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Comparative Analysis of *Pseudomonas syringae* pv. actinidiae and pv. phaseolicola Based on Phaseolotoxin-Resistant Ornithine Carbamoyltransferase Gene (*argK*) and 16S-23S rRNA Intergenic Spacer Sequences

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Pseudomonas syringae **pv. phaseolicola, which causes halo blight on various legumes, and pv. actinidiae, responsible for canker or leaf spot on actinidia plants, are known as phaseolotoxin producers, and the former possesses phaseolotoxin-resistant ornithine carbamoyltransferase (ROCT) which confers resistance to the toxin. We confirmed that the latter is also resistant to phaseolotoxin and possesses ROCT, and we compared the two pathovars by using sequence data of the ROCT gene and the intergenic spacer region located between the 16S and 23S rRNA genes (16S-23S spacer region) as an index. It was found that the identical ROCT gene (*argK*) is contained not only in bean isolates of *P. syringae* pv. phaseolicola in Mexico and the United States but also in bean isolates in Japan and Canada, and that it is also distributed in the kudzu (*Pueraria lobata*) isolates of *P. syringae* pv. phaseolicola. Moreover, the kiwifruit and tara vine isolates of *P. syringae* pv. actinidiae were also found to possess the identical *argK*. On the contrary, the 16S-23S spacer regions showed a significant level of sequence variation between *P. syringae* pv. actinidiae and pv. phaseolicola, suggesting that these two pathovars evolved differently from each other in the phylogenetic development. The fact that even synonymous substitution has not occurred in *argK* among these strains despite their extreme differences in phylogenetic evolution and geographical distribution suggests that it was only recently in evolutionary time that *argK* was transferred from its origin to *P. syringae* pv. actinidiae and/or pv. phaseolicola.**

Pseudomonas syringae is differentiated into more than 40 types of pathovars with different host ranges (2, 25, 42). Many ice nucleation-active bacteria that are considered to belong to *P. syringae* have also been found on the surface of plant bodies (9, 16). Having adapted to various environments, *P. syringae* has numerous varieties with differentiated properties and is abundant in genetic diversity. Nevertheless, research on the evolution and systematics of *P. syringae* is hindered by an insufficient understanding of the actual conditions of this diversity (2, 42). Given this situation, we are pursuing research with the aim of clarifying the actual conditions of the diversity by comparing *P. syringae* pathovars based on various genetic markers including pathogenicity genes (29a). In this study we selected and compared *P. syringae* pv. actinidiae (30, 34, 39, 40), which causes canker or leaf spot on actinidia plants, and pv. phaseolicola (2, 8), responsible for halo blight on various legumes, out of the many pathovars which exist in *P. syringae*. Both of these pathovars are phaseolotoxin producers (28, 35).

Phaseolotoxin is a non-host-specific toxin that is active not only in plants but in microorganisms including *Escherichia coli* (reviewed in reference 28). The enzyme that is the target of the toxin is ornithine carbamoyltransferase (OCTase) in the biosynthetic pathway of arginine. This means, in turn, that the biosynthesis of L-citrulline from L-ornithine and carbamoylphosphate is inhibited by the toxin. This is thought to be the cause of both the accumulation of L-ornithine and the deficiency of L-citrulline and L-arginine (19, 20, 27, 29). *P. syringae* pv. phaseolicola, which produces phaseolotoxin, possesses phaseolotoxin-resistant OCTase (ROCT), as well as phaseolotoxin-sensitive OCTase (SOCT). Due to the action of ROCT, which is not inhibited by phaseolotoxin, *P. syringae* pv. phaseolicola is resistant to phaseolotoxin produced by itself (4, 14, 32, 36).

In this paper, we confirm that *P. syringae* pv. actinidiae also possesses ROCT, and we sought to compare *P. syringae* pv. phaseolicola and pv. actinidiae by using sequence data of the ROCT genes, which play an important role in phaseolotoxin producers, as an index. Specifically, we used PCR to clone the gene which encodes ROCT from *P. syringae* pv. actinidiae, determined its sequence by direct sequencing, and compared it with the sequences of the ROCT gene (*argK*) of *P. syringae* pv. phaseolicola isolated from diverse sources. In addition, to clarify the phylogenetic relationships between the two pathovars, the sequences of the intergenic spacer region located between the 16S and 23S rRNA genes (16S-23S spacer region) were also determined by direct sequencing and compared, because this region is known to exhibit a great deal of sequence and length variation which is useful for determining the intraspecific relationships of prokaryotes (1, 6, 7, 15).

MATERIALS AND METHODS

Bacterial strains. The sources of bacterial strains and their relevant characteristics are shown in Table 1. Taxonomic positions of *P. syringae* strains were confirmed by identifying their phenotypic features according to standard methods (13, 34).

