Molecular Analysis of Avirulence Gene *avrRpt2* and Identification of a Putative Regulatory Sequence Common to All Known *Pseudomonas syringae* Avirulence Genes

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Received 3 March 1993/Accepted 13 May 1993

The *avrRpt2* locus from *Pseudomonas syringae* pv. *tomato* causes virulent strains of *P. syringae* to be avirulent on some, but not all, lines of *Arabidopsis thaliana* and *Glycine max* (soybean). We determined the DNA sequence of the *avrRpt2* locus and identified the *avrRpt2* gene as a 768-bp open reading frame encoding a putative 28.2-kDa protein. Deletion analysis and transcription studies provided further evidence that this open reading frame encodes AvrRpt2. We found that the *avrRpt2* gene also has avirulence activity in *P. syringae* pathogens of *Phaseolus vulgaris* (common bean), suggesting that disease resistance genes specific to *avrRpt2* are functionally conserved among diverse plant species. The predicted AvrRpt2 protein is hydrophilic and contains no obvious membrane-spanning domains or export signal sequences, and there was no significant similarity of AvrRpt2 to sequences in the GenBank, EMBL, or Swiss PIR data bases. A comparison of the *avrRpt2* DNA sequence to nine other *P. syringae* avirulence genes revealed a highly conserved sequence, GGAACCNA-N₁₄-CCACNNA, upstream of the translation initiation codon. This motif is located 6 to 8 nucleotides upstream of the transcription start site in all four *P. syringae* avirulence genes for which a transcription start site has been determined, suggesting a role as a binding site for a novel form of RNA polymerase. Regulation of *avrRpt2* was similar to other *P. syringae* avirulence genes; expression was high in minimal medium and low in rich medium and depended on the *hrpRS* locus and an additional locus at the opposite end of the *hrp* cluster of *P. syringae* pv. *tomato*.

The interaction of plants and pathogens is often characterized by a gene-for-gene relationship (15). In such interactions, resistance of a plant to a given pathogen is dependent on a specific resistance gene in the host and a corresponding dominant avirulence gene in the pathogen. Loss of either member of this gene pair results in loss of resistance. Avirulence genes thus limit the host range of plant pathogens. The mechanism of this interaction is not understood. Several bacterial avirulence genes have been cloned and characterized (24, 25), but no plant resistance gene with avirulence gene specificity has been isolated.

Gene-for-gene resistance is usually manifested phenotypically as a rapid necrosis of the plant tissue localized to the site of pathogen ingress. This rapid response to infection is referred to as the hypersensitive response (HR). The mechanism of HR production is poorly understood, but it is always associated with cessation of pathogen growth (27).

Induction of the plant HR by avirulent bacterial pathogens requires a second set of bacterial genes in addition to avirulence genes. These genes have been designated *hrp* genes, because mutations in these genes prevent bacterial pathogens from inducing a hypersensitive response in resistant plants and they also abolish pathogenicity on susceptible plants (35). Such *hrp* genes have been identified in a wide variety of plant pathogens and typically are organized in a cluster containing 9 to 11 complementation groups spanning 20 to 30 kb of DNA (58). Similarity between specific *hrp* genes and virulence genes of animal pathogens has been reported (12, 16, 20). The sequence similarities suggest that these *hrp* genes may be part of a protein secretion system,

which has led to the proposal that *avr* gene products may be secreted via a *hrp* gene mediated mechanism (12, 16).

At least two *hrp* loci are known to be required for transcription of the avirulence genes *avrB* and *avrPto* from *Pseudomonas syringae* (21, 47), which indicates a regulatory interaction between some *hrp* and *avr* genes. The functional significance of these interactions for either *hrp* gene function or *avr* gene function has remained enigmatic.

We and others are now attempting to molecularly clone and analyze a matched resistance gene-avirulence gene pair. To facilitate cloning of plant resistance genes, we are pursuing this goal in the plant *Arabidopsis thaliana* (56). *A. thaliana* is a host for several bacterial, viral, and fungal pathogens (5, 6). The bacterial pathogen *P. syringae* pv. *tomato* strain DC3000 is virulent on most ecotypes of *A. thaliana* (56). We previously described the isolation of a 1.5-kb piece of DNA from *P. syringae* pv. *tomato* strain JL1065 that converted strain DC3000 to avirulence on *A. thaliana* ecotype Col-0 (56). This locus was designated *avrRpt2*. Here we present the sequence of *avrRpt2* and analyze *avrRpt2* gene regulation. Comparison of the 5' region of *avrRpt2* to other *P. syringae* *avr* genes revealed a striking sequence conservation that we propose may be a consensus avirulence gene promoter.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are described in Table 1. Derivatives of *P. syringae* pv. *tomato* strain DC3000 containing transposon insertions in the *hrp* region were constructed by C. Boucher, D. Dahlbeck, and B. J. Staskawicz and will be reported elsewhere. *P. syringae* strains were

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics ^a	Source or reference
<i>E. coli</i>		
DH5- α	F ⁻ <i>lacZ</i> Δ M15 <i>endA1 recA1 hsdR17 supE44 thi-1 gyrA relA1</i> λ ⁻	Bethesda Research Laboratories
TG-1	<i>supE hsd</i> Δ 5 <i>thi</i> Δ (<i>lac-proAB</i>) F'(<i>traD36 proAB⁺ lacI^f lacZ</i> Δ M15]	48
<i>P. syringae</i> pv.		
<i>glycinea</i> race 0	Rif ^r	R. E. Stall (52)
<i>glycinea</i> race 4	Rif ^r	N. Keen (28)
<i>maculicola</i> 4326	Rif ^r	ICMP ^b
<i>phaseolicola</i> NPS3121	Rif ^r	N. J. Panopoulos (35)
<i>tomato</i>		
Bmg13	Rif ^r	D. Cuppels
DC3000	Rif ^r	D. Cuppels
JL1065	Rif ^r	J. Lindeman
PT-7	Rif ^r	M. Schroth
PT-23		N. Keen (2)
T1	Rif ^r	D. Cuppels
3435		ICMP ^b
3455		ICMP ^b
Phage (M13K07)	Helper phage	55
Plasmids		
pLAFR3	Tc ^r , broad-host-range vector	52
pLAFR6	Tc ^r , broad-host-range vector with transcriptional terminators	4a
pRK2013	Km ^r , Tra ⁺ , helper plasmid	13
pUC119	Ap ^r , cloning and sequencing vector	55
pBluescript-SK ⁺	Ap ^r , used for riboprobe generation	Stratagene (La Jolla, CA)
p4-24	~25-kb fragment containing <i>avrRpt2</i> in pLAFR3	56
pABL18	3.6-kb fragment containing <i>avrRpt2</i> in pLAFR3	56
pABL30	3.7-kb fragment containing <i>avrRpt2</i> in pLAFR3	56
pLH12	1.4-kb <i>Hind</i> III fragment from pABL30 containing <i>avrRpt2</i> in pLAFR3	56
pLH12:: Ω	pLH12 with an Ω fragment inserted in <i>avrRpt2</i>	56
pRSR0	1.4-kb <i>Sac</i> I fragment from pABL30 containing <i>avrRpt2</i> in pUC119	This work
pRSL0	As pRSR0, but inserted in opposite orientation	This work

