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Spontaneous and Induced Mutations in a Single Open Reading Frame Alter Both Virulence and Avirulence in *Xanthomonas campestris* pv. *vesicatoria avrBs2*

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Molecular characterization of the *avrBs2* locus from *Xanthomonas campestris* pv. *vesicatoria* has revealed that expression of this gene triggers disease resistance in *Bs2* pepper (*Capsicum annuum*) plants and contributes to virulence of the pathogen. Deletion analysis and site-directed mutagenesis established the *avrBs2* gene as a 2,190-bp open reading frame encoding a putative 80.1-kDa protein. Two classes of *Xanthomonas* pathogens evading *Bs2* host resistance and displaying reduced fitness were found to be specifically mutated in *avrBs2*. Members of one class contained a 5-bp insertion, while the second class was distinguished by a divergent 3′ region of *avrBs2*; both mutant classes were complemented in *trans* by a plasmid-borne copy of *avrBs2*. A divergent *avrBs2* homolog was cloned from the Brassica pathogen *X. campestris* pv. *campestris*. The predicted AvrBs2 proteins from the two *Xanthomonas* pathovars were strongly conserved and had predicted sequence similarity with both *Agrobacterium tumefaciens* agrocinopine synthase and *Escherichia coli* UgpQ, two enzymes involved in the synthesis or hydrolysis of phosphodiester linkages. On the basis of homology with agrocinopine synthase and UgpQ and the dual phenotype of avirulence and virulence, several models for the function of AvrBs2 are proposed.

Pathogen recognition is key to establishing a timely and effective defense in resistant plants. In gene-specific resistance, single host resistance (*R*) genes govern recognition of pathogens carrying particular avirulence (*avr*) genes (29). This gene-for-gene complementarity has led to the cloning of *R* genes, the products of many of which have predicted similarity to proteins involved in ligand binding and signal transduction (52). The translated sequences of most *avr* genes, on the other hand, have not provided obvious clues as to their function (11). In many *avr-R* gene interactions, the *avr* gene determinant is essential in triggering host defense (29, 51), but among the more than 30 bacterial *avr* genes reported to have been cloned (11), only *avrD* has a proposed biochemical function (35, 40, 49).

One *avr* gene of particular interest is the *avrBs2* locus of *Xanthomonas campestris*, which is involved not only in delivering an avirulence signal to the host but also in promoting pathogen virulence (27). The *avrBs2* gene was originally identified in *X. campestris* pv. *vesicatoria* (Doidge) Dye, the causal agent of bacterial spot disease in pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.) (21, 39). Bacteria expressing *avrBs2* are specifically recognized by pepper cultivars carrying the dominant resistance locus, *Bs2* (39). Mutation at the *avrBs2* locus results in both the loss of *Bs2* recognition and attenuated bacterial growth in susceptible plants (27). This attribute has since been described for a handful of other *avr* genes (33, 42, 60). However, *avrBs2* has the additional feature of being present in nearly all *X. campestris* pathovars examined, representing a diverse host range. Not only is there strong apparent DNA homology, but *avrBs2* alleles recovered from other pathovars can complement *X. campestris* pv. *vesicatoria* mutants in *Bs2* pepper interactions (27), arguing for the maintenance of functional conservation.

The dual phenotype of avirulence and virulence associated with the *avrBs2* locus holds promise for durable *Bs2* resistance in the field. Three dominant resistance genes, *Bs1*, *Bs2*, and *Bs3*, have been identified and introgressed into the susceptible pepper cultivar Early Cal Wonder (ECW) (21, 39). The genes *Bs3* and *Bs1* confer resistance to *X. campestris* pv. *vesicatoria* race 1 (*avrBs3*) and race 2 (*avrBs1*), respectively (39); both of these resistance genes have been overcome in the field, as loss of the avirulence factor has no apparent effect on pathogen fitness (5, 28). In contrast, *Bs2* provides resistance to *X. campestris* pv. *vesicatoria* races 1, 2, and 3, since they all contain *avrBs2* activity.

In this report, we demonstrate that a single open reading frame (ORF) at the *avrBs2* locus is required both for the production of the avirulence signal detected by the *Bs2* resistant host and for full pathogen fitness. Field isolates lacking *avrBs2* activity are specifically disrupted or divergent in this putative protein. The predicted AvrBs2 protein is homologous to enzymes that synthesize or hydrolyze phosphodiester linkages between carbohydrates or phospholipids. On the basis of predicted protein similarities and the dual phenotype of avirulence and virulence contribution, we propose several alternative models for the action of AvrBs2 in *Xanthomonas* species.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this study are described in Table 1. All *Xanthomonas* strains were provided by R. E. Stall. *X. campestris* and *Escherichia coli* strains were cultured as described previously (58), using the following concentrations of antibiotics: tetracycline, 10 μg/ml; rifampin, 100 μg/ml; spectinomycin, 100 μg/ml; ampicillin, 50 μg/ml; kanamycin, 25 μg/ml; and gentamicin, 50 μg/ml. Broad-host-range plasmids were mobilized from *E. coli* into recipient *Xanthomonas* strains by triparental mating (13, 17). *Xanthomonas* exconjugates were verified by reisolation of the plasmid DNA followed by restriction analysis.

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TABLE 1. Bacterial strains, vectors, and plasmid constructions used