Confirmation of phaseolotoxin production. *E. coli* test plates were prepared by mixing 10 ml of M9 medium (1.5% agar) kept at 65°C with 200 μl of a log-phase *E. coli* DH1 grown in M9. Test strains were spotted onto the *E. coli* test plates and incubated at 18°C for 3 days. After the incubation period, the diameter of the inhibition zone formed around the spot was recorded. Whether inhibition was reversed by L-arginine, L-citrulline, or L-ornithine was determined by applying 20

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TABLE 1. Bacterial strains used in this study and their ROCT gene and 16S-23S spacer DDBJ accession numbers

Strain	Host plant and location	Source and reference[a]	DDBJ accession no.	
			ROCT gene	16S-23S spacer
P. syringae pv. actinidiae				
Kw-11 (pathotype strain)	*Actinidia deliciosa* Liang et Ferguson (kiwifruit); Shizuoka, Japan	Y. Takikawa (34)	D86356	D86357
MAFF 302091	*A. deliciosa* Liang et Ferguson (kiwifruit); Kanagawa, Japan	MAFF (41, 38)	D86356	D86357
MAFF 302092	*A. deliciosa* Liang et Ferguson (kiwifruit); Kanagawa, Japan	MAFF (41, 38)	D86356	D86357
MAFF 302145	*A. deliciosa* Liang et Ferguson (kiwifruit); Wakayama, Japan	MAFF (38)	D86356	D86357
MAFF 302133	*Actinidia arguta* Planch. (tara vine); Kanagawa, Japan	MAFF (39, 40)	D86356	D86357
P. syringae pv. phaseolicola				
MAFF 302282 (pathotype strain)	*Phaseolus vulgaris* L. (bean); Canada	MAFF	M94049[b]	D86377
MAFF 301673	*P. vulgaris* L. (bean); Ibaraki, Japan	MAFF (23)	M94049	D86377
MAFF 301022	*P. vulgaris* L. (bean); Hokkaido, Japan	MAFF	M94049	D86377
MAFF 301616	*P. vulgaris* L. (bean); Hokkaido, Japan	MAFF	M94049	D86377
KUZ 1	*Pueraria lobata* (Willd) Ohwi (kudzu); Ibaraki, Japan	M. Sato (8)	M94049	D86377
MAFF 301766	*P. lobata* (Willd) Ohwi (kudzu); Ibaraki, Japan	MAFF	M94049	D86379
KUZ 8	*P. lobata* (Willd) Ohwi (kudzu); Yamagata, Japan	M. Sato (8)	M94049	D86378
TMR 327	*P. lobata* (Willd) Ohwi (kudzu); Shizuoka, Japan	Y. Takikawa	M94049	D86378
P. syringae pv. coronafaciens				
MAFF 302257 (pathotype strain)	*Avena sativa* L. (oat); United Kingdom	MAFF		
P. syringae pv. glycinea				
MAFF 302260 (pathotype strain)	*Glycine max* Merrill (soybean); New Zealand	MAFF		
P. syringae pv. maculicola				
MAFF 302264 (pathotype strain)	*Brassica oleracea* L. (cauliflower); New Zealand	MAFF		
P. syringae pv. mori				
MAFF 302279 (pathotype strain)	*Morus alba* L. (white mulberry); Hungary	MAFF		
P. syringae pv. morsprunorum				
MAFF 302280 (pathotype strain)	*Prunus domestica* L. (European plum); ?	MAFF		
P. syringae pv. syringae				
MAFF 302155 (type strain)	*Syringa vulgaris* L. (lilac); United Kingdom	MAFF		
P. syringae pv. tabaci				
MAFF 302270 (pathotype strain)	*Nicotiana tabacum* L. (tobacco); Hungary	MAFF		
P. syringae pv. tomato				
MAFF 302272 (pathotype strain)	*Lycopersicon esculentum* Mill. (tomato); United Kingdom	MAFF		

[a] Abbreviations: MAFF, Ministry of Agriculture, Forestry and Fisheries, Tsukuba, Ibaraki, Japan; Y. Takikawa, Yuichi Takikawa, Faculty of Agriculture, Shizuoka University, Shizuoka, Japan; M. Sato, Mamoru Sato, National Institute of Sericultural and Entomological Science, Tsukuba, Ibaraki, Japan.

[b] All 8 strains of *P. syringae* pv. phaseolicola used in this study contained the identical ROCT gene with *argK* whose sequence is represented by the accession no. M94049 submitted by Mosqueda et al. (21) and Hatziloukas and Panopoulos (11).

μl of these solutions (2 μg/μl) to a paper disk (0.8-cm diameter) placed near the spot of the test strain.

Phaseolotoxin isolation. Phaseolotoxin was isolated from liquid cultures of test strains by the procedure described by Mitchell (18). The toxin was eluted with methanol-chloroform-aqueous ammonia solution from charcoal and evaporated to dryness. The resulting dry material was resuspended in 50 mM Tris-acetate buffer (pH 8.3) and centrifuged, and the supernatant was kept at −20°C as crude toxin preparation. The activities of crude toxin preparations were tested by means of the microbiological assay (31), using *E. coli* DH1 as the indicator strain, and the concentration of the toxin preparation was adjusted to form an inhibition zone with a diameter of 20 mm when a 20-μl volume of the preparation was added to the paper disk placed on the assay plate.

Confirmation of phaseolotoxin resistance. A growth inhibition assay was performed by using toxin plates containing 10 ml of M9 medium (1.5% agar) plus 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and 50 μl of the crude toxin preparation which was isolated from *P. syringae* pv. phaseolicola MAFF 301673 and whose concentration was adjusted as described above. Toxin plates were inoculated by transfer of the test strain with a sterilized toothpick and were incubated at 25°C for 3 days.

