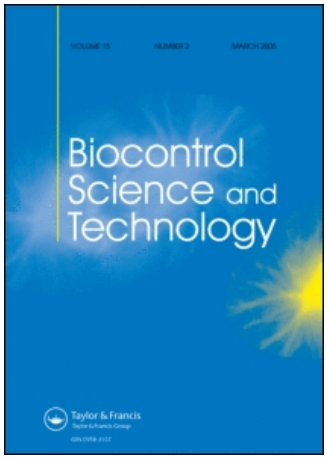

Biocontrol Science and Technology
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713409232

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Online Publication Date: 01 August 2004
To cite this Article: Otsu, Y., Matsuda, Y., Mori, H., Ueki, H., Nakajima, T., Fujiwara, K., Matsumoto, M., Azuma, N., Kakutani, K., Nonomura, T., Sakuratani, Y., Shinogi, T., Tosa, Y., Mayama, S. and Toyoda, H. (2004) 'Stable phylloplane colonization by entomopathogenic bacterium **Pseudomonas fluorescens** KPM-018P and biological control of Phytophagous ladybird beetles **Epilachna vigintioctopunctata** (Coleoptera: Coccinellidae)', Biocontrol Science and Technology, 14:5, 427 - 439
To link to this article: DOI: 10.1080/09583150410001683538
URL: http://dx.doi.org/10.1080/09583150410001683538

Biocontrol Science and Technology (August 2004), Vol. 14, No. 5, 427–439

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Stable Phylloplane Colonization by Entomopathogenic Bacterium *Pseudomonas fluorescens* KPM-018P and Biological Control of Phytophagous Ladybird Beetles *Epilachna vigintioctopunctata* (Coleoptera: Coccinellidae)

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(*Received 9 July 2003; accepted 9 September 2003*)

An entomopathogenic bacterium was isolated from tomato leaves and used as a microbial agent to control larvae of phytophagous ladybird beetles Epilachna vigintioctopunctata. *The isolate was identified as* Pseudomonas fluorescens *KPM-018P on the basis of its bacteriological characteristics. KPM-018P produced extracellular chitinase to form a transparent zone around their colonies by hydrolyzing chitin in a minimal medium. Pale-yellow colonies turned red after a change of incubation temperature. These characteristics were availed as markers for tracking KPM-018P. The bacteria produced biosurfactants that enabled the bacteria to stably colonize the hydrophobic leaf surface; they were recovered without any considerable decrease even after a suspension of KPM-018P was sprayed onto leaves. KPM-018P, transformed with the* gfp *gene and observed with fluorescence microscopy, stably dwelled in the junctions of epidermal cells of bacteria-sprayed leaves. Ingestion of KPM-018P-sprayed leaves by the larvae caused prompt death of these insects to eventually suppress their pupation. This method is thus effective for decreasing the population of larvae and adult insect pests in the subsequent generation. The study provides an experimental basis for the biocontrol of herbivorous insect pests using a leaf-inhabiting, entomopathogenic strain of* P. fluorescens.

Keywords: gfp-*marking, phylloplane bacterium, monitoring, pest control,* Pseudomonas fluorescens, Epilachna vigintioctopunctata

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ISSN 0958-3157 (print)/ISSN 1360-0478 (online)/04/050427-13
DOI: 10.1080/09583150410001683538

INTRODUCTION

Microorganisms that stably colonize the surface of plant leaves are potential biological agents to suppress foliar pathogens or insect defoliators (Andrews, 1992). In our previous work (Otsu *et al*., 2003a,b), we isolated chitinolytic phylloplane bacteria from greenhouse-grown tomato and used bacteria-entrapped alginate gel beads to suppress leaf feeding and oviposition by phytophagous ladybird beetles. Suppression was mainly due to enzymatic degradation of the chitinous peritrophic membrane in the midguts of insect pests that had fed on bacteria-treated tomato leaves (Otsu *et al*., 2003b). This application was easy and effective, but only adult ladybird beetles were suppressed (Otsu *et al*., 2003a). Unfortunately, in our cultivation system for tomato, the plants suffered severely from both larvae and adults feeding on leaves in the seedling stage before transplanting. We therefore needed to screen further for an effective biocontrol agent, focusing on bacterial habitants of leaves of tomato seedlings at the pre-planting stage. Here, we describe the isolation and identification of the phylloplane bacterium *Pseudomonas fluorescens* from tomato leaves and its efficacy as an entomopathogen of larvae of phytophagous ladybird beetles.

