

In *Pseudomonas syringae* pv. phaseolicola, Expression of the *argK* Gene, Encoding the Phaseolotoxin-Resistant Ornithine Carbamoyltransferase, Is Regulated Indirectly by Temperature and Directly by a Precursor Resembling Carbamoylphosphate

Karina López-López,¹ José Luis Hernández-Flores,¹ Marisa Cruz-Aguilar,² and Ariel Alvarez-Morales^{1*}

Departamento de Ingeniería Genética, Centro de Investigación y de Estudios Avanzados (CINVESTAV) del Instituto Politécnico Nacional (IPN), Unidad Irapuato, Irapuato, Guanajuato, C.P. 36500,¹ and Departamento de Biomedicina Molecular, CINVESTAV IPN, Mexico D.F.,² Mexico

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Pseudomonas syringae pv. phaseolicola synthesizes a non-host-specific toxin, phaseolotoxin, and also synthesizes a phaseolotoxin-resistant ornithine carbamoyltransferase (ROCT) to protect itself from its own toxin. ROCT is encoded by *argK*, which is expressed coordinately with phaseolotoxin synthesis at 18°C. To investigate the regulatory mechanisms of this system, null mutants were constructed for *argK*, *argF* (encoding the phaseolotoxin-sensitive OCTase [SOCT]), and *amtA* (encoding an amidinotransferase involved in phaseolotoxin synthesis). The *argF* mutant did not exhibit arginine auxotrophy when grown in M9 medium at 28°C, because under this condition SOCT was replaced by ROCT. This loss of thermoregulation of *argK* was apparently caused by accumulation of carbamoylphosphate, one of the substrates of SOCT. Carbamoylphosphate, which has a structure similar to that of the inorganic moiety of phaseolotoxin, was used in induction assays with wild-type *P. syringae* pv. phaseolicola and was shown to be able to induce *argK* expression in M9 medium at 28°C. These results indicate that *argK* expression is independent of temperature and is regulated directly by a compound resembling the inorganic moiety of phaseolotoxin.

Pseudomonas syringae pv. phaseolicola is the causal agent of halo blight disease in beans (*Phaseolus vulgaris* L.). Disease symptoms are typically water-soaked lesions that are surrounded by a chlorotic zone or halo (19). This halo is believed to result from the action of a non-host-specific toxin known as phaseolotoxin [N⁶-(N'-sulfodiaminophosphinyl)-ornithyl-alanyl-homoarginine] (18, 20). Phaseolotoxin is produced when bacteria are grown in minimal medium at temperatures between 18 and 20°C, and it is not detected at 30°C (7, 16, 22, 33).

Phaseolotoxin is a reversible inhibitor of the enzyme ornithine carbamoyltransferase (OCTase; EC 2.1.3.3) (5), which catalyzes the formation of citrulline from ornithine and carbamoylphosphate in the sixth step of the arginine biosynthetic pathway. In planta, phaseolotoxin is readily cleaved by peptidases to release N⁶-(N'-sulfodiaminophosphinyl)-L-ornithine (PSOrn), the major toxic chemical species present in diseased leaf tissue (19). Phaseolotoxin is an effective inhibitor of OCTase activity from plant, mammalian, and bacterial sources and causes a phenotypic requirement for arginine. To protect itself from its own toxin, *P. syringae* pv. phaseolicola synthesizes a phaseolotoxin-resistant OCTase (ROCT) (6, 10, 11, 25, 34).

The ROCT, which is the product of the *argK* gene, is expressed under conditions leading to the synthesis of phaseolo-

toxin, such as growth in minimal medium at 16 to 18°C (6, 8, 21, 34). It has been postulated that in *P. syringae* pv. phaseolicola the *argK* gene may be regulated under negative control by a repressor protein synthesized at 28°C (21). This repressor protein may bind to specific motifs (thermoregulatory region [TRR] motifs) that have been postulated to be involved in thermoregulation of phaseolotoxin synthesis (27, 28). It has been observed that Tox⁻ mutants do not seem to express phaseolotoxin immunity, which indicates a coordinated mode of regulation (4, 25). Coordinated regulation of phaseolotoxin synthesis and *argK* expression could be explained by assuming that a precursor molecule of phaseolotoxin or phaseolotoxin itself could act as an inducer of *argK* (21).

When *P. syringae* pv. phaseolicola is grown under conditions that do not promote phaseolotoxin production, the synthesis of arginine is mediated by a phaseolotoxin-sensitive OCTase (SOCT) encoded by the *argF* gene. SOCT appears to be irrelevant to pathogenicity and to exert a housekeeping function; it is not clear whether SOCT is expressed at 18°C and what relationship (if any) it has with ROCT. Of the two forms of the OCTase present in *P. syringae* pv. phaseolicola, only SOCT has been shown to be regulated by arginine and complex media (35).