Strain, vector, or plasmid	Relevant characteristics	Source or reference
E. coli		
DH5-α	F^- λ^- *recA1* Δ(*lacZYA-argF*) *U169 hsdR17 thi-1 gyrA96 supE44 endA1 relA1* ϕ80d*lacZ*Δ*M15*	Bethesda Research Laboratories
TG-1	*supE hsd*Δ*5 thi* Δ(*lac-proAB*) F′ (*traD36 proAB* + *lac*I^q *lacZ*Δ*M15*)	45
X. campestris pv. *vesicatoria*		
82-8 race 1	Rif^r *avrBs2*$^+$	39
82-8 Δ*avrBs2*	Rif^r Gm^r, marker exchange *Cla*I-*Nde*I *avrBs2*	This study
90-61, 90-62, 90-60	Field isolates, Rif^r *avrBs2*	R. E. Stall
Race 4, race 5	Field isolates, Rif^r *avrBs2*	R. E. Stall
X. campestris pv. *campestris* 8004	Rif^r *avrBs2*$^+$	12
Vectors		
pLAFR3	Tc^r, broad-host-range vector	51
pLAFR6	Tc^r, broad-host-range vector with transcriptional terminators	22
pRI40	Sp^r, broad-host-range vector	23
pUC118, pUC119	Ap^r, cloning and sequencing vector	57
pUC-K18	Km^r, cloning and sequencing vector	R. Jorgensen
pUC-C18	Cm^r, cloning and sequencing vector	R. Jorgensen
pBluescript (SK^+)	Ap^r, cloning and sequencing vector	Stratagene
pRK2013	Km^r Tra^+, helper plasmid	17
Plasmids		
pEC815	pLAFR3 clone from *X. campestris* pv. *vesicatoria* 75-3 race 1 with *avrBs2* activity	39
p81533, p81534	pUC118 with *avrBs2* 2.35-kbp *Sph*I fragment from pEC815 in both orientations	This study
pUCavrBs2	pUC118 with 5 kbp including *avrBs2*	This study
pUCavrBs2:Gm	pUCavrBs2 with Gm^r cassette replacing *avrBs2*	This study
p81538	pLAFR3 clone of p81533	This study
p81546	pRI40 clone of p81533 2.35 kbp with *avrBs2* activity	This study
p815	pLAFR3 clone containing 20 kbp with *avrBs2* activity	This study
p815avrBs2:Gm	pLAFR3 with Gm^r cassette exchanged into *avrBs2* on p815	This study
p9-47	pLAFR3 with containing 20 kbp *X. campestris* pv. *campestris avrBs2*	This study
pBlue:avrBs2	pBluescript KS^+ with *avrBs2* as 2.36-kbp *Bam*HI fragment	This study
pUC-C18:avrBs2	pUC-C18 with *X. campestris* pv. *vesicatoria avrBs2* 2.36-kbp *Bam*HI fragment	This study
pUC-K18:avrBs2	pUC-K18 with *X. campestris* pv. *vesicatoria avrBs2* 2.36-kbp *Bam*HI fragment	This study
pR1, pR2, pR3, pR4, pR5, pL1	pLAFR6 deletion derivatives of *X. campestris* pv. *vesicatoria avrBs2*	This study
pL2	pRI40 deletion derivative of *X. campestris* pv. *vesicatoria avrBs2*	This study
pKstopA, pKstopB, pKstopC, pKstopD, pKstopE, pKstopF	pRI40 *X. campestris* pv. *vesicatoria avrBs2* with stop codons	This study

An exchange mutant at the *avrBs2* locus was constructed in *X. campestris* pv. *vesicatoria* 82-8 (*Xcv* 82-8 Δ*avrBs2*; Table 1) by using the following strategy. The *avrBs2* region of pUCavrBs2 (ampicillin resistance [Ap^r]) from *Nde*I to *Cla*I was replaced with the gentamicin resistance (Gm^r) cassette to generate pUCavrBs2:Gm. The mutagenized *avrBs2* region from pUCavrBs2:Gm was inserted in the homologous site on the cosmid p815 by selecting on tetracycline and gentamicin, testing for Ap^s. The resulting mutagenized cosmid, p815:avrBs2:Gm, then provided enough homologous flanking DNA surrounding the *avrBs2* locus to allow exchange of the mutated allele into the corresponding chromosomal site in *X. campestris* pv. *vesicatoria* 82-8 to generate *Xcv* 82-8 Δ*avrBs2*. Loss of *avrBs2* was verified by DNA gel blot analysis.

Plant growth, inoculation procedures, and growth curves. *C. annuum* plants were cultivated as described previously (54). Seeds of ECW (*bs2*/*bs2*) and the near-isogenic resistant cultivar (ECW20R; *Bs2*/*Bs2*) were provided by R. E. Stall (9, 21). Greenhouse-grown plants were shifted to a growth chamber with a 16-h photoperiod at 24°C for 24 h prior to experimental use. Host response to pathogen was tested by inoculating a suspension of bacteria (10^8 CFU ml^{-1} in 10 mM $MgCl_2$) into expanded pepper leaves, using a plastic transfer pipette (54). The resistant response was scored after 24 h, and the susceptible disease reaction was scored after 72 h (39). Bacterial growth in planta was measured as described by Kearney and Staskawicz (27).

Recombinant DNA techniques and sequence analysis. Standard techniques were used for most cloning procedures (46). DNA gels were blotted onto Hybond N (Amersham) and hybridized as instructed by the manufacturer. Posthybridization washes were performed at 65°C, with the final wash in 0.1% (wt/vol) sodium dodecyl sulfate, 0.2× SSPE (high stringency), or 2× SSPE (low stringency) (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, and 1 mM EDTA [pH 7.7]).

The 25-kb cosmid pEC815 containing *avrBs2* activity (39) was partially digested with *Sau*3A, ligated into *Bam*HI-digested pLAFR3, and transformed into *E. coli* DH5α. Clones were mobilized into an *X. campestris* pv. *vesicatoria* 82-8 *avrBs2* strain (13, 17, 39), and exconjugants were screened for the ability to activate a hypersensitive reaction (HR) in *Bs2*/*Bs2* pepper. The *avrBs2* active 2.36-kb *Sph*I DNA fragment was blunt-end cloned into pUC118 in both orientations (p81533 and p81534 [57]), and a nested exonuclease III deletion series was created. These derivatives were sequenced manually, using a Sequenase 2.0 kit from U.S. Biochemical Corp. (Cleveland, Ohio) or by automatic sequencing using a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing kit (Applied Biosystems DNA Sequencing System, models 373A and 377; Applied Biosystems, Foster City, Calif.).

The *avrBs2* allele from *X. campestris* pv. *campestris* 8004 was isolated by constructing a cosmid library in pLAFR3 and screening with radiolabeled *X. campestris* pv. *vesicatoria avrBs2* (2.36-kb *Sph*I fragment). Positive cosmids were tested for *avrBs2* avirulence activity by conjugating into *Xcv* 82-8 Δ*avrBs2*, infiltrating exconjugates into *Bs2* pepper leaves by using a pipette, and scoring for HR induction. The cosmid p9-47 containing *avrBs2* activity was identified and subjected to *Sau*3A partial digestion, and DNA fragments of approximately 4 kb were gel fractionated, eluted, and cloned into the *Bam*HI site of pLAFR3. A 3.5-kb clone that displayed *avrBs2* DNA homology and *avrBs2* avirulence activity in *X. campestris* pv. *vesicatoria* 82-8 was further subcloned into pUC118 and sequenced.

