Isolation and Characterization of the Gene Coding for the Amidinotransferase Involved in the Biosynthesis of Phaseolotoxin in *Pseudomonas syringae* pv. *phaseolicola*

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Pseudomonas syringae pv. *phaseolicola* is the causal agent of the “halo blight” disease of beans. A key component in the development of the disease is a nonhost-specific toxin, N^δ-(N′-sulphodiaminophosphinyl)-ornithyl-alanyl-homoarginine, known as phaseolotoxin. The homoarginine residue in this molecule has been suggested to be the product of L-arginine:lysine amidinotransferase activity, previously detected in extracts of *P. syringae* pv. *phaseolicola* grown under conditions of phaseolotoxin production. We report the isolation and characterization of an amidinotransferase gene (*amtA*) from *P. syringae* pv. *phaseolicola* coding for a polypeptide of 362 residues (41.36 kDa) and showing approximately 40% sequence similarity to L-arginine:inosamine-phosphate amidinotransferase from three species of *Streptomyces* spp. and 50.4% with an L-arginine:glycine amidinotransferase from human mitochondria. The cysteine, histidine, and aspartic acid residues involved in substrate binding are conserved. Furthermore, expression of the *amtA* and *argK* genes and phaseolotoxin production occurs at 18°C but not at 28°C. An amidinotransferase insertion mutant was obtained that lost the capacity to synthesize homoarginine and phaseolotoxin. These results show that the *amtA* gene isolated is responsible for the amidinotransferase activity detected previously and that phaseolotoxin production depends upon the activity of this gene.

Additional keywords: arginine, gene mapping, homoarginine biosynthesis, ornithine metabolism.

Pseudomonas syringae pv. *phaseolicola* is the causal agent of the “halo blight” disease of beans (*Phaseolus vulgaris* L.), which is characterized by a discrete necrotic lesion surrounded by a chlorotic halo. A key component in the development of the disease is a nonhost-specific toxin produced by the bacterium known as phaseolotoxin. This toxin, N^δ-(N′-sulphodiaminophosphinyl)-ornithyl-alanyl-homoarginine is a

tripeptide with an inorganic moiety (Mitchell 1976; Mitchell 1984; Moore et al. 1984). Phaseolotoxin is hydrolyzed by plant peptidases at the ornithyl-alanyl and alanyl-homoarginine peptide bonds and a smaller molecule, N^δ-(N′-sulphodiaminophosphinyl)-ornithine (PSorn), is produced (Mitchell 1984; Mitchell 1991; Mitchell and Bielecki 1977). PSorn is the most active form of the toxin (Templeton et al. 1985). In *Escherichia coli*, the toxin is transported into the cell via an oligopeptide permease (Staskawicz and Panopoulos 1980).

The target for phaseolotoxin and PSorn is the enzyme ornithine carbamoyltransferase (OCTase: E.C.2.1.3.3.) (Ferguson and Johnston 1980), which catalyses the conversion of ornithine to citrulline in the pathway leading to the biosynthesis of arginine. An effect on the enzyme ornithine decarboxylase of plants also has been reported (Bachmann et al. 1998). Phaseolotoxin is not specific for plant OCTases, and in plants and bacteria, this toxin causes a phenotypic requirement for arginine by efficiently inhibiting OCTase and, as a consequence, stopping arginine biosynthesis, a property that has led to the development of rapid bioassay for phaseolotoxin (Staskawicz and Panopoulos 1979).

Phaseolotoxin is produced at 18 to 20°C, with no detectable synthesis at 28 to 30°C (Goss 1940; Mitchell 1978; Staskawicz and Panopoulos 1979). The mechanism by which *P. syringae* pv. *phaseolicola* is immune to its own toxin is through the synthesis of a phaseolotoxin-resistant OCTase, whose expression is coupled with phaseolotoxin production (Durbin and Langston-Unkefer 1988; Ferguson et al. 1980; Peet and Panopoulos 1987; Staskawicz et al. 1980; Templeton et al. 1986). The phaseolotoxin-resistant OCTase (ROCT) from *P. syringae* pv. *phaseolicola* is the product of the *argK* gene, and bacteria expressing this gene are rendered resistant to phaseolotoxin (Mosqueda et al. 1990). Likewise, transgenic tobacco plants expressing *argK* acquire resistance to phaseolotoxin (De la Fuente-Martinez et al. 1992; Hatziloukas and Panopoulos 1992).

