Pseudomonas solanacearum: Plasmid pJTPS1 Mediates a Shift from the Pathogenic to Nonpathogenic Phenotype

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To elucidate the role of a small plasmid, pJTPS1, harbored by a spontaneous nonpathogenic mutant (M4S) of *Pseudomonas solanacearum* (U-7), a recombinant plasmid pJTPS2, expressing a hygromycin resistance gene inserted in pJTPS1, was transformed into the parental pathogenic strain, U-7. The hygromycin-resistant transformant (U-7T1) formed nonfluidal colonies similar to M4S and lost pathogenicity on tobacco and tomato. Moreover, the amount of extracellular polysaccharide (EPS) production and the level of endoglucanase (EG) activity in U-7T1 were decreased to levels lower than those of M4S. Furthermore, a pJTPS2-cured mutant isolated from U-7T1 formed fluidal colonies with high EPS production and EG activity and regained pathogenicity. These data suggest that pJTPS1 reduces or eliminates pathogenicity of the bacterium upon tobacco and tomato.

Additional keyword: nonpathogenicity.

Pseudomonas solanacearum, the causal agent of bacterial wilt of solanaceous plants, is one of the most destructive plant pathogens in the world. *P. solanacearum* spontaneously loses pathogenicity under certain conditions. Genes governing and/or controlling spontaneous mutation have been elucidated by several groups. Brumbley and Denny (1990) showed that a functional *phcA* gene was required to maintain the pathogenic phenotype as well as to produce extracellular polysaccharide (EPS) and the high levels of endoglucanase (EG) activity that probably contribute to pathogenicity. Huang and Sequeira (1990) also described a genomic locus that may determine the shift to nonpathogenicity. According to their report, the locus is present in the genome of both wild-type and spontaneous nonpathogenic mutants, but they postulated that the shift of phenotype results from the increased level of expression of this locus in the mutants. Studies of plasmids in connection with the loss of pathogenicity have been reported, but no detectable changes were reported in plasmid number and size in nonpathogenic mutants compared to the parental strains (Currier and Morgan 1981; Morales and Sequeira 1985).

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We reported previously that pJTPS1 exists in *P. solanacearum* M4S (Negishi *et al.* 1990), a spontaneous nonpathogenic mutant of *P. solanacearum* U-7 (Tanaka 1985). This plasmid hybridized with whole genomic DNA and may have originated by excision from genomic DNA or megaplasmid DNA in the parental pathogenic strain U-7 (Negishi *et al.* 1990). It is not clear whether the presence of pJTPS1 is involved in the shift of the phenotype from pathogenicity to nonpathogenicity.

To determine if pJTPS1 is related to the shift to nonpathogenicity, we constructed pJTPS2 with a gene for hygromycin resistance inserted in pJTPS1, transformed pJTPS2 into the parental pathogenic strain U-7, and assayed the transformant and pJTPS2-cured strain for pathogenicity. The results support the hypothesis that pJTPS1 plays a role in the phenotypic shift of *P. solanacearum* to nonpathogenicity.

RESULTS

Copy number of pJTPS1.

Purified pJTPS1 (28 μg) was obtained from 4.6×10^{12} cells of M4S. The copy number of pJTPS1 was calculated as 0.84/cell and was assumed to be approximately one/cell.

Direct transformation of pJTPS2 into U-7.

The recombinant plasmid pJTPS2, which carries the *hph* gene in pJTPS1, was constructed and transformed into the wild-type strain U-7 (Fig. 1). The transformation efficiency was 1.5×10^4 colonies per microgram of DNA. Five colonies were randomly selected from TZCH plates, and plasmids were isolated and hybridized with pJTPS1 and the *Bam*HI-*Hin*dIII fragment of pTOM1. The sizes of the restriction fragments by digestion with *Hin*dIII and *Eco*RV were 6.4 and 2.3 kb as expected. This recombinant plasmid was designated as pJTPS2 (Fig. 1).

Pathogenicity and hypersensitivity.

Bacterial wilt in tobacco or tomato did not result in plants inoculated with the transformant (U-7T1) or M4S (Table 1), while plants inoculated with U-7 or U-7R exhibited a severe wilt symptom 2 wk after inoculation.

