JOURNAL OF BACTERIOLOGY, Jan. 1990, p. 143–148
0021-9193/90/010143-06$02.00/0

Vol. 172, No. 1

Characterization of IS*476* and Its Role in Bacterial Spot Disease of Tomato and Pepper

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Received 31 July 1989/Accepted 28 September 1989

IS*476* is an endogenous insertion sequence present in copper-tolerant strains of *Xanthomonas campestris* pv. *vesicatoria*. Sequence analysis has revealed that the element is 1,225 base pairs in length, has 26-base-pair inverted repeats, and causes a 4-base-pair target site duplication upon insertion into the avirulence gene *avrBs1*. Comparison of the full-length sequence with sequences in the National Biomedical Research Foundation and National Institutes of Health data bases showed that one of the predicted IS*476* proteins is partially homologous to the putative transposase of IS*3* from *Escherichia coli*, and the inverted repeats of IS*476* have significant homology to the inverted repeats of the IS*51* insertion sequence of *Pseudomonas syringae* pv. *savastanoi*. A transposition assay based on the insertional inactivation of the *sacRB* locus of *Bacillus subtilis* was used to demonstrate that one of the three copies of IS*476* residing on the 200-kilobase copper plasmid pXVCU1 is capable of transposition in several strains of *Xanthomonas campestris*. The position of IS*476* insertion in several *avrBs1* mutants was established and was shown to influence both induction of hypersensitivity and bacterial growth in planta.

Xanthomonas campestris pv. *vesicatoria* (Doidge) Dye causes bacterial spot disease of pepper (*Capsicum annuum* L.) and tomato (*Lycopersicon esculentum* Mill.) (4, 5). Expression of disease resistance is controlled by specific avirulence genes in the pathogen that correspond to specific resistance genes in the plant, consistent with the gene-for-gene hypothesis (4, 5, 14, 22, 27, 29). Inoculation of an avirulent pathogen induces a hypersensitive defense reaction in a resistant host, a response characterized by rapid necrosis of host tissue and localization of the pathogen at the site of inoculation (18). The host range of the pathogen is limited in part by its complement of avirulence genes; the pathogen can cause disease only on plants that do not contain resistance genes corresponding to its avirulence genes. Loss or inactivation of an avirulence gene extends the host range of the pathogen to include plants previously resistant because they contained the corresponding resistance gene.

Numerous resistance genes have been described for pepper (3, 4, 14, 17), and the nature of their corresponding *X. campestris* pv. *vesicatoria* avirulence genes has been studied (22, 27, 29). The insertion sequence IS*476* was discovered in the study of the interaction between pepper cultivar ECW10R, which contains the resistance gene *Bs1*, and *X. campestris* pv. *vesicatoria* pepper race 2 strains that contain the avirulence gene *avrBs1* (22, 25, 27). Previous experiments have shown that *X. campestris* pv. *vesicatoria* strains carrying *avrBs1*, normally avirulent on ECW10R, spontaneously mutated to virulence at a frequency of 5×10^{-4} (6). In each case studied, the mutation occurred at the *avrBs1* locus, and the inactivated avirulence gene contained a 1.2-kilobase (kb) insertion of DNA (16, 27). Structural and sequence analysis of the region revealed the presence of an insertion sequence-like element, given the name IS*476* by the Central Plasmid Registry (1). In the following report, the entire nucleotide sequence of the element is presented and experiments are described that demonstrate IS*476* transposition, its effect on the hypersensitive defense reaction induced by *avrBs1*, and its effect on the growth of *X. campestris* pv. *vesicatoria* in planta.

