Effector Genes of *Xanthomonas axonopodis* pv. *vesicatoria* Promote Transmission and Enhance Other Fitness Traits in the Field

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ABSTRACT

Establishing durable disease resistance in agricultural crops, where much of the plant defense is provided through effector-***R*** gene interactions, is complicated by the ability of pathogens to overcome ***R*** gene resistance by losing the corresponding effector gene. Many proposed methods to maintain disease resistance in the field depend on the idea that effector gene loss results in a fitness cost to the pathogen. In this article we test for fitness costs of effector gene function loss. We created directed knockouts of up to four effector genes from the bacterial plant pathogen *Xanthomonas axonopodis* pv. *vesicatoria* (*Xav*) and examined the effect of the loss of a functional gene product on several important fitness parameters in the field. These traits included transmission, lesion development, and epiphytic survival. We found that the products of all four effector genes had significant and often additive effects on fitness traits. Additional greenhouse tests revealed costs of effector gene loss on *in planta* growth and further showed that the effects on lesion development were separable from the effects on growth. Observable fitness effects of the three plasmid-borne effector genes were dependent upon the loss of functional *avrBs2*, indicating that complex functional interactions exist among effector genes with *Xav*.

Plants have evolved a complex surveillance system, mediated by plant resistance (**R**)** genes, that is capable of recognizing and responding to the presence of many different plant pathogens. During infection, bacterial plant pathogens inject proteins directly into plant cells via the type III secretion system (*Collmer et al.* 2000; *Casper-Lindley et al.* 2002). These pathogen-delivered “effector” proteins presumably alter plant cell function to create a more favorable environment for the pathogen. **R** genes monitor the plant cell for the presence of pathogen-secreted effector proteins and, upon detection, activate rapid and robust plant defense responses. Associated with these is the hypersensitive response, a rapid localized form of programmed cell death that is readily assayed in whole leaves and that severely reduces the ability of a pathogen to grow and cause disease (*Dangl and Jones* 2001; *Bonas and Lahaye* 2002). Historically, any pathogen effector protein that was recognized by an **R** gene was termed an “avirulence” (**avr**) gene, since the presence of the effector gene in the pathogen prevents it from successfully infecting a host plant with the matching **R** gene. Plants carry many different **R** genes, and pathogens harbor a diverse array of effector genes, but the interaction between an effector and an **R** gene is quite specific. If either the **R** gene or its matching effector gene is not present, then no **R** gene-mediated defense response occurs during an infection, and disease progresses. Because the presence of both plant **R** genes and pathogen effector genes varies within populations, neither disease nor resistance is assured during infection (*Jarosz and Berdon* 1991; *Holub et al.* 1994; *Stahl et al.* 1999).

Pathogens of crop plants have usually been able to overcome gene-for-gene (major gene) resistance through the loss of the effector genes, leading to a general lack of **R** gene durability in the field (*Leach et al.* 2001; *Stuiver and Custers* 2001; *Brown* 2002; *McDonald and Linde* 2002; *Stuthman* 2002). Thus, there is keen interest in identifying ways to slow down pathogen adaptation and thereby preserve **R** gene effectiveness. One proposed method is to target effector genes critical to pathogen virulence, so that any pathogen that regains virulence through loss of an effector gene would be dramatically less fit (*Laue et al.* 1998; *Vera Cruz et al.* 2000). Other proposed methods to maintain **R** gene resistance include using mixtures of host plant varieties in the field (*Zhu et al.* 2000; *Mudr* 2002), pyramiding major resistance genes (*McDonald and Linde* 2002), and relying more on quantitative (minor gene) resistance (*Stuiver and Custers* 2001; *McDonald and Linde* 2002). Most of these methods assume that pathogens incur a cost to virulence, *i.e.*, that most, if not all, effector genes are beneficial for pathogen fitness.

To date, roughly half of all effector genes cloned from phytopathogenic bacteria have been tested for fitness contributions (reviewed by *Leach et al.* 2001; *van’t Slot and Knogge* 2002). For many effector genes (42%), there appears to be no associated fitness benefit. Indeed,
one effector gene, \textit{avrBs1}, has been shown to produce a fitness cost when present in the pathogen (O’Garro et al. 1997). If a large fraction of effector genes are entirely dispensable to the pathogen, then many of the strategies currently proposed to maintain \textit{R} gene durability must be reexamined. On the other hand, it is unlikely that effector genes lack a beneficial function since selection is not expected to maintain genes that incur a high cost with no counteracting benefit (Frank 1992, 1993). Furthermore, the recent demonstration that several effector proteins actively interfere with the host plant’s resistance pathways, better enabling the pathogen to cause disease, casts doubt on the idea that effector genes do not actively benefit the pathogens that harbor them (Abramovitch et al. 2003; Antell and Staskawicz 2003; Hauck et al. 2003; Mackey et al. 2003).

There are several reasons why fitness benefits may have been overlooked for some effector genes. First, interactions among effector genes in bacterial pathogens can be very complex. Assemblages of effector genes that interact both additively and redundantly (Lorang et al. 1994; Yang et al. 1996) or in complex relationships have been identified (Jackson et al. 1999; TsaiM et al. 2000). Due to these complex interactions, the fitness contributions of an effector gene may be obscured when the presence/absence of only a single gene is tested. Second, very subtle changes in disease phenotypes, which are difficult to detect and even more difficult to quantify, may be dismissed as not significant to the pathogen. It is important to remember that as the effective size of a population increases, smaller fitness effects are subject to selection (Kimura 1983). The relatively large sizes of bacterial populations imply that even a very small fitness change can have a significant effect on the evolution of the pathogen.

When fitness benefits associated with effector genes have been detected (58%), all but one study (Vera Cruz et al. 2000) was restricted to laboratory/greenhouse assays of \textit{in planta} bacterial growth and/or symptom development (Kearney and Staskawicz 1990; Yang et al. 1994; Ritter and Dangl 1995; Chang et al. 2000; Guttman and Greenberg 2001). While controlled, indoor experiments such as these undoubtedly provide valuable data on plant-pathogen interactions, how well these results extrapolate to determining disease dynamics in the field is unknown. Indeed, none of these studies measure directly the ability of a pathogen to transmit and cause new infection, even though transmission is the most important determinant of pathogen fitness (Anderson and May 1992). If we are to rely on gene-for-gene interactions in agriculture, then we must know how an effector gene contributes to pathogen fitness under field conditions.