Some strains of *P. fluorescens*, widely investigated as endophytic biocontrol agents, had antimicrobial activity against plant pathogenic bacteria and fungi by inducing host resistance (Hallmann *et al*., 1997; Ramamoorthy *et al*., 2001). Fluorescent pseudomonads can also influence the growth and development of insects at various stages of their development (Ramamoorthy *et al*., 2001). In fact, certain strains of *P. fluorescens* were used to control insect pests such as Colorado potato beetles (*Leptinotarsa decemlineata*) and rice leaf folder (*Cnaphalocrocis medinalis*) by feeding the pests a diet of bacteria-treated plant tubers (Costanzo *et al*., 1998) and foliage (Commare *et al*., 2002). Beattie and Lindow (1995) noted that bacteria could be localized on particular sites of leaf surface, such as trichomes, stomata and epidermal cell wall junctions. In attempts to biologically control foliar plant pathogens with antagonistic strains of *P. fluorescens*, the investigators sprayed powder-formulated bacterial cell suspensions on plant leaves to obtain sustainable leaf colonization of the bacteria (Gnanamanickam *et al*., 1992; Chatterjee *et al*., 1996; Vidhyasekaran *et al*., 1997).

Our approach for applying bacteria is also based on the ability of the bacteria to stably survive on the surface of tomato leaves that insect pests attack during feeding. Recent molecular techniques, especially the use of the green fluorescent protein gene (*gfp*) as a reporter to visualize *in planta* colonization of target bacteria (Unge & Jansson, 2001), enabled us to genetically mark and monitor bacteria with reporter genes (Tombolini *et al*., 1997; Firth, 1999). The *gfp*-marking of bacteria is also successfully used in our system to track sustainable bacterial colonization of treated leaves. Using these approaches, we evaluated the feasibility of using the entomopathogenic strain of *P. fluorescens* against larvae of phytophagous ladybird beetles.

MATERIALS AND METHODS

Plant and Insect

Germinated seeds of tomato (*Lycopersicon esculentum* Mill, cv. Moneymaker) were sown in 12-cm pots containing vermiculite and placed in a growth box ($2.5 \times 5.0 \times 1.5$ m) that had upper and lateral sides covered with transparent vinyl film to block UV spectrum shorter than 390 nm. The growth box was set in the greenhouse controlled at $26 \pm 4^{\circ}$C. The daily fluctuation of relative humidity in the box was between 50 and 90%.

Adult 28-spotted ladybird beetles *Epilachna vigintioctopunctata* (Coleoptera: Coccinellidae) were collected from field tomatoes and reared on 1-month-old seedlings of tomato (Moneymaker) in the growth chamber ($26 \pm 1^{\circ}$C, 16 h photoperiod). Third and fourth instars of ladybird beetles were fed on bacteria-sprayed tomato leaves (Otsu *et al*., 2003a).

Isolation and Identification of Entomopathogenic Bacteria

Fully developed leaves were collected at random from tomatoes (cv. Moneymaker) grown for 2 months in the greenhouse under humid conditions ($26 \pm 6^{\circ}$C, $70 \pm 20\%$ relative humidity).

The upper surface of detached leaves was pressed onto M9 minimal agar medium (12.8 g $Na_2HPO_4 \cdot 7H_2O$, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl and 4 g glucose in 1000 mL of water) and left for 2 min. Subsequent bacterial colonies growing on the medium were transferred to M9 medium containing colloidal chitin (Chi-M9 medium). Colloidal chitin was prepared by the method of Hirano and Nagao (1988). After incubating at 26°C for 3 days, bacterial colonies that formed a halo (transparent zone in the medium around the colonies from the hydrolysis of chitin by bacteria-secreted chitinase) were transferred to Chi-M9 media supplemented with antibiotics at standard concentrations; tetracycline at 20 μg/mL, ampicillin at 50 μg/mL, streptomycin at 50 μg/mL, kanamycin at 50 μg/mL and chloramphenicol at 100 μg/mL. Based on colony color and sensitivities to antibiotics, the isolates were classified into 18 types. The classified bacteria were then separately suspended in distilled water and tested for pathogenicity to larvae of ladybird beetles. Isolate KPM-018P, having entomopathogenic activity, was characterized according to *Bergey's Manual of Determinative Bacteriology* (9th edn.).

Phylogenetic Analysis of 16S rRNA

Chromosomal DNA was extracted from KPM-018P to amplify 16S rRNA-coding region by polymerase chain reaction (PCR), using the primers (5′-TGAAGAGTTTGATCATGGC-3′ and 5′-GGTTACCTTGTTACGACTT-3′) designed by Weisburg *et al*. (1991). PCR was performed according to the following protocols; 30 cycles for denaturing (94°C, 60 s), annealing (61°C, 60 s) and extension (72°C, 90 s) followed by a final extension cycle of 120 s at 72°C. The amplified DNA was inserted in a pGEM-T easy vector for sequencing (Promega, Madison, WI, USA). The nucleotide sequence of the amplified region was determined using the Big Dye Terminator Cycle Sequencing Ready Reaction Kit from Applied Biosystems (Tokyo, Japan) on an ABI Prism 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems, Tokyo, Japan) in the Pharmaceutical Research and Technology Institute, Kinki University, Japan. The nucleotide sequence determined was registered to the DNA data bank of Japan (DDBJ); the accession number was AB091837 for 16S rRNA of KPM-018P. For phylogenetic analysis, the 16S rRNA sequence data for some bacteria were retrieved from the DDBJ database; three strains ATCC17482 (AF094728), CCUG32456A (AY321588) and VUN10011 (AF068010) of *P. fluorescens*, *P. aeruginosa* (PAZ76651), *P. alcaligenes* (PAZ76653), *P. amygdali* (PAZ76654), *P. aureofaciens* (PAZ76656), *P. coronafaciens* (PCZ76660), *P. ficuserectae* (PFZ76661), *P. fragi* (D84014), *P. putida* (D86000), *P. stutzeri* (PSU26262), and *P. tolaasii* (PTZ76670). All data were initially aligned using the Clustal W of DNA Space Version 3.5 (Hitachi Software Engineering, Yokohama, Japan), and a phylogenetic tree was inferred using the neighbour-joining method (Saitou & Nei, 1987). The resultant tree topologies were evaluated in bootstrap analysis of the neighbour-joining method based on 1000 re-samplings.