MATERIALS AND METHODS

Bacterial strains and plasmids. *X. campestris* pv. *vesicatoria* strains 81-23, 71-21, and 75-3 and *X. campestris* T55 were provided by R. E. Stall. Isolation of spontaneous mutants M1, M2, M4, and M13 was described previously (15, 16). Plasmid pXVCU1 is a native 200-kb plasmid from *X. campestris* pv. *vesicatoria* E3C5 that carries *avrBs1*, three copies of the transposon IS*476*, and a gene encoding resistance to copper (16, 20, 25). *X. campestris* T55 is a nonpathogenic strain that carries no copies of *avrBs1* or IS*476*. *X. campestris* T55(pXVCU1) resulted from a mating of T55 and *X. campestris* pv. *vesicatoria* E3C5 as described previously (16). Plasmid pXV2M101 contains the mutated *avrBs1* gene cloned from M1 on a 7.1-kb *Pst*I-*Sac*I fragment (16). C. I. Kado provided pUCD800, which contains the levansucrase gene *sacRB* (11). Plasmid pL3SAC was constructed by digesting pUCD800 with *Pst*I and *Bam*HI and cloning the resulting 2.7-kb fragment containing the *sacRB* gene into the wide-host-range cosmid pLAFR3 (see Fig. 3) (26). Whenever an *Escherichia coli* host was necessary, strain DH5α (BRL Life Technologies, Inc., Gaithersburg, Md.) was used.

Plant culture and in planta growth curves. Pepper cultivar ECW10R was provided by R. E. Stall (4, 14). Sixteen hours before infiltration with bacteria, the plants were taken from the greenhouse and placed in a growth chamber. Subsequent growing conditions included 16-h day length at 30°C and 20,000-lux light intensity; 8-h night at 22°C; and 90% relative humidity. Fresh overnight cultures of the wild-type strain 81-23 and mutants M1, M2, M4, and M13 were suspended to approximately 10^5 CFU/ml in 10 mM $MgCl_2$ and vacuum-infiltrated into 6-week-old ECW10R plants. At 1 h after infiltration and subsequently once each 24 h, three replicates of three 7-mm leaf disks were taken from each plant, pulverized in 10 mM $MgCl_2$, serially diluted, and plated on nutrient yeast-glycerol agar (NYGA) (7).

Transposition of IS*476*. Plasmid pL3SAC was conjugated

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into *X. campestris* pv. *vesicatoria* 81-23, E3C5, and 71-21 and *X. campestris* T55(pXVCU1) by using the helper plasmid pRK2013 in triparental matings (8). Seven single colonies of each mating were grown to log phase and plated on NYGA supplemented with tetracycline (10 μg/ml) and 5% sucrose. When appropriate, the medium was also supplemented with $CuSO_4$ (200 μg/ml). Plasmid DNA from a single sucrose-viable mutant of each original single colony was isolated and transformed into *E. coli* DH5α, selecting on LB agar (21) supplemented with tetracycline (10 μg/ml). Plasmid DNA was isolated from each *E. coli* transformant, digested with *Pst*I and *Bam*HI, Southern blotted, and probed with either a 350-base-pair (bp) *Sal*I-*Sma*I fragment of pXV2M101 specific to IS*476* or the 2.7-kb *Pst*I-*Bam*HI fragment of pL3SAC containing *sacRB*. A 1.25-kb change in the mobility of the *sacRB* fragment and hybridization of the new fragment to IS*476* indicated capture of the transposon.

Sequencing IS*476*. IS*476* and flanking *avrBs1* DNA sequences were cloned from *X. campestris* pv. *vesicatoria* M1 on a 2.1-kb *Eco*RV fragment into the *Sma*I site of pUC118 as described previously (15). Unidirectional exonuclease III deletions were made for both strands (13), and dideoxy sequencing was performed by the method of Sanger et al. (23).

Molecular techniques. DNA isolations, ligations, transformations, and Southern blotting were performed essentially as described by Maniatis et al. (19). Radiolabeled probes were prepared by random hexamer labeling (9).

