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Research Notes

A Gene from *Xanthomonas campestris* pv. *vesicatoria* that Determines Avirulence in Tomato Is Related to *avrBs3*

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Strains of *Xanthomonas campestris* pv. *vesicatoria* that were avirulent in tomato leaves but virulent in pepper leaves were identified. A cloned gene, *avrBsP*, from one of the strains, Xv 87-7, converted a virulent strain in tomato to avirulent in tomato. A 1.7-kb subclone containing the avirulence gene cross-hybridized with the avirulence gene, which determines race 1 within the pepper group of strains (*avrBs3*). However, the two avirulence genes differ in their biological activity. The base sequences of the two avirulence genes were almost identical through the 1.7-kb segment of *avrBsP*, with significant differences only in some bases in the repeat region.

Additional keywords: bacterial spot, disease resistance.

Strains of *Xanthomonas campestris* pv. *vesicatoria* that cause the bacterial spot disease in pepper plants, but not in tomato plants have been described. Such strains were first isolated in Brazil and collectively referred to as the "Rio strain." These strains were reported to cause a hypersensitive reaction (HR) on two cultivars of tomato, Santa Cruz and Grothen's Globe. (Rodriquez-Neto *et al.* 1984; Reifschneider *et al.* 1985; Bongiolo-Neto *et al.* 1986). In another report (Minsavage *et al.* 1990a), the putative HR caused by strains of this group could not be distinguished consistently in greenhouse tests from a susceptible reaction if time to necrosis after infiltration of tomato leaves with high inoculum was used as a criterion. However, an incompatibility in tomato of strains isolated from pepper plants was recognized consistently by swabbing Carborundum-containing inoculum onto immature tomato leaflets. Thus, the incompatibility of strains of *X. c.* pv. *vesicatoria* in tomato plants seemed to be different from other described incompatibilities of the pathogen and its hosts (Minsavage *et al.* 1990a). The basis of the host specificity of the strains was of interest and is the subject of this note.

Two hundred and fifty strains of *X. c.* pv. *vesicatoria* were tested for incompatibility in tomato. Ninety-one of the strains had been isolated from pepper. The strains represented wide temporal and geographical varieties of the bacterium and had been maintained in sterile tap-water.

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Nucleotide and/or amino acid sequence data are to be submitted to GenBank, EMBL, and DDBJ as accession number JO3705.

A description of some of the strains and plasmids used in the cloning work is in Table 1.

For inoculations, strains were grown in nutrient broth with shaking for 24 hr at 30° C, harvested by centrifugation, and resuspended in sterile tap-water to an optical density (OD) of 0.3 at 600 nm (about 3×10^8 cfu/ml; high inoculum). Except for cultivar screening, tests were completed with plants of the tomato cultivar, Bonny Best. Plants grown in a steamed peat-vermiculite mix in 20-cm pots were used in inoculations. All plants were grown in a greenhouse at ambient temperature and treated biweekly with a 20-20-20 (N-P-K) soluble fertilizer at 0.4 g/pot. During inoculations, either a bacterial suspension containing 600-grit Carborundum was lightly rubbed over the surface of not fully expanded leaflets with a cotton swab, or fully expanded leaflets were infiltrated with a hypodermic syringe fitted with a 26-gauge needle. The plants were kept on a greenhouse bench after inoculation or in a growth chamber kept at 24°, or 30° C, depending on the experiment.

Compatible strains of *X. c.* pv. *vesicatoria* caused typical symptoms of bacterial spot in tomato leaflets after inoculation by the Carborundum-swab method. Incompatible strains produced either no symptoms or small flecks. All strains from tomato were compatible in tomato, whereas 41 of the 91 strains from pepper were rated as incompatible in tomato. All strains that were incompatible in tomato were virulent in leaves of the pepper cultivar, Early Calwonder. After classification of the 91 strains from pepper for race as described by Minsavage *et al.* (1990a), it was found that 24 of the 26 strains of race 1, two of the 27 strains of race 2, and 15 of the 38 strains of race 3 were incompatible in tomato.