Here we report the results of experiments designed to test for fitness costs associated with the loss of functional protein for four effector genes, alone and in combination, under field conditions. We used the economically important and well-defined system \textit{Xanthomonas axonopodis pv. vesicatoria} (\textit{Xav}; formerly known as \textit{X. campestris pv. vesicatoria}), the causal agent of bacterial spot disease in peppers and tomatoes, and the host plant \textit{Capsicum annuum} (bell pepper). We examined the fitness effects of four effector genes in \textit{Xav}: \textit{avrBs1}, \textit{avrBs2}, \textit{avrBs3}, and \textit{avrBs4} (also known as \textit{avrBs3-2} or \textit{avrBsP}). We report the effect of these effector genes on several important epidemiological traits in the field, including transmission, epiphytic survival, and the degree of lesion development. Follow-up greenhouse experiments also allowed us to compare effects of effector gene loss on lesion development between the field and the greenhouse, as well as to examine the correlation between lesion development and \textit{in planta} growth rates.

Each of the four effector genes examined in this study was previously identified and cloned (\textit{avrBs1}, Ronald and Staskawicz 1988; \textit{avrBs2}, Kearney and Staskawicz 1990; \textit{avrBs3}, Bonas et al. 1989; \textit{avrBs4}, Bonas et al. 1993). \textit{avrBs4} is recognized by an \textit{R} gene found in tomato, while the other three effector genes are recognized by \textit{R} genes found in pepper.

\textit{avrBs2} is found in many \textit{Xanthomonas} species and is functionally conserved within this genus, implying that it performs an essential function (Kearney and Staskawicz 1990). It is located chromosomally, and its removal via marker-exchange mutagenesis resulted in reduced \textit{Xav} growth \textit{in planta} but no change in symptom development (Kearney and Staskawicz 1990; Swords et al. 1996). \textit{avrBs2} has homology to two other bacterial genes that are known to be involved in the synthesis and hydrolysis of phosphodiester bonds (Swords et al. 1996), although \textit{avrBs2}'s fundamental biochemical function is still unknown.

The remaining effector genes (\textit{avrBs1}, \textit{avrBs3}, and \textit{avrBs4}) are found on large endogenous plasmids, and their presence within an individual bacterium is variable. The presence of \textit{avrBs1} in \textit{Xav} has been found to decrease the ability of the pathogen to survive in soil and dead plant material (O’Garro et al. 1997); no compensating fitness benefit has yet been reported. \textit{avrBs3} and \textit{avrBs4} belong to a large, highly conserved effector gene family (the \textit{avrBs3} family), which is distributed widely throughout the genus \textit{Xanthomonas}. \textit{avrBs3} and \textit{avrBs4} are 97% identical at the sequence level, but they are recognized by different plant \textit{R} genes (Bonas et al. 1993; Ballyora et al. 2001). All members of this gene family contain three nuclear localization signals and an acidic activation domain (similar to eukaryotic transcription factors) in the C-terminal end of the protein (Yang and Gabriel 1995; Van den Ackerveken et al. 1996; Zhu et al. 1998), and \textit{avrBs3} has been shown to stimulate new gene transcription in host plants (Marois et al. 2002). Loss of the nuclear localization signals from \textit{avrBs3} has been shown to result in an inability to trigger a defense response in resistant plants (Van den Ackerveken et al. 1996; Zhu et al. 1998).
TABLE 1
Genotypes of all Xav bacterial lines used in this study, with respect to the effector genes

<table>
<thead>
<tr>
<th>Strain name</th>
<th>avrBs1</th>
<th>avrBs2</th>
<th>avrBs3</th>
<th>avrBs4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Xav19R (wt)</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
<td>wt</td>
</tr>
</tbody>
</table>

Mutant bacterial genotypes

- 1<sup>a</sup> wt wt wt wt
- 1<sup>b</sup> wt wt wt wt
- 2    wt wt   wt wt
- 21<sup>a</sup> wt wt   wt wt
- 23    wt wt   - wt
- 231   wt wt   - wt
- 2314  wt wt   - -

Restored bacterial genotypes

- 2+2<sup>a</sup> wt + wt  wt
- 21+21<sup>a</sup> + + wt  wt
- 23+23<sup>a</sup> wt + + + wt
- 231+231<sup>a</sup> + + + + wt
- 2314+23<sup>a</sup> - + + -

The restored bacterial lines were made from the corresponding mutant lines and used the same process (described in MATERIALS AND METHODS) to reinsert wild-type effector gene sequence. wt, original gene never altered; +, mutant gene replaced with original wild-type sequence; −, frameshifting (null) mutation inserted into wild-type gene.

<sup>a</sup> Used only in greenhouse experiments, not in field experiments.

MATERIALS AND METHODS

Plant lines: All pepper seed (C. annuum) used in this study was obtained from David Ritchie. Experiments testing effector fitness used the pepper cultivar early calwonder (ECW), which is fully susceptible to Xav19R. Three near-isogenic lines of ECW (ECW10R, ECW20R, and ECW30R; HIBBERD et al. 1987), each containing an R gene corresponding to avrBs1, avrBs2, or avrBs3, respectively, were used to test for the presence or absence of functional effector gene activity.

Bacterial culturing: All mutant Xanthomonas strains used in this study were derived from strain Xav19, which was isolated from a diseased pepper plant in North Carolina by David Ritchie. Xav19 is known to carry four effector genes: avrBs1, avrBs2, avrBs3, and avrBs4. We derived a streptomycin-resistant (stre^<p>^<p>) strain of Xav19 (Xav19R, which we refer to as "wild type") that had a growth curve similar to that of the original Xav19 and used this for all subsequent strain construction. All bacterial strains created in this study are listed in Table 1.

Xanthomonas was grown on nutrient yeast glycerol (NYG) media at 30°C and Escherichia coli was cultured on standard Luria-Bertani (LB) media. NYG and LB were supplemented with the appropriate antibiotics at the following concentrations: streptomycin (NYG, 25 μg/ml), kanamycin (NYG, 10 μg/ml), and ampicillin (LB, 50 μg/ml). Bacteria washed from pepper leaves were plated on TWEEN A media that allowed for growth of Xav19R, but inhibited growth of fungi and other plant-associated bacteria (adapted from McGUIRE et al. 1986). The modifications affected only the antibiotics added to the media, which were 100 μg/ml cycloheximide, 64 μg/ml cephalaxin, 25 μg/ml streptomycin, and 12 μg/ml 5-fluorouracil.

For long-term storage, all bacterial stocks were kept at −80°C in 15% glycerol. For short-term storage, bacteria were kept in 10 mM sterile MgCl<sub>2</sub> at 4°C. Measurements of the concentration of bacteria both in and on leaves were done using a spiral plater (Whitley automatic spiral plater, Du Scientific) and the associated plate reader (Protocol).