Arginine is a key amino acid in phaseolotoxin synthesis, since it is substrate of an amidinotransferase that catalyzes the synthesis of homoarginine, a constituent of the organic moiety of phaseolotoxin (13, 26). This amidinotransferase is encoded by *amtA*, whose expression is coordinated with phaseolotoxin synthesis and *argK* expression. A null mutant of this gene

* Corresponding author. Mailing address: Cinvestav Unidad Irapuato, Km. 9.6 Libramiento Nte. Carretera Irapuato-Leon, Apdo. Postal 629, Irapuato, Gto., CP 36500, Mexico. Phone: 52 462 62 39600. Fax: 52 462 62 45849. E-mail: aalvarez@ira.cinvestav.mx.

TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Relevant characteristic	Source or reference
Strains		
<i>E. coli</i>		
JM103	Commercial strain used for cloning	36
DH5 α F'	Commercial strain used for cloning	BRL
TOP10F'	Commercial strain used for cloning	Invitrogen
<i>P. syringae</i> pv. phaseolicola		
NPS3121	Wild type	29
UIKcat	<i>argK::cat</i> mutant of NPS3121; Tox ⁺	This study
UIFtet	<i>argF::tet</i> mutant of NPS3121; Tox ⁺	This study
AT3	<i>amtA::aph</i> mutant of NPS3121; Tox ⁻	9
Plasmids		
pUIKL1	<i>argK::cat</i> in pU1511 for gene replacement	This study
pUIMC1	<i>argF::tet</i> in pUIGM1 for gene replacement	This study
pMW4	Chloramphenicol cassette	19
pUIRM504	Tn5 <i>cat</i> delivery plasmid; Tc ^r Km ^r	14
pUI511	<i>argK</i> cloned into pUC19	21
pUIGM1	<i>argF</i> cloned into pUC19	G. Mosqueda, personal communication
pUC19	<i>bla lacZ'</i> Ap ^r	36

results in a Tox⁻ phenotype (9). The *argK* and *amtA* genes are located on a 270-kbp *PmeI* fragment of the *P. syringae* pv. phaseolicola chromosome and have a low G+C content compared with the bacterial genome (2, 9). These characteristics and other evidence suggest that the genes involved in phaseolotoxin biosynthesis, together with the *argK* gene, may have been acquired by *P. syringae* pv. phaseolicola by horizontal transfer at some point during evolution (31, 32).

Previously reported Tox⁻ mutants obtained by chemical mutagenesis have been shown to be unable to synthesize phaseolotoxin and also to lack ROCT (4, 25). We have obtained a well-characterized *amtA* mutant with a stringent Tox⁻ phenotype, and we sought to analyze the expression of *argK* in this mutant, in order to investigate whether expression of *argK* had been compromised. Contrary to our expectations, *argK* was overexpressed in the *amtA* mutant. Therefore, to understand this result and to further investigate the regulation of the system, we decided to construct *argK* and *argF* null mutants and to analyze their behavior in terms of growth, phaseolotoxin production, and expression of *argK* at 18 and 28°C.

We confirmed that ROCT, which is coded by *argK*, is essential for *P. syringae* pv. phaseolicola under conditions of phaseolotoxin synthesis, as it ensures an optimal supply of the arginine required for growth and for phaseolotoxin synthesis. Additionally, we demonstrated that carbamoylphosphate is able to induce *argK* expression, bypassing the temperature control. Carbamoylphosphate is a compound resembling the inorganic moiety of phaseolotoxin, *N'*-sulfodiaminophosphinyl, which strongly suggests that *argK* is being directly regulated by a molecule that may be a precursor of phaseolotoxin and only indirectly by temperature, which directly regulates genes involved in the synthesis of phaseolotoxin.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *P. syringae* pv. phaseolicola was grown on King's B medium (KB) (29) or M9 medium (30) at 18 or 28°C. *Escherichia coli* was grown in Luria-Bertani medium at 37°C. For phaseolotoxin production, *P. syringae* pv. phaseolicola was grown in M9 medium

at 18°C for 48 h. When required, the following supplements were added (with quantities given in micrograms per milliliter): carbenicillin, 100; tetracycline, 10; kanamycin, 50; chloramphenicol, 50; and rifampin, 50.

Construction of the *P. syringae* pv. phaseolicola mutants. Mutants were obtained by double recombination events, replacing the wild-type gene of interest with the interrupted allele in the *P. syringae* pv. phaseolicola chromosome.

(i) **UIKcat.** The *argK* gene in plasmid pUI511 (21) was interrupted at the *MluI* site with a 1.9-kbp *SalI-SmaI* fragment containing the chloramphenicol resistance gene (*cat*) from pWM4 (15). The resulting plasmid, pUIKL1, was introduced by electroporation into *P. syringae* pv. phaseolicola, and chloramphenicol was used to select for double recombination events. The fidelity of the double recombination was confirmed by Southern blot hybridization.

(ii) **UIFtet.** The *argF* gene in plasmid pUIGM1 was interrupted at the *NruI* site with a 1.9-kbp *DraI-HpaI* fragment containing the tetracycline resistance gene (*tet*) from pUIRM504 (14). The resulting plasmid, pUIMC, was introduced by electroporation into *P. syringae* pv. phaseolicola, and tetracycline was used to select for double recombination events. The fidelity of the double recombination was confirmed by Southern blot hybridization.

