JOURNAL OF BACTERIOLOGY, Dec. 1987, p. 5789–5794
0021-9193/87/125789-06$02.00/0

Vol. 169, No. 12

Molecular Characterization of Cloned Avirulence Genes from Race 0 and Race 1 of *Pseudomonas syringae* pv. *glycinea*

BRIAN STASKAWICZ,[1]* DOUG DAHLBECK,[1] NOEL KEEN,[2] AND CAROLYN NAPOLI[1]†

Department of Plant Pathology, University of California, Berkeley, California 94720,[1] and Department of Plant Pathology, University of California, Riverside, California 92521[2]

Received 20 April 1987/Accepted 4 September 1987

A wide-host-range cosmid cloning vector, pLAFR3, was constructed and used to make cosmid libraries of partially digested *Sau*3A DNA from race 0 and race 1 of *Pseudomonas syringae* pv. *glycinea*. Two avirulence genes, $avrB_0$ and *avrC*, cloned from race 0, elicited the hypersensitivity reaction (HR) on specific cultivars of soybean. Race 4 transconjugants containing $avrB_0$ induced a dark brown necrotic HR within 24 h on the soybean cultivars Harosoy and Norchief, whereas race 4 transconjugants containing *avrC* induced a light brown necrotic HR within 48 h on the soybean cultivars Acme, Peking, Norchief, and Flambeau. An additional avirulence gene, $avrB_1$, cloned from race 1, appeared to be identical to $avrB_0$ from race 0. The $avrB_0$ and *avrC* genes from race 0 were characterized by restriction enzyme mapping, Southern blot analysis, Tn*5* transposon mutagenesis, and site-directed gene replacements. The effects of these three genes on the in planta bacterial growth of race 4 transconjugants have also been examined. The identification and cloning of $avrB_1$ provides genetic evidence for a gene-for-gene interaction in the bacterial blight disease of soybean, as $avrB_1$ from race 1 interacts with the soybean disease resistance locus, *Rpg1*.

Bacterial blight of soybeans, a disease caused by *Pseudomonas syringae* pv. *glycinea*, provides a model system to elucidate the molecular genetic and biochemical events that determine recognition specificity and the subsequent induction of disease resistance in a plant-bacterium interaction (9). The phenotype of disease resistance in this plant-pathogen interaction is typified by the plant defense response known as the hypersensitivity reaction (HR) and can be observed as a rapid necrosis of leaf mesophyll cells near the invading pathogen (11). Past studies (1, 9, 17) have demonstrated that variation for the ability to induce the HR exists in different strains of *P. syringae* pv. *glycinea*. Thus, one strain of *P. syringae* pv. *glycinea* may cause disease (i.e., be virulent) on one cultivar of soybean but may induce a HR (be avirulent) on another cultivar. Natural variants that induce the HR or cause disease on an inoculated set of differential cultivars have been grouped into races (1, 12, 13).

The existence of differential cultivars of soybean and distinct races of *P. syringae* pv. *glycinea* is suggestive of a gene-for-gene interaction (4, 5). In a gene-for-gene interaction, the expression of disease resistance is specifically determined by the genotypes of both the host and the pathogen and is often controlled by a single dominant locus in both interacting partners. The dominant locus in the plant is usually referred to as a resistance gene, and the dominant locus in the pathogen has been termed an avirulence gene. In simple terms, the avirulence gene has been shown to condition or control the ability of a specific race of a pathogen to induce a defense response (HR) only on a particular plant cultivar that contains a specific, and thus corresponding, resistance gene. On the other hand, if a particular race of a pathogen does not contain a specific avirulence gene or if the host has a recessive allele at the corresponding resistance locus, disease resistance does not occur and disease results. Thus, the various combinations of avirulence genes in the pathogen and resistance genes in the plant result in a pattern of race-cultivar specificity that is observed in the bacterial blight disease of soybean. Since these phenotypes are controlled by both of the interacting partners, the terms incompatible reaction (expression of disease resistance) and compatible reaction (expression of disease susceptibility) have been introduced to describe the interaction that results from a plant-pathogen interaction.

Molecular genetic evidence for the existence of a dominant avirulence gene in *P. syringae* pv. *glycinea* has been previously demonstrated by constructing a genomic library of race 6 and identifying a cosmid clone that specifically converted a race 4 transconjugant to the race-specific HR-induction pattern of the wild-type race 6.

The molecular characterization of the avirulence gene from race 6, designated *arvA*, has revealed that it contains an open reading frame of 2,721 base pairs encoding a putative 100-kilodalton protein (17). However, as pointed out by Crute (2), the disease resistance locus in soybean that corresponds to the *P. syringae* pv. *glycinea avrA* gene has not been genetically mapped. The only genetic locus that has been identified is a single dominant locus, *Rpg1*, in the soybean cultivars Harosoy and Norchief, that specifically confers resistance to race 1 (16). The corresponding avirulence gene in race 1 has not been genetically identified, precluding the establishment of genetic proof for a gene-for-gene interaction in the bacterial blight disease of soybean.

