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# At Least Six Avirulence Genes Are Clustered on a 90-Kilobase Plasmid in *Xanthomonas campestris* pv. *malvacearum*

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**A total genomic library of a strain of *Xanthomonas campestris* pv. *malvacearum* with multiple avirulence genes was introduced into strains of *X. c.* pv. *malvacearum* that are virulent on cotton lines containing the blight resistance genes $B_2$, $B_4$, $b_6$, $b_7$, or $B_{In}$. Fourteen cosmid clones that conferred host gene-specific avirulence on at least one of the virulent strains were isolated. Eight of the clones carried more than one avirulence activity. Restriction analyses and DNA hybridization studies showed that all of the cloned fragments overlapped, and all were derived from a 90-kb plasmid in *X. c.* pv. *malvacearum* strain H (XcmH). Subcloning experiments resulted in the separation and partial localization of four avirulence genes, designated *avrB4*, *avrb6*, *avrb7*, and *avrBIn*. These four appeared to interact in a gene-for-gene manner with the corresponding resistance genes, but these were not exclusive interactions. Subcloning also demonstrated the presence of two additional avirulence genes, designated *avrB101* and *avrB102*, on the XcmH plasmid. All six *avr* genes in *X. c.* pv. *malvacearum* induced a strong hypersensitive response on cotton cultivar 101-102B (contains $B_2$, $B_3$, and $B_{Sm}$) and all but *avrBIn* in *X. c.* pv. *malvacearum* induced a weak hypersensitive response on cultivar Acala-B2. These observations represent possible exceptions to the gene-for-gene hypothesis. Genes *avrb6* and *avrb7* increased the ability of some *X. c.* pv. *malvacearum* strains to water soak the susceptible cultivar Acala-44. Spontaneous Avrb6$^-$ mutants of *X. c.* pv. *malvacearum* were reduced in water-soaking ability, suggesting a possible role of at least one of the *avr* genes in conditioning virulence of the pathogen on a susceptible host.**

*Additional keywords*: cotton blight, horizontal resistance, specificity, vertical resistance.

Angular leaf spot of cotton is an economically important, world-wide disease caused by *Xanthomonas campestris* pv. *malvacearum* (Smith) Dye. The genetics of resistance to this pathogen are well described, with at least 16 specific resistance (*R*) genes identified (Endrizzi *et al.* 1985). Race specific avirulence of *X. c.* pv. *malvacearum* and resistance in cotton is thought to be gene-for-gene, and presumably different *X. c.* pv. *malvacearum* races contain different combinations of *avr* genes. Gene-for-gene interactions are usually assayed in terms of relative incompatibility in comparisons with the most compatible interactions known. Incompatible plant-bacterial interactions are manifested as a relatively faster and more intense necrotic (hypersensitive response or HR) reaction than the compatible necrotic reaction (normosensitive response or NR) (Klement 1982). Incompatible interactions with different *R* genes in congenic cotton lines are different (Gabriel *et al.* 1986). All compatible and some incompatible interactions result in water-soaked lesions that may persist for variable periods of time, depending on the environment and host genotype before the NR or HR.

Most individual plant *R* genes are overcome by new pathogenic races when cultivars containing the *R* genes are planted on a large scale, presumably due to mutations of specific *avr* genes (Watson 1970; Day 1974; Vanderplank 1968). Most genes for resistance to bacterial cotton blight are similarly "defeated" by new races (Brinkerhoff 1970). The search for resistance genes that would be effective against all strains of a pathogen has been generally unsuccessful (Ellingboe 1981; Ellingboe 1975), although some individual *R* genes have remained "undefeated" in field use for considerable periods of time (Brinkerhoff *et al.* 1984; Roelfs 1988; Nelson 1978). Such genes have not been shown to be race-specific or to follow a gene-for-gene pattern of recognition. A few genes have been recognized and used by cotton breeders to create cotton lines that are essentially immune to bacterial blight (Brinkerhoff *et al.* 1984). One such gene is blight resistance gene $B_2$, which has not been defeated by attempts to create virulent mutants (Brinkerhoff *et al.* 1978) and which is one of several genes carried in the immune cotton line 101-102B (Brinkerhoff 1970).

The genetic basis for the longevity of certain *R* genes and not others is largely unknown at present. Speculation has long hinged on the idea that some *avr* genes may pleiotropically encode essential metabolic or virulence functions, and recent evidence supports this idea in a few cases (reviewed in Gabriel and Rolfe 1990). An alternative, not mutually exclusive, hypothesis is that some *R* genes have a much wider specificity than others; that is, they react with more than one *avr* gene in a gene-for-genes manner (Gabriel 1989). Such *R* genes would not necessarily be effective against all pathogenic strains or races, but they would be statistically more difficult to defeat by *avr* gene mutations. Evidence for this has not yet been reported.

In the process of subcloning fragments from previously reported *avr* gene clones from *X. c.* pv. *malvacearum* (Gabriel *et al.* 1986), we observed that no avirulence activity

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was evident on any of the cosmids originally reported to carry *avrB5* or *avrb6*, except pUFA704, which reacts with multiple plant host lines. This prompted us to remake the *X. c.* pv. *malvacearum* strain H library in the newly created cosmid vector, pUFR034 (De Feyter *et al.* 1990), and re-screen for avirulence. We report here the identification and cloning of six new *X. c.* pv. *malvacearum avr* genes, all resident on a previously cryptic plasmid. In addition to their specificity for other cotton cultivars, all six *avr* genes in *X. c.* pv. *malvacearum* induced a strong HR on the "immune" cotton cultivar 101-102B, and all but one induced an HR on Acala-B2, thought to carry only the $B_2$ resistance gene.

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1. Antibiotic resistant *X. c.* pv. *malvacearum* strains were selected by spreading approximately $10^9$ cells on peptone, yeast extract, glycerol, MOPS (PYGM) agar (De Feyter *et al.* 1990) containing rifampicin or spectinomycin. After 3 days of incubation at 30° C, colonies were selected that had grown to a size similar to that of the parental strain on the same medium without antibiotics.

**Media.** *Escherichia coli* strains were routinely grown in LB medium (Maniatis *et al.* 1982) at 37° C, and *X. c.* pv. *malvacearum* strains in PYGM medium (De Feyter *et al.* 1990) at 30° C. Agar was added at 15 g/L to solidify media. Antibiotics were used at the following final concentrations (μg/ml): kanamycin (Km), 20; neomycin (Nm), 20: rifampicin (Rif), 75; streptomycin (Sm), 150; spectinomycin (Sp), 50; tetracycline (Tc), 15.

