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Watersoaking Function(s) of XcmH1005 Are Redundantly Encoded by Members of the *Xanthomonas avr/pth* Gene Family

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Xanthomonas campestris pv. _malvacearum_ strain XcmH1005 is the source of 6 plasmidborne _avr_ genes that are members of the _Xanthomonas_ avirulence (_avr_)/ pathogenicity (_pth_) gene family, but only one of these highly homologous genes is known to affect its pathogenicity (Yang et al., 1994, Mol. Plant-Microbe Interact. 7:345-355). Since XcmH1005 contained six additional DNA fragments that hybridized to gene family members, these additional fragments were cloned, characterized, and assayed for avirulence and pathogenicity functions. Four of the six fragments exhibited avirulence function; three are new genes and one (_avrBn_) is previously described but now recognized as a member of the gene family. Marker-eviction mutagenesis of XcmH1005 and complementation tests revealed that at least seven of the 10 XcmH1005 _avr/pth_ family members exhibited pleiotropic pathogenicity (watersoaking) function on cotton, but only five _avr/pth_ genes were needed for full watersoaking. The contribution of each gene to pathogenicity was additive, and five of the 10 _avr/pth_ genes appeared to be redundant. One XcmH1005 mutant, in which seven members of the gene family were eliminated, was asymptomatic on susceptible cotton and 1,600 times less mutant bacteria were released onto the leaf surface in comparison with the wild-type strain. However, the in planta growth rate and yield of this mutant on susceptible plants were identical to those of the wild type. We conclude that pathogenicity of XcmH1005 on cotton, but not growth in planta, requires multiple specific members of the _Xanthomonas avr/pth_ gene family.

Additional keywords: angular leaf spot, cotton blight, gene-for-gene, host range, specificity.

Many microbial plant pathogens carry avirulence (*avr*) genes that act as negative factors to trigger plant defenses on hosts carrying specific resistance (*R*) genes. Experimental removal of most *avr* genes tested increases host range to include otherwise resistant host cultivars, and such strains (lacking the *avr* genes) are usually not impaired in pathogenicity on susceptible hosts (Gabriel and Rolfe 1990; Keen 1990). There are some notable exceptions to this rule; *avr* genes that have been shown to pleiotropically contribute to virulence on susceptible hosts are: *avrBs2* of *Xanthomonas campestris* pv. *vesicatoria* (Kearney and Staskawicz 1990), *pthA* of *X. citri* (Swarup et al. 1992), *avrb6* of *malvacearum* (Yang et al. 1994), *avrRpm1* of *Pseudomonas syringae* pv. *maculicola* (Ritter and Dangl 1995) and *avrA* and *avrE* of *P. syringae* pv. *tomato* (Lorang et al. 1994). It is argued that *avr* genes must have selective value in order to be maintained in a pathogen population (for example, Keen and Staskawicz 1988; Lorang et al. 1994), but this concept of "stabilizing selection" is an old one in plant pathology for which there is no general evidence (for example, refer to Bronson and Ellingboe 1986). With the exception of the few *avr* genes listed out of more than 30 cloned and published to date (Gabriel et al. 1993), the presence of most *avr* genes in pathogens remains enigmatic (Keen 1990; Ritter and Dangl 1995).

Both *pthA* and *avrb6* are members of a large *Xanthomonas* avirulence (*avr*)/ pathogenicity (*pth*) gene family (Yang and Gabriel 1995; De Feyter et al. 1993). This gene family comprises nearly all published *Xanthomonas avr* genes and constitutes over 1/3 of all *avr* genes reported cloned to date. Other published members of the *avr/pth* gene family include *avrB4, avrb7, avrBIn, avrB101,* and *avrB102* of *X. campestris* pv. *malvacearum* (De Feyter and Gabriel 1991a; De Feyter et al. 1993), *avrBs3*, *avrBs3-2* (syn. *avrBsP*) of *X. campestris* pv. *vesicatoria* (Bonas et al. 1989, 1993; Canteros et al. 1991) and *avrXa5, avrXa7,* and *avrXa10* of *X. oryzae* (Hopkins et al. 1992). All family members sequenced to date are 95 to 98% identical to each other (Bonas et al. 1993; Hopkins et al. 1992; De Feyter et al. 1993; Yang and Gabriel 1995). Most members of this gene family were isolated as avirulence genes and without evidence of pathogenicity function.

Xanthomonas campestris pv. *malvacearum* (Smith) Dye is the causal agent of angular leaf spot of cotton, an economically important disease with worldwide distribution. All strains examined of *X. campestris* that attack cotton carry at least 4 to 12 DNA fragments that hybridize with members of the *Xanthomonas avr/pth* gene family (De Feyter et al. 1993).

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One of these nearly identical gene family members, *avrb6*, is known to contribute to the watersoaked, pathogenic symptoms produced by *X. campestris* pv. *malvacearum* strain XcmH1005, but other genes are also clearly involved (Yang et al. 1994). We speculated that other members of the *avr/pth* gene family, besides *avrb6,* were necessary for pathogenicity of XcmH1005, but insertional inactivation of only a few members of this gene family in XcmH1005 failed to provide evidence to support this idea (Yang et al. 1994). Southern hybridization analysis revealed at least five additional DNA fragments in XcmH1005 with the potential to encode members of the gene family, besides the six plasmidborne *avr* genes previously cloned (De Feyter et al. 1993). The purpose of this research was to identify and experimentally destroy all members of the *avr/pth* gene family in XcmH1005 in an effort to clarify the role in pathogenicity of the many members of the gene family found in this one strain.

RESULTS

Cloning of six potential *avr/pth* genes from XcmH1005 by hybridization.

In addition to the six members of the *Xanthomonas avr/pth* gene family previously cloned from plasmid pXcmH of strain XcmH1005 (De Feyter and Gabriel 1991a), Southern blot analyses revealed the presence of six more DNA fragments in XcmH1005 that hybridized to an internal *Bam*HI fragment from *avrb6* (Fig. 1). All of the hybridizing fragments appeared to be large enough to carry functional members of the *avr/pth* gene family. Based on a lower intensity of hybridization and the fact that XcmH1005 appeared to carry only a single plasmid (pXcmH; De Feyter and Gabriel 1991a), the additional hybridizing fragments appeared to be chromosomal.