To isolate spontaneous mutations at *avrBs2*, a single colony of *X. campestris* pv. *vesicatoria* 82-8 wild-type strain (rifampin resistant [Rif^r]) was grown in liquid culture overnight, washed, and diluted to 10^4 CFU ml^{-1} in 10 mM $MgCl_2$. Resistant *Bs2*/*Bs2* plants were submerged into the bacterial suspension, and the bacteria were introduced into the leaf intercellular spaces by using a vacuum. Host tissue was recovered, and the initial bacterial concentration was approximately 100 cells cm^{-2}. After 6 to 10 weeks of greenhouse growth, rare water-soaked disease lesions appeared on the leaves of infiltrated resistant plants. Rif^r *X. campestris* pv. *vesicatoria* bacteria recovered from lesions were complemented with p81538 (*avrBs2*$^+$), and exconjugate growth was evaluated in *bs2*/*bs2* and *Bs2*/*Bs2* plants. The *avrBs2* loci from two independent greenhouse spontaneous mutants and from race 4 and race 6 field isolates were PCR amplified, cloned, and sequenced.

Deletion and site-directed mutagenesis. Deletions in the *avrBs2* 2.36-kb *Sph*I fragment were created by endonuclease digestion of p81533 with *Cla*I, *Nru*I, *Stu*I, *Nco*I, and *Eco*RI, yielding R1, R2, R3, R6, and L2, respectively. Deletion R4 resulted from site-directed mutagenesis using DD2 primer (55). Deletion R5 was

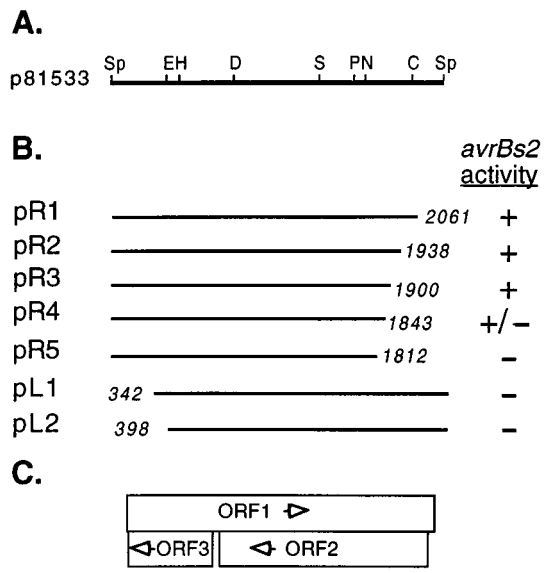


FIG. 1. (A) Restriction map of *avrBs2*. The 2.36-kb *Sph*I fragment containing *avrBs2* was blunt-end cloned into pUC118 (*Sma*I) to produce p81533. Sp, *Sph*I; E, *Eco*RI; H, *Hin*dIII; D, *Dra*III; S, *Sca*I; P, *Pvu*II; N, *Nco*I; C, *Cla*I. (B) Localization of *avrBs2* activity. A deletion series was generated from p81533 and subcloned into wide-host-range vectors. The nucleotide position marking the deletion endpoint is indicated. Exconjugates carrying deletion derivatives were tested for avirulence activity on the pepper cultivar ECW20R (*Bs2*/*Bs2*). +, active; +/−, weakly active; −, inactive by HR induction. (C) Positions of ORF1, ORF2, and ORF3 identified by DNA sequence analysis.

generated by exonuclease III digestion of p81533. All derivatives were subcloned into pLAFR6. Deletion L1 was PCR generated by using Ksp13 (forward) and Ksp9 (reverse) primers, and the PCR product was used to replace the 5′ end of the wild-type *avrBs2* locus; the truncated allele was cloned into pRI40 (pL1).

PCRs were performed in 100-μl volumes containing 20 mM Tris-HCl (pH 8.5), 10 mM $(NH_4)_2SO_4$, 2 mM $MgCl_2$, 100 μg of bovine serum albumin per ml, 0.1% Triton X-100, 20 μM each deoxynucleoside triphosphate, 1 μM each primer, 2.5 U of recombinant *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.), and 100 ng of p81533 *avrBs2* target DNA, using the following conditions: 1 min at 94°C, 1 min at 52°C, and 2 min at 72°C for 35 cycles. The following primers were used: Ksp1 (5′-CCGTGTAGTACTTGGCAT-3′), Ksp4 (5′-ATGCCAAGTACTACA CGGGCGGCTTTGAC(TC)(AT)ATTCC-3′), Ksp6 (5′-TCGCGGGCCGGCA CCTCGTGGCCGTGCTAGCG-3′), Ksp7 (5′-AGGCAAGCTTGCCACGCAT C(TA)(TA)GCGCAG-3′), Ksp9 (GATGATTGCGCAACAGCACTTGCTTG-3′), Ksp12 (5′-GCCAATTGTCATATGCCACGCGCTTGACTGGC-3′), Ksp13 (5′-CCGCTGGATCCGTTGAACGACAGCGCGATGAC-3′), Mrp3 (5′-AAT CCGTCTCCGTCTGCCTGG-3′), Mrp4 (5′-AACGCAGGCATCGTTTCGCA -3′), BK5 (5′-GGACTTTTCCGTGCCGCGCGCCAATCATGACAAGGAC-3′), and DD2 (5′-GCGCGCGGCACTGAAAAGT-3′). Restriction sites used in replacement of mutagenic PCR fragment are underlined; mutagenic nucleotides are boldfaced and underlined. We used the following strategies to introduce independent stop codons in each of the predicted open reading frames at *avrBs2*. For stop A, PCR product generated with Ksp12 (forward) and Ksp9 (reverse) was replaced in the corresponding site in pBlue:avrBs2. For stop B, PCR product generated with Mrp4 (forward) and Ksp6 (reverse) was replaced in pUC-C18: avrBs2. For stop C and stop E, PCR product generated with Ksp4 (forward) and Mrp3 (reverse) was replaced in pUC-K18:avrBs2; these derivatives were distinguished by sequencing. For stop D, PCR product generated with BK5 (forward) and Mrp5 (reverse) was cloned into pBlue:avrBs2. For stop F, PCR product generated with Ksp7 (forward) and Ksp1 (reverse) was replaced as in stop B. The PCR-derived region (306 to 386 bp) of each mutant was sequenced, and the allele was recovered as a *Bam*HI fragment and subcloned into pRI40 (23) to generate pKstopA, pKstopB, pKstopC, pKstopD, pKstopE, and pKstopF. Mutagenized loci were mobilized into *Xcv* 82-8 Δ*avrBs2*, exconjugates were inoculated into *Bs2* pepper leaves using a pipette, and avirulence activity was scored. At least three independent PCR products from each mutant were analyzed.