**Bacterial conjugation.** The *X. c.* pv. *malvacearum* gene library in cosmid pUFR034 was maintained in *E. coli* strain HB101. Transfer of helper plasmid pRK2073 and modifier plasmid pUFR054 (De Feyter and Gabriel, in press) into each clone of the library was achieved by triparental con-

**Table 1.** Bacterial strains and plasmids used in this study

| Bacterial strain or plasmid | Relevant characteristics | Reference |
|---|---|---|
| *E. coli* | | |
| HB101 | F⁻, *hsdS20(hsdR hsdM)*, *recA13*, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20*(Sm$^R$), *xyl-5*, *mtl-1*, *supE44*, λ⁻ | Boyer and Roulland-Dussoix 1969 |
| DH5∝ | F⁻, *endA1*, *hsdR17* ($r_k^-m_k^+$), *supE44*, *thi-1*, *recA1*, *gyrA*, *relA1*, *ϕ80dlacZ* ΔM15, Δ(*lacZYA-argF*)U169 | Bethesda Research Laboratories |
| ED8767 | *recA*, *met* | Murray *et al.* 1977 |
| *Xanthomonas campestris* pv. *malvacearum* | | |
| A | Race 3, Avrb7⁻ | Gabriel *et al.* 1986 |
| H | Race 1, widely avirulent | Gabriel *e al.* 1986 |
| HSp | Spc$^R$ derivative of H | De Feyter *et al.* 1990 |
| N | Widely virulent | Gabriel *et al.* 1986 |
| Xcm1003 | Spc$^R$, Rif$^R$ derivative of N | This study |
| Xcm1004 | Rif$^R$ derivative of A | This study |
| Xcm1102 | Spc$^R$, Avrb6⁻ AvrBIn⁻ deriv. of HSp | This study |
| Xcm1103 | Rif$^R$ derivative of Xcm1102 | This study |
| Xcm1113 | Spc$^R$, AvrBIn⁻ deriv. of HSp | This study |
| Xcm1177 | Rif$^R$ derivative of Xcm1113 | This study |
| Xcm1192 | AvrB4⁻ derivative of Xcm1102 | This study |
| Xcm1198 | Rif$^R$ derivative of Xcm1192 | This study |
| Plasmids | | |
| pUFR030 | IncW, Nm$^R$, Mob$^+$, *mob*(P), *lacZ*α$^+$ | De Feyter *et al.* 1990 |
| pUFR031 | IncW, Nm$^R$, Mob$^+$, *mob*(P), *lacZ*α$^+$, Par$^+$ | This study |
| pUFR033 | IncW, Nm$^R$, Mob$^+$, *mob*(P), *lacZ*α$^+$, *cos* | De Feyter *et al.* 1990 |
| pUFR034 | IncW, Nm$^R$, Mob$^+$, *mob*(P), *lacZ*α$^+$, Par$^+$, *cos* | De Feyter *et al.* 1990 |
| pUFR038 | IncW, Nm$^R$, Gm$^R$ (weak), Mob$^+$, *mob*(P), *lacZ*α$^+$, *cos* | This study |
| pUFR039 | IncW, Nm$^R$, Gm$^R$, Mob$^+$, *mob*(P), *lacZ*α$^+$, *cos* | This study |
| pUFR040 | IncW, Nm$^R$, Gm$^R$, Mob$^+$, *mob*(P), *lacZ*α$^+$, Par$^+$ | This study |
| pUFR042 | IncW, Nm$^R$, Gm$^R$, Mob$^+$, *mob*(P), *lacZ*α$^+$, Par$^+$, *cos* | This study |
| pUFR051 | IncP, Tc$^R$, *cos* (pLAFR3 derivative containing *XbaI* and *KpnI* sites in polylinker) | De Feyter and Gabriel 1991 |
| pUFR054 | IncP, Tc$^R$, *cos*, M.*Xma*I, M.*Xma*III in pUFR051 | De Feyter and Gabriel 1991 |
| pUFR101 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR102 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR103 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR104 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR105 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR106 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR107 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR108 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR109 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR110 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR111 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR112 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR113 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR114 | Avr$^+$ cosmid clone, see Figure 2 | This study |
| pUFR115 | *Eco*RI deletion from pUFR101 | This study |
| pUFR116 | 18-kb *Eco*RI fragment from pUFR106 in pUFR042 | This study |
| pUFR117 | *Eco*RI deletion from pUFR112 | This study |
| pUFR118 | *Eco*RI deletion from pUFR114 | This study |
| pUFR119 | *Eco*RI deletion from pUFR114 | This study |
| pUFR120 | *Hin*dIII deletion from pUFR114 | This study |
| pUFR121 | *Eco*RI deletion from pUFR103 | This study |
| pUFR122 | *Eco*RI-*Bgl*II fragment from pUFR106 in pUFR042 | This study |
| pUFR127 | 5.0-kb *Eco*RI fragment from pXcmH in pUFR042 | This study |
| pUFR128 | 5.0-kb *Eco*RI fragment from pXcmH in pUFR042 | This study |
| pUFR130 | 9.5-kb *Eco*RI fragment from pXcmH | This study |
| pMON7051 | Gm$^R$, RSF1010-derived replicon | Barry 1988 |
| pRK2073 | Tra$^+$, Mob$^+$, Sp$^R$, ColEl replicon (helper plasmid) | Leong *et al.* 1982 |

jugations. The HB101 (pUFR034 derivatives) were patched onto LB plates. Mid-exponential phase cultures of ED8767 (pRK2073) and HB101 (pUFR054) were harvested by centrifugation, washed free of antibiotics, each was resuspended in one volume of LB broth, and mixed in a 1:1 ratio. Ten-microliter aliquots of the mixture were spotted onto the patched strains, the liquid was allowed to soak into the plates, and the plates were incubated overnight at 37° C. Selection for transconjugants was achieved by streaking cells from the patches onto medium containing Sm, Sp, Tc, and Nm.

Transfer of pUFR034- or pUFR042-derived plasmids into rifampicin-resistant *X. c.* pv. *malvacearum* recipients was as follows. Mid-exponential phase cultures of *X. c.* pv. *malvacearum* were harvested by centrifugation, resuspended in 0.05 vol of PYGM medium, and 10 μl vol was spotted onto dry PYGM plates, 44 spots per plate. Colonies of the tri-plasmid *E. coli* donors HB101(pRK2073, pUFR054, pUFR034) were transferred to the *X. c.* pv. *malvacearum* spots by using sterile toothpicks, and the plates were incubated at 30° C overnight to allow for transfer. Selection for transconjugants was achieved by transferring cells from the patches onto PYGM plates containing Rif and Km at 30° C. Transconjugants appeared as yellow colonies after 3 days of growth.

**Plasmid isolation and manipulation.** Plasmid DNA was prepared from *E. coli* cultures by alkaline lysis (Birnboim and Doly 1979), and from *X. c.* pv. *malvacearum* strain H (XcmH) as follows. Cells were harvested from 2 L of mid-exponential phase culture, grown in PYGM broth, and resuspended in 300 ml of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0. The following procedure was carried out at 4° C. Sodium dodecyl sulfate was added to 1% while stirring the suspension, and 10 M NaOH was added to pH 12.4, lysing the cells. After 10 min, 160 ml of 3 M potassium acetate (titrated to pH 4.8 with formic acid) was added. After 30 min, cell debris was removed by centrifugation, and the supernatant was extracted once with phenol/chloroform. Nucleic acid was recovered by precipitation with isopropanol, washed once with 70% ethanol, and, without drying, resuspended in 10 mM Tris, 0.5 mM EDTA, pH 8.0 (TE) buffer. Plasmid-DNA was then purified by two cycles of CsCl-EtBr gradient fractionation.

All recombinant DNA procedures were by standard methods (Maniatis *et al.* 1982).