Colony hybridizations were carried out to isolate all hybridizing clones from a genomic library of XcmH1005 (De Feyter and Gabriel 1991a) by probing with the internal *Bam*HI fragment from *avrb6*. Twenty-three out of 72 cosmid clones identified by colony hybridization were found to carry DNA fragments not found on pXcmH. These new, presumably chromosomal DNA fragments were readily distinguished from the DNA fragments that form pXcmH by restriction fragment length polymorphisms (RFLPs) (Fig. 1A; and data not shown). Six different chromosomal DNA fragments that hybridized with *avrb6* were cloned and identified as representing the six additional DNA fragments observed on the Southern blots of XcmH1005 (Fig. 1B). These DNA fragments were arbitrarily named according to size as Hc1 to 6. Six cosmid clones were selected for further study: pXcm1.12 carried Hc1 and Hc3; pXcm1.21 carried Hc2; pXcm2.23 carried Hc3; pXcm2.12 carried Hc4 and Hc5; pXcm2.11 carried Hc5 only; and pXcm1.22 carried Hc6 (Fig. 1). Restriction digests of these clones revealed that Hc1, Hc2, and Hc3 were tightly linked, and that Hc4 and Hc5 were tightly linked.

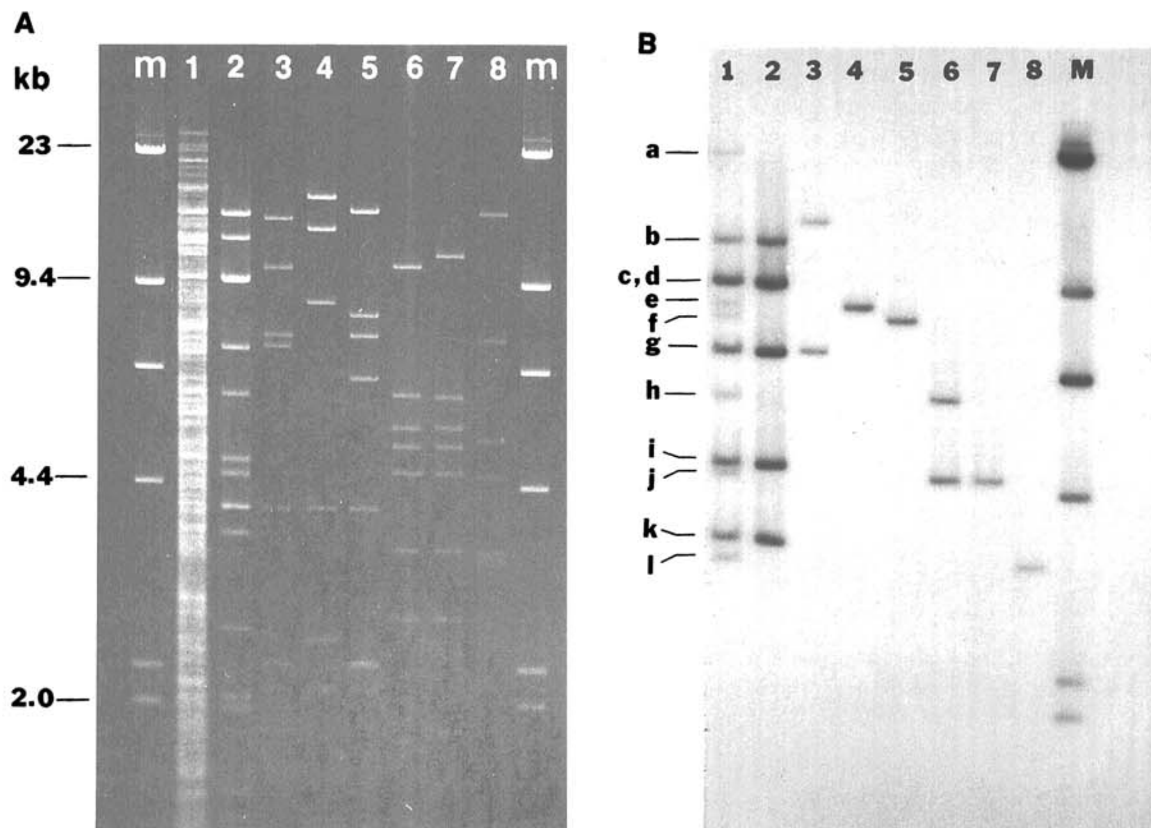

Fig. 1. Southern hybridization of cosmid DNAs containing six chromosomal *avr* genes after *Eco*RI and *Sst*I double digestion. The blot was probed with a 2.9-kb, ^{32}P-labeled internal *Bam*HI fragment of *avrb6*. **A,** Ethidium bromide stained gel; **B,** Autoradiograph resulting from Southern hybridization: lane 1, XcmH1005 genomic DNA; lane 2, pXcmH; lane 3, pXcm1.12; lane 4, pXcm1.21; lane 5, pXcm2.23; lane 6, pXcm2.12; lane 7, pXcm2.11; lane 8, pXcm1.22; M, molecular marker. a, Hc1; b, *avrB4*; c, *avrB101*; d, *avrB102*, e, *avrB103*; f, *avrBn*; g, *avrBIn*; h, *avrB104*; i, *avrb7*; j, *avrB5*; k, *avrb6*; l, Hc6.

Phenotypic activity of hybridizing fragments in Xcm1003. All six cosmid clones identified by hybridization as potentially carrying members of the *avr/pth* gene family were conjugally transferred to the widely virulent *X. campestris* pv. *malvacearum* African strain Xcm1003 for activity assays. Transconjugants were tested on cotton cv. Acala 44 (Ac44) and nine cotton resistance lines, congenic with Ac44 and each carrying one of the following resistance genes: *B1, B2, B4, B5a, B5b, b6, b7, BIn,* or *BIn3*. Transconjugants were also tested on cotton line 20-3, carrying *Bn* and *BSm*. None of the cosmid clones conferred increased watersoaking to Xcm1003, but all except pXcm1.22 conferred *R* gene-specific avirulence. To localize avirulence activity, all cosmid clones with avirulence activity were directionally subcloned into pUFR047 by *Eco*RI and *Hin*dIII digestion. pXcm1.12 was subcloned to separate fragments Hc1 and Hc3, generating pUFY36.8 (Hc1) and pUFY36.26 (Hc3). Fragment Hc2 on pXcm1.21 was subcloned to form pUFY31.46. pXcm2.12 was subcloned to generate pUFY37.62, still carrying Hc4 and Hc5 on a single, 11-kb *Eco*RI/*Hin*dIII DNA fragment. Fragment Hc4 on pUFY37.62 was further subcloned to form pUFY38.1. Fragment Hc5 on pXcm2.11 was subcloned to form pUFY33.19.