**Strain construction:** Four effector genes were PCR amplified out of Xav19R and either cloned into pUC19 (avrBs1 and avrBs2) or ligated to the PCR-amplified vector backbone of plasmid pPSG49a (avrBs3 and avrBs4). The vector backbone of pPSG49a contains the region from the R6K origin to the ampicillin-resistance gene of plasmid pBSL118 (ALEXEYEV et al. 1995). Table 2 lists all primers and PCR conditions. Frameshift mutations were engineered in each cloned effector gene by digesting the gene with a restriction enzyme (that cut only once within the first one-third of the coding sequence), treating with T4 DNA polymerase, and religating. Clones with an altered restriction pattern were sequenced to determine the nature of the mutation (Table 3). Sequencing reactions used Big Dye (Applied Biosystems/ABI, Foster City, CA) version 3.0 and were run on an ABI 3700 sequencer. PCR primers also doubled as sequencing primers. Both mutated and wild-type effector genes were subsequently cloned into the vector pSD800 to be used for chromosomal gene exchange, using the protocol of GASSMANN et al. (2000). Briefly, pSD800 carrying a single, frameshifted effector gene was introduced via electroporation (Xav19R is resistant to triparental mating): fresh electrocompetent Xav cells were made using the protocol of WHITE and GONZALEZ (1991) and between 250 and 750 ng of plasmid DNA was added to a 50-μl aliquot of fresh electrocompetent cells; transferred to a 1-mm electroporation cuvette; electroporated at 25 μF, 200 Ω, and 1400 V; and allowed to recover for 2 hr at 30°C with mild shaking. Kanamycin-resistant colonies were picked, grown without selection, and plated on NYG plates amended with 5% sucrose. After replica plating to NYG-kanamycin plates, cells that were both kanamycin sensitive and sucrose resistant were picked and tested on pepper lines containing the appropriate R gene to test for loss of effector gene function, defined here as the inability to elicit a hypersensitive response on resistant plants. To screen for mutations in avrBs4, we used colony PCR to amplify the region containing the Psd site and digested 8 μl of the PCR product with Psd. Colonies causing disease on resistant plants or, in the case of avrBs4, possessing an altered restriction pattern, were selected, and the effector gene was PCR amplified and sequenced to confirm that our engineered mutation had replaced the wild-type sequence. Southern blots were also performed to verify that no pPSG49a vector sequences remained in the mutant strains (data not shown).

The effector genes were mutated sequentially, so that each genotype containing an additional mutant effector gene was derived from the previous mutant genotype. The mutant bacterial genotypes are therefore not independent of each other and are referred to as bacterial lines or strains. The name of the mutant line indicates the order in which the genes were removed. Wild-type effector genes were restored into all mutant bacterial lines listed in Table 1 via the same process of chromosomal gene exchange described above. The resultant "restored" lines contain any random mutation acquired during line construction, as well as additional mutations created during the restoration, but contain only wild-type effector gene sequences in their native loci.
### Table 2

**PCR conditions and primers used to amplify and clone effector genes for fitness analysis**

<table>
<thead>
<tr>
<th>Gene target</th>
<th>Primer name</th>
<th>Primer sequence (5'-3')</th>
<th>Restriction site</th>
<th>Annealing temperature</th>
<th>Extension time</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full length</td>
<td>avrBs1-642U</td>
<td>TGAGCTCCTATGACGGACTTGTGCTCG</td>
<td>SacI5</td>
<td>58°C</td>
<td>2 min</td>
</tr>
<tr>
<td>avrBs1(ORF2)</td>
<td>avrBs1-2069L</td>
<td>TGCATGGCTGGCGGATCTTCTTCTCT</td>
<td>SphI</td>
<td>58°C</td>
<td>2 min</td>
</tr>
<tr>
<td>Full length</td>
<td>avrBs2</td>
<td>TGCATGCGAACGCTTGATGGGGAAGGT</td>
<td>XmaI</td>
<td>53°C</td>
<td>4 min</td>
</tr>
<tr>
<td>avrBs3</td>
<td>avrBs3-187U</td>
<td>TCCCGGGCCACGTCGATTCTGCT</td>
<td>XmaI</td>
<td>53°C</td>
<td>4 min</td>
</tr>
<tr>
<td>Full length</td>
<td>avrBs4</td>
<td>avrBs4-50U</td>
<td>XmaI</td>
<td>58°C</td>
<td>2 min</td>
</tr>
<tr>
<td>avrBs4</td>
<td>avrBs4-3963L</td>
<td>TCCCGGGCCACGTCGATTCTGCT</td>
<td>XmaI</td>
<td>58°C</td>
<td>50 sec</td>
</tr>
<tr>
<td>backbone</td>
<td>TransFL</td>
<td>TCCCGGGCCACGTCGATTCTGCT</td>
<td>—</td>
<td>50°C</td>
<td>50 sec</td>
</tr>
<tr>
<td>Internal</td>
<td>avrBs1-1643L</td>
<td>TACAGTACAGTACAGTACAGT</td>
<td>—</td>
<td></td>
<td></td>
</tr>
<tr>
<td>avrBs2</td>
<td>avrBs2-28U</td>
<td>GGCAACGCGTCCAAACAAC</td>
<td>—</td>
<td>50°C</td>
<td>50 sec</td>
</tr>
<tr>
<td>avrBs3</td>
<td>avrBs3-187U(noX)</td>
<td>CGACGGTATGGGTAAGTTG</td>
<td>—</td>
<td>58°C</td>
<td>50 sec</td>
</tr>
<tr>
<td>avrBs4</td>
<td>avrBs4-375L</td>
<td>CATCGACGCTAGTTCGTT</td>
<td>—</td>
<td>58°C</td>
<td>50 sec</td>
</tr>
</tbody>
</table>

All PCRs had an initial denaturation time of 3 min at 95°C, followed by 35 cycles of 30 sec at 95°C, 30 sec at the specified annealing temperature, and 72°C for the specified extension time, with a final 5-min extension at 72°C.

a PCR reactions included both Taq and Pwo polymerase (Roche Applied Science), each at 0.05 units per microliter per reaction.

b PCRs used Pfu turbo (Stratagene, La Jolla, CA) according to manufacturer’s recommendations.

c Primers are the same sequence.