RESULTS

Sequence analysis. The sequence of IS*476* was determined and is shown in Fig. 1. The element is 1,225 bp in length and has 26-bp imperfect inverted repeats. Upon insertion, IS*476* causes a 4-bp target site duplication, as assayed by sequence analysis of four independent insertions into *avrBs1* (15). Sequence analysis revealed two open reading frames (ORFs). ORF1 predicts a 346-amino-acid protein, starting at position 156 and stopping at position 1193. ORF2 resides on the opposite DNA strand but is found in the same frame as ORF1. ORF2 starts at position 923 and stops at position 330, predicting a 198-amino-acid protein. A computerized search of the National Biomedical Research Foundation (NBRF) protein data base (24) found significant homology between the predicted protein from ORF1 and the putative transposase of IS*3* from *E. coli* (28) (Fig. 2A). Over a 233-amino-acid stretch, there was 24% identity between the IS*476* and IS*3* putative proteins. When conservative amino acid changes were considered, the homology became 31%. The two proteins were 46 to 60% homologous over short stretches. The small size of ORF2 and the lack of significant homology between the ORF2 predicted protein and the NBRF data base suggests that ORF1 probably encodes the transposase for IS*476* transposition.

Computerized search of the National Institutes of Health DNA sequence data base (24) revealed significant homology between the inverted repeats of IS*476* and IS*51*, a transposable element found in *Pseudomonas syringae* pv. *savastanoi* (2, 30) (Fig. 2B). The two elements were roughly the same size, and like IS*476*, IS*51* had 26-bp imperfect inverted repeats. Sixteen of the 26 bp in the left inverted repeats of IS*476* and IS*51* were identical, suggesting common ancestry (Fig. 2B). It is not known whether the transposase of either element can activate transposition of the other. Computer analysis found no significant homology between the internal sequences of the two elements, and this lack of homology was confirmed by Southern analysis (data not shown).

IS*476* transposition. IS*476* was originally classified as a transposable element by sequence analysis. The inverted repeats, target site duplication, and size of the insertion led us to conclude that the mutation in M1 was caused by IS*476* transposition into *avrBs1* (15). However, we did not know whether IS*476* insertion into *avrBs1* was a random or specific event. To answer the question, a transposition assay was developed to capture IS*476*. The "landing pad" used was the *Bacillus subtilis* gene *sacRB*, which encodes the enzyme levansucrase (10–12). In certain gram-negative organisms, including *E. coli* and *Agrobacterium tumefaciens* (11), levansucrase is lethal when expressed in bacteria grown on medium containing 5% sucrose. As such, the gene provides a positive selection for its own inactivation and has been used as a trap for transposons in *Agrobacterium* spp. (11).

To test the lethality of levansucrase to *X. campestris* pv. *vesicatoria*, the *sacRB*-containing plasmid pUCD800 (11) was conjugated into strains 81-23 and E3C5. Kanamycin-resistant colonies of the strains were isolated, and plasmids with restriction patterns identical to that of pUCD800 were recovered from the transconjugants. However, viability of the transconjugants was not reduced when grown on medium containing 5% sucrose (data not shown). Concurrently, the 2.7-kb *Pst*I-*Bam*HI fragment of pUCD800 containing *sacRB* was cloned into pLAFR3, a vector that replicates well in *X. campestris* pv. *vesicatoria* (26). The resulting construct, pL3SAC, is shown in Fig. 3. The viability of transconjugants containing pL3SAC was significantly reduced when grown on medium containing 5% sucrose from a frequency of 10^{-4} in pepper race 2 strains to 10^{-7} in pepper race 1 strains (data not shown).