The biosynthesis of phaseolotoxin is not fully understood, and much of it remains speculative (Bender et al. 1999). The synthesis of the unusual amino acid homoarginine (HArg) present in the phaseolotoxin molecule, however, was postulated to involve an enzyme with amidinotransferase (AT) ac-

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tivity. In fact, biochemical activity corresponding to an AT was detected in *P. syringae* pv. *phaseolicola* extracts (Markisch and Reuter 1990; Reuter 1989). The AT activity detected was shown to catalyze the transfer of an amidino group from arginine to lysine to produce one molecule of homoarginine and one molecule of ornithine, both being precursors in the biosynthesis of phaseolotoxin (Fig. 1). This activity is related closely to phaseolotoxin biosynthesis because i) the highest rates of enzyme activity corresponded with the highest rates of phaseolotoxin production during the growth cycle; ii) a *P. syringae* pv. *phaseolicola* mutant producing low amounts of the toxin exhibited low levels of AT activity; iii) only those pathovars of *P. syringae* known to produce phaseolotoxin had any detectable AT activity; and iv) the highest AT activity was observed when cultures were grown at 18°C, whereas the lowest was observed at 30°C, following the temperature-dependent production of phaseolotoxin. Further characterization of this AT indicated an apparent molecular weight of 200 kDa and a K_m of 7.7 mM for arginine and 4.2 mM for lysine (Markisch and Reuter 1990; Reuter 1989).

Amidino transferases catalyze the reversible transfer of an amidino group from L-arginine to the amino moiety of the acceptor compounds (Walker 1973). L-arginine:glycine amidino transferases have been involved in the biosynthesis of creatine in vertebrates (humans, rats, and pigs). In plants of the *Fabaceae* family such as *Galega officinalis* and *Lathyrus sativus*, L-arginine:glycine AT activity has been detected. In *G. officinalis*, this enzyme is involved in the synthesis of guanidino acetic acid, a precursor of galegine. In microorganisms, AT activity has been reported in strains of *Streptomyces* spp., which are known to synthesize streptomycin or hydroxystreptomycin. This activity is involved in the biosynthesis of N,N'-diamidino-streptamine-P, one of the precursors of streptomycin (Markisch and Reuter 1990; Walker 1973).

Human mitochondrial AT (L-arginine:glycine amidino transferase; E.C.2.1.4.1.) and the AT from *Streptomyces griseus* (StrB1; L-arginine:inosamine-phosphate amidino transferase; E.C.2.1.4.2.) are thought to occur naturally as dimers with monomeric subunits of 386 amino acids for the mature polypeptide of human mitochondrial AT and 348 amino acid residues for the polypeptide of StrB1 (Fritsche et al. 1998; Humm et al. 1997).

Amidino transferases are well conserved, showing over 80% sequence similarity among the StrB1 proteins from *S. griseus*, *Streptomyces galbus*, and *Streptomyces glaucescens*. This overall similarity decreases when any of the StrB1 products is compared with StrB2, and it decreases further when compared with mammalian amidino transferases. Nevertheless, in mammalian and bacterial amidino transferases, specific cysteine (Cys), histidine (His), and aspartic acid (Asp) residues have been implicated in substrate binding and are conserved among the different AT sequences available, with the exception of StrB2 of *S. griseus*, which seems to lack enzymatic activity (Fritsche et al. 1998; Humm et al. 1994; Humm et al. 1997).

The high degree of conservation among amidino transferases suggested the possibility of designing specific primers for polymerase chain reaction (PCR)-amplification of sequences of *P. syringae* pv. *phaseolicola* corresponding to an AT. This strategy made it possible to amplify a 718 bp specific fragment from total DNA from *P. syringae* pv. *phaseolicola*, which in turn was used as a probe for the identification of clones carrying the complete chromosomal region of *P. syringae* pv. *phaseolicola* corresponding to this sequence. The nucleotide sequence of the cloned fragment (GenBank accession no. AF186235) shows significant sequence similarity with different amidino transferases from *Streptomyces* spp. and human mitochondrial AT. Furthermore, specific amino acid residues involved in substrate binding also are present in this sequence. A role for this gene in the synthesis of phaseolotoxin has been shown conclusively by interrupting the gene in vitro, exchanging the allele in the wild type *P. syringae* pv. *phaseolicola*, and demonstrating that the mutant lacks the capacity to synthesize phaseolotoxin under permissive conditions. We propose the name *amtA* (amidino transferase) for this gene, which to our knowledge is the first gene of *P. syringae* pv. *phaseolicola* that is described with a direct role in the biosynthesis of phaseolotoxin.

