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Journal

Molecular plant-microbe interactions : MPMI, 1(5)

ISSN

0894-0282

Authors

Ronald, P C
Staskawicz, B J

Publication Date

1988

Peer reviewed

The Avirulence Gene *avrBs₁* from *Xanthomonas campestris* pv. *vesicatoria* Encodes a 50-kD Protein

Pamela C. Ronald and Brian J. Staskawicz

Department of Plant Pathology, University of California, Berkeley, 94720 U.S.A.
Received 12 May 1988. Accepted 29 June 1988.

A gene cloned from *Xanthomonas campestris* pv. *vesicatoria* race 2, *avrBs₁*, specified avirulence on pepper cultivars containing the resistance gene *Bs₁*. A series of exonuclease III deletions were made on a 3.2-kbp DNA fragment that determined full avirulence activity, observed as hypersensitive response (HR) induction. The deletion products were subcloned into the broad host range cloning vector pLAFR3, conjugated into a virulent *X. c.* pv. *vesicatoria* race 1 strain, 82-8, and scored for their ability to induce a HR on a pepper cultivar (ECW10R) containing the resistance gene *Bs₁*. A span of approximately 1.8 kbp of DNA was necessary for full induction of the HR. The nucleotide sequence revealed two open reading frames (ORFs) capable of encoding proteins of 12.3 and 49.8 kD, designated ORF1 and ORF2, respectively. Deletions into ORF1 altered the HR-inducing activity to give an

intermediate phenotype. Deletions into ORF2 completely destroyed activity. When the ORF2 coding region was driven by the *lacZ* promoter on plasmid pLAFR3 (placD), full avirulence activity was restored, indicating that ORF2 alone can induce the HR. Antisera raised to a β -galactosidase-ORF2 fusion protein reacted with a 50-kD protein in *X. c.* pv. *vesicatoria* race 1 (placD) transconjugants. The deduced amino acid sequence of ORF2 had \approx 47% overall homology to the carboxyl terminus of the avirulence gene, *avrA*, isolated from *Pseudomonas syringae* pv. *glycinea* race 6, and 86% homology over a region of 49 amino acids. *P. s.* pv. *glycinea*, however, did not induce an HR on ECW10R plants. Coding sequences for *avrBs₁* were also present in *X. campestris* pathovars *vitiensis* and *campestris*; both of these pathovars induced a HR on ECW10R plants.

Additional keywords: disease resistance, gene-for-gene, incompatibility, race specificity.

The factors involved in specifying disease resistance in plants are poorly understood. In most cases, expression of the resistant phenotype requires the interaction of a host resistance gene and a pathogen avirulence gene, as was first shown by the classic genetic studies of flax and flax rust (Flor 1956). This gene-for-gene pattern has also been shown to function in several bacterial-plant systems (Staskawicz *et al.* 1984; Gabriel *et al.* 1986; Staskawicz *et al.* 1987; Swanson *et al.* 1988). Considerable progress has been made in isolating the bacterial genes involved in the interaction. However, the gene products encoded by these loci have not yet been characterized.

The phytopathogenic bacterium *Xanthomonas campestris* pv. *vesicatoria*, the causal agent of bacterial spot disease on peppers and tomatoes, provides a model system for addressing this problem. The host's resistance genes, *Bs₁*, *Bs₂*, and *Bs₃* (Cook and Stall 1963; Cook and Guevara 1984; Kim and Hartmann 1985), each corresponding to a specific avirulence gene in the bacterium (*X. c.* pv. *vesicatoria*), have been genetically defined. The resistance genes are inherited independently (Hibberd *et al.* 1987), and the phenotype of the hypersensitive response (HR) associated with each resistance interaction can be distinguished by both the intensity and the timing of the response. The gene *Bs₁* controls a HR to *X. c.* pv. *vesicatoria* race 2 strains of the pathogen (Cook and Stall 1963) containing the avirulence gene, *avrBs₁*, whereas the *Bs₂* and *Bs₃* loci correspond to the *avrBs₂* and *avrBs₃* genes in *X. c.* pv. *vesicatoria* race 1 strains (Minsavage *et al.*, unpublished).

A 200-kbp self-transmissible plasmid present in *X. c.* pv. *vesicatoria* race 2 was found to confer both copper resistance and avirulence to bacteria inoculated into pepper plants (ECW10R) containing the *Bs₁* gene (Stall *et al.* 1986). A 5.3-kbp clone was isolated from the megaplasmid carrying copper resistance that, when conjugated into *X. c.* pv. *vesicatoria* race 1 and inoculated into the pepper cultivar ECW10R, specifically converted the bacterial phenotype from virulence to avirulence. The avirulence gene, designated *avrBs₁*, complemented 13 race 2 mutants that had spontaneously become virulent on ECW10R plants (Swanson *et al.* 1988). To further characterize the gene product(s) encoded by *avrBs₁* and to begin to understand the biochemical basis of recognitional specificity, we have determined the nucleotide sequence of *avrBs₁*. This locus encodes two ORFs. Only the ORF2-encoded protein is required to induce an HR. Antisera raised to ORF2 react specifically with a 50-kD protein in protein extracts of *Escherichia coli* and *X. c.* pv. *vesicatoria* expressing *avrBs₁*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and bacterial conjugations. Races of *X. c.* pv. *vesicatoria* used (race 1 strain 82-8rif and race 2 81-23rif) and their reactions on pepper are described by Swanson *et al.* (1988). Strains 81-23m13 and 81-23m1 are spontaneous mutants of *X. c.* pv. *vesicatoria* race 2 containing the insertion element IS476 in the 12.5-kbp and 824-bp *EcoRV* fragments, respectively, of *avrBs₁* (Kearney *et al.* 1988). Both of these mutants cause watersoaking when inoculated into ECW10R plants containing the resistance gene *Bs₁*. *X. campestris* pathovars were obtained from R. E. Stall. *Pseudomonas syringae* pv. *glycinea* race 0 and race 6 were supplied by Noël Keen. A rifampicin-resistant (rif)

Nucleotide and/or amino acid sequence data is to be submitted to GenBank as accession number J03672.

isolate of *X. c. pv. vitians* was obtained as described previously (Staskawicz *et al.* 1984).