Neither M4S nor U-7T1 caused typical hypersensitive reactions on tobacco leaves, as characterized by a rapid

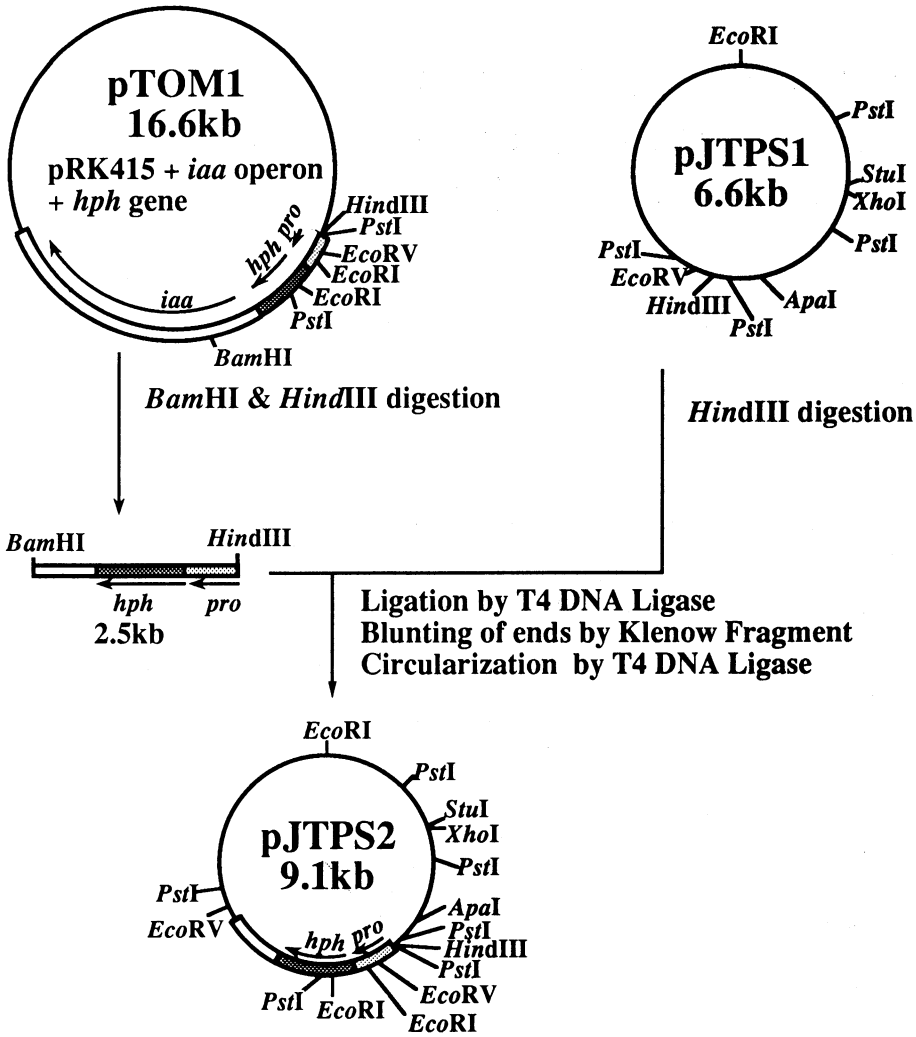

Fig. 1. Schematic diagram of the construction of pJTPS2. Restriction maps of pJTPS1 (Negishi *et al.* 1990) and pTOM1 (Yamada *et al.* 1991). Abbreviations are: *iaa, Pseudomonas syringae* pv. *savastanoi iaa* genes; *hph*, hygromycin B phosphotransferase gene; pro, *iaa* promoter.

death of mesophyll cells in contact with incompatible bacteria (Lozano and Sequeira 1970). Leaves infiltrated with all strains remained symptomless 24 hr after the inoculation. Necrotic symptoms, however, developed in all leaves 48 hr after the inoculation. After that, no wilt symptoms developed on the leaves inoculated with M4S or U-7T1, but leaves inoculated with U-7 began to wilt 4 days after infiltration, resulting in the wilting of entire plants (Figs. 2 and 3).

Morphology and phenotype of the transformants and the revertant.

U-7T1 formed nonfluidal colonies similar to those of the spontaneously derived nonpathogenic strain M4S on TZC medium (Fig. 4) and grew on a minimal medium (MM) agar plates (Boucher *et al.* 1985). U-7R formed fluidal colonies on TZC medium similar to those of the wild-type U-7.