The indicated plasmids were transferred to Xcm1003 and tested on the cotton lines. The Hc1 and Hc6 DNA fragments conferred no detected phenotypic alteration to Xcm1003 when inoculated on any of the cotton lines and pUFY36.8 and pXcm1.22 were not investigated further. The other four DNA fragments conferred *R* gene-specific avirulence and were mapped by restriction enzyme and Southern hybridization analyses to confirm that they carried members of the *Xanthomonas avr/pth* gene family (data not shown). Restriction sites for *Bam*HI, *Stu*I, *Pst*I, *Bal*I, *Hinc*II, and *Sst*I were present in identical positions within each of the genes as have been found in other members of the *avr/pth* gene family, and each gene exhibited a *Bal*I ladder, indicative of the presence of 102-bp tandem repeats characteristic of the family. The Hc2 and Hc4 DNA fragments conferred the same gene-for-genes (plural) avirulence specificity to Xcm1003 as did clones carrying *avrB101* (De Feyter et al. 1993): the transconjugants elicited a hypersensitive response (HR) on AcB5a and AcBIn3. Since these DNA fragments were physically different from each other and from the fragment that carries *avrB101,* we conclude that Hc2 and Hc4 carried two new members of the gene family, named *avrB103* and *avrB104*, respectively. The HR elicited by transconjugants carrying these chromosomal fragments was weaker than that elicited by isogenic strains carrying *avrB101* on the same vector.

The Hc3 DNA fragment on pXcm2.23 conferred avirulence to Xcm1003 on cotton lines containing resistance genes *Bn* or *BIn3*. Gene *avrBn* had previously been cloned from XcmH1005 on pUFA-H1 (Gabriel et al. 1986), but *avrBn* was not then recognized as a member of the *avr/pth* gene family. Based on phenotypic assays and restriction fragment and Southern hybridization analyses (not shown) *avrBn* was found to be independently cloned on both pUFA-H1 and pXcm2.23. The Hc5 DNA fragment conferred avirulence to Xcm1003 on AcB5b and AcBIn3 and we conclude that Hc5 carried a new member of the gene family, named *avrB5*. Except for *avrB101* and *avrB102*, which are carried on identically sized *Eco*RI/*Sst*I fragments, each of the 10 XcmH1005 *avr* genes was placed on a uniquely sized, clearly identifiable *Eco*RI/*Sst*I DNA fragment (Fig. 1B).

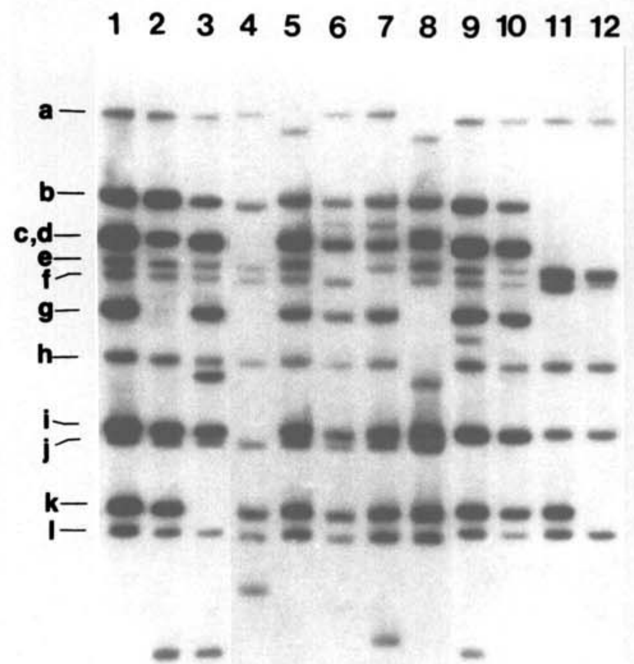

Fig. 2. Southern hybridization of *Eco*RI and *Sst*I double-digested genomic DNAs of XcmH1005 mutants. The blot was probed with ^{32}P-labeled internal *Bam*HI fragment of *avrb6*. Lane 1, XcmH1005; lane 2, HM1.10; lane 3, HM1.15; lane 4, HM1.38S; lane 5, HM1.26; lane 6, HM1.36; lane 7, HM1.34; lane 8, HM1.32S; lane 9, HM1.20; lane 10, HM1.20S; lane 11, HM2.2; lane 12, HM2.2S. a through l as per Figure 1 legend.

Table 1. Phenotypes of marker-exchange mutants of *Xanthomonas campestris* pv. *malvacearum* strain XcmH1005 on cotton

	Cotton cv. Acala-44 congenic lines						
Strain	**Ac44**	**AcB4**	**AcB5b**	**Acb6**	**Acb7**	**AcBIn**	**AcBIn3**
XcmH1005	+ + +[a]	–	–	–	–	–	–
HM1.10 (*avrBIn::nptI-sac*)	+ + +	–	–	–	–	+ + +	–
HM1.15 (*avrb6::nptI-sac*)	+ +	–	–	+ +	–	–	–
HM1.20S (*avrB5*⁻, *avrb6*⁻)	+ +	–	+ +	+ +	–	–	–
HM1.38S (*avrB4*⁻, *avrb7*⁻, *avrBIn*⁻, *avrB101*⁻, *avr102*⁻)	+ +	+ +	–	+ +	+ +	+ +	–
HM2.2S (*avrB4*⁻, *avrB5*⁻, *avrb6*⁻, *avrBn*⁻, *avrBIn*⁻, *avrB101*⁻, *avr102*⁻)	0	0	0	0	0	0	±

[a] + + + = strong watersoaking; + + = moderate watersoaking; 0 = no watersoaking and null phenotype at lowinoculum levels; – = hypersensitive response (HR); ± = weak HR.

Inactivation of multiple *avr/pth* genes in XcmH1005.