All *E. coli* strains and vectors used are described in Table 1 and plasmid constructions described in Table 2. Clones of *X. c. pv. vesicatoria* DNA maintained in *E. coli* were mobilized to the appropriate *X. c. pv. vesicatoria* race by employing the helper plasmid pRK2013 in triparental matings (Ditta *et al.* 1980). The identity of plasmids from all *X. c. pv. vesicatoria* transconjugants was verified by recovering plasmid DNA, transforming it into *E. coli*, reisolating, and digesting with the appropriate restriction endonucleases.

Growth of plants, plant inoculations, and media. The maintenance and inoculation of pepper cultivars ECW and the near-isogenic ECW10R containing the resistance gene *Bs₁* are described by Swanson *et al.* (1988). *X. c. pv. vesicatoria* was routinely subcultured on nutrient yeast agar (NYGA; Daniels *et al.* 1984). *E. coli* strains were cultured on Luria-Bertani media (LB; Miller 1972). The following concentrations of antibiotics were used: tetracycline, 10 µg/ml; ampicillin, 50 µg/ml; and rifampicin, 100 µg/ml.

Recombinant DNA techniques. Cloning procedures, restriction enzyme digestions, agarose gel electrophoresis, Southern hybridization, and DNA small-scale preparations were essentially as described by Maniatis *et al.* (1982). Genomic DNA was isolated from *Xanthomonas* and *Pseudomonas* as described previously (Staskawicz *et al.* 1984). DNA restriction endonuclease fragments were ³²P-labeled using random primers (Feinberg and Vogelstein 1983).

Exonuclease III deletion analysis. The 5' termini of a 3.2-kbp *Bgl*II-*Pvu*II DNA fragment from pXV2007 were filled in with T4 DNA polymerase and cloned in both orientations into the *Sma*I site of pUC18, creating the

plasmids pXV2108 and pXV2109. DNA of each plasmid was digested with *Bam*HI and *Pst*I followed by exonuclease III and S1 nuclease treatment as described by Henikoff (1984). DNA was then treated with T4 polymerase to ensure formation of blunt ends. After ethanol precipitation, the samples were resuspended in 10 µl of a solution containing 0.05 M Tris, pH 7.6, 0.01 M MgCl₂, 0.01 M DTT, 0.1 mg/ml of bovine serum albumin, 1 mM ATP, 0.2 mM dNtps, and 1 unit of T4 DNA polymerase and incubated 30 min at room temperature. The samples were ligated to 0.25 µg of unphosphorylated *Bam*HI linkers at 12° C overnight, diluted to 100 µl in TE (Maniatis *et al.* 1982), ethanol precipitated, and resuspended in TE containing 0.1 M NaCl. Cohesive ends were allowed to anneal by heating to 65° C for 5 min and then cooling slowly to 4° C. Thirty µl of this mix was used in transformation of *E. coli* DH5α.

Defining endpoints of *avrBs₁* activity. Deletion products in pUC18 or pUC118 were digested with *Bam*HI and *Eco*RI, cloned into the *Bam*HI and *Eco*RI sites of pLAFR3, transformed into *E. coli*, and conjugated into *X. c. pv. vesicatoria* race 1 strain 82-8. All DNA fragments were oriented such that the *lacZ* promoter in pLAFR3 did not provide transcriptional readthrough into the ORFs as later determined by sequence analysis. The transconjugants were inoculated into ECW and ECW10R and checked for HR-inducing activity.

Nucleotide sequencing. Two deletions that still retained full *avrBs₁* activity, pXV2108e8 and pXV2109e8, initially made in pUC18, were subcloned into the *Bam*HI and *Eco*RI sites of pUC118, and a series of exonuclease III deletions were created. Single-stranded templates were prepared as described by Viera and Messing (1987) and sequenced by using the dideoxy chain-termination sequencing method

Table 1. Bacterial strains and vectors

Strain or vector	Relevant characteristics	Source
<i>E. coli</i>		
DH5α	F ⁻ <i>recA thil lacZ</i> ΔM15	Bethesda Research Laboratories
AR68	λ lysogen, TS <i>cI</i> , htpR ⁻	Shatzman and Rosenberg 1986
MM294	λ lysogen <i>cI</i> ⁺	Shatzman and Rosenberg 1986
JM107	F ⁺ , <i>recA</i>	Stephen Elledge, unpublished
MV1193	Δ (<i>lacproAB</i>) Tc ^r <i>thi</i> , [F ⁺ (<i>lacI</i> ^r ZΔM15)]	Messing, unpublished
Vectors		
pUR278, 288, 289	<i>lacZ</i> fusion vectors	Rüther and Müller-Hill 1983
pOTS	bacteriophage λ P _L promoter vector under thermo-inducible control of a thermolabile CI represser	Shatzman and Rosenberg 1986
pLAFR3	pLAFR1 containing <i>Hae</i> II of pUC8 Tc ^r Tra ⁻ Mob ⁺ , RK2 replicon	Staskawicz <i>et al.</i> 1987
pUC18	ColEI replicon	Norrande <i>et al.</i> 1983
pUC118	IG region of M13 in pUC18	Viera and Messing 1987
pRK2013	Km ^r Tra ⁺ Mob ⁺ , ColEI replicon	Figurski and Helinski 1979