^a Rif, rifampin; Tc, tetracycline; Km, kanamycin; Ap, ampicillin. ^r indicates resistance.

^b ICMP, International Collection of Micro-organisms from Plants, Auckland, New Zealand.

routinely cultured at 30°C on King's medium B (KB) (26). For studies on *avrRpt2* expression, we used a minimal medium (MM; 13 mM potassium phosphate, 17 mM sodium chloride, 30 mM ammonium sulfate, 2.8 mM magnesium sulfate, pH 5.5) supplemented with either citrate (10 mM), fructose (10 mM), or both (10 mM each) as the carbon source. *Escherichia coli* strains were grown at 37°C on Luria-Bertani medium (37). The plasmid pRK2013 (Table 1) was used in triparental matings (13) to mobilize broad-host-range vectors from *E. coli* into *P. syringae*. Matings were conducted on nutrient yeast glycerol agar (8). Antibiotics (Sigma) were used for selection at the following concentrations (milligrams per liter): ampicillin, 100; tetracycline, 16 to 20; rifampin, 100; spectinomycin, 40; streptomycin, 30; cyclohexamide, 50.

Analysis of *Pseudomonas* plasmid profiles. The identity of *P. syringae* strains was periodically confirmed by checking the number and sizes of native plasmids. Plasmids were analyzed by a modification of the in-gel lysis procedure of Eckhardt (10). *P. syringae* cells were scraped off a fresh overnight plate and resuspended in 1 mM MgCl₂. Approximately 6 × 10⁷ CFU were pelleted in a microcentrifuge tube and resuspended in 20 μ l of freshly made lysis buffer (1× TBE electrophoresis buffer [1], 10% [wt/vol] sucrose, 100 μ g of lysozyme per ml, 10 μ g of RNase per ml). The cell suspension was immediately loaded into the well of a horizontal 1× TBE–0.5% sodium dodecyl sulfate (SDS)–0.7%

agarose gel. Lysis was initiated by electrophoresing at approximately 0.3 V/cm for 15 min. Plasmids were then separated by electrophoresis at 5 V/cm for 2 h. Sizes of *P. syringae* plasmids were estimated by comparison to the plasmid pLAFR3, which forms a ladder of multimers (monomer, dimer, trimer, tetramer, etc.) when isolated from *P. syringae* by the above procedure. To determine whether *avrRpt2* was located on a plasmid, an Eckhardt gel was blotted onto a nylon membrane as described below and hybridized with a ³²P-labeled DNA probe.

Plant growth and inoculation procedures. *A. thaliana* ecotype Col-0 plants were grown from seed in growth chambers under an 8-h photoperiod at 24°C as described previously (56). Bean (*Phaseolus vulgaris*) cultivar Bush Blue Lake and soybean (*Glycine max*) cultivar Centennial were grown from seed in greenhouses and transferred 1 day before inoculation to a growth chamber with a 12-h photoperiod at 24°C.

Plant leaves were infiltrated with *P. syringae* strains with a plastic transfer pipette as described previously (53). Two cell concentrations were used for *A. thaliana* inoculations, 1 × 10⁶ and 2 × 10⁷ CFU/ml in 10 mM MgCl₂. For soybean and bean inoculations, 1 × 10⁷- and 2 × 10⁸-CFU/ml suspensions were used. Cell concentrations were estimated by the optical density at 600 nm. An optical density at 600 nm of 0.5 was found to be approximately 5 × 10⁸ CFU/ml. Following inoculation, plants were returned to growth cham-

FIG. 1. (A) Restriction map of *avrRpt2*. The 1.5-kb *SacI* fragment encoding *avrRpt2* was subcloned from pABL30 (56). The *SacI* site at the left end is derived from vector sequences in pABL30. This fragment was cloned in both orientations into pUC119 prior to exonuclease treatments and sequence analysis, to produce clones pRSR0 and pRSL0. The position of the ORF identified by DNA sequence analysis is indicated by the horizontal arrow. The region used as a hybridization probe is also indicated. (B) Localization of *avrRpt2* activity. Clone pLH12 contains a 1.4-kb *HindIII* fragment subcloned into pLAFR3 from pABL30 (56). The Ω fragment was inserted into the indicated *SacI* site in pLH12. The three pL6 clones contain the indicated portions of the 1.5-kb *SacI* fragment in the vector pLAFR6. The number of nucleotides deleted is indicated by Δ and the adjacent number. These clones were tested for avirulence activity on *A. thaliana* ecotype Col-0, soybean cultivar Centennial, and bean cultivar Bush Blue Lake. +, active; -, inactive as defined by induction of an HR.

bers, and disease phenotypes were monitored for 5 days. For inoculations at the lower cell concentration, disease symptoms were scored on day 5. For inoculations at the higher cell concentration, the presence of a visible HR was scored at approximately 24 h after inoculation. Growth of *P. syringae* pv. *tomato* strains within *A. thaliana* was determined as previously described (56).

Recombinant DNA techniques and DNA sequence analysis. Standard techniques were used for plasmid preparations, DNA subcloning, and gel electrophoresis (1). ³²P-labeled DNA probes were prepared by random primer extension with the Multiprime DNA labeling system (Amersham). Southern hybridizations were done with Hybond-N (Amersham) nylon membranes following the manufacturer's recommendations. Posthybridization washes were done at 65°C, with a final wash in 0.1% SDS-0.1× SSPE (1× SSPE is 0.15 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.7).