Phaseolotoxin bioassays. Phaseolotoxin production by *P. syringae* pv. phaseolicola and mutants was assayed by the *E. coli* growth inhibition assay (33) as previously described (9, 32).

Molecular biology techniques. Routine techniques were performed as described previously (30). Plasmids and DNA from agarose gels were purified with Qiagen (Valencia, Calif.) columns and kits. Restriction enzymes were used according to the instructions provided. Chromosomal DNA from *P. syringae* pv. phaseolicola was obtained as described previously (1). DNA fragments used as probes for Southern blotting were labeled with fluorescein by use of the ECF Random Primer Labeling kit, and signal detection was performed with the ECF Signal Amplification system, both from Amersham Pharmacia Biotech (Piscataway, N.J.). For image processing and quantification, a Molecular Dynamics (Sunnyvale, Calif.) Storm 860 apparatus with ImageQuant version 1.1 software was used.

Northern blot analysis. Total RNA was extracted from *P. syringae* pv. phaseolicola cells by using the Trizol reagent according to the supplier's instructions (GIBCO). Genomic DNA was removed by digestion with RNase-free DNase (Roche). Samples of total RNA (20 μ g) were denatured by treatment with formamide and separated by electrophoresis using 1.5% agarose gels that contained formaldehyde. The RNA was transferred to nylon membranes (Hybond N+; Amersham) and cross-linked by exposure to UV radiation. For hybridization of the nylon membranes, Church buffer was used (30). Hybridization was performed by using the following DNA probes: a 1.2-kbp *HincII-EcoRV* fragment of *argF* from plasmid pUIGM1 and a 1.0-kbp PCR-amplified fragment of *argK* from plasmid pUI511. The probes were labeled with [α -³²P]dCTP by using the RediPrime Random Primer labeling kit (Amersham). Hybridization was carried out overnight at 65°C. The membranes were washed with 2 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate)-0.1% sodium dodecyl sulfate for 3 min at room temperature, followed by a wash with 1 \times SSC-0.1% sodium

dodecyl sulfate for 3 min at 65°C. Densitometry of the Northern blotting results was carried out by considering the bands plus the smear as the positive signal. The relative transcript level is related to the value of the positive control, which was defined as 100%.

Induction assays with carbamoylphosphate. *P. syringae* pv. phaseolicola grown overnight at 28°C was inoculated into 50 ml of M9 medium to an initial optical density at 600 nm of 0.1. The culture was grown at 28°C to the end of the log phase. At this point, carbamoylphosphate (carbamyl phosphate disodium salt; Sigma) was added to the culture at different concentrations. Samples were collected at the appropriate times and were centrifuged, and the bacterial pellet was used to isolate RNA. The supernatant was used to perform phaseolotoxin assays.

RESULTS

The *argK* gene is overexpressed in the *amtA* mutant. AT3, a mutant in which the *amtA* gene (coding for the amidinotransferase involved in the synthesis of homoarginine) is affected, and which does not synthesize phaseolotoxin, was obtained previously (9). To determine whether *argK* was expressed in this mutant background, AT3 was grown under conditions allowing phaseolotoxin production. Total RNA was extracted from this strain and used to identify *argK*-specific transcripts by Northern blot analysis. Hybridization revealed two different transcripts of around 1.2 and 2.3 kbp as well as a smear, all *argK* specific; the shorter transcript had the size expected of a monocistronic *argK* message (Fig. 1). This pattern of hybridization has been observed previously, and alternative transcription start sites have been postulated as a possible explanation (8). The results obtained indicated that despite the Tox^- phenotype of strain AT3, not only was *argK* expressed, but the level of expression was higher than those observed for the wild-type strain.

Effect of an *argK* mutation in *P. syringae* pv. phaseolicola grown under conditions of phaseolotoxin synthesis. In order to further investigate the regulation of the system, an *argK*-null mutant, UIKcat, was constructed. To assess its phenotype, this strain was grown under conditions allowing phaseolotoxin production. It might be expected that this mutant strain would not be viable under these conditions because of the loss of immunity due to the absence of a functional ROCT. However, it was observed that the mutant could grow, although at a lower rate than that for the wild-type strain (Fig. 2). Addition of 20 mM arginine to the medium restored growth to wild-type levels, suggesting that the major cause for growth retardation was limitation of this amino acid.

A possible explanation for the observed phenotype was that UIKcat was producing little or no phaseolotoxin, allowing some SOCT activity to be retained. To test this idea, we conducted a bioassay to detect phaseolotoxin in the supernatant of UIKcat grown at 18°C in M9 medium. A small and diffuse inhibition halo was detected (Fig. 3B); this halo was the product of phaseolotoxin, since inhibition could be reversed by the addition of 10 mM arginine to the assay medium.

Northern blot analysis revealed that *argF* was strongly expressed in the UIKcat mutant under conditions of phaseolotoxin production (Fig. 3A), supporting the view that residual SOCT activity at suboptimal phaseolotoxin concentrations allowed substantial, although limiting, arginine synthesis.