The levels of EPS production and EG activity that might contribute to the pathogenicity in *P. solanacearum* were determined in U-7, U-7T1, U-7R, and M4S. No measurable EPS was detected in the culture filtrate of the transformant (U-7T1), but the pJTPS2-cured mutant (U-7R) regained the ability to produce EPS at even higher level than that of U-7 (993.2 μg/ml) (Table 1). EPS produced by M4S was 36.2 μg/ml, which is about one tenth of that by U-7 (345.8 g/ml).

Table 1. Phenotypes of *Pseudomonas solanacearum* strains and mutants

Bacteria	Pathogenicity[a]		EPS production (μg/ml)[b]	Endoglucanase activity (U/ml)[c]
	Tobacco	Tomato		
U-7	100	80	345.8 ± 1.38 a	2.70 ± 0.02 a
M4S	0	0	36.2 ± 1.65 b	1.84 ± 0.12 b
U-7T1	0	0	ND[d]	ND
U-7R	100	100	993.2 ± 8.56 c	2.56 ± 0.04 a

[a] Percentage of wilted plants. Twenty tomato and three tobacco were inoculated with each strain.
[b] Dialyzed culture supernatants assayed with an Elson and Morgan reaction. Average of five samples ± standard error. Values followed by same letter do not differ significantly at $P < 0.05$ according to Duncan's multiple range test.
[c] 1 nmol product/ml/min by measuring reducing sugar. Average of five samples ± standard error. Values followed by same letter do not differ significantly at $P < 0.05$ according to Duncan's multiple range test.
[d] Not detected.

No EG activity was detected in the culture filtrate of the transformant (U-7T1), and that of M4S was lower than that of U-7 or U-7R (Table 1).

Multiplication of the transformants *in planta*.

The multiplication of bacteria was examined after inoculation. The number of the cells of all tested strains rapidly increased 24 hr after infiltration, and the cell number (measured as cfu/plate) was significantly greater than each original number (Student's t test, $P = 0.05$). The number of U-7 cells at 48 hr after inoculation remained the same as that of 24 hr after inoculation, but those of M4S and U-7T1 decreased significantly 48 hr after inoculation (Student's t test, $P = 0.05$). The numbers of

