Table 1*a* and *b*). In the Portuguese samples (Table 2), high d-infection levels were not recorded in the pathogenic phase, but in the saprophytic phase d-infection of the supergroup increased from ~44% at the front (Tomar) to ~67% (small sample) at the older site, whereas that of the heterogeneous component declined from ~38% to ~21%. As sexual outcrossing would tend to restrict the spread of d-factors by generating new vc groups and producing d-factor free ascospores, debilitation of the frontal vc clones by high levels of mycovirus infection could be a prime cause of the rapid change in population structure. The contrasting low level of d-infection in the dominant vc group at Avila (Table 1*c*) could therefore be due to the high frequency of A-mating types within it.

The Tomar/Mafra samples also support the view that the supergroups are products of selection maintained by asexual propagation. This is indicated primarily by their having a very uniform colony phenotype, whereas the heterogeneous component isolates are extremely variable. Second, in a pathogenicity test on a 4 m Commelin elm in June 1987, ten supergroup isolates (5 T+5 M) were significantly more pathogenic than ten heterogeneous component isolates, causing 69 and 49% mean defoliation at 12 weeks, respectively ($P < 0.001$). Third, strong selection between the saprophytic and pathogenic phases is indicated (Table 2) by: (1) the smaller heterogeneous component in the pathogenic phase at both sites ($P < 0.001$); (2) the lower frequency of less pathogenic A-types[9,11] in the pathogenic phase at Mafra ($P < 0.001$); (3) the lower frequency of the weakly pathogenic non-aggressive subgroup[6,9] in the pathogenic phase at Tomar ($P < 0.01$) and (4) the lower frequency of diseased or d-infected isolates in the pathogenic phase at both sites ($P < 0.001$ for supergroup; $P < 0.01$ for heterogeneous component). The supergroup B types may therefore have a selective advantage over many heterogeneous component genotypes in the pathogenic phase, at least during the early stages of an outbreak. This in turn would enhance the supergroup genotype's success by increasing its feedback[9,21] from the pathogenic phase into the saprophytic, so promoting its dispersal by the next generation of vector beetles. The population frequency of a supergroup may therefore depend in part upon a balance between its fitness advantage and the disadvantage of heavy d-infection. Other environmental changes as an epidemic progresses, such as the ensuing collapse of the elm and beetle vector populations[5,9], may also favour an increase in pathogen diversity and so contribute to the decline of the supergroups.

Further research is in progress on the comparative fitness and genetic structure of the dominant vc clones, on the selective impact of d-factors and on the processes underlying the appearance of A-mating types and rapid generation of variability. The latter could involve pseudo-selfing[14,15] or gene-flow from the resident, but almost totally genetically isolated[5,9], non-aggressive subgroup. Although the emergence of 'pathotype' clones in biotrophic pathogens of arable crops is well known[22,23], the plasticity shown by *O. ulmi* suggests a potential for rapid evolution under intense selection, and for the emergence of fitted clones in more ecologically complex necrotrophic pathogens of perennial hosts. Such selection might occur during epidemic spread in a geographically new and susceptible host population as here, during exposure to widespread crop monoculture, or other environmental pressure. Nonetheless, a clone could also be particularly vulnerable to further changes in selection, including the spread of a mycovirus. Indeed, there is evidence to suggest that both the decline of the chestnut blight epidemic in Europe during the 1950s (refs 24-26), and the decline of the first epidemic of Dutch elm disease in the 1920s to 1940s (A. G. Mitchell and C.M.B., unpublished results) could have followed the spread of mycoviruses in dominant vc clones. In the current epidemics of Dutch elm disease, however, the diversification of the *O. ulmi* supergroups, if a response to d-factor pressure, may have occurred too rapidly for the latter to limit the initial impact of the disease.

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# Molecular basis for evasion of plant host defence in bacterial spot disease of pepper

**Brian Kearney, Pamela C. Ronald, Douglas Dahlbeck & Brian J. Staskawicz**

Department of Plant Pathology, University of California, Berkeley, California 94720, USA

**A fundamental objective in studying host–pathogen interactions is to determine how a pathogen evolves to overcome the defences of a previously resistant host. In mammalian systems, evolution of a pathogen population is often directed towards avoiding recognition by an immune system[1–3]. Although plants lack circulating antibodies and the memory response of mammalian immune systems, it is becoming increasingly clear that active resistance in plants involves recognition of the pathogen by its host[4,5]. In this paper we present the first molecular evidence to indicate that plant pathogens evolve to overcome resistance by evading host recognition and response. The bacterial pathogen *Xanthomonas campestris* pathovar *vesicatoria* mutates to overcome genetically defined resistance in pepper, *Capsicum annuum*, by the transposon-induced mutation of *avrBs*$_1$, a bacterial gene that provokes the plant's resistance response.**