The plasmid pL3SAC was conjugated into *X. campestris* pv. *vesicatoria* pepper race 2 strains 81-23 and E3C5 and pepper race 1 strain 71-21 and nonpathogenic *Xanthomonas campestris* T55 carrying pXVCU1. Seven single colonies of each exconjugant were grown to log phase and plated on NYGA containing 5% sucrose. Plasmid DNA from one sucrose-viable mutant of each original colony was purified and transformed into *E. coli* to isolate the putatively mutated *sacRB* genes on pL3SAC away from the numerous copies of IS*476* in the 81-23 and E3C5 backgrounds. Plasmid DNA was isolated from each *E. coli* transformant and examined in Southern analysis. Since the seven individual colonies of a particular strain showed identical hybridization patterns (data not shown), only hybridization to a single representative from each strain is presented (Fig. 4). Wild-type *sacRB* resided on a 2.7-kb *Pst*I-*Bam*HI fragment (Fig. 4A, lane 1). Mutation to sucrose viability was accompanied by insertion of 1.2 kb of DNA into the fragment containing *sacRB* (lanes 2 to 4). Hybridization with an IS*476* probe (Fig. 4B) revealed that mutagenesis in each case was accompanied by the appearance of a copy of IS*476* on the same size fragment as the mutated *sacRB* (Fig. 4B, lanes 2 to 4), indicating that mutation of the *sacRB* genes in each case was caused by insertion of IS*476*.

Our data demonstrate that IS*476* transposition is not specific for *avrBs1* and that transposition can occur both in *X. campestris* pv. *vesicatoria* pepper race 2 strains and in a nonpathogenic strain of *X. campestris* (T55). Since T55 harbors no copies of IS*476* except those introduced on pXVCU1, it is evident that at least one of the three copies of IS*476* carried on the native copper plasmid (16) is a mobile transposable element. When pL3SAC was recovered from sucrose-viable colonies of pepper race 1 strain 71-21, no gross genetic changes were noted in *sacRB*, suggesting that some mutagenic process other than IS*476* transposition was