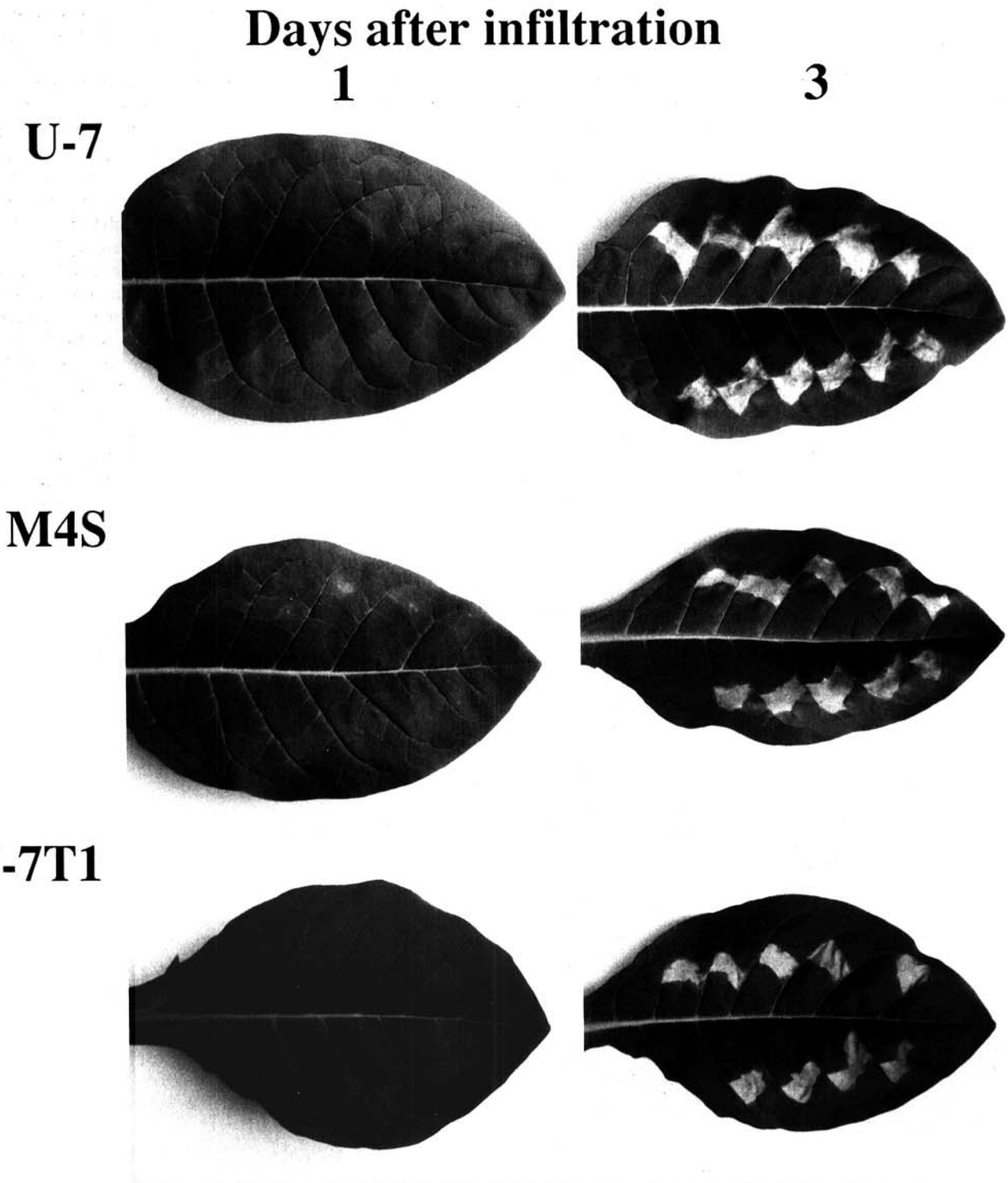

Fig. 2. Tobacco leaves (cv. Burly 21) infiltrated with *Pseudomonas solanacearum* suspensions (U-7, M4S, or U-7T1). Photographs taken 1 or 3 days after infiltration.

all strains 72 hr after inoculation were smaller than were those 48 hr after inoculation (Fig. 5). Isolated bacteria from plant tissues inoculated with M4S or U-7T1 formed nonfluidal colonies on GS medium or GSH medium, respectively. There was no significant difference between the number of the reisolated colonies of U-7T1 on GS medium and that on GSH medium, but no growth of U-7 and M4S was obtained on TZCH medium.

DISCUSSION

In this study we demonstrated that the plasmid pJTPS1 present in a spontaneous nonpathogenic mutant M4S may have some role in changing the phenotype of *P. solanacearum,* since all the transformants formed nonfluidal colonies and lost pathogenicity on tobacco and tomato in a manner similar to M4S. The transformant U-7T1 lost the ability to produce EPS and EG, which is believed to play an important role in pathogenicity of the bacterium (Denny and Baek 1991; Denny *et al.* 1988). Furthermore, pJTPS2-cured mutants regained the ability to cause wilt symptoms on tomato and regained EPS production and EG activity.

Although pJTPS1 may have some role in the spontaneous mutation to nonpathogenicity on M4S, the possible function of genes on this plasmid may be different from that of an avirulence gene (*avrA*) in *P. solanacearum* reported by Carney and Denny (1990). M4S and U-7T1 are not pathogenic to major host plants of race 1 of *P. solanacearum*, and they do not cause a typical hypersensitive reaction. *avrA* is a species-specific avirulence gene and could be solely responsible for the hypersensitive reaction elicited on tobacco and for the restriction of the host range of *P. solanacearum* strains. pJTPS1 does not have these characteristics.

Gene(s) on pJTPS1 may be free from the control or regulation mechanism that suppresses the expression of the corresponding gene(s) on the genome or megaplasmid, ultimately resulting in the phenotypic change of wild-type. Brumbley and Denny (1990) have postulated that the expression of *phcA* gene is required to maintain the wild-type phenotype in *P. solanacearum* (ability to cause wilt symptoms and the production of EPS and EG that may contribute to the pathogenicity) and phenotypic conversion results from a loss of *phcA* gene expression or the function of its gene product. There is little possibility that pJTPS1 affects *phcA* by gene dose effect, which masks the presence of a low copy number of plasmid mediated by the low copy number megaplasmid or chromosome, because the copy number of pJTPS1 is small (approximately one/cell). We cannot rule out the possibility that the excision of pJTPS1 sequences from the chromosome or megaplasmid in the pathogenic strain might accompany the loss of the functional *phcA* gene in the bacterium. It would be necessary to investigate the homology of pJTPS1 to *phcA*. Huang and Sequeira (1990) described a locus that normally is present in the genomes of both wild-type and spontaneously nonpathogenic mutants, and they assumed that the loss of virulence resulted from the increased level of expression of this locus in the mutants. Furthermore,