The ornithine carbamoyltransferase assay employed is based on that of Pastra-Landis et al. (26) and Nunome (24). To prepare bacterial extracts for OCTase assays, test strains were grown in Luria-Bertani (LB) medium containing 0.1 mM IPTG at 24°C. Cells were harvested, washed three times with 50 mM Tris-acetate buffer (pH 8.3), and sonicated for 3 min on ice. Cell pellets were removed by centrifugation, and supernatants were used immediately in the assays. The reaction mixture (1 ml) contained the bacterial extract, 4.8 mM carbamoylphosphate, 5 mM L-ornithine, and various amounts of the crude toxin preparation isolated from *P. syringae* pv. phaseolicola MAFF 301673 in 50 mM Tris-acetate buffer (pH 8.3). After the bacterial extract was preincubated with the toxin at 25°C for 10 min, the reaction was initiated by adding the substrates of OCTase. Following a 15-min incubation at 25°C, 20 μl of the reaction mixture was spotted onto Silica gel 60 F_{254} a thin-layer chromatography (TLC) plate (Merck) and separated by TLC by using a phenol-water (3:1, vol/vol) solvent system. L-Citrulline was visualized by spraying plates with ninhydrin reagent.

PCR amplification of ROCT gene. Cell lysates of the test strains were prepared by using an InstaGene DNA purification matrix (Bio-Rad Laboratories) according to the supplier's instructions. Before use, the lysates were centrifuged at 12,000 rpm for 3 min, and the resulting supernatants were subjected to PCR as templates.

The following oligonucleotide primers were designed from the published *P. syringae* pv. phaseolicola sequence (21) and used to target the ROCT gene: OCTF, 5′-gtgctgcag TATTACCCTGATGAGCTCGA-3′; OCTR, 5′-cacggatcc GATG ATCGACCTTGTTGACCTCCCG-3′. Lower-case sequences represent linkers with restriction sites, i.e., *Pst*I for OCTF and *Bam*HI for OCTR. The relative priming positions of the primers are indicated in Fig. 2.

PCR amplification of the target sequence was performed in a total volume of 100 μl of the following reaction mixture: 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 μM (each) deoxynucleoside triphosphate, 20 pmol of each primer, 5 μl of template, and 5 U of *Taq* DNA polymerase (TOYOBO). PCR was performed in a DNA thermal cycler 480 (Perkin-Elmer Cetus) by using the following protocol: initial denaturation at 95°C for 2.5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C

for 1 min, and extension at 72°C for 2 min, and then an additional extension at 72°C for 10 min. After PCR, an aliquot of the reaction mixture (2 μl) was separated by electrophoresis in a 2% agarose gel in Tris-acetate buffer, and the amplified DNA fragment was visualized by ethidium bromide staining and transillumination. Every time ROCT genes were amplified by PCR, a negative control (*E. coli* and/or *P. syringae* pv. tabaci) was always used to check for contamination. As a result, absolutely no amplification was detected with the negative control in the experiments presented in this paper.

For Southern blots, PCR products of the test strains were electrophoresed and transferred to a nylon membrane (Hybond-N; Amersham International). The entire fragment of the ROCT gene amplified from *P. syringae* pv. phaseolicola MAFF 301673 was used as a probe. Southern hybridization and detection of the hybridized DNA were carried out with the DIG DNA labeling and detection kit (Boehringer Mannheim) as specified by the manufacturer.

Cloning of amplified ROCT gene. The PCR product was extracted with phenol-chloroform and precipitated with ethanol. The DNA pellet was dissolved in Tris-EDTA buffer and digested with *Pst*I and *Bam*HI. The DNA fragment was obtained by agarose gel electrophoresis followed by excision of the fragment and subsequent purification by the Gene-Clean procedure (Bio 101).

The fragment was ligated to *Pst*I- and *Bam*HI-digested pUC19, and the recombinant molecule was transformed into *E. coli* DH1 by standard methods (17). Plasmids were prepared from selected transformants by the alkaline lysis method (17).

Sequence analysis of ROCT gene. To prepare templates for sequence reaction, ROCT genes were amplified by using OCTF and biotinylated OCTR as PCR primers. The biotinylated PCR products were immobilized onto streptavidin-coated paramagnetic beads (Dynabeads M-280 Streptavidin; DYNAL), and single-stranded DNA templates were prepared following the manufacturer's instructions. Both strands were then sequenced directly by cycle sequencing, using an ALFred autocycle sequencing kit (Pharmacia Biotech) and the following four Cy5-labeled primers (see Fig. 2): OCTF-01, 5′-TTACCCTGATGAGCTCGATAATTCAATG-3′; OCTF-02, 5′-GACCGTCAAGGAAGAATTCGGGCGC-3′; OCTR-01, 5′-CGACCTTGTTGACCTCCCGGAGGTC-3′; and OCTR-02, 5′-ACCGAACTTGAGCGCCCCGATGGCC-3′. The relative priming positions of these primers are shown in Fig. 2. The sequencing products were loaded onto a 6% polyacrylamide gel, and the separation was monitored on-line with ALFred DNA sequencer (Pharmacia Biotech).