The pleiotropic pathogenicity and avirulence functions of *avrb6, avrb7,* and *avrBIn* had previously been investigated by marker-exchange inactivation of each gene individually (Yang et al. 1994). In an attempt to inactivate or delete the remaining seven *avr* genes, marker-eviction mutagenesis was carried out in XcmH1005 in a two-step process. The first step involved semirandom marker exchange using an *nptI-sac* cartridge inserted in the tandem repeat region of homologue *pthA*. Marker-exchange mutants were recovered with *nptI-sac* insertions in *avrBIn* (HM1.10), *avrb6* (HM1.15), fragment Hc1 (HM1.26), *avrB103* (HM1.36), *avrBn* (HM1.34), and *avrB5* (HM1.20) (Fig. 2). In the second step, marker-exchange mutants were grown in the presence of sucrose, resulting in selection of *nptI-sac* eviction mutants that sometimes lost multiple *avr* genes (e.g., HM1.38S, HM1.32S, and HM1.20S; refer to Figure 2). More than 140 mutants were generated and 60 of them were tested on the Acala congenic resistance lines and analyzed by Southern hybridization.

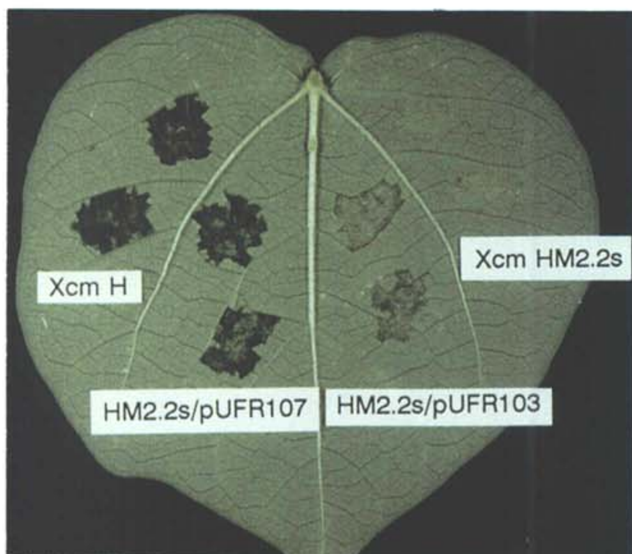

Fig. 3. Effect of *avr* genes on watersoaking symptoms caused by *Xanthomonas campestris* pv. *malvacearum* on susceptible cotton line Ac44. The indicated strains were inoculated at 10^8 CFU/ml. XcmH is the wild-type parent of spontaneous rifamycin mutant XcmH1005; both exhibit identical pathogenic symptoms.

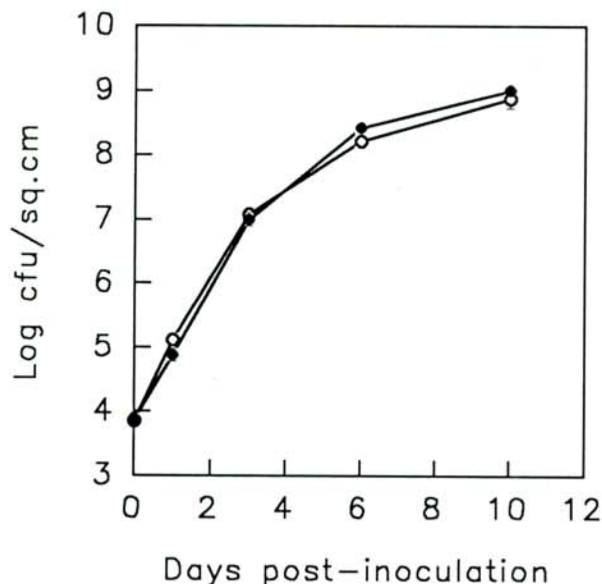

Fig. 4. Inactivation of multiple *avr* genes in *Xanthomonas campestris* pv. *malvacearum* did not affect bacterial growth in planta on cotton susceptible line Ac44. Filled circle, XcmH1005; open circle, HM2.2S. Plants were initially inoculated at 10^6 CFU/ml.

Based on hybridizations with the *Bam*HI internal fragment of homologue *avrb6*, marker-eviction mutants affecting all 10 *avr/pth* genes were obtained. Plasmid pXcmH suffered extensive deletions/rearrangements, but in no case was the entire plasmid cured. No mutant carrying fewer than four hybridizing fragments was obtained. In some cases, members of the family appeared to rearrange to form new members, as evidenced by the appearance of new hybridizing DNA fragments, and often accompanied by the appearance of a new avirulence specificity (data not shown). Also in all cases, at least one member remained plasmidborne, as evidenced by comparatively strong hybridization intensities characteristic of the plasmidborne genes.

Table 1 lists selected mutants that exhibited altered phenotypes on cotton. As predicted by gene-for-gene theory, mutations of *avrBIn* (HM1.10), *avrb5* (HM1.20S), *avrb6* (HM1.15 and HM1.20S), *avrB4,* or *avrb7* (HM1.38S) resulted in the loss of *avr* specificity on the indicated cotton lines. As expected, mutation of the *avrB103* (HM1.36), *avrB104* (HM1.32S), *avrBn* (HM1.34), or Hc1 (HM1.26) fragments resulted in no alteration of avirulence or pathogenic phenotypes, presumably because of other, epistatic, gene-for-gene interactions (see De Feyter et al. 1993). No marker exchanges affecting fragment Hc6 were recovered.

All mutations or mutation combinations involving *avrb6* (e.g., HM1.15 and HM1.20S) reduced watersoaking ability on susceptible cotton lines. (Although in Figure 2 HM1.2S appears to have a band of similar size to the one carrying *avrb6*, additional Southern analyses using other restriction enzymes reveal that the original *avrb6* band is missing and that this "k" band is slightly smaller than the original *avrb6* band; data not shown.) By contrast, mutations eliminating all five of the other pXcmH *avr* genes except *avrb6* did not affect watersoaking symptoms on susceptible cotton lines. Based on plasmid profile (not shown), Southern blot analysis (Fig. 2), and plant tests (Table 1), HM1.38S had lost *avrB4, avrb7, avrBIn, avrB101,* and *avrB102* and remained fully pathogenic. To evict as many *avr* genes as possible, several marker-evicted mutants were subjected to a second round of marker-exchange mutagenesis. Marker-evicted mutant HM2.2S was derived from HM1.20S (*avrB5*$^-$, *avrb6*$^-$) and had lost at least seven *avr* genes (*avrB4, avrB5, avrb6, avrBn, avrBIn, avrB101,* and *avr102*). By contrast with HM1.38S, however, HM2.2S also lost all watersoaking ability on mature leaves of the susceptible line Ac44 (Table 1; Fig. 3). HM2.2S was asymptomatic (nonpathogenic) on susceptible cotton leaves when inoculated at 10^6 CFU/ml, and nearly asymptomatic (but no watersoaking) when inoculated at high levels (10^8 CFU/ml; Fig. 3). Interestingly, when inoculated at high levels on AcBIn3 plants, a slight HR was observed.