**Construction of pUFR042.** Plasmid pUFR031 was constructed by inserting the 1.3-kb *Bam*HI fragment containing *par*A$^+$ from pUFR037 into the *Bgl*II site of pUFR030 (De Feyter *et al.* 1990). A gene specifying resistance to gentamicin was inserted into pSa-derived broad host range cloning vectors as follows. The 1.9-kb *Eco*RI-*Sph*I fragment of pMON7051 (Barry 1988) containing the gene for Gm$^R$ was inserted into one of the two *Xba*I sites of pUFR033 (De Feyter *et al.* 1990), after both DNAs had been treated briefly with *Bal*31, forming pUFR038. The *Bal*31 digestion removed approximately 0.3 kb of DNA in total from the ends of the DNA fragments. This plasmid conferred resistance to gentamicin at 1 μg/ml, but not at 5 μg/ml. A derivative, pUFR039, which expressed stronger gentamicin resistance, was isolated by plating DH5α/pUFR038 onto media containing 5 μg/ml of gentamicin and by selecting a larger colony. The plasmid isolated from this colony, pUFR039, is the same size as pUFR038, appeared to replicate at the same number of copies per cell, and conferred resistance to 5 μg/ml of gentamicin in DH5α. The smaller *Sal*I fragment from pUFR039 was joined to the larger *Sal*I fragment of pUFR031 forming pUFR040. One of the two *Sal*I sites of pUFR040 was deleted by partial digestion of the plasmid with *Sal*I, treatment with Klenow fragment and dNTPs, and recircularization with DNA ligase. The resulting 8.5-kb plasmid was designated pUFR042 and is shown schematically in Figure 1. This plasmid has unique restriction sites for *Eco*RI, *Bam*HI, *Sst*I, *Kpn*I, and *Sal*I.

**Plant inoculations.** The cotton lines used were derived from those originally described (Hunter *et al.* 1968; Hunter and Brinkerhoff 1961). Cotton plants were inoculated with suspensions ($10^8$–$10^9$ cfu/ml) of *X. c.* pv. *malvacearum* strains in sterile tap water, and pathogenic reactions were scored as previously described (Gabriel *et al.* 1986). Plants were maintained in growth chambers at 29° C with a 14-hr light period, and plant reactions were evaluated periodically between 2 and 8 days after inoculation. For the isolation of race-change mutants of *X. c.* pv. *malvacearum*, six-week-old cotton plants were spray-inoculated with a cell suspension ($10^4$ cfu/ml) of an avirulent strain. Ten to 12 days after inoculation, bacteria were recovered from water-soaked lesions with a fine wire point and streaked onto PYGM plates. Single colony isolates were checked for their response (either HR or water soaking) on cotton cultivars after inoculation as described above.

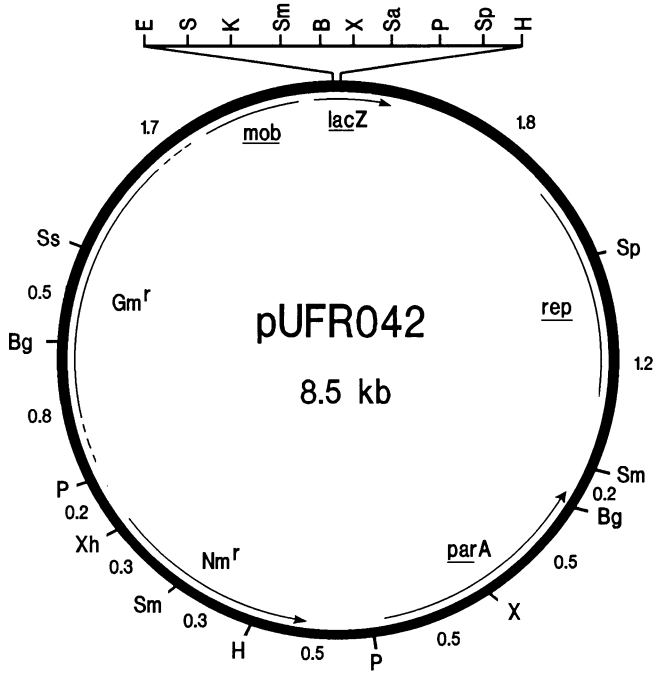


**Fig. 1.** Schematic representation of pUFR042, which was constructed as described in Materials and Methods. Arrows indicate the direction of transcription where known. Unique sites are: *Eco*RI, *Sst*I, *Kpn*I, *Bam*HI, and *Sal*I. Abbreviations: *rep*, replication origin from plasmid pSa; *par*A, partition locus; *mob*, conferring plasmid mobilization ability; Gm$^R$, resistance to gentamycin; Nm$^R$, resistance to neomycin; E, *Eco*RI; S, *Sst*I; K, *Kpn*I; Sm, *Sma*I; B, *Bam*HI; X, *Xba*I; Sa, *Sal*I; P, *Pst*I; Sp, *Sph*I, H, *Hin*dIII; Bg, *Bgl*II; Xh, *Xho*I; Ss, *Sst*II.

## RESULTS

**Isolation of race-change mutants of *X. c.* pv. *malvacearum*.** To isolate virulent race-change mutants of *X. c.* pv. *malvacearum*, the avirulent strain HSp was spray-inoculated onto cotton cultivar Ac44-BIn containing the single blight resistance gene $B_{In}$. Twelve days after inoculation, sprayed leaves showed numerous tiny, brown lesions and a few angular, water-soaked spots (ratio approximately 1,000:1). Single-colony isolates of bacteria from the water-soaked spots were tested for virulence by reinoculation onto Ac44 and Ac44-BIn plants, and 66 strains (each from a different lesion) that were virulent on both cultivars were retained. By comparison, the parental strain HSp induces an incompatible HR on Ac44-BIn, and a virulent, water-soaked response on the susceptible Ac44 host.

Inoculation of a series of cotton cultivars with the 66 HSp mutants virulent on Ac44-BIn showed that all except one (Xcm1102) remained unaltered in their reactions on cultivars other than Ac44-BIn (Table 2). Strain Xcm1102, however, gave compatible water-soaked lesions on cultivars Ac44-b6, Stoneville 2B-S9, and Gregg, in addition to Ac44-BIn. It also appeared to be reduced slightly in virulence on Ac44 because inoculated zones became water-soaked more slowly than for the parental strain, HSp.

Derivatives of Xcm1102 that were virulent on Ac44-B4 were obtained similarly by spray inoculation of this cultivar with suspensions of Xcm1102. One derivative that gave water-soaked lesions on Ac44-B4 was retained and designated Xcm1192. This strain was identical to Xcm1102 in its reactions on cultivars other than Ac44-B4. Complementation of the virulence mutations in Xcm1102 and other strains is the subject of the rest of this paper.

**Construction of a gene library of *X. c.* pv. *malvacearum* strain H.** XcmH was chosen to make a gene library because this strain induces a strong HR and is avirulent on at least nine cotton cultivars, each thought to contain different resistance genes (Gabriel *et al.* 1986). The cosmid pUFR034 (De Feyter *et al.* 1990) was used for the library construction. This plasmid can be mobilized from *E. coli* to *X. c.* pv. *malvacearum* at a frequency of approximately $1 \times 10^{-6}$ per recipient, is stably maintained in *X. c.* pv. *malvacearum* in the absence of selection pressure, and was therefore a suitable vector. It confers resistance to neomycin and kana-