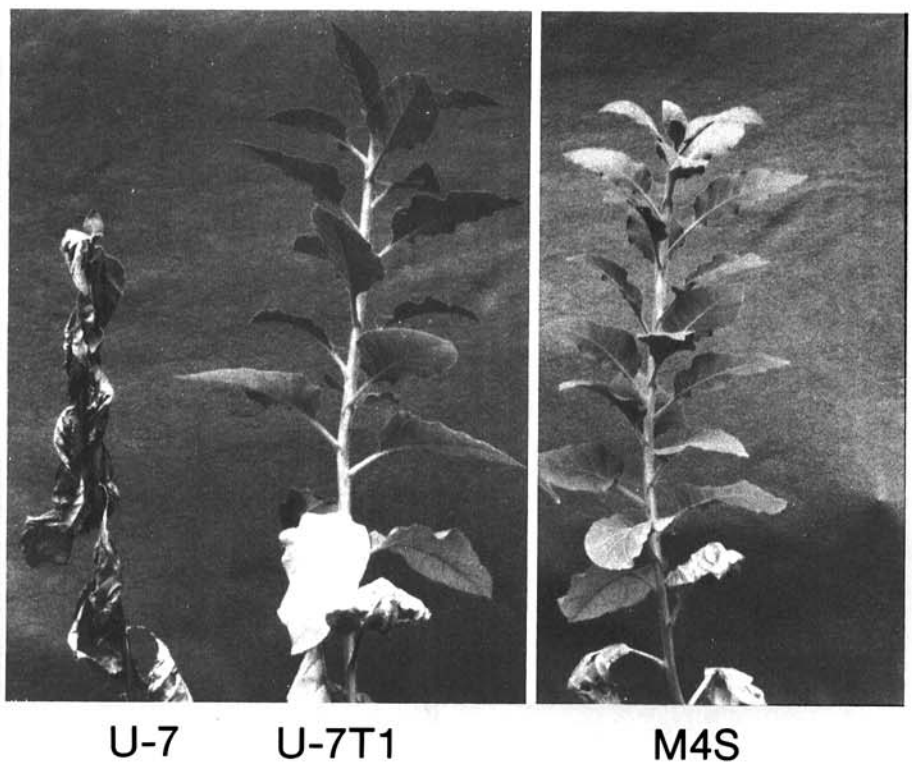

Fig. 3. Tobacco plants inoculated with bacterial suspensions of *Pseudomonas solanacearum* by leaf infiltration. Photographs taken 17 days after infiltration.

introduction of a DNA fragment from the nonpathogenic mutant B1 (*epsR* region) mimicked the shift in phenotype change that occurs spontaneously in the pathogenic strain K60 (Gosti *et al.* 1991). The possible function of pJTPS1 might be rather similar to this *epsR*. It would be interesting to investigate the homology of pJTPS1 to this gene, *epsR*. Studies are under way to determine the DNA sequence of pJTPS1, but a sequence homologous to *epsR* has not yet been detected in pJTPS1 (data not shown).

Transposon mutagenesis is probably one of the most useful methods for the investigation of the function of pJTPS1 or the detection of regions of pJTPS1 involved in the phenotype shift of *P. solanacearum*. In a preliminary transposon mutagenesis experiment, DNA/RNA blot hybridization analysis was performed using total RNA isolated from U-7, U-7T1, and M4S and pJTPS1 as a probe. Some transcripts were expressed in U-7T1 and M4S but not in U-7 (data not shown).

Although pJTPS1 may play an important role in the loss of pathogenicity in U-7, it is not clear whether other mechanisms are involved in the loss of pathogenicity of *P. solanacearum* in general. Studies are under way to determine the entire DNA sequence of pJTPS1 to determine the gene(s) carried by pJTPS1 and the role of these genes in spontaneous mutation in pathogenicity.

MATERIALS AND METHODS

Bacterial strains and plasmids.

Bacterial strains and plasmids used in this study are listed in Table 2. *P. solanacearum* was grown on TZC agar medium (Kelman 1954) at 30° C and stored in sterilized distilled water at room temperature.

Chemicals and reagents.