To determine the sequence of *avrRpt2*, we subcloned a 1.5-kb *SacI* fragment that contained *avrRpt2* activity from pABL30 (56) into pUC119 in both orientations. The resulting plasmids, pRSR0 and pRSL0 (Fig. 1A), were then used in exonuclease III reactions to generate a set of nested deletions from each end of the 1.5-kb *SacI* fragment. The exonuclease III reactions were done with the Erase-a-Base kit from Promega Corp. (Madison, Wis.). The deletion derivatives of pRSR0 and pRSL0 were transformed into *E. coli* TG-1, and single-stranded DNA was generated with the helper bacteriophage M13K07 (55). Dideoxy sequencing of single-stranded DNA templates was done with the Sequenase 2.0 kit from U.S. Biochemical Corp. (Cleveland, Ohio). The entire length of both strands was sequenced. Deletion derivatives with endpoints that flanked the *avrRpt2* open reading frame were subcloned from pUC119 into pLAFR6 and conjugated into *P. syringae* strains to test for avirulence activity (Fig. 1B).

Induction of *avrRpt2* and RNA isolation. *P. syringae* cells were grown in KB broth to an optical density at 600 nm of 0.3 to 0.6, pelleted by centrifugation, and washed in 10 mM MgCl₂. The washed pellet was resuspended in either KB, 10 mM MgCl₂, or MM supplemented with the carbon sources indicated in figure legends. The cells were then shaken at

28°C for 3 h before being pelleted by centrifugation. The cell pellet was resuspended in lysis buffer (50 mM Tris-HCl [pH 9.0], 50 mM EDTA, 300 mM sodium acetate, 0.625% [wt/vol] SDS) and incubated in a boiling water bath for 30 s. The lysate was then extracted two times with 65°C phenol and two times with chloroform before being precipitated with an equal volume of isopropyl alcohol. Visible clumps of DNA were hooked out before the precipitate was pelleted. The pellet was then resuspended in deionized water, and an equal volume of 4 M lithium acetate was added. The RNA was pelleted after a 60-min incubation on ice. The pellet from the lithium acetate precipitation was resuspended in water and precipitated a second time with 4 M lithium acetate. This pellet was resuspended in water, and the nucleic acids were precipitated with ethanol. This final pellet was resuspended in water. RNA concentration and integrity were evaluated by running aliquots on nondenaturing agarose gels.

Analysis of *avrRpt2* transcripts. Relative abundance of *avrRpt2* message in *P. syringae* cells was determined by RNA gel blot analysis. RNA samples of approximately 10 μg were resuspended in loading buffer (8 M urea, 10 mM Tris [pH 8.0], 1 mM EDTA, 0.1% [wt/vol] SDS, 0.25% [wt/vol] bromophenol blue, 0.25% [wt/vol] xylene cyanol) and run on a 4% polyacrylamide-8 M urea gel with 1× TBE electrophoresis buffer (1). Alternatively, RNA samples were run on formaldehyde-agarose gels (48). RNA was transferred from polyacrylamide gels to nylon membranes with an electroblotter and 25 mM NaPO₄ (pH 6.5) as a transfer buffer. Transfer from agarose gels was done by capillary blotting with 10× SSC (1.5 M NaCl plus 150 mM sodium citrate, pH 7.0) as the transfer buffer. RNA was fixed to the membrane by cross-linking with UV light. To normalize for any differences in loading or transfer, we hybridized nylon membranes with a DNA probe homologous to the 16S rRNA genes of *P. syringae* pv. *tomato*. This probe was generated by polymerase chain reaction amplification of the 16S rRNA genes of *P. syringae* pv. *tomato* strain DC3000. The primers used were 5'-GTGCCAGCAGCCGCG-3' and 5'-GGTTACCTTGTTACGACTT-3', which correspond to universally conserved regions of 16S rRNA genes (34). Hybridizations to DNA probes and posthybridization washes were performed

For primer extension analysis, approximately 10 µg of RNA from *P. syringae* pv. *tomato* strain DC3000(p4-24) was annealed in an aqueous hybridization buffer (1.0 M NaCl, 0.167 M HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 7.5], 0.33 mM EDTA) with 10⁵ cpm of a ³²P-end-labeled 30-mer oligonucleotide (5'-GCTGAGCGGG CTGTGATTTATGGCAACTGG-3'). This oligonucleotide is complementary to the coding strand beginning at position 39 of the *avrRpt2* sequence (Fig. 2). Extension reactions were performed according to section 4.8 of Ausubel et al. (1), and products were analyzed on a 6% polyacrylamide-8 M urea gel. To provide a size standard, a ³⁵S-labeled dideoxy sequence ladder was generated with the above oligonucleotide as a primer.

RNase protection assays were performed with the RPAII kit of Ambion (Austin, Tex.). To generate a riboprobe for these protection experiments, we subcloned the insert from deletion clone pRSL8 (endpoint indicated in Fig. 2) as a *SalI*-*SacI* fragment into the vector pBluescript-SK⁺, which contains T3 and T7 phage polymerase promoters that flank the polylinker. The resulting plasmid was linearized at the *SalI* site, and in vitro transcription was done with T3 polymerase and [³²P]UTP, following the protocol provided by the Ambion MAXIScript kit. Full-length transcripts were gel purified from a 5% polyacrylamide-8 M urea gel (1). The resulting transcript had a predicted size of 328 nucleotides. The riboprobe was hybridized to approximately 10 µg of *P. syringae* RNA. RNA samples were treated with RNase-free DNase prior to hybridization with the riboprobe to prevent hybridization of the riboprobe to any contaminating DNA.

FIG. 2—Continued.

Hybridizations and digestion with RNase A/RNase T₁ were performed according to the manufacturer's instructions. Protected fragments were analyzed on a 5% polyacrylamide-8 M urea gel alongside a dideoxy sequencing ladder.

Nucleotide sequence accession number. The nucleotide sequence of *avrRpt2* has been deposited in GenBank under accession number L11355.