**Table 2.** Phenotypes resulting from interactions of *Xanthomonas campestris* pv. *malvacearum* strains or transconjugants, and cotton host lines

| Strain or transconjugant | Cotton cultivar and known *R* gene(s)[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Ac44 ... | B2 $B_2$ | B4 $B_4$ | b6 $b_6$ | b7 $b_7$ | BIn $B_{In}$ | 2BS9 $B_{Sm}$ | Gregg ? | 1-10B[b] ($B_{Sm}$), $B_{In}$ | 20-3[b] ($B_{Sm}$), $B_N$ | 101-102B $B_{Sm}$, $B_2$, $B_3$ |
| HSp | + | − | − | − | − | − | − | − | − | − | − |
| Xcm1004 | + | − | − | − | + | − | − | − | − | − | − |
| Xcm1102 | + | − | − | + | − | + | + | + | + | − | − |
| Xcm1113 | + | − | − | − | − | + | − | − | − | − | − |
| Xcm1177 | + | − | − | − | − | + | − | − | − | − | − |
| Xcm1192 | + | − | + | + | − | + | + | + | + | − | − |
| Xcm1198[c] | + | − | + | + | − | + | + | + | + | − | − |
| Xcm1003[c] | + | + | + | + | + | + | + | + | + | + | + |
| Xcm1004/pUFR112[d] | + | − | − | − | − | − | − | − | − | − | − |
| Xcm1198/pUFR101 | + | − | − | − | − | + | − | − | − | − | − |
| Xcm1198/pUFR102 | + | − | − | + | − | + | + | + | + | − | − |
| Xcm1198/pUFR103 | + | − | − | − | − | + | − | − | − | − | − |
| Xcm1198/pUFR104 | + | − | − | − | − | + | − | − | − | − | − |
| Xcm1198/pUFR105 | + | − | − | − | − | + | − | − | − | − | − |
| Xcm1198/pUFR106 | + | − | − | − | − | + | − | − | − | − | − |
| Xcm1198/pUFR107 | + | − | + | − | − | − | − | − | − | − | − |
| Xcm1198/pUFR108 | + | − | + | + | − | − | + | + | − | − | − |
| Xcm1198/pUFR109 | + | − | + | + | − | − | + | + | − | − | − |
| Xcm1198/pUFR110 | + | − | + | + | − | − | + | + | − | − | − |
| Xcm1198/pUFR111 | + | − | + | + | − | − | + | + | − | − | − |
| Xcm1198/pUFR112 | + | − | + | + | − | − | + | + | − | − | − |
| Xcm1003/pUFR101 | + | ± | − | − | + | + | − | − | − | − | − |
| Xcm1003/pUFR102 | + | ± | − | + | + | + | + | + | + | + | − |
| Xcm1003/pUFR103 | + | ± | − | − | + | + | − | − | − | − | − |
| Xcm1003/pUFR104 | + | ± | − | − | + | + | − | − | − | − | − |
| Xcm1003/pUFR105 | + | ± | − | − | + | + | − | − | − | − | − |
| Xcm1003/pUFR106 | + | ± | − | − | + | + | − | − | − | − | − |
| Xcm1003/pUFR107 | + | ± | + | − | + | − | − | − | − | − | − |
| Xcm1003/pUFR108 | + | ± | + | + | + | − | + | + | − | ± | − |
| Xcm1003/pUFR109 | + | ± | + | + | + | − | + | + | − | ± | − |
| Xcm1003/pUFR110 | + | ± | + | + | + | − | + | − | − | ± | − |
| Xcm1003/pUFR111 | + | ± | + | + | + | − | + | + | − | ± | − |
| Xcm1003/pUFR112 | + | ± | + | + | − | − | + | + | − | + | − |
| Xcm1003/pUFR113 | + | ± | + | + | − | + | + | + | + | + | − |
| Xcm1003/pUFR114 | + | ± | + | + | − | + | + | + | + | + | − |

[a] + Indicates a compatible, water-soaked reaction; − indicates an incompatible, hypersensitive response; ± indicates an intermediate reaction, weakly incompatible.
[b] The "polygenes" of 1-10B and 20-3 are here assumed to be $B_{Sm}$, but see discussion.
[c] We have not attempted to indicate the altered virulence phenotype of strains Xcm1003, Xcm1198, and their transconjugants (refer text).
[d] Results for Xcm1004/pUFR113 and Xcm1004/pUFR114 were identical to those for Xcm1004/pUFR112.

mycin on both *E. coli* and *X. c.* pv. *malvacearum*.

To construct the library, *Sau*3A fragments (32–40 kb) of strain H DNA were ligated to *Bam*HI-cut pUFR034 DNA and packaged *in vitro* into phage λ heads. When the packaged library was introduced into *E. coli* HB101, numerous neomycin-resistant colonies were obtained, containing plasmids with an average insert size of 36 kb. Approximately 1,000-fold fewer colonies were obtained when strain DH5α was used as the host, and many of the plasmids contained very small or no insert DNA, suggesting a restriction of *X. c.* pv. *malvacearum* DNA in this strain (De Feyter and Gabriel, in press). Five hundred and forty clones in HB101 were retained, representing a twofold coverage of the XcmH genome. In an earlier experiment, a partial genomic library was made from XcmH DNA by ligating *Sau*3A fragments (15–20 kb) into *Bam*HI cut pUFR034 DNA and introducing these into HB101 by transformation. This partial library contained 100 clones and was examined together with the larger library for avirulence gene clones.

**Transfer of *X. c.* pv. *malvacearum* library and complementation of race-change mutations with cloned avirulence genes.** Initially we sought to complement mutations in two virulent strains of *X. c.* pv. *malvacearum*, converting them to avirulence. These were strain A, an isolate from race 3 which is virulent on Ac44-b7, and the race-change mutant Xcm1192, which is virulent on Ac44-B4, Ac44-b6, and Ac44-BIn. To introduce the plasmids from the library into these *X. c.* pv. *malvacearum* strains, we used a modifier plasmid to increase the transfer frequency. The modifier plasmid, pUFR054, contains the *Xma*I and *Xma*III DNA methylase genes, and when present in the *E. coli* donor strains enhances plasmid transfer into *X. c.* pv. *malvacearum* from approximately $1 \times 10^{-12}$ per recipient without the modifier to $1 \times 10^{-7}$ (De Feyter and Gabriel, in press). Presumably, methylation of the DNA before transfer reduces *Xma*I- and *Xma*III-mediated restriction on entry into *X. c.* pv. *malvacearum*.

The 640 plasmid clones in the two libraries were introduced into the rifampicin-resistant derivatives Xcm1004 and Xcm1198 (from XcmA and Xcm1192, respectively). Transconjugants from each of the conjugations with Xcm1004 were inoculated onto cotton cultivar Ac44-b7. Of the 640 plasmids tested, three (pUFR112, pUFR113, pUFR114) converted the phenotype of the *X. c.* pv. *malvacearum* recipient from compatible to incompatible on this host, giving a strong necrotic HR by 48 hr after inoculation. When these three plasmids were examined by restriction digestion, they yielded several fragments in common, indicating they probably contained overlapping segments of the *X. c.* pv. *malvacearum* genome. Three other plasmids in the *X. c.* pv. *malvacearum* library conferred a weak avirulence on the *X. c.* pv. *malvacearum* recipient, but this avirulence was not cultivar-specific and, for in-