Table 2. Plasmid constructions

Plasmid	Vector	Insert
pXV2007	pLAFR3	5.3-kbp <i>Pst</i> - <i>Sst</i> I fragment cloned from <i>Xanthomonas campestris</i> pv. <i>vesicatoria</i> race 2 containing the <i>avrBs₁</i> gene (Swanson <i>et al.</i> 1988)
pXV2108	pUC18	3.2-kbp <i>Bgl</i> II- <i>Pvu</i> II fragment of pXV2007 cloned into <i>Sma</i> <i>Sma</i> I site of vector
pSV2109	pUC18	As above, cloned in opposite orientation
pXV2110	pLAFR3	3.2-kbp <i>Bam</i> HI- <i>Eco</i> RI fragment from pXV2108
pSV2111	pUC18	2.3-kbp <i>Sph</i> I fragment from pXV2109
pXV2112	pLAFR3	2.3-kbp <i>Pst</i> - <i>Bam</i> HI fragment from pXV2111
placA	pLAFR3	Transcriptional fusion of the <i>Bam</i> HI- <i>Eco</i> RI subclone from pXV2108e8 to the <i>lacZ</i> promoter
placD	pLAFR3	Transcriptional fusion of the <i>Bam</i> HI- <i>Eco</i> RI subclone from pXV2108e28 to the <i>lacZ</i> promoter
placE	pLAFR3	Transcriptional fusion of the <i>Bam</i> HI- <i>Eco</i> RI subclone from pXV2108e1 to the <i>lacZ</i> promoter
pOTS A	pOTS	<i>Bam</i> HI- <i>Eco</i> RI subclone of pXV2108e8 cloned in opposite orientation to the P _L promoter
pOTS B	pOTS	Transcriptional fusion of the <i>Bam</i> HI- <i>Eco</i> RI subclone from pXV2108e8 to the bacteriophage P _L promoter
pUR 6	pUR289	<i>avrBs₁</i> -ORF2- <i>lacZ</i> fusion protein; <i>Bam</i> HI subclone of placE
pXV2m105a	pLAFR3	<i>Sph</i> I subclone of an <i>avrBs₁</i> mutant (81-23ml, Kearney <i>et al.</i> 1988) containing the transposon IS476 clone in the direction of vector <i>lacZ</i> promoter

(Sanger *et al.* 1977). Both strands of a 2,061-bp fragment of *X. c. pv. vesicatoria* race 2 containing full avirulence activity were sequenced.

Construction of transcriptional fusions to *avrBs₁*. Transcriptional fusions of three deletions, pXV108e8, pXV2108e28, and pXV2108e1, were constructed to the β -galactosidase promoter in pLAFR3. The plasmids were digested with *EcoRI*, the 5' termini filled in with T4 polymerase and ligated to *BamHI* linkers. The resulting plasmids were then digested with *BamHI*, and the DNA fragments were cloned into the *BamHI* site of pLAFR3 and named *placA*, *placD*, and *placE* (Fig. 1C and Table 2).

β -galactosidase fusions and preparation of antisera. The *E. coli* β -galactosidase-*avrBs₁* ORF2 fusion plasmid was constructed by using the pUR series of vectors containing polylinker sequences at the carboxyl terminus of the β -galactosidase gene (Rüther and Müller-Hill 1983). Plasmid pXV2108e1 (Fig. 1B) was cleaved with *EcoRI*, the 5' extended termini were filled in with T4 DNA polymerase, ligated to unphosphorylated *BamHI* linkers, cleaved with *BamHI*, and the DNA fragment ligated to *BamHI* cleaved pUR289. The plasmid orientation producing an in-frame fusion protein approximately 50 kD greater than β -galactosidase was called pUR6 (Table 2). The construct

pUR6 contained all but the first eight amino acids of the coding sequence of ORF2 fused to the 3' end of the *lacZ* gene. The preparative purification of the bacterial fusion protein aggregates and immunization of rabbits was accomplished as described by Rio *et al.* (1986). The crude antisera were affinity purified according to the method of Smith and Fisher (1984), and the β -galactosidase specific antibodies were removed by absorption of the affinity purified antisera to an *E. coli* extract containing β -galactosidase.

β -galactosidase fusions were also constructed to a *BamHI-EcoRI* DNA fragment from pXV2108e8 containing both ORFs in all three pUR vectors. These constructs gave the expected size fusion proteins as predicted by the DNA sequence for translational stops in all three frames (data not shown).

Protein preparations. *E. coli* carrying plasmids with the β -galactosidase fusion proteins were grown in LB and 100 μ g/ml of ampicillin. One-ml cultures were induced with 1 mM isopropylthiogalactoside (IPTG) at $A_{590} \approx 0.2-0.4$ and grown 2 hr at 37° C following induction. The bacteria were harvested, resuspended in 100 μ l of sodium dodecyl sulfate (SDS) gel sample buffer (Laemmli 1970), and boiled for 90 sec. After a 10-min centrifugation, 10 μ l was run on a 10%

