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The Global Arginine Regulator ArgR Controls Expression of *argF* in *Pseudomonas syringae* pv. phaseolicola but Is Not Required for the Synthesis of Phaseolotoxin or for the Regulated Expression of *argK*†

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In *Pseudomonas syringae* pv. phaseolicola the enzyme ornithine carbamoyltransferase (OCTase), encoded by *argF*, is negatively regulated by *argR*, similar to what has been reported for *Pseudomonas aeruginosa*. However, production of the phaseolotoxin-resistant OCTase encoded by *argK*, synthesis of phaseolotoxin, and infectivity for bean pods occur independently of the ArgR protein.

Pseudomonas syringae pv. phaseolicola causes halo blight disease in common bean (*Phaseolus vulgaris* L.), which is characterized by water-soaked lesions surrounded by chlorotic haloes. Chlorosis is caused by a non-host-specific toxin known as phaseolotoxin [N^{δ}-(N'-sulfodiaminophosphinyl)-ornithyl-alanyl-homoarginine] (15, 16), which is produced ex planta when bacteria are grown in minimal medium at temperatures between 18 and 20°C and is not detected at 28°C (7, 14, 19, 26). The target of phaseolotoxin is the enzyme ornithine carbamoyltransferase (OCTase) (EC 2.1.3.3) (4), which catalyzes the conversion of ornithine and carbamoylphosphate to citrulline in the arginine biosynthesis pathway.

In *P. syringae* pv. phaseolicola the *argF* and *argK* genes code for phaseolotoxin-sensitive OCTase and phaseolotoxin-resistant OCTase, respectively (5, 9, 10, 27). *argK* is expressed at 18°C and ensures a supply of arginine for both cell growth and phaseolotoxin synthesis, since arginine is a substrate for an amidinotransferase encoded by *amtA* (8) that catalyzes the synthesis of homoarginine and ornithine (12). *argK* and *amtA* have been shown to be located close to each other on the chromosome of *P. syringae* pv. phaseolicola (8), and their relative proximity, as well as their G+C content, which differs from that of other genes in this bacterium, is consistent with the hypothesis that the genes involved in phaseolotoxin synthesis were horizontally acquired (24, 25).

There is evidence that *argK* is negatively regulated at 28°C, and it has been suggested that the repressor of *argK* binds to specific sequences designated the thermoregulatory region (TRR) (22, 23). It has been shown previously that in fact *argK* is not directly regulated by temperature but most likely is regulated by a precursor of phaseolotoxin resembling carbamoylphosphate (11). In *Pseudomonas aeruginosa argF* is repressed when growth occurs in media that provide high levels of exogenous arginine, such as King's medium B (KB), together with the repressor protein ArgR, which also represses the *carAB* operon involved in carbamoylphosphate biosynthesis (20, 21). ArgR is a global regulator of the AraC/XylS family (3, 6, 20) and is also required for expression of genes involved in arginine catabolism (*aru*) and uptake and transport (*aot-argR* operon) (18, 21). The following findings are interesting: (i) both *argF* and *argK* are negatively regulated; (ii) these two genes code for enzymes that catalyze the same enzymatic reaction; (iii) the repressor molecule involved in *argK* regulation seems to be capable of interacting with carbamoylphosphate (11); (iv) an open reading frame with homology to a regulatory molecule belonging to the AraC family has been reported to be in the phaseolotoxin gene cluster (28); and (v) in *Pseudomonas*, ArgR is a global regulator that negatively regulates *argF* and is also involved in regulation of carbamoylphosphate synthesis.

Could the ArgR product of *P. syringae* pv. phaseolicola be involved in the regulation of *argK* or the synthesis of phaseolotoxin? By using *argR* from *P. aeruginosa* as a probe, the corresponding gene from *P. syringae* pv. phaseolicola NPS3121 was identified and isolated from a genomic library. Analysis of this sequence with BLAST-N (1) and BLAST-X showed that it exhibited 54.5% similarity with *argR* from *P. aeruginosa* PAO1 at the nucleotide level and that the level of similarity of the translation products is 81.5% (*argR* from *P. syringae* pv. phaseolicola, GenBank database accession number AAL35898).