RESULTS

Primer selection and amplification of a putative AT from *P. syringae* pv. *phaseolicola* DNA.

To design primers for PCR amplification of an AT sequence, amino acid sequences from nine different amidino-

Fig. 1. Synthesis of homoarginine. The amidino group from L-arginine is transferred to the amino group of L-lysine to produce homoarginine plus ornithine in the reaction catalyzed by L-arginine:lysine amidino transferase.

transferases were aligned and analyzed to locate regions described to be important for enzyme activity and maintaining a high degree of conservation. These were human Arg–Gly AT (GenBank accession no. S41734), rat Arg–AT (GenBank accession no. AAA21250), pig Gly–AT (GenBank accession no. S40296), *S. griseus strB1* (GenBank accession no. B269984), *S. griseus strB2* (GenBank accession no. XJSMIG), *S. galbus* AT (GenBank accession no. S44227), *S. bluensis* IP–AT (GenBank accession no. S44224), *S. glaucescens strB1* (GenBank accession no. S44229), and *S. glaucescens strB2* (GenBank accession no. S44235). Regions that included an Asp and a Cys residue were chosen for the forward and reverse primer, respectively. Both amino acid residues have been implicated in substrate binding (Humm et al. 1997), and the adjacent residues showed a high degree of conservation.

A consensus for each region was derived from the nine sequences after alignment, and this consensus was used to derive a degenerate oligonucleotide sequence. Degenerate primers were designed from this information. The resulting 23-base forward (5′–3′) primer was TA(C/T)TGCCICGIGA (C/T)GGI(C/T)TI(C/T)T and the reverse (3′–5′) 22-base primer was A(A/G)GT(A/G)AC(A/G)CGITGI(A/G)AICT (A/G)CA.

Previously, we demonstrated that the *argK* gene of *P. syringae* pv. *phaseolicola* is located within a 270-kbp chromosomal fragment derived from a *PmeI* digestion (fragment “I”) (De Ita et al. 1998), and we thought it was possible that the AT gene could be linked to *argK*. With the use of the AT primers in a PCR reaction, either with total DNA or DNA from the *PmeI* I fragment of the *P. syringae* pv. *phaseolicola* chromosome as template, it was possible to amplify a 718-bp DNA fragment. The amplification product was cloned into the pCR2.1 vector (Invitrogen, San Diego, CA, U.S.A.) and sequenced. Comparison of the sequence from the 718 bp fragment to the sequences in the different databases resulted in high scores of similarity with sequences from amidinotransferases. To find out whether the 718 bp fragment corresponded to an AT involved in the biosynthesis of phaseolotoxin, expression of the corresponding gene was assayed at 18 and 28°C. To this end, *P. syringae* pv. *phaseolicola* was grown at 18 and 28°C in minimal medium. Phaseolotoxin production was assayed for both cultures, although production of the toxin occurred only in the culture grown at 18°C. Total RNA was extracted from both *P. syringae* pv. *phaseolicola* cultures, separated on duplicate gels, and blotted to obtain two similar membranes.

When one of these Northern blots was probed with a labeled fragment from the *argK* gene, a signal was observed from the culture grown at 18°C and not from the culture grown at 28°C (Fig. 2). A similar result was obtained on the second membrane with the 718 bp PCR product as probe, suggesting that the gene from which the PCR product had been derived was transcribed in a temperature-dependent manner and followed the pattern of *argK* expression and phaseolotoxin production. The migration of the signal suggested a mRNA of approximately 1.4 kb.

Southern blot analysis of total DNA from *P. syringae* pv. *phaseolicola* digested with different enzymes such as *HindIII* and probed at low stringency with the 718 bp PCR-product revealed a single hybridization signal, suggesting that there was a single AT gene in *P. syringae* pv. *phaseolicola* as well as a single copy of it. Therefore, the PCR-amplified sequence

could correspond to a sequence derived from the postulated AT enzyme involved in phaseolotoxin synthesis.

Isolation and comparative analysis of the chromosomal sequence for the putative amidinotransferase from *P. syringae* pv. *phaseolicola*.

Total DNA from *P. syringae* pv. *phaseolicola* was obtained and digested with *HindIII*. The digestion products were separated on a preparative 0.8% agarose gel. A narrow strip of gel was cut off, transferred to a nylon membrane, and probed with the 718 bp PCR product. The corresponding area of the preparative gel where the hybridization signal was observed was excised and the DNA was purified. This material was cloned into *HindIII*-digested and dephosphorylated pUC19_{Mob} vector (pUC19_{Mob} is pUC19 with a 2.1 kbp fragment containing the *oriT* and *Mob* region from RP4 cloned into the unique *SapI* site) (A. Alvarez-Morales, unpublished).