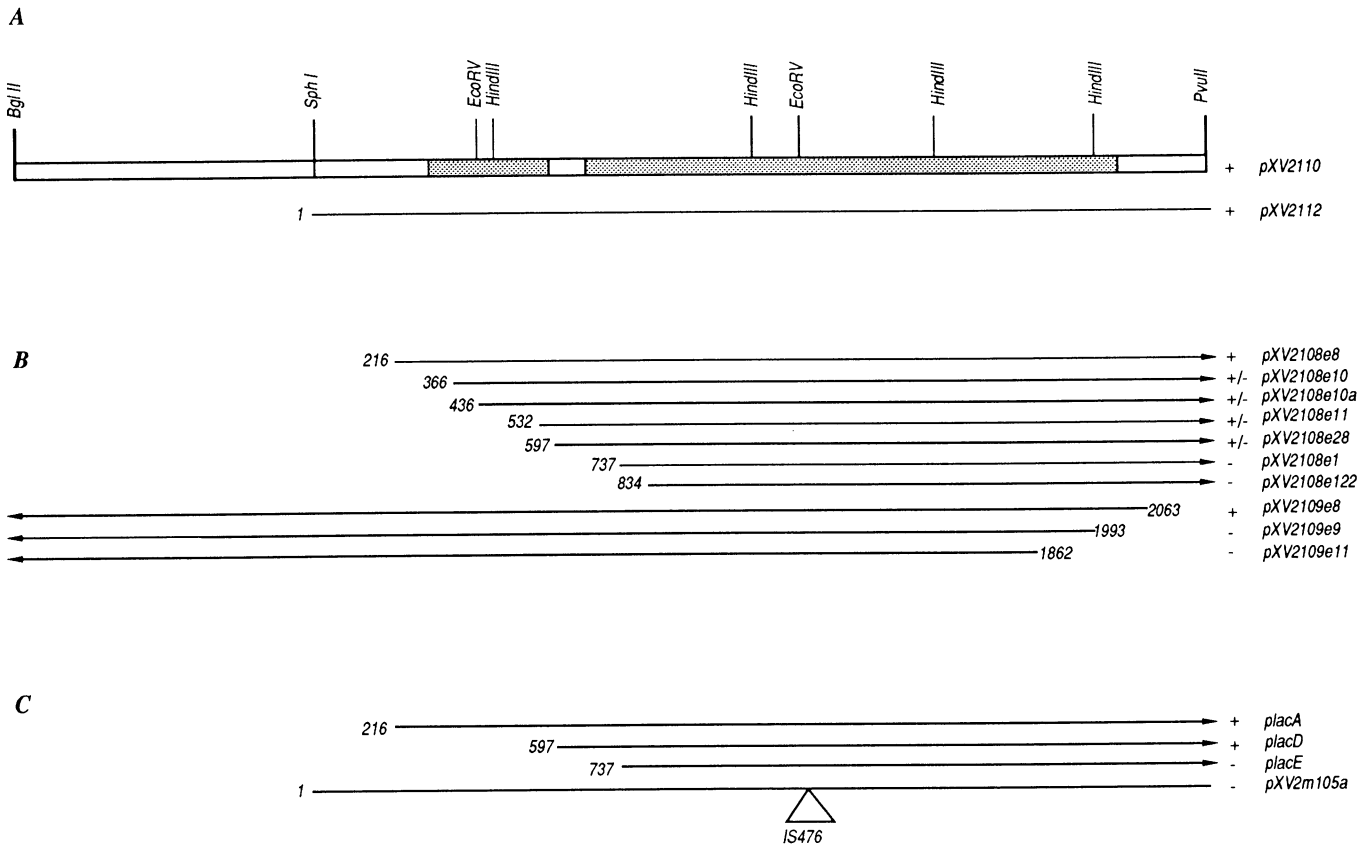


Fig. 1. A, Restriction map and position of open reading frames (ORFs) of *avrBs₁* on the 3.2-kbp subclone pXV2110. The first shaded region designates ORF1 (bp 308–619), and the second designates ORF2 (bp 713–2,047). Both ORFs are in the same reading frame directed from left to right. The 2.3-kbp *SphI-PvuII* subclone pXV2112 is also shown. **B,** Deletion analysis of *avrBs₁*. Exonuclease III deletions made from the 5' end of the 3.2-kbp subclone are indicated by arrows directed right (–). Deletions made from the 3' end are indicated by (–). The deletions are oriented in pLAFR3 such that the *lacZ* promoter drives transcription in the opposite direction to the presumed transcription of *avrBs₁*. **C,** Transcriptional fusions of *avrBs₁* to the *lacZ* promoter. The *lacZ* promoter transcribes from the left into *avrBs₁* coding sequences. The triangle indicates the insertion site of IS476. Numbering starts with position 1 at the *SphI* site and corresponds to the sequence in Figure 2. All plasmids indicated on the right were conjugated into *X. c. pv. vesicatoria* race 1 and inoculated into ECW10R plants. The ability of the transconjugants to induce an HR are shown as +, full activity; +/-, intermediate activity; and –, watersoaking.

denaturing discontinuous polyacrylamide gel and stained with Coomassie blue R-250 (Laemmli 1969). *X. c. pv. vesicatoria* cells carrying plasmids were grown to saturation in NYG broth containing 100 µg/ml of rifampicin and 10 µg/ml of tetracycline. One-ml cultures were pelleted and resuspended in 200-µl sample buffer.

pOTS vector constructs. To overexpress the cloned gene *avrBs₁* in *E. coli*, a derivative of the vector system pAS, pOTS (Shatzman and Rosenberg 1986), was used. This system provides the strong transcriptional promoter P_L from the bacteriophage λ genome, and the plasmids constructed are under thermoinducible control of the *cI* thermolabile repressor. The plasmid pXV2108e8 was digested with *EcoRI*, the 5' termini filled in with T4 DNA polymerase and ligated to *BamHI* linkers. The plasmid was then digested with *BamHI*, and the 2.1-kbp *BamHI* DNA fragment containing ORF1 and ORF2 was cloned in both orientations into the *BamHI* site of the pOTS vector. The plasmid pOTSB is a transcriptional fusion such that translation of ORF2 must initiate from the *avrBs₁* sequence. Translation initiating from the ribosome binding site present in the vector will terminate upstream from ORF2

due to translational stops in all three frames. All cloning experiments were carried out in a *cI⁺* lysogen (MM294cI⁺) to maximize stability of the vector. To express the cloned gene, pOTSA and pOTSB were transformed into a λ lysogen (AR68) carrying a temperature-sensitive mutation in its repressor gene (*cI*). Host cells were protease-deficient to ensure stability of the translated product (Baker *et al.* 1984). The transformed cells were grown in LB containing ampicillin at 30° C until mid-log phase. At this time the cells were moved to 42° C and incubated for an additional hour. The cells were then harvested and the proteins prepared for gel electrophoresis as described above.

Western blotting. SDS-PAGE and electrophoretic transfer to nitrocellulose were performed as described by Towbin *et al.* (1979). The blots were reacted overnight with a 1:500 dilution of the affinity purified, β-galactosidase depleted antibody. After washing, the blot was reacted with ¹²⁵I-labeled protein A for 2 hr before autoradiography.