Construction of an *argR*-defective mutant. A 60-bp fragment from the middle of the *argR* structural gene was replaced with a 1.8-kbp DraI/HpaI tetracycline resistance cassette from pUIRM504 (13). Double recombinants were selected as tetracycline-resistant clones and were confirmed by Southern blotting (data not shown). One mutant, UILH13R, was analyzed further, and its phenotype was identical to that reported for the *argR* mutant of *P. aeruginosa*. Thus, UILH13R was not able to use arginine as a sole carbon source (20), and it constitutively expressed *argF*; in KB and M9 medium the OCTase specific activities of the *P. syringae* pv. phaseolicola wild-type strain

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† This work is dedicated to the memory of our friend and colleague Esther de Ita Morales.

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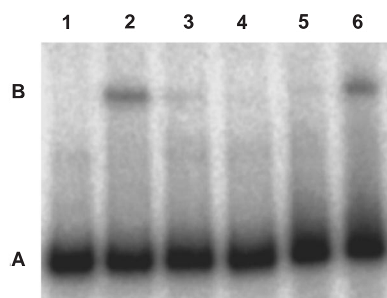

FIG. 1. Gel retardation assay performed with extracts from the *P. syringae* pv. phaseolicola wild-type strain and the *argR* mutant UILH13R. Lane 1, free *argF* promoter probe; lane 2, *argF* promoter probe plus extract from the wild-type strain grown in rich medium at 28°C; lane 3, *argF* promoter probe plus extract from the wild-type strain grown in minimal medium at 28°C; lane 4, *argF* promoter probe plus extract from UILH13R grown in rich medium at 28°C; lane 5, competition with nonlabeled *argF* promoter probe; lane 6, competition with nonlabeled *argK*. "B" indicates the position of retardation signals observed in lanes 2 and 6 and very weak signals in lanes 3 and 5; no retardation signal was observed in lane 4. "A" indicates the position of free probe.

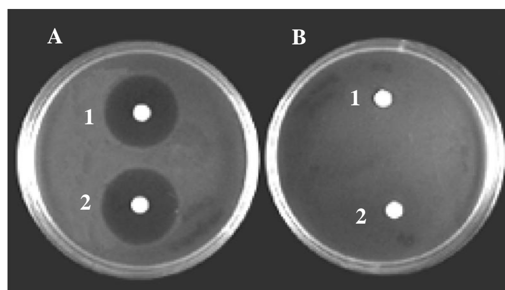

FIG. 2. Phaseolotoxin bioassay. The assay was performed by using supernatants from *P. syringae* pv. phaseolicola (spot 1) and UILH13R (spot 2) grown in minimal medium at 18°C (A) and 28°C (B).

were 0.37 ± 0.09 and 0.95 ± 0.44 nmol of citrulline produced per μg of protein per min (mean ± standard deviation; $n = 3$) at 37°C, respectively, whereas the specific activities of the UILH13R *argR* mutant were 1.23 ± 0.34 and 1.42 ± 0.06 nmol of citrulline produced per μg of protein per min at 37°C, respectively. These values were determined by measuring OCTase specific activity essentially as described by Ceriotti (2) by using 5-ml cultures of both *P. syringae* pv. phaseolicola and the UILH13R mutant grown overnight at 28°C in KB or M9 medium. Cells were disrupted with a VirTis sonicator (model VirSonic 60). For the assay we used 3 μl of crude extract. The reaction mixtures were incubated for 20 min at 37°C, and OCTase activity was determined. The activity measured corresponded to the product of *argF*, the phaseolotoxin-sensitive OCTase, and not to the phaseolotoxin-resistant OCTase encoded by *argK*, since addition of a phaseolotoxin-containing supernatant to the reaction mixture and preincubation for 25 min before OCTase activity was determined eliminated OCTase activity from the samples (data not shown).

Binding of ArgR to the promoter region of *argF*. Gel retardation experiments were carried out by using a 275-bp DNA probe from the *argF* promoter region containing the −35 and −10 regions and the ArgR binding sequence TGTCGCN$_8$AA (21). The reaction mixture [20 mM Tris (pH 8.0), 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, 10 ng of poly(dI-dC) μl^{-1}, 5% glycerol, 5 μg of crude extract] was preincubated for 10 min at room temperature, the radioactively labeled probe ([γ-^{32}P]dATP) was added, and the mixture was incubated for 15 min at room temperature. Samples were loaded onto a 5% polyacrylamide gel in 0.5× Tris-borate-EDTA and electrophoresed for 60 min at 18 mA. A clear retardation signal of the *argF* promoter probe was observed when crude extract from the wild-type strain grown at 28°C in rich medium was added to the retardation mixture, indicating that the ArgR repressor was bound to the operator of *argF* (Fig. 1). As expected, when crude extract from the wild-type strain grown in minimal medium at the same temperature was used, only a weak signal was observed. When crude extract from UILH13R grown in rich medium was used, no retardation signal was detected, confirming that the protein binding to *argF* was the product of *argR*, which is not present in this strain. Specific binding of ArgR to *argF* was demonstrated when the nonlabeled *argF* promoter probe efficiently outcompeted labeled *argF* for binding and the retardation signal disappeared almost completely.