Assay for Entomopathogenic Activity of Bacteria

For preliminary screening of entomopathogenicity of KPM-018P, fourth-instar larvae were isolated in a desiccator kept on 14–15% of relative humidity for a 12-h starvation, then placed for 30 min onto a paper soaked with bacterial suspension (sterile water suspension at 10^9 cells/mL, determined with a hemacytometer and phase-contrast microscopy). After ingestion of bacteria evident by the swelling of larval abdomens, the insects were kept in a Petri dish containing fresh detached leaves and incubated until the end of the subsequent pupal stage (another 10 days).

Isolate KPM-018P was tested by the direct-injection method. Namely, starved larvae were laid on their backs on agar (1.5%, w/v) on a glass slide, and 2 μL of bacterial suspension (sterile water suspension) was directly injected into the mouths of larvae with a micropipette (tip diameter, 10 μm) under a stereomicroscope. The bacteria-ingested insects were similarly kept in a Petri dish of leaves for 10 days. *Escherichia coli* HB101/amp$^+$ (HB101 transformed

with pUC119) and *Alcaligenes paradoxus* KPM-012A were similarly used as negative control bacteria. KPM-012A was previously obtained from the tomato leaf surface (Otsu *et al.*, 2003a).

Estimation of Enzymatic Activities in Bacteria-infected Larvae

Chitinase activity was measured by the method of McCreath and Gooday (1992), using 4-methylumbelliferyl-β-d-*N*,*N*′,*N*″-triacetylchitotrioside [4MU-$(GlcNAc)_3$] (Sigma) as a substrate. Bacteria-inoculated larvae or noninoculated larvae were homogenized in distilled water, and the homogenate (100 μL) was clarified by centrifugation and mixed with 900 μL of McIlvaine's buffer (pH 7, 17.8 mL of 0.1 mol/L citric acid and 82.2 mL of 0.2 mol/L dibasic sodium phosphate). The reaction was initiated by adding 50 μL of 4MU-$(GlcNAc)_3$ at 0.134 mg/mL. After incubation at 37°C for 10 min, the reaction was terminated by the addition of 1.2 mL of 1 mol/L glycine/NaOH buffer (pH 10.6) and the fluorescence of the released 4-methylumbelliferon (4MU) was monitored using a spectrofluorophotometer (RF-5000; Shimazu, Kyoto, Japan). The amount of 4MU was estimated according to a calibration curve made with authentic 4MU in the same buffer at pH 7.0.

Proteolytic activity was measured according to the method of Bowen *et al*. (2000), using azocasein (Sigma) as a substrate. The larvae were homogenized with 50 mM Tris-buffered saline (pH 8.0), and the clarified homogenate (20 μL) was added to 500 μL of the same buffer containing azocasein at 10 mg/mL, then incubated at 30°C for 4 h on an orbital shaker. Non-hydrolyzed azocasein was precipitated by adding 500 μL of 10% trichloroacetic acid to the sample. The mixture was centrifuged, and the absorbance (at 440 nm) of the resulting supernatant was determined. Increased absorbance indicated proteolytic activity.

Assay for Biosurfactant Production by Bacteria

The production of biosurfactants by KPM-018P was assessed by the rapid drop-collapsing test (Jain *et al*., 1991). Bacteria were shake-cultured in liquid M9 medium (without agar) at 25°C, and 10 μL of the bacterial culture or the supernatant of centrifuged culture were dropped onto a hydrophobic membrane (Parafilm) or tomato leaf surface to examine the shape of droplets. As a control, water droplets containing a synthetic surfactant (10%, v/v, Tween 20) were placed on the membrane. Two-day-old cultures of *E. coli* HB101/amp$^+$ and *A. paradoxus* KPM-012A, bacteria that do not produce biosurfactants, were also used as negative controls.

Genetic Marking of Bacteria with the *gfp* Gene for Monitoring

The *gfp* gene was excised from the original plasmid pUT*gfp* (Tombolini *et al*., 1997) by digestion with *Not* I and inserted into the pBluescript (Stratagene, CA, USA) containing the ampicillin resistance gene as the selection marker. The newly constructed plasmid (pBlue/*gfp*) was introduced into KPM-018P by electroporation according to the method described previously (Toyoda *et al*., 1991). Colonies growing on L broth with ampicillin at 150 μg/mL were examined for production of green fluorescence under UV irradiation. Two-day-old culture (10^9 cells/mL) of GFP-marked bacteria (KPM-018P/*gfp*) was sprayed onto leaves of the seedlings and placed in the growth box. After 7 days of incubation, sprayed leaves were detached and directly observed with an Olympus fluorescence microscope BX-60 (IB excitation with BP460-490 excitation filter and BA510IF absorption filter).