Effect of mutational loss of pathogenicity on endophytic growth of HM2.2S.

Since *avrb6*$^-$ mutants of XcmH1005 that had lost significant watersoaking ability on Ac44 were shown to be unaffected in growth in planta (Yang et al. 1994), we evaluated growth of HM2.2S on Ac44 in comparison with the parental strain XcmH1005. The growth rate and yield in planta of HM2.2S were no different from those of XcmH1005 (Fig. 4). However, the amount of HM2.2S released onto the leaf surface was 1,600 times less than that of XcmH1005. Only 0.007% ($8.8 \pm 3.0 \times 10^4$ CFU/cm^2 out of $1.18 \pm 0.24 \times 10^9$ CFU/cm^2) of HM2.2S bacteria present in the infected leaf tissue was released onto the leaf surface, whereas 9.2% ($1.42 \pm 0.15 \times 10^8$ CFU/cm^2 out of $1.55 \pm 0.23 \times 10^9$ CFU/cm^2) of the XcmH1005 bacteria was released onto the surface. Therefore, mutant HM2.2S appeared to be unaffected in growth in cotton.

Additive complementation of pathogenicity defect in HM2.2S by *avr/pth* genes.

Complementation tests were carried out to further analyze the potential pathogenicity function(s) of all 10 members of the *avr/pth* gene family isolated from XcmH1005 and to search for potential pathogenicity function(s) encoded on pXcmH, but not encoded by the six *avr/pth* genes found on the plasmid. Plasmids carrying individual family members *avrb6* (pUFR127), *avrB101* (pUFR142), *avrBIn* (pUFR156), and *avrb7* (pUFR163) conferred a very slight watersoaking ability to HM2.2S. Plasmids carrying the other six individual *avr/pth* genes or the Hc1 or Hc6 fragments were not observed to even partially complement the watersoaking defect in HM2.2S. Pathogenicity assays of DNA fragments in regions outside the *avr/pth* genes on pXcmH (the extent of these large genes is indicated by arrows in Figure 5) did not reveal any watersoaking activity when introduced into HM2.2S (Fig. 5).

Plasmids carrying any combination of two or more members of the *avr/pth* gene family encoded on pXcmH conferred watersoaking activity to HM2.2S (Fig. 5). Most *avr/pth* genes encoded on pXcmH appeared to act synergistically to confer a level of watersoaking that appeared to be greater than the sum of the individual genes. For example, strains containing *avrBIn* and *avrB101* together exhibited nearly wild-type levels of watersoaking activity (on pUFY058), but individually they conferred barely detectable watersoaking. Similarly, *avrb7* and *avrB102* together conferred much stronger watersoaking than either alone; adding a third gene (e.g., *avrBIn* on