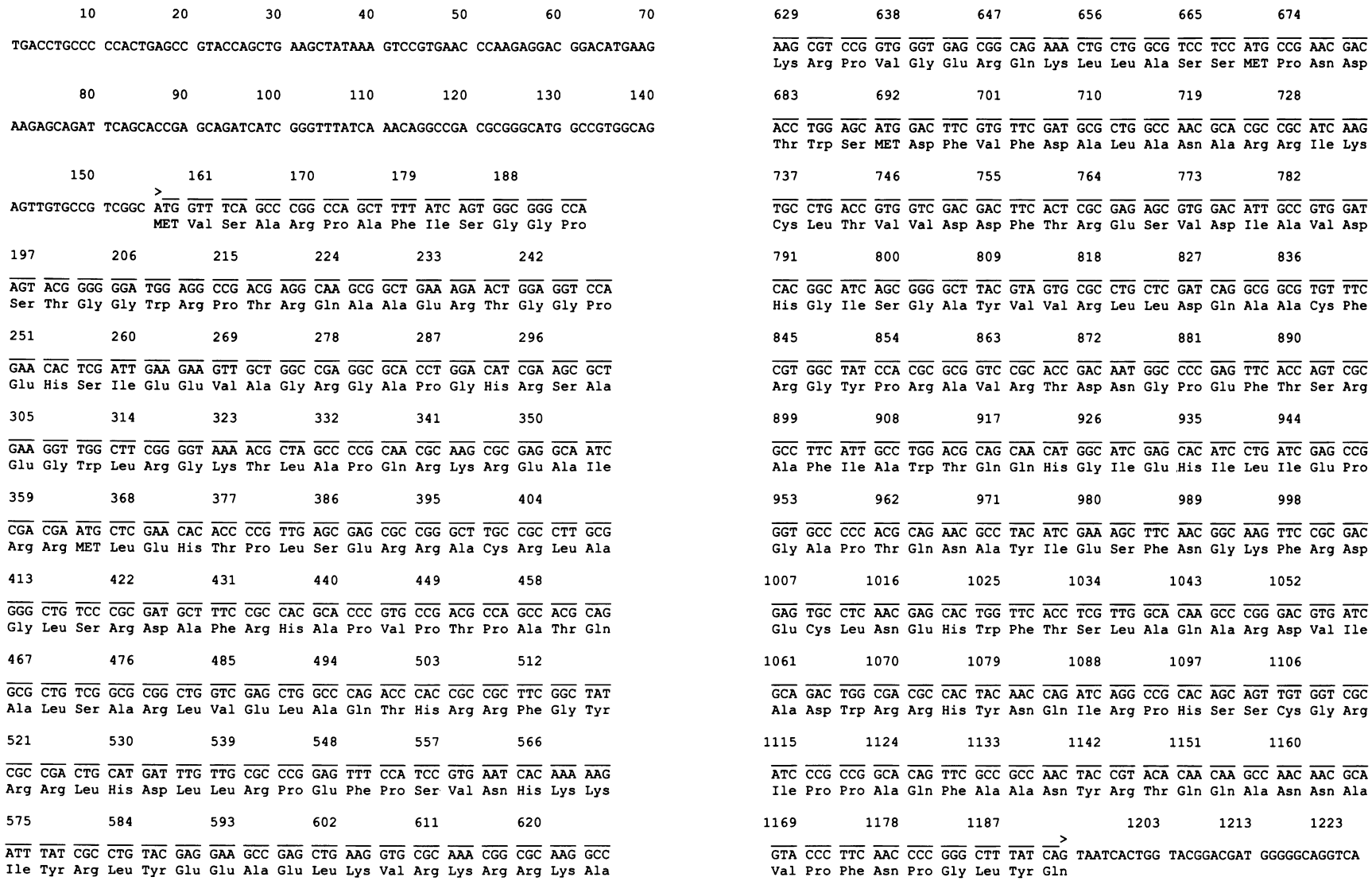

FIG. 1. Nucleotide sequence of IS*476*. Translation of ORF1 is shown, starting at position 156 and extending for 346 amino acids. ORF2 resides on the opposite DNA strand, starting at position 923 and extending for 198 amino acids.

A.

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          60        70        80        90       100       110
IS476  KTLAPQRKREAIRRMLEHTPLSERRACRLAGLSRDAFRHAPVPTPATQALSARLVELAQT
                                                          :     :
IS3    MKYVFIEKHQAEFSIKAMCRVLRVARSGWYTWCQRRTRISTRQQFRQHCDSVVLAAFTRS
              10        20        30        40        50        60

          120       130       140       150       160           170
IS476  HRRFGYRRLHDLLRPEFPSVNHKKIYRLYEEAELKVRKRRK------AKRPVGERQKLLA
       : :  :: : ::     : : .       :. .  ::          .          ::
IS3    KQRYGAPRLTDELRAQGYPFNVKTVAASLRRQGLRAKASRKFSPVSYRAHGLPVSENLLE
              70        80        90       100       110       120

              180       190       200       210       220
IS476  ----SSMPNDTWSMDFVFDALANARRIKCLTVVDDFTRESVDIAVDHGISGAYVVRLLDQ
           : ::  :  :              . :.:  .:  .     .       :
IS3    QDFYASGPNQKWAGDITYLRTPEGWLYLAV-VIDLWSRAVIGWSMSPRMTAQLACDALQM
              130       140       150       160       170

          230         240       250       260       270       280
IS476  AACFRGYPRA--VRTDNGPEFTSRAFIAWTQQHGIEHILIEPGAPTQNAYIESFNGKFRD
       :   :  ::   : :: :   :   :   :  : .       :    :: .:::    .
IS3    ALWRRKRPRNVIVHTDRGGQYCSADYQAQLKRHNLRGSMSAKGCCYDNACVESFFHSLKV
       180       190       200       210       220       230

          290       300       310       320       330
IS476  ECLNEHWFTSLAQARDVIADWRR-HYNQIRPHSSCGRIPPAQFAANYRT
       ::.     : :    :  .      ::  : :: :: . : ::
IS3    ECIHGEHFISREIMRATVFNYIECDYNRWRRHSWCGGLSPEQFENKNLA
       240       250       260       270       280
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B.