Kucharek and Stall (12) recently described a new race of *P. syringae* pv. *glycinea* that appeared in Florida during the 1982 growing season. This race, which has been designated *P. syringae* pv. *glycinea* race 0, is virulent on the cultivar Centennial but is avirulent on other soybean cultivars tested. Since race 0 was avirulent on most soybean cultivars tested, we were interested to determine if it contained one or more avirulence genes that specified incompatibility on the soybean cultivars tested. In this paper, we describe the construction of a new wide-host-range cosmid cloning vector and demonstrate its utility in identifying and cloning two avirulence genes from race 0 and a single avirulence gene

* Corresponding author.
† Present address: Advanced Genetic Sciences, Oakland, CA 94608.

from the previously described race 1. We have analyzed these avirulence genes by restriction enzyme mapping, deletion analysis, Tn*5* transposon mutagenesis, site-directed gene replacements, and Southern blot analysis. Subclones of these avirulence genes have also been biologically characterized by studying their effects on the timing and intensity of host-cell necrosis during the induction of the HR and their effect on bacterial growth in planta. The identification and cloning of the avirulence gene from race 1 provides formal genetic evidence for a gene-for-gene relationship in the soybean-*P. syringae* pv. *glycinea* interaction.

(A preliminary report of these findings has appeared as an extended abstract [20]).

MATERIALS AND METHODS

Bacterial strains and media. The bacterial strains used and their relevant characteristics are shown in Table 1. Rifampin-resistant strains were cultured on King medium B (10) containing 100 μg of rifampin per ml. *Escherichia coli* strains were routinely subcultured on LB medium (15). Antibiotics incorporated into the above media were kanamycin, 25 μg/ml; tetracycline, 20 μg/ml; and ampicillin, 50 μg/ml. Antibiotics were purchased from Sigma Chemical Co., St. Louis, Mo.

Growth of plants, inoculation of bacteria into plants, and bacterial growth curves. The growth of soybean plants and inoculation techniques were as previously described (19). In planta growth of race 4 transconjugants, containing either $avrB_0$, $avrB_1$, or *avrC*, was performed as previously described (13, 19).

Recombinant DNA techniques. Restriction enzyme digestions, subcloning procedures, plasmid extractions, Southern blot analysis, and colony hybridizations were carried out as described by Maniatis et al. (14).

Hybridizations were performed in a solution of 6× SSPE, 0.01 M EDTA, 5× Denhardt solution, 0.5% sodium dodecyl sulfate, 100 μg of denatured salmon sperm DNA per ml, and 10^6 cpm of ^{32}P-labeled probe at 65°C for 16 h with gentle rocking (14). Probed filters were washed at room temperature in 2× SSPE–0.5% sodium dodecyl sulfate for 5 min, in 2× SSPE for 5 min at room temperature, and in 0.1× SSPE–0.5% sodium dodecyl sulfate for 2.5 h at 65°C. Filters were dried, and autoradiography was carried out at −80°C with Kodak XAR-5 film and Du Pont Cronex Lightning-Plus intensifying screens.

Construction of the wide-host-range cosmid pLAFR3. pLAFR1 (6) DNA was digested with the restriction enzyme *Eco*RI and treated with 20 U of S1 nuclease at 37°C for 20 min according to Maniatis et al. (14). The plasmid pUC8 was digested with the restriction enzyme *Hae*II. The resulting 454-base-pair *Hae*II fragment, containing the multiple restriction enzyme cloning sites and α-complementation for β-galactosidase activity, was preparatively isolated from an agarose gel by electroelution and treated with S1 nuclease. The *Eco*RI-digested, S1 nuclease-treated pLAFR1 plasmid DNA was ligated with the electroeluted, S1 nuclease-treated *Hae*II fragment from pUC8 and subsequently transformed into *E. coli* HB101, selecting for Tc^r cells. Plasmid DNA was isolated by a rapid alkaline lysis plasmid extraction method (8) from several hundred transformants grown up as a mix and used to transform *E. coli* JM83 plated on medium containing tetracycline and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Of 80 transformants, 1 was Tc^r and was blue on X-Gal-containing medium. The orientation of the *Hae*II fragment and the restriction enzyme sites of pLAFR3 are depicted in Fig. 1.

Construction of genomic libraries of race 0 and race 1. Total genomic DNAs from race 0 and race 1 were isolated as previously described (19). The DNA was partially digested with the enzyme *Sau*3A, treated with bacterial alkaline phosphatase, and size fractionated on a 5 to 20% linear sucrose gradient. Sucrose gradient fractions, enriched for fragments in the 25- to 30-kilobase (kb) range, were diluted five times and ethanol precipitated. This served as the insert DNA (Fig. 1). The strategy used for cosmid cloning was a modification of the method of Ish-Horowicz and Burke (8) (Fig. 1).