Clones carrying the chromosomal region corresponding to the 718 bp PCR product were detected by colony hybridization. Two positive colonies were isolated and plasmid DNA prepared from them and subjected to restriction analysis. Both clones were identical, each carrying a 4.2 kbp insert. One

Fig. 2. Temperature-dependent expression of the putative amidinotransferase gene from *Pseudomonas syringae* pv. *phaseolicola*. RNA was isolated from *P. syringae* pv. *phaseolicola* cells growing in liquid M9 medium at 18 and 28°C. The AT probe was the 718-bp polymerase chain reaction product. The *argK* probe was a 964-bp *SstI*–*SphI* fragment generated from the plasmid pUI511 (Mosqueda et al. 1990). The molecular weight marker used was the 0.29–9.5 kb RNA ladder (Life Technologies, Rockville, MD, U.S.A.).

clone, pUIGH1, was chosen, and a preliminary restriction map obtained. This map allowed us to position the 718 bp PCR product within this clone and choose suitable restriction fragments for sequencing directly from within this fragment. The restriction map and sequence obtained are shown in Figure 3.

A sequence of 1,475 residues was analyzed with WU-BLASTX+BEAUTY software (Baylor College of Medicine, Houston, TX, U.S.A.). High scores were obtained with amidinotransferases from eukaryotes and *Streptomyces* spp. (data not shown). The analysis for the sequence showed an open

Fig. 3. Restriction map of the *Hind*III fragment containing the AT gene. **A**, The approximate size of the clone is 4.3 kbp and the putative open reading frame for the amidinotransferase is indicated by the black arrow. The *Pst*I-*Bgl*III fragment (black line) was used as a probe in subsequent hybridization assays. The thin narrow line corresponds to the 1,475-pb fragment sequenced and submitted to GenBank (accession no. AF186235). **B**, *Bam*HI; *Bg*, *Bgl*III; *E*, *Eco*RI; *H*, *Hind*III; *Nc*, *Nco*I; *P*, *Pst*I; *S*, *Sal*I; *Sc*, *Scal* and *Sm*, *Sma*I. **B**, The nucleotide sequence shown is that submitted to GenBank, together with the corresponding translation product of the cloned amidinotransferase. The putative Shine-Dalgarno and terminator sequences are underlined.

reading frame (ORF) of 1,089 bp, with a coding capacity of 362 amino acids (41.36 kDa). The deduced amino acid sequence was then aligned with known amidinotransferases from human mitochondria and *S. galbus* StrB1 with MegAlign software (DNASTAR, Madison WI, U.S.A.) (Fig. 4). The results of the alignment indicated that the AT from *P. syringae* pv. *phaseolicola* had significant residue identities with *S. galbus* and human mitochondria (31.6 and 46.7%, respectively). Among these identities are residues found on the *P. syringae* pv. *phaseolicola* polypeptide such as Asp 195, His 244, and Cys 346 that belong to the proposed catalytic triad; Asp 117, Met 243, Asp 246, Arg 263, and Ser 294, which are residues involved in hydrogen bond formation with the arginine substrate; and a Cys residue at position 34 (Humm et al. 1997).

To investigate the possibility that the gene coding for the AT of *P. syringae* pv. *phaseolicola* could be involved in the

but not from the AT3 mutant. AT activity was measured by its ability to synthesize homoarginine and ornithine from the substrates lysine and arginine, although ornithine also may be synthesized through alternative pathways (Table 1). The observation that homoarginine is being detected, even in conditions when the substrate only contained arginine and not lysine, can be explained, assuming that the lysine already present in the cell-free extract is used. The increase in the amount of homoarginine obtained when lysine and arginine are used as substrate may not be substantial (50.65%). We believe that this may be a consequence of the low concentrations of amino acids used (10 mM) to avoid the high background that resulted with higher levels of these amino acids.

The production of phaseolotoxin followed a similar pattern. With the use of 50 µl of supernatant, the plate bioassay showed a large zone of inhibition of growth of the *E. coli* strain JM103 with supernatant from the wild-type culture, whereas no inhibition was observed when supernatant from the AT3 mutant was used. When growth inhibition was measured in the microtiter plate assay, the results of the plate assay were confirmed. In both assays, inhibition could be totally reversed by adding arginine to a final concentration of 10 mM to either the M9 plates or the M9 liquid medium, thus showing that inhibition is a consequence of the produced phaseolotoxin.