High inocula of strain Xv 87-7 (incompatible in tomato and race 1 of the pepper group of strains) were swabbed onto and infiltrated into leaves of tomato cultivars, Homestead 500, Sunny, Euromech, Saturn, Walter, Floradade, Manalucie, Malentha, Marglobe, CRA-66, Florida MH-1,

Table 1. Bacterial strains and plasmids used in this study

Designation	Description	Hosts	Reference
Xanthomonas campestris pv. *vesicatoria*[a]			
Xv 87-7	*avrBs3*, *avrBsP*	Pepper	R. E. Stall
Xv 56	*avrBsT*	Tomato	R. E. Stall
E-3	*avrBs1*	Tomato, pepper	R. E. Stall
Xv 87-56	*avrBs3*, *avrBsP*	Pepper	R. E. Stall
Xv 87-69	*avrBs3*, *avrBsP*	Pepper	R. E. Stall
Xv 82-4j		Tomato, pepper	R. E. Stall
Escherichia coli			
DH5α	F⁻ *recA thil lacZ* M15		Bethesda Research Laboratories
Plasmids			
pLAFR3	pLAFR1 containing *HaeII* of pUC8 Tc^r Tra^- Mob^+ RK2 replicon		Staskawicz *et al.* 1987
p965	*avrBsP* clone with 29.1 kb insert		This study
p965-2	*avrBsP* clone with 11.4 kb insert		This study
p965-2-4	*avrBsP* clone with 1.7 kb insert		This study
p965-2-4::Tn*5*	clone p965-2-4 with Tn*5* insert that inactivates *avrBsP*		This study

[a] All strains of *X. c.* pv. *vesicatoria* used in this study have *avrBs2*.

and 19 primitive stocks obtained from the Plant Introduction collection of the U.S. Department of Agriculture. Reactions similar to that observed in Bonny Best were observed in all plants, except that tissue collapse after infiltration of leaflets of Florida MH-1 was delayed with respect to the other tomato lines.

Patterns for increase in population and electrolyte leakage in inoculated tomato leaves were determined for strain Xv 87-7 by methods previously published (Hibberd *et al.* 1987). For population determinations, inoculum of 1×10^4 cfu/ml was used, and inoculated plants were kept in a greenhouse in which the night and day temperatures fluctuated from 22° to 35° C. For electrolyte leakage, the high inoculum was used, and inoculated plants were maintained in a growth chamber kept at 30° or 24° C, depending on the experiment.

The population of strain Xv 87-7 increased to 10^6 cfu/cm^2 of leaf tissue by 4 days in tomato and then the population decreased after 6 days. In contrast, the same strain reached a population of 10^8 cfu/cm^2 of leaf tissue by 6 days in pepper, and that population level remained stable at least until day 14. At 30° C, conductivity values of water that contained tomato tissue inoculated with strain Xv 87-7 increased at an earlier time after inoculation than water that contained pepper tissue inoculated with the same strain (data not shown).

Reactions of the incompatible strain, Xv 87-7, were compared with a virulent strain, Xv 56, in tomato leaves. Strain Xv 87-7 increased in population to 9×10^6 cfu/cm^2 within 4 days and then decreased to 2×10^5 cfu/cm^2 by 10 days after inoculation. The population of Xv 56 increased exponentially in tomato leaves to 2×10^7 cfu/cm^2 within 4 days. The population then increased gradually to 6×10^7 cfu/cm^2 over the next 6 days (data not shown). At 30° C, an increase in electrolyte leakage began between 12 and 24 hr after infiltrations with the incompatible strain and between 36 and 48 hr with the virulent strain (data not shown). When inoculated plants were kept at 24° C, an increase in electrolyte leakage with the incompatible strain again began between 12 and 24 hr after inoculation, but with the virulent strain no significant increase in electrolyte leakage occurred by 48 hr after inoculation (Fig. 1). No tissue collapse occurred in leaves inoculated with the virulent strain by 3 days after inoculation. Thus, the reaction in tomato of strain Xv 87-7 was typical hypersensitivity, and the strain is now considered to be avirulent in tomato.