PCR amplification of 16S-23S spacer region. PCR amplification of the 16S-23S spacer region was performed as described above, by using the PCR primers (space F-B and space R) which we designed by inspecting the sequences of 16S and 23S rRNA genes conserved in various *Pseudomonas* species. Primer space F-B was immediately adjacent to the 16S-23S spacer region; the sequence of this oligonucleotide was 5′-TTTCACACAGGAAACAGCTATGACGGGGTGAAGTCGTAACAAGGTAGCCG-3′, which corresponded to a conserved sequence at the 3′ end of the 16S rRNA (from position 1486 to position 1511 on *E. coli* numbering [10]). The sequence of primer space R was deduced from an alignment of 23S rRNA 5′ sequences (from position 50 to position 26 on *E. coli* numbering [10]); its sequence was 5′-CGCCAGGGTTTTCCCAGTCACGACATCGCCTCTGACTGCCAAGGCATCC-3′. Both the PCR primers contained the sequences of Cy5-labeled primers for cycle sequencing reactions, which are indicated by an underline (the sequence of primer ALFred M13 reversal was added to primer space F-B, and that of primer ALFred M13-40 was added to primer space R).

Sequence analysis of the 16S-23S spacer region. Using primers space R and biotinylated space F-B, biotinylated PCR products were obtained from which single-stranded DNA templates were prepared and sequenced as mentioned above by using the following Cy5-labeled primers: primer ALFred M13-40 (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′) and primer ALFred M13 reversal (5′-TTTCACACAGGAAACAGCTATGAC-3′).

The sequences obtained were aligned with the CLUSTAL V program (12), and the alignments were then adjusted manually.

Nucleotide sequence accession numbers. The ROCT gene and 16S-23S spacer sequences which we determined have been deposited in the DDBJ (DNA Data Base of Japan, Mishima, Japan) under the accession numbers shown in Table 1. The same sequences within a pathovar are represented by one accession number.

RESULTS AND DISCUSSION

Confirmation of the phaseolotoxin production of the test strains. Many researchers have already confirmed that *P. syringae* pv. phaseolicola produces phaseolotoxin (reviewed in reference 28). Tamura et al. (35) reported that *P. syringae* pv. actinidiae also produces phaseolotoxin. We examined whether the 13 test strains which belong to *P. syringae* pv. actinidiae and pv. phaseolicola (Table 1) actually produce the toxin.

As a result of a bioassay using the *E. coli* test plates, these test strains were confirmed to inhibit the growth of *E. coli* DH1, which is highly sensitive to phaseolotoxin. Further, this inhibiting action was reversed by adding either L-arginine or L-citrulline (data not shown). In addition, a toxic active substance was isolated from all the test strains in accordance with the phaseolotoxin extraction method (18). The administration of this substance resulted in the formation of a yellow halo on a kiwifruit leaf (data not shown). It was further investigated whether this active substance inhibits the citrulline synthesis by OCTase of *E. coli* DH1. The active substance was added along with the substrates of OCTase to bacterial extracts obtained by sonicating *E. coli* DH1. The extracts were developed by using TLC, and citrulline was detected by the ninhydrin reaction. No citrulline was produced by *E. coli* DH1 (Fig. 1, lane 2), which showed that the active substance inhibits the citrulline synthesis of *E. coli* DH1. Thus, all 13 strains of *P. syringae* pv. actinidiae and pv. phaseolicola tested were confirmed to produce phaseolotoxin.

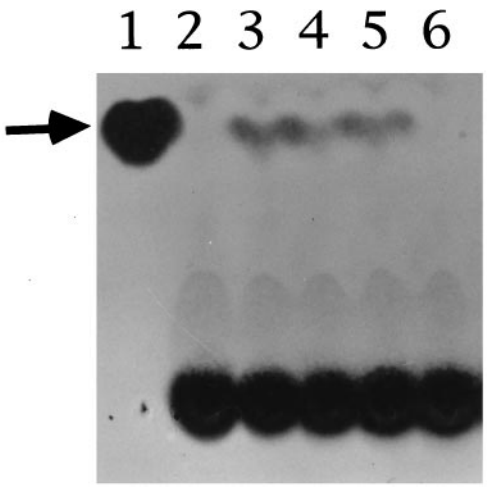

FIG. 1. Ornithine carbamoyltransferase activities in sonic extracts of test strains assayed in the presence of phaseolotoxin. Enzyme assays were performed as described in the text. Citrulline visualized by spraying ninhydrin reagent is indicated by arrow. Lanes: 1, L-citrulline (Wako Pure Chemical) (R_f, approximately 0.38); 2, *E. coli* DH1; 3, *P. syringae* pv. actinidiae Kw-11; 4 and 5, *E. coli* DH1 which harbored pUC19 carrying PCR products amplified from *P. syringae* pv. actinidiae by using the OCTF-OCTR primer set; 6, *E. coli* DH1 (pUC19).