Recovery of Bacteria Sprayed onto Leaves

One-month-old tomato seedlings (six leaves with 20 leaflets) were sprayed with KPM-018P suspension (2-day-old culture, 10^9 cells/mL) for a bacterial density of 10^4 to 10^5 cells/cm^2 leaf. Leaves were detached daily from bacteria-sprayed tomato seedlings during the experimental period of 7 days, homogenized and diluted with sterilized water and spread onto Chi-M9

medium supplemented with antibiotics to monitor the survival of the sprayed bacteria. After 2 days of incubation, the number of bacterial colonies formed was determined as colony-forming units (cfu)/cm^2 leaf. The bacteria were distinguished on the basis of their colony appearance, chitin-hydrolytic capability and antibiotic resistance. KPM-018P had ampicillin- and tetracycline-resistant, red colonies, positive for chitin hydrolysis; KPM-012A had kanamycin-resistant, pale-yellow colonies, positive for chitin hydrolysis; and *E. coli* HB101/amp$^+$ had ampicillin-resistant, white colonies, negative for chitin hydrolysis.

Ingestion of Bacteria-treated Leaves by Insects

Ten third-instar larvae of ladybird beetles (per seedling) were released onto leaves of 1-month-old seedlings sprayed with KPM-018P suspension according to the method mentioned above. The seedlings with ladybird beetles were separately placed in a transparent box ($25 \times 25 \times 40$ cm) in the growth cabinet. The seedlings were exchanged daily with newly sprayed ones during the experiment (2 weeks). Survival of larvae and leaf feeding by the larvae were recorded daily. Suspensions of *E. coli* HB101/amp$^+$ and *A. paradoxus* KPM-012A were also used as nonpathogenic controls.

To evaluate the degree of leaf feeding by the ladybird beetles, the ingested area on all leaves of each plant was scored on a 0–4 scale: 0, no feeding; 1, less than 25% of leaf area; 2, less than 50%; 3, less than 75%; 4, 75–100%. Feeding severity of each plant was determined using the following formula:

$$[(0A + 1B + 2C + 3D + 4E)/(A + B + C + D + E)]$$

where A, B, C, D and E are the number of leaves corresponding to the scores 0, 1, 2, 3 and 4, respectively. Leaves of all seedlings supplied during the larval stage were scored at the end of this stage.

RESULTS

The phytophagous ladybird beetles were collected from our tomato fields and successfully maintained on tomato leaves under laboratory conditions, as shown in our previous paper (Otsu *et al*., 2003a). Third and/or fourth instars were used for the experiments because of their active, synchronous feeding of tomato leaves.

Tomato leaves (500) were collected at random from 100 tomato plants and used for stamp culture. By gently stamping leaves onto M9 medium, 820 bacterial and 7110 yeast (budding cell-forming) colonies were obtained from 98 and 315 leaves, respectively. All bacterial colonies were transferred to the antibiotic-supplemented Chi-M9 medium, and chitin-degrading bacteria were preliminarily classified into 18 bacterial types on the basis of colony appearance and degree of sensitivity or resistance to antibiotics. Of the bacteria obtained, isolate KPM-018P had lethal activity against fourth-instar larvae in the preliminary test; $70.5 \pm 21.5\%$ (means of three separate experiments) of bacteria-ingested larvae being killed before pupation. Other isolated bacteria did not cause any lethal effect on the ingested larvae. Thus, KPM-018P was adopted as an entomopathogenic bacterium and used for the subsequent experiment.

KPM-018P formed smooth, round, fluorescent, glossy, pale-yellow colonies at 37°C and turned red when transferred to 25°C. The colonies of KPM-018P became fluidal when cultured on the solid medium for 3 days, from production of exopolysaccharides (EPS) (Allison *et al*., 1998). The isolate was tolerant to ampicillin, tetracycline and chloramphenicol and sensitive to kanamycin and streptomycin. Secretion of chitinase by KPM-018P was high, producing a clearly transparent zone around their colonies by digesting colloidal chitin in the medium (Figure 1A). According to these properties (colony color change, halo formation and antibiotics responsibilities), KPM-018P was specifically detected when