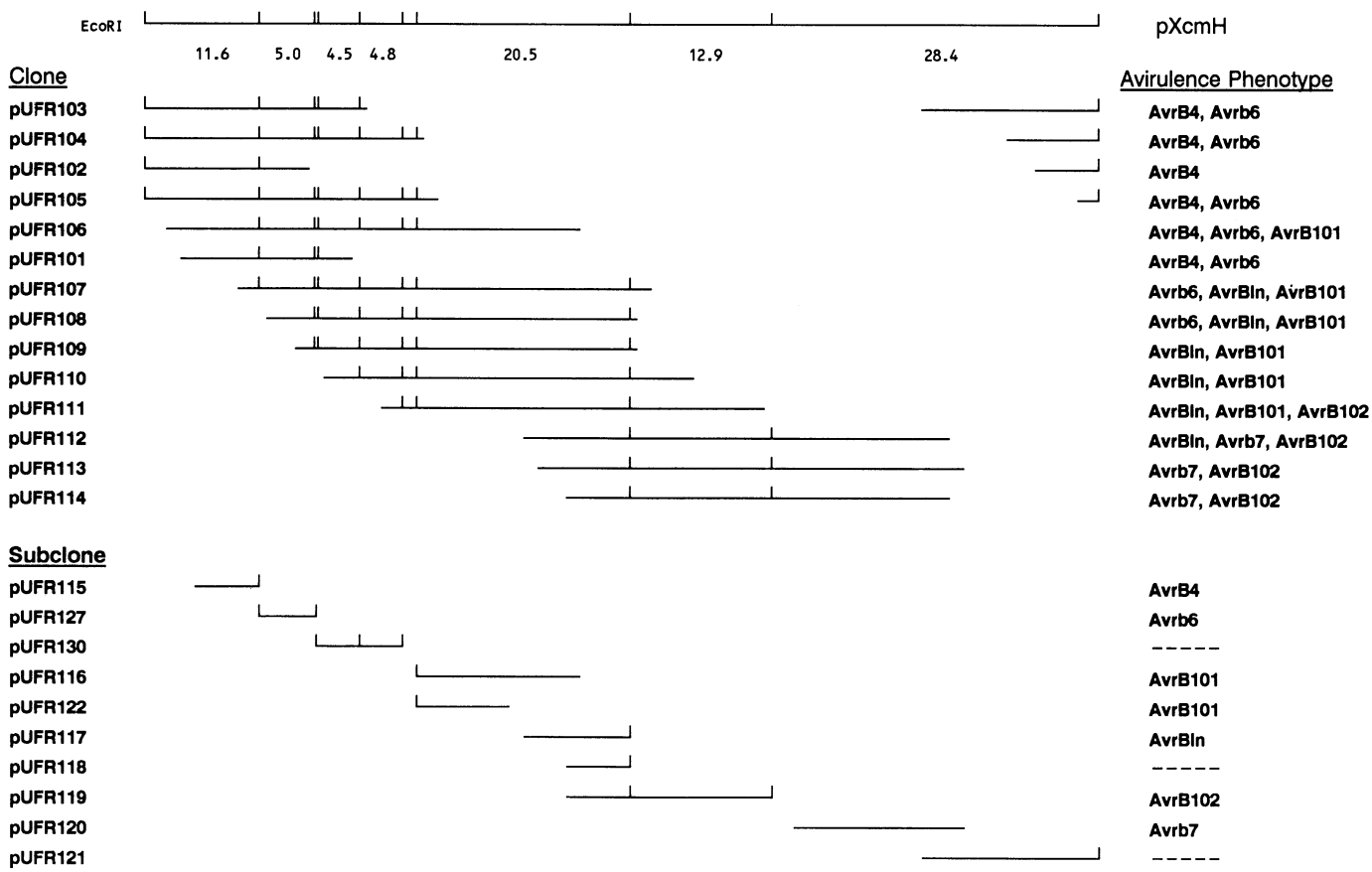


**Fig. 2.** Physical map of pXcmH showing *Eco*RI sites, the original cosmid clones used to identify the *avr* genes, and subclones used to localize the avirulence genes.

stance, occurred on the susceptible cultivar Ac44. Therefore, they were not examined further.

Strain Xcm1198 is virulent on cotton cultivars Ac44-B4, Ac44-b6, Ac44-BIn, Stoneville 2B-S9, and Gregg. Transconjugants of Xcm1198 were therefore inoculated into these cultivars. Twelve plasmids (pUFR101–112), which converted the recipient to avirulence on at least one of the cultivars (Table 2), but not on Ac44, were identified. Importantly, some of the plasmids conferred avirulence on more than one of the cultivars Ac44-B4, Ac44-b6, and Ac44-BIn, which are known to contain the different indicated *R* genes. Other plasmids conferred avirulence on only one of these cultivars. Plasmids that gave avirulence on Ac44-b6 in every case also conferred avirulence on cultivar Gregg and on all cultivars known to contain $B_{Sm}$ or "polygenes" (refer to Discussion), suggesting that these might contain the same *R* gene. Restriction analysis of the twelve plasmids indicated that they were overlapping (Fig. 2). One of the overlapping plasmids (pUFR112) had been shown to confer avirulence to Xcm1004 on cultivar Ac44-b7. Therefore, based on the restriction analysis, at least four *avr* genes appeared clustered in the XcmH genome.

The 14 plasmids (pUFR101–114) were also introduced into Xcm1003, a rifampicin-resistant derivative of strain XcmN, isolated originally from West Africa and virulent on every cotton cultivar we have examined (Table 2). Transconjugants of Xcm1003 were tested for avirulence activity, and in every case pUFR101–114 conferred the same avirulence activities as had been observed when using Xcm1004 and Xcm1198 as recipients of the plasmids. Therefore, strain Xcm1003 was used as the recipient in further analyses of subclones of the plasmids. Strains Xcm1004 or Xcm1198 were used to confirm that plasmids containing subclones could confer avirulence.

**These avirulence genes are plasmid-borne in *X. c.* pv. *malvacearum*.** XcmH contains a single plasmid of approximately 90 kb, which we designated pXcmH. Plasmids pUFR101–114 and pXcmH were compared by digestion with *Eco*RI and *Hin*dIII and agarose gel electrophoresis. Fragments of 21, 13, 12, 5.0, 4.8, 4.5, and 2.1 kb were in common in digests of several plasmids (data not shown). All of these fragments were also present in the digest of pXcmH. Moreover, when pXcmH DNA was radiolabeled and used to probe a Southern blot of the gel under stringent hybridization conditions, all of the fragments bound the probe except those derived from the vector, pUFR034. We concluded that pUFR101–114 were derived from pXcmH DNA, and that the avirulence genes reported here are plasmid-borne in XcmH.

Plasmids pUFR103, pUFR107, and pUFR114 overlapped and together contain the entire plasmid pXcmH. Cloned *Eco*RI and *Sst*I fragments derived from these plasmids were analyzed with various restriction enzymes to derive a restriction map of pXcmH. A map for 10 restriction enzymes is presented in Figure 3. By summation of individual fragment sizes, the size of pXcmH was about 90.4 kb.

**Preliminary localization of the avirulence genes and evidence for two more on pXcmH.** The location of the genes conferring avirulence on cultivars Ac44-B4, Ac44-