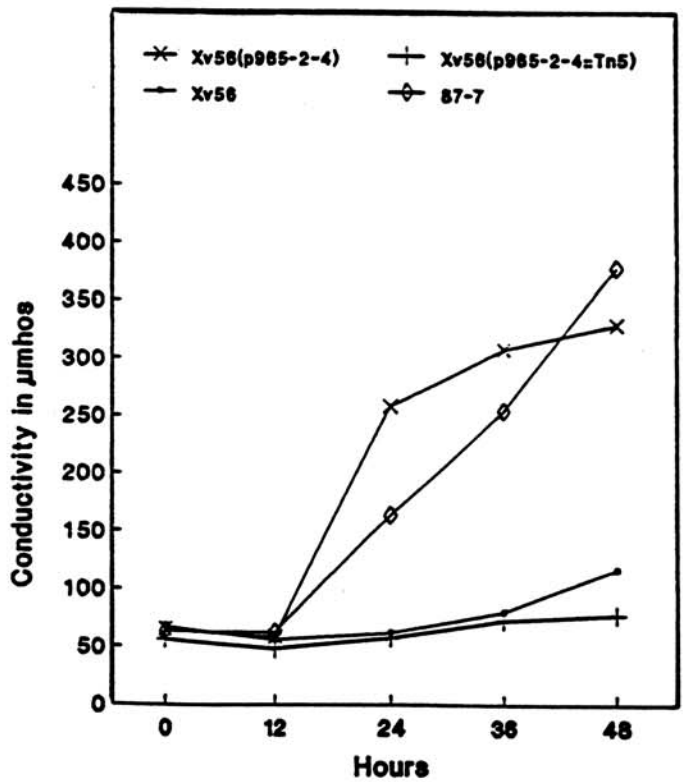

Fig. 1. Electrolyte leakage patterns from tomato leaves. Strains of *Xanthomonas campestris* pv. *vesicatoria* that were avirulent (Xv 87-7), virulent (Xv 56), virulent containing clone with *avrBsP* activity (Xv 56 [p965-2-4]), and virulent containing clone with Tn*5* insertion in *avrBsP* (Xv 56 [p965-2-4::Tn*5*]) were infiltrated into the leaves. Each inoculum contained cells at a concentration of 3×10^8 cfu/ml. Inoculated plants were kept at 24° C.

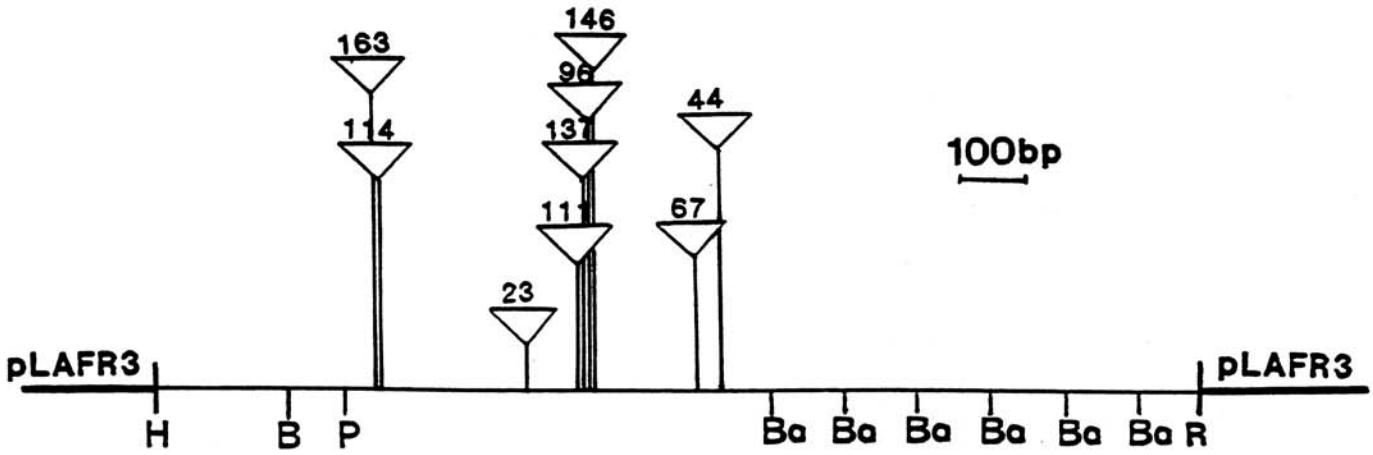

Fig. 2. Restriction map of the 1.7-kb region of DNA containing *avrBsP* activity. The positions of nine Tn*5* insertions that inactivated *avrBsP* activity are indicated by triangles. H = *Hin*dIII; R = *Eco*RI; P = *Pst*I; B = *Bam*HI; Ba = *Bal*I. The *Hin*dIII and *Eco*R1 sites are vector sites.