Restriction endonucleases were obtained from Nippon Gene, Inc. (Tokyo, Japan) and Takara, Inc. (Kyoto, Japan). T4 DNA ligase and *E. coli* DNA polymerase I (large fragment) were obtained from Takara, Inc. ECL Gene Detection System was obtained from Amersham Japan, Inc. (Tokyo). DIG Nucleic Acid Labeling and Detection System were obtained from Boehringer Mannheim Yamanouchi, Inc. (Tokyo). The Southern-Light DNA detection system was obtained from Tropix, Inc. (Bedford, MA). Noncharged nylon membrane filter, Biodine A, was obtained from Pole Co. (East Hills, NY). Lysozyme, RNase A, and hygromycin B (Hm) were obtained from Sigma Chemical Co. (St. Louis, MO).

Plasmid isolation.

P. solanacearum was cultured in CPG liquid medium (Kelman 1954) at 30° C. Plasmids were extracted from

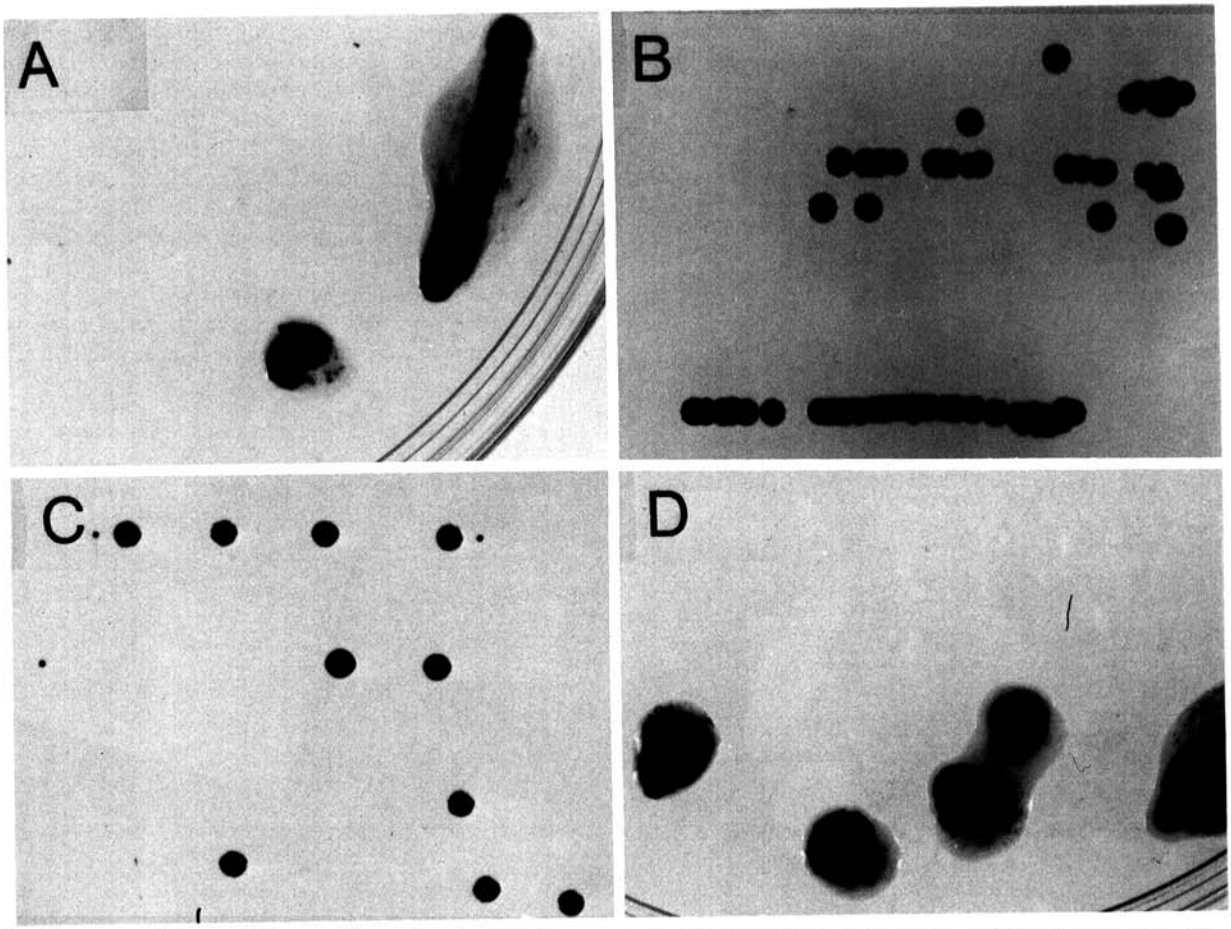

Fig. 4. Colony morphology of the transformant and pJTPS2-cured strain. Bacterial strains were grown on TZC for 72 hr at 28° C. **A,** Wild-type (U-7); **B,** nonpathogenic spontaneous mutant (M4S); **C,** transformant (U-7T1); and **D,** pJTPS2-cured U-7T1 (U-7R). Photographs were prepared at the same magnification.

