Molecular Analysis of Thermoregulation of Phaseolotoxin-Resistant Ornithine Carbamoyltransferase (*argK*) from *Pseudomonas syringae* pv. *phaseolicola*

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The phaseolotoxin-resistant ornithine carbamoyltransferase (ROCT) and phaseolotoxin are produced by *Pseudomonas syringae* pv. *phaseolicola* at 18°C but not at 28°C. At 28°C, the pathogen produces a protein(s) that binds (in vitro) to a 485-bp fragment (thermoregulatory region, TRR) from a heterologous clone from the pathogen genomic library, which in multiple copies overrides thermoregulation of phaseolotoxin production in wild-type cells (K. B. Rowley, D. E. Clements, M. Mandel, T. Humphreys, and S. S. Patil, Mol. Microbiol. 8:625-635, 1993). We report here that DNase I protection analysis of the 485-bp fragment shows that a single site is protected from cleavage by the protein in the 28°C extract and that this site contains two repeats of a core motif G/C AAAG separated by a 5-bp spacer. Partially purified binding protein forms specific complexes with a synthetic oligonucleotide containing four tandem repeats of this motif. A 492-bp upstream fragment from *argK* encoding ROCT also forms specific complexes with the protein in the 28°C crude extract, and a 260-bp subfragment from the TRR containing the binding site cross competes with the *argK* fragment, indicating that the same protein binds to nucleotides in both fragments. DNase I protection analysis of the fragment from *argK* revealed four separate protected sequence elements, with element III containing half of the core motif sequence (CTTTG), and the other elements containing similar sequences. Gel shift assays were done with DNA fragments from which one or all of the sites were removed as competitor DNAs against the *argK* probe. The results of these experiments confirmed that the binding sites (in *argK*) are necessary for the protein to bind to the *argK* fragment in a specific manner. Taken together, the results of studies presented here suggest that in cells of *P. syringae* pv. *phaseolicola* grown at high temperature *argK* may be negatively regulated by the protein produced at this temperature.

Additional keywords: DNA-binding protein, footprinting, gel retardation, halo blight, transcription factor.

Toxigenic strains of *Pseudomonas syringae* pv. *phaseolicola*, the causal agent of halo blight of bean (*Phaseolus vulgaris* L.), produce a nonspecific phytotoxin, phaseolotoxin, Nδ(N'-sulfo-diaminophosphinyl)-ornithyl-alanyl-homoarginine (Moore et al. 1984), at temperatures between 18 and 20°C (Rudolph and Stahmann 1966). The toxin inhibits ornithine carbamoyltransferase (OCT), a key enzyme in the arginine biosynthetic pathway (Patil et al. 1970), and causes chlorosis, ornithine accumulation, and growth inhibition in infected, susceptible, bean plants (Ferguson and Johnston 1980; Mitchell and Bielecki 1977; Patel and Walker 1963; Rudolph and Stahmann 1966). In addition to increasing the virulence of the pathogen, phaseolotoxin is necessary for the systemic invasion of the plant by the bacteria (Patil et al. 1974).

OCT from many bacterial strains, including closely related pathovars of *P. syringae* and nontoxigenic strains of *P. syringae* pv. *phaseolicola*, is inhibited by phaseolotoxin (Mitchell 1978). However, toxigenic strains of *P. syringae* pv. *phaseolicola* produce a unique, toxin-resistant OCT (ROCT) in addition to a toxin-sensitive OCT (SOCT). ROCT protects the bacterium from the deleterious effects of phaseolotoxin (Ferguson and Johnston 1980; Staskawicz et al. 1980; Jahn et al. 1985) at temperatures conducive to toxin production. Phaseolotoxin and ROCT production are coordinately regulated in response to changes in temperature (Goss 1940; Mitchell 1978; Peet and Panopoulos 1987; Staskawicz et al. 1980); phaseolotoxin and ROCT are produced at 18 to 20°C, but not at the optimum growth temperatures (28 to 30°C). Our laboratory has been interested in elucidating the molecular mechanism of this thermoregulation.

Thermoregulation has been reported in two other phyto-bacteria besides *P. syringae* pv. *phaseolicola*, *P. syringae* pv. *glycinea* (Bender et al. 1999) and *Agrobacterium tumefaciens*. In *P. syringae* pv. *glycinea*, coronatine production is positively regulated at the transcriptional level by temperature; toxin is produced at 18°C but not at 28°C (Rangaswamy et al. 1997). Ullrich et al. (1995) reported the existence of a modified two-component system composed of three genes—*corS*, *corP*, and *corR*—involved in coronatine synthesis. Of these three genes only *corS*, which encodes the sensor protein, was activated at 18°C. Recently, Budde et al. (1998) showed that in three other genes (the *cmaABT* operon) in the coronamic acid cluster

promoter activity and transcription rates were highest at 18°C. The possible existence of a temperature-sensing, two-component system has also been reported in *A. tumefaciens* (Charles et al. 1992).