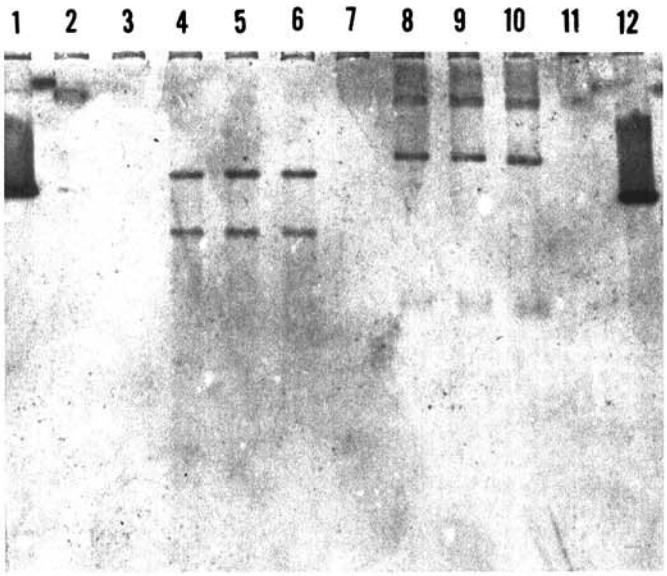

Fig. 3. Hybridization of the 1.7-kb insert DNA of p965-2-4 to genomic DNA (lanes 3, 4, 5, and 6) and plasmid DNA (lanes 7, 8, 9, and 10) of strains of *Xanthomonas campestris* pv. *vesicatoria*. Genomic DNA was digested with *Eco*RI. The plasmids are 80 kb and 40 kb in size. Lane: 1, 12 = p965-2-4 linearized with *Hin*dIII; 2, 11 = blank; 3, 7 = strain E-3; 4, 8 = strain Xv 87-56; 5, 9 = strain Xv 87-69; 6, 10 = strain 87-7. All strains except E-3 are avirulent on tomato.

At 24° C, the differential in time to tissue collapse after inoculation of leaves with high inoculum of avirulent and virulent strains is sufficient for identifying an HR in tomato caused by a pepper group of strains of *X. c.* pv. *vesicatoria*.

A genomic library of strain Xv 87-7 was constructed in the cosmid, pLARF3, for isolation of a putative avirulence gene (Staskawicz *et al.* 1987). Strain DH5α of *Echerichia coli* was used as the recipient for *in vitro* packaged DNA. Clones were conjugated into strain Xv 56 as described previously (Minsavage *et al.* 1990a). Standard procedures for cosmid cloning, enzyme digestion, ligation, map construction, subcloning, Southern transfer, plasmid alkaline lysis, and agarose gel electrophoresis were followed (Maniatis *et al.* 1982).

Of 1,195 library clones, 1,102 independent conjugations were successful with cells of strain Xv 56. After high inoculum of each transconjugant was infiltrated into tomato leaflets, three clones (p629, p965, and p1119) with inserts of 6.4, 29.1, and 25.2 kb, respectively, converted cells of strain Xv 56 to avirulence in tomato. Each of the three clones caused identical phenotypes in inoculated tomato leaves and had overlapping sequences as determined by endonuclease digestion. A fragment of DNA of about 0.7 kb occurred in digests of each clone with *Pst*I and *Bal*I endonucleases (see Fig. 2). Thus, the three clones probably contained the same avirulence gene. The avirulence locus was designated *avrBsP*.