Plasmid mobilizations and constructions. Cosmid clones or subclones contained in pLAFR3 or pWB5A were mobilized from *E. coli* strains to various races of *P. syringae* pv. *glycinea* by triparental matings using the plasmid pRK2013

TABLE 1. Bacterial strains, transposons, and plasmids

Strain, phage, or plasmid	Relevant characteristics	Source or reference
P. syringae pv. *glycinea*		
Race 0	Rif^r	R. E. Stall
Race 1	Rif^r	N. Keen
Race 4	Rif^r	N. Keen
E. coli		
HB101	F$^-$ *hsdS20* (r_B^- m_B^-) *recA13 ara-14 proA2 lacY1 galK2 rpsL20* (Str^r) *xyl-5 mtl-1 supE44* λ$^-$ *thi leu*	N. Panopoulos
DHα	F$^-$ *endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA96 relA1* φ80d*lacZM15*	Bethesda Research Laboratories, Inc. (Gaithersburg, Md.)
DH1	F$^-$ *recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+ *supE44* λ$^-$	S. Long
JM83	*ara* (*lac proAB*) *rpsL* φ80d*lacZM15* (r_K^+ m_K^+)	C. Napoli
Phage		
λ467::*b221 rex*::Tn*5*	*c*I875 *O*am29 *P*am80	R. Riedel
Plasmids		
pLAFR1	pRK290 containing *cos* site	S. Long
pLAFR3	pLAFR1 containing *Hae*II fragment of pUC8	This study
pWB5A	pRK290 containing the *Eco*RI polylinker of πVX	W. Buikema; 14
pRK2013	IncP, Km^r Tra Rk2$^+$ *repRK2 repE1*	3
pLVC18	Tc^r Ap^r pBR322 with *mob bom* region of pRSF1010	G. Warren

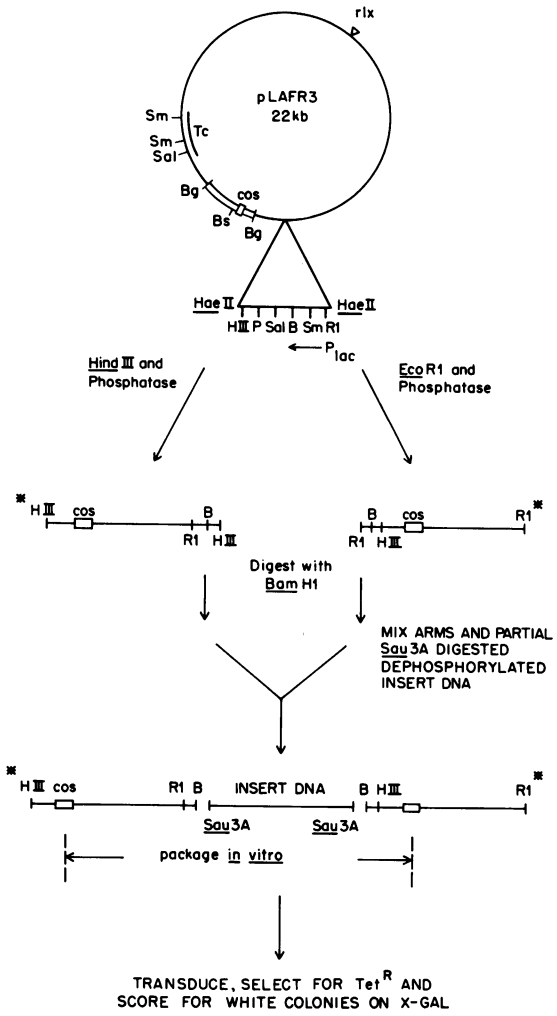

FIG. 1. Strategy used to construct genomic libraries of *P. syringae* pv. *glycinea* in pLAFR3. The circular restriction enzyme map of pLAFR3 is shown with the orientation of the pUC8 454-base-pair *Hae*II fragment relative to the tetracycline resistance locus and *cos* site. Size-fractionated, partially digested *Sau*3A DNA of *P. syringae* pv. *glycinea* was mixed with the individual arms treated with phosphatase, ligated, and packaged in vitro by previously described procedures (8). *E. coli* DH5α or HB101 was transduced with this mixture, and colonies were selected on LB agar plates containing tetracycline and X-Gal. Abbreviations for restriction enzyme sites are as follows: B, *Bam*HI; Bs, *Bst*EII; Bg, *Bgl*II; HIII, *Hin*dIII; P, *Pst*I; Sal, *Sal*I; Sm, *Sma*I; RI, *Eco*RI.

(3). Table 2 describes the plasmid constructions used in this study.