bacterial cells by the method of Comai and Kosuge (1982) and purified by isopycnic centrifugation in CsCl with ethidium bromide. Plasmid copy numbers were calculated from lysed cell numbers and yield of purified plasmid.

Construction of pJTPS2.

Plasmid pTOM1, containing *iaa* promoter sequences and hygromycin B phosphotransferase gene (*hph*) for hygromycin B phosphotransferase (Yamada *et al.* 1991), was digested with *Bam*HI and *Hin*dIII and subjected to electrophoresis through a 0.7% low temperature melting agarose gel in Tris-borate-EDTA (TBE) buffer (Fig. 1). A 2.5-kb *Bam*HI-*Hin*dIII fragment was excised from the gel and melted by heating at 68° C with the addition of two volumes of TE (10 mM TrisCl, pH 7.5, and 1 mM EDTA). DNA was purified from a NACS column (Bethesda Research Labs, Gaithersburg, MD) according to the manufacturer's specifications. The *Hin*dIII end of extracted DNA fragment was ligated into the *Hin*dIII site of pJTPS1. After overnight incubation at 12° C, the partially ligated DNA sample was treated with phenol-chloroform to inactivate T4 ligase followed by ethyl ether extraction, to remove chloroform and phenol, and then precipitated with ethanol. The DNA mixture was treated with *E. coli* DNA polymerase I (large fragment) to fill in the sticky ends of the *Bam*HI end in the vector and the remaining *Hin*dIII end in the vector. After phenol-chloroform extraction, the DNA was extracted again with ethyl ether and precipitated. The precipitate was dissolved in H_2O and ligated with T4 DNA ligase. The ligated DNA was transformed into pathogenic *P. solanacearum* U-7 by the procedure described below.

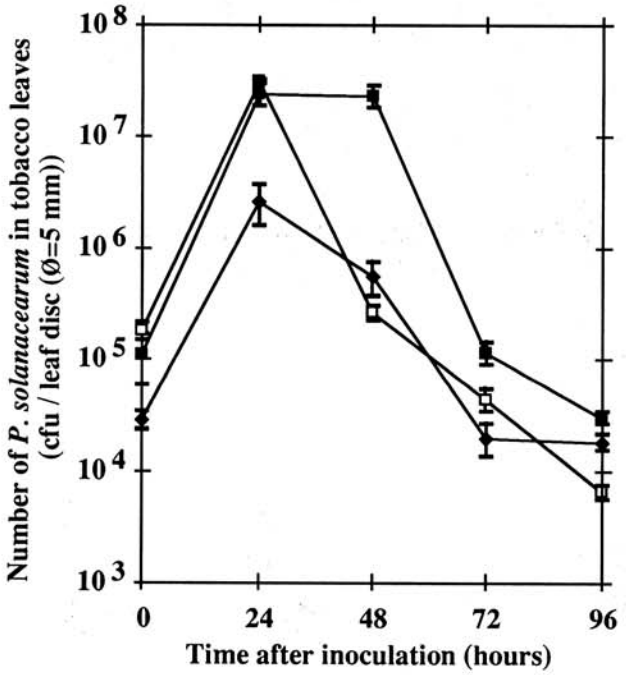

Fig. 5. Time course of the cell multiplication in host plant. Bacterial suspensions (OD_{600} = 1.0) were infiltrated into the leaves of 10-wk-old tobacco (cv. Burley 21). Leaf disks (5 mm in diameter) from the inoculated portions were macerated in sterilized distilled water and the number of *P. solanacearum* (cfu) cells was determined. ■: U-7, □: M4S, ▲: U-7T1. Bar represents standard error ($N = 3$).

Transformation in *P. solanacearum*.