Computer analysis. The DNA sequence and deduced protein sequence of *avrBs₁* (Fig. 2) were compared to the National Biomedical Research Foundation and NIH Genbank libraries by using the FASTP program available



Fig. 2. Nucleotide sequence of a 2,061-bp segment of DNA and amino acid translation of the two ORFs. The sequence shown begins at the *SphI* site and extends to deletion pXV2109e8. The predicted molecular weight of products of the first ORF is 12.3 kD; that of the second is 49.8 kD.

from the NIH-sponsored Bionet National Computer Resource for Molecular Biology (Smith *et al.* 1986; Lipman and Pearson 1985). The algorithm of Hopp and Woods (1981) was used to produce the hydropathy patterns from the deduced proteins of *avrBs*₁ and *avrA*. The alignment of *avrA* and *avrBs*₁ in Figure 3 was accomplished by using the Bionet program FASTA (Lipman and Pearson 1985).

RESULTS

Defining endpoints of *avrBs*₁ activity. Previous work by Swanson *et al.* (1988) showed that a 5.3-kbp DNA fragment contained *avrBs*₁ activity. We further subcloned this DNA fragment to define better the segment of DNA necessary for activity. *X. c. pv. vesicatoria* race 1 (82-8) transconjugants containing plasmids pXV2110 or pXV2112 (Fig. 1A and Table 2) gave a HR when inoculated into ECW10R, indicating that the 2.3-kbp *Sph*I-*Pvu*II DNA fragment contained the gene *avrBs*₁. Exonuclease III deletions made on the 5' and 3' ends of the 3.2-kb *Bgl*III-*Pvu*II subclone were cloned into pLAFR3, conjugated into *X. c. pv. vesicatoria* race 1, and inoculated into ECW10R. The phenotypes of the reactions are shown in Figure 1 as full avirulence activity (+), intermediate avirulence activity (+/-), and watersoaking (-). The intermediate phenotype is characterized by partial watersoaking and partial necrosis. *X. c. pv. vesicatoria* race 1 transconjugants containing plasmid pXV2108e8, a 2.1-kbp subclone beginning at bp 216 and extending to the *Pvu*II site, gave full *avrBs*₁ activity (Fig. 1B). Transconjugants containing plasmid pXV2108e10, which contains a deletion extending 8 bp into ORF1, gave the intermediate phenotype. *X. c. pv. vesicatoria* race 1 transconjugants containing deletions further downstream into ORF1 (pXV2108e10a, pXV2108e11, pXV2108e28) also gave an intermediate reaction. Deletion products pXV2109e9 and pXV2109e11 extend into the 3' side of

ORF2 and abolish the ability of pXV2109 to express avirulence activity. Deletion product pXV2109e8 maintained activity. Sequence analysis (see below) revealed that this deletion maintained the ORF2 stop codon. Thus, deletion analysis delineated a span of approximately 1.8 kbp of DNA that was necessary for avirulence activity.

Sequence analysis. The nucleotide sequence of a 2,061-bp fragment of DNA containing full avirulence activity as defined by deletion analysis was determined (Fig. 2). The ORF1 began 308 bp downstream from the *Sph*I site and terminated at bp 622 (Fig. 1A). There were four more methionine codons downstream from the first ATG that could also serve as translation initiation sites. The largest peptide encoded in ORF1 was 12.3 kD. ORF2 began at bp 713 and extended to bp 2,047. A second ATG at position 722 and a third at 1,247 could also serve as translational starts. The largest protein encoded by ORF2 was 445 amino acids with a calculated molecular weight of 49.8 kD. DNA sequences upstream from ORF1 and ORF2 showed no homology to the *E. coli* consensus sequence for a ribosome binding site (Shine and Dalgarno 1974). An AG-rich Shine-Dalgarno consensus sequence was present preceding the ATG at position 1,247; however, translation initiating at this nucleotide produced a protein of only 30 kD. Sequences resembling the *E. coli* -10 and -35 promoter elements were found upstream of ORF1 at bp 246 and 224, respectively. A -10-like sequence at bp 702 preceded ORF2, but no -35 sequence was present.

To determine if the first ORF was necessary for the function of *avrBs*₁ or simply supplied transcriptional signals necessary for ORF2 transcription, we constructed plasmids that used a β -galactosidase promoter to drive ORF2. When the deletion fragment from plasmid pXV2108e28 was subcloned into pLAFR3 such that the *lacZ* promoter could provide transcriptional readthrough (placD, Fig. 1C), full avirulence activity was conferred to *X. c. pv. vesicatoria* race 1 (placD) transconjugants. In contrast, no avirulence activity was present in transcriptional fusions to constructs with deletions in ORF2 (placE, Fig. 1C) or insertions in ORF2 (pXV2m105a, Fig. 1C). Transconjugants containing a larger DNA fragment in plasmid pLAFR3 containing both ORFs gave full avirulence activity, in the presence or absence of the *lacZ* promoter (placA, Fig. 1C; and pXV2108e8, Fig. 1B).

Homology of *avrBs*₁ with *avrA*. Considerable homology was observed between the deduced amino acid sequences of *avrBs*₁ and *avrA*, an avirulence gene from race 6 of *P. s. pv. glycinea* (Napoli and Staskawicz 1987). Comparison of the 908 amino acids of *avrA* with *avrBs*₁ revealed extensive homology at the *avrA* carboxyl terminus with the *avrBs*₁ deduced protein sequence (Fig. 3). Forty-seven percent identity was observed between the amino acids of the carboxyl terminus of *avrA* and the *avrBs*₁ ORF2 coding regions. More significant homology (57%) was shown by allowing for conservative amino acid changes rather than perfect matches. Two large regions of the peptides were highly homologous. Forty-nine amino acids (amino acids 748-796 in *avrA*; and amino acids 285-333 in *avrBs*₁) shared 86% homology. An additional region spanning 28 amino acids (*avrA* 606-633, *avrBs*₁ 142-169) shared 75% homology. The DNA sequence homology stopped completely after the *avrA* stop codons (data not shown). Hydropathy analysis of *avrBs*₁ ORF1 and ORF2 indicated