Transposon Tn*5* mutagenesis and site-directed gene replacements. Bacteriophage λ::Tn*5* mutagenesis of *E. coli* HB101, carrying avirulence gene subclones, was carried out according to Ruvkun and Ausubel (18). Tn*5*-mutagenized subclones (pPSG0003::Tn*5* and pPSG0102::Tn*5*) were introduced into the wild-type *P. syringae* pv. *glycinea* race 0 on the nonreplicating vector pLVC18 by pRK2013-assisted mobilization. Transconjugants were selected on King medium B containing kanamycin, tetracycline, and rifampin. The resolution of the *cis*-merodiploids was accomplished by four to five rounds of growth in King B broth containing kanamycin and rifampin. Double-crossover recombinants were identified by replica plating and scoring for Km^r Tc^s colonies. Verification of the gene replacements was accomplished by Southern blot analysis (data not shown).

RESULTS

Phenotypic variation of the HR in soybean cultivars inoculated with race 0. *P. syringae* pv. *glycinea* race 0 was avirulent and elicited a HR on all cultivars of soybeans tested, except cv. Centennial (Table 3). Close visual inspection of the HR phenotypes revealed two distinct classes of HR. The HR observed on the cultivars Harosoy and Norchief appeared in 18 to 24 h and was characterized by a dark brown necrosis (Table 3). On the cultivars Acme, Peking, and Flambeau, the HR developed only after 36 to 48 h and the necrosis was light brown. These observations suggested that race 0 might have more than one avirulence gene. To test this hypothesis, we constructed a genomic library of race 0 and mobilized individual clones to race 4 (virulent on all tested soybean cultivars) and inoculated the differential soybean cultivars to search for avirulence genes that specified the different classes of HR.

Construction of the wide-host-range cosmid cloning vector pLAFR3 and genomic libraries of races 0 and 1. The strategy depicted in Fig. 1 was used to construct partial *Sau*3A DNA genomic libraries of race 0 and race 1 in the cosmid vector pLAFR3. The use of this protocol resulted in efficient library constructions of both races. Greater than 10^5 transductants per μg of insert DNA were obtained. Analysis of random clones revealed the presence of insert DNA that ranged from 25 to 30 kb (data not shown).

Identification and cloning of two race-specific avirulence genes from race 0. Eight hundred independent pLAFR3 cosmid clones, each containing approximately 25 to 30 kb of race 0 DNA, were individually conjugated by triparental matings to race 4. The race 4 transconjugants were inoculated on the differential soybean cultivars (Table 3). A single cosmid clone, pPSG0001, was identified that specifically converted race 4 to avirulence only on the cultivars Harosoy and Norchief. An additional cosmid clone, designated pPSG0100, was identified that converted race 4 to avirulence on the cultivars Acme, Peking, Norchief, and Flambeau (Table 3). Clone pPSG0001 reproducibly induced a rapid, dark HR, whereas clone pPSG0100 induced a slower and lighter HR. These results demonstrated that race 0 contains two separate avirulence genes that differ in their interaction with resistance genes present in the soybean differential hosts. These avirulence genes were designated $avrB_0$ and *avrC*, respectively.

Physical and molecular genetic characterization of $avrB_0$ and *avrC*. To map physical locations for the two avirulence loci, the cosmid clones were digested with the enzyme *Pst*I and the resulting fragments were subcloned in the vector pWB5A. A 2.2-kb *Pst*I fragment from pPSG0001 was subcloned in the vector pWB5A (designated pPSG0002) and conferred the same race specificity and HR phenotype as the original cosmid clone containing $avrB_0$ (Table 3). Likewise, a 2.7-kb *Pst*I subclone from pPSG0100, designated pPSG0101, conferred the same specificity and phenotype of the HR as the parent cosmid containing *avrC* (Table 3). The restriction enzyme maps of $avrB_0$ and *avrC* are shown in Fig. 2a and 3a, respectively.

Tn*5* mutagenesis of both $avrB_0$ and *avrC* was carried out to further characterize these loci. Single Tn*5* insertions in both genes are also shown in Fig. 2a and 3a. The mobilization of these mutagenized subclones to race 4 failed to elicit the HR on any of the differential soybean cultivars tested (Table 3).

TABLE 2. Plasmid constructions

Plasmid	Vector	Insert
pPSG0001	pLAFR3	27-kb partial *Sau*3A insert of race 0 cloned into the *Bam*HI site of pLAFR3
pPSG0002	pWB5A	2.2-kb *Pst*I subclone of pPSG0001 cloned into the *Pst*I site of pWB5A
pPSG0100	pLAFR3	28-kb partial *Sau*3A insert of race 0 cloned into the *Bam*HI site of pLAFR3
pPSG0101	pWB5A	2.7-kb *Pst*I subclone of pPSG0100 cloned into the *Pst*I site of pWB5A
pPSG1000	pLAFR3	28.5-kb partial *Sau*3A insert of race 1 cloned into the *Bam*HI site of pLAFR3
pPSG1001	pWB5A	2.2-kb *Pst*I subclone of pPSG1000 cloned into the *Pst*I site of pWB5A
pPSG0002::Tn*5*	pWB5A	Tn*5*-mutagenized 2.2-kb *Pst*I fragment of pPSG0001 (Fig. 2a)
pPSG0101::Tn*5*	pWB5A	Tn*5*-mutagenized 2.7-kb fragment of pPSG0100 (Fig. 3a)
pPSG0003::Tn*5*	pLVC18	Tn*5*-mutagenized 2.2-kb *Pst*I fragment of pPSG0002
pPSG0102::Tn*5*	pLVC18	Tn*5*-mutagenized 2.7-kb *Pst*I fragment of pPSG0101
pPSG0004	pWB5A	*Cla*I-*Pst*I subclone of pPSG0002 (Fig. 2a)
pPSG0005	pWB5A	*Pst*I-*Eco*RI subclone of pPSG0002 (Fig. 2a)
pPSG0006	pWB5A	*Pst*I-*Hin*dIII subclone of pPSG0002 (Fig. 2a)
pPSG0103	pWB5A	*Pst*I-*Eco*RI subclone of pPSG0101 (Fig. 3a)
pPSG0104	pWB5A	*Eco*RI-*Pst*I subclone of pPSG0101 (Fig. 3a)