Thermoregulation of virulence genes in human- and animal-pathogenic bacteria, including toxin genes, and survival genes encoding porins and pili has been studied extensively; however, the exact mechanisms involved remain unclear (Hurme and Rhen 1998). A number of factors have been implicated in the thermoregulation of these pathogens. Two-component regulatory systems are involved in the thermoregulation of toxin and other virulence genes in *Bordetella pertussis* (Ackerley et al. 1992; Miller et al. 1989; Roy and Falkow 1991) and *Vibrio cholerae* (DiRita 1992). Other factors thought to be important in thermoregulation in human and animal pathogens include DNA-binding activator and repressor proteins (Higgins 1992; Skurnik and Toivanen 1992; Lambert de Rouvroit et al. 1992; Yao and Palchaudhuri 1992), histone-like proteins, DNA supercoiling (Dorman et al. 1990; Goransson et al. 1990; Higgins et al. 1990; Hromockyj et al. 1992), and N-terminal acetylase (White-Ziegler and Low 1992). Recently, Hurme et al. (1997) described an autoregulatory repressor protein in *Salmonella typhimurium* 1275 that senses temperature shifts directly and modulates the extent of transcription repression. A global regulatory protein known as the leucine-responsive protein (Lrp) has been implicated in the thermoregulation of the pili and porin genes in *Escherichia coli* (Ferrario et al. 1995; Nou et al. 1993). Homologs of this protein have been detected in several gram-negative bacteria, including *P. cepacia*, *P. aeruginosa*, and *P. putida* (Madhusudhan et al. 1995).

Previously, our laboratory reported that when present in multiple copies a clone from the *P. syringae* pv. *phaseolicola* genomic library (pDC938) and a 485-bp fragment from this

clone override the thermoregulation of phaseolotoxin production by wild-type cells grown at 28°C (Rowley et al. 1993). The nucleotide sequence of the fragment showed that it did not harbor a gene but rather contained inverted and direct repeats, indicative of DNA-binding sites. Gel-shift assays showed that the fragment formed a specific DNA-protein complex with a protein(s) found in extracts of cultured cells of the wild-type cells (G-50-1) grown at 28°C, but only a trace of the DNA-binding protein(s) was found in the cells grown at 18°C (Rowley et al. 1993). However, because the fragment, which was designated as the "thermoregulatory region" (TRR), is not part of any gene in the phaseolotoxin gene cluster (Kamdar et al. 1991), it was not clear whether this protein(s) was directly involved in thermoregulation of genes involved in ROCT or phaseolotoxin production.

The nucleotide sequence of the ROCT gene from *P. syringae* pv. *phaseolicola* was initially reported by Mosqueda et al. (1990), who named it *argK*. Subsequently, Hatziloukas and Panopoulos (1992) determined the transcription initiation site for the ROCT gene. They also showed that transcription of *argK* was partially repressed when cells were grown at 28°C. A comparison of the upstream sequences of *argK* and TRR (Rowley et al. 1993) showed that *argK* harbored sequences that had similarities to putative DNA-binding sites in TRR and to known DNA-binding sites in other bacteria. In preliminary studies, Rowley et al. (1993) also showed that a 490-bp *argK* upstream fragment formed complexes with a protein(s) present in the extracts of *P. syringae* pv. *phaseolicola* cells grown at 28°C. Based on these results, they proposed that the protein(s) produced by *P. syringae* pv. *phaseolicola* at 28°C negatively controls *argK* and the coordinately controlled genes involved in phaseolotoxin biosynthesis.

The objectives of this study were to determine whether, like the TRR sequence, the *argK* sequence forms specific complexes with the thermoregulatory protein(s) produced by *P. syringae* pv. *phaseolicola* at 28°C and whether TRR cross-competes with the *argK* sequence for binding of this protein(s), and to identify the specific bases in the TRR and *argK* fragments that are protected by the protein(s) from DNase I cleavage.

RESULTS

Mobility shift assays of the upstream region of the *argK* gene.

A 492-bp *EcoRI* fragment upstream of the start codon of the *argK* gene was subcloned (pKRK490) from the plasmid pRCPI7 (Peet et al. 1986) as described in Materials and Methods, and used as a probe in mobility shift assays (Fig. 1). Based on the transcription start site of *argK* (Hatziloukas and Panopoulos 1992), this 492-bp fragment (which contains a 143-bp sequence that encodes the leader transcript) does not contain the promoter region. The partially purified protein extract was prepared from cultures of *P. syringae* pv. *phaseolicola* G50-1 grown at 28°C as previously described (Rowley et al. 1993). A significant reduction in the mobility of the probe occurred in the presence of the 28°C extract (Fig. 1, lane 2) due to the formation of probe-protein complexes. In the presence of cold, specific (S) competitor DNA (pKRK490 plasmid DNA) at concentrations of 0.07, 0.35, or 0.7 µg (Fig. 1, lanes 3, 4, 5, respectively), the gel retardation progressively de-

Fig. 1. Mobility shift assay with the 492-bp *EcoRI* fragment, 5' end-labeled with [α^{32} P]dATP from the *argK* gene (pKRK490) as the probe. The labeled fragment (approximately 4 ng, 6,000 cpm) was incubated with 64 µg of 5 to 40% ammonium sulfate precipitate from the crude extract of G-50-1 cells grown at 28°C, 300 µg of bovine serum albumin (BSA), 30 ng of pT7/T3 α -19, 1.0 µg of poly(dI-dC) • poly(dI-dC), and varying amounts of competitor DNA. Unbound fragments (F) were separated from DNA-protein complexes (C) by electrophoresis through a 4.0% nondenaturing polyacrylamide gel as previously described (Rowley et al. 1993). Lane 1, probe alone; lane 2, probe + 64 µg of protein. Probe + 64 µg of protein + cold specific (S) competitor DNA (pKRK490) at lane 3, 0.07; lane 4, 0.35; or lane 5, 0.7 µg. Probe + 64 µg of protein plus cross-specific (CS) DNA (pKRK260 from the thermoregulatory region [TRR]) at lane 6, 0.06; lane 7, 0.30; or lane 8, 0.60 µg. Probe + 64 µg of protein plus nonspecific competitor DNA (T3/T7 α -19) at lane 9, 0.2; lane 10, 1.0; or lane 11, 2.0 µg.