When the correlation between phaseolotoxin production and gene expression from *argK* and the AT gene was measured, mRNA from both genes could be detected only when the plate assay for phaseolotoxin started to show the presence of toxin by inhibiting *E. coli* growth (Fig. 5). Taken together, these results show that the gene we cloned corresponds to an amidinotransferase, and that phaseolotoxin production is dependent upon the activity of this enzyme.

Probing for the presence of AT gene coding in different strains and pathovars of *P. syringae*.

To test whether the AT gene cloned was present in other *P. syringae* pathovars or strains, we hybridized an AT probe to total DNA from *P. syringae* pathovars *atropurpurea*, *glycinea*, *maculicola*, FF5, and *tomato* and to the *P. syringae* pv. *phaseolicola* Mexican isolate Texcoco at high and low stringency. The result of the Southern blot analysis is shown in Figure 6. Only phaseolotoxin-producing pathovars had an AT gene, which in turn suggests that there is no other role for this enzyme, except to produce homoarginine and, perhaps, ornithine to be used in the synthesis of phaseolotoxin.

Locating the AT gene on the physical map of *P. syringae phaseolicola*.

A physical map for *P. syringae* pv. *phaseolicola* was obtained previously in this laboratory. It includes 15 *PacI* frag-

ments (1 to 15) and 16 *PmeI* fragments (A to P) (De Ita et al. 1998). The *argK* gene coding for the phaseolotoxin-resistant OCTase maps within *PacI* fragment 8 and *PmeI* fragment I. To localize the putative AT gene cloned in the physical map of *P. syringae* pv. *phaseolicola*, we hybridized the AT probe to *PacI*- or *PmeI*-digested chromosomal DNA at low and high stringency. The AT probe hybridized to fragments 7 and I of the *PacI* and *PmeI* digestions, respectively. Fragment 7 is contiguous to fragment 8 and overlapping band I (Fig. 7). This result showed that the *argK* gene and the gene coding for the AT activity involved in phaseolotoxin biosynthesis are located within the 270-kbp I fragment.

DISCUSSION

It was proposed that an enzyme with AT activity was involved in the synthesis of the homoarginine residue present as one of the three amino acids in the organic moiety of phaseolotoxin (Markisch and Reuter 1990; Reuter 1989). We sought

Fig. 5. Correlation of growth, phaseolotoxin production, and gene expression from *argK* and the AT gene. *Pseudomonas syringae* pv. *phaseolicola* was grown in M9 at 18°C and aliquots were taken every 5 h to detect phaseolotoxin production in the supernatant with the plate assay (see text). Growth was followed measuring absorbance at 600 nm (empty circles). Phaseolotoxin production (filled circles) was measured as the size of the growth-inhibition zone (cm). The AT and *argK* messengers were detected as described in text. The 23S rRNA image corresponds to the ethidium-bromide stained gel. The asterisk corresponds to t_0 because the t_5 sample was not measured.

Table 1. Measurement of amidinotransferase activity in cell-free extracts of *Pseudomonas syringae* pv. *phaseolicola*

Strain	Temperature	Homoarginine (µmoles) ^a		Ornithine (µmoles) ^a	
		R substrate	RK substrate	R substrate	RK substrate
<i>P. syringae</i> pv. <i>phaseolicola</i> wt	18°C	0.77	1.16	18.36	36.12
	28°C	0.00	0.00	16.91	26.80
<i>P. syringae</i> pv. <i>phaseolicola</i> AT3	18°C	0.00	0.00	20.20	20.85
	28°C	0.00	0.00	19.92	15.22

^a The values are means of at least two repeats.

to identify and isolate the gene responsible for this AT activity by generating an AT-specific probe by PCR with primers derived from conserved regions of different AT sequences derived from *Streptomyces* spp., human, rat, and pig. The resulting primers were very specific because, when annealed to total DNA from *P. syringae* pv. *phaseolicola* at 56°C, a single 718 bp PCR amplification product was detected.

When this fragment was labeled and used to probe RNA obtained from *P. syringae* pv. *phaseolicola* grown at 18 and 28°C, it was clear that expression from the corresponding gene was thermoregulated, showing significant expression only at the lower temperature and following a pattern of expression similar to that obtained for *argK*, the gene coding for the phaseolotoxin-insensitive OCTase. This data strongly suggested that we had obtained a fragment from the AT gene involved in homoarginine biosynthesis.