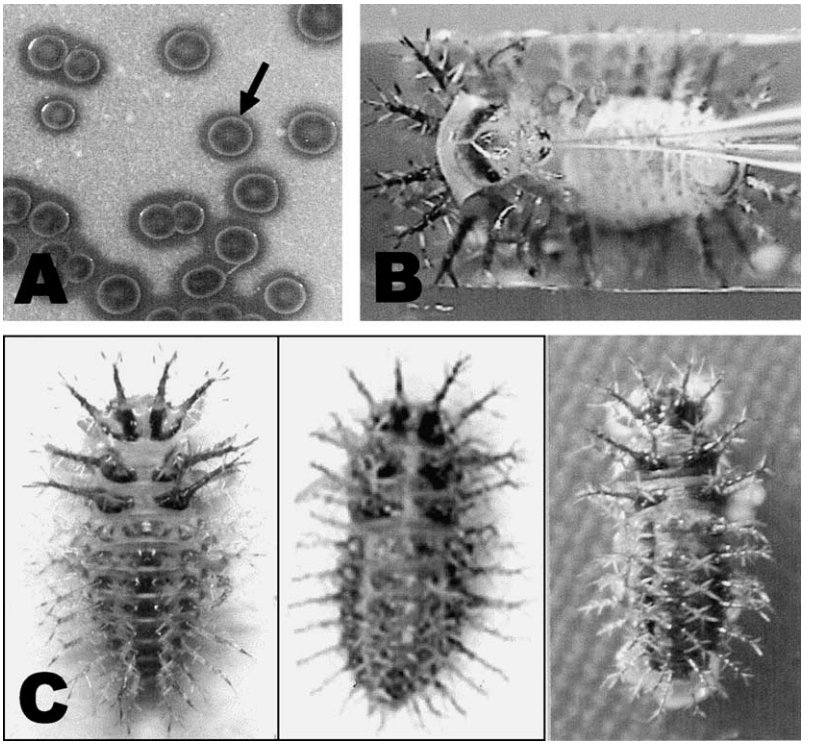

FIGURE 1. Entomopathogenic strain of *P. fluorescens* KPM-018P and larvae of ladybird beetle *E. vigintioctopunctata.* (A) Colonies of KPM-018P with transparent zone (arrow) resulting from hydrolysis of chitin in medium by bacterium-secreted chitinase (2 days after incubation on Chi-M9 medium). (B) Fourth-instar larva into which bacterial suspension was orally injected with a micropipette. (C) KPM-018P-infected larvae before (left) and 24 h (center) after injection. Note the change in body color to red or pale brown in the infected larva. Infected larva (72 h after injection) (right) placed on M9 medium. Note KPM-018P exuding from infected larva (48 h after transfer to the medium).

cultured first at 37°C for 1 day and then 25°C for 1 day on antibiotics-supplemented Chi-M9 agar.

Using criteria in *Bergey's Manual of Determinative Bacteriology*, KPM-018P was characterized as Gram-negative, motile, rod-shaped (0.6–0.7 μm wide) with one polar flagellum. Optimum temperature for growth was 25–30°C. The bacteria were aerobic, positive in the catalase reaction, nitrate reduction, arginine dihydrolase reaction, gelatin hydrolysis and negative in oxidase reaction, starch hydrolysis, and oxidation of ethanol to acetic acid. In addition, the bacteria produced acid aerobically from D-glucose and utilized D-glucose, D-mannitol and L-histidine as a carbon source. Based on these data, we propose the assignment of our strain as *Pseudomonas fluorescens* KPM-018P.

Additionally, the phylogenetic relationship between KPM-018P and other bacteria was analyzed on the basis of 16S rRNA nucleotide sequences. Figure 2 shows the phylogenetic tree of these bacteria, where KPM-018P and the strains of *P. fluorescens* were positioned in the same clade. This result confirmed the validity for identification of KPM-018P as *P. fluorescens* obtained by the conventional test for bacteriological characteristics based on the Bergey's criteria.

To further clarify the relationship between the density of KPM-018P and lethality to bacteria-ingested insects, we introduced the bacterial suspension into the mouth of fourth-

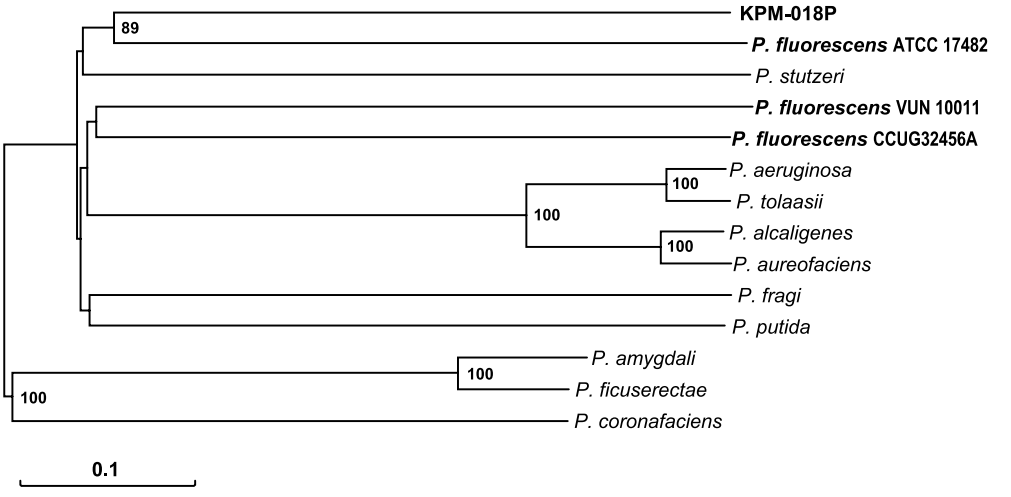

FIGURE 2. Phylogenetic position of isolate KPM-018P within the clade containing *Pseudomonas fluorescens*. The tree was constructed using neighbour-joining analysis based on unambiguously aligned 16S rRNA positions. The numbers at the nodes are the levels of bootstrap support based on neighbour-joining analysis of 1000 re-sampled data sets. Scale bar indicates 0.1 nucleotide substitution per position.