### Field experiments:

Field experiments were initiated on June 5, 2001, at the University of Illinois Pharmacognacy Field Station in Downer’s Grove, Illinois. Pepper plants were grown from seed at the University of Chicago greenhouse for 6 weeks prior to transplantation to the field. All field experiments were performed blindly; bacterial phenotypes were not matched to treatment genotypes until after data were recorded. Plants were identified by numbers, with the specific treatment assigned to that number determined randomly prior to planting in the field. Patches were separated by 3 m of bare earth. Each bacterial genotype had 18 replicates and in addition there were 10 control patches (center plant not treated), for a total of 100 patches. A border of 3 m of bare earth was also maintained around each site. No fertilizer or irrigation was applied to plants in the field, and watering was done by hand when necessary. A hard rain occurred late on the planting day (June 5, 2001) and continued throughout the second day. In all field experiments, we tested four mutant bacterial lines, along with wild-type (Xav19R) and control plants (Table 1).

### Table 3

**Description of engineered effector gene mutations used for fitness analysis**

<table>
<thead>
<tr>
<th>avr gene</th>
<th>Restriction site</th>
<th>Wild-type sequence</th>
<th>Frameshift mutation</th>
<th>Nature of mutation</th>
<th>Position of cut site</th>
<th>Total length of coding sequence (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>avrBs1</td>
<td>SphI</td>
<td>ACTAGT</td>
<td>ACTAGCTAG</td>
<td>4-bp insertion</td>
<td>412</td>
<td>1338</td>
</tr>
<tr>
<td>avrBs2</td>
<td>NotI</td>
<td>GGGGGGCGC</td>
<td>GGGGGGCGGCGCGCG</td>
<td>4-bp insertion</td>
<td>434</td>
<td>2145</td>
</tr>
<tr>
<td>avrBs3</td>
<td>PstI</td>
<td>CGACTGAGATC</td>
<td>CGATC</td>
<td>7-bp deletion</td>
<td>84</td>
<td>3495</td>
</tr>
<tr>
<td>avrBs4</td>
<td>PstI</td>
<td>CGACTGAGATC</td>
<td>CGAGATC</td>
<td>4-bp deletion</td>
<td>84</td>
<td>3483</td>
</tr>
</tbody>
</table>

The restriction site mutated in each of the four effector genes occurs only once within the coding sequence and cloned 5' flanking region of that gene. The position of the cut site is listed from the start of the protein coding sequence. The base pairs comprising the original restriction sequences are underlined.
were kept under plastic cages for 3 days in the greenhouse to maintain high humidity and then transplanted to the field on the fourth day postinfection (June 5, 2001).

Symptom development of the center, infected plant was monitored daily between days 3 and 6 in the field (days 6–9 postinfection). Infected leaves started falling off plants 9 days after infection. Disease symptoms began with small water-soaked lesions that were visible only on the underside of the leaves. These lesions grew and coalesced into large, necrotic lesions visible on the upper leaf surfaces. Symptom development was grouped into three categories: very little/no disease (no symptoms or only a few pinpricks of disease observable), moderate disease (many small water-soaked lesions visible only on the underside of the leaf), and full disease (large, necrotic lesions visible on the top of the leaf). Data on disease symptom expression are reported for day 8 postinfection for both field and greenhouse experiments.

Transmission was calculated as the total area of visible disease symptoms on the top of all previously untreated leaves on all plants (both center and uninfected ring), done by overlaying a transparent grid of 1-mm squares on plant leaves. Transmission was recorded on days 14, 18, and 22 in the field. On the second transmission collection date, to confirm that the bacterial genotype causing infection matched the original treatment genotype, diseased tissue was also collected (~1 cm²) from 20 plants showing high levels of new disease. Tissue was sampled from all five genotype treatments. Tissue was surface sterilized in 70% ethanol and then macerated in 200 μl of sterile 10 mM MgCl₂ and plated on NYG with 25 μg/ml streptomycin. A subsample of 20 colonies from each of the 20 tissue samples (400 colonies total) was randomly picked from the plates and stored in sterile water for several months to await genotyping.

Plot 2, epiphytic survival: A second field plot examined the epiphytic survival cost associated with the loss of effector gene function. A bacterial suspension (3 × 10⁶ cfu/ml) of a single genotype was sprayed onto the upper leaves of a plant, until the liquid ran off the leaves. Each plant received ~5 ml of bacterial suspension. Plants were allowed to dry overnight in the greenhouse and were transplanted to the field the following day (June 5, 2001).

The epiphytic experiment consisted of 100 patches of plants spaced 5 m apart on bare ground. Patches consisted of four plants of the same bacterial treatment planted at the corners of a square with 20-cm sides. Each of the bacterial genotypes (five total) was replicated 18 times, in addition to 10 control patches (not treated). One leaf per patch was randomly selected from the patch canopy on days 3, 9, 18, 25, and 46 in the field. Leaves >6 cm long were not chosen due to size constraints in the washing process.

Prior to the field experiment, the correlation between leaf area and leaf length was calculated for ECW plants: cell area (cm²) = −6.802 + 3.21 × length (cm) (r = 0.947, P < 0.001). The amount of bacteria removed per leaf was normalized by leaf area using the equation above. A single leaf was placed into a sterile, brown paper lunch bag in a chilled cooler and brought back to the lab for immediate processing. Leaves were placed into sterile 25-ml flasks with 10 ml of sterile potassium phosphate buffer (10 mM KH₂PO₄, pH 7.0) with 0.05% peptone, washed at 250 rpm for 2 hr at room temperature, and plated. (The limit of detection for bacteria on a leaf was ~10⁰ cfu/leaf.) A subsample of 400 colonies (20 colonies from 20 different plants) was randomly picked off plates from the day 25 field sample and stored in sterile water for several months to await genotyping.

Symptom development in the greenhouse: The costs associated with the loss of effector gene function seen in the field were repeated under greenhouse conditions using the same methods, except that plants were kept in the greenhouse instead of being transplanted to the field. Three additional mutant genotypes (−I, −I₃, and −I₂; Table 1) were used in the greenhouse disease emergence experiment, with 15 replicates of each genotype. Symptom development was monitored from days 5 through 8 postinfection.

Symptom development and in planta growth in the greenhouse: The relationship between symptom development and bacterial growth in planta was assayed with additional experiments in the greenhouse. The use of restored lines in these experiments was used to confirm the role of effector genes in symptom development and bacterial growth. A small patch near the tip of fully expanded leaves was infiltrated with a bacterial suspension (3 × 10⁵ cfu/ml) using a needle-less syringe. Infected plants were kept in the greenhouse under humidity cages for 3 days. Measures of the number of bacteria in leaf tissue were taken on days 0, 5, and 7 postinfection. Greenhouse conditions were set at a minimum temperature of 24° during the day and 22° at night, with 16 hr of light, although temperatures often exceeded 24° during the day.