creased, compared with the probe-protein complexes (lane 2). This indicated that increasing concentrations of specific competitor DNA increasingly titrated the probe. pKR260 plasmid DNA (which contained a 260-bp fragment harboring the binding site from the TRR) was used as the cross-specific (CS) competitor at concentrations similar to S DNA to determine whether the same protein that forms TRR-protein complexes is involved in the formation of *argK*-protein complexes. At 0.06, 0.3, or 0.6 μg (Fig. 1, lanes 6, 7, 8, respectively), the shift distance progressively decreased except that at 0.6 μg of CS DNA the shift distance was reduced even more than at the comparable concentration of S DNA, indicating that the CS

DNA has a higher binding affinity for the protein than S DNA. In preliminary experiments, we observed that when nonspecific (NS) DNA (T3/T7 ϕ -19 plasmid DNA) was used as a competitor at concentrations comparable to S and CS DNA, it did not affect the shift distance of the probe-protein complexes. In this experiment, NS DNA was used (0.2, 1.0, or 2.0 μg ; Fig. 1, lanes 9, 10, 11, respectively) at roughly three times the concentration used in the case of S or CS DNA (Fig. 1, lanes 3, 4, 5 and 6, 7, 8, respectively). However, in spite of higher competitor concentrations, the shift distances were equal to or less than those for the S or CS DNA, indicating that the binding of the protein to the S and CS DNA is specific.

Fig. 2. DNase I protection analysis of the 260-bp fragment from the thermoregulatory region (TRR). **A**, The 260-bp fragment from the TRR was digested with *Hind*III and *Eco*RI and the 5' end of the + strand was labeled with [γ - ^{32}P]ATP with T4 polynucleotide kinase at the *Eco*RI site. Labeled fragment (2×10^4 cpm; 0.1 pmol) was mixed with 1 μg of poly(dI-dC) • poly(dI-dC) and lanes 1 and 6, 0; lane 2, 25; lane 3, 50; lane 4, 75; or lane 5, 100 μg of 28°C extract, and DNase I (1.25 $\mu\text{g}/\text{ml}$). **B**, The (-) strand of the 260-bp fragment from the TRR was digested with *Eco*RI-*Pst*I and labeled at the *Eco*RI end with [α - ^{32}P]dATP with the Klenow fragment as described above except that approximately 18 fmol of the labeled probe (20,000 cpm) was used with lane 1, 0; lane 2, 25; lane 3, 50; lane 4, 75; lane 5, 100; or lane 6, 150 μg of the 28°C extract, and DNase I (2.5 $\mu\text{g}/\text{ml}$). **A and B**, A Maxam-Gilbert G + A chemical cleavage reaction (Maxam and Gilbert 1978) of the fragment was used as the marker (lane 7). **C**, Sequence of a portion of the TRR on both strands protected by the 28°C extract. Numbering is with reference to the sequence published in Rowley et al. (1993); (+) refers to strand of the published sequence; (-) is the complementary strand. Boxes refer to the bases protected by the protein. Sequence used to synthesize a tetramer of the binding site is underlined.

Mobility shift assays of the *argK* fragment with extracts from cultures of *P. syringae* pv. *phaseolicola* grown at the permissive temperature (18 to 20°C) were done to determine whether the detection of the *argK*-binding protein is dependent on the temperature at which the culture was grown. No *argK*-protein complexes with the 18°C extract were detected (data not shown). Thus, the presence of both the *argK*-binding and TRR-binding proteins is strongly dependent on the temperature of the culture, suggesting that the same protein may be involved in binding to both probes. However, whereas a trace of the binding protein was detected with the TRR probe, none was detected with the *argK* probe.

The above results suggested that transconjugants containing multiple copies of the *argK* fragment should abolish thermoregulation, as previously reported for TRR (Rowley et al. 1993). Therefore, the 492-bp *argK* fragment was cloned into the broad-host-range vector pNP483 to form pKRK492 and mobilized into wild-type *P. syringae* pv. *phaseolicola* G50-1 by electroporation. A 3.2-kb fragment containing the entire *argK* gene including the promoter region (Hatziloukas and Panopoulos 1992) was also subcloned in pNP483 to form pKRK32, and mobilized into G50-1 by electroporation. Transconjugants containing pKRK492 or pKRK32 were tested for their ability to produce toxin at 18 and 28°C with the microbial bioassay. Neither transconjugant produced toxin at the nonpermissive temperature (28°C) although, as expected, both produced toxin at the permissive temperature (18°C).

Analysis of the binding sites in TRR.

DNase I footprinting of the protein-DNA complexes (Lee et al. 1987) was first performed to determine which nucleotides in the TRR sequence were protected from cleavage by DNase I. The 260-bp fragment from the TRR that retains protein-binding ability was isolated as a *Hind*III-*Eco*RI fragment from the 485-bp insert of pKR482 (Rowley et al. 1993). The fragment was labeled on the (+) strand at the *Eco*RI end with

[γ^{32} P]ATP and T4 polynucleotide kinase and the (–) strand was labeled with [α^{32} P]dATP and the Klenow fragment of DNA polymerase. The labeled fragments were subjected to partial cleavage with DNase I in the presence of increasing amounts of the protein extract. The region of the nucleotide sequence protected from cleavage by DNase I appears as a gap on the sequencing gel. The identity of the protected nucleotides was determined from the Maxam and Gilbert (1980) G+A ladders (Fig. 2A, lane 7, and B, lane 7). The sequences protected from DNase I cleavage on the (+) strand (Fig. 2A) and on the (–) strand (Fig. 2B), 22 bp (Fig. 2C, nucleotides 143 to 165) and 26 bp (Fig. 2C, nucleotides 134 to 160) in length, respectively, revealed a single site (Fig. 2C, boxed sequence). The protected site contains two repeats of a core motif G/C AAAG (CTTT C/G, on the complementary or (–) strand) separated by a 5-bp spacer, placing the core motifs on the same side of the DNA helix.