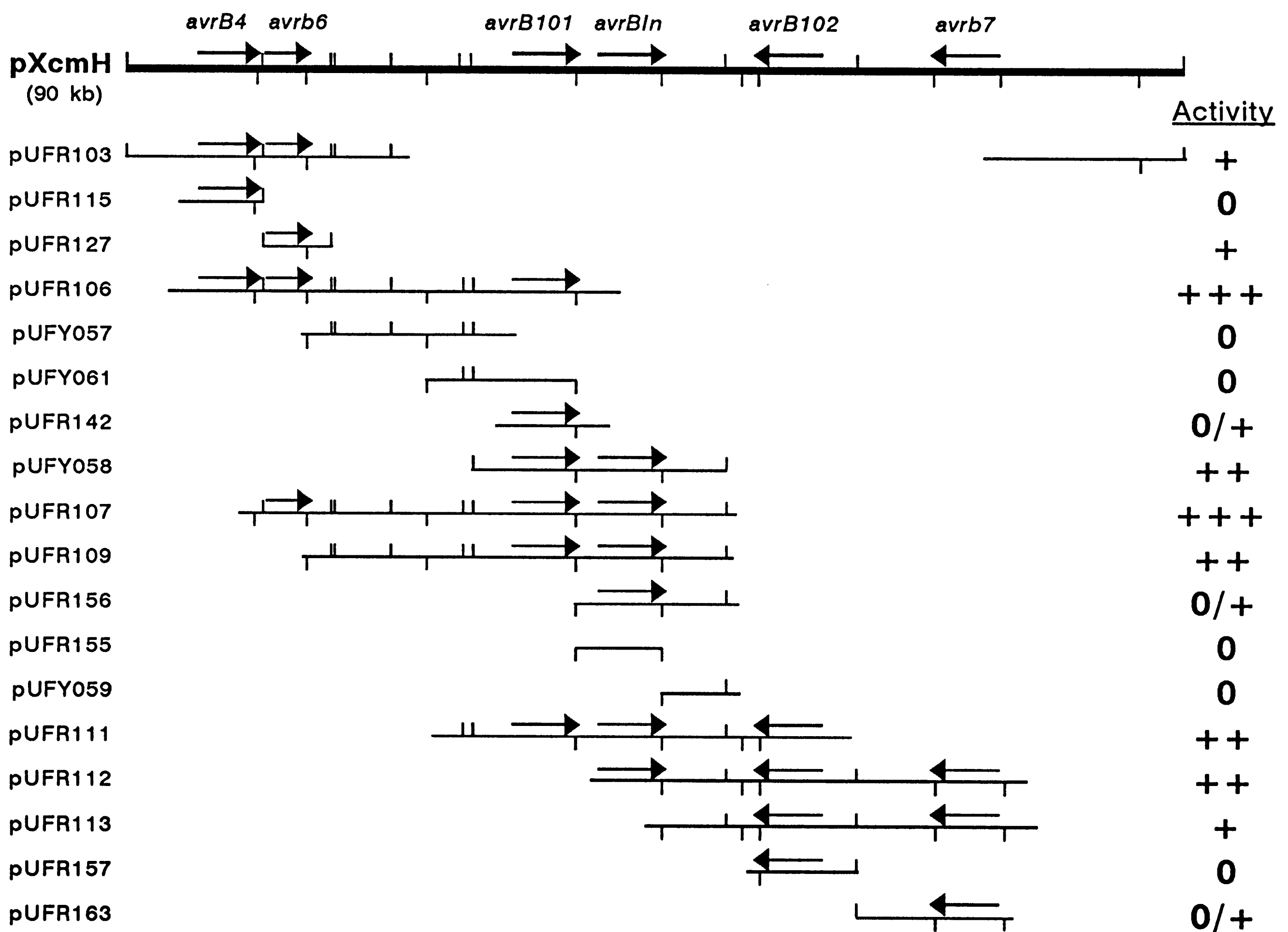

Fig. 5. Watersoaking activity of pXcmH DNA fragments in mutant HM2.2S. Arrows indicate the location, size, and direction of transcription of the *avr/pth* genes on pXcmH. HM2.2S was asymptomatic on susceptible cotton line Ac44: 0 = no detected watersoaking activity; +/0 = very weak watersoaking activity; + = weak watersoaking activity; ++ = moderate watersoaking activity; +++ = full watersoaking activity (as wild-type XcmH1005; refer to Figure 3). *Eco*RI sites are indicated by upper vertical bars; *Sst*I sites are indicated by lower vertical bars.

pUFR112) again added strongly to the visible watersoaking effect. This apparent synergism allowed detection of watersoaking activity contributed by *avrB102* (on pUFR113 by comparison with pUFR163) and by both *avrB5* and *avrB104* (on pXcm2.12 by comparison with plasmids carrying each gene alone). Cosmid clones (pUFR106 and pUFR107) carrying *avrb6* and at least two other *avr* genes fully restored the watersoaking defects of HM2.2S. No evidence was found for pathogenicity function of *avrB4*, *avrB103,* and *avrBn*, but these genes were not cloned in a combination that might have allowed detection of very weak function.

DISCUSSION

With the exception of *pthA* of *X. citri* (Swarup et al. 1991, 1992), all of the other published members of the *Xanthomonas avr/pth* gene family were identified and cloned as *avr* genes. Six plasmidborne members of this gene family were previously isolated from *X. campestris* pv. *malvacearum* strain XcmH1005 (De Feyter and Gabriel 1991a). Two of these (*avrb6* and *avrb7*) were noted to confer enhanced watersoaking to another strain on susceptible hosts (De Feyter et al. 1993). We have now cloned all 12 DNA fragments found in XcmH1005 that hybridize with members of the gene family, and have found that 10 of these carry functional members of the gene family. Four new members are reported here. Based on hybridization intensity and lack of evidence for a second plasmid in XcmH1005, these four new members appear to be chromosomally encoded. One of these is the previously reported *avrBn* (Gabriel et al. 1986), which is now recognized as a member of this *avr/pth* gene family. The three others are previously undescribed *avr* genes (*avrB5, avrB103,* and *avrB104*).

Mutations affecting all 10 *avr* genes in XcmH1005 were achieved by marker exchange–eviction mutagenesis. Mutation of *avrb6* was known to result in a significant loss of watersoaking ability of XcmH1005 (Yang et al. 1994), but mutations of other individual members of the *avr/pth* gene family in XcmH1005 had no obvious effect on watersoaking on susceptible cotton lines. In fact, mutation of all five other members (except *avrb6*) encoded on native plasmid pXcmH (e.g., in HM1.38S) also had no effect. Surprisingly, however, mutation of *avrb6* plus six additional *avr/pth* family members in XcmH1005 resulted in the complete loss of pathogenic symptoms (e.g., in HM2.2S) on susceptible cotton leaves. Since HM2.2S suffered deletions of seven *avr/pth* genes and adjacent DNA fragments, mutational analysis alone did not rule out the potential involvement of other closely linked pathogenicity genes. However, complementation tests did rule out the involvement of other closely linked genes and revealed that at least seven of the 10 members of the *avr/pth* family could additively contribute to pathogenicity, but that only three members were needed to fully restore the watersoaking defect of HM2.2S (Fig. 5). We conclude that (i) many of the XcmH1005 *avr* genes that are members of the *Xanthomonas avr/pth* gene family are also functional as *pth* genes, (ii) the contribution of each member to pathogenicity of cotton is quantitative, and (iii) XcmH1005 carries redundant *pth* homologues that are not necessary for full pathogenicity.

In contrast to *avrBs2* of *X. campestris* pv. *vesicatoria* (Kearney and Staskawicz 1990), *pthA* in *X. citri* (Swarup et al. 1991), and *avrA* and *avrE* of *P. syringae* pv. *tomato* (Lorang et al. 1994), there was no evidence that the *avr/pth* genes of XcmH1005 were even slightly involved in bacterial growth in planta. Instead, they were involved in the induction of watersoaking symptoms and associated release of 9.2% of the total bacteria of the leaf onto the leaf surface, presumably aiding in dispersal. Mutant HM2.2S, with at least seven *avr/pth* genes destroyed, released 1,600 times less bacteria to the leaf surface (0.007% of the total) than the wild type. Although HM2.2S was asymptomatic on susceptible cotton when inoculated at 10^6 CFU/ml, it grew endophytically at the same rate and to the same level as the wild-type strain. Therefore, inactivation of multiple *avr/pth* genes in *X. campestris* pv. *malvacearum* created what appears to be a nonpathogenic endophyte of cotton. In the case of *X. campestris* pv. *malvacearum*, pathogenicity and host tissue destruction may be beneficial for the reproductive fitness of the pathogen. Since *X. campestris* pv. *malvacearum* spreads primarily by rain splash, it seems doubtful that a nonpathogenic endophyte could disseminate efficiently in natural field situations. The lack of correlation between disease symptoms and growth in planta has been observed with other plant pathogens. Indole acetic acid–deficient strains of *Pseudomonas syringae* pv. *savastanoi* failed to elicit gall on oleander but exhibited similar growth patterns as the gall-eliciting wild-type strain (Smidt and Kosuge 1978). The *lemA* gene, which encodes a two-component regulator, is required by *P. syringae* pv. *syringae* for disease lesion formation on bean plants, but not bacterial growth within or on the leaves of bean (Hrabak and Willis 1992; Willis et al. 1990). Mutation of the fungal pathogen *Colletotrichum magna* resulted in a strain that is not able to induce pathogenic symptoms, but that grows in host tissue as an endophyte and retains the wild-type host range (Freeman and Rodriguez 1993).