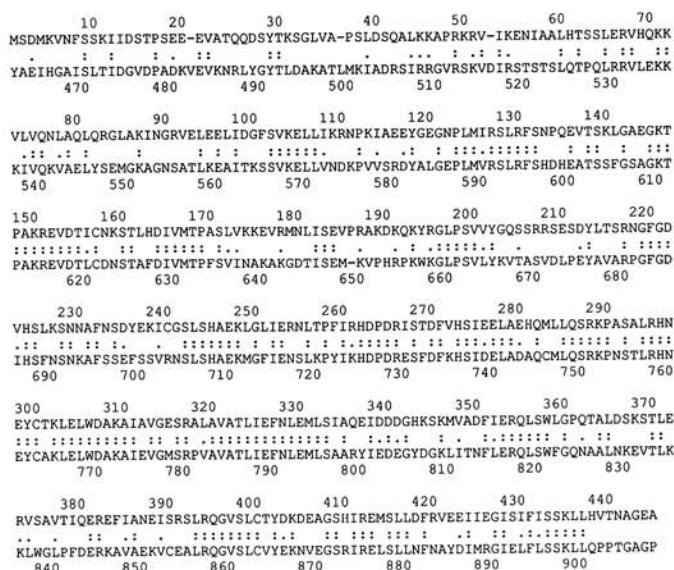


Fig. 3. Alignment of amino acids deduced from the nucleotide sequences of *avrBs*₁ from *X. campestris* pv. *vesicatoria* race 2 (top) and *avrA* from *Pseudomonas syringae* pv. *glycinea* race 6 (bottom). The optimized alignment is denoted by a colon for an identity and a dot for a conservative replacement. Insertions made during optimization are marked with a dash.

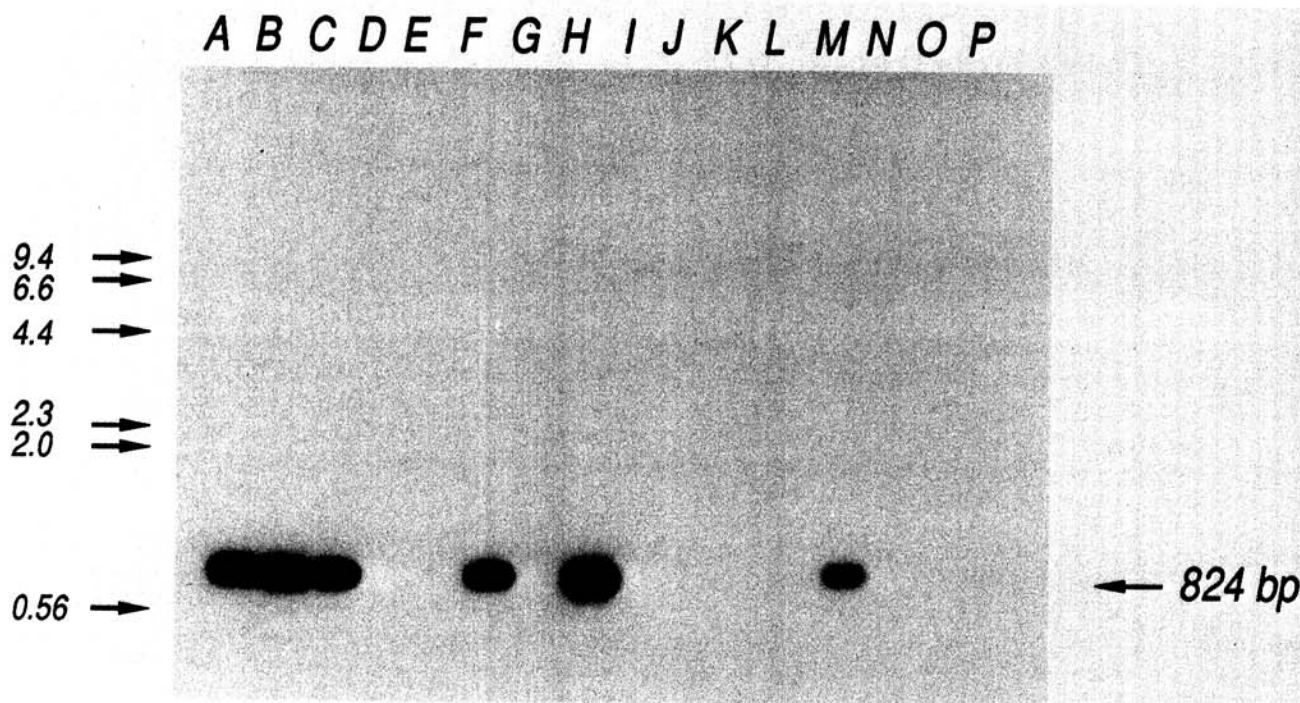


Fig. 4. Southern blot analysis of various xanthomonads and pseudomonads probed with an internal fragment to *avrBs*₁. Total genomic DNA (including plasmid DNA) was isolated and digested with *EcoRV*. Each lane was loaded with approximately 2 µg of DNA and electrophoresed in 0.7% agarose. The DNA was transferred to Nytran and probed with a ³²P-labeled 824-bp *EcoRV* fragment from pXV2-108. Lane A, *X. c. pv. vesicatoria* race 2 81-23; B, *X. c. pv. vesicatoria* race 2 85-10; C, *X. c. pv. vesicatoria* race 2 81-23m13; D, *X. c. pv. vesicatoria* race 3 68-1; E, *X. c. pv. vesicatoria* race 1 82-8; F, tomato race *X. c. pv. vesicatoria* 75-3; G, *X. campestris* T55; H, *X. c. pv. vitians* 164; I, *X. c. pv. malvacearum*; J, *X. c. pv. holcicola*; K, *X. c. pv. vignicola*; L, *X. c. pv. glycines*; M, *X. c. pv. campestris*; N, *X. c. pv. phaseoli*; O, *P. s. pv. glycinea* race 0; P, *P. s. pv. glycinea* race 6. Lambda DNA, digested with *HindIII*, was used as molecular weight markers.