The *Hin*dIII fragments of p965 were subcloned into pLAFR3, transformed into *E. coli* strain DH5α, and then conjugated into cells of strain Xv 56 to screen for the avirulence phenotype. Clone p965-2, with an insert of 11.4 kb, conferred *avrBsP* activity. Another round of subcloning was performed as above, except the DNA insert of p965-2 was partially digested with *Sau*3A, and fragments were ligated into the *Bam*HI site of pLAFR3. Cells of strain Xv 56 that contained clone p965-2-4 induced HR in tomato. The 1.7-kb insert in p965-2-4 was characterized by restriction mapping (Fig. 2).

The avirulence gene in clone p965-2-4 was inactivated by Tn*5* mutagenesis by the procedure of Ruvken and Ausubel (1981). Different derivatives of p965-2-4, each containing a Tn*5* insertion (Fig. 2.), were conjugated from *E. coli* into strain Xv 56. Insertions of Tn*5* into the vector that did not inactivate *avrBsP* were found, but all nine independent insertions into the DNA from Xv 87-7 inactivated the gene. A time course of electrolyte leakage at 24° C from tomato leaves infiltrated with cells of strain Xv 56 containing the clone (p965-2-4), the clone mutagenized with a Tn*5* insertion (p965-2-4::Tn*5*), and with cells of the wild type strains, Xv 56 and Xv 87-7, demonstrated the activity of the clone and the inactivation of it by a Tn*5* insertion (Fig. 1).

Hybridization of the DNA insert of p965-2-4 to plasmid DNA and to genomic DNA digested with *Eco*RI from selected strains of *X. c.* pv. *vesicatoria* was tested. Genomic

DNA extractions were by the method of Boucher *et al.* (1987), and plasmid DNA was extracted by the method of Minsavage *et al.* (1990b). All hybridization experiments were performed on nylon membranes. The GENIUS kit was used for nonradioactive DNA labeling and detection (Boehringer Mannheim, Indianapolis, IN). No hybridization occurred with the DNAs from strain E-3, which is virulent in tomato, but strong hybridization occurred with DNAs from tomato-avirulent strains, Xv 87-7, Xv 87-56, and Xv 86-69. Hybridization occurred with two plasmids, classes I (38–46.9 kb) and F (80–119.9 kb), and with two bands in the genomic DNA from each of the three avirulent strains (Fig. 3). The two bands in the genomic DNA are assumed to be the plasmid DNA, and the fragments are smaller because the genomic DNA was digested with *Eco*RI.

The avirulence gene, *avrBs3* from strains of race 1 of *X. c.* pv. *vesicatoria* also hybridizes to plasmids of classes I and F (Bonas *et al.* 1989), and a very high percentage of race 1 strains induce an HR in tomato plants. Therefore,

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GATCCTCCATGCGGGTCCGTGATCGCCTTCATGTCTGCGCCTCACCCTGGTCGTCGAGGGTTGCCAGGATCACCCGAAGTTGTGTACTGCCATGCGGCCT   100
....................................................................................................

                                                                   M  D  P  I  R  S  R  T  P  S  P  A
CGGAAGCTATGTAGGAACCACAGACCGCTAGTCTGGAGGCGACCATGTAAAGAGGTATGCCTGATGGATCCCATTCGTTCGCGCACACCAAGTCCTGCCC   200
....................................................................................................

R  E  L  L  P  G  P  Q  P  D  G  V  Q  P  T  A  D  R  G  V  S  P  P  A  G  G  P  L  D  G  L  P  A  R
GCGAGCTTCTGCCCGGACCCCAACCCGATGGGGTTCAGCCGACTGCAGATCGTGGGGTGTCTCCGCCTGCCGGCGGCCCCCTGGATGGCTTGCCCGCTCG   300
....................................................................................................

 R  T  M  S  R  T  R  L  P  S  P  P  A  P  S  P  A  F  S  A  G  S  F  S  D  L  L  R  Q  F  D  P  S
GCGGACGATGTCCCGGACCCGGCTGCCATCTCCCCCTGCCCCCTCACCTGCGTTCTCGGCGGGCAGCTTCAGTGACCTGTTACGTCAGTTCGATCCGTCA   400
....................................................................................................

 L  F  N  T  S  L  F  D  S  L  P  P  F  G  A  H  H  T  E  A  A  T  G  E  W  D  E  V  Q  S  G  L  R
CTTTTTAATACATCGCTTTTTGATTCATTGCCTCCCTTCGGCGCTCACCATACAGAGGCTGCCACAGGCGAGTGGGATGAGGTGCAATCGGGTCTGCGGG   500
....................................................................................................

