanthomonas*. The latter possibility is intriguing and is supported by studies in *Yersinia*. For Yop proteins, a modular structure has been reported, i.e., presence of an N-terminal secretion signal, a translocation signal located downstream, and a C-terminal effector domain (2). Whether only the secretion signal, but not the translocation signal of heterologous proteins, is conserved and recognized in the complex type III secretion system is a possibility that awaits further experimentation.

In conclusion, this study opens an important route to the identification of additional substrates of the secretion machinery of *X. campestris* pv. *vesicatoria*, especially pathogenicity factors.

Note Added in Proof. Type III-dependent *in vitro* secretion of another bacterial avirulence protein, AvrRpt2 from *P. syringae* pv. *tomato*, has recently been demonstrated by Mudgett and Staskawicz (53).

We thank Christian Boucher, Hans Wolf-Watz, Jorge Galán, Eric Marois, and Jeff Dangl for kindly providing plasmids and antisera used in this study and Matthieu Arlat for testing functionality of the pLAZ13 construct in *R. solanacearum*. We are grateful to John Mansfield and Thomas Lahaye for critical reading of the manuscript. This work was funded in part by an Action Concertées Coordonnées–Sciences du Vivant 6 grant from the French Ministère de l'Education Nationale et de la Recherche and European Community Grant BIO4-CT97–2244 (to U.B.) O.R. was supported by a grant from the Ministère de l'Education Nationale et de la Recherche, and K.W. was supported by the Human Capital and Mobility program of the European Union.

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