RESULTS

Sequence analysis of *avrRpt2*. Previous work by Whalen et al. (56) localized *avrRpt2* activity to a 1.5-kb *SalI* fragment from clone pABL30 (Fig. 1A). To further characterize the *avrRpt2* locus, we determined the nucleotide sequence of the entire 1.5-kb fragment (Fig. 2). An open reading frame (ORF) of 768 bp was identified, and the predicted protein product is indicated in Fig. 2. The ORF begins with two adjacent ATGs. A canonical ribosome binding site (GA GGU) is separated from the first ATG by only 2 bp; thus, the second ATG is located in a preferred position for initiation of protein synthesis (50). A perfect 13-bp inverted repeat that could form a stem-and-loop structure was identified in the 3' sequence flanking the 768-bp ORF (Fig. 2).

Translation of the ORF produces a predicted protein of 255 amino acids with a molecular mass of 28.2 kDa and an

isoelectric point of 7.16. Hydropathy analysis (33) indicated that the predicted protein is very hydrophilic, with no likely membrane-spanning domains. Furthermore, the predicted protein contains no similarity to N-terminal signal peptides or C-terminal hemolysin-like secretion signals (36). A search of the Protein Information Resource (PIR), EMBL, and translated GenBank data bases revealed no proteins with significant sequence similarities.

Deletion analysis of *avrRpt2*. To determine whether the large ORF identified by sequence analysis corresponded to *avrRpt2* activity, we assayed several deletion clones of *avrRpt2* for the ability to convert *P. syringae* pv. *tomato* strain DC3000 to avirulence when assayed on *A. thaliana* ecotype Col-0 (Fig. 1). Exonuclease deletions of *avrRpt2* generated for the sequence analysis were subcloned from pUC119 into the broad-host-range vector pLAFR6 as *HindIII*-*EcoRI* fragments (Fig. 1). The pLAFR6 plasmid contains transcriptional terminators from an Ω insertion mutagenesis element (43) flanking the multiple cloning site of the vector (4a). Thus, transcription from vector sequences into the *avrRpt2* subclones should be minimal. The pLAFR6 clones were conjugated into strain DC3000 and inoculated into *A. thaliana* ecotype Col-0. Inoculations were done at both high (2×10^7 CFU/ml) and low (1×10^6 CFU/ml) bacterial concentrations to allow detection of an HR and

prevention of disease symptoms, respectively. Production of an HR and prevention of disease symptoms typify a resistant response. The phenotypes (resistant or susceptible) of the resulting plant reactions are summarized in Fig. 1. Deletion clone pL6-RSL2, which contains DNA from 171 bp 5' of the large ORF to 138 bp 3' of the ORF (Fig. 2), retained full *avrRpt2* activity, inducing a resistant response on ecotype Col-0. In contrast, deletion clone pL6-RSR1, in which the first 89 bp of the ORF are deleted (Fig. 2), had no *avrRpt2* activity. Likewise, deletion clone pL6-RSL3, in which 72 bp from the 3' end of the ORF are deleted (Fig. 2), also lost all *avrRpt2* activity. These experiments defined a 1-kb region carried on subclone pL6-RSL2 that is required for induction of an HR in *A. thaliana*. As the vector pLAFR6 contains transcription termination signals that flank the polylinker cloning site, the 1-kb fragment present in pRSL2 likely contains regulatory sequences as well as the coding region of *avrRpt2*. Thus, the required 5' elements must be within 170 bp of the putative translation start site (Fig. 2).

The location and size of the ORF shown in Fig. 2 correlate well with the deletion analysis described above. All deletions that had no effect on *avrRpt2* activity mapped outside the designated ORF, while all deletions that abolished *avrRpt2* activity deleted at least part of the ORF (Fig. 1 and 2). An insertional mutation (pLH12::Ω) that disrupts *avrRpt2* function (56) mapped to the middle of the ORF (Fig. 1 and 2). In addition, no other ORFs longer than 165 nucleotides are present in the region defined by the deletion analysis to be necessary for *avrRpt2* function. Thus, the *avrRpt2* locus appears to contain a single gene, which is encoded by the ORF depicted in Fig. 2. This statement is further supported by the size of the *avrRpt2* transcript (850 to 960 nucleotides) and the location of the transcription initiation site (see below). We therefore refer to this ORF as the *avrRpt2* ORF.

Activity of *avrRpt2* on soybean and bean. A 1.5-kb region carrying *avrRpt2* was previously shown to confer avirulence activity to *P. syringae* pv. *glycinea* on specific cultivars of soybean (56). To determine whether the *avrRpt2* ORF is responsible for this activity, the deletion clones described above were also conjugated into *P. syringae* pv. *glycinea* race 4 and tested for avirulence activity on soybean cultivar Centennial. Avirulence activity was scored by assaying for induction of an HR when bacterial suspensions were infiltrated into the leaf mesophyll at high concentrations (approximately 2×10^8 CFU/ml). As described above with *A. thaliana*, only clone pL6-RSL2 conferred avirulence activity, indicating that the *avrRpt2* ORF is responsible for triggering resistant reactions in both soybean and *A. thaliana*.

We also tested *P. vulgaris* (common bean) for the ability to respond to bacteria expressing *avrRpt2*. We chose this species because its interaction with *P. syringae* strains has been extensively studied and genes conferring resistance to specific strains of *P. syringae* pv. *phaseolicola* have been identified (23, 35, 51). The deletion clones described above were conjugated into the bean pathogen *P. syringae* pv. *phaseolicola* strain NP3121 and tested for avirulence activity on bean cultivar Bush Blue Lake. Clone pL6-RSL2, as well as the full 1.5-kb *avrRpt2* clone, but not pL6-RSL3 or pL6-RSR1, conferred the ability to induce an HR on bean. Thus, common bean also responds to the *avrRpt2* ORF.

Distribution of *avrRpt2* among *P. syringae* pathovars. The ability of *avrRpt2* to induce resistant reactions in multiple plant species led us to ask whether *avrRpt2* is present in pathovars of *P. syringae* with different host specificities. We used an internal fragment from the *avrRpt2* ORF as a

hybridization probe (Fig. 1; nucleotides 117 through 653 in Fig. 2) to search for homologous sequences in strains from three other pathovars and in several other strains of pathovar *tomato*. HindIII-digested genomic DNA from the *P. syringae* strains described in Table 1 was hybridized to this *avrRpt2* probe under moderately stringent hybridization conditions (see Materials and Methods). No hybridization was detected in non-*P. syringae* pv. *tomato* pathovars (data not shown). Among *P. syringae* pv. *tomato* strains, only strains JL1065 (the original source of *avrRpt2*), BMG13, and T1 gave detectable hybridization signals, and the hybridizing restriction fragment was the same in all three (approximately 4.1 kb in size; data not shown). The hybridization to strain T1 was of interest because we had previously identified a cosmid, pT1390, from strain T1 that had avirulence activity on *Arabidopsis* sp. (56). Hybridization of the above *avrRpt2* probe to clone pT1390 indicated that this cosmid contains the T1 homolog of *avrRpt2*. Interestingly, when the entire 1.5-kb *Sall* fragment containing *avrRpt2* was used as a hybridization probe, multiple hybridizing fragments were detected in strains 1065, BMG13, and T1 and in several additional strains, including non-*P. syringae* pv. *tomato* pathovars. This result indicates that there are repeated DNA sequences on this fragment conserved among the different pathovars. Hybridization with additional subclones revealed that the repeated sequence is 3' of the *avrRpt2* ORF (data not shown).