Binding of the synthetic oligomer to the partially purified thermoregulatory protein.

An oligonucleotide containing four tandem repeats of the synthetic binding site was cloned (pKRE4) as described in Materials and Methods and was used to determine whether the partially purified binding protein binds to it. The insert from pKRE4 was isolated as an *Eco*RI-*Hind*III fragment and used as the probe in a mobility shift assay with the partially purified protein (Materials and Methods). A gradual disappearance of the probe (approximately 4 ng per lane) was detected with increasing amounts (120 ng to 1.56 μ g) of Heparin-Agarose (Hep-Ag) purified protein (Fig. 3, lanes 2 to 6). Concomitantly, one to four new bands appeared with increasing amounts of protein, showing that as the concentration of the protein increased additional sites in the probe were occupied by the protein (the higher the shift distance, the larger the molecular mass of the complex). This result confirmed that G/C AAAG is the core motif in the TRR.

Analysis of the binding sites in the *argK* fragment.

We previously reported that a comparison of the published sequence (Mosqueda et al. 1990; Hatziloukas and Panopoulos 1992) of the region from the *Eco*RI upstream fragment of the *argK* gene showed sequences with similarity to the sequences in the TRR (Rowley et al. 1993). However, reexamination of the *argK* upstream sequence, to determine whether or not motifs with similarity to the core motif identified in TRR existed, revealed that similar motifs were present on both the (+) and the (–) strands of this region. This observation and the results of the cross-competition experiment required that we determine the specific nucleotides in the 492 (pKRK490)-bp fragment to which the protein binds. Therefore, the 492-bp fragment containing this region was subcloned as follows. To differentially label one end of the *argK* fragment, the 3' *Eco*RI site was eliminated by digesting pKRK490 with *Bal*I. The religated plasmid (pKRK490 Δ E) contained a 12-bp deletion at the 3' end and a single *Eco*RI site at the 5' end of the fragment. This fragment retained the protein-binding ability as determined by the mobility shift assay and could be differentially labeled at the 5' *Eco*RI site and the vector *Pst*I site at the 3' end. However, only the 5' end-labeled probe was used in the foot printing experiment, because experiments with the 3' labeled strand were not successful. The labeled probe was in-

Fig. 3. DNA binding of partially purified protein to a probe containing four repeats of the synthetic binding site. Uncomplexed fragment (F) was separated from DNA-protein complexes (B1–B4) by electrophoresis, as described for Figure 1. Labeled purified insert DNA from pKRE4 (2×10^4 cpm; 50 fmol) was incubated with 20 ng of pT7/T3 α -19, 1 μ g poly(dI-dC) • poly(dI-dC), and lane 1, 0 ng; lane 2, 120 ng; lane 3, 240 ng; lane 4, 0.6 μ g; lane 5, 1.2 μ g; or lane 6, 1.56 μ g of partially purified protein as described in Materials and Methods.

cubated with approximately 190 μg of the extract of cells grown at 28°C and digested with increasing amounts of DNase I (Fig. 4A, lanes 1 to 4). At this protein concentration, no free probe was detected in mobility shift assays. The identity of the protected bases was determined from the Maxam and Gilbert (1980) G + A ladder (Fig. 4A, lane 5). Results of these assays revealed four sequence elements (Fig. 4B, shaded sequences I to IV) that are clearly protected from digestion with DNase I. Element I contains the sequence TAAAG, which is inverted (CTTTA) in element II. The distance between the central nucleotides in these two elements is 20 nucleotides or one turn of the DNA helix, placing the sequences on the same side of the DNA helix. The protected sequence in element III contains half of the core motif, CTTTG, and a partial repeat of this sequence (CTTT) in element II. Element III also contains the sequence GTTTTG, which is similar to element IV (GTTTTTC), except that the G is replaced by a C. Attempts to footprint the fragment at lower protein concentrations were not successful.

Fig. 4. DNase I protection analysis of the insert from pKR490 ΔE (*argK*). **A**, Labeled noncoding strand of the 478-bp fragment was digested with *EcoRI* and *PstI* and labeled at the *EcoRI* end with [$\alpha^{32}\text{P}$]dATP with the Klenow fragment. Labeled fragment (20,000 cpm; approximately 25 fmol) was mixed with 20 ng of pT7/T3 α -19 DNA; 1 μg of poly(dI-dC) • poly(dI-dC), 185 μg of 28°C extract, and lane 1, 1.25; lane 2, 2.5; lane 3, 5; or lane 4, 10 $\mu\text{g}/\text{ml}$ of DNase I. Identity of the protected bases was determined from the G+A ladder shown in Figure 4A (lane 5). **B**, A portion of the sequence and the bases protected (shaded boxes) by the protein in the 28°C extract from DNase I cleavage. The -35, -10 promoter regions of the *argK* gene are underlined. Transcription begins at a T (boxed), and the start codon (ATG) for the open reading frame is shown. *EcoRI*, *SalI*, and *SstI* restriction sites are marked.