Confirmation of the ROCT activity of the test strains. It was investigated whether *P. syringae* pv. actinidiae actually possesses ROCT as *P. syringae* pv. phaseolicola does (4, 14, 32, 36). First of all, by using M9 medium which contained phaseolotoxin, the resistance of *P. syringae* pv. actinidiae to phaseolotoxin was examined. All the strains of *P. syringae* pv. actinidiae even grew on the M9 medium (10 ml) which contained 50 μl of a crude preparation of phaseolotoxin (data not shown). *E. coli* DH1 tested for comparison, however, did not grow on these toxin plates. Whether phaseolotoxin affects the citrulline synthesis of *P. syringae* pv. actinidiae was then examined by the method described in the previous section. *P. syringae* pv. actinidiae produced citrulline in this assay (Fig. 1, lane 3), showing that the citrulline synthesis of the test strains is free from any inhibition. Thus, *P. syringae* pv. actinidiae was regarded to be resistant to phaseolotoxin and to possess ROCT.

Design of primers for amplifying the ROCT-coding region. Given that the sequence of the ROCT gene (*argK*) of *P. syringae* pv. phaseolicola has already been identified (11, 21), we considered using this sequence for designing primers to clone the amplified products of PCR. Figure 2 shows the *argK* sequence and neighboring sequences of *P. syringae* pv. phaseolicola (21). A forward primer (OCTF) was designed on the basis of the sequence around the Shine-Dalgarno region which pre-

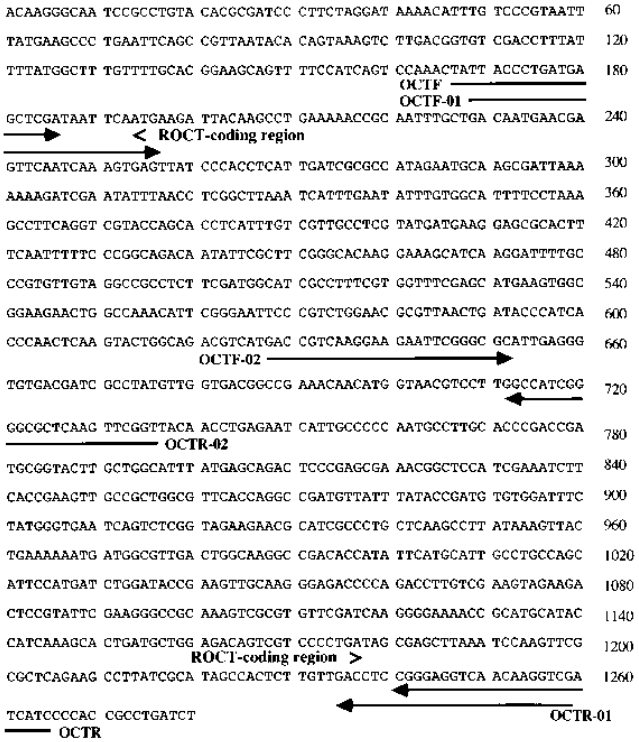

FIG. 2. Nucleotide sequence of the ROCT gene (*argK*) and its neighborhood from *P. syringae* pv. phaseolicola characterized by Mosqueda et al. (21) and Hatziloukas and Panopoulos (11). The first nucleotide shown corresponds to position 1 of Mosqueda et al. (21). The ROCT-coding region (984 bp) is indicated by the angle brackets. Arrows indicate the relative priming positions of the primers used in the PCR and the sequencing analysis of the amplified products. Primers OCTF and OCTR were utilized for amplification, and primers OCTF-01, OCTF-02, OCTR-01, and OCTR-02 were used for sequencing. All 13 test strains of *P. syringae* pv. actinidiae and pv. phaseolicola used in this study (see Table 1) were found to possess the identical ROCT-coding region as that of *argK* shown in this figure.

cedes the start codon of *argK*. Then, a reverse primer (OCTR) was designed on the basis of the sequence around the stem-and-loop terminator structure on the 3′ end of *argK*. For the cloning of the PCR products in succeeding experiments, one *Pst*I or one *Bam*HI restriction site was added to OCTF or OCTR, respectively.

As a result of the PCR conducted with the set of these primers (OCTF and OCTR), PCR products that coincided with the expected size (1,098 bp) were obtained from all 13 strains of *P. syringae* pv. phaseolicola and pv. actinidiae tested (Fig. 3). The degree of amplification and the size of amplified products did not differ among the strains. On the other hand, no amplification was obtained in *E. coli* DH1, DH1 (pUC19), *P. syringae* pv. coronafaciens MAFF 302257, *P. syringae* pv. glycinea MAFF 302260, *P. syringae* pv. maculicola MAFF 302264, *P. syringae* pv. mori MAFF 302279, *P. syringae* pv. morsprunorum MAFF 302280, *P. syringae* pv. syringae MAFF 302155^T, *P. syringae* pv. tabaci MAFF 302270, or *P. syringae* pv. tomato MAFF 302272 (Fig. 3). Using the PCR product obtained from *P. syringae* pv. phaseolicola MAFF 301673 as a probe, Southern hybridization analysis was conducted on the PCR products obtained from the test strains of *P. syringae* pv. actinidiae and pv. phaseolicola. This revealed that every PCR product hybridized with the probe (data not shown).