Samples of infected leaf tissue were excised using a standard paper hole puncher (1.2 cm²), surface sterilized in 70% ethanol, and then macerated in 200 μl of sterile 10 mM MgCl₂, serial diluted, and plated on NYG with 25 μg/ml streptomycin. Symptom development was monitored between days 5 and 8 postinfection. Each experiment had eight replicates per bacterial genotype (11) per time point (3), for a total of 264 individual infections. Two leaves per plant were infected, each leaf with a different genotype, for a total of 192 plants. The experiment was repeated three times.

Statistical analysis: All analyses were performed using Stastistica version 6.0 (StatSoft, Tulsa, OK). Data from all time points for both disease transmission and epiphytic growth were analyzed using a multivariate approach to repeated-measures ANOVA, in which data from each date are considered a separate variable. In addition, to test for differences among bacterial genotypes at the start of the experiment, a one-way ANOVA was performed on data from the first sample date (day 3 in the field) for the epiphytic experiments. All disease transmission and epiphytic growth data were transformed using the equation \( y = \log(x + 1) \). To compare performance of individual bacterial genotypes, contrast analysis was performed using the genotype by time interaction, with the three time points given equal weight. For all contrast analyses, a modified Bonferroni correction known as the Dunn–Sidak method (Sokal and Rohlf 1995) was employed to determine the correct \( \alpha \). The Dunn–Sidak equation is \( \alpha' = 1 - (1 - \alpha)^{1/k} \), in which \( \alpha = 0.05 \) and \( k \) is the number of contrasts performed.

One-way ANOVAs involving all genotypes were performed on in planta growth data on days 0 and 5. Data from each time point were transformed using the Box-Cox method: \( y = x^k \), where \( s \) is 1 minus the slope of the regression of the ln(median) vs. ln(interquartile spread) for each treatment (Steidl and Thomas 2001). Comparisons of individual bacterial genotypes to wild type for each time point used contrast analysis, corrected for multiple contrasts as discussed above.

For all data sets involving the development of disease symptoms, two analyses were performed. First, we calculated an overall chi-square statistic on a contingency table of bacterial genotype and disease severity. Second, we performed a correspondence analysis to compare individual genotypes. Correspondence analysis is analogous to a principal components analysis, but it is performed on two-way or multway tables. No P values or significance tests are associated with this analysis. Instead, it is a visual representation of the similarities/differences in frequencies for all rows (bacterial genotypes) in the
contingency table. The distances between points in the final plot of a correspondence analysis are analogous to a weighted chi-square value; the further apart two points are in a correspondence analysis, the more dissimilar are the frequencies in the contingency table (Statistica 6.0 manual, StatSoft).

RESULTS

Disease emergence and transmission in the field:
When the transmission experiment was transplanted to the field (day 4 postinfection), all treated leaves showed early visible disease symptoms (tiny lesions visible on the underside of leaves). By the third day in the field (day 6 postinfection), a dramatic difference was already observable between plants infected with wild-type Xav-19R vs. mutant genotypes. Leaves infected with wild type had progressed in disease severity, with most leaves showing full disease, while disease on leaves infected with the mutant genotypes either had not progressed or had disappeared (the water-soaked lesions were no longer visible). Disease symptoms slowly redeveloped and progressed, and a clear trend was observed among the mutant genotypes for the rates at which disease reemerged and the final levels of disease severity (Figure 1). Bacterial genotypes with fewer effector genes redeveloped disease more slowly, and the most severe mutant genotypes (−23I and −23I4) never reached full disease before the leaves fell off the plants on day 6 in the field (day 9 postinfection).

Because Xav is not a vascular pathogen, transmission of bacteria to new parts of the plant depends primarily on rain splash. A hard rain occurred several hours after our experiment had been transplanted into the field and continued through the next day. Thus, it is likely that most transmission occurred during the first 2 days in the field. When initially planted, all genotypes were showing moderate disease on their infected leaves (due to the high humidity treatment in the greenhouse). It was not until the rain passed (and the relative humidity dropped) on the third day in the field that differences in the level of visible disease among genotypes became noticeable. After 2 weeks in the field, full bacterial spot disease lesions were detected on the upper, untreated, leaves of the center plants. New disease was then spread over all the untreated leaves, rather than being restricted to the lowest leaves. Almost no transmission to the ring of untreated plants surrounding the center plant was observed, and control plants remained clear of all disease.

A repeated-measures, multivariate ANOVA on transmission revealed significant effects of bacterial genotype and date, but the interaction between bacterial genotype and date was not significant (Figure 2). These patterns were robust in an analysis that excluded the wild-type bacteria. In the graph of transmission in Figure 2, wild-type bacteria were best able to transmit, with the mutant lines −2 and −23I4 second best, and mutant lines −23 and −23I least able to transmit. Contrast analysis on the individual genotypes confirmed this pattern. Contrast analysis of wild type vs. all four mutant genotypes revealed a significant difference (P < 0.001; for all transmission contrast analyses, α′ = 0.0127, k = 4). Mutant lines −23 and −23I had transmission rates indistinguishable from each other (P = 0.87), just as line −23I4 was indistinguishable from line −2 (P = 0.83). A contrast analysis of mutant lines −2 and −23I4 vs. mutant lines −23 and −23I revealed these two groups to be significantly different (P < 0.001).

Samples of plant tissue showing new disease con-

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Figure 2.—Effect of effector gene removal on disease transmission in the field. Transmission was measured as the total area of new disease lesions (full disease, visible on the upper surface of the leaf) on untreated leaves, with 18 replicates per genotype per time point. Data were analyzed by a repeated-measures ANOVA, using the multivariate statistic, Roy’s greatest root (R). Lowercase letters denote significant differences among genotypes over the course of the experiment, as determined by contrast analysis. Error bars represent one standard error. ANOVA tables are given for both an analysis on all bacterial genotypes in the field and an analysis on mutant genotypes only.

Figure 3.—Effect of effector gene removal on epiphytic growth in the field. One leaf per replicate was removed at each time point and brought back to the lab for immediate processing; the bacteria were washed off the leaf, plated, and counted. Each genotype had 18 replicates in the field. Data were analyzed by a repeated-measures ANOVA, using the multivariate statistic Roy’s greatest root (R). Lowercase letters denote significant differences among genotypes over the course of the experiment, as determined by contrast analysis. Error bars represent one standard error. ANOVA tables are given for both an analysis of all bacterial genotypes in the field and an analysis of mutant genotypes only.

tained almost exclusively bacteria able to grow on 25 μg/ml streptomycin that looked identical to Xav19R. Of the 400 colonies collected, ~200 colonies (50%), representing 19 different patches, were recovered after prolonged storage at 4°C. All recovered colonies caused disease in the susceptible pepper line ECW, and all but 9 colonies matched the original genotype treatment for the particular plant. Of the 9 colonies that did not match the original bacterial genotype treatment for the plant, 7 came from a single tissue sample. This infected tissue was collected from a plant originally treated with wild-type Xav19R, but half of the colonies tested from this tissue appeared to have lost avrBs3 gene function. Further testing of these 7 deviant colonies by PCR and restriction digest showed that the avrBs3 loci had not been lost entirely, and they did not contain our engineered mutation. It appears that some other mutation spontaneously arose within avrBs3 during transmission and infection of the leaf. The remaining 2 of the 9 deviant colonies did contain our engineered mutations, but did not correspond to the original bacterial treatment for that plant, giving an estimated cross-contamination rate of 1% in the transmission experiment.