RESULTS

Molecular analysis of the *avrBs2* locus. The *avrBs2* avirulence activity was localized to a 2.36-kb *Sph*I DNA fragment, shown in Fig. 1A. To determine the minimum amount of DNA required for avirulence activity, a deletion series of the *Sph*I fragment was constructed. Derivatives were cloned into wide-host-range vectors, and plasmids were mobilized by triparental mating into the *avrBs2* exchange mutant, *Xcv* 82-8 Δ*avrBs2*, which has no detectable avirulence activity (see Materials and Methods). The avirulence activity of the exconjugates was assessed by inoculation of a bacterial suspension (10^8 CFU ml^{-1}) into resistant *Bs2*/*Bs2* leaves. In resistant plants, bacteria carrying an active *avrBs2* gene stimulate the HR in the zone of inoculation, which appears as a brown, papery lesion after 24 h. Bacteria carrying an inactive copy of *avrBs2* do not trigger the HR and instead give rise to a water-soaked disease lesion after 72 h (39). As controls, bacteria were inoculated into the leaves of susceptible *bs2*/*bs2* plants to evaluate disease symptoms.

avrBs2 avirulence activity in resistant pepper was scored as either active (HR^+) or inactive (HR^-) (Fig. 1B). Bacteria carrying pR3 induced an HR comparable to that of wild-type controls, delimiting 1,900 bp of the 2,366-bp DNA fragment as important for avirulence activity. A weak HR was induced by pR4 (1,843 bp), while activity was completely lost in pR5 (1,812 bp). Deletions beyond position 1812 were inactive. In the case of the inactive fragments, avirulence activity could not be restored when the fragment was driven by the strong β-galactosidase *lac* promoter (data not shown). All exconjugates caused water-soaked disease symptoms in the susceptible host.

To further characterize the *avrBs2* locus, the entire nucleotide sequence of the 2,366-bp *Sph*I fragment was determined (Fig. 2). Three potential open reading frames (ORFs) were identified (Fig. 1C). On one DNA strand, ORF1 extended from positions 134 to 2323, spanning a total of 2,190 bp. On the opposite strand, ORF2 encompassed 1,154 bp (positions 2022 to 868), while ORF3 spanned 528 bp (positions 656 to 128). With respect to one another, ORF1 and ORF2 are in the +2 and −2 reading frames, while ORF3 is in the −1 reading frame. Our deletion analysis indicated that ORF1 and ORF2 could be partially truncated without loss of avirulence signaling (pR3; Fig. 1B), prompting us to create site-directed mutations in each of the predicted ORFs.

ORF1 is required for avirulence and virulence. Using a PCR-based mutagenesis scheme, we introduced independent stop codons in all three ORFs to determine which were essential for *avrBs2* function. The introduced stop codons did not disrupt the opposite-strand ORF. The positions of mutations A to F are summarized in Fig. 3. The mutations were sequenced, subcloned into the wide-host-range vector pRI40, and conjugated into *Xcv* 82-8 Δ*avrBs2*. Exconjugants carrying the mutant derivatives were inoculated into *Bs2*/*Bs2* leaves, and the ability of the resulting strain to elicit an HR was determined.

The positions and resulting avirulence activities of introduced translational stop mutations are summarized in Fig. 3. Stop D in ORF2 maintained the serine in the predicted ORF1 sequence, whereas stop E changed a predicted glutamine to a leucine. Stop F positioned in ORF3 also induced an amino acid alteration in the overlapping ORF1 sequence, changing a leucine to a histidine. Despite amino acid substitutions in the overlapping ORF1, however, bacteria carrying any of these three mutations were able to trigger a strong HR that was indistinguishable from that of wild-type *avrBs2*. These results indicate that ORF2 and ORF3 are not involved in the production of the avirulence signal.

Mutagenesis of ORF1 was accomplished by introducing three independent stop codons at predicted amino acid positions 5 (stop A), 231 (stop B), and 440 (stop C; Fig. 2 and 3). Stop A changed a predicted lysine amino acid to a glutamine in