To verify that the protected regions were due to specific binding, a mobility shift assay was done with, as competitor DNAs, the unlabeled 490 ΔE fragment (Fig. 5B) containing all four of the protected sites (Fig. 5A, lanes 3 to 7), a deletion of the 490 ΔE fragment, 470 (Fig. 5B), containing three sites (Fig. 5A, lanes 8 to 12), or a second deletion fragment, 380 (Fig. 5B), with all sites deleted (Fig. 5A, lanes 13 to 17). Labeled 490 ΔE fragment was used as the probe (F; Fig. 5A, lane 1) and lane 2 (Fig. 5A) contains probe plus protein only. The competitor fragments were isolated, purified, and quantitated, as described in Materials and Methods. When the unlabeled 490 ΔE fragment (containing all four sites) or the 470-bp fragment (containing three of the four sites) was used as the competitor DNA, three new bands with decreasing shift distances appeared gradually with increasing concentrations of competitor DNA. At a fivefold excess of specific competitor DNA (490 ΔE fragment) over the probe concentration, band 4 disappeared and a new band at position 3 appeared (Fig. 5, lane 3). In the presence of a 100-fold excess of competitor DNA (Fig. 5, lane 7) only a slight mobility shift (position 1),

Fig. 5. Deletion analysis of the upstream sequence in *argK* for protein binding. **A**, Mobility shift assays with the labeled 490 ΔE fragment (500 cpm; 16 fmol) as the probe (F) were done in the lane 1, absence; or lanes 2–17, presence of 144 μg of 28°C extract. Purified fragments from 490 ΔE and deletion fragments from 490 ΔE were used as competitor DNAs, as follows: 490 ΔE (containing protected sites I–IV; lanes 3–7); 470 bp (containing sites II–IV; lanes 8–12), and 380 bp (containing no binding sites; lanes 13–17). These were added at lanes 3, 8, and 13, fivefold; lanes 4, 9, and 14, 10-fold; lanes 5, 10, and 15, 20-fold; lanes 6, 11, and 16, 50-fold; and lanes 7, 12, and 17, 100-fold concentrations of molar excess, compared with the probe concentration, respectively. Positions of the DNA-protein complexes in the absence of (lane 2) or with increasing concentrations of competitor fragments are indicated by “1–4” to the left. Roman numerals I–IV indicate protected binding sites present in the competitor DNA. **B**, Diagram showing probe (490 ΔE) and competitor fragments (490 ΔE , 470, and 380) used. Roman numerals I–IV and filled rectangles indicate positions of sequences identified by DNase I footprinting that are protected by the protein in the crude extract. Positions of restriction enzyme sites *EcoRI*, *SalI*, and *SstI* and junction between the *BallI* site in the *argK* gene and the *SmaI* site of the pT7/T3 α -18 vector are shown.

compared with the free probe, was detected. A 50-fold excess of the 470-bp competitor fragment (with site 1 deleted; 10 times more 470-bp competitor than 490ΔE competitor), is required before the band at position 4 is no longer detected (Fig. 5, lane 11). When the 470-bp fragment was used as competitor DNA, a band at position 2 appeared after a 20-fold excess was added (Fig. 5A, lane 10), but a band at position 3 was not detected; a band at position 1 was detected when a 50-fold excess was added, although at much lower intensity than in the presence of the 490ΔE competitor DNA (Fig. 5, lane 11). The 380-bp fragment that contains none of the sites (Fig. 5B) protected by DNase I was not effective as competitor DNA. The band at position 4 is still apparent even in the presence of 100-fold excess of the 380-bp competitor DNA (Fig. 5A, lane 17). Furthermore, when mobility shift assays were done with the 380-bp fragment as the probe, nearly all of the fragment remained unbound in the presence of the protein extract (data not shown). The results of the mobility shift assay (Fig. 5A) confirm the evidence that the sites protected in the DNase I footprinting experiment were the result of the formation of specific *argK*-protein complexes. The bands at positions 1, and (particularly) 2 and 3 were diffuse. This could be due to differing affinities of the binding protein to different sites, which in turn cause variations in the sites occupied per DNA molecule.

DISCUSSION

Several lines of evidence obtained in the course of these studies suggest that a protein produced by *P. syringae* pv. *phaseolicola* at 28°C that binds specifically to both the TRR and the *argK* fragments could be involved in the negative regulation of the *argK* gene in vivo at this temperature. First, mobility shift assays showed that there is a protein, detected only in cells grown at 28°C, that binds specifically to both the TRR and the *argK* fragments. Second, the cross-competition data shows that the TRR fragment efficiently and specifically competes with the *argK* fragment for binding of the protein (Fig. 1, lanes 6, 7, and 8). This suggests that the same protein is involved in the binding to both fragments. Third, the sites in the *argK* fragment that are protected from DNase I cleavage harbor conserved or semiconserved core motifs found in the TRR. Even though we do not have direct evidence that the protein binds to these sites and represses transcription of the *argK* gene in vivo, such an assumption is corroborated by the results of Hatziloukas and Panopoulos (1992), which showed that *phoA* expression from the *argK* promoter in *P. syringae* pv. *phaseolicola* cells grown at 28°C was 60% lower than in cells grown at 21°C. Further in vivo experiments on the transcription of *argK* are necessary to determine whether our hypothesis is correct.