Epiphytic survival in the field: Our experiment on epiphytic survival found substantial variation in the distribution (over a log scale) of bacteria on leaves, consistent with published studies of bacteria on leaf surfaces (Hirano et al. 1982; Hirano and Upper 1993). A one-way ANOVA performed on the samples taken 2 days after transplantation showed no differences among genotypes in the initial amount of bacteria on leaf surfaces (F = 0.27, d.f. = 4.85, P = 0.90). A repeated-measures, multivariate ANOVA on data over the course of the first month of the experiment revealed significant effects of bacterial genotype, date, and the interaction term on epiphytic survival (Figure 3).

An examination of only the mutant bacterial genotypes found no significant differences among the four mutant bacterial genotypes and no significant effect of the bacterial genotype by date interaction for either...
multivariate statistic. Date, however, was found to be significant (Figure 3). Contrast analyses of the wild-type bacteria with each individual mutant genotype showed that the epiphytic survival of Xav19R was significantly higher than that of each mutant. Contrast values were significant at $P < 0.001$ in all cases ($\alpha' = 0.0127$, $k = 4$).

By day 25 in the field, the leaves treated with bacteria were becoming too large to sample. Three weeks were allowed to pass before another sample was taken (day 46 in the field) to see if colonization of new, untreated leaves occurred. Data from this sample revealed that most new leaves contained almost no Xanthomonas; only 14 leaves (15.6% of patches) contained any detectable Xanthomonas. The distribution of leaves containing Xanthomonas was spread evenly among all five genotypes ($5 \times 2$ contingency table, $\chi^2 = 3.61$, d.f. = 4, $P = 0.46$). Control plants never yielded any detectable Xanthomonas in the samples taken through day 25 in the field, although one control leaf sampled on day 46 did contain Xanthomonas.

Of the 400 colonies picked from plates and stored at $4^\circ$ for genotyping, only 25 (6%), representing 11 different patches, were recovered. All colonies caused disease in the susceptible pepper line ECW, and 2 colonies did not match the original genotype sprayed onto the plant, yielding an estimated cross-contamination rate of 8%. Both of the mismatched colonies came from a plant sprayed with the bacterial line $\text{avrBs2314}$, from which no other colonies were recovered to test.

**Disease emergence in the greenhouse:** We found that the disease emergence phenotypes observed in the field could be duplicated under greenhouse conditions (Figure 4). Mutant genotypes with fewer effector genes were generally less able to cause disease symptoms, although in this greenhouse experiment, there was no difference between the two mutant genotypes $\text{-2}$ and $\text{-23}$. Three additional genotypes were tested in the greenhouse. Among these, the $\text{-21}$ genotype was slightly less robust than $\text{-23}$ (and $\text{-2}$), but it still had higher fitness than the genotypes with more $\text{avr}$ genes removed ($\text{-231}$ and $\text{-2314}$). Interestingly, the mutant genotypes $\text{-1}$ and $\text{-13}$ developed full disease just as well as wild type (Figure 4). Even though removal of $\text{avrBs1}$ and $\text{avrBs3}$ in the mutant $\text{avrBs2}$ background showed clear effects in the field and greenhouse, we did not observe these effects in the presence of wild-type $\text{avrBs2}$.

**Growth vs. symptom development in the greenhouse:** Because we found that the disease emergence pattern could be replicated in the greenhouse, we next examined if there was an association between in planta growth and symptom development. In these experiments, we used only the mutant bacterial genotypes for which we had previously detected deficiencies in symptom development, namely genotypes $\text{-2}$, $\text{-21}$, $\text{-23}$, $\text{-231}$, and $\text{-2314}$. We also used the corresponding restored bacterial lines (Table 1) to determine if the phenotypic differences previously observed could be ascribed to the loss of effector genes.

We observed the same pattern of symptom development among mutant lines (Figure 5) that we saw in the field and greenhouse experiments. Namely, as more effector genes were removed, the pathogen was less able to form disease lesions. The wild-type bacteria had the most robust disease development, followed closely by all the restored lines. Although the restored lines did not return symptom development to the same level as wild type, they were nearly as robust and clearly showed increased fitness relative to their corresponding mutants (Figure 5).

By day 8 postinfection, most of the full disease seen...
Figure 6.—Effect of effector genes on in planta bacterial growth. Leaf tissue was excised on day 5 postinfection using a paper hole puncher, surface sterilized, macerated, and plated, and the bacteria were counted. Each bacterial genotype was replicated eight times. Lowercase letters represent significant differences between genotypes. All lines were compared to wild type, plus each bacterial mutant genotype was compared against its corresponding restored line (\(H_9251/<H_11005>0.0034, k=15\)). The significant difference seen here between /H11002 231 and /H11002 2314 was not consistently observed among replicate experiments. No significant differences were observed among bacterial genotypes on day 0 of the experiments (data not shown).

Figure 5.—Effect of effector genes on disease emergence during bacterial growth experiments, including both mutant genotypes and their corresponding restored lines. Data shown are for day 8 postinfection. (A) Contingency table of the number of plants showing full, moderate, or little/no disease when infected with different bacterial genotypes. The chi-square statistic is for the entire contingency table. (B) Correspondence analysis for the contingency table shown in A.

Figure 6 revealed a significant growth difference for only the most severe effector gene knockout lines: \(-231 (P=0.000003)\) and \(-2314 (P=0.0000001)\); \(P\) values for the other three mutant lines (-2, -21, and -23) ranged from 0.170 to 0.827. For all bacterial growth contrast analyses, \(\alpha' = 0.0054 (k=15\) total comparisons). Further contrast analysis comparing the amount of bacterial growth of each restored line to wild type failed to detect significant growth differences; contrast \(P\) values ranged from 0.216 to 0.780. Finally, comparisons of each restored line to its corresponding mutant genotype found a significant growth difference for \(-231 vs. -231 + 231 (P=0.001)\) and \(-2314 vs. -2314 + 23 (P=0.00002)\); significance of the three other mutant/ restored line contrast analyses ranged from \(P=0.105\) to \(P=0.984\). This experiment was repeated a total of three times with similar results, with one exception. As shown in Figure 6, the bacterial genotypes \(-231\) and \(-2314\) are significantly different from each other; however, in some cases, these two mutant lines were not statistically distinguishable (although they always had significantly less growth than wild type).