The apparent enigma of why pathogens carry *avr* genes at all, and why some pathogens, such as XcmH1005, carry so many of them, appears to have several valid explanations. First, some *avr* genes have obvious selective value (see introductory paragraphs, above). Second, some of them may have only weak selective value that is difficult to assay, but the genes quantitatively contribute a selective value. In the case of *X. campestris* strains that attack cotton, at least 10 *avr/pth* family members can contribute quantitatively and synergistically to pathogenicity, and some can contribute more than others. Third, some *avr* genes may simply be redundant. Deletion of up to five of the 10 *avr/pth* genes in XcmH1005 did not appear to affect pathogenicity, release of the pathogen, and, presumably, the fitness of the pathogen on cotton. It is also possible that some *avr* genes have selective value on plants not known to be in the host range of the pathogen. However, since *Xanthomonas* strains are highly host-specific, since the pathogenicity functions of *avrb6* and *pthA* appear to be host-specific (Yang et al. 1994), and since cotton blight is well studied, it is difficult to argue that some of these XcmH1005 *avr/pth* genes have selective value on some hypothetical host other than cotton.

Of course, the apparent enigma of why pathogens carry dispensable *avr* genes is also explicable by stochastic events, such as horizontal gene transfer and mutation. At least some members of the *Xanthomonas avr/pth* gene family are found on self-mobilizing plasmids (for example *avrBs3* of *X. cam-*

pestris pv. vesicatoria; Bonas et al. 1989). Horizontal, intraspecific transfer from one *X. campestris* pathovar to another may be due to a coincidental linkage with another (selected) factor, such as copper resistance, on the same plasmid (e.g., refer to Stall et al. 1986). Members of the *Xanthomonas* *avr/pth* gene family are not found in all strains of *X. campestris* pv. *vesicatoria*, which evidently does not need or utilize members of the *Xanthomonas avr/pth* gene family. Maintenance or even increase of unnecessary (duplicate, mutant, or horizontally transferred) genes in a population does not re-

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
Escherichia coli		
DH5∝	F^-, *endA1*, *hsdR17* ($r_k^-m_k^+$), *supE44*, *thi-1*, *recA1*, *gyrA*, *relA1*, *ϕ80dlacZ ΔM15*, *Δ(lacZYA-argF)*U169	Gibco-BRL, Gaithersburg, MD
HB101	*supE44*, *hsdS20* ($r_k^-m_k^+$), *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*, *xyl-5*, *mtl-1*	Boyer and Roulland-Dussoix 1969
ED8767	*supE44*, *supF58*, *hsdS3* ($r_k^-m_k^+$), *recA56*, *galK2*, *galT22*, *metB1*	Murray et al. 1977
Xanthomonas campestris pv. *malvacearum*		
XcmH	Natural race 4 isolate from cotton; carries six plasmid-borne *avr* genes plus at least four others	De Feyter and Gabriel, 1991a; Gabriel et al. 1986
XcmH1005	Spontaneous Rif^r derivative of XcmH	Yang et al. 1994
HM1.10	*avrBIn::nptI-sac*, marker-exchange mutant of XcmH1005.	This study
HM1.15	*avrb6::nptI-sac*, marker-exchange mutant of XcmH1005.	This study
HM1.20	*avrB5::nptI-sac*, marker-exchange mutant of XcmH1005.	This study
HM1.20S	*avrB5*⁻, *avrb6*⁻ marker-evicted mutant derived from HM1.20.	This study
HM1.26	Hc1::*nptI-sac*, marker-exchange mutant of XcmH1005.	This study
HM1.32S	Hc1⁻, *avrBIn*⁻, *avrB104*⁻ marker-evicted mutant of XcmH1005.	This study
HM1.34	*avrBn::nptI-sac*, marker-exchange mutant of XcmH1005.	This study
HM1.36	*avrB103::nptI-sac*, marker-exchange mutant of XcmH1005.	This study
HM1.38S	*avrB4*⁻, *avrb7*⁻, *avrBIn*⁻, *avrB101*⁻, *avrl02*⁻ marker-evicted mutant of XcmH1005	This study
HM2.2	*avrB4*⁻, *avrB5*⁻, *avrb6*⁻, *avrB101::nptI-sac avrBIn*⁻, *avrl02*⁻), marker exchange mutant of HM1.20S.	This study
HM2.2S	*avrB4*⁻, *avrB5*⁻, *avrb6*⁻, *avrBn*⁻, *avrBIn*⁻, *avrB101*⁻, *avrl02*⁻), marker-evicted mutant derived from HM2.2S.	This study
XcmN	Natural isolate from cotton from Upper Volta, Africa; virulent on all tested Upland cotton lines	Gabriel et al. 1986
Xcm1003	Spc^r Rif^r derivative of XcmN	De Feyter and Gabriel 1991a
Plasmid		
pRK2073	pRK2013 derivative, npt::Tn7,Kms, Sp^r, Tra^+, helper plasmid	Leong et al. 1982
pUFR004	ColE1, Mob^+, Cm^r	De Feyter et al. 1990
pUFR034	IncW, Km^r, Mob^+, lacZα⁺, Par^+, *cos*	De Feyter et al. 1990
pUFR042	IncW, Km^r, Gm^r, Mob^+, *lac*Zα⁺, Par^+	De Feyter and Gabriel 1991a
pUFR047	IncW, Gmr, Apr, Mob+, *lac*Zα⁺, Par^+	De Feyter et al. 1993
pUFR054	IncP, Tc^r, Mob^+, containing methylases *Xma*I and *Xma*III	De Feyter and Gabriel 1991b
pUFR103	Cosmid clone carrying *avrB4, avrb6*	De Feyter and Gabriel 1991a
pUFR106	Cosmid clone carrying *avrB4, avrb6, avrB101*	De Feyter and Gabriel 1991a
pUFR107	Cosmid clone carrying *avrb6, avrB101, avrBIn*	De Feyter and Gabriel 1991a
pUFR109	Cosmid clone carrying *avrB101, avrBIn*	De Feyter and Gabriel 1991a
pUFR111	Cosmid clone carrying *avrB101, avrBIn, avrB102*	De Feyter and Gabriel 1991a
pUFR112	Cosmid clone carrying *avrBIn, avrB102, avrb7*	De Feyter and Gabriel 1991a
pUFR113	Cosmid clone carrying *avrB102, avrb7*	De Feyter and Gabriel 1991a
pUFR115	7.5-kb fragment containing *avrB4* in pUFR042	De Feyter and Gabriel 1991a
pUFR127	5-kb fragment containing *avrb6* in pUFR042	De Feyter and Gabriel 1991a
pUFR142	9-kb fragment containing *avrB101* in pUFR047	De Feyter et al. 1993
pUFR156	12.9-kb fragment containing *avrBIn* in pUFR042	De Feyter et al. 1993
pUFR157	11-kb fragment containing avrB102 in pUFR042	De Feyter et al. 1993
pUFR163	10-kb fragment containing *avrb7* in pUFR042	De Feyter et al. 1993
pUFY1.48	8.4-kb fragment carrying *pthA::npt-sac* in pUFR047	Yang and Gabriel 1995
pUFY10.1	8.1-kb fragment carrying *pthA::npt-sac* in pUFR004	Yang and Gabriel 1995
pUFY31.46	8.5-kb fragment carrying *avrB103* in pUFR047	This study
pUFY33.19	5-kb fragment carrying *avrB5* in pUFR047	This study
pUFY36.8	8.6-kb fragment carrying Hc1 in pUFR047	This study
pUFY36.26	5-kb fragment carrying *avrBn* in pUFR047	This study
pUFY37.62	11-kb fragment carrying *avrB104* and *avrB5* on pUFR047	This study
pUFY38.1	8-kb fragment carrying *avrB104* in pUFR047	This study
pUM24	*nptI-sac* cartridge in a 3.8-kb *Bam*HI fragment	Ried and Collmer 1987
pXcm1.12	Cosmid clone carrying Hc1 and *avrBn*	This study
pXcm1.21	Cosmid clone carrying *avrB103*	This study
pXcm2.23	Cosmid clone carrying *avrBn*	This study
pXcm2.12	Cosmid clone carrying *avrB104* and *avrB5*	This study
pXcm2.11	Cosmid clone carrying *avrB5*	This study
pXcm1.22	Cosmid clone carrying Hc6	This study
pZit45	4.5-kb fragment containing *pthA* in pUFR047	Swarup et al. 1992