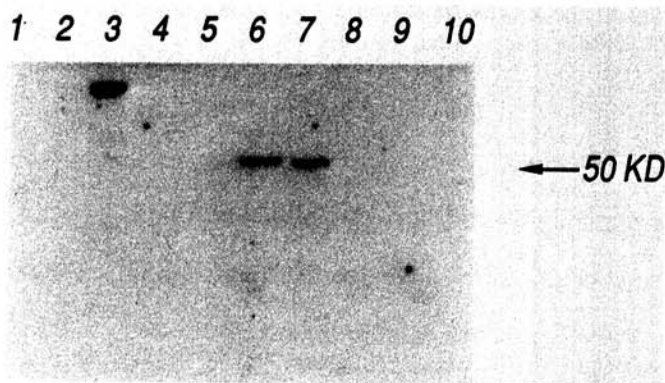


Fig. 5. Autoradiogram of western blot analysis of *avrBs*₁ ORF2. Protein samples were electrophoresed on a 10% sodium dodecyl sulfate polyacrylamide gel and the proteins electrophoretically transferred to nitrocellulose. The blot was first reacted with affinity purified, β-galactosidase depleted anti-ORF2 antisera (1:500 dilution) and then with ¹²⁵I-labeled protein A. Lanes are as follows: 1, Sigma molecular weight markers; 2, *E. coli* JM107recA (pUR289) IPTG treated; 3, *E. coli* JM107recA (pUR6) IPTG treated; 4, *E. coli* AR68 (pOTS) 42° C; 5, *E. coli* AR68 (pOTS) 42° C; 6, *E. coli* AR68 (pOTSB) 42° C; 7, *X. c. pv. vesicatoria* 82-8 (placD); 8, *X. c. pv. vesicatoria* 82-8; 9, *X. c. pv. vesicatoria* 81-23; 10, *X. c. pv. vesicatoria* 81-23m13.

that the putative proteins were mostly hydrophilic (data not shown). As expected, the hydropathy profiles of the last 400 amino acids of the *avrA* protein and *avrBs*₁ ORF2 were similar. No homology to other DNA sequences or protein sequences in the NBRF or Genbank data bases was found.

Homology of *avrBs*₁ to other *X. campestris* pathovars. A Southern blot of total genomic DNA from various races and pathovars of *X. campestris* and *P. s. pv. glycinea* was probed with a ³²P-labeled 824-bp *EcoRV* fragment containing 188 bp of ORF1 and 545 bp of ORF2 (Fig. 1A). The 824-bp fragment hybridized to DNA of *X. c. pv. vesicatoria* race 2 and tomato race (Fig. 4, lanes A, B, and F) and to *X. c. pv. vesicatoria* race 2 81-23m13 (lane C) that contained an intact 824-bp *EcoRV* fragment. As shown previously (Swanson *et al.* 1988), there was no hybridization to *X. c. pv. vesicatoria* race 1 (lane E), race 3 (lane D), or to a nonpathogenic epiphyte, *X. campestris* strain T55 (lane G). Interestingly, two pathovars of *X. campestris*, *vitians* and *campestris*, also contained an 824-bp *EcoRV* DNA fragment that hybridized to the probe (lanes H and M). These two pathovars also gave a HR when inoculated into ECW10R (Bs₁) plants. *P. s. pv. glycinea* races 0 and 6 showed no homology to the probe.

Western blot analysis of *avrBs*₁. No reaction of the *avrBs*₁ ORF2-specific antisera was observed to the β-galactosidase marker (lane 1, Fig. 5) or to *E. coli* extracts containing the pUR289 vector (lane 2, Fig. 5). A 165-kD protein in extracts containing the *lacZ*-ORF2 fusion protein (lane 3) reacted with the antibody. The antibody did not react with *E. coli* extracts containing the pOTS vector alone (lane 4) nor with the pOTS vector containing an *avrBs*₁ ORF2 insert in the opposite orientation to the bacteriophage λ P_L promoter (lane 5). *E. coli* extracts containing pOTSB abundantly expressed a 50-kD protein, after temperature induction at 42° C, that reacted with the antisera (lane 6) and that comigrated with a protein from race 1 (placD) extracts (lane

7). No reaction with the antibody was observed with *X. c. pv. vesicatoria* race 1 (lane 8), 81-23m13 (lane 10), *X. c. pv. vitians*, *X. c. pv. campestris*, or *P. s. pv. glycinea* race 6 (data not shown). Unexpectedly, wild-type *X. c. pv. vesicatoria* race 2 strain 81-23 containing *avrBs₁* (lane 9) did not react with the antibody.

DISCUSSION

Recent work on the *avrBs₁* locus of *X. c. pv. vesicatoria* has shown that a 5.3-kbp fragment of DNA is necessary to specify the ability to induce an HR on pepper plants carrying the resistance gene *Bs₁* (Swanson *et al.* 1988). In this paper, we further characterize the *avrBs₁* locus and show that a 1.8-kbp fragment of DNA is necessary for full avirulence activity.

Exonuclease III deletion analysis in conjunction with sequence analysis defined two ORFs in *avrBs₁*. An intermediate phenotype was observed when portions of the first ORF were deleted. However, when the first ORF was replaced with the *lacZ* promoter (Fig. 1C, placD), full avirulence activity was restored, indicating that only the second ORF is necessary for full activity. Deletion analysis of the 3' end of *avrBs₁* ORF2 showed that the carboxyl terminus of the protein was necessary for avirulence activity. A deletion 13 bp downstream from the TAA stop codon had no effect on avirulence activity, whereas a deletion of the last 18 amino acids of the ORF2 protein prevented the *X. c. pv. vesicatoria* transconjugants from inducing a HR. These data corroborate a previous study that shows that insertions at the *avrBs₁* locus disrupt the HR-inducing phenotype (Kearney *et al.* 1988).