We previously reported that transconjugants of the wild-type cells containing multiple copies of the 485-bp fragment from the TRR produced phaseolotoxin at 28°C, thus overriding thermoregulation (Rowley et al. 1993). However, neither multiple copies of the 490-bp fragment from the *argK* gene nor the intact *argK* gene were able to override thermoregulation of toxin production by *P. syringae* pv. *phaseolicola*. The cross-competition mobility shift assay revealed that the 260-bp TRR fragment titrated the probe-protein complexes more efficiently than the *argK* fragment at comparable concentrations (Fig. 1, lanes 7 and 8 versus 4 and 5), suggesting that the pro-

tein has a higher affinity for the binding site in the TRR than for all four sites in the 490ΔE fragment combined. Thus, the negative results of toxin bioassays could be due to incomplete "titration" resulting from the lower affinity of the protein for the 490ΔE fragment. Indeed, as previously reported (Rowley et al. 1993), even multiple copies of the TRR are not sufficient to completely override thermoregulation of toxin production at 28°C in wild-type cells of *P. syringae* pv. *phaseolicola*.

DNase I protection analysis of the 260-bp TRR fragment identified a single protected site. The sequence contained a core motif, G/C AAAG (CTTT C/G on the complementary strand), repeated once with a 5-bp spacer between the repeats (Fig. 2C). Thus, the central nucleotides in each motif are separated by 10 bp, equivalent to one complete turn of the DNA helix. This positions the core motifs on the same side of the double helix and may represent half of a site for a homodimer, a factor that may be important for DNA binding and could explain why the protein appears to bind to the TRR motif with high affinity. Data in Figure 3 show that the thermoregulatory protein binds to the synthetic oligomer containing four tandem repeats of the core motif, confirming the results of the DNase I protection analysis of the 260-bp fragment. We have not yet identified the gene that harbors this regulatory site. Southern blot experiments have previously shown that the clone (pDC938) harboring the 260-bp fragment does not hybridize to restriction digests of the clone (pHK120) containing the phaseolotoxin gene cluster (Kamdar et al. 1991). Therefore, there must be additional genes, as yet unidentified, that are thermoregulated by the same protein that regulates *argK*, suggesting the existence of a regulon.

DNase I protection studies of the 490ΔE fragment from the *argK* gene revealed a much different pattern of protection, compared with the TRR fragment. Four separate but adjacent regions (Fig. 4A and B, regions I to IV) were protected from cleavage. This is corroborated by the results of mobility shift assays in which four different bands were detected in the presence of a competitor fragment containing the four sites, but only three were detected when one of the sites was deleted (Fig. 5). Region III contains half of the core motif identified in the protected site in the TRR and other regions contain sequences with similarity to the core motif. None of the regions contains the complete core motif. Given the similarities in these sequences, the same protein probably binds to these sites in the *argK* gene. There are many examples of the same transcription factor binding to multiple sites in a gene to repress or induce that gene. For example, the leucine-responsive protein (Lrp) of *E. coli* negatively regulates the *ilvGMEDA* operon of *E. coli* by binding to three sites in the leader region of this operon 226 bp downstream of the transcriptional start site (Rhee et al. 1996). If one protein binds to all four sites in the *argK* fragment then it must have different affinities for different sites, as has been demonstrated for the Lrp protein (Wang and Calvo 1993). This could explain why the protein appears to have a lower affinity for the *argK* fragment, compared with TRR, for only one of the sites contains part of the core motif found in TRR, to which it appears to bind with high affinity.

Even though the 492-bp upstream fragment from *argK* did not contain the promoter region, we selected it for the mobility shift studies because we noticed potential binding sites in this region (Rowley et al. 1993). The results reported here show that this fragment forms specific complexes with the

protein in the 28°C extract and that the TRR cross-competes with this fragment. Therefore, we chose the 492-bp fragment to do the footprinting experiments. It is possible that additional binding sites exist in the -10 and -35 regions because the 490ΔE fragment did not contain these regions. Although it is common to find single primary regulatory sites for transcription factors near the promoters in prokaryotic genes (Gralla and Collado-Vides 1996; Choy and Adhya 1996), the four binding sites in the *argK* fragment are located between the transcription start site and the start codon of this gene. Although it is less common for regulatory sequences to be located in regions downstream of the transcriptional start, a number of operons involved in the biosynthesis of amino acids contain protein-binding sites in this region that are involved in attenuation or negative regulation of transcription (Choy and Adhya 1996; Gralla and Collado-Vides 1996). The Lrp protein mentioned above binds to multiple sites located in different regions and regulates, both negatively and positively, a number (75 at last count) of diverse genes (Wang and Calvo 1993; Wang et al. 1994). For example, Lrp binds in a highly cooperative manner at multiple sites that are located either in the leader-attenuator region (*ilvGMEDA* operon) (Rhee et al. 1996) or upstream of the promoter (*ilvIH* promoter region) (Marasco et al. 1994). Another example of the regulator binding to multiple areas upstream of the promoter is the OmpR protein of *E. coli*, which represses transcription of *ompF* by binding to the regulatory sequences from nucleotides -40 to -100 and -350 to -380 (Pratt and Silhavy 1995; Tsung et al. 1989).

The results presented here indicate that the binding protein negatively regulates *argK* at 28°C. This gene is part of the cluster of genes involved in phaseolotoxin production and is coordinately regulated by temperature (Peet et al. 1986) with phaseolotoxin genes. Therefore, the same transcription factor may be involved in regulating both the *argK* and phaseolotoxin genes. If this is the case, one would expect to find regulatory sites in genes involved in phaseolotoxin synthesis as well. We previously sequenced the largest locus (*phtE*) (Zhang et al. 1993) in the phaseolotoxin gene cluster; however, this operon is not regulated by temperature (Zhang and Patil 1997). We have recently sequenced six additional loci in the toxin cluster, and preliminary analysis shows that at least two genes harbor sequences showing similarity to the core motif (L. Ratnapala and S. S. Patil, unpublished data).