A  A  D  A  P  P  P  T  M  R  V  A  V  T  A  A  R  P  P  R  A  K  P  A  P  R  R  R  A  A  Q  P  S  D
CAGCCGACGCCCCCCCACCCACCATGCGCGTGGCTGTCACTGCCGCGCGGCCGCCGCGCGCCAAGCCGGCGCCGCGACGACGTGCTGCGCAACCCTCCGA   600
....................................................................................................

 A  S  P  A  A  Q  V  D  L  R  T  L  G  Y  S  Q  Q  Q  Q  E  K  I  K  P  K  V  R  S  T  V  A  Q  H
CGCTTCGCCGGCCGCGCAGGTGGATCTACGCACGCTCGGCTACAGCCAGCAGCAACAGGAGAAGATCAAACCGAAGGTTCGTTCGACAGTGGCGCAGCAC   700
....................................................................................................

 H  E  A  L  V  G  H  G  F  T  H  A  H  I  V  A  L  S  Q  H  P  A  A  L  G  T  V  A  V  K  Y  Q  D
CACGAGGCACTGGTCGGCCATGGGTTTACACACGCGCACATCGTTGCGCTCAGCCAACACCCGGCAGCGTTAGGGACCGTCGCTGTCAAGTATCAGGACA   800
....................................................................................................

M  I  A  A  L  P  E  A  T  H  E  A  I  V  G  V  G  K  Q  W  S  G  A  R  A  L  E  A  L  L  T  V  A  G
TGATCGCAGCGTTGCCAGAGGCGACACGAAGCGATCGTTGGCGTCGGCAAACAGTGGTCCGGCGCACGCGCCCTGGAGGCCTTGCTCACGGTGGCGGG   900
.........................................................................T..........................

 E  L  R  G  P  P  L  Q  L  D  T  G  Q  L  L  K  I  A  K  R  G  G  V  T  A  V  E  A  V  H  A  W  R
AGAGTTGAGAGGTCCACCGTTACAGTTGGACACAGGCCAACTTCTCAAGATTGCAAAACGTGGCGGCGTGACCGCAGTGGAGGCAGTGCATGCATGGCGC  1000
....................................................................................................

 N  A  L  T  G  A  P  L  N  L  T  P  E  Q  V  V  A  I  A  S  N  I  G  G  K  Q  A  L  E  T  V  Q  A
AATGCACTGACGGGTGCCCCCCTGAACCTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGACGGTGCAGGCGC  1100
...........................................................C.CGA...C..............................CG

L  L  P  V  L  C  Q  A  H  G  L  T  P  D  Q  V  V  A  I  A  S  N  G  G  G  K  Q  A  L  E  T  V  Q  R
TGTTGCCGGTGCTGTGCCAGGCCCATGGCCTGACCCCGGACCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTGCAGCG  1200
.....................................C.G............................................................

 L  L  P  V  L  C  Q  A  H  G  L  T  P  E  Q  V  V  A  I  A  S  N  I  G  G  K  Q  A  L  E  T  V  Q
GCTGTTGCCGGTGCTGTGCCAGGCCCATGGCCTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGACGGTGCAG  1300
........................................C..........................GC...............................

 R  L  L  P  V  L  C  Q  A  H  G  L  T  P  E  Q  V  V  A  I  A  S  N  I  G  G  K  Q  A  L  E  T  V
CGGCTGTTGCCGGTGCTGTGCCAGGCCCATGGCCTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGACGGTGC  1400
....................................................................GGC.............................

Q  R  L  L  P  V  L  C  Q  A  H  G  L  T  P  E  Q  V  V  A  I  A  S  N  G  G  G  K  Q  A  L  E  T  V
AGCGGCTGTTGCCGGTGCTGTGCCAGGCCCATGGCCTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGT  1500
.....................................................................ATT............................

 Q  R  L  L  P  V  L  C  Q  A  H  G  L  T  P  E  Q  V  V  A  I  A  S  N  G  G  G  K  Q  A  L  E  T
GCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCATGGCCTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACG  1600
....GC.................................................................ATT..........................