Two mutants of *avrBs₁* that had completely overcome resistance encoded by *Bs₁* contained the insertion element IS476 located in ORF2 (Kearney *et al.* 1988). These mutants caused watersoaked lesions on *Bs₁* plants identical to those observed for deletions into the second ORF. A mutant containing IS476 integrated into the central portion of *avrBs₁* ORF1 gave an intermediate reaction when inoculated into ECW10R (Kearney *et al.* 1988). The same reaction was observed for *X. c. pv. vesicatoria* race 1 transconjugants containing plasmids with deletions into ORF1.

Although the first ORF had the coding capacity for a 12.3 kD protein, it is not known if this region is transcribed and translated. ORF1 was preceded by sequences resembling *E. coli* consensus promoter sequences; however, the function of these sequences in *Xanthomonas* is unknown. It is possible that deletions that gave the intermediate phenotype altered the level of *avrBs₁* transcription and thereby reduced the active ORF2 protein product. Information on transcription initiation is necessary before one can conclude if the ORF1 sequences actually function as transcriptional start signals.

The similarity of an avirulence gene from *X. c. pv. vesicatoria*, a pathogen of pepper, with an avirulence gene from *P. s. pv. glycinea*, a pathogen of soybean, suggests that the proteins encoded by these two genes may function in an analogous manner to specify disease resistance in their respective hosts. Both *avrA* and *avrBs₁* encode proteins that are hydrophilic, have similar hydropathy profiles, and have long stretches of conserved amino acids. In addition, neither protein contains recognizable transit signal sequences

(Oliver 1985). Although other avirulence genes have been shown to be conserved among races (Tamaki *et al.* 1988) and between pathovars (Kobayashi and Keen 1986), this is the first report of conservation of avirulence genes between genera. *X. c. pv. vesicatoria* race 1 transconjugants containing *avrA* do not elicit an HR when inoculated into ECW10R plants (data not shown). Similarly, *P. s. pv. glycinea* race 4 transconjugants containing *avrBs₁* clones (pXV2112 or placD; Table 2) do not induce an HR when inoculated into a soybean cultivar that recognizes *avrA*. It is unknown if the avirulence genes are expressed in the transconjugants. Although the avirulence gene proteins are highly conserved, the gene products still maintain specificity for their respective hosts in a gene-for-gene manner. No other proteins in the Bionet database have significant homology to *avrBs₁*.

We have also shown that the *avrBs₁* 824-bp DNA fragment is homologous to a fragment of the same size in *X. campestris* pathovars *vitians* and *campestris*. Both these pathovars cause an HR characteristic of the *avrBs₁-Bs₁* interaction when inoculated into ECW10R plants. *X. c. pv. vitians* causes watersoaked lesions on ECW plants. *X. c. pv. campestris* gives a light HR on ECW that can be clearly distinguished from the HR induced on ECW10R plants. We are constructing *avrBs₁* marker exchange mutants in *X. campestris* pathovars *vitians* and *campestris* to verify that *avrBs₁* functions to specify avirulence when these pathovars are inoculated into *Bs₁* plants.

The anti-ORF2 antisera showed no reaction with proteins of *X. c. pv. vesicatoria* race 2 grown in broth, indicating that the *avrBs₁* ORF2 protein is not abundant under the conditions tested. The regulation of expression of this avirulence gene appears to be different from that of other avirulence genes so far studied. Preliminary evidence suggests that the *avrBs₁* protein is not induced when *X. c. pv. vesicatoria* is grown *in planta*, as has been shown for the avirulence genes *avrB* and *avrC* from *P. s. pv. glycinea* (T. Huyunh, D. Dahlbeck, and B. J. Staskawicz, unpublished; S. Tamaki, personal communication). We are currently investigating more sensitive methods for detecting the protein in wild-type race 2 cells as well as other growth conditions in which the protein may be more highly expressed.

Western blots of *E. coli* and *X. c. pv. vesicatoria* cells expressing transcriptional fusions to *avrBs₁* show that a 50-kD protein is produced and recognized by the antisera specific to *avrBs₁* ORF2. The protein is stable in an *E. coli* protease deficient strain and in *X. c. pv. vesicatoria* race 1. It appears that *avrBs₁* ORF2 is efficiently translated both in *E. coli* and *X. c. pv. vesicatoria* despite the lack of homology to the *E. coli* consensus sequence for a ribosome binding site. The 50-kD protein detected by the antisera correlates well with the 49.8 kD size protein predicted by the DNA sequence of *avrBs₁* ORF2. In addition, *X. c. pv. vesicatoria* race 1 transconjugants that express the 50-kD protein also induce a HR on the host ECW10R. *X. c. pv. vesicatoria* race 1, and *X. c. pv. vesicatoria* race 1 containing transcriptional fusions to the insertionally mutated subclone in pLAFR3 (pXV2m105a), do not express a 50-kD protein and do not induce a HR when inoculated in ECW10R (data not shown). These data are consistent with the hypothesis that *avrBs₁* ORF2 encodes a 50-kD protein necessary for eliciting the HR; however, it is currently unknown if this

protein acts directly or in conjunction with other molecules to induce the resistant plant response in the gene-for-gene interaction.

ACKNOWLEDGMENTS

We thank Thanh Huynh and Doug Dahlbeck for valuable discussions; Roger Innes, Brian Kearney, Gretchen Kuldau, Maureen Whalen, and Patty Zambryski for critical readings of the manuscript; and Chris Hodgkinson for typing the manuscript.

This work was funded by National Science Foundation Grant DMB-8502642. Pamela Ronald was supported by a McKnight Foundation fellowship.

We acknowledge the computer programs funded by BIONET NIH Grant 1-U41-RR-01685-05.

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