The determination of the minimum number of *argK* sites to which the protein must bind to effect repression, and the elucidation of the relative affinity of the putative transcription factor for each of the four sites, must await purification of the protein. Xu et al. (1999) recently reported preliminary results on the purification of the binding protein with Hep-Ag chromatography and Southwestern blotting. These will be reported in a subsequent communication.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

DH5α (F80d *lacZ*ΔM15 Δ[*lacZYA-argF*]U169 *recA1 endA1 hsdR17*[r_K,m_K⁺] *supE44* *thi-1 gyrA relA1*), which was used for DNA isolation and subcloning, was grown in Luria-Bertani (LB) broth (Maniatis et al. 1982). *P. syringae* pv. *phaseolicola* strains G50-1 (Race 2, Tox⁺, Rif^r) and transconjugants of G50-1 were cultured in King's B (KB) medium

(King et al. 1954) at 18 or 28°C. When necessary, the medium was supplemented with ampicillin: 100 μg/ml (for strains containing pT7/T3 plasmid derivatives); 50 μg/ml (for pNP483 derivatives).

Toxin bioassay, electroporation, and recombinant techniques.

Toxin production by *P. syringae* pv. *phaseolicola* strains and transconjugants was assayed by the *E. coli* growth-inhibition assay (Staskawicz and Panopoulos 1979) as previously described (Rowley et al. 1993). Plasmids were transferred into *P. syringae* pv. *phaseolicola* by electroporation as previously described (Rowley et al. 1993). Plasmid DNA for sequencing, fragment isolation, and electroporation was prepared either by the alkaline lysis method (Birnboim and Doly 1979) or with the protocol and reagents from Qiagen (Chatsworth, CA, U.S.A.). Mini-prep plasmid DNAs to verify subcloned constructs were prepared by the boiling method of Holmes and Quigley (1981).

Construction of subclones.

The construction of plasmids pKR260 and pKR480 is described elsewhere (Rowley et al. 1993). The plasmid pKRK490 was constructed by cloning a 492-bp *EcoRI* fragment from a 3.2-kb *HindIII-PvuII* insert (Hatziloukas and Panopoulos 1992) isolated from pRCP17 (Peet and Panopoulos 1987) into pT7/T3α-18 expression vector (Bethesda Research Laboratories, Bethesda, MD, U.S.A.) also digested with *EcoRI* and treated with shrimp alkaline phosphatase. The plasmid pKRK490ΔE was prepared by digesting pKRK490 with *BalI* and *SmaI* and religating. This resulted in a deletion of 12 bp, and the removal of one of the two *EcoRI* sites from the 3' end of the fragment. The plasmid pKRK492 was prepared by isolating the 492-bp fragment from pKRK490 as an *EcoRI* fragment and ligating it into the pNP483 vector DNA, digested with *EcoRI* and treated with shrimp alkaline phosphatase. The plasmid pKRK32 was constructed by isolating the 3.2-kb *HindIII-PvuII* fragment from the plasmid pRCP17 and cloning the fragment into pT7/T3α-19 (Bethesda Research Laboratories), digested with *HindIII* and *SmaI*. The entire insert was re-isolated as an *HindIII-KpnI* fragment and cloned into pNP483 (Rowley et al. 1993) for electroporation into *P. syringae* pv. *phaseolicola*.

Preparation of DNA binding site probes and competitor DNAs.

The *argK* probes and unlabeled competitor fragments were prepared by digesting pKRK490 or pKRK490ΔE with appropriate enzymes (Fig. 5B). The fragments were separated from the vector by electrophoresis through a 1.2 to 1.4% low melting point agarose in Tris-acetic acid-EDTA (TAE) buffer, and purified with USBioclean glass beads (US Biochemical, Cleveland, OH, U.S.A.). The probes were labeled with either [³²P]ATP (NEN Life Science Products, Boston, U.S.A.) with T4 polynucleotide kinase (Promega, Madison, WI, U.S.A.) or with [α³²P]dATP (NEN Life Science Products) with the Klenow fragment. The unincorporated nucleotides were removed with USBioclean glass beads or G50 Quick spin columns (Boehringer Mannheim, Indianapolis, IN, U.S.A.), with protocols provided by the manufacturers.

The TRR probe was prepared by digesting the plasmid pKR260 with *EcoRI*, treating the *EcoRI*-digested ends with

shrimp alkaline phosphatase (US Biochemicals), and digesting with *Hind*III to release the fragment labeled at one end. The *Eco*RI end-labeled fragment was isolated after electrophoresis through a 5% polyacrylamide gel and diluted in 10 mM Tris-HCl (pH 8.0), and 1 mM EDTA (TE buffer). Fragments labeled with [α^{32} P]dATP were digested with two different restriction enzymes (unless specified), isolated from 1.2% low-gelling agarose (US Biochemicals) in TAE buffer and purified with glass beads and kits provided by US Biochemicals or Qiagen. The fragment was selectively labeled at one end, unless specified, with either [α^{32} P]dATP or [α^{32} P]dCTP with the Klenow fragment. After labeling, the remaining nucleotides of the overhang were filled in by adding a solution containing dGTP, dATP, dTTP, and dCTP (0.2 mM concentrations of each) 5 to 10 min prior to stopping the reaction. The fragment was separated from the unincorporated nucleotides by passage through a Quick-Spin column (Boehringer-Mannheim). Fragments used as competitors in mobility shift assays were prepared in the same manner as the probe, except that they were not labeled with radioactive nucleotides.