quire selection for the gene itself. Linkage with other, selected, factors can lead to rapid increases or decreases of a given trait; in the case of asexually reproducing prokaryotes, the entire genome is linked and clonally reproduced. Therefore, the frequent epidemics and pandemics of individual pathogen clones must result in major shifts in frequency and distribution of large numbers of unselected genes in the overall population, including *avr* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and matings.

The bacterial strains and plasmids used in this study are listed in Table 2. Strains of *Escherichia coli* were grown in Luria-Bertani (LB) medium (Sambrook et al. 1989) at 37°C. Strains of *Xanthomonas* were grown in PYGM (peptone–yeast extract–glycerol-MOPS [morpholinepropanesulfonic acid]) medium at 30°C (De Feyter et al. 1990). For culture on solid media, agar was added at 15 g/liter. Antibiotics were used as previously described (Yang et al. 1994). To transfer plasmids from *E. coli* strains HB101 or DH5α to *X. campestris* pv. *malvacearum*, helper plasmid pRK2073 and modifier plasmid pUFR054 were used as described (De Feyter and Gabriel 1991a, 1991b).

Recombinant DNA techniques.

Total DNA isolation from *Xanthomonas* was as described (Gabriel and De Feyter 1992). Plasmids were isolated from *E. coli* by alkaline lysis methods (Sambrook et al. 1989). Restriction enzyme digestion, alkaline phosphatase treatment, DNA ligation, and random priming reactions were performed as recommended by the manufacturers. Southern hybridization was performed by using nylon membranes as described (Lazo and Gabriel 1987). Otherwise, standard recombinant DNA procedures were used (Sambrook et al. 1989).

Gene replacement and marker eviction.

To carry out marker-exchange mutagenesis, a 3.8-kb *Bam*HI fragment containing a *nptI-sac* cartridge from pUM24 (Ried and Collmer 1987) was randomly ligated into a *Bal*I site of *pthA* on pZit45. Recombinant plasmids were screened for an insertion in the middle of the tandemly repeated region, and pUFY1.48 was selected, in which the *nptI-sac* cartridge was found in the *BalI* site of repeat number 10 of *pthA*. An *Sst*I fragment carrying the *pthA*::*nptI-sac* fusion from pUFY1.48 was recloned into the suicide vector pUFR004 (De Feyter et al. 1990), forming pUFY10.1. Marker-exchange mutants (*avr*::*nptI-sac*) were created by transferring pUFR10.1 to XcmH1005 and selecting for colonies resistant to kanamycin (15 μg/ml) and sensitive to chloramphenicol (35 μg/ml) and sucrose (5%). Marker-exchange mutants were plated on PYGM medium containing 5% sucrose to evict the *nptI-sac* marker. Some marker-evicted strains were selected to for further rounds of marker eviction mutagenesis.

Plant inoculations and bacterial growth in planta.

Cotton (*Gossypium hirsutum* L.) lines used were Acala-44 (Ac44) and its congenic resistance lines AcB1, AcB2, AcB4, AcB5a, AcB5b, Acb6, Acb7, AcBIn, and AcBIn3 as described (Swarup et al. 1992; De Feyter et al. 1993). Cotton line 20-3 carries both *Bn* and *Bsm* resistance genes (De Feyter and Gabriel 1991a). Cotton plants were grown in the greenhouse, transferred to growth chambers before inoculation, and maintained under conditions as described (De Feyter and Gabriel 1991a). Bacterial suspensions of *X. campestris* pv. *malvacearum* (10^8 CFU/ml) in sterile tap water were gently pressure infiltrated into leaves of 4- to 6-week-old cotton plants. Pathogenic symptoms were observed periodically 2 to 7 days after inoculation.

The growth of *X. campestris* pv. *malvacearum* in the susceptible cotton line Ac44 was determined as described (Yang et al. 1994). In planta growth experiments were performed three times. Data points shown in Figure 4 were the mean and standard error of three samples from one experiment. Bacterial populations released onto the leaf surface were quantified as described (Yang et al. 1994). These data are reported as the mean and standard error of three samples. Similar results were obtained in two independent experiments.

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