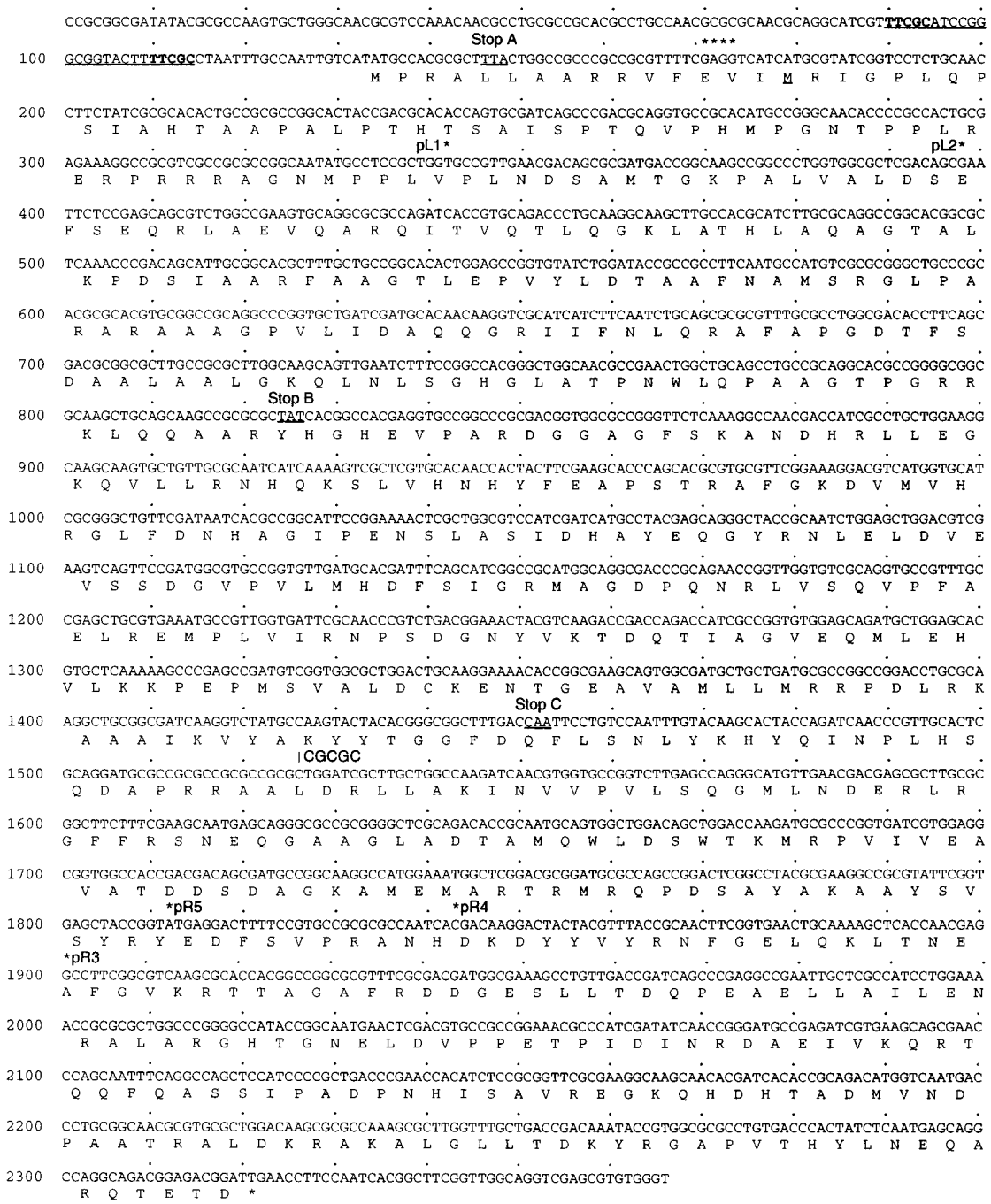


FIG. 2. Nucleotide sequence of *avrBs2*. Translation of ORF1 is shown, beginning at position 134 and extending for 730 aa. On the opposite strand reside ORF2 and ORF3, which start at position 344 (385 aa) and position 1710 (176 aa), respectively. The endpoints for the deletion derivatives pL1 and pL2 on the 5′ side and pR3, pR4, and pR5 on the 3′ side are indicated. Codons subjected to site-directed mutagenesis are underlined (stops A, B, and C). The potential initiation Met shared by the *X. campestris* pv. *vesicatoria* and *X. campestris* pv. *campestris* alleles is underlined, and the conserved upstream purine-rich sequence is marked with asterisks. The putative promoter element (PIP box [16]) is boldfaced and underlined.

ORF3, stop B changed an isoleucine to a valine in ORF2, and stop C maintained the predicted leucine in ORF2. In this series of mutations, only stop A yielded a mutant that was still fully active. Translational stops positioned further downstream at either amino acid (aa) 231 (stop B) or aa 440 (stop C) caused complete loss of *avrBs2* activity. These experiments demonstrate that the predicted product of ORF1 is essential for *avrBs2* avirulence activity. However, alignments with a divergent *avrBs2* homolog cloned from the Brassica pathogen, *X. campestris* pv. *campestris* (discussed below), suggested use of the second methionine as an initiation codon (Fig. 2).

Given that the expression of *avrBs2* both increases pathogen virulence in susceptible plants and triggers defense responses in resistant plants, we evaluated the contribution of ORF1 to these functions by measuring bacterial growth in planta as shown in Fig. 4. Mutant or wild-type *avrBs2* genes were cloned

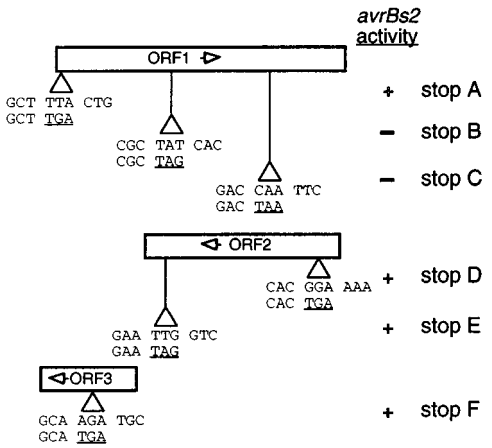


FIG. 3. Positions of introduced stop codons. Independent stop codons were created by PCR site-directed mutagenesis; the top line is wild-type sequence, and the lower line indicates the mutation position (stop codon is underlined). Each mutagenized fragment was replaced into the corresponding site in *avrBs2*, sequenced, and subcloned into pRI40. Exconjugants (*Xcv* 82-8Δ*avrBs2*) carrying each allele were tested for avirulence activity in the pepper cultivar ECW20R (*Bs2*/*Bs2*). +, active; −, inactive as defined by HR induction.

into pRI40 (24), as this vector is stable in *Xanthomonas* species over the course of growth experiments (54a). A suspension of *Xcv* 82-8Δ*avrBs2* (10^5 CFU ml^{-1}) carrying *avrBs2* plasmid derivatives was uniformly infiltrated into resistant *Bs2*/*Bs2* or susceptible *bs2*/*bs2* pepper leaves. Leaf disks were recovered over a period of 6 days, and bacterial growth was determined (27). In resistant plants (Fig. 4A), bacteria carrying wild-type *avrBs2* were restricted in growth as a result of the recognition by *Bs2*, reaching a maximum level of about 10^5 CFU cm^{-2}. Final growth of the ORF1 mutant (stop C) was equivalent to that measured in the exchange mutant (*Xcv* 82-8Δ*avrBs2*/pRI40 [vector control]), verifying the loss of avirulence (Fig. 4A). In susceptible plants (Fig. 4B), growth of bacteria carrying the stop C mutation was reduced 15-fold below that of the bacteria carrying the wild-type locus to a level similar to that measured with the exchange mutant and in agreement with results previously reported for a spontaneous *avrBs2* mutant (27). Therefore, the product of ORF1 is essential for triggering *Bs2* resistance and for pathogen fitness in susceptible plants.

Translation of the *X. campestris* pv. *vesicatoria* ORF1 produces a predicted protein of 730 aa with a molecular mass of 80.1 kDa. Hydropathy analysis (31) indicated that the predicted protein is largely hydrophilic, with no significant hydrophobic protein subdomains. A purine-rich nucleotide sequence, GAGG, that could serve in ribosome binding (Fig. 2 and reference 48) lies 5 bp upstream from the second methionine. A perfect PIP (plant-inducible promoter) box, associated with plant-induced genes of *X. campestris* pv. *vesicatoria* (16), is present 44 bp upstream of the first potential start codon (Fig. 2).

Spontaneous *Xanthomonas* isolates that evade *Bs2* resistance are altered in ORF1. To begin to understand the consequences of mutation at *avrBs2*, we analyzed a number of spontaneous mutants recovered from controlled experiments and from the field. In greenhouse trials, we used resistant plants to identify *avrBs2* mutants by infiltrating *Bs2* leaves with a low titer of wild-type *X. campestris* pv. *vesicatoria* 82-8 (Rif^r). After 6 to 10 weeks, rare disease lesions appeared, from which Rif^r *X. campestris* pv. *vesicatoria* was recovered. These spontaneous mutants were no longer able to trigger an *avrBs2*-specific HR and displayed lower growth in the susceptible cultivar. Both phenotypes were complemented in *trans* with plasmid-borne wild-type *avrBs2* (growth curves not shown). In addition, two novel *X. campestris* pv. *vesicatoria* races isolated from Australia also showed *avrBs2*-specific mutations (21a, 27). Designated race 4 and race 6 (30, 44), these field isolates had growth characteristics comparable to those of *Xcv* 82-8 Δ*avrBs2* and the spontaneous mutants described above, with pathogen proliferation enhanced and avirulence signaling restored with plasmid-borne *avrBs2* (reference 27 and data not shown).