The ligation mixture (7.65 g) was transformed into a pathogenic strain U-7 according to the procedure of Boucher *et al.* (1985). Transformants were selected on TZC medium containing 30 mg/L hygromycin B (TZCH medium). To verify the presence of the recombinant plasmid in the transformants and the orientation of inserted DNA fragment, plasmid DNA was isolated, digested with restriction enzymes, and analyzed by DNA/DNA hybridization using labeled pJTPS1 or the *Bam*HI-*Hin*dIII fragment of pTOM1 as a probe. For DNA labeling, DNA hybridization and detection, the ECL Gene Detection System was used according to the manufacturer's specification.

Isolation of pJTPS2-cured mutant from the transformant.

A single cell culture of the transformant (U-7T1) stored in sterilized distilled water at room temperature for over 2 yr was streaked on the TZC medium and hygromycin-susceptible colonies (U-7R) were picked. To confirm curing of the plasmid, plasmid DNA was isolated from the hygromycin-susceptible strain as described above.

Inoculation tests.

Bacteria grown on TZC medium or TZCH medium for 3 days at 28° C were transferred to sterilized water, and the concentration was adjusted to 10^9 cfu/ml.

Four-week-old-seedlings of tomato (*Lycopersicon esculentum* Mill. 'Houkin', Tohoku Shubyo Co., Tokyo) were grown at 30° C in a growth chamber. Five milliliters of bacterial suspension was added to each pot containing a tomato plant whose roots had been previously injured with a knife (Winstead and Kelman 1952). Twenty plants were inoculated with each strain (U-7, M4S, U-7T1, and U-7R) and disease incidence was determined 2 wk after inoculation.

Ten-week-old plants of tobacco (*Nicotiana tabacum* L. 'Xanthi') were inoculated with the bacterial suspensions by leaf infiltration as described by Sequeira and Hill (1974).

Table 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
P. solanacearum		
U-7	Vir$^+$, wild-type, isolated in East Japan	Tanaka 1983
M4S	Vir$^-$, NF, spontaneous derivative of U-7	Tanaka 1985
U-7T1	Vir$^-$, NF, transformants of U-7 with pJTPS2	This paper
U-7R	Vir$^+$, F, pJTPS2-cured U-7T1	This paper
Plasmids		
pJTPS1	Isolated from M4S	Negishi *et al.* 1990
pJTPS2	pJTPS1 with *iaa*[b] promoter and *hph*[c]	This paper
pTOM1	pTET40 (pRK415 with *iaa* operon) with *hph*	Yamada *et al.* 1991

[a] Vir$^+$: pathogenic to tobacco and tomato; Vir$^-$: nonpathogenic to tobacco and tomato; NF = nonfluidal colony on TZC medium. F = fluidal colony on TZC medium.
[b] *Pseudomonas syringae* pv. *savastanoi iaa* genes.
[c] Hygromycin B phosphotransferase gene.

Three plants were inoculated with each strain (U-7, M4S, and U-7T1), and disease incidence was determined 2 wk after the inoculation.

The multiplication of the transformants in leaf tissues was examined after infiltrating tobacco leaves with bacterial suspensions (OD_{600}=1.0, about 2×10^9 cfu/ml). Leaf disks (5 mm in diameter) from the inoculated portions of the eighth, ninth, and tenth leaves of 10-wk-old tobacco (cv. Burley 21, one disk from each leaf, three plants for each strain) were macerated in sterilized distilled water, and the number of *P. solanacearum* (cfu) was determined by plating on a selective GS medium as described by Tanaka and Fukuda (1982), a modification of SM-1 medium (Granada and Sequeira 1983) with or without hygromycin B (30 mg/L, GSH). At the same time, the strains were assayed for their ability to cause disease, damage the host, and induce the hypersensitive reaction.

Extracellular polysaccharide (EPS) production in culture.

Bacteria were grown in a 50-ml Erlenmeyer flask containing 10 ml of CPG medium on a rotary shaker at 150 rpm at 28° C for 3 days. Cells were removed by centrifugation at 8,500 *g* for 10 min at 4° C. After filtration (0.45 m), the supernatant solution was dialyzed against large volumes of water at 4° C for 3 days. The sample was hydrolyzed in 3 N hydrochloric acid at 110° C for 30 min (Akiyama *et al.* 1986), and the liberated amino sugars were measured colorimetrically using *N*-acetylgalactosamine as a standard (Gatt and Berman 1966).

Enzyme assays.

The enzyme activity of endoglucanase (EG) in culture supernatant fluids was determined as described by Denny *et al.* (1988).

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