Bacterial spot disease of peppers is caused by *X. c.* pv. *vesicatoria* (*Xcv*)[6–8]. Peppers carrying the gene $Bs_1$ are resistant to Race 2 strains of *Xcv*. Inoculation of the cultivar ECW10R ($Bs_1Bs_1$) with *Xcv* Race 2 causes a hypersensitive response[9] (HR) in the region of infection. When ECW10R is inoculated

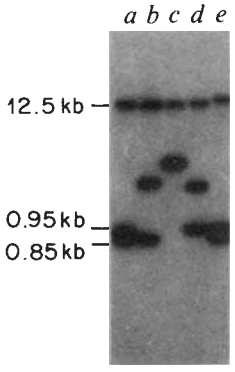


**Fig. 1** Southern blot of *Xcv* strains probed with $avrBs_1$. Lane *a*, DNA from *Xcv* Race 2, strain 81-23. Three hybridizing bands appear at 12.5, 0.95 and 0.85 kb. Lanes *b–e*, DNA from M1, M2, M4 and M13 respectively. Each mutant displays a mobility shift in one hybridizing band of ~1.2 kb when compared with the wild-type isolate. In M2 both the 0.85 kb and 0.95 kb bands disappear and a new band appears at 3.0 kb, which can be explained by a 1.2 kb insertion into the *Eco*RV site in between the two smaller bands.
**Methods.** Total genomic DNA was digested with *Eco*RV, separated electrophoretically through an 0.8% agarose gel, transferred to Nytran membrane and probed with a $^{32}$P-labelled *Pst*I/*Bgl*II fragment of pXV2007[12] containing $avrBs_1$. The blots were washed at high stringency, essentially as described[19].

with *Xcv* Race 1 water-soaked lesions occur, and the pathogen is able to multiply to high titre[10].

To study how bacterial pathogens become virulent on previously resistant hosts, thirteen independent spontaneous mutants of *Xcv* Race 2 were selected that had become virulent on ECW10R and were thus able to overcome $Bs_1$[11,12]. We have previously identified and cloned a genetic locus ($avrBs_1$) from *Xcv* Race 2 that is responsible for specifically inducing a HR on pepper plants carrying the resistance gene $Bs_1$. When a plasmid containing $avrBs_1$ was conjugated into each of the 13 spontaneous mutants, the transconjugants caused a HR on pepper plants containing $Bs_1$[12]. We therefore concentrated our study on the $avrBs_1$ locus of each mutant.

Four mutants were chosen for detailed study. Of the four, two (M1 and M13) had completely overcome the resistance encoded by $Bs_1$ and induced fully water-soaked lesions when inoculated onto ECW10R plants. The other two mutants caused intermediate reactions on $Bs_1$ plants. M4 develops a HR on ECW10R ~24 h later than the wild-type pathogen. M2 causes an intermediate reaction that is partially necrotic and partially water-soaked.

The $avrBs_1$ locus of each mutant was examined and compared to the wild-type gene from *Xcv* Race 2, strain 81-23. Southern analysis of *Eco*RV-digested DNA revealed that ~1.2 kilobases (kb) of DNA was inserted into different regions of $avrBs_1$ in each mutant (Fig. 1). Southern analysis of the remaining nine mutants demonstrated that 1.2 kb of DNA was inserted into $avrBs_1$ in these cases as well (data not shown).

To further analyse the mutant avirulence genes, a library of *Xcv* Race 2, mutant M1, was made in the cosmid pLAFR3 essentially as described previously[13]. Two clones containing the mutant $avrBs_1$ gene were identified by colony hybridization using a 5.3 kb *Pst*I/*Bgl*II fragment containing $avrBs_1$ as a probe to screen the library. To clone the $avrBs_1$ locus from the other three mutants under detailed study, total genomic DNA was digested with *Pst*I and *Sst*I, size fractionated on an agarose gel, and 6–9 kb fragments were cloned into pUC118 and pUC119[14]. The clones containing the mutant $avrBs_1$ loci from M2, M4 and M13 were also identified using the wild-type 5.3 kb fragment containing $avrBs_1$ as a $^{32}$P-labelled probe.

Restriction-enzyme analysis of the cloned loci confirmed that each mutation was accompanied by an insertion of 1.2 kb into $avrBs_1$. Furthermore, the DNA inserted in each case had an identical restriction map (Fig. 2*a*), suggesting the independent mutations were caused by the same element.

The complete DNA sequence of the insertion from M1 was determined. The element is 1,225 base pairs (bp) long and displays the structures of a typical bacterial insertion sequence. It has 26 bp imperfect inverted repeats and is flanked by a 4 bp target-site duplication. Two open reading frames (1,037 and 593 bp) are present in opposite orientations in the same frame. The element has been listed with the Central Plasmid Registry[15] and has been named IS476.