When a genomic clone from *P. syringae* pv. *phaseolicola* was identified, a 1,475 nucleotide fragment was sequenced. This fragment contains a single ORF of 1,089 bp whose translation product corresponded to an AT. Analysis of a sequence located at the 3' end showed the potential to form a stem-and-loop structure ($\Delta G' = -3.02$ at 37°C), which suggests a Rho-independent terminator. The observation that the mRNA signal observed in the Northern blots corresponds to a molecule of approximately 1.4 kb suggests that the gene is transcribed as a single operon. Because we do not know how this mRNA is being degraded, however, there is still the possibility that the AT gene may be part of a larger operon.

The translation product of the 1,089-bp ORF corresponded to a protein composed of 362 amino acid residues with an

apparent molecular weight of 41.36 kDa. The number of amino acids found in this ORF correlates with those obtained for other ATs such as the mature polypeptide of human mitochondrial AT and StrB1, with 386 and 348 amino acid residues, respectively. The difference in the apparent molecular weight of 200 kDa, initially determined for the AT of *P. syringae* pv. *phaseolicola* by comparison with elution volumes of reference proteins (Markisch and Reuter 1990), and the 41.36 kDa corresponding to the AT we have isolated remains to be resolved.

The unusual fact that the AT sequence shows a higher similarity to the human mitochondrial enzyme than to the *Streptomyces* spp. AT could be a result of the nature of the recipient molecule involved in the reaction. In the case of the *Streptomyces* spp. enzyme, the molecule is inosamine-phosphate, an intermediate in the synthesis of streptomycin, whereas in the case of the *P. syringae* pv. *phaseolicola* and human mitochondrial enzymes, the recipients are both the amino acids lysine and glycine, respectively. Furthermore, inosamine-phosphate ATs are reported to have a rather broad substrate specificity for the amidino donor and recipient (Fritsche et al. 1998), which may account for the differences observed between the *Streptomyces* spp. and *P. syringae* pv. *phaseolicola* ATs at the amino acid sequence level.

When expression of the cloned AT gene was analyzed in the context of phaseolotoxin production, it was clear that expression of this gene was thermoregulated in a similar fashion to that observed for *argK* (Fig. 4). Furthermore, expression of these genes was observed prior to or immediately after the onset of phaseolotoxin production, which started 15 h after

Fig. 6. Identification of the amidinotransferase gene in *Pseudomonas syringae* pv. *phaseolicola* pathovars. The pictures are the ethidium bromide-stained gel and the corresponding Southern blot of chromosomal DNA from different *P. syringae* strains, which were digested with *Hind*III, electrophoresed, and hybridized at low-stringency conditions with the AT probe as described in text. 3121, *P. syringae* pv. *phaseolicola* NPS3121; Tx, *P. syringae* pv. *phaseolicola* Texcoco; M, 1 kbp ladder; a, *atropurpurea* 1304; g, *glycinea* 18a/90; m, *maculicola* 438; p, *pisi*; FF5 a noncharacterized isolate; t, *tomato* DC3000.

incubation at 18°C. An *argK*-specific transcript is detectable 10 h after growth at the permissive temperature for phaseolotoxin production, and the signal increases rapidly in the next 5 h and continues to increase, but at a very low rate. AT expression, however, seems to lag behind *argK* expression, and its mRNA is detectable only after 15 h, showing a maximum 10 h later and then decreasing. Whether this pattern of expression is a true indication of the regulation of the system or is influenced strongly by mRNA turnover, medium composition, or any other factors remains to be investigated. What is clear from the results shown in Figure 4, however, is that expression of the cloned gene is related closely to *argK* expression and phaseolotoxin production.

We obtained a loss-of-function mutant to show unambiguously that the gene coded for an AT involved in the synthesis of phaseolotoxin. The resulting AT⁻ mutant lost the ability to synthesize homoarginine and phaseolotoxin when grown in M9 medium at 18°C, conditions in which the wild-type strain efficiently synthesized both of these compounds. This result showed conclusively that the gene we cloned was an AT and was directly involved in the synthesis of phaseolotoxin.

An additional test of our conclusion regarding the identity of the cloned gene was the search for a similar gene in other *P. syringae* pathovars (Fig. 5), where we found the gene coding for AT activity was present only in the *P. syringae* pv. *phaseolicola* pathovars, which suggests that this gene plays a role only in the synthesis of phaseolotoxin.