Mobility shift assays and DNase I footprinting.

Mobility shift assays were performed as previously described (Rowley et al. 1993), except that the amount of the probe, protein extracts, and competitor DNAs varied (Fig. 1 and 5, captions).

The DNA-binding reactions and DNase I (Promega) digestion were carried out in a 50- μ l volume with 20,000 cpm of the end-labeled fragment, 1 μ g of poly(dI-dC) • poly(dI-dC) competitor DNA (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.), and varying amounts of crude protein extract in a buffer (final concentration 25 mM HEPES (*N*-[2-hydroxyethyl] piperazine-*N'*-[2-ethanesulfonic acid]) [pH 7.6], 10% [wt/vol] glycerol, 0.05 mM ZnSO₄, 50 mM KCl, 0.05 mM dithiothreitol [DTT], and 0.05% Nonidet-P40 [NP40; US Biochemicals]), modified from that described in Lee et al. (1987). Where specified, 25 ng of pT7/T3- α 19 competitor DNA was added. The DNase I reactions for the 260-bp fragment from the TRR were incubated on ice for 15 min followed by a 1-min incubation at room temperature. After dilution with an equal volume of a mixture containing 5 mM CaCl₂ and 10 mM MgCl₂ the fragment was digested for 20 s with 2 ml of freshly diluted DNase I at a concentration of 2.5 μ g/ml in the presence of varying amounts of crude protein extract (1.25 μ g/ml, in the absence of protein). Reactions for the *argK* fragment were incubated for 5 min at 30°C followed by a 1-min incubation at room temperature. Digestion with DNase I was carried out as described above except that varying amounts of DNase I were mixed with 185 μ g of the crude protein extract after concentrating with a centricon-30 spin column (Millipore, Bedford, MA, U.S.A.). The reaction was stopped by adding a solution containing 20 mM EDTA (pH 8.0), 1% (wt/vol) sodium dodecyl sulfate (SDS), 0.2 M NaCl, and glycogen (250 μ g/ml; Boehringer-Mannheim). Protein was removed by digestion with 25 μ g of proteinase K (Sigma Chemical, St. Louis, MO, U.S.A.) for 5 min followed by one phenol/chloroform extraction. To localize the binding-site sequences, the G+A sequence ladder, prepared from the DNA probe by the method described by Maxam and Gilbert (1980), was electrophoresed adjacent to the footprint reactions in which the same probe was used.

Preparation of the synthetic oligonucleotide.

Two complementary synthetic oligonucleotides (5'-AAAA-ATTCGAGAGAAAGGGATCCAAAGTTAATCTTGTGCGACGGGG-3') and (5'-CCCCCGTCGACAAGATTAACCTTTGATCCCTTTCTCTCGAATTTT-3') containing the sequence of core motif and surrounding bases were synthesized with an Applied Biosystems (Branchburg, NJ, U.S.A.) 380A DNA Synthesizer. The oligonucleotides were annealed to form a single complete synthetic thermoregulatory site with a *Taq*I restriction site at either end. The annealed fragment was digested with *Taq*I (Promega) and ligated to form multimers of the binding site by the procedure described by Kadonaga and Tjian (1986). The few additional nucleotides upstream and downstream of the core motif were included in the synthetic binding site so that, when the *Taq*I ends were ligated, the center of the core sequence was separated from the center of the core sequence in the next repeat by 20 bases, or two full turns of the double helix. The ligated mixture was cloned into pUC19 digested with *Acc*I, (which has a 5' overhang complementary to the *Taq*I overhang), and transformed into *E. coli* DH5 α . A clone, pKRE4, was isolated. To verify the sequence of the construct, both strands of the insert in pKRE4 were sequenced with the automated DNA sequencer (model 373A; Perkin-Elmer-Applied Biosystems, Branchburg, NJ, U.S.A.) with a linear template, (pKRE4 digested with *Eco*RI), *Taq*I polymerase (Perkin Elmer, Foster City, CA, U.S.A.), and the reverse primer. The sequence showed three complete head to tail repeats of the synthetic oligonucleotide and a partial sequence of the fourth repeat before termination occurred (data not shown). We concluded that the insert contained four repeats of the binding site, and that the abrupt termination of DNA replication may have been due to secondary structure formation in the template.

Partial purification of the binding protein.

The 28°C extract (37 mg) was prepared as previously described (Rowley et al. 1993) and was applied to a Hep-Ag affinity (Bio-Rad, Richmond, CA, U.S.A.) column equilibrated in HA buffer (25 mM Tris [pH 8.0], 0.1 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM DTT, and 10% glycerol) containing 50 mM KCl. After washing with 12 ml of HA buffer, the proteins that were specifically bound to Hep-Ag were eluted with HA buffer in 0.1 M incremental steps (6 ml per step) of KCl, from 0.1 M to 0.8 M. Fractions eluted from the affinity columns were assayed for specific binding activity with the mobility shift analysis (data not shown). The fractions containing activity (0.2 to 0.5 M KCl) were pooled, precipitated by adding (NH₄)₂SO₄ (0.39 g/ml), and dialyzed in HA buffer containing 50 mM KCl and 1.25 mg of bovine serum albumin (BSA). Protein concentrations were quantitated by the method of Bradford (1976).

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