Many avirulence genes have been localized to plasmids (39). The relative scarcity of *avrRpt2* among *P. syringae* strains suggested that *avrRpt2* might also reside on a plasmid. We thus attempted to determine whether *avrRpt2* was located on plasmids in strains JL1065, BMG13, and T1. Southern analysis of Eckhardt gels (see Materials and Methods) with the internal *avrRpt2* probe (Fig. 1) showed hybridization only to DNA trapped in the well and faint hybridization to a diffuse band of sheared DNA (data not shown). We determined that the latter hybridization was not due to a plasmid comigrating with the sheared DNA band by increasing the agarose concentration to 0.8%, which affects the relative mobility of linear DNA differently than covalently closed circular DNA. These analyses suggest that *avrRpt2* is located in the chromosome of the above *P. syringae* pv. *tomato* strains, but we cannot rule out the possibility that our gels failed to detect a very large plasmid. The largest plasmid visible was approximately 100 kb in size and was present in all three strains.

Response of *avrRpt2* gene expression to growth conditions. Several *P. syringae* avirulence genes and *hrp* genes have been shown to be inducible by growth in minimal media (21, 45, 47, 59). We therefore tested whether *avrRpt2* could be induced in vitro under similar conditions. We initially tried using minimal medium formulations as given in references 45 and 59, but found that *P. syringae* pv. *tomato* strains JL1065 and DC3000 grew poorly on these media. These strains grew well in the minimal medium described in reference 47, however, and this medium (MM) was used for subsequent analyses. To allow testing of various carbon sources, we did not include sodium citrate in the medium unless indicated (see Materials and Methods). Cells were grown in rich medium (KB; pH 7.0) until the early log phase and then transferred to MM and incubated for 3 h. The 3-h incubation period was chosen because *hrp* genes in *P. syringae* pvs. *syringae* and *phaseolicola* are maximally induced in MM between 3 and 5 h after transfer from KB (45, 59). In addition, longer incubation periods were avoided because these resulted in significant pH increases in MM containing

FIG. 3. Effect of media on *avrRpt2* message abundance in *P. syringae* pv. *tomato* strains. The RNA gel blot was hybridized to a DNA probe containing only internal portions of the *avrRpt2* ORF (Fig. 1; nucleotides 262 through 795 of Fig. 2). Lanes 1 to 6, strain DC3000(p4-24); lane 7, blank; lane 8, strain JL1065. Media were KB (pH 7.0) (lane 1), KB (pH 5.5) (lane 2), MM with no carbon source (lane 3), MM plus fructose (lane 4), MM plus fructose and citrate (lanes 5 and 8), and MM plus citrate (lane 6). RNA was separated on a 4% polyacrylamide-8 M urea gel. Sizes of predominant transcripts (in nucleotides) were estimated by comparison to RNA size standards (Bethesda Research Laboratories 0.16- to 1.77-kb RNA ladder). Equivalent loading and transfer of RNA in each lane was verified by stripping the above blot and hybridizing to a 16S rRNA probe (data not shown).

citrate as a carbon source. Gene expression was monitored by RNA gel blot analysis of total RNA.

The *avrRpt2* message in strain DC3000(p4-24) was strongly induced when this strain was incubated in MM containing a metabolizable carbon source (Fig. 3). Using an internal *avrRpt2* probe (Fig. 1; nucleotides 117 through 653 in Fig. 2), we detected two strongly hybridizing bands of approximately 850 and 960 nucleotides in RNA isolated from cells incubated in MM that contained 10 mM citrate or 10 mM citrate plus 10 mM fructose (Fig. 3, lanes 5 and 6). A faint band of approximately 1.2 kb was also visible in these samples. Incubation in MM containing 10 mM fructose and no citrate resulted in significantly lower accumulation of *avrRpt2* message (Fig. 3, lane 4). Growth rate in this medium was much lower than in MM plus citrate or MM plus fructose and citrate (data not shown). Almost no message was detected in cells incubated in MM that lacked a carbon source, or when cells were incubated in rich medium (KB) at pH 7.0 (Fig. 3, lanes 3 and 1, respectively; bands were visible only after exposing the blot 20 times longer; data not shown). Interestingly, almost no *avrRpt2* message was detected in strain JL1065 under any growth condition [Fig. 3, lane 8 and data not shown; faint bands of the same size as observed in DC3000(p4-24) were visible after a 20-times-longer exposure].

The pH of MM is 5.5; thus, the induction observed in strain DC3000(p4-24) by incubation in MM could be due to pH changes rather than nutrient changes. We therefore tested the effect of transferring cells from KB at pH 7.0 to KB at pH 5.5. Only a small amount of *avrRpt2* message was detected in these cells (Fig. 3, lane 2), indicating that the pH change alone could not account for the observed induction.