To analyze the possibility that ArgR binds to *argK* DNA, we obtained an *argK* promoter probe containing both the core motif G/CAAAG of the putative binding domain (TRR) involved in temperature-mediated regulation and identified in *argK* (23) and a sequence (TGTCG) similar to the core sequence of the binding site of ArgR (TGTCGCN$_8$AA) (21) located 80 bp upstream of the ATG initiation codon (17). The nonlabeled *argK* promoter probe failed to compete the binding of *argF* by ArgR, clearly indicating that the postulated protein that binds to the *argK* promoter is not the product of *argR*.

It could be argued that the inability of *argK* to compete for binding of ArgR to *argF* could have been predicted because it has been proposed that the repressor would bind to the TRR. However, only weak binding of the putative repressor protein to the TRR sites in *argK* has been postulated (23). Furthermore, the TRR domains in *argK* may not be involved in its regulation since we have shown that this gene is not directly regulated by temperature but most likely is regulated through induction mediated by a precursor of phaseolotoxin resembling carbamoylphosphate (11). Therefore, there seem to be two proteins that bind to the TRR, one protein which binds tightly to strictly temperature-regulated promoters and a different protein which acts as a repressor for *argK* with only weak binding to the TRR and which is not temperature regulated.

Synthesis of phaseolotoxin. Forty-milliliter portions of M9 medium were inoculated with the wild-type strain and mutant UILH13R to obtain an initial optical density at 600 nm of 0.1 and incubated at 18°C. Parallel controls were grown at 28°C. Supernatants were collected after 60 h and used for phaseolotoxin bioassays (26). Supernatants from the wild-type strain and UILH13R grown at 18°C produced similar inhibition patterns in the bioassay (Fig. 2), whereas cultures grown at 28°C did not produce phaseolotoxin, indicating that the absence of ArgR did not have any qualitative or quantitative effect on phaseolotoxin synthesis.

Role of the ArgR regulatory protein in pathogenicity. Bean pod infection assays were carried out by using the wild-type strain and the *argR* mutant. The results clearly showed that

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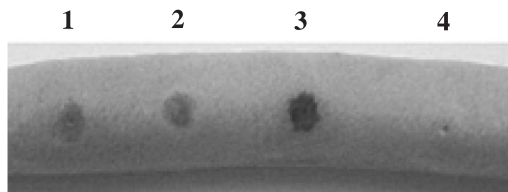

FIG. 3. Pod inoculation assay. Fully developed green pods from susceptible bean plants (*P. vulgaris* cv. Flor de Mayo) were inoculated by puncturing them with toothpicks soaked in fresh cultures of the UILH13R mutant (spot 1), *P. syringae* pv. phaseolicola (spot 2), *P. syringae* pv. tomato DC3000 (spot 3), and sterile distilled water (spot 4). A typical hypersensitive response was observed in spot 3, and no difference in lesion formation was observed between wild-type bacteria (spot 2) and the *argR* null mutant (spot 1). Inoculated pods were incubated inside a sealed plastic container with a wet paper towel in a growth room at 28°C. At this temperature, phaseolotoxin production was not expected to occur, but lesion development was clear. The lesions were examined 4 days postinoculation.

symptom development with the wild-type strain and symptom development with UILH13R were similar (Fig. 3). *P. syringae* pv. tomato DC3000 was included in the assay to observe a typical hypersensitive response. The lack of ArgR in strain UILH13R did not lead to a hypersensitive response in the plants.

The fact that ArgR does not act upon *argK*, the fact that this protein is not involved in the synthesis of phaseolotoxin, and the fact that its absence does not interfere with pathogenicity support the idea that phaseolotoxin synthesis genes were gained by horizontal gene transfer during evolution (24, 25), and therefore there seems to be no metabolic link between the acquired set of genes for phaseolotoxin biosynthesis and the resident genes involved in arginine metabolism.

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