**DISCUSSION**

In any host-pathogen interaction, pathogen transmission is the most important fitness parameter (Anderson and May 1992; McCallum *et al.* 2001; Fenton *et al.*
To our knowledge, this is the first study designed to look explicitly at the loss of effector genes on pathogen transmission. Most previous studies have inferred that the costs of effector gene removal seen in the greenhouse, i.e., reduced *in planta* bacterial growth (Swords et al. 1996), symptom development (Bai et al. 2000), or amount of bacteria released onto leaf surfaces (Yang et al. 1994), would directly translate to reduced pathogen transmission in the field. A notable exception is the study done by Vera Cruz et al. (2000) on the rice pathogen *X. oryzae pv. oryzae*. Although not a direct measurement of transmission, this study found that pathogen strains carrying mutations in the *avrXa7* gene were less aggressive and less persistent in the field. In addition, two articles by Kousik and Ritchie (1996, 1998) have looked at the persistence and spread (transmission) of several races of *Xav* on different pepper cultivars in the field; however, it is difficult to extrapolate the costs/benefits of effector genes from these studies because such comparisons would be confounded by different genetic backgrounds.

Our experiments utilized frameshifted effector genes that lead to severely altered and early truncated proteins, and hence full effector gene activity is assumed to be abolished in our mutant strains, since it is highly likely that the improperly folded proteins are degraded (Wickner et al. 1999). However, since Western blots were not performed, we cannot rule out the possibility that the correct N-terminal protein fragment encoded before the frameshift could still be delivered into plant cells and affect the host-pathogen interaction. Therefore, the results we discuss below may be conservative. In particular, if residual activity of any effector genes occurred, then a complete deletion mutation would likely have a greater effect on pathogen fitness.

In our field studies of *Xav* transmission, the wild-type genotype, *Xav19R*, caused more new disease on host plants than did any of the mutant effector genotypes (Figure 2). Removal of *avrBs2* resulted in significantly less new disease, and in general, subsequent removal of additional effector genes further reduced pathogen transmission. The transmission costs of effector gene removal in *Xav19R* therefore appear to be largely additive. Additive fitness effects are probably a common feature of effector genes and have been reported in several other plant pathogen species (Lorang et al. 1994; Yang et al. 1996).

One surprising result of the transmission experiment was the ability of the most severe mutant genotype, −2314H, to transmit new disease at the same rate as the −2 mutant genotype. It is difficult to understand the meaning of a higher transmission rate for the −2314 genotype relative to the −23 and −231 mutants. Either there is a beneficial effect for the pathogen of removing *avrBs4*, or the data are somehow anomalous. Subsampling of newly infected plant tissue revealed only a 1% level of cross-contamination in the transmission experiment. An examination of spatial location in the field also showed that plants containing the highest disease transmission were scattered throughout the field, negating the possibility that spatial position may have had an inadvertent effect on transmission. In addition, further tests (discussed below) showed that among all the mutant genotypes, −2314 had the lowest ability to form lesions and had the lowest bacterial *in planta* growth. Interpretation of the −2314 transmission data must therefore be left for further investigation.

Transmission combines many important aspects of a pathogen’s life cycle, such as the ability to survive outside the host prior to infection (epiphytic survival), the ability to reproduce in the host (*in planta* growth), and the ability to escape the host (develop lesions). In an attempt to decipher the driving forces behind the observed transmission cost of *avr* gene loss, we individually tested these three components of transmission. In examining the effect of effector genes on the pathogen’s epiphytic fitness, we found that the wild-type genotype was much more fit than all the mutant effector genotypes (Figure 3). Since all mutant effector genotypes tested in the field contained the *avrBs2* mutation, and the mutant genotypes were indistinguishable from each other, the observed epiphytic fitness cost can be attributed to the loss of functional *avrBs2* alone. The low recovery of epiphytic colonies stored temporarily at 4°C prevented an extensive test of cross-contamination among patches. Nevertheless, our small sample revealed that 8% of recovered colonies were mismatched with the host plant treatment. This level of bacterial genotype cross-contamination would lead to homogenization among treatments and might explain why we did not observe any differences among the mutant genotypes.

Our findings support the work of two other studies that examined the effect of the type III secretion system on epiphytic survival; both Roine et al. (1997) and Hernando et al. (1999) found that removal of the type III secretion system in different pathovars of *Pseudomonas syringae* led to dramatically less epiphytic growth in the field. Although neither of these studies explicitly looked at the effect of effector genes, they postulate that the ability to secrete effector molecules is important for a pathogen’s epiphytic fitness.

We also examined the effect of effector gene removal on the ability of the pathogen to form disease lesions in the field. Previous work has shown that the rate of lesion expansion can play a very important role in plant disease epidemics (Berger et al. 1997). We found that all effector genes examined (*avrBs1, avrBs2, avrBs3*, and *avrBs4*) had a significant effect on the ability to develop disease lesions (Figure 1). Removal of functional *avrBs2* reduced the rate at which the pathogen developed disease, and within the *avrBs2*-deficient background, subsequent removal of one, two, or three more effector genes had additive costs on both the rate of disease develop-
ment and the extent to which disease symptoms developed. Repeating the disease emergence experiment in the greenhouse showed that the cost of effector gene removal on disease development could be reproduced indoors (Figure 4), which supports the argument that effector gene fitness costs found in the greenhouse can be translated to costs in the field. The result that several effector genes affected the ability of the pathogen to form disease lesions was not surprising given that many other effector genes had previously been shown to have an effect on symptom development during disease: avrB6 (Yang et al. 1994, 1996), pthA (Swarup et al. 1991), avrRpm1 (Ritter and Dangl 1995), avrE and avrA (Lorang et al. 1994), avrXa7 and avrXa5 (Bai et al. 2000), avrRpt2 (Chang et al. 2000; Guttman and Greenberg 2001), and avrPto (Chang et al. 2000; Shan et al. 2000).