Interaction of *avrRpt2* with *hrp* genes. Expression of the *P. syringae* avirulence genes *avrB* and *avrPto* requires function of at least two *hrp* loci (21, 47). We therefore tested whether expression of *avrRpt2* was also dependent on *hrp* genes. Plasmid pABL18, which carries *avrRpt2*, was conjugated into several derivatives of strain DC3000 that contain transposon insertions in various *hrp* genes (2a). These *hrp* mutants have lost the capacity to induce an HR on tobacco plants and to cause disease on tomato and *A. thaliana* plants. We grew these transconjugants overnight in MM containing 1.7 mM citrate and 10 mM fructose and assayed for accumulation of *avrRpt2* transcript. RNA gel blot anal-

FIG. 4. Effect of *hrp* mutations on *avrRpt2* message abundance. Lanes: 1, *P. syringae* pv. *tomato* strain DC3000(pABL18); 2 to 14, DC3000 *hrp* mutants containing pABL18 (2, CB153; 3, CB337; 4, CB59; 5, CB2; 6, CB176; 7, CB23; 8, CB215; 9, CB183; 10, CB200; 11, CB217; 12, CB312; 13, CB319; 14, CB191). Strain CB59 (lane 4) contains a transposon insertion in the *hrp* region, but is *hrp*⁺ in phenotype (2a). All strains were grown in MM plus fructose (pH 7.0) supplemented with 1.7 mM citrate. RNA was separated on a formaldehyde-agarose gel. Cells were harvested in the early stationary phase. The size (in nucleotides) of the predominant transcript is indicated.

ysis revealed that the majority of these strains were unaffected in production of the *avrRpt2* transcript (Fig. 4). However, mutations in the *hrpSR* locus (lanes 2 and 14) abolished production of a detectable *avrRpt2* transcript, as does a mutation at the other end of the *P. syringae* pv. *tomato* *hrp* cluster (lane 3). In *P. syringae* pv. *phaseolicola*, this area contains the *hrpL* locus (44). These same three mutations also block accumulation of message from the *avrPto* gene in strain DC3000 (47).

Identification of transcription start site of *avrRpt2*. The above results demonstrated that regulation of *avrRpt2* is similar to that of other characterized *P. syringae* *avr* and *hrp* genes. We thus wished to identify 5' regulatory sequences that might be shared by these genes. To identify a potential promoter sequence for *avrRpt2*, we first determined the transcription start site. We did this using both primer extension and RNase protection analyses.

We performed the primer extension analysis on RNA that had been isolated from *P. syringae* pv. *tomato* strain DC3000(p4-24) to include extensive 5' sequence and minimize vector effects. Cosmid p4-24 contains an approximately 25-kb insert of the *P. syringae* pv. *tomato* strain JL1065 genome in the vector pLAFR3 (Table 1). Restriction endonuclease analysis indicated that *avrRpt2* was at least 3 kb distant from any vector sequences (data not shown). RNA from strain JL1065 was not used because RNA gel blot analysis (Fig. 2) indicated that this strain contained only small amounts of *avrRpt2* message.

The results of the primer extension analysis are shown in Fig. 5A. RNA from DC3000(p4-24) incubated in MM plus fructose and citrate (lane 1) or MM plus citrate (lane 4) produced a clear primer extension product. The position of the major primer extension product relative to the DNA sequence ladder generated with the same primer indicated that the transcription start site is at position 1 in Fig. 2. No primer extension products of greater size were detected, even after longer exposures, suggesting that the two prominent *avrRpt2* transcripts detected by RNA gel blot analysis (Fig. 3) have the same 5' end. The transcription start site is thus 26 nucleotides upstream of the putative translational start site.

To confirm the results of the primer extension analysis, we used an RNase protection assay. RNase protection analysis offers the advantage that the signal generated does not drop off with distance from a primer. We synthesized a riboprobe complementary to the *avrRpt2* message that contained nucleotides -146 to +157 shown in Fig. 2. The undigested riboprobe (328 nucleotides) migrated at position 316 of a

FIG. 5. Determination of the *avrRpt2* transcription start site. (A) Primer extension analysis. Lanes labeled GATC contain a ^{35}S -labeled sequencing ladder generated with a single-stranded DNA template and the same primer as used for the primer extension reactions. Lanes 1 to 4 contain primer extension products with the following RNA as the template: lane 1, DC3000(p4-24) incubated in MM plus fructose plus citrate; lane 2, DC3000(p4-24) incubated in KB (pH 7.0); lane 3, *S. cerevisiae*; lane 4, DC3000(p4-24) incubated in MM plus citrate; lane 5, blank. Nucleotides listed on the right indicate the DNA sequence complementary to the sequencing ladder and can be read top to bottom as 5' to 3'. The arrow indicates the major primer extension product. (B) RNase protection analysis. Lanes labeled GATC contain a DNA sequencing ladder used to estimate sizes of the protected RNAs. Lanes 1 to 4, RNase protection products produced by the following RNAs: lane 1, *P. syringae* pv. *tomato* strain JL1065 (incubated in MM plus citrate and fructose); lane 2, DC3000(p4-24) (incubated in MM plus citrate plus fructose); lane 3, DC3000(p4-24) (incubated in MM with no carbon source); lane 4, 5 mg of yeast RNA. Lane 5, undigested riboprobe. Sizes in nucleotides are indicated alongside the DNA ladder. Note that the undigested riboprobe (328 nucleotides) migrated through the gel at the same speed as a 316-nucleotide DNA molecule.

DNA sequencing ladder (Fig. 5B, lane 5); thus, we used a correction factor of 1.04 to estimate the size of the protected fragment. A fragment of approximately 148 nucleotides was strongly protected by RNA isolated from DC3000(p4-24) cells incubated in MM plus fructose and citrate (Fig. 5B, lane 2). Fainter bands up to 2 nucleotides larger were also apparent. Assuming that vector sequences present on the riboprobe were removed by the RNase treatment, the 148-nucleotide fragment corresponds to a transcriptional start site at position +9 in Fig. 2. This difference in predicted start sites is small and likely reflects inaccuracies inherent in sizing RNA molecules with a DNA ladder and/or digestion at the ends of RNA-RNA hybrids. The RNase protection also confirmed the results of the primer extension analysis in suggesting that the two prominent transcripts visible in the RNA gel blot have the same 5' end. A weak, but reproducible band of about 302 nucleotides was also observed. A fragment of this size would be protected if there was an RNA species present that extended 5' of the riboprobe. This fragment may indicate that there is a weak transcription start site 5' of the primary start site. This possibility is supported by RNA gel blot analysis, which revealed a low-abundance transcript of approximately 1.2 kb (i.e., 240 nucleotides longer than the largest prominent transcript; Fig. 3, lane 5). No fragments were protected by RNA isolated from *P. syringae* pv. *tomato* strain JL1065, *P. syringae* pv. *tomato* strain DC3000(p4-24) incubated in MM without a carbon source, or *Saccharomyces cerevisiae* (Fig. 5B, lanes 1, 3, and 4, respectively).