It is interesting to note that although we could obtain the AT⁻ mutant through in vitro mutagenesis with relative ease, earlier attempts to obtain Tox⁻ mutants through the use of wild-type and modified transposon mutagenesis apparently

never succeeded in disrupting this gene nor any other gene directly involved in the synthesis of phaseolotoxin (Marsch-Moreno et al. 1998; Peet et al. 1986; our unpublished data). The target sequence for Tn5 is A-GNTYWRANC-T (Goryshin et al. 1998), we were unable to locate such sequences in the fragment corresponding to the AT gene. This could explain why it was not possible to obtain a Tn5 insertion in this gene, although it still remains to be explained why, at least in our hands, Tn5 mutagenesis never yielded a full Tox⁻ mutant, despite the fact that more genes involved in the synthesis of the tripeptide and the inorganic moiety of the toxin should be required to be fully functional for *P. syringae* pv. *phaseolicola* to synthesize phaseolotoxin.

Recently it was shown that animal-pathogen bacteria such as *Salmonella* spp., *Yersinia* spp., or *E. coli* have genes involved in pathogenicity that are clustered in what is known as "pathogenicity islands" (Blanc-Potard et al. 1999; Buchrieser et al. 1998; Perna et al. 1998). The same is true for *P. syringae* pv. *phaseolicola* strain 1449B, which carries virulence and avirulence genes clustered as a pathogenicity island on a large plasmid (Jackson et al. 1999). We mapped the AT gene to a 270 kbp *PmeI* fragment of the *P. syringae* pv. *phaseolicola* chromosome. The *argK* gene also is located within this fragment, although we do not yet know the distance between both genes. We are currently sequencing the chromosomal region between both genes, however, searching for other gene(s) that may be involved in the biosynthesis of phaseolotoxin and will try to determine whether they constitute a cluster of pathogenicity genes or a pathogenicity island.

The AT gene we identified is the first gene of *P. syringae* pv. *phaseolicola* involved directly in the synthesis of phaseolotoxin, which has been isolated and described and that we propose to be called *amtA*. At the moment, our laboratory is directing its efforts to investigate how this gene is regulated compared with *argK*.

MATERIALS AND METHODS

Media, antibiotics, and supplements.

Pseudomonas syringae pv. *phaseolicola* NPS3121 (Peet et al. 1986), also referred to as the Riverside strain (De Ita et al. 1998), as well as other strains of *P. syringae* (pathovars *atro-purpurea* 1304, *glycinea* 18a/90, *maculicola* 438, *phaseolicola* isolate Texcoco, *pisi*, FF5, and *tomato* DC3000) were routinely grown on King's B medium (KB) (King et al. 1954) or M9 minimal medium (Sambrook et al. 1989) at 28°C. For phaseolotoxin production, *P. syringae* pv. *phaseolicola* was grown in M9 minimal medium at 18°C for 36 h. The *E. coli* strains DH5 α (Life Technologies, Rockville, MD, U.S.A) and JM103 (Messing 1983) were routinely grown in Luria-Bertani and M9 media at 37°C. Supplements added were 50 μ g of kanamycin per ml, 10 mM arginine, 40 μ M isopropyl- β -D-thiogalactopyranoside, and 30 μ g of X-Gal per ml.

Growth condition for phaseolotoxin production.

Sterile 250-ml Erlenmeyer flasks containing 50 ml of M9 medium were inoculated with an overnight culture of *P. syringae* pv. *phaseolicola* to an initial optical density (OD) at 600 nm of 0.1. These flasks were incubated at 18°C at 200 rpm. At the appropriate times, flasks were taken and the entire culture centrifuged at 7,000 rpm for 8 min in a Sorvall SL-50T rotor.

Fig. 7. Localization of the AT gene in the physical map of *Pseudomonas syringae* pv. *phaseolicola*. The amidinotransferase gene was located by hybridization to bands 7 and I of the *PacI* and *PmeI* digestions of total DNA from *P. syringae* pv. *phaseolicola*. The actual distance between *argK* and the AT gene has not yet been determined.

The bacterial pellet was used to isolate RNA (Aiba et al. 1981) or to produce cell-free extracts to determine AT activity. The supernatant was used to determine phaseolotoxin.

Phaseolotoxin assays.

Production of phaseolotoxin routinely was assessed by the *E. coli* growth inhibition assay (Staskawicz and Panopoulos 1979) and modified by Sawada et al. (1997) with *E. coli* JM103 as an indicator strain. Plates with 10 mM arginine were included to reverse the effect of phaseolotoxin, and 50 µl of each supernatant was placed onto 0.8-cm diameter Whatman paper (Clifton, NJ, U.S.A.), dried in a laminar-flow chamber, and placed on the respective M9 plate. Alternatively, a microtiter plate assay was used to confirm the presence or absence of phaseolotoxin in the supernatant of the different strains assayed. In this case, M9 medium was inoculated (2%) with a fresh overnight culture of the *E. coli* strain JM103, and 200 µl of this suspension was added to the wells plus 0 to 35 µl of the supernatants from the *P. syringae* pv. *phaseolicola* cultures to be assayed. The plate was incubated at 37°C under agitation, and growth of *E. coli* was followed every hour by measuring the OD₄₉₂.