Site-directed gene replacements with the Tn*5*-mutagenized subclones in race 0 altered plant reactions upon infection. The elimination of the $avrB_0$ gene from race 0 extended its pathogenicity to the cultivar Harosoy, but it elicited incompatible reactions on the other cultivars (Table 3). However, the incompatible reaction of the cultivar Norchief was altered from type I^B to I^C (Table 3). From this data, we assume that the slowly developing lighter HR was revealed only because the HR controlled by $avrB_0$ is characterized by a more rapid and darker necrosis and masked its appearance.

To determine the relationship of $avrB_0$ and *avrC* to other races of *P. syringae* pv. *glycinea*, we examined homology by fragment hybridization. Preliminary Southern blot experiments using the entire ^{32}P-labeled *Pst*I subclones of pPSG0002 and pPSG0101 revealed multiple hybridizing bands when probed against DNAs of all *P. syringae* pv. *glycinea* races digested with the enzyme *Eco*RI (data not shown). To define which DNA fragments were responsible for the multiple hybridizing bands, individually electroeluted restriction enzyme fragments from the subclones were used as ^{32}P-labeled probes in Southern blot experiments (Fig. 2b and 3b). These hybridizations are gene specific, as *avrB* and *avrC* did not cross-hybridize with each other at the high stringencies used in this study (data not shown). In the case of $avrB_0$, fragment A contained the majority of the multiple hybridizing sequences. Restriction fragments B and C only hybridized to race 0 and race 1, whereas fragment D hybridized strongly to races 0 and 1 and weakly to races 4, 5, and 6 (Fig. 2b). Deletion analysis of the $avrB_0$ clone demonstrated that fragments A and D were not necessary for full avirulence activity, therefore suggesting that $avrB_0$ is present only in races 0 and 1. Similar experiments using individual electroeluted ^{32}P-labeled fragments of *avrC* also revealed multiple hybridizing bands of fragments A through E (Fig. 3b). In the case of *avrC*, there were hybridizing bands present in the other races. Whether these bands represent recessive or nonfunctional alleles is currently unknown.

The hybridizing bands observed with fragments B and C of $avrB_0$ suggested that race 1 also contained *avrB*. In fact, the race specificity of the wild-type race 1 is identical to race 4 transconjugant containing $avrB_0$ (Table 3).

Identification and molecular cloning of $avrB_1$ from race 1. To test whether race 1 contained *avrB*, a cosmid clone was isolated from the race 1 library by colony hybridization using fragment B of $avrB_0$ as a ^{32}P-labeled probe (Fig. 2b). The cosmid clone pPSG1000 was identified from the pLAFR3 race 1 library. The cosmid clone was mobilized from *E. coli* DH5α to race 4, and when it was inoculated on the cultivars

TABLE 3. Compatibility phenotypes of *P. syringae* pv. *glycinea*-soybean interactions

Race	Plasmid	Reaction with soybean cultivar[a]					
		Harosoy	Acme	Peking	Norchief	Flambeau	Centennial
0		I^B	I^C	I^C	I^{BC}	I^C	C
1		I^B	C	C	I^B	C	C
4		C	C	C	C	C	C
4	pPSG0001	I^B	C	C	I^B	C	C
4	pPSG1000	I^B	C	C	I^B	C	C
4	pPSG0002	I^B	C	C	I^B	C	C
4	pPSG1001	I^B	C	C	I^B	C	C
4	pPSG0002::Tn*5*	C	C	C	C	C	C
4	pPSG0100	C	I^C	I^C	I^C	I^C	C
4	pPSG0101	C	I^C	I^C	I^C	I^C	C
4	pPSG0101::Tn*5*	C	C	C	C	C	C
0	*avrB*::Tn*5*	C	I^C	I^C	I^C	I^C	C
0	*avrC*::Tn*5*	I^B	C	C	I^B	C	C

[a] I, Incompatible reaction which results in disease resistance expression by the plant (HR) and avirulence expression by the pathogen; C, compatible reaction which results in disease susceptibility in the plant and virulence expression by the pathogen; I^B, phenotype of HR is characterized by dark necrosis first appearing after 18 to 24 h; I^C, phenotype of HR is characterized by light necrosis first appearing after 36 to 48 h; I^{BC}, both HR classes can be observed on the cv. Norchief when the race 0 *avrB*::Tn*5* mutant is inoculated.