The precise location of the insertion in each mutant was determined by sequencing outward into $avrBs_1$ from the ends of IS476. A 20-nucleotide primer was synthesized for each inverted repeat. Small differences in the sequences of the left and right repeats were exploited to make specific 3′ ends of the primers (Fig. 2*b*). The location of IS476 in the mutants is shown

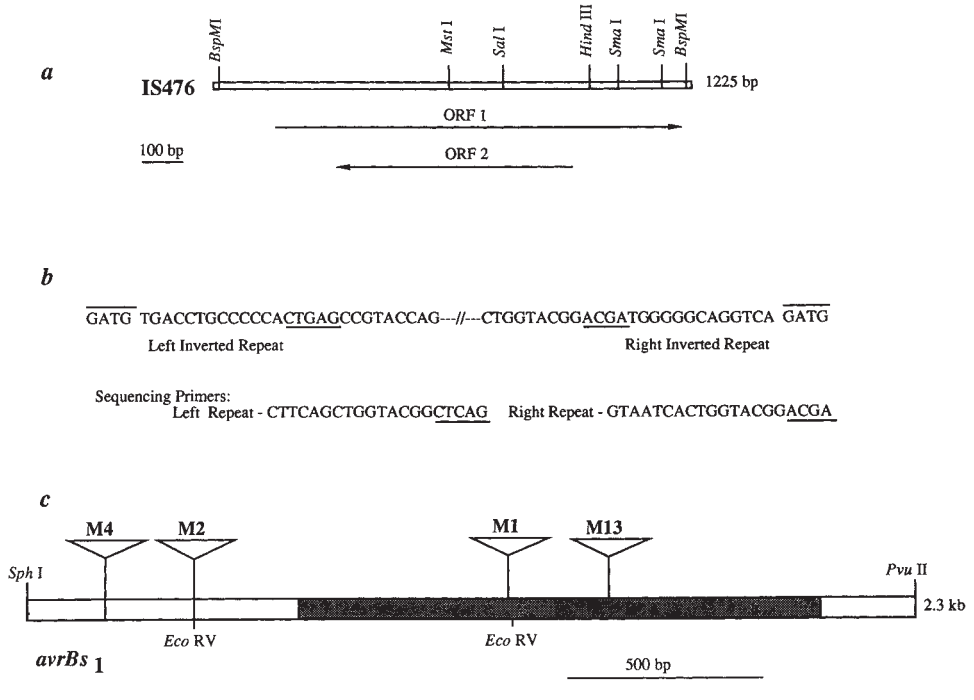


**Fig. 2** *a*, Restriction enzyme map of IS476. For all the sites tested, the restriction map of IS476 in mutants M1, M2, M4 and M13 is identical. *b*, The 26 bp inverted repeats and the target site duplication (GATG) from mutant M1 are shown. The regions underlined indicate the differences between the left and the right repeats. The sequencing primers synthesized to localize the position of insertion of IS476 in each mutant are also shown. The underlined bases at the 3′ ends of each primer correspond to the areas of difference between the inverted repeats. *c*, Restriction map of $avrBs_1$ showing the position of insertion of IS476 in each mutant as determined by sequencing analysis. The shaded region of the map contains the open reading frame necessary for $avrBs_1$ activity (P.C.R. *et al.*, manuscript in preparation). In M1 and M13 IS476 inerted into the coding region of $avrBs_1$. In M4 and M2, IS476 inserted far upstream of the $avrBs_1$ coding region, suggesting that the mutations are polar or lie in regulatory regions necessary for normal $avrBs_1$ activity.

in Fig. 2c. In M1 and M13 (which completely lose $avrBs_1$ function), IS476 is integrated into the coding region of $avrBs_1$ (shaded area in Fig. 2c; P.C.R. *et al.*, manuscript in preparation). In both M4 and M2, IS476 is inserted upstream of the $avrBs_1$ coding region. As mentioned previously, the HR caused by M4 on $Bs_1$ plants is delayed by 24 hours and M2 gives an intermediate HR/water-soaking phenotype on $Bs_1$ plants. These intermediate reactions may result from lowered protein levels due to IS476 insertion into an $avrBs_1$ regulatory region.

In bacterial spot disease of pepper, the stability of resistance is a function of the mutation frequency of the avirulence genes in the pathogen, and, to date, all naturally occurring mutations isolated in $avrBs_1$ have been caused by IS476. We do not know if transposon mutagenesis is a universal mechanism by which pathogens overcome host resistance as too few data are available to make any conclusion. Presumably any kind of mutation that enables host recognition to be avoided would have the same effect.