instar larvae with a micropipette (Figure 1B). This approach enabled us to quantify the volume (2 μL) of the suspension injected into each larva. The survival rate decreased with an increase of KPM-018P cells in the ingested suspension (Table 1), while no lethal effect was detected in the larvae into which nonpathogenic bacteria or distilled water was similarly introduced, indicating that the pipette introduction did not mechanically damage the larvae. Histopathological changes in the bacteria-ingested larvae were highly synchronous. The larvae ceased moving 36–40 h after injection, then their bodies turned from yellow to dark-pink or brown (Figure 1C), especially when bacteria at 10^7 cells/larva were introduced. The introduced bacteria first decreased in number, then multiplied gradually, then vigorously soon after the larvae became discolored (Figure 3). This initial decrease of bacteria was due to excretion with fecal pellets after injection. KPM-018P (between 10^3 and 10^6 bacteria/pellet) was recovered from the pellets (30) collected at random 12 h after injection, and the bacteria in the 24-h pellets were less than 10. The bacterial population reached 10^{11} cells/

TABLE 1. Lethal effect of *Pseudomonas fluorescens* KPM-018P on fourth-instar larvae of ladybird beetles as assessed by injection of bacterial suspension with oral pipette

			Number of larvae killed					Number of	
Bacteria used	Number of injected bacteria/larva	Number of tested larvae	1	2	3	4[a]	Total	Pupated larvae	Emerged adults
Sterilized water		30	0	0	0	0	0 (0[b])	30	30
KPM-018P	10^5	30	0	1	2	5	8 (8)	22 (10[c])	12
	10^6	30	0	3	10	4	17 (17)	13 (9)	4
	10^7	30	1	24	5	0	30 (30)	0	0
A. paradoxus	10^7	30	0	0	0	0	0 (0)	30	29
E. coli	10^7	30	0	0	0	0	0 (0)	30	29

[a]Days after injection of bacteria.
[b]Number of larvae in which the injected bacteria were recovered as a result of multiplication after inoculation.
[c]Number of pupae with disease symptoms followed by multiplication of bacteria during the pupal stage.

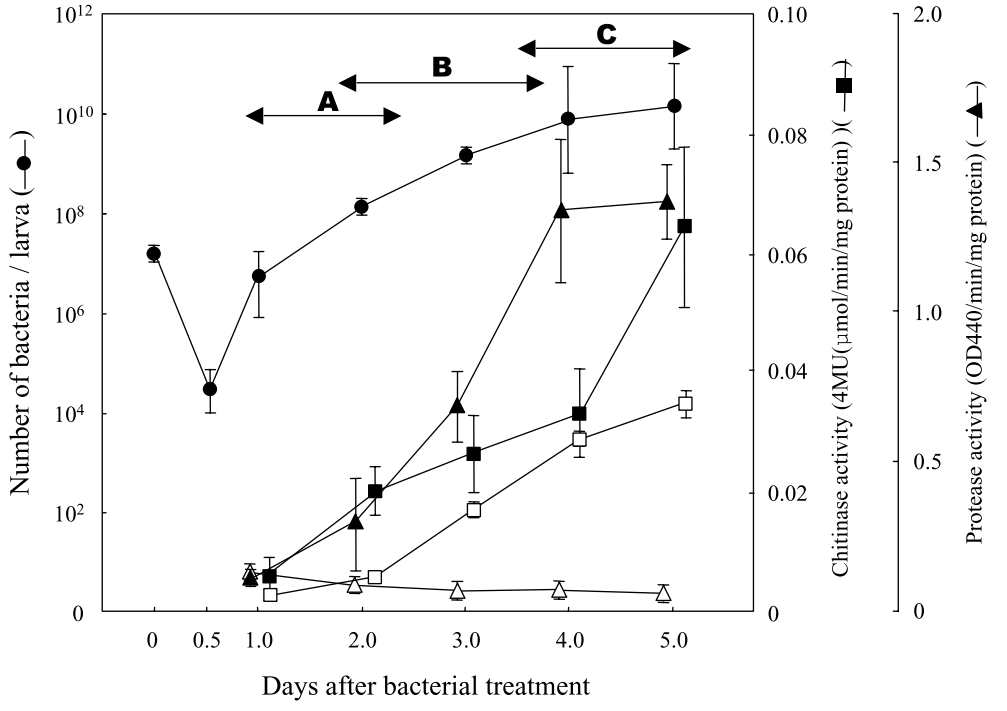

FIGURE 3. Growth of *P. fluorescens* KPM-018P in ladybird beetle larvae after micropipette injection and increase of chitinase and protease activities. A, B and C represent the duration of pathological changes in the infected larvae; the cessation of insect movement, discoloration of insect, and softening of larval shell followed by exudation of bacteria, respectively. Open squares and triangles represent the activities of chitinase and protease in non-inoculated larvae, respectively.

larva by 4 days after injection. At this stage, the larvae were soft, and bacteria gushed out of infected larvae (Figure 1C). The enzymatic activities of chitinase and protease in infected larvae considerably increased in parallel with the rapid multiplication of KPM-018P.