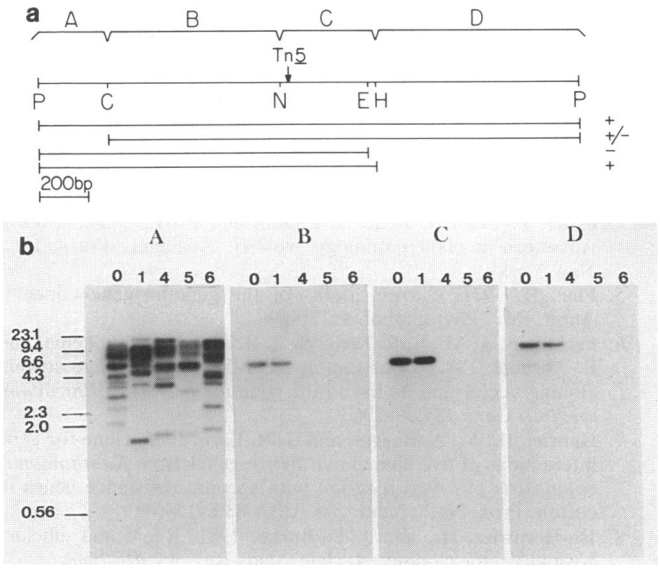

FIG. 2. (a) Restriction enzyme map and deletion analysis of $avrB_0$. The locations of several restriction enzyme sites in the 2.2-kb *Pst*I fragment which contains *avrB* are shown. The location of the Tn*5* insert that inactivates the avirulence gene is depicted by the arrow. The activities of various deletions (bars represent amount of DNA present) are shown by the following: +, full activity; +/−, delayed activity; −, no activity. The abbreviations for restriction enzyme sites were as follows: P, *Pst*I; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; N, *Nru*I. bp, Base pairs. (b) Southern blot analysis of five *P. syringae* pv. *glycinea* races probed with individually electroeluted fragments from $avrB_0$. Total genomic DNA was isolated from races 0, 1, 4, 5, and 6 of *P. syringae* pv. *glycinea* and digested with the restriction endonuclease *Eco*RI. Each lane was loaded with 2 μg of DNA and electrophoresed in 0.7% agarose. The DNA was transferred to nitrocellulose and probed with the individually electroeluted ^{32}P-labeled fragments A, B, C, and D shown in panel a.

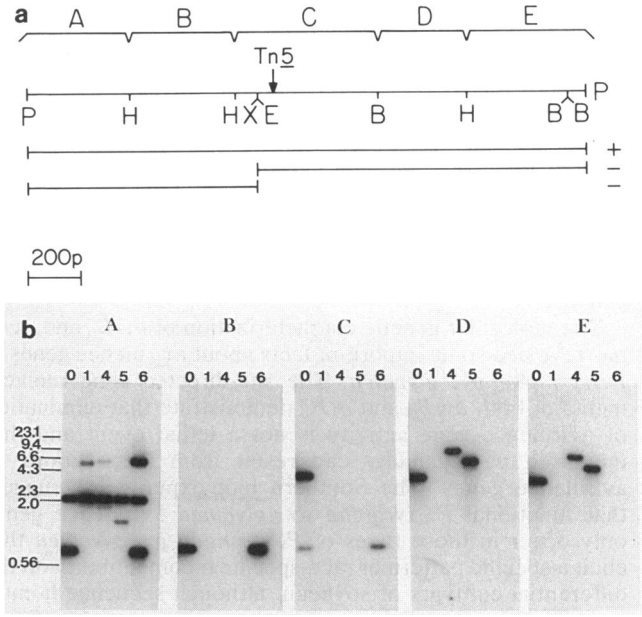

FIG. 3. (a) Restriction enzyme map and deletion analysis of *avrC*. The locations of several restriction enzyme sites in the 2.7-kb *Pst*I fragment which contains *avrC* are shown. Tn*5* insertional inactivation of *avrC* activity is shown by the arrow. Both deletions that were tested resulted in no *avrC* activity (−), whereas the complete fragment had full activity (+). The abbreviations for restriction enzyme sites were as follows: P, *Pst*I; B, *Bam*HI; H, *Hin*dIII; X, *Xba*I; E, *Eco*RI. p, Base pairs. (b) Southern blot analysis of five *P. syringae* pv. *glycinea* races probed with individually electroeluted probes from *avrC*. Total genomic DNAs were isolated from races 0, 1, 4, 5, and 6 of *P. syringae* pv. *glycinea* and digested with the restriction endonuclease *Eco*RI. Each lane was loaded with 2 μg of DNA and electrophoresed in 0.7% agarose. The DNAs were transferred to nitrocellulose and probed with the individually electroeluted ^{32}P-labeled fragments A, B, C, D, and E shown in panel a.