 V  Q  R  L  L  P  V  L  C  Q  A  H  G  L  I
GTGCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCATGGCCTGATC
......GC...................................C.
                                      1640
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Fig. 4. Nucleotide sequence of the 1.7-kb fragment of DNA that has *avrBsP* activity (top line). The lower line is the sequence of *avrBs3* through the 1,640 bases of *avrBsP*. Each dot of the *avrBs3* sequence means no change from *avrBsP*. The amino acid equivalents of triplets are listed above the sequences starting with methionine at position 163. Possible promoters are underlined before the open reading frame. The first repeat at position 1,027 is underlined.

avrBsP was tested for cross-hybridization with *avrBs3,* and the 1.7-kb insert DNA of p965-2-4 hybridized strongly with a cosmid containing *avrBs3* (pBs3). The cross-hybridization probably accounts for the two hybridization bands that occurred with DNAs from the tomato-avirulent strains, which were all strains of race 1 of the pepper group and therefore contained *avrBs3*.

When pBs3 (*avrBs3*) was conjugated into strain Xv 82-4j, which is virulent on pepper and tomato, the transconjugant did not cause HR in tomato leaves. The transconjugant did cause HR in leaves of the pepper cultivar ECW-30R, which contains the *Bs*3 gene for resistance to strains of race 1. The transconjugant of Xv 82-4j containing plasmid p965-2-4 caused HR in leaves of tomato, but did not in leaves of ECW-30R. Thus, the two avirulence genes cross-hybridized, but conferred different biological activities.

The sequence of the 1.7-kb subclone was determined by the dideoxy method using T7 DNA polymerase (Bonas *et al.* 1989). The biologically active insert in clone p965-2-4 contained a 1,645-base pair (bp) sequence with very high similarity to the 5′ end of *avrBs3* (Bonas *et al.* 1989) (Fig. 4). The similarities include an identical 163-bp region 5′ to the start codon and 864 bp, with only a T-C transition in *avrBsP* at bp 874, following the start codon. Sequences 3′ to this highly conserved region are six sets of a generally conserved 102-bp repeat, which occurs as 17 sets in *avrBs3*. Sequences of the *avrBsP* repeats and the order of the repeats differed from *avrBs3*. Repeats 1 and 2 of *avrBsP* are distinct, repeats 3 and 4 are identical, and repeats 5 and 6 are identical. Repeat 1 is represented in *avrBs3* as repeats 5, 6, and 7; repeats 2, 3, and 4 are not represented in *avrBs3*, and repeats 5 and 6 are represented as repeat 4 of *avrBs3*. An insertion of T at 1,644, or a C-T transition, at the beginning of a seventh copy, is not found in any of the 17 repeats of *avrBs3*, and resulted in a *Sau*3A site used in cloning of *avrBsP*. The 1.7-kb region would encode a 493 residue polypeptide.

The 1.7-kb fragment may not represent the entire *avrBsP* gene because no stop codon was found in the sequence. We assume transcription of the clone terminated in the vector, pLAFR3, which has a stop codon in the cloning region. We also assume that some of the repeats are necessary for biological activity because complete removal of them by digestion with the restriction enzyme *Bal*I eliminated biological activity (data not shown).

A region of 3.6–3.7 kb is needed for activity of *avrBs3* (Bonas *et al.* 1989), whereas a region of 1.7 kb is sufficient for activity of *avrBsP*. However, the complete *avrBsP* gene may be equally as large as *avrBs3*. To determine this, a second library clone would have to be subcloned for sequencing, because a *Sau*3A cleavage occurred in the repeat region in the original cloning that gave rise to p965. If the complete gene is as large as *avrBs3*, exonuclease digestion should provide more answers regarding the portion of the gene that is necessary for activity.

DNA homology among avirulence genes is uncommon. So far, six avirulence genes from *X. c.* pv. *vesicatoria* have been cloned (Minsavage *et al.*, 1990a; Whalen *et al.* 1988) and only *avrBs3* and *avrBsP* are similar as determined by DNA hybridization. The biochemical similarity of these genes provides an opportunity to study the specific sequences involved in differential biological activity.

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