Effect of an *argF* mutation in *P. syringae* pv. phaseolicola grown at 28°C. To investigate in more detail any possible involvement of *argF* in the regulatory mechanisms involved in

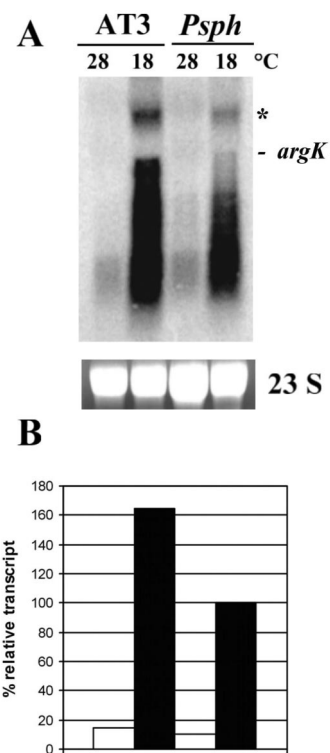


FIG. 1. Expression of the *argK* gene in the *amtA* mutant background. (A) Northern blot of total RNA from *P. syringae* pv. phaseolicola (*Psph*) and the *amtA* mutant (AT3) grown in rich medium (KB) at 28 and 18°C. *P. syringae* pv. phaseolicola grown in rich medium (KB) at 18°C was used as a positive control. The blot was hybridized with a radioactively labeled 1-kbp PCR product of *argK*. 23S rRNA stained with ethidium bromide is shown as a loading control. The signal corresponding to the monocistronic *argK* message is marked. Asterisk indicates the position of a band of approximately 2.3 kbp corresponding to a possible alternative *argK* transcript (see the text for details). (B) Densitometric analysis of the Northern blot. The relative transcript level is expressed as a percentage of the transcript level for *P. syringae* pv. phaseolicola grown in KB at 18°C, which was set at 100%.

the synthesis of phaseolotoxin, a null mutant was obtained by interrupting the *argF* gene. After selection of double recombination events for homogenization, preliminary screening of putative mutants was performed by replicating and growing them in M9 medium at 28°C on the premise that, since *argK* should not be expressed at this temperature, ROCT would not be present and the mutants should behave as arginine auxotrophs. However, all presumptive double recombinants grew under these conditions. Five independent double recombinants were chosen and retested for their ability to grow in solid and liquid M9 media at 28°C. They were all capable of substantial growth, with a growth rate comparable to that normally observed for the wild-type strain. Their genotypes were also confirmed by Southern blot hybridization, showing that all mutants were bona fide double recombinants (data not shown). One strain, UIFtet, was chosen for further analysis.

If UIFtet was growing at 28°C in M9 medium, then for some reason the ROCT would have to be replacing the function of SOCT, suggesting that a second mechanism, in addition to thermoregulation, was available to allow expression of *argK* under otherwise nonpermissive conditions.

To test this hypothesis, expression from *argK* in UIFtet

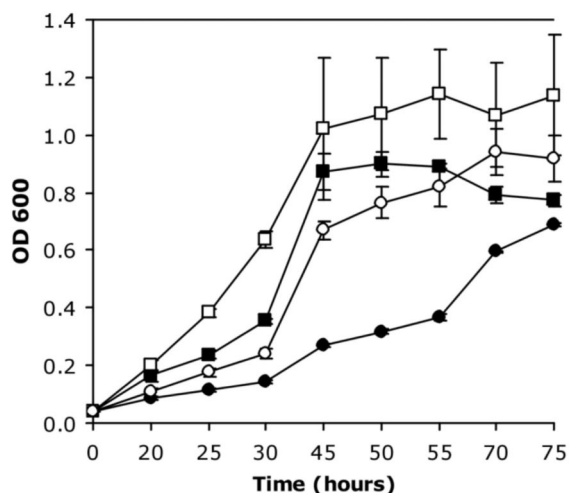


FIG. 2. Phenotype of the *argK* mutant grown under conditions of phaseolotoxin synthesis. *P. syringae* pv. phaseolicola and the *argK* mutant (UIKcat) were grown in M9 liquid medium at 18°C. Growth was monitored by measuring absorbance at 600 nm. Squares, *P. syringae* pv. phaseolicola; circles, UIKcat; solid symbols, growth in unsupplemented M9 medium; open symbols, growth in M9 medium supplemented with 20 mM arginine. Each data point is the average from triplicate experiments. OD 600, optical density at 600 nm.

grown at 28°C in M9 medium was analyzed by Northern blot analysis. The results obtained confirmed that *argK* was indeed being transcribed under these conditions (Fig. 4); however, no phaseolotoxin was detected by use of the growth inhibition assay (data not shown).