Cloning of the PCR product and analysis of its function. Given that PCR products could be obtained by using the set of OCTF and OCTR primers, we attempted to clone the product. The PCR product obtained from the pathotype strain (Kw-11) of *P. syringae* pv. actinidiae was ligated to pUC19 which had been treated with *Pst*I and *Bam*HI and then transformed into *E. coli* DH1; this resulted in 16 clones of an ampicillin-resistant transformant. Plasmids were then extracted from these transformants and subjected to *Pst*I and *Bam*HI treatment; every plasmid was shown to contain approximately 1.1 kb of insert (data not shown).

The functions of the obtained recombinants were examined to confirm whether the PCR products were actually the targeted ROCT genes. First of all, it was of interest whether they could grow on M9 medium containing isopropyl-β-D-thiogalactopyranoside and crude preparation of phaseolotoxin. Contrary to DH1 and DH1 (pUC19) which showed no growth, all the recombinants obtained did grow, showing resistance to phaseolotoxin (data not shown). Then, the citrulline synthesis in the presence of phaseolotoxin was examined to learn whether this toxin inhibits the OCTase activity of the recombinants. The result was that the citrulline synthesis of *E. coli* DH1 and DH1 (pUC19) was inhibited by phaseolotoxin (Fig. 1, lanes 2 and 6) but that of the recombinants was not subject to such inhibition (Fig. 1, lanes 4 and 5). Thus, the ROCT genes were confirmed to be amplifiable from *P. syringae* pv. actinidiae by using the set of OCTF and OCTR primers.

Sequence analysis of ROCT genes. We amplified the ROCT genes by using the set of OCTF and OCTR primers, the effectiveness of which was confirmed as described above, and determined their sequences by direct sequencing. The ROCT genes were first amplified from each test strain of *P. syringae* pv. actinidiae and pv. phaseolicola (Table 1) by using OCTF and biotinylated OCTR as primers. Then, after preparing single-stranded DNA from the biotinylated PCR products, their sequences were directly determined in both directions by cycle sequencing. OCTF-01, OCTF-02, OCTR-01, and OCTR-02 installed inside the PCR products were used as the primers for sequencing (Fig. 2).

It was determined that all the sequences of the open reading frame of 984 bp commonly recognized in the PCR products of the 13 test strains coincided completely with each other (Fig. 2). This sequence was further compared with the sequence of *argK* (Fig. 2) which had already been identified by Mosqueda et al. (21) and Hatziloukas and Panopoulos (11) and was found to

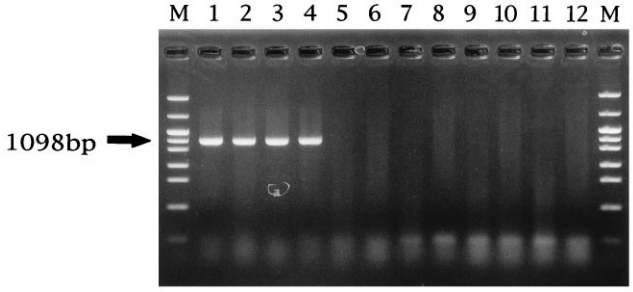

FIG. 3. Agarose gel (2%) showing PCR products of approximately 1.1 kb in length amplified from *P. syringae* pv. actinidiae and pv. phaseolicola with OCTF and OCTR as primers. Lanes: M, pHY marker (bands from top to bottom: 4,870; 2,016; 1,360; 1,107; 926; 658; 489; and 267 bp) (Takara); 1, *P. syringae* pv. phaseolicola MAFF 301673; 2, *P. syringae* pv. phaseolicola TMR 327; 3, *P. syringae* pv. actinidiae Kw-11; 4, *P. syringae* pv. actinidiae MAFF 302133; 5, *P. syringae* pv. coronafaciens MAFF 302257; 6, *P. syringae* pv. glycinea MAFF 302260; 7, *P. syringae* pv. maculicola MAFF 302264; 8, *P. syringae* pv. mori MAFF 302279; 9, *P. syringae* pv. morsprunorum MAFF 302280; 10, *P. syringae* pv. syringae MAFF 302155^T; 11, *P. syringae* pv. tabaci MAFF 302270; 12, *P. syringae* pv. tomato MAFF 302272.