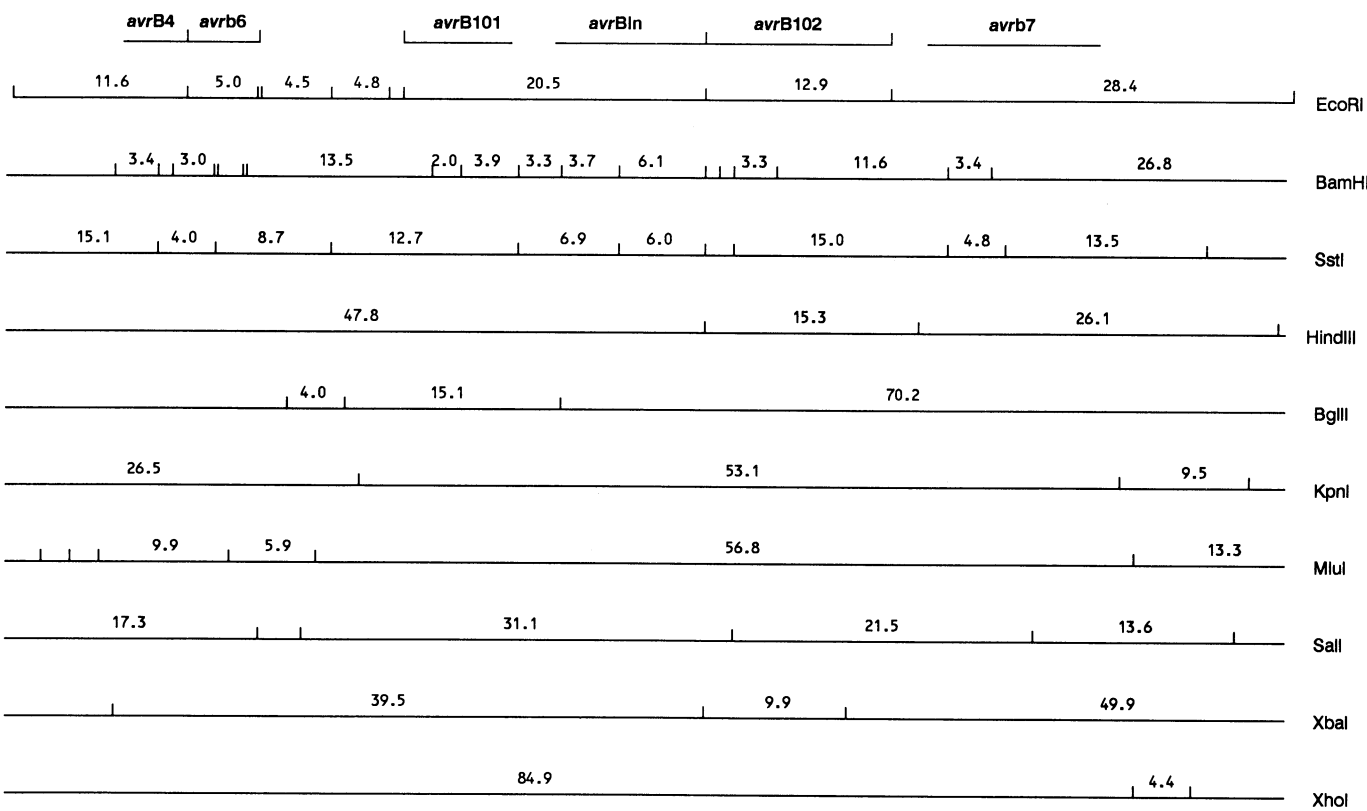


**Fig. 3.** Physical map of pXcmH with 10 restriction enzymes, and showing the location of the cloned *avr* genes, based on the data in Figure 2.

b6, Ac44-b7, and Ac44-BIn could be inferred to be in the overlapping regions of plasmids in the series pUFR101–114 having these activities in common. We designated these genes as *avrB4*, *avrb6*, *avrb7*, and *avrBIn*, respectively. To further localize these *avr* genes, deletion derivatives of pUFR013, pUFR112, and pUFR114 were generated by *Eco*RI or *Hin*dIII digestion followed by recircularization with DNA ligase. In addition, some *Eco*RI fragments of pXcmH were inserted into pUFR042 (Table 1). All deletion derivatives were introduced into Xcm1003, and transconjugants were analyzed for avirulence activity on the appropriate cotton cultivars.

The gene *avrB4* was mapped to the 10.5-kb region of overlap between plasmids pUFR101 and pUFR102. To confirm this, four *Eco*RI fragments were deleted from pUFR101, leaving 7.5 kb of insert DNA and forming pUFR115 (Fig. 2). This plasmid still conferred full avirulence activity on Ac44-B4 but not on Ac44-b6, and therefore *avrB4* lay within this 7.5 kb of *X. c.* pv. *malvacearum* DNA. In similar fashion, gene *avrb6* was mapped to the 8-kb region of overlap between pUFR101 and pUFR107. The 5.0-kb *Eco*RI fragment within this region was isolated from pXcmH and inserted in both orientations into pUFR042, forming pUFR127 and pUFR128. Both plasmids conferred full avirulence activity to Xcm1003 when inoculated onto Ac44-b6. In addition, Xcm1003 (pUFR127) was avirulent on cotton cultivar Gregg and on cultivars Stoneville 2B-S9, 1-10B, 20-3, and 101-102B. The latter four cultivars are known to contain a "polygenic" complex. The complex from Stoneville 2B lines is named $B_{Sm}$; $B_{Sm}$ is known to be present in 101-102B (Brinkerhoff 1970; Innes *et al.* 1974). From this work, the polygenic complex in all of these lines may be $B_{Sm}$, and it appeared to carry the recessive resistance gene $b_6$.

The cosmid clone pUFR114 conferred avirulence to Xcm1003 on two cotton cultivars, Ac44-b7 and 101-102B. To localize *avrb7*, two *Hin*dIII fragments were deleted from pUFR114 to make pUFR120, containing approximately 16 kb of *X. c.* pv. *malvacearum* DNA (Fig. 2). This plasmid conferred full avirulence to Xcm1003 on both Ac44-b7 and 101-102B. The two other cosmid clones identified as carrying *avrb7*, pUFR112 and pUFR113, also contain this region of pXcmH. Deletion of the 18-kb *Eco*RI fragment, including the *avrb7* region, of pUFR114 formed the plasmid pUFR119. As expected, pUFR119 did not confer avirulence on Ac44-b7, but, curiously, this plasmid retained the ability to confer avirulence on cultivar 101-102B. This activity was not present when a second *Eco*RI fragment (12.9 kb) of pUFR114 was deleted, forming pUFR118 (Fig. 2). This indicated that another avirulence gene, which we designated *avrB102* lay at least in part on the 12.9-kb *Eco*RI fragment of pXcmH. Apparently, the presence of *avrB102* on pUFR114 had been masked by *avrb7*, also present on pUFR114, because both genes conferred avirulence to Xcm1003 on cultivar 101-102B. Because the inserts in the subclones pUFR119 and pUFR120 do not overlap, the activities must represent separate genes.

A similar situation was found with subclones covering the 20.5-kb *Eco*RI fragment of pXcmH. All plasmids containing this fragment conferred avirulence on Xcm1003 when inoculated on Ac44-BIn. To define the *avrBIn* region further, pUFR117 was constructed from pUFR112 by deletion of two fragments with *Eco*RI. Both pUFR117 and pUFR112 conferred an AvrBIn$^+$ phenotype, demonstrating that *avrBIn* lay within the 9 kb of DNA at the right-hand end of the 20.5-kb *Eco*RI fragment (Fig. 2). Plasmid pUFR113 contains the rightward 7 kb of this 9-kb region and yet did not confer the AvrBIn$^+$ phenotype, indicating a contribution of the additional 2 kb in the left-hand end of *avrBIn*. The right-hand end of *avrBIn* was defined by the AvrBIn$^-$ phenotype of both pUFR106 and its *Eco*RI deletion derivative, pUFR116, both of which contain the leftward 4.2kb of the 9-kb *avrBIn* region (Fig. 2). Therefore, activity of the *avrBIn* gene required a region of at least 2.2 kb of DNA.

Although Xcm1003 (pUFR116) was virulent on Ac44-BIn, it unexpectedly was strongly avirulent on cultivar 101-102B and weakly avirulent on 20-3. The gene specifying this activity was clearly distinct from *avrBIn,* and we designated it *avrB101*. Activity of *avrB101* had been masked by *avrBIn* on plasmids such as pUFR112, and by *avrb6* on other plasmids (Tables 2 and 3).