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IS476  TGACCTGCCCCCA-TGAGCCGT--ACCAG
       ::::: :::::   :   ::  :::
IS51   TGACC-GCCCCGGGTTTCTCGGAGACC
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FIG. 2. (A) Protein homology between the predicted transposase of IS*3* and ORF1 of IS*476*. Overall homology is 24%, 31% when conservative changes are taken into account. In the regions from IS*476* amino acids 120 to 131, 231 to 243, and 271 to 280, the two proteins range from being 46 to 60% homologous. Identical amino acids are shown with two dots, and conservative changes are shown with one dot. (B) DNA homology between the inverted repeats of IS*476* and IS*51*. Sixteen of the 26 bp are identical between the two.

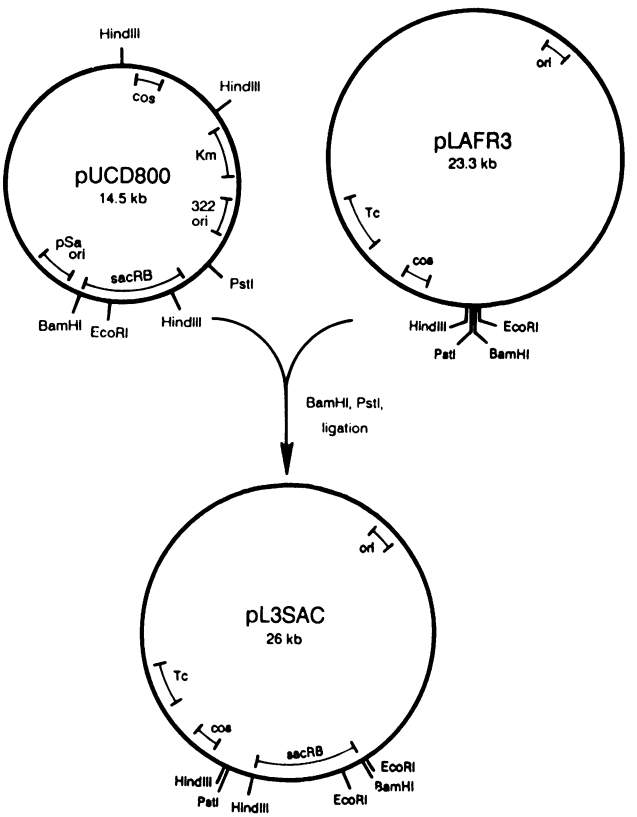

FIG. 3. Construction of pL3SAC. DNA from pUCD800 (11) was concurrently digested with *Pst*I and *Bam*HI. The 2.7-kb fragment containing *sacRB* was purified and ligated into the *Pst*I and *Bam*HI sites of pLAFR3 (26).

responsible for sucrose viability in these cases (data not shown).

Effect of IS*476* insertion on growth in pepper. In the initial screen for *X. campestris* pv. *vesicatoria* pepper race 2 mutants that caused disease on ECW10R plants, three classes of mutants were observed. Each class causes a distinct phenotypic reaction when inoculated at 10^8 CFU/ml into the intercellular space of leaves of ECW10R plants (15, 16). Wild-type strain 81-23 causes a hypersensitive reaction. M13 is fully virulent and causes confluent water soaking. M4 causes a hypersensitive reaction that is delayed by approximately 24 h, and M2 causes localized tissue collapse and necrosis around the center of inoculation surrounded by a water-soaked, spreading halo (data not shown).

The phenotype of the interaction is correlated with the position of insertion of IS*476* into the *avrBs1* locus of each mutant. In the fully virulent mutants (e.g., M13), IS*476* resides in the 50-kilodalton (kDa)-protein coding region of *avrBs1*. In the other two classes (e.g., M2 and M4), IS*476* is in the upstream, untranslated regions of *avrBs1* (15, 22). These intermediate reactions have also been observed in strains carrying deletion mutations of *avrBs1* (22).