Harosoy and Norchief, a HR resulted which was indistinguishable from that of the race 0 cosmid clone, pPSG0001 (Table 3). Further characterization of this cosmid clone revealed that it contained a *Pst*I fragment that was identical to the $avrB_0$ gene from *P. syringae* pv. *glycinea* race 0. This was determined by cutting both *Pst*I fragments from race 0 and race 1 individually with the restriction enzyme *Sau*3A and observing the restriction fragment pattern on an ethidium bromide-stained 8% polyacrylamide gel. We have designated the plasmid containing this 2.2-kb subclone pPSG1001, and the avirulence gene it contains was designated $avrB_1$.

TABLE 4. Effect of $avrB_0$, $avrB_1$, and *avrC* on the growth of *P. syringae* pv. *glycinea* race 4 inoculated into soybean leaves

Avirulence gene	Plasmid	Cultivar	Plant reaction[a]	Bacterial growth[b] (10^9 CFU/g)
None	None	Harosoy	C	22
$avrB_0$	pPSG0002	Harosoy	I^B	0.09
$avrB_1$	pPSG1001	Harosoy	I^B	0.15
avrC	pPSG0101	Harosoy	C	25
None	None	Acme	C	24
$avrB_0$	pPSG0002	Acme	C	21
$avrB_1$	pPSG1001	Acme	C	23
avrC	pPSG0101	Acme	I^C	4.1

[a] C, Compatible interaction; I^B, incompatible reaction characterized by dark necrosis first appearing after 18 to 24 h; I^C, incompatible reaction characterized by light necrosis first appearing after 36 to 38 h.

[b] Bacterial growth was determined 48 h postinfiltration as described previously (19).

Bacterial growth curves of race 4 containing $avrB_0$, $avrB_1$, and *avrC*. The mobilization of each of the three avirulence gene subclones into race 4 and their subsequent effect on bacterial populations in inoculated soybean leaves are shown in Table 4. Both $avrB_0$ and $avrB_1$ specified incompatible reactions on the cultivar Harosoy and reduced the growth of race 4 carrying these genes by 2 orders of magnitude after 48 h. On the other hand, *avrC* reduced the growth of race 4 by 1 order of magnitude when inoculated on the cultivar Acme after 48 h. Moreover, the two different classes of HR specified by $avrB_0$, $avrB_1$, and *avrC* were correlated with different bacterial population sizes within the inoculated tissue. The rapid and darker necrosis of the HR specified by both $avrB_0$ and $avrB_1$ was always accompanied by significantly smaller bacterial populations than the slower and lighter HR specified by the *avrC* gene.

DISCUSSION

We have used pLAFR3 to construct genomic libraries of *P. syringae* pv. *glycinea* and have shown that race 0 contains two separate avirulence genes, $avrB_0$ and *avrC*, which determine race-specific incompatibility on different cultivars

of soybean (Table 3). Significantly, $avrB_0$ and *avrC* not only specify incompatibility in a qualitative race-specific manner, but they also quantitatively control the phenotype of the HR by affecting the temporal induction and intensity of browning and necrosis which occur during the plant defense reaction. A similar result was observed by Gabriel et al. (7), as they demonstrated that different avirulence gene-resistance gene pairs specified unique phenotypes of the HR. Furthermore, the phenotypes specified by both $avrB_0$ and $avrB_1$ resulted in significantly lower in planta multiplication of race 4 transconjugants than that of race 4 containing the cloned *avrC* gene.

The molecular genetic characterization of $avrB_0$ and *avrC* has revealed some important facts about avirulence genes in *P. syringae* pv. *glycinea*. The site-directed gene replacements of both $avrB_0$ and *avrC* demonstrate that elimination of avirulence gene activity is not a lethal event and that increased pathogenicity can result from inactivations of avirulence genes. The Southern blot experiments suggest that functional *P. syringae* pv. *glycinea* avirulence genes only occur in those races of *P. syringae* pv. *glycinea* that elicit a specific pattern of race-specific incompatibility on the differential cultivars of soybean, although sequence homology of *avrC* with race 6 suggests a recessive or nonfunctional allele. This result differs from the experiments of Gabriel et al. (7), as they suggest that recessive alleles for avirulence occur in *Xanthomonas campestris* pv. *malvacearum*. The significance of the multiple hybridizing sequences that flank $avrB_0$ and *avrC* is currently unknown.

The identification and cloning of $avrB_1$ from race 1 demonstrated that race 1 contains an avirulence gene that is indistinguishable from the $avrB_0$ gene cloned from race 0 by restriction enzyme site analysis. Furthermore, the $avrB_1$ gene from race 1 corresponds to the genetically characterized *Rpg1* resistance locus in the cultivars Norchief and Harosoy, demonstrating that this interaction is a bona fide gene-for-gene relationship.