AT assay.

Initially, we tried a method, essentially as described previously (Van Pilsum et al. 1970), except that L-arginine and cadaverine were used as the amidino donor and acceptor, respectively. The natural amidino acceptor, lysine, for the AT of *P. syringae* pv. *phaseolicola* could not be used in this assay because it developed a strong color on reaction with ninhydrin under the conditions of the assay. It has been reported previously, however, that the AT activity present in the extracts of *P. syringae* pv. *phaseolicola* could utilize cadaverine as an amidino acceptor with a specific activity comparable to the level of the arginine-lysine combination (Markisch and Reuter 1990). We found that cadaverine did not react with ninhydrin under the conditions of the assay, although with our use of cadaverine as substrate, the results were quite inconsistent. Therefore, cell-free extracts from cultures grown at 18 or 28°C were prepared as described (Van Pilsum et al. 1970) and incubated at 37°C for 1 h with an Arg-substrate buffer (L-arginine 40 mM) or an Arg-Lys-substrate buffer (L-arginine and L-lysine, 10 mM each). The reaction was stopped with 0.6 N perchloric acid. To identify homoarginine and ornithine, samples were derivatized with phenyl-isothiocyanate (Bidlemeier et al 1984) and passed through a PicoTag column coupled to high pressure liquid chromatography (HPLC) (Waters, Milford, MA, U.S.A.), with an acetonitrile:water (3:2 vol/vol) mixture as eluting solvent.

Molecular techniques.

Routine techniques were performed as described previously (Sambrook et al. 1989). Plasmids and DNA from agarose gels were purified with Qiagen (Valencia, CA, U.S.A.) columns and kits. Restriction enzymes were used according to the manufacturer's instructions. Chromosomal DNA from *Pseudomonas* spp. was obtained as described (Chen and Kuo 1993). DNA fragments used as probes were labeled with fluorescein with the ECF Random primer labeling kit, and signal detection was performed with the ECF signal amplification system (both from Amersham Pharmacia Biotech, Piscataway,

NJ, U.S.A.) in a Storm 860 apparatus with Image Quant 1.1 software (both from Molecular Dynamics, Sunnyvale, CA, U.S.A.) for image processing.

Pulsed-field gel electrophoresis (PFGE) conditions were as described previously (De Ita et al. 1998). After staining, the area corresponding to the *PacI* I fragment was excised, stored in Tris-EDTA buffer at 4°C, and used as DNA template for PCR.

Sequencing was performed with the ABI 377 Perkin Elmer automatic sequencing system (Applied Biosystems, Foster City, CA, U.S.A.) and the Thermo Sequenase kit with dye terminator (Amersham Life Sciences, Little Chalfont, Buckinghamshire, U.K.) on overlapping clones from specific primers designed from the sequence data that was obtained. Both strands were sequenced on a 1,475-bp fragment containing the AT gene (Fig. 2).

PCR conditions.

Chromosomal DNA from *P. syringae* pv. *phaseolicola* was digested with *PmeI*, and the digestion products were separated on a low-melting-point agarose gel after PFGE. The 270-kb I fragment was extracted, and approximately 1 mm³ from the agarose strip was used as DNA template. Alternatively, 1 ng of chromosomal DNA also was used as template. A "hot start" was used, followed by 30 cycles of 1 min at 94°C, annealing 1 min at 50°C, and polymerization 1 min at 72°C, with a final polymerization step of 5 min at 72°C.

Hybridization techniques.

For Southern blots, 5 µg of chromosomal DNA was routinely digested with the appropriate restriction enzyme. The fragments were separated in 0.8% agarose gels, and DNA was transferred to a Hybond membrane (Amersham) and cross linked by exposure to UV. The probe concentration was approximately 10 ng per ml.

For Northern blots, 5 µg of RNA was loaded per lane. The RNA was transferred to Hybond membranes and hybridized with the AT or *argK* probes. The membranes were washed with 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) for 15 min at room temperature, followed by a second wash with preheated 0.5× SSC and 0.1% SDS at 60°C for 5 min.

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