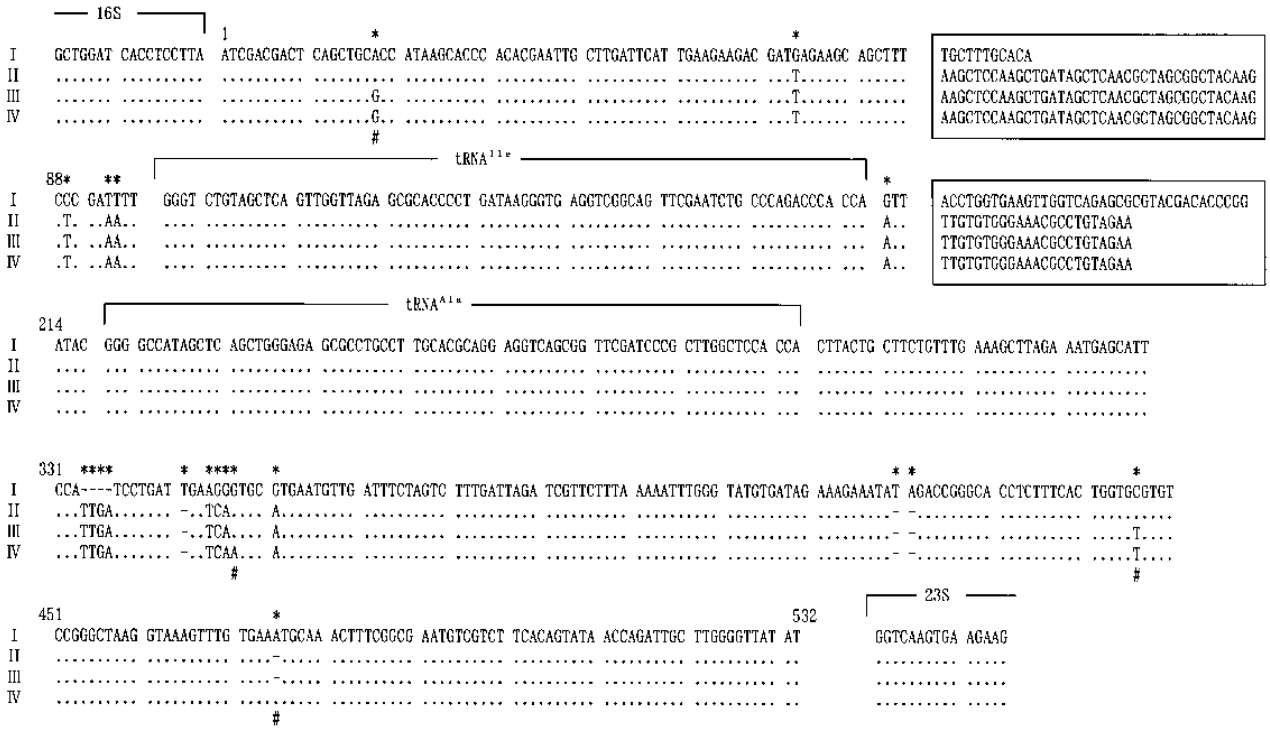

FIG. 4. Sequence alignment of the intergenic spacer regions located between the 16S and 23S rRNA genes in the RNA operon of *P. syringae* pv. actinidiae and pv. phaseolicola. The sequences are aligned with the sequence of *P. syringae* pv. actinidiae. Only the nucleotides that differ from those of *P. syringae* pv. actinidiae are shown; identical nucleotides are indicated by dots and deletions are indicated by dashes. Asterisks indicate nucleotides different between *P. syringae* pv. actinidiae and pv. phaseolicola, and pound signs (#) indicate nucleotides different among *P. syringae* pv. phaseolicola strains. The numbers above the sequence of *P. syringae* pv. actinidiae indicate positions from the 5′ end of the 16S-23S spacer of *P. syringae* pv. actinidiae. Positions 77 to 87, and 177 to 213 (*P. syringae* pv. actinidiae numbering) are not aligned, and the sequences of these two regions (highly variable regions) are enclosed in the boxes on the right. The 3′ region of the 16S rRNA gene and the 5′ region of the 23S rRNA gene are shown, together with the sequence of the 16S-23S spacer. The location of two tRNA genes contained within the 16S-23S spacer is also indicated. Rows: I, *P. syringae* pv. actinidiae Kw-11, MAFF 302091, MAFF 302092, MAFF 302145, and MAFF 302133; II, *P. syringae* pv. phaseolicola MAFF 302282, MAFF 301673, MAFF 301022, MAFF 301616, and KUZ 1; III, *P. syringae* pv. phaseolicola MAFF 301766; and IV, *P. syringae* pv. phaseolicola KUZ 8 and TMR 327.

completely coincide with the ROCT-coding region of *argK*. This means, in turn, that *P. syringae* pv. actinidiae and pv. phaseolicola isolated from various hosts in Japan and Canada (Table 1) were proved to possess ROCT genes that are exactly the same as those of bean-originated *P. syringae* pv. phaseolicola in Mexico and the United States.

Sequence comparison of 16S-23S spacer regions. Experiments were then conducted to determine the phylogenetic relationships between these two pathovars which share the identical *argK*. Selected as an index for this was the intergenic spacer region which exists between the 16S and 23S rRNA genes. The reason for this selection is that the evolution of the 16S-23S spacer region is believed to be faster than that of rRNA genes and so this region is greatly variable (1, 6, 7, 15); therefore, we can expect its sequence to be a suitable index for making comparisons at the pathovar level within a species. 16S-23S spacer regions were amplified with PCR for the 13 strains belonging to *P. syringae* pv. actinidiae and pv. phaseolicola by using primers space F-B and space R. The complete sequences were then determined by direct sequencing with primers ALFred M13-40 and M13 reversal.

Figure 4 shows the alignment of the sequences of the 16S-23S spacer regions determined. The 3′ end of the 16S rRNA gene and the 5′ end of the 23S rRNA gene were deduced from the corresponding sequences of *E. coli* (10). The sizes of the 16S-23S spacer regions for the 13 strains tested were 532- to 546-bp long. The sequence analysis revealed that they encode $tRNA^{Ile}$ and $tRNA^{Ala}$ in the order of 16S rRNA-$tRNA^{Ile}$-$tRNA^{Ala}$-23S rRNA. These tRNA species are also observed in the *E. coli* rRNA operon (*rrnA*, *rrnD*, and *rrnH*) and in many other eubacteria (3, 5, 22, 37). The sequences of both spacer tRNA genes in the 13 strains tested are identical, suggesting that the spacer tRNA genes are highly conserved. In all of the 3′ ends of the spacer tRNA genes, the 3′-terminal CCA sequence existed, which is the same as in the *E. coli* tRNA genes reported so far (5, 37).