Further experiments designed to tease apart the relationship between symptom development and in planta bacterial growth revealed that deficiencies in lesion development did not correspond to deficiencies in bacterial growth (Figures 5 and 6). Although the mutant genotypes −2, −21, and −23 were consistently less able than wild-type bacteria to cause disease symptoms, these same genotypes had in planta growth rates indistinguishable from wild type. Only the most severe mutant genotypes, −231 and −2314, had a significant reduction in in planta growth relative to wild type. We conclude from these experiments that within the avrBs2-deficient background of Xav19R, avrBs1 and avrBs3 must each have very small effects on in planta growth, but that these small effects are additive. In addition, we conclude that all four effector genes have much larger and separable additive effects on disease symptom development. Although it has been already shown that many effector genes have an effect on both disease development and bacterial growth (i.e., pthA, avrRpm1, avrE, avrA, avrXa7, avrXa5, avrRpt2, and avrPto; see references above), to our knowledge this is the first report that it is possible to separate an effector gene’s contribution to in planta growth from its contribution to disease development.

Within the in planta growth and lesion development experiments, use of the restored lines allowed us to attribute the observed loss of fitness to the removal of effector genes in the mutant genotypes. Not only did reinstating the wild-type effector gene sequences restore growth to wild-type levels in those mutant lines that had reduced growth (−231 and −2314; Figure 6), but also the restored lines were dramatically better in forming disease lesions than were their respective mutant genotypes (Figure 5). Residual impairment of the restored lines in their ability to cause disease symptoms is presumably due to accumulation of unknown random mutations during the repeated rounds of strain creation. Although not tested directly under field conditions, our ability to restore bacterial performance under greenhouse conditions makes it highly likely that the loss of effector genes, not other random mutations, was the primary cause of fitness reduction in the epiphytic and transmission experiments as well.

The inclusion of additional mutant bacterial genotypes in some of the greenhouse experiments (specifically lines −1 and −13) revealed novel complexity among effector genes. In our experiments, we found that the loss of avrBs1 and avrBs3 in an avrBs2-deficient background reduced the ability of the pathogen to cause disease symptoms. However, we found that the −1 and −13 mutant lines (which have a wild-type avrBs2 gene) developed disease at the same rate and severity as wild-type Xav19R. In other words, it appears that complex interactions exist among the effector genes in Xav19R, such that the loss of functional avrBs2 seems to amplify or result in fitness costs associated with the loss of functional avrBs1 and avrBs3. The in planta bacterial growth experiments also revealed a complex interaction between avrBs4 and avrBs2 within Xav19R. In the growth experiments, the most severe mutant line, −2314, was only partially restored (−2314+23), ultimately resulting in a −14 mutant genotype. We found that the fitness of this partially restored, −14 genotype was the same (growth) or nearly the same (symptom development) as that of wild type, indicating that the loss of avrBs4 and/or avrBs1 does not appear to incur a fitness cost in the presence of wild-type avrBs2. Therefore, in Xav19R, whether or not fitness costs are seen when plasmid-borne effector gene function is lost depends on the presence or absence of functional avrBs2. In other words, functional avrBs2 seems to mask, or alleviate, the costs of losing the other effector gene functions. This complex pattern of effector gene interaction is similar to that observed in P. syringae pv. phaseolicola (Jackson et al. 1999; Tsiamis et al. 2000), in which effector genes can interact with each other to change their effects on virulence. The disproportionately large effect of avrBs2 on pathogen fitness is also similar to a recent finding that avrPto on its own is able to affect expression of 80% of the Arabidopsis genes normally affected by invading P. syringae pv. tomato DC3000 (Hauck et al. 2003), although this ability was also redundantly encoded by at least one other, unidentified effector gene(s) in the pathogen.

It is noteworthy to point out that avrBs2 was the first effector gene found to contribute to pathogen fitness, due to its effect on in planta growth when removed from Xav (Kearney and Staskawicz 1990; Swords et al. 1996). For this reason, we expected all mutant genotypes lacking functional avrBs2 to have reduced growth during infection. Surprisingly, mutant genotypes −2, −21, and −23 all had levels of growth indistinguishable from wild type. This discrepancy could be due to our use of a different pathogen strain and/or the effect of environment. In particular, previous studies have used greenhouse conditions without supplemental humidity,
whereas we kept plants at 100% humidity for 3 days. Subsequent greenhouse experiments designed to mimic previously published work succeeded in revealing a cost of \textit{avrBs2} removal, although the results were somewhat inconsistent across experiments, perhaps due to fluctuations in ambient humidity in the greenhouse.

The results of our experiments on the fitness effects of effector genes support the belief that \textit{avrBs2} is a crucial gene in the life cycle of \textit{Xav} and that removal of \textit{avrBs2} should have significant consequences on both virulence and pathogenicity (Kearney and Staskawicz 1990; Tai \textit{et al.} 1999). Historically, strains of \textit{Xav} collected from the field consistently contained functional \textit{avrBs2}. However, strains of \textit{Xav} carrying mutated \textit{avrBs2} alleles are now being detected in the field, due to intense selection pressure from the widespread use of resistant pepper plants carrying the corresponding \textit{R} gene, \textit{Bs2} (Kousik and Ritchie 1996, 1998; Gassmann \textit{et al.} 2000). Other field and greenhouse studies have shown that strains that contain mutated \textit{avrBs2} genes are still pathogenic and can cause significant disease and subsequent crop loss (Kousik and Ritchie 1996, 1998; Gassmann \textit{et al.} 2000); our findings are not inconsistent with these data. We found that even upon removal of \textit{avrBs2}, the \textit{−2} genotype can cause disease and transmit in the field reasonably well, although at rates significantly reduced from those of wild type.

In this study, the three plasmid-borne effector genes, when in association with an \textit{avrBs2} mutant, had significant and additive fitness effects on nearly all the important epidemiological traits examined. Even though all of the mutant strains examined were less fit than wild-type \textit{Xav} (including \textit{−2}), it was only after the loss of several (three or four) effector genes that we saw large fitness costs in lesion development and \textit{in planta} growth, which contributed to a drastically reduced ability to transmit in the field. These results concur with the findings that single \textit{R} genes, even those targeting effector genes that are important to pathogen fitness, such as \textit{avrBs2}, do not provide durable resistance (Vera Cruz \textit{et al.} 2000; McDonald and Linde 2002). Instead, our results suggest that durable resistance is more likely to be achieved through pyramiding \textit{R} genes and/or using several plant varieties in a field (Zhu \textit{et al.} 2000; McDonald and Linde 2002; Mundt 2002). These methods require that many different effector genes be removed before the pathogen can become widely virulent and thus should drastically reduce the pathogen’s fitness. In addition, the requirement for a pathogen to lose several effector genes decreases the probability that a single second-site compensatory mutation may restore pathogen fitness. These conclusions are further supported by the recent study of virulence in a fungal pathogen on wild flax in the field (Thrall and Burdon 2003). The authors found that, on average, a strain that could infect many host genotypes produced fewer spores during an infection; in other words, as a strain became more widely virulent, its fitness was proportionally reduced.

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