Identification of a putative 5' regulatory motif conserved among *P. syringae* avirulence genes. Identification of the transcriptional start site of *avrRpt2* directed our attention to the sequence between -146 and +1 in Fig. 2 to search for potential regulatory sequences. This region was compared with nine other *P. syringae* avirulence genes for which there is sequence information available. We identified a consensus sequence motif, GGAACCNA-N₁₄-CCACNNA, that is highly conserved among all nine of the *P. syringae* avirulence genes (Table 2). The 3'-terminal A of this motif is located 7 nucleotides upstream of the transcriptional start site of *avrRpt2* (Fig. 2). This approximate spacing is also conserved for *avrB*, *avrPto*, and *avrD* (Table 2). The position of this conserved motif relative to transcriptional initi-

TABLE 2. Putative promoter sequences for *P. syringae* avirulence genes

Gene ^a	Putative promoter region	Position relative to transcription start ^b	Position relative to first ATG of ORF ^b
<i>avrRpt2</i>	GGAACCCATTTCATTTTGGAAACCACCAA	-7	-30
<i>avrA</i>	TGAAACCGAAACGGCGTTGCTTGCCACACA	ND ^c	-335
<i>avrB</i>	TGGAACCTAATTGAGGGTAAATGCCACACA	-6 ^d	-104
<i>avrC</i>	TGGAACCGTTCTGCAACTCGT-GCCACTAA	ND	-224
<i>avrD₈</i>	TGGAACCAAATCCGTCCCAAAGGCCACACA	ND	-84
<i>avrD₁</i>	TGGAACCAAATCCGTCCCAAAGGCCACACA	-7; -53 ^e	-94
<i>avrPto</i>	TGGAACCGATCCGCTCCGTATGACCACTCA	-8 ^e	-39
<i>avrPph3</i>	TGGAACCGAATGGGT-CAGCTGGACACTTA	ND	-72
<i>avrPpi2</i>	GGAACCTCATTCTTCT-TTTAAACCACACA	ND	-32
<i>avrRpm1</i>	GGAACCTCATTCTTCT-TTTAAACCACACA	ND	-32
Consensus	tGGAACCNA ^a _{tc} NN ^a _{tc} NN ^a _{tc} NgCCACNca		

^a Complete DNA sequences for the listed genes can be found in the following references: *avrPto* (47), *avrA* (42), *avrB* and *avrC* (54), *avrD₁* (29), *avrD₈* (30), *avrPph3* (23), *avrPpi2* and *avrRpm1* (7). *avrD₁* and *avrD₈* are homologous genes from *P. syringae* pv. *tomato* and *glycinea*, respectively.

^b Numbers refer to position of the 3' A in the putative promoter sequence.

^c ND, not determined.

^d Reference 8a.

^e As reported in reference 49 for *avrD₁* and reference 47 for *avrPto*.

ation suggests that this sequence functions as a promoter for avirulence gene expression.

DISCUSSION

We sequenced the *avrRpt2* locus from *P. syringae* pv. *tomato* strain JL1065 and found that it contains an ORF encoding a putative protein of 28 kDa. This locus can convert *P. syringae* strains from virulence to avirulence on specific varieties of *A. thaliana*, soybean, and bean. The predicted AvrRpt2 protein does not contain recognizable secretion signals or significant hydrophobic regions. Thus, the subcellular localization of AvrRpt2 cannot be predicted. The other nine *P. syringae* avirulence genes that have been sequenced are also hydrophilic, and lack obvious secretion signals (7, 23, 29, 30, 42, 47, 54). It is still possible that these avirulence gene products are being secreted, however, as secretion of proteins without recognizable secretion signals has been reported in *Yersinia* spp. (38).

RNA gel blot analysis of the *avrRpt2* transcript revealed two abundant species, one of 850 nucleotides and one of 960 nucleotides. The primer extension and RNase protection experiments indicate that these species have the same 5' end. The 3' end of the 850-nucleotide fragment would thus be at position 850 in Fig. 2, which places it immediately 3' of a potential stem-loop structure. Such stem-loop structures can act to stabilize messages by protecting against 3' exonucleolytic degradation (41). A nearly identical sequence is also found 3' of an ORF in the *P. aeruginosa* plasmid R100 and Tn501 mercuric resistance operons (3). A potential stem-loop structure is also found just 3' of the *avrPto* ORF (47). The sizes of the *avrRpt2* and *avrPto* transcripts indicate that these genes are monocistronic; thus, the location of these potential stem-loops is consistent with such a role. The 960-nucleotide *avrRpt2* transcript may represent unprocessed transcript or an alternative transcription termination site.

The RNase protection analysis of the *avrRpt2* transcript indicated that there may be a weak promoter upstream of the region we characterized. A faint band corresponding to the full-length riboprobe (minus vector sequences) was reproducibly observed (Fig. 5B). This band was also observed in RNA isolated from strain JL1065 in some experiments (data not shown). The significance of this second transcript is unclear, as it is not required for functional expression of *avrRpt2*; subclone pL6-RSL0 (Fig. 1) lacks the start site of this putative transcript, yet confers a full *avrRpt2* phenotype.

The transcript abundance of *avrRpt2* in strain JL1065, which was the original source of *avrRpt2*, was much lower than in strain DC3000(p4-24). The low level of *avrRpt2* transcript in strain JL1065 may account for the extremely weak HR that this strain produces on *A. thaliana* (9, 56). Strain DC3000(p4-24) induces a much stronger HR. Although the higher level of *avrRpt2* expression in strain DC3000 can be partially attributed to the copy number of the vector, we observed that *avrRpt2* expression was low in strain JL1065, even when *avrRpt2* was carried on a plasmid (pABL18; data not shown).

The sequence upstream of the *avrRpt2* transcription start site revealed a putative promoter that is highly conserved among all 10 of the *P. syringae* avirulence genes sequenced (Table 2). Jenner et al. (23) have previously noted that a portion of this sequence, GGAACC, is common to *avrD*, *avrB*, and *avrPph3*. Subsequently, Salmeron and Staskawicz (47) reported that this sequence is also present in *avrPto*.