Examination of the plasmid DNA content of some of the spontaneous race-change mutants of strain HSp revealed that pXcmH had suffered deletions of up to 7 kb and/or rearrangements in some, but not all cases.

**Complementation of the apparent virulence defect of Xcm1102.** Xcm1102, derived as a race-change mutant from

**Table 3.** Summary table of phenotypes resulting from transconjugants of Xcm1003 carrying single avirulence genes inoculated onto cotton cultivars with the indicated *R* genes

| | Cotton cultivar and known *R* gene(s)[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **Transconjugant** | **Ac44** ... | **B2** $B_2$ | **B4** $B_4$ | **b6** $b_6$ | **b7** $b_7$ | **BIn** $B_{In}$ | **2BS9** $B_{Sm}$ | **Gregg** ? | **1-10B**[b] ($B_{Sm}$), $B_{In}$, | **20-3**[b] ($B_{Sm}$), $B_N$ | **101-102B** $B_{Sm}$, $B_2$, $B_3$ |
| Xcm1003/pUFR115 (*avrB4*) | + | ± | − | + | + | + | + | + | + | + | − |
| Xcm1003/pUFR127 (*avrb6*) | + | ± | + | − | + | + | − | − | − | − | − |
| Xcm1003/pUFR120 (*avrb7*) | + | ± | + | + | − | + | + | + | + | + | − |
| Xcm1003/pUFR117 (*avrBIn*) | + | + | + | + | + | − | + | + | − | + | − |
| Xcm1003/pUFR122 (*avrB101*) | + | ± | + | + | + | +[c] | +[c] | + | + | ± | − |
| Xcm1003/pUFR119 (*avrB102*) | + | ± | + | + | + | + | + | + | + | + | − |
| Xcm1003/pUFR042 (*vector*) | + | + | + | + | + | + | + | + | + | + | + |

[a] + Indicates a compatible, water-soaked reaction; − indicates an incompatible, hypersensitive response; ± indicates an intermediate reaction, usually involving a slowly developing hypersensitive response after initial water soaking.
[b] The "polygenes" of 1-10B and 20-3 are here assumed to be $B_{Sm}$, but see discussion.
[c] A slight hypersensitive response detected in some experiments.

HSp, had a reduced ability to water soak the susceptible cultivar Ac44, in addition to its virulent phenotype on cultivars Ac44-b6 and Ac44-BIn. The apparent reduction in virulence of this strain was seen on all cotton cultivars tested, including a delayed and weakened induction of the HR in incompatible reactions. We tested whether cloned *avrb6* or *avrBIn* might complement this altered phenotype. Plasmids pUFR127 (*avrb6*) and pUFR117 (*avrBIn*) were introduced into the rifampicin-resistant derivative Xcm1103, and transconjugants were inoculated onto Ac44, Ac44-b6, and Ac44-BIn. The transconjugant carrying *avrb6* showed a restored, wild-type water-soaking ability on Ac44 and Ac44-BIn, whereas the *avrBIn* transconjugant retained the reduced virulence phenotype. Both plasmids conferred the expected avirulence activities. This implicates the 5.0-kb *Eco*RI fragment containing *avrb6* as also conditioning water-soaking ability in XcmH.

The natural isolate XcmN also shows slightly reduced water-soaking ability on Ac44 compared to XcmH. The six cloned *avr* genes were each introduced into Xcm1003 and transconjugants inoculated onto cotton Ac44. The transconjugants containing pUFR127 (*avrb6*) and pUFR120 (*avrb7*) both showed slightly increased water-soaking ability compared to the parental strain. Work is being done to determine whether the altered water-soaking phenotypes observed are correlated with altered growth rates of *X. c.* pv. *malvacearum in planta.*

## DISCUSSION

We report here six new avirulence genes isolated from *X. c.* pv. *malvacearum* strain H plasmid DNA. Strain H is avirulent on at least nine cotton cultivars, each thought to carry a different *R* gene, and therefore might be expected to contain at least nine avirulence genes (Gabriel *et al.* 1986). Each of the cloned genes converted virulent strains of *X. c.* pv. *malvacearum* to avirulence in a cultivar-specific manner. By using the widely virulent strain Xcm1003 or mutant strain XcmH derivatives as recipients, the cloned genes *avrB4*, *avrb6*, *avrBIn*, and *avrb7* caused a hypersensitive reaction on cotton hosts with specific single *R* genes (Table 3). These four genes and two other avirulence genes, designated *avrB101* and *avrB102*, caused strong HR reactions of otherwise virulent *X. c.* pv. *malvacearum* strains on cultivar 101-102B. Five of the six genes caused weak HR reactions of otherwise virulent *X. c.* pv. *malvacearum* strains on cultivar Ac44-B2. Genes *avrB101* and *avrB102* could be distinguished by a differential *X. c.* pv. *malvacearum* reaction on cultivar 20-3 (Table 3).

Several potential exceptions to the gene-for-gene pattern were noticed. The first was that the presence of *avrb6* resulted in an HR on all lines known to carry a polygene complex, including Stoneville 2B-S9, Acala 1-10B, 20-3, and 101-102B. The other five *avr* genes reported here did not cause such a reaction. Polygenes are thought to be compound loci that give very weak resistance alone, but which strongly enhance or modify the resistance phenotype of other genes (Brinkerhoff *et al.* 1984; Brinkerhoff 1970; Innes *et al.* 1974). The polygenic complex found in Stoneville 2B lines is designated $B_{Sm}$ (Bird and Hadley 1958), and this same complex is found in 101-102B (Innes *et al.* 1974). Although to our knowledge it is untested that the polygenes present in Acala 1-10B and 20-3 are the same $B_{Sm}$ polygenes found in Stoneville 2B-S9 and 101-102B, such an interpretation is consistent with our results. The data are most easily explained if polygenes ($B_{Sm}$) in all four lines are assumed to either include $b_6b_6$, or be synonymous with $b_6$. Indeed, the $b_6$ gene was first reported to be a strong enhancer-modifier that conferred no detectable resistance when alone (Brinkerhoff 1970). In the absence of specific combinations of races or cloned genes, such as *avrbb*, recessive or weak resistance loci are difficult to evaluate genetically. To our knowledge loci are difficult to evaluate genetically. To our knowledge the $B_{Sm}$ locus has not been tested for homology with the $b_6$ locus.

Two other observations are more difficult to explain. First, all six *avr* genes caused strong incompatibility in otherwise compatible strains on cultivar 101-102B. This cultivar contains the blight resistance genes $B_2$ and $B_3$ in addition to $B_{Sm}$ (Brinkerhoff 1970; Innes *et al.* 1974), and it was expected to react with *X. c.* pv. *malvacearum* carrying any one of several *avr* genes, including *avrb6* (the latter because the $B_{Sm}$ complex appeared from this work to contain $b_6b_6$). However, 101-102B was not expected to react with *X. c.* pv. *malvacearum* strains carrying any of the *avr* genes, particularly those strains carrying *avrB4*, *avrb7*, or *avrBIn*, which exhibited gene-for-gene reactions on plants with $B_4$, $b_7b_7$, or $B_{In}$. The second observation was that the Ac44-B2 line, which is thought to carry only resistance gene *B2*, also reacted with otherwise virulent *X. c.* pv. *malvacearum* strains carrying *avrb6*, *avrb7*, *avrB4*, *avrB101,* or *avrB102*. The reactions were quantitatively different in intensity of the HR (Ac44-B2 was relatively quite weak compared with 101-102B), and therefore it becomes difficult to argue that the same five *R* genes, two of which are recessive, are all together in two different plant lines, and cause entirely different reactions. If the hypothesis is that $B_4$, $b_6b_6$ and $b_7b_7$ are all present in cultivar Ac44-B2, it is difficult to explain why this cultivar reacts only weakly with *X. c.* pv. *malvacearum* strains carrying *avrB4*, *avrb6*, or *avrb7*, whereas these same strains elicit such strong reactions in cultivars Ac-B4, Ac-b6, and Ac-b7. Most cotton blight *R* genes are additive when combined (Brinkerhoff 1970). It is unlikely that the two recessive genes plus three dominant genes are present in one background by accidental outcrossing.