To determine whether the phenotype of ECW10R foliar inoculations is correlated with the ability of the pathogens to grow in the plant, in planta growth curves were determined for one representative of each class of mutants (Fig. 5). In approximately 5 days, mutants that gave fully watersoaked reactions grew from a level of 10^4 CFU/cm^2 to 10^9 CFU/cm^2, a difference of 5 logs. In the same time, the growth of wild-type strain 81-23 increased only 3 logs. Similar to 81-23, M4's growth was severely restricted in the ECW10R plant compared with the fully virulent mutants. The growth of M2 and M13 was virtually indistinguishable in ECW10R plants (Fig. 5), despite the significant difference in the symptoms caused by each pathogen on ECW10R.

DISCUSSION

X. campestris pv. *vesicatoria* pepper race 2 strains spontaneously mutate to overcome the resistance encoded by the pepper gene *Bs1*. The mutation occurs at the *avrBs1* locus, and in all the mutants examined, mutation was caused by IS*476*. IS*476* is a small insertion sequence (1,225 bp) with 26-bp imperfect inverted repeats that cause a 4-bp target site duplication upon insertion. The element has been found only in copper-resistant strains of *X. campestris* pv. *vesicatoria* (unpublished observations). Plasmid pXVCU1 is a 200-kb self-transmissible plasmid from *X. campestris* pv. *vesicatoria* pepper race 2 strain E3C5. The plasmid contains *avrBs1*, a gene for copper resistance, and three copies of IS*476*. At least one copy of IS*476* on pXVCU1 is active, since transposition of IS*476* was observed in *X. campestris* T55 (pXVCU1), whose only copies of IS*476* are found on the introduced copper plasmid.

The homology between the inverted repeats of IS*476* and IS*51* from *P. syringae* pv. *savastanoi* implies a common origin. Furthermore, the small but significant homology between ORF1 and the putative transposase of IS*3* from *E. coli* strongly suggests that ORF1 encodes a transposase.

IS*476* transposition is not specific for *avrBs1*. Transposition into the plasmid-borne *sacRB* gene of *B. subtilis* was also observed. We assume that IS*476* transposes extensively

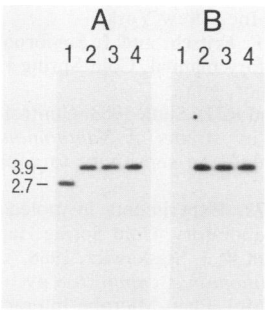

FIG. 4. Southern blot of pL3SAC recovered from sucrose-viable mutants of *X. campestris* pv. *vesicatoria*. Lane 1 contains pL3SAC DNA. Lanes 2 to 4 contain pL3SAC DNA recovered from sucrose-viable strains 81-23 and E3C5 and *X. campestris* T55(pXVCU1), respectively. The filter in panel A was probed with the 2.7-kb *Bam*HI-*Pst*I fragment containing *sacRB*. The control lane in panel A confirms that *sacRB* resides on a 2.7-kb fragment. In the pL3SAC plasmid recovered from each mutant, the size of the *sacRB* fragment is 3.9 kb. Panel B was probed with a 350-bp *Sal*I-*Sma*I fragment of IS*476*. No homology to IS*476* was observed in the native *sacRB* fragment, but the fragment recovered from each mutant hybridized to the IS*476* probe. In each case, then, mutation of *sacRB* was accompanied by insertion of IS*476* into the 2.7-kb fragment.

throughout the genome, and the effect of transposition on *X. campestris* pv. *vesicatoria* virulence is a random rather than directed event.

Insertions into different regions of *avrBs1* have different effects in terms of both inoculation phenotype and in planta growth of the *X. campestris* pv. *vesicatoria* strains. Presumably, insertion into different regions causes differential expression of *avrBs1*. Consequently, we believe that the level of *avrBs1* activity is rate-limiting in inducing the hypersensitive reaction on pepper hosts.