Sequence analysis of the subcloned *avrBs2* fragment revealed that the Australian race 4 and 6 isolates and two of the greenhouse-recovered spontaneous mutants all contained a 5-bp insertion in ORF1. This insertion at position 1526 of CGCGC followed the sequence CGCGC CGCGC CGCGC and thus appeared to induce a fourth reiteration of the previous repeats (Fig. 2). The mutation would introduce a premature stop codon in the predicted AvrBs2 protein sequence (aa 478) far upstream of the deletion endpoint known to abolish avirulence activity.

A second class of field isolates lacking *avrBs2* activity was recovered from bacterial spot-diseased pepper fields in Barbados (50a). In bacterial growth curve experiments, three independent isolates, *X. campestris* pv. *vesicatoria* 90-60, 90-61, and 90-62, were found to have reduced in planta growth and to lack *avrBs2* avirulence activity (data not shown). As demonstrated for the 90-60 isolate in Fig. 5, both virulence and avirulence could be restored by introduction of wild-type *avrBs2*, indicating that this class of mutants did not contain a functional *avrBs2* gene. Further evidence for this conclusion was obtained by performing DNA Southern analysis (Fig. 6). For a large number of *Xanthomonas* pathovars, DNA hybridization of

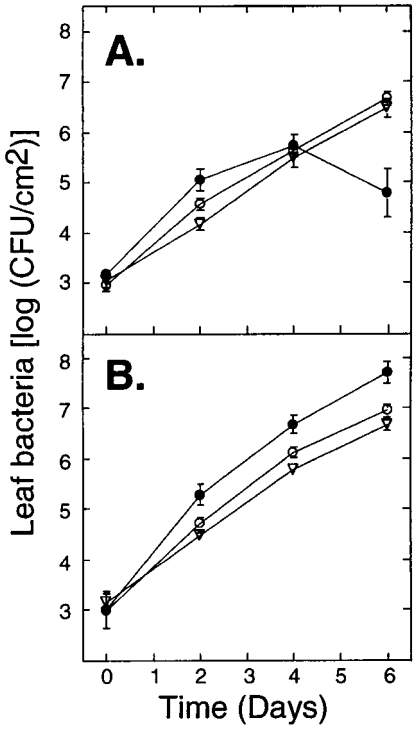


FIG. 4. Growth of *X. campestris* pv. *vesicatoria* strains in pepper cultivars ECW20R (*Bs2*/*Bs2*) (A) and ECW (*bs2*/*bs2*) (B). Values are means from three samples, each consisting of three leaf disks. Error bars represent standard errors. ●, *Xcv* 82-8 Δ*avrBs2*/p81546 (*avrBs2*); ○, *Xcv* 82-8 Δ*avrBs2*/pRI40 (vector control); ▽, *Xcv* 82-8 Δ*avrBs2*/pKstopC. Similar results were obtained in three independent trials.

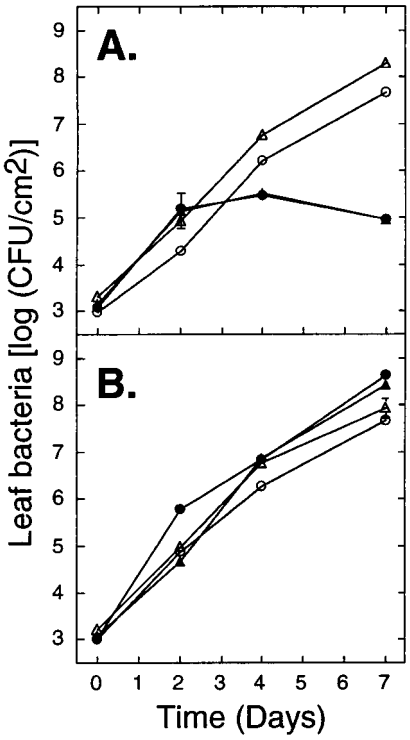


FIG. 5. Growth of *X. campestris* pv. *vesicatoria* strains in pepper cultivars ECW20R (*Bs2*/*Bs2*) (A) and ECW (*bs2*/*bs2*) (B). Values are means from three samples, each consisting of three leaf disks. Error bars represent standard errors. ●, *Xcv* 82-8 Δ*avrBs2*/p81546 (*avrBs2*); ○, *Xcv* 82-8 Δ*avrBs2*/pRI40 (vector control); ▲, *X. campestris* pv. *vesicatoria* 90-60/p81546 (*avrBs2*); △, *X. campestris* pv. *vesicatoria* 90-60/pRI40 (vector control). Duplicate experiments gave similar results.

*Sph*I digests visualize a 2.4-kb fragment when the 2.4-kb *Sph*I probe from *X. campestris* pv. *vesicatoria* is used (25, 27). This 2.4-kb fragment is also visualized when smaller *X. campestris* pv. *vesicatoria* DNA probes are used, exemplified by *X. campestris* pv. *vesicatoria* 82-8 in Fig. 6 (lanes 1). The field isolates from Barbados, however, gave a very different Southern profile. In *Sph*I DNA digests of the 90-60, 90-61, and 90-62 isolates, a smaller 1.5-kb fragment was detected with the full length 2.4-kb *X. campestris* pv. *vesicatoria avrBs2* probe (data not shown). This smaller 1.5-kb band was also visualized with a radioactively labeled 0.8-kb 5′ terminal fragment of *avrBs2* (probe A [Fig. 6A, lanes 2 to 4]), but no hybridization was detected with the 3′-terminal probe (probe B [Fig. 6B, lanes 2 to 4]). These results suggested that the C terminus of the putative AvrBs2 protein is either strongly divergent or absent in these field isolates.

Active *avrBs2* alleles from *X. campestris* pv. *campestris* have ORF1 homology. Unlike many of the *X. campestris* pathovars examined (27), DNA isolated from the Brassica pathogen, *X. campestris* pv. *campestris*, displayed weak Southern homology and nonconservation of DNA restriction sites compared with *X. campestris* pv. *vesicatoria avrBs2* (Fig. 6; compare lanes 5 with lanes 1). Despite the apparent low DNA homology, hand inoculation of the Brassica pathogen into *Bs2* pepper leaves stimulated an HR-like response. Further testing on near-isogenic pepper cultivars carrying either *Bs1*, *Bs2*, or *Bs3* revealed that the HR-like response was specific for *Bs2* (10a), suggesting that *X. campestris* pv. *campestris* contained an *avrBs2* homolog. To investigate the divergence of this gene, we cloned the *X. campestris* pv. *campestris avrBs2* locus from a pLAFR3 cosmid library (see Materials and Methods). The recovered cosmid clone from the Brassica pathogen restored *Bs2*-specific avirulence activity and enhanced virulence to *Xcv* 82-8Δ*avrBs2*, demonstrating functional conservation (reference 27 and data not shown).