Induction of *argK* with carbamoylphosphate in cultures grown at 28°C. From the results obtained, it appeared that induction of *argK* was being mediated by a temperature-independent regulatory mechanism. A model of regulation had been proposed previously in which, at the permissive temperature for phaseolotoxin production, an inducer molecule could bind the postulated repressor protein of *argK* to release it from the *argK* operator and allow expression of this gene. It had also been proposed that such an inducer could be a precursor of phaseolotoxin or phaseolotoxin itself (21). Therefore, it was possible to imagine that in the mutant growing at 28°C, citrulline synthesis would be blocked and both precursor molecules, ornithine and carbamoylphosphate, could accumulate. On the other hand, the chemical structure of carbamoylphosphate resembled the proposed structure of the inorganic moiety of phaseolotoxin, *N'*-sulfodiaminophosphinyl (35) (Fig. 5). This idea prompted us to test whether carbamoylphosphate could be used as an inducer of *argK* expression.

To verify this, *P. syringae* pv. phaseolicola was grown in M9 medium at 28°C until the end of the logarithmic phase. At this point, carbamoylphosphate was added to the culture at final concentrations of 1, 5, and 10 mM. The cultures were further incubated for 30, 60, and 120 min under agitation at 28°C. Total RNA was extracted from each culture and used to perform Northern blot analysis by using a fragment of the *argK* gene as the probe. The *argK* transcript was detected under all the conditions tested (data not shown). Additionally, growth inhibition bioassays were conducted for each sample, but no phaseolotoxin was detected in any case. To investigate any

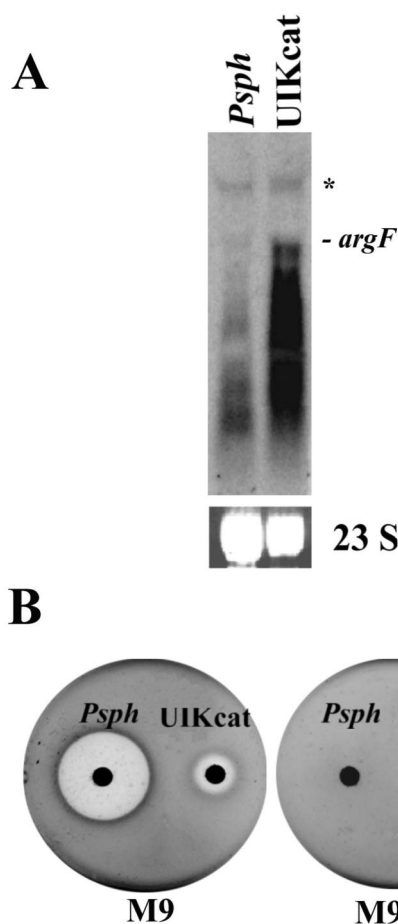


FIG. 3. (A) Expression of *argF* in the *argK* mutant background. Shown is a Northern blot of total RNA from *P. syringae* pv. phaseolicola (*PspH*) and the *argK* mutant (UIKcat) grown in M9 medium at 18°C. The blot was hybridized with a radioactively labeled 1.2-kbp fragment of *argF*. 23S rRNA stained with ethidium bromide is shown as a loading control. The signal of the size expected for a monocistronic *argF* message is marked. Asterisk indicates the position of a secondary band of ≈ 2.2 kbp that also hybridizes with *argF*. (B) Phaseolotoxin production by the *argK* mutant. Phaseolotoxin produced by *P. syringae* pv. phaseolicola and the *argK* mutant grown in M9 medium at 18°C was evaluated by using the *E. coli* growth inhibition assay. M9, *E. coli* JM103 plus M9 medium; M9 + R, *E. coli* JM103 plus M9 medium supplemented with 10 mM arginine.

possible dose-response effect, we conducted a second induction experiment exposing wild-type *P. syringae* pv. phaseolicola to carbamoylphosphate at concentrations ranging from 1 mM to 10 nM for 30 min. These results show a dose-response pattern, indicating that expression from *argK* is indeed induced by carbamoylphosphate. The highest level attained was only approximately 60% of the level observed for the wild type growing at 18°C, a finding consistent with carbamoylphosphate functioning as a replacement instead of the original inducer (Fig. 6).

DISCUSSION

It was previously reported that *Tox⁻* mutants of *P. syringae* pv. phaseolicola also showed impaired ROCT synthesis, suggesting a coordinated response of genes coding for enzymes