The importance of IS476 in bacterial spot disease may result from its linkage to copper resistance in *Xanthomonas*. Southern analysis has shown that the 200 kb plasmid (pXvCu1) (ref. 16) in *Xcv* that carries $avrBs_1$ also carries three copies of IS476 and a gene for copper resistance. We have recently determined that at least one copy of IS476 on pXvCu1 is an active transposable element, and preliminary data suggest that mutation rates in *Xcv* backgrounds containing IS476 are approximately an order of magnitude higher than in backgrounds lacking the element (B.K. *et al.*, manuscript in preparation). We have also determined that pXvCu1 is self-transmissible in bacteria grown both in culture[12,16] and in plants when maintained under copper selection (data not shown). Copper has been a primary chemical defence against bacterial spot disease in Florida for years[17,18], and now almost all strains of *Xcv* isolated from Florida are copper resistant (R. E. Stall, personal communication). Southern blot analysis of DNA isolated from a 20-year collection of *Xcv* strains has hown that IS476 is found only in copper-resistant strains (data not shown). This evidence suggests that IS476 is spread by the copper-selected transmission of pXvCu1. It is ironic that by trying to control *Xcv* pathogenesis with copper sprays, a highly mutagenic element has been propagated that enables the pathogen to overcome genetically defined disease resistance.

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# DNA typing from single hairs

**Russell Higuchi, Cecilia H. von Beroldingen*, George F. Sensabaugh* & Henry A. Erlich**

Department of Human Genetics, Cetus Corporation, Emeryville, California 94608, USA
* Forensic Sciences Program, University of California, Berkeley, California 94720, USA

**The characterization of genetic variation at the DNA level has generated significant advances in gene and disease mapping[1], and in the forensic identification of individuals[2–6]. The most common method of DNA analysis, that of restriction fragment length polymorphism (RFLP), requires microgram amounts of relatively undegraded DNA for multi-locus typing, and hundreds of nanograms for single-locus comparisons[7]. Such DNA frequently cannot be obtained from forensic samples such as single hairs and blood stains, or from anthropological, genetic or zoological samples collected in the field. To detect polymorphic DNA sequences from single human hairs, we have used the polymerase chain reaction (PCR), in which specific short regions of a gene can be greatly amplified *in vitro*[8–10] from as little as a single molecule of DNA[10]. We have detected genetically variable mitochondrial and nuclear DNA sequences from the root region of shed, as well as freshly-plucked, single hairs; mitochondrial DNA (mtDNA) sequences have been detected in a sample from a single hair shaft. We have used three different means of DNA typing on these samples: the determination of amplified DNA fragment length differences, hybridization with allele-specific oligonucleotide probes, and direct DNA sequencing.**

Most of the DNA in hair is located in the root and surrounding sheath cells[11]. As measured by the fluorescence of DNA-dye complexes in crude lysates of hairs, the root end of freshly-plucked hairs may contain as much as 0.5 μg DNA, whereas hair shafts contain too little DNA to determine its quantity and condition[11]. Our recovery of purified DNA, however, has rarely been more than 200 ng from freshly-plucked hairs and has usually been less than 10 ng from shed hairs. DNA 'fingerprint' analysis with multi-locus probes has been done on DNA pooled from multiple, freshly-plucked hairs of the same individual[3] and a single-locus analysis could presumably be performed on a single, freshly-plucked hair[7]. The hairs found at the scene of a crime, however, may derive from different individuals, and are usually shed hairs. Therefore, the typing of single, shed hairs would be much preferable to that of pooled, freshly-plucked samples.

Figure 1 shows electrophoresis of the amplification products from DNA isolated from the separated root and shaft portions of single, freshly-plucked head hairs from two individuals. The Klenow fragment of DNA polymerase I was used for these amplifications[8,9]. A previously characterized length polymorphism in human mtDNA[12,13] distinguishes the samples from the different individuals; DNA sequencing of the mtDNA of individual 1 had shown a 9 base-pair (bp) deletion in this region[13]. This deletion may be Asian-specific. PCR with oligonucleotide primers flanking this region results in a DNA fragment that includes the length variable region and is either 111 or 120 bp depending on whether this deletion is present (this difference could, therefore, be deemed a '(PCR)FLP'). Amplification products of the appropriate sizes for each individual are obtained from both the root and shaft portions of the hairs. Thus even the shaft portion contains enough copies of mtDNA to be detected using PCR. Electrophoresis can also distinguish amplification products that differ in their pattern of restriction enzyme sites[3,9,14].

Figure 2 shows typings of a nuclear gene amplified from the root portions of single, shed hairs from six different individuals. 'Single-copy' nuclear genes, in this case the class II HLA gene