The present paper provides some evidence for possible characteristics that enable stable colonization of leaf surface by KPM-018P. Figure 4A shows the change in shape of droplets placed on a hydrophobic membrane. Water droplets containing Tween 20 at more than 1% (v/v) decreased spherical tension with an increase in surface activity. A similar effect was observed for droplets of the 48-h culture and supernatant of KPM-018P, indicating that the bacteria secreted surfactants into the culture. On the other hand, neither *E. coli* nor *A. paradoxus* produced enough surfactant to collapse the spherical shape of the droplet on the membrane. A similar result was also obtained when the cultures were dropped onto tomato leaves (Figure 4A). Because of these results, a 48-h culture of KPM-018P was applied as a foliar spray, and colonization on leaves was checked at various stages after spraying. Figure 4B shows *gfp*-marked bacteria (KPM-018P/*gfp*) living on tomato leaves 7 days after spraying. Fluorescing bacteria were detected frequently in the junctions of epidermal cells of bacteria-sprayed leaves. Moreover, KPM-018P was successfully recovered from leaves by stamping them onto the medium (Figure 4C). The number of bacterial colonies recovered from the stamped culture was $5.2 \pm 1.1 \times 10^3$ and $3.8 \pm 2.6 \times 10^3$ colonies/leaf 30 min and 7 days after spraying, respectively. In addition, the number of bacteria recovered from the homogenates of bacteria-sprayed leaves was between $4.6 \pm 1.8 \times 10^4$ and $2.3 \pm 1.2 \times 10^4$

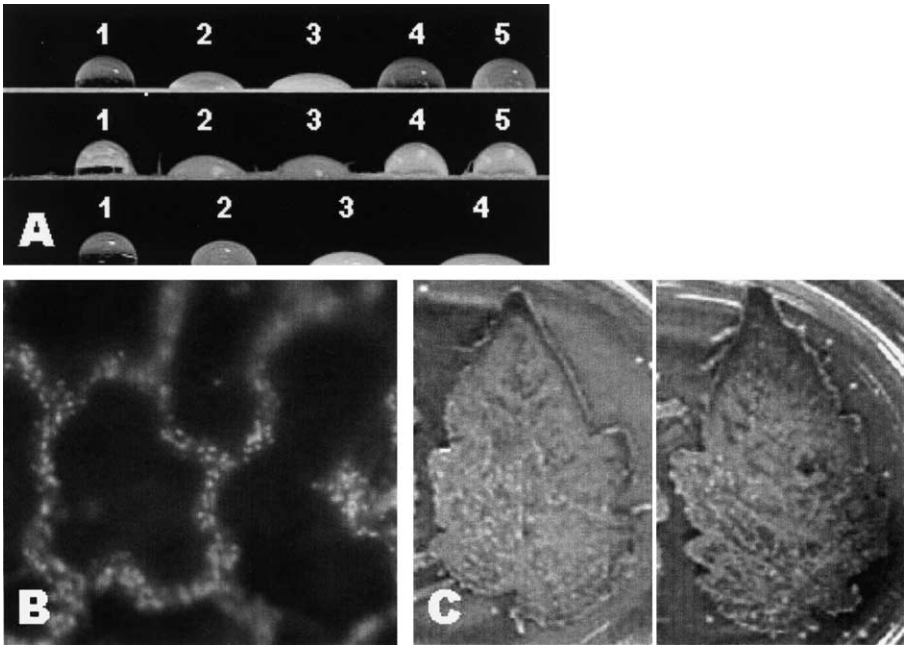

FIGURE 4. Test for production of surfactant by entomopathogenic strain of *P. fluorescens* KPM-018P and bacterial colonization of tomato leaves. (A) Droplets of bacterial suspension placed on a hydrophobic membrane (upper and lower) and tomato leaf (middle) for testing production of surfactants by bacteria. Upper and middle: 1, water droplet; 2, water droplet containing 10% (v/v) Tween 20; 3 to 5, 48-h cultures of bacteria KPM-018P (3), *A. paradoxus* KPM-012A (4), *E. coli* HB101 (5). Lower: 0 h (1), 24 h (2), 48-h culture of KPM-018P (3), 48-h culture filtrate of KPM-018P (4). (B) GFP-fluorescing bacteria (KPM-018P/*gfp*) colonizing leaf surface at the epidermal cell junctions (7 days after spraying). (C) Colonies of KPM-018P recovered from bacteria-sprayed tomato leaves by stamp culture, 30 min (left) and 7 days after spraying (right).

during the experimental period (Figure 5). These results suggest that the bacterial population on leaves did not significantly decrease within 7 days after inoculation.