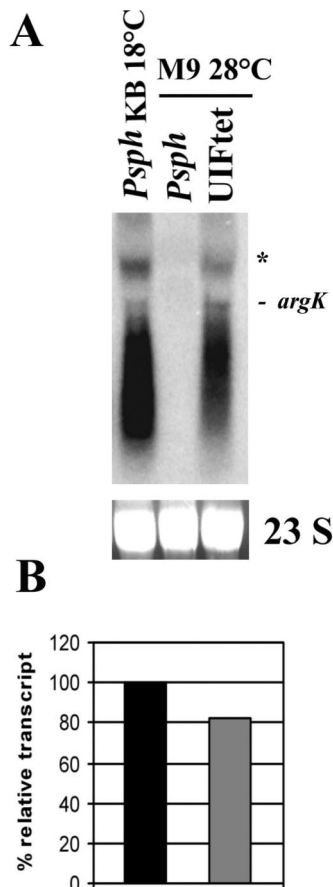


FIG. 4. Expression from *argK* in the *argF* mutant background. (A) Northern blot of total RNA from *P. syringae* pv. phaseolicola (*Psph*) and the *argF* mutant (UIFtet) grown in M9 medium at 28°C. *P. syringae* pv. phaseolicola grown in rich medium (KB) at 18°C was used as a positive control. The blot was hybridized with a 1-kbp PCR product of *argK* labeled with [α - 32 P]dCTP. 23S rRNA stained with ethidium bromide is shown as a loading control. The signal that has the size expected for a monocistronic *argK* message is marked. Asterisk indicates the position of a secondary transcript of \approx 2.3 kbp. (B) Densitometric analysis of the Northern blot. The relative transcript level is expressed as a percentage of the level of the positive control, which was defined as 100%.

involved in the synthesis of phaseolotoxin and the *argK* gene, coding for the ROCT (3, 25). Hernández-Guzmán and Alvarez-Morales had previously obtained a well-characterized Tox^- mutant that was unable to synthesize the amidinotrans-

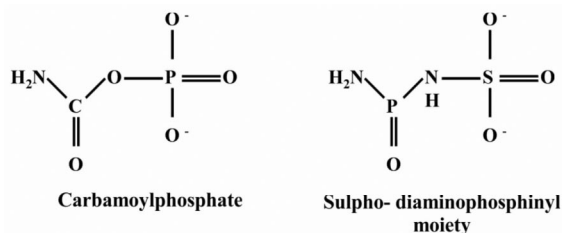


FIG. 5. Carbamoylphosphate and the inorganic moiety of phaseolotoxin (*N'*-sulfodiaminophosphinyl) present very similar molecular structures, and both compete for the carbamoylphosphate binding site of OCTase.

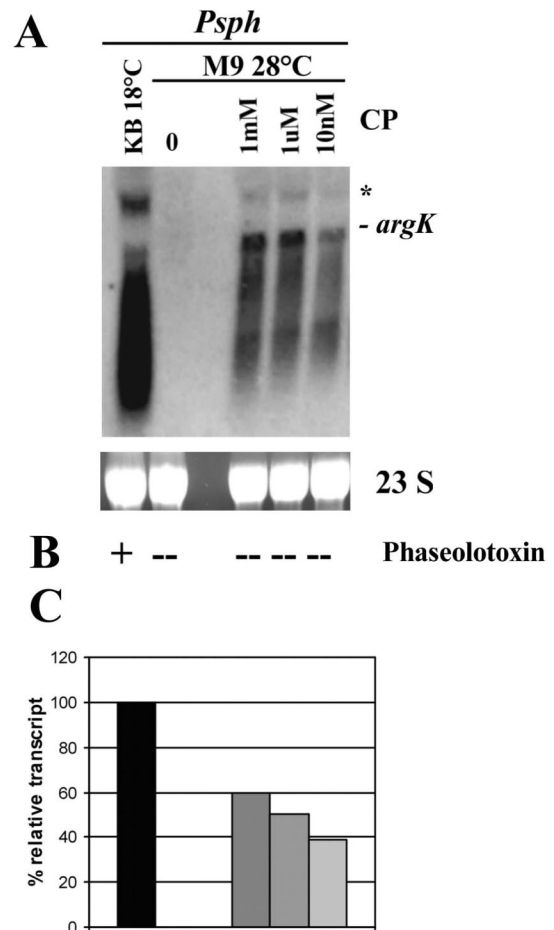


FIG. 6. Induction of *argK* expression by carbamoylphosphate under nonpermissive conditions for the synthesis of phaseolotoxin. (A) Northern blot of total RNA from *P. syringae* pv. phaseolicola (*Psph*) grown at 28°C in M9 medium supplemented with different concentrations of carbamoylphosphate (CP) (none, 1 mM, 1 μ M, and 10 nM) and induced for 30 min. *P. syringae* pv. phaseolicola grown in rich medium (KB) at 18°C was used as a positive control. The blot was hybridized with a 1-kbp PCR product of *argK* labeled with [α - 32 P]dCTP. 23S rRNA stained with ethidium bromide is shown as a loading control. The signal that has the size expected for a monocistronic *argK* message is marked. Asterisk indicates the position of a secondary transcript of \approx 2.3 kbp. (B) Phaseolotoxin synthesis in the supernatant was evaluated by using the *E. coli* growth inhibition assay. +, presence of phaseolotoxin; -, absence of phaseolotoxin. (C) Densitometric analysis of the Northern blot. The relative transcript level is expressed as a percentage of the level of the positive control, which was defined as 100%.

ferase required for the biosynthesis of homoarginine, and possibly unable to synthesize enough ornithine, both molecules being required for the synthesis of the tripeptide moiety of phaseolotoxin (9). When we grew this mutant under conditions of phaseolotoxin synthesis to assess the expression of *argK*, it was clear that not only was the gene expressed, but expression occurred at higher levels than those in the wild-type strain. This result led us to revise other assumptions we had made about this system.