The 16S-23S spacer sequences of all five strains of *P. syringae* pv. actinidiae including tara vine isolates (Kw-11, MAFF 302091, MAFF 302092, MAFF 302145, and MAFF 302133 sequences) were found to be perfectly identical (Fig. 4, sequence I), which clearly showed that *P. syringae* pv. actinidiae is a homogeneous group.

On the other hand, a close comparison of the 16S-23S spacer sequences of the eight strains belonging to *P. syringae* pv. phaseolicola showed they had very slight variations. Four strains originating from beans (MAFF 302282, MAFF 301673, MAFF 301022, and MAFF 301616) had the identical 545-bp sequence (Fig. 4, sequence II) and one of the four kudzu isolates (KUZ 1) had the same sequence as the bean isolates. However, the sequences of kudzu-originated MAFF 301766 (Fig. 4, sequence III), KUZ 8, and TMR 327 (Fig. 4, sequence IV) differed from that of the bean isolates (Fig. 4, sequence II) at 2, 4, and 4 positions, respectively. Therefore, the total number of sequence variations are four positions within *P. syringae* pv. phaseolicola (indicated by "#" in Fig. 4). It should be noted, however, that aside from these four positions, the 16S-

23S spacer sequences of the eight strains belonging to *P. syringae* pv. phaseolicola were completely identical, including the highly variable regions mentioned below. As a result, the levels of the sequence similarity of the 16S-23S spacers for the eight *P. syringae* pv. phaseolicola strains are high (99.3 to 99.6%; gaps are calculated as 1-base mismatches), showing high preservability within the pathovar.

In contrast, a comparison of the 16S-23S spacer sequences between *P. syringae* pv. actinidiae and pv. phaseolicola showed that two highly variable regions existed where the high frequency of insertion-deletion events prevented accurate sequence alignment (shown inside the boxes in Fig. 4). In all, there were 48 bases contained in the highly variable regions of *P. syringae* pv. actinidiae (11 in the upstream region and 37 in the downstream region) (Fig. 4) and 61 bases in the highly variable regions of pv. phaseolicola (38 and 23 in the upstream and downstream region, respectively). In addition, outside the two highly variable regions, there were 17 to 20 positions where the sequences were different between the two pathovars (indicated by asterisks in Fig. 4). As a result, the sequence similarities of the 16S-23S spacers between the pathovars are low (82.7 to 83.2%). The discovery of such large discrepancies in the 16S-23S spacer sequences clearly indicates that the two pathovars evolved differently from each other during phylogenetic development.

Conclusions. In a comparison of the amino acid sequences of ROCT and SOCT of *P. syringae* pv. phaseolicola, amino acid substitution was recognized even in the important parts deemed to be involved with the functions of enzymes (11, 21). The homology of the entire ROCT and SOCT sequences can be as low as 35.6%. Furthermore, the G+C content of the SOCT gene (*argF*) of *P. syringae* pv. phaseolicola was 57.3%, being roughly the same as the 56% G+C content of its entire genome (11). The G+C content of ROCT gene (*argK*), on the other hand, was 49.4%, markedly lower than the above values. Thus, it is assumed that *argK* did not evolve from *argF* of *P. syringae* pv. phaseolicola but is of another far-removed prokaryote origin and has immigrated into *P. syringae* pv. phaseolicola from the origin (11). Recently, Takikawa et al. (33) demonstrated that a high degree of homology is seen over all regions of the *tox* clusters containing *argK* between *P. syringae* pv. actinidiae and pv. phaseolicola, whereas no homology was apparent between the two pathovars outside the cluster regions, a finding which supports the transfer of the *tox* cluster among strains.

The results obtained in this study proved that the identical *argK* is present not only in bean isolates of *P. syringae* pv. phaseolicola in Mexico and the United States but also in bean isolates in Japan and Canada, and that it is also distributed in the kudzu (*Pueraria lobata*) isolates of *P. syringae* pv. phaseolicola which can be distinguished from bean-originated *P. syringae* pv. phaseolicola by their ability to produce ethylene (8). We further found that the kiwifruit and tara vine isolates of *P. syringae* pv. actinidiae also possess the identical *argK*. On the contrary, our data obtained by sequencing the 16S-23S spacer regions clearly demonstrated that *P. syringae* pv. actinidiae and pv. phaseolicola evolved differently from each other during phylogenetic development. The facts that the sequences of *argK* possessed by these strains are completely the same and even synonymous substitution has not occurred among them despite their extreme differences in phylogenetic evolution and geographical distribution suggest that it was only recently in evolutionary time that *argK* or the *argK-tox* cluster was transferred from another prokaryote, its origin, to *P. syringae* pv. actinidiae and/or pv. phaseolicola. This supports the hypothesis of Hatziloukas and Panopoulos (11) and can be useful data in studying the time and the mechanism of the transfer of the *tox* cluster containing *argK*.

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