The identification and cloning of these three avirulence genes, in addition to the previously cloned *avrA* gene from *P. syringae* pv. *glycinea* race 6, allows the design of experiments to elucidate the molecular and biochemical events that determine specificity in the bacterial blight disease of soybean. Experiments are in progress to sequence the $avrB_0$ and *avrC* genes, raise antisera to their respective gene products, and study the expression of these avirulence genes in *P. syringae* pv. *glycinea* cells grown in broth and in planta.

ACKNOWLEDGMENTS

We thank G. Warren for providing the plasmid pLVC18, Sharon Long for providing the plasmids pWB5A and pLAFR1, and Bob Stall for providing the race 0 isolate of *P. syringae* pv. *glycinea*. We also thank Maureen Whalen, Milt Schroth, Thanh Huynh, and Andy Jackson for critical reviews of the manuscript.

This work was supported by U.S. Department of Agriculture grants 84-CRCR-1-1441 and 86-CRCR-1-2086 to Brian J. Staskawicz.

LITERATURE CITED

1. **Cross, J. E., B. W. Kennedy, J. W. Lambert, and R. L. Cooper.** 1966. Pathogenic races of the bacterial blight pathogen of soybeans, *Pseudomonas glycinea*. Plant Dis. Rep. **50**:557–560.
2. **Crute, I. R.** 1986. Investigations of gene-for-gene relationships: the need for genetic analyses of both host and parasite. Plant Pathol. **35**:15–17.
3. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **27**:7347–7351.
4. **Ellingboe, A. E.** 1984. Genetics of host-parasite relations: an essay, p. 131–151. *In* D. S. Ingram and P. H. Williams (ed.), Advances in plant pathology, vol. II. Academic Press, Inc., New York.
5. **Flor, H.** 1971. Current status of the gene-for-gene concept. Annu. Rev. Phytopathol. **9**:275–296.
6. **Freidman, A. M., S. R. Long, S. E. Brown, W. J. Buikema, and F. Ausubel.** 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium meliloti*. Gene **18**:289–296.
7. **Gabriel, D. W., A. Burges, and G. R. Lazo.** 1986. Gene-for-gene interactions of five cloned avirulence genes from *Xanthomonas campestris* pv. *malvacearum* with specific resistance genes in cotton. Proc. Natl. Acad. Sci. USA **83**:6415–6419.
8. **Ish-Horowicz, D., and J. F. Burke.** 1981. Rapid and efficient cosmid vector cloning. Nucleic Acids Res. **9**:2989–2998.
9. **Keen, N. T., and M. J. Holiday.** 1982. Recognition of bacterial pathogens by plants, p. 179–217. *In* M. S. Mount and G. H. Lacey (ed.), Phytopathogenic procaryotes, vol. II. Academic Press, Inc., New York.
10. **King, E. O., M. K. Ward, and D. E. Raney.** 1954. Two simple media for the demonstration of phycocyanin and fluorescein. J. Lab. Clin. Med. **44**:301–307.
11. **Klement, Z.** 1982. Hypersensitivity, p. 149–177. *In* M. S. Mount and G. H. Lacy (ed.), Phytopathogenic procaryotes, vol. II. Academic Press, Inc., New York.
12. **Kucharek, T., and R. E. Stall.** 1985. A bacterial leafspot disease of soybean caused by a new race of *Pseudomonas syringae* pv. *glycinea*. Proc. Soil Crop Sci. Soc. Fla. **44**:174–177.
13. **Long, M., P. Barton-Willis, B. J. Staskawicz, D. Dahlbeck, and N. T. Keen.** 1985. Further studies on the relationship between glyceollin accumulation and the resistance of soybean leaves to *Pseudomonas syringae* pv. *glycinea*. Phytopathology **75**: 235–239.
14. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
16. **Mukherjee, D., J. W. Lambert, R. L. Cooper, and B. W. Kennedy.** 1966. Inheritance of resistance to bacterial blight in soybeans. Crop Sci. **6**:324–326.
17. **Napoli, C., and B. Staskawicz.** 1987. Molecular characterization and nucleic acid sequence of an avirulence gene from race 6 of *Pseudomonas syringae* pv. *glycinea*. J. Bacteriol. **169**:572–578.
18. **Ruvkun, G. B., and F. Ausubel.** 1981. A general method for site-directed mutagenesis in prokaryotes. Nature (London) **289**:85–89.
19. **Staskawicz, B. J., D. Dahlbeck, and N. T. Keen.** 1984. Cloned avirulence gene of *Pseudomonas syringae* pv. *glycinea* determines race-specific incompatibility on *Glycine max*. Proc. Natl. Acad. Sci. USA **81**:6024–6028.
20. **Staskawicz, B. J., D. Dahlbeck, and C. Napoli.** 1986. Molecular genetics of race-specific avirulence in *Pseudomonas syringae* pv. *glycinea*, p. 109–114. *In* I. Sussex, A. Ellingboe, M. Crouch, and R. Malmberg (ed.), Current communication in molecular biology: plant cell/cell interactions. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.