Since regulation of the *argK* gene, coding for the ROCT, had been proposed to be directly mediated by temperature (28), it

became interesting to obtain an *argK*-null mutant and to assess its phenotype when it was grown under conditions promoting phaseolotoxin synthesis. Under such conditions, this mutant might have been expected to be lethal. Also, it was possible that a null mutation in *argF*, encoding SOCT, would cause the mutant to behave as an arginine auxotroph when grown at 28°C.

Testing these ideas, we found that an *argK* mutant unable to produce ROCT was capable of growing and producing phaseolotoxin when cultured in minimal medium at 18°C, although at reduced levels compared to those of the wild-type strain. Northern blot data for this strain showed that expression of the *argF* gene was taking place under these conditions; this could account for the phenotype observed. It is known that in *Pseudomonas aeruginosa* the *argF* gene is negatively regulated by arginine and the repressor molecule ArgR (23, 24); the same situation has been shown to occur in *P. syringae* pv. phaseolicola (J. L. Hernández-Flores, K. Lopez-Lopez, R. Garcidueñas-Piña, A. Jofre-Garfias, and A. Alvarez-Morales, unpublished data). Therefore, when the *argK* mutant UIKcat is grown at 18°C, there is no ROCT to provide the arginine required to sustain growth; thus, the levels of this amino acid must be low and the *argF* operon should be derepressed. However, even though the enzyme is being synthesized, its enzymatic activity is severely reduced due to the presence of phaseolotoxin, and the consequence of this reduction is a steady low level of arginine and a constant state of derepression for *argF*, which we observe in Fig. 3.

When we tested the phenotype of the *argF* mutant strain UIFtet, cultured in minimal medium at 28°C, it did not behave as we expected, that is, like an arginine auxotroph. Suppression selected by the culture conditions, such as a second mutation in the repressor molecule negatively regulating *argK*, could also result in such a phenotype. However, suppressors are expected to appear at very low frequencies, whereas the phenotype observed represented the behavior of the whole mutant population.

The fact that strain UIFtet was capable of growing at 28°C indicated that *argK* was escaping thermoregulation and being expressed. Northern blot experiments using an *argK*-derived probe showed that indeed the *argK* gene was being expressed to levels that could account for the phenotype observed.

Why was *argK* derepressed at 28°C? We had previously postulated that a precursor of phaseolotoxin, or the toxin itself, could act as an inducer that would bind to the postulated repressor molecule when cells were grown at 18°C, relieving repression. The fact that *argK* was being expressed in the *amtA* mutant indicated that homoarginine could be ruled out as an inducer, since this molecule is not produced by this strain. Ornithine and alanine, the other two amino acids present in the organic moiety, were not likely candidates, because of their ubiquity. A molecule that could play this role might be the inorganic moiety of phaseolotoxin, the *N'*-sulfodiaminophosphinyl group. The chemical structure of this molecule strongly resembles the structure of carbamoylphosphate, and this molecule, when bound to ornithine to form PSOrn, results in a transition state analogue of an unstable intermediate catalyzed by OCTase (12, 17).

We assumed that in the *argF* mutant grown at 28°C, lacking SOCT, the reaction synthesizing citrulline from carbamoyl-

phosphate and ornithine would not proceed and these precursors would then accumulate. Could derepression of *argK* be induced by the accumulated carbamoylphosphate? As expected, *P. syringae* pv. phaseolicola growing in minimal medium at 28°C did not show any expression from *argK*; however, upon addition of carbamoylphosphate to the medium, *argK* was derepressed even though the temperature was kept at 28°C and phaseolotoxin could not be detected. This finding indicates that carbamoylphosphate has an effect only on the *argK* gene, not on those genes involved in biosynthesis of the toxin.

This result strongly suggests the following: (i) *argK* is subject to induction with negative control, where the inducer molecule appears to be the *N'*-sulfodiaminophosphinyl group resembling carbamoylphosphate; (ii) *argK* is not directly regulated by temperature, but coordination with phaseolotoxin synthesis is mediated through the synthesis of the inducer, which occurs at the lower permissive temperatures.

Based on this idea, we can then attempt to explain the phenotype of *argK* overexpression in the *amtA* mutant when it is cultured at 18°C. Amidinotransferase is an enzyme that transfers the amidino group of L-arginine to L-lysine to produce homoarginine and ornithine (13); both of these amino acids are present in the tripeptide moiety of phaseolotoxin. Therefore, in the absence of amidinotransferase, the *N'*-sulfodiaminophosphinyl group may accumulate because of the lack of homoarginine and possibly limiting amounts of ornithine. The accumulated *N'*-sulfodiaminophosphinyl molecule could then be responsible for the continuous expression of *argK*, resulting in overexpression of *amtA* in this mutant.