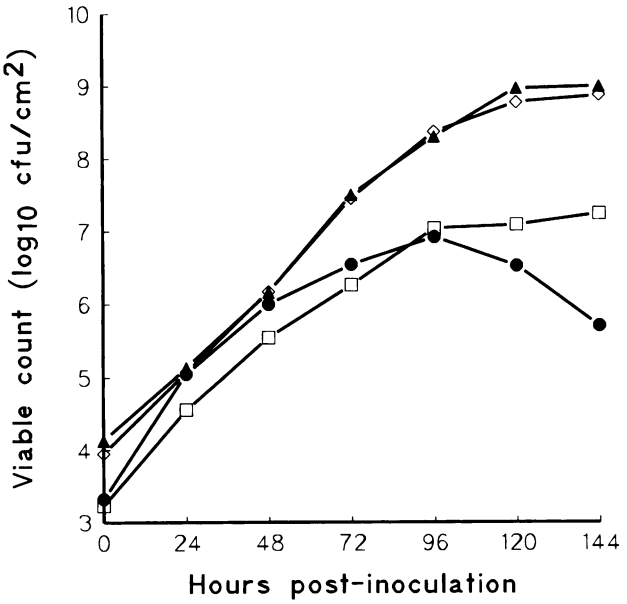

FIG. 5. Growth of *X. campestris* pv. *vesicatoria* strains 81-23 (●), M2 (◇), M4 (□), and M13 (▲) in ECW10R (*Bs1Bs1*) plants. Growth of avirulent strain 81-23 was restricted to 3 logs in 5 days. In the same time, growth of virulent strains M13 and M2 increased 5 logs. The growth of M4 paralleled the growth of 81-23 until 96 h postinoculation. At that time, growth of M4 plateaued while that of 81-23 declined. For each point, the standard error is ≤ 0.10 $\log_{10}$ CFU/cm^2. The growth curve was repeated twice with essentially the same results.

The onset of hypersensitivity varies between resistance gene-avirulence gene pairs in *X. campestris* pv. *vesicatoria*-pepper interactions. Hypersensitivity caused by the resistance gene *Bs1* can be observed within 8 h of inoculation (4). On the other hand, hypersensitivity encoded by the pepper resistance gene *Bs3* may take 48 h to become visible (17). The 24-h delay in onset of hypersensitivity upon inoculation of M4 compared with wild-type reactions demonstrates that timing of the hypersensitive reaction is dependent on avirulence gene activity.

It has often been argued whether hypersensitivity is a cause or a result of resistance (18). In this interaction, M2 induces hypersensitive necrosis when inoculated at high cell density and is still able to multiply effectively in the plant when infiltrated at low cell density. The virtually indistinguishable growth of M2 and fully virulent M13 despite very different symptoms suggests that hypersensitivity and resistance may be separable.

The association of active copies of IS*476* and a gene for copper resistance on a self-transmissible plasmid has raised the question of whether spraying copper on peppers for microbial control is counterproductive (15). In pepper race 2 strains, all the spontaneous mutations to wider host range recovered were caused by IS*476*. It is possible, then, that spread of pXVCU1 caused by selection of copper-resistant strains will increase the ability of pathogens to spontaneously mutate to overcome genetically defined resistance. It is too early to tell, however, whether subsequent distribution of IS*476* will reduce the stability of single-gene disease resistance. Our laboratory has isolated numerous avirulence genes that limit host range in *X. campestris* pv. *vesicatoria*. We have molecular probes for these genes, for IS*476*, and for pXVCU1. It should be possible, then, to study the evolution of pathogen populations in the field when plants with specific resistance genes are released.

ACKNOWLEDGMENTS

We thank R. E. Stall for providing the original *X. campestris* pv. *vesicatoria* strains and the pepper cultivar ECW10R. C. I. Kado kindly provided pUCD800. Maureen Whalen, Pamela Ronald, Roger Innes, Milt Schroth, and Yves Dessaux read the manuscript and made useful suggestions. Doug Dahlbeck provided valuable technical help.

This work was supported by National Science Foundation grant DMB 88-05123 to B.J.S. B.K. was supported by a National Science Foundation graduate fellowship.

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