Third-instar larvae were released on bacteria- or water-sprayed seedlings to examine the effectiveness of our control approach. Table 2 shows the lethal rates of larvae at the third- and fourth-instar stages, the rates for pupation and adult emergence, and the severity of leaf feeding by the larvae during the experiment. The KPM-018P treatment effectively killed almost all larvae that ingested bacteria-sprayed leaves, resulting in low pupation without adult emergence. At the same time, leaf-feeding by the larvae considerably decreased. On the other hand, neither *E. coli* nor *A. paradoxus* killed any larvae or suppressed subsequent pupation and adult emergence; the effects were comparable to those in the water-sprayed treatment.

DISCUSSION

A new outbreak of powdery mildew on greenhouse-grown tomatoes has been reported in Japan (Matsuda *et al*., 2001; Kashimoto *et al*., 2003). This pathogen also heavily infested young seedlings of tomato in our greenhouse, especially when chemical control was insufficient (Matsuda *et al*., 2001). To completely prevent the disease, therefore, repeated application of fungicides was necessary. However, this was contradictory to our university policy requiring reduced use of agricultural chemicals. Abiko (1978) examined optimal

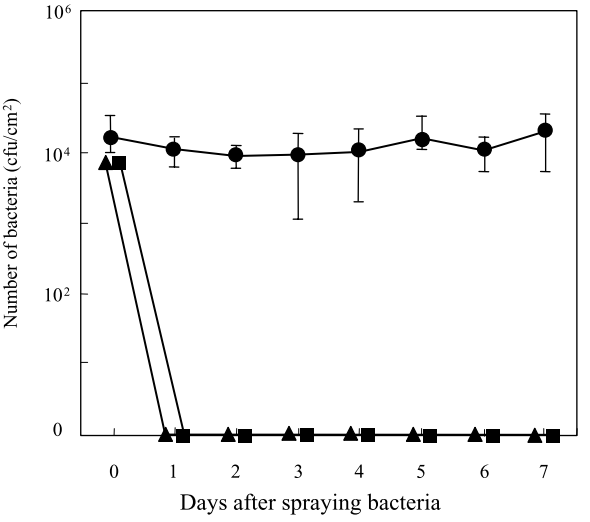

FIGURE 5. Recovery of bacteria from leaves of tomato seedlings after spraying suspensions of *P. fluorescens* KPM-018P (●), *A. paradoxus* (■) and *E. coli* HB101/pUC119 (▲). Five bacteria-sprayed leaves were detached daily, homogenized with sterilized water and spread onto Chi-M9 medium containing antibiotics. The data are means and standard deviations of three separate experiments.

conditions for colonization and sporogenesis of tomato powdery mildew and reported that high humidity (100% relative humidity) was suppressive to mycelial growth and spore production by the pathogen. Based on these investigations, the seedlings were cultivated at high humidity until transplanting. To maintain high humidity, the seedlings were placed in a growth box surrounded by transparent vinyl film that absorbed UV spectrum shorter than 390 nm, which is essential for photosporogenesis and rapid colonization on tomato by gray mold (*Botrytis cinerea*) (Honda & Yunoki, 1978). Although pathogen-free tomato seedlings could be successfully cultivated in the humid growth box, the box was ineffective for eliminating feeding by insect pests. In fact, it rather favored insect defoliators such as

TABLE 2. Effects of bacteria-treatment on larval mortality and leaf feeding, pupation and adult emergence by third and fourth instars of ladybird beetles

Treatments	Number of 3rd-instar larvae[a]	Number of mortal larvae			Indices for leaf feeding[b]	% Pupation	% Adult emergence
		3rd instar	4th instar	Total (%)			
Water-sprayed (control)	90	2	1	3 (3.3)	2.14±0.25	96.7	94.4
Bacteria-sprayed							
P. fluorescens KPM-018P	90	22	65	87 (96.7)	0.97±0.51	3.3	0
A. paradoxus KPM-012A	90	3	2	5 (5.6)	2.13±0.19	94.4	94.4
E. coli HB101	90	3	1	4 (4.4)	2.64±0.41	95.5	95.5

[a]Bacterial suspension (10^9 cells/mL) was sprayed onto tomato seedlings to make 10^5 cells/cm^2 leaf and ingested by the larvae to reach the pupal stage.

[b]The indices for severity of leaf feeding by third- and fourth-instar larvae were examined at the end of larval stage according to the formula indicated in the text.

Ten third instars were used in each experiment, and data were given as means and standard deviation (for leaf-feeding index) of eight or nine replications.

phytophagous ladybird beetles. Therefore, our strategy focused on controlling phytophagous insect pests, especially using a biocontrol agent.

The phytophagous ladybird beetle *E. vigintioctopunctata* is a pest insect of solanaceous plants (Shirai & Katakura, 1999) and naturally occurs in our greenhouses and fields. These ladybird beetles