It has previously been suggested that the postulated repressor molecule that acts on *argK* may also exert a regulatory action on other genes involved in the synthesis of phaseolotoxin, because mobility shift experiments show that a protein present only in cells growing at 28°C binds specifically both to the TRR motifs and to fragments derived from *argK* (28). DNA fragments carrying the TRR motif have been shown to override thermoregulation, presumably due to the titration of the repressor molecule (27, 28). However this is not the case when only fragments derived from *argK* are used; in this case, lower affinity of the repressor for these DNA regions in *argK* has been postulated as responsible for this lack of titration (28). Our results indicate that *argK* is not directly thermoregulated, whereas some other genes involved in the synthesis of phaseolotoxin seem to respond to temperature. Therefore, it is possible that more than one repressor protein is found in cells growing at 28°C: one species recognizing the TRR motif and responding to temperature changes, and a second species, a repressor molecule, acting on the *argK* gene, presumably with an allosteric site for recognition of the inorganic moiety of phaseolotoxin and with only a limited affinity for sequences similar to the TRR motif.

From the data obtained as part of this work, we propose a working model for the induction of genes involved in phaseolotoxin synthesis in *P. syringae* pv. phaseolicola growing at 18°C. This model includes the following steps (Fig. 7). (i) Temperature downshifts are sensed through an as yet unidentified mechanism. The signal is processed and transduced to some effector molecule that will act upon the repressor (R_1 in Fig. 7) of phaseolotoxin genes. (ii) At 28°C, genes involved in the synthesis of phaseolotoxin, such as *amtA*, are negatively

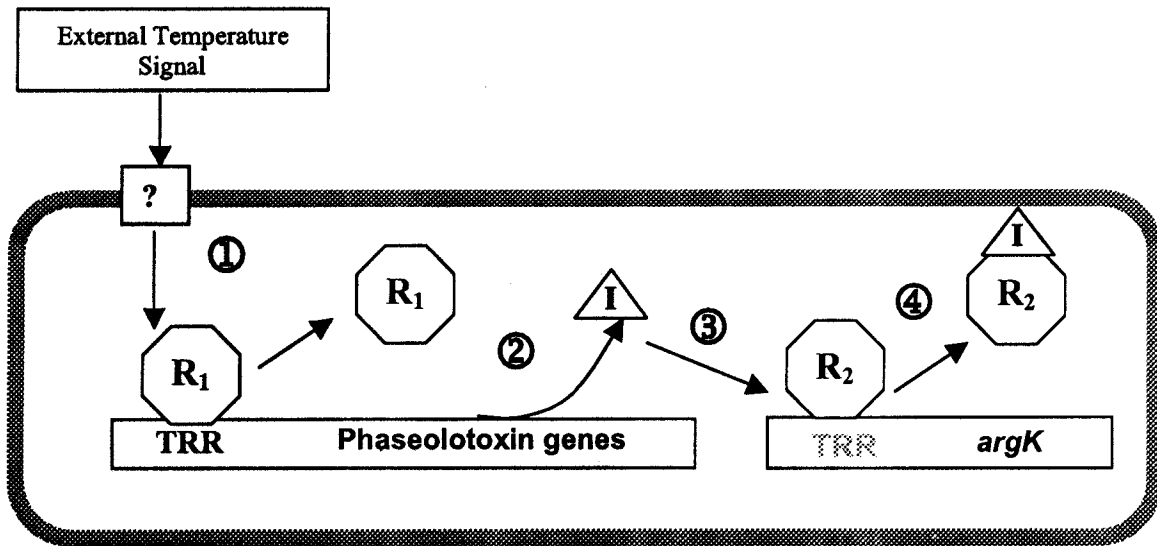


FIG. 7. Model for the induction of genes involved in phaseolotoxin synthesis in *P. syringae* pv. *phaseolicola* growing at 18°C. The question mark represents an unknown signal-transducing element; TRR, specific motifs that have been postulated to be involved in the thermoregulation of phaseolotoxin synthesis; R₁, a repressor molecule that may bind to TRR motifs; I, inorganic moiety of phaseolotoxin (*N*'-sulfodiaminophosphinyl); R₂, an *argK*-specific repressor molecule. For an explanation of the model, see the text.

regulated by a repressor molecule (R₁) that may bind to TRR motifs; when the temperature is downshifted to 16 to 18°C, the signal is processed and relieves repression mediated by R₁. These genes are then actively expressed. (iii) The gene products from the phaseolotoxin genes synthesize both the inorganic (I) and peptide moieties of phaseolotoxin. (iv) The inorganic moiety resembling carbamoylphosphate acts as an inducer of *argK* by binding to an *argK*-specific repressor molecule (R₂) and removing it from its DNA target sequence. (v) Expression of *argK* takes place, and ROCT is synthesized to provide arginine and, via the amidinotransferase reaction, homoarginine and ornithine for cell growth and phaseolotoxin synthesis.

This model can account for the results we have obtained and discussed in the present work, as well as for other observations, such as the phenotypes observed for some Tox⁻ mutants that do not produce ROCT. In this case the mutations may be affecting the synthesis of the inorganic moiety, and therefore no inducer is available to relieve repression of *argK*.

Further work to investigate the thermoregulation of genes involved in the synthesis of phaseolotoxin, as well as to characterize genes that may be involved in the synthesis of the inorganic moiety, is currently under way in our laboratory.

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