One simple explanation is that cultivars with certain *R* genes, such as cultivars Ac44-B2 and 101-102B, react with *X. c.* pv. *malvacearum* strains carrying any one of several *avr* genes, in violation of Flor's gene-for-gene (Flor 1946; Flor 1947) hypothesis. The $B_2$ resistance gene is a good candidate example for this for several reasons. First, Ac44-B2, thought to contain only one *R* gene ($B_2$), reacted with *X. c.* pv. *malvacearum* strains carrying all but one of the *avr* genes here reported. Second, $B_2$ is the only cotton blight *R* gene tested that could not be used to isolate virulent race-change mutants (Brinkerhoff *et al.* 1978). Finally, the $B_2$ gene is considered to be an essential part of the "immunity" phenotype of cultivar 101-102B, effective against all known North American strains (but not some African strains, such as XcmN) of *X. c.* pv. *malvacearum* (Brinkerhoff *et al.* 1984). We hypothesize that the broad-

spectrum, long-lasting resistance of immune cotton lines is due to the presence of one or more *R* genes, such as $B_2$, that recognizes, in a genetic sense, multiple *avr* genes. In the presence of an intensifier gene such as $B_{Sm}$, the otherwise weak, but evidently broad-spectrum, resistance of $B_2$ may become strong and broad-spectrum. If gene-for-genes (plural) recognition is correct, it would explain why some *R* genes are useful in field situations longer than others—not on the basis of qualitative differences (e.g., the horizontal resistance concept; refer to Ellingboe 1981; Ellingboe 1975), but rather on a statistical basis. If a pathogen carried five *avr* genes, all of which cause the pathogen to induce an HR on plants carrying only one *R* gene, the probability of selecting virulence mutations in all five genes simultaneously is low. Segregation tests of the cotton lines are in progress to test this gene-for-genes hypothesis.

XcmH contains a single plasmid of 90.4 kb, designated pXcmH. Restriction enzyme digests and DNA hybridization analyses were used to localize the six *avr* genes isolated in this study on pXcmH. Additional subcloning experiments and transposon insertion mutagenesis have further localized and defined the six *avr* genes from pXcmH (R. De Feyter and D. Gabriel, in preparation). The presence of plasmid-borne avirulence genes in other *Xanthomonas* and *Pseudomonas* strains has been noted (Swanson *et al.* 1988; Bonas *et al.* 1989; Minsavage *et al.* 1990), although other avirulence genes appear to be chromosomally borne (Gabriel *et al.* 1986; Minsavage *et al.* 1990). This is the first case reported, however, where several avirulence genes appear to be closely linked. The significance of such clustering is unclear. It is conceivable the avirulence genes were introduced together into XcmH on a transmissible plasmid.

The role of plasmids in the pathogenicity of *X. c.* pv. *malvacearum* has been studied (Lazo and Gabriel 1987). A collection of 32 *X. c.* pv. *malvacearum* strains isolated from cotton in either the United States or West Africa were examined for plasmid content, and all were found to contain one or two plasmids. In that study, plasmid DNAs from all but one of the 32 strains (including XcmN and XcmH) strongly cross-hybridized with one another in Southern analyses, indicating extensive homology. We have never observed a plasmidless and pathogenic *X. c.* pv. *malvacearum* strain, nor have we been successful at curing XcmH or derivatives of its plasmid (unpublished results), suggesting that some essential function(s) are encoded by the plasmid(s) of *X. c.* pv. *malvacearum*. The strains from the United States all contained one plasmid in the size range 80–120 kb, while strains from Africa had one or two plasmids in the size range 30–55 kb, but none in the larger size range. Chromosomal DNA from strains of the two sources are also closely related, as shown by the almost identical hybridization patterns found in Southern hybridization experiments with several chromosomally derived probes (Gabriel, unpublished results). Nevertheless, XcmH (U.S. origin) has at least six avirulence genes, while XcmN (probable African origin) is virulent on all resistant cotton cultivars tested (Table 2). The apparent absence of avirulence genes in XcmN could be explained by deletions of the regions containing avirulence genes from pXcmH, by point mutations at all *avr* gene loci, or by a general suppressor.

Studies in many plant-pathogen systems have shown that inoculation of the resistant plant with an avirulent strain of the pathogen may occasionally give rise to virulent variants of the pathogen, in which the plant apparently acts as the selective agent for the virulent strains (Watson 1970). The rapid appearance of new, virulent races of *X. c.* pv. *malvacearum* on some (but not all) resistant cotton cultivars in the field (Brinkerhoff 1970) is reflected in the easy selection of spontaneous race-change mutants of *X. c.* pv. *malvacearum* on some cotton lines in the laboratory (Sun 1987; McNally 1990; McNally *et al.* 1987). It should be noted that isolation of such mutants *in planta* involves selection for mutations of cultivar-specific avirulence genes, and retention of virulence of the pathogen. Spontaneous race-change mutants of *X. c.* pv. *vesicatoria* on pepper have been isolated at a relatively high frequency (Dahlbeck and Stall 1979); mutations of $avrBs_1$ invariably contained a 1.2-kb insertion element within the gene (Kearney *et al.* 1988). Although a similar sized insertion element has been found in XcmH (McNally 1990), insertion elements did not appear to be involved in the spontaneous mutations of the *avr* genes reported here. Some spontaneous *X. c.* pv. *malvacearum* race-change mutations have been previously associated with size changes of the strain's resident plasmids (Sun 1987). In some of the race-change mutants of *X. c.* pv. *malvacearum* reported here, the plasmid pXcmH appeared to have suffered deletions of up to 7 kb, spanning part or all of the mutated *avr* genes (results not shown).

As a general rule, avirulence genes appear to be dispensable, readily mutable, and do not appear to confer any obvious selective advantage to plant pathogens (Gabriel 1989; Gabriel and Rolfe 1990). There are exceptions in which some of the genes may have pleiotropic function (Kearney and Staskawicz 1990). With regard to this, one of the 66 race-change mutants (Xcm1102) isolated from Ac44-BIn plants was also virulent on Ac44-b6 and was simultaneously delayed and reduced in its water-soaking response on all compatible cotton cultivars, including the susceptible line Ac44. We have not yet determined if this reduced pathogenicity is reflected in a reduced growth of Xcm1102 on cotton. The mutant phenotype was complemented by a 5-kb *Eco*RI fragment containing *avrb6*, raising the possibility that this avirulence gene functions in conditioning virulence in addition to its role in avirulence. This was supported by the observation that cloned DNA fragments carrying either *avrb6* or *avrb7* conferred increased water-soaking to Xcm1003. None of the other four *avr* genes conferred this pleiotropic phenotype.

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