This sequence has been designated the harp box, because a similar sequence is present upstream of several *hrp* genes in *P. syringae* (11, 20). Significantly, Salmeron and Staskawicz (47) determined that the transcription start site of *avrPto* is located 32 nucleotides 3' of the harp box. Here we report that a larger consensus sequence can be derived that includes a CCACNNA motif 15 to 16 nucleotides 3' of the harp box. In addition, we determined that the transcription start site of *avrRpt2* is 7 nucleotides 3' of the CCACNNA sequence (31 nucleotides 3' of the harp box), which is consistent with the relative positions of the transcription start sites of *avrPto*, *avrB*, and *avrD* (Table 2). However, Shen and Keen (49) have recently reported that the *avrD* gene has a second transcription start site located 74 nucleotides 3' of the harp box. The significance of this second start site for *avrD* is not clear. Deletion or alteration of the harp box of *avrD* abolished induction in MM, indicating that the harp box plays an important regulatory role, regardless of which start site is used (49). Similarly, Salmeron and Staskawicz (47) found that deletion of the first 2 nucleotides of the harp box (and all other sequences 5' of this position) blocked expression of *avrPto*.

The position of the transcription start site relative to the harp box and the CCACNNA motif in *avrRpt2*, *avrB*, *avrD*, and *avrPto* suggests that these conserved sequences are acting as a recognition site for RNA polymerase. This presumptive promoter is not similar to consensus promoters of *E. coli* genes transcribed by either sigma 70- or sigma 54-associated RNA polymerases (4, 32) nor to various *Pseudomonas putida* promoters (22, 40, 46). Thus, if these conserved sequences are acting as a promoter, a novel regulatory pathway is likely to be involved.

Consistent with the apparent conservation of promoter sequences, we found that *avrRpt2* transcription was regulated in a manner similar to that reported for other *P. syringae* avirulence genes and for *P. syringae* *hrp* genes. Expression of *avrRpt2* required function of the *hrpRS* locus and an additional locus at the opposite end of the *P. syringae* pv. *tomato* *hrp* cluster. Huynh et al. (21) demonstrated that expression of *avrB* in *P. syringae* pv. *glycinea* is also dependent on at least two different *hrp* loci. We used the same *hrp* mutants for our analyses of *hrp* gene dependence as those used by Salmeron and Staskawicz (47) to study *avrPto* expression. Our results are in agreement with their results; the mutations that blocked *avrPto* expression also blocked *avrRpt2* expression. This pattern of regulation has also been observed for several *hrp* loci in *P. syringae* pv. *phaseolicola* (44, 45).

The *hrpS* gene has sequence similarity to the *ntrC* family of transcriptional regulators and includes a putative DNA binding domain (18). This observation is of particular interest because this family of transcriptional regulators is known to interact specifically with sigma 54-type RNA polymerase (32). The putative *avrRpt2* promoter, however, does not contain the canonical sigma 54-type -12/-24 promoter sequence (32), nor does the *avrB* 5' region (54). Several *hrp* genes in *P. syringae* pv. *phaseolicola* require the sigma 54 gene (*ntrA*) for expression (11). Additional work is needed to determine whether transcription of *avrRpt2* is also dependent on sigma 54. It is possible that the requirement for *hrpS* is indirect; *hrpS* could mediate expression of a second transcription factor that in turn regulates expression of *avrRpt2*.

Mutations in most of the *hrp* loci do not affect *avr* gene expression (Fig. 4) (21, 47), yet completely abolish avirulence activity. The proposed role of *hrp* genes in secretion of

elicitor molecules could explain this result (12, 16). Virulence-avirulence factors may not be properly secreted in such *hrp* mutants.

Growth conditions that induce *avrRpt2* expression also induce expression of other *P. syringae* *avr* genes and *hrp* genes. Hybridization of the RNA gel blot shown in Fig. 3 with an *avrPto* probe revealed an expression pattern similar to *avrRpt2*; expression was highest in MM that contained citrate or citrate plus fructose as the carbon sources and was not detectable in cells grown in KB at pH 7.0 (data not shown). Other investigators have also reported that *avr* and *hrp* genes are induced during growth in minimal media (21, 45, 59); however, some minor differences were observed. For example, expression of *avrB* in MM was reported to be induced by carbon sources such as fructose and sucrose and repressed by dicarboxylic acids such as citrate and succinate (21), while the *hrpIII* locus of *P. syringae* pv. *syringae* is induced in MM with succinate as a carbon source (59). While conducting the present experiments, we discovered that *P. syringae* strains make the growth medium alkaline when sodium citrate is used as a carbon source for extended periods. In the experiments described by Huynh et al. (21), the pH values of the various media tested were not equivalent at the time of sampling. The apparent repression by dicarboxylic acids may have been partially or completely due to changes in the pH of the medium. Rahme et al. (45) have recently reported that alkaline pH causes a reduction in *hrp* gene expression in *P. syringae* pv. *phaseolicola*. Taken together, these reports have established that *avr* and *hrp* genes from *P. syringae* are inducible by transferring from rich medium to MM, with optimal expression at pH 5.5 (21, 45, 47, 59, this work). These inductive conditions are similar to the environment encountered by *P. syringae* in the leaf apoplast, which is typically about pH 5.5 (17) and limited in nutrients (19). The regulation of these genes in vitro is thus consistent with their role in plant-pathogen interactions.

Finally, it is worth noting that many, and perhaps most, avirulence genes can be recognized by multiple plant species. The *avrRpt2* gene can convert virulent strains of *P. syringae* to avirulence on *A. thaliana*, soybean, and bean. Such cross-species recognition has been reported for several other avirulence genes from *P. syringae* and *Xanthomonas campestris* (7, 14, 28, 57). Thus, functional specificity of resistance genes appears to be conserved across very diverse species of plants, which suggests that the physical structure of resistance genes may also be conserved. The resistance gene in *A. thaliana* that is specific to *avrRpt2* has been genetically mapped and has been designated *RPS2* (31). Molecular cloning of *RPS2* from *A. thaliana* will allow us to determine whether *RPS2* is physically conserved in other plant species and should provide further insight into how specificity in resistance gene-avirulence gene interactions is controlled.

ACKNOWLEDGMENTS

We thank Noel Keen for sharing results prior to publication.

R.W.I. and A.F.B. were National Science Foundation postdoctoral fellows in plant biology during part of this work. B.N.K. was a D.O.E.-energy biosciences postdoctoral fellow of the Life Sciences Research Foundation. This work was supported by Public Health Service grant R29 GM 46451 from the National Institutes of Health to R.W.I. and by U.S. Department of Energy grant DE-FG03-88ER13917 to B.J.S.

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