Subsequent sequence analysis of the *X. campestris* pv. *campestris avrBs2* locus showed that it potentially encodes a 720-aa, 77.9-kDa protein. As illustrated in Fig. 7A, *X. campestris* pv. *vesicatoria* AvrBs2 and its *X. campestris* pv. *campestris* homolog are 67% identical (84% similar). The N-terminal domains of the two sequences have a lower degree of identity, with optimal alignment requiring the introduction of gaps into the *X. campestris* pv. *campestris* sequence. Beyond the first 120-aa block, the remaining 610 aa are 76% identical (92% similar). The two predicted proteins align from the second methionine in the *X. campestris* pv. *vesicatoria* sequence, downstream from the active stop A mutation but upstream of the inactive stop B mutation, suggesting that this Met is a likely candidate for translation initiation (Fig. 7A). DNA sequences from both pathovars have a potential ribosome-binding site associated with the second methionine (48), and the *X. campestris* pv. *campestris* sequence retains an imperfect PIP sequence (16) at the same site as in *X. campestris* pv. *vesicatoria*.

AvrBs2 has predicted homology with ACS and UgpQ. Database searches were conducted with the predicted amino acid sequence of *X. campestris* pv. *vesicatoria* ORF1. Comparisons with the Protein Information Resource, EMBL, Swiss-Protein, and translated GenBank databases revealed striking sequence similarity between the C-terminal half of the putative AvrBs2 protein and enzymes synthesizing or hydrolyzing phosphodiester linkages. Using the BLASTP program (1), we found that AvrBs2 had strongest homology with agrocinopine synthase (ACS) of *Agrobacterium tumefaciens* (41). As depicted in Fig. 7B, the entire length of ACS aligned with AvrBs2 (aa 188 to 646) and had overall identity of 22% (48% similarity). A second enzyme, glycerophosphoryl diester phosphodiesterase (UgpQ) from *E. coli* (56), also displayed homology with AvrBs2 (26% identity and 51% similarity) over the same re-

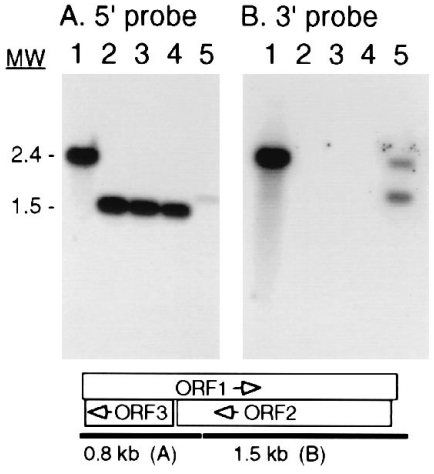
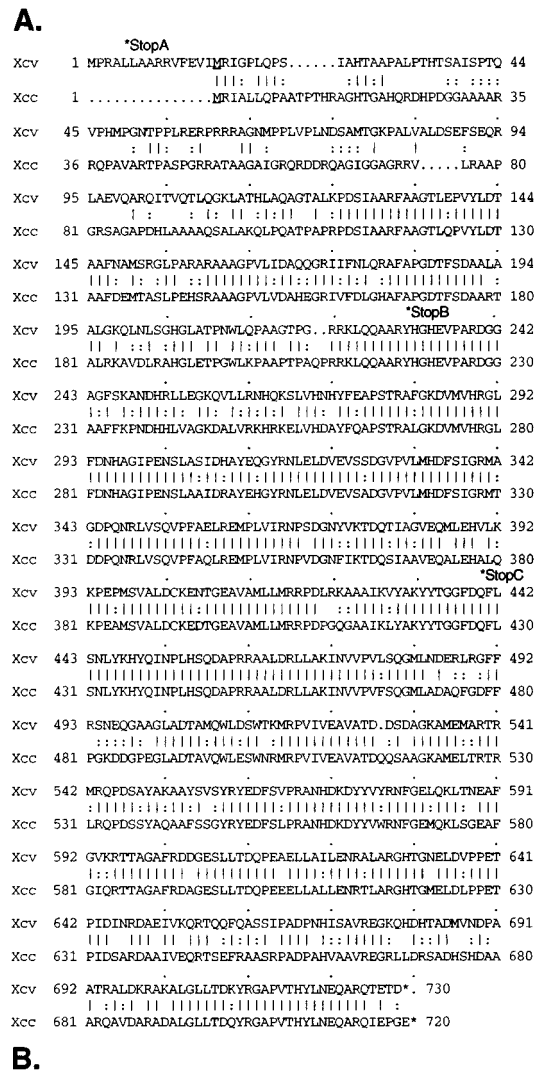


FIG. 6. Genomic DNA gel blots of total DNAs isolated from different *X. campestris* strains. Approximately 2 μg of *Sph*I-digested DNA was loaded per lane and resolved on a 0.75% agarose gel. Duplicate blots were hybridized with *avrBs2* DNA probe A (nucleotides 1 to 833) or B (nucleotides 834 to 2366). Lanes: 1, *X. campestris* pv. *vesicatoria* race 1 82-8; 2, *X. campestris* pv. *vesicatoria* 90-60 field isolate; 3, *X. campestris* pv. *vesicatoria* 90-61 field isolate; 4, *X. campestris* pv. *vesicatoria* 90-62 field isolate; 5, *X. campestris* pv. *campestris* 8004.



gion as ACS, although optimal alignment required introduction of gaps into the shorter UgpQ sequence (data not shown). Strongest contiguous homology was between the N-terminal 60 aa of UgpQ and AvrBs2 (aa 280 to 340), where identity rose to 37% (68% similarity).

DISCUSSION

The recent isolation of plant resistance genes opens new potentials for engineering disease resistance in heterologous species. The ultimate usefulness of a particular resistance gene, however, hinges on the behavior and distribution of the cognate avirulence gene harbored in the pathogen. Here we describe the analysis of the *avrBs2* locus from *X. campestris* pv. *vesicatoria*, which promotes pathogen growth in susceptible pepper plants while creating a positive avirulence signal that triggers *Bs2* resistance. From our mutation analysis, we conclude that a single 2,190-bp ORF potentially encoding an 80.1-kDa protein is required for both functions.

X. campestris pv. *vesicatoria* field isolates virulent on *Bs2* pepper were specifically altered in *avrBs2*. In greenhouse trials, our recovery of *avrBs2* spontaneous mutants was considerably lower than our recovery of *avrBs1* mutants from *Bs1* resistant plants in parallel experiments. Mutation at *avrBs1* is due to insertion of a transposon and, by fluctuation analysis, was estimated to occur at a frequency of 5×10^{-4} (10, 26). Since *Bs2* plants tolerate a high level of *avrBs2*-containing bacteria before activating defense, an accurate measure of in planta mutation is difficult to obtain. However, on the basis of our work with *avrBs1*, we estimate a spontaneous mutation frequency at *avrBs2* of less than 10^{-6} (10a). The 5-bp insertion of CGCGC identified in the Australian and greenhouse *avrBs2* mutants may be due to DNA polymerase slippage (32, 53) that could be promoted during nutrient deprivation (18).

The C-terminal half of the predicted AvrBs2 protein has sequence homology with enzymes that either synthesize or hydrolyze phosphodiester linkages, which may give clues of *avrBs2* function in *Xanthomonas* species. AvrBs2 displays strongest contiguous identity with the *A. tumefaciens* enzyme ACS (41). ACS is encoded by one of a cluster of *Agrobacterium* genes residing between the T-DNA borders that are transformed into the *Agrobacterium*-infected host (41). Plant-expressed ACS catalyzes a phosphodiester linkage between L-arabinose and sucrose to create agrocinopine A (43). The codon bias of AvrBs2, however, does not appear to be skewed toward those preferred in plants. The same C-terminal AvrBs2 domain was also similar to the *E. coli* protein UgpQ (56). A member of the Ugp operon, UgpQ hydrolyzes the phosphodiester linkage during uptake of glycerophosphoryl diesters, liberating *sn*-glycerol-3-phosphate and the corresponding alcohol (7). Comparison of UgpQ and a related *E. coli* enzyme, GlpQ, suggests that the catalytic domains of these hydrolyases reside in the N termini of both proteins (56) through the region of strongest identity with AvrBs2. AvrBs2 may represent a protein that shares elements of ACS and UgpQ, as neither of these enzymes identifies the other in global protein searches. Alignment between the putative AvrBs2 protein and the afore-

FIG. 7. (A) Comparisons of deduced amino acid sequence of ORF1 from *X. campestris* pv. *vesicatoria* 82-8 (Xcv) and *X. campestris* pv. *campestris* 8004 (Xcc). Overall identity is 67% (84% similarity). Between *X. campestris* pv. *campestris* aa 120 and 730, identity is 76% (92% similarity). (B) Alignment between the predicted *X. campestris* pv. *vesicatoria* ORF1 (Xcv) protein and *A. tumefaciens* ACS shows 22% identical amino acids (48% similarity). Sequences were aligned by BESTFIT (50). Identical amino acids are indicated with vertical bars; conserved changes (PAM 250) are indicated with colons.

mentioned enzymes ends near the 3′ endpoint of *avrBs2* required for avirulence activity.

On the basis of homology with ACS and UgpQ and their defined substrate preferences, one class of molecule, β-1,2-glucans, may be a possible enzymatic target for AvrBs2. Cyclic β-1,2-glucans in *Agrobacterium*, *Rhizobium*, and *Xanthomonas* species are synthesized under hypo-osmotic conditions (2, 6, 19, 38) and have been implicated in both osmotic adaptation and plant host signaling (6, 8, 14). In *Rhizobium meliloti* and *A. tumefaciens*, the majority of neutral glucans receive anionic substitutions via a phosphodiester linkage (3, 6), the most common being *sn*-glycerol-3-phosphate derived from phosphatidylglycerol (36). On the basis of predicted protein similarities between ACS and UgpQ, we propose that AvrBs2 may be an enzyme that catalyzes the phosphodiester addition of *sn*-glycerol-3-phosphate to the glucan backbone. The modified glucan may promote *Xanthomonas* adaptation to the plant intercellular space, with mutation of *avrBs2* reflected as a decline of pathogen growth. Furthermore, β-glucans appear to be important for *Agrobacterium* attachment and establishment of functional *Rhizobium* nodules (6, 8) and, in a purified form, are capable of triggering a plant defense response (37). Thus, β-glucans may help phytopathogenic bacteria adapt to the host environment while providing signals that mediate early bacterium-plant recognition events, a dual phenotype shared by *avrBs2*.

The detection of bacteria carrying an *avr* gene depends on functional bacterial *hrp* genes (5, 22), possibly reflecting the involvement of *hrp* loci in the regulation of *avr* genes (22, 23, 45, 47) or in the delivery of avr-elicitors (15, 20). In *X. campestris* pv. *vesicatoria*, the avirulence activity of *avrBs2* is dependent on functional *hrp* loci (10a). The recently identified sequence motif, the PIP box, upstream from a number of *Xanthomonas hrp* genes (16), the *Xanthomonas* avirulence gene *avrRxv* (58), and *avrBs2* suggests that *Xanthomonas avr* loci may be under the same environmentally sensitive regulation as the *hrp* genes (59). By analogy, many *Pseudomonas syringae avr* genes possess a harp box sequence motif upstream of transcriptional start sites (23) and are dependent on the *hrp* transcriptional regulators for expression (22, 23, 45, 47). Alternatively, the elicitor produced by *avrBs2* may require a *hrp* secretory apparatus for presentation to the *Bs2* host (15, 16). One interesting possibility is that the *hrp* complex delivers AvrBs2 to the plant, where it conditions the host environment to favor *Xanthomonas* growth while generating the *avrBs2* elicitor. This hypothesis is being tested through the transient expression of *avrBs2* in *Bs2* pepper. Such an elicitor is not likely to be identical to agrocinopine A, however, as a crude agrocinopine extract from *Agrobacterium*-induced galls does not stimulate an HR when infiltrated into *Bs2* leaves (data not shown).

The utility of the *Bs2* resistance gene in the field is dependent on the mutability of *avrBs2* and the fitness of such mutants. We have characterized two distinct classes of *X. campestris* pv. *vesicatoria* field isolates that evade *Bs2* recognition. Both the spontaneous 5-bp insertion class, represented by the Australian race 4 and 6 and independent greenhouse mutants, and isolates from Barbados are altered in *avrBs2* and no longer trigger host defense. Because of the concomitant reduction of bacterial virulence, such mutant strains may not severely diminish crop productivity. However, if *Bs2* resistance is widely used high selection pressure may result in mutations that render the loss of *avrBs2* signaling without compromising virulence, perhaps by mutating loci unlinked to *avrBs2*. We are exploring the possibility of generating second-site mutations to determine the potential for such occurrences in the field. Thus far, the correlation between *avrBs2* avirulence signaling and enhanced pathogen virulence supports the potential of *Bs2* as a source of durable *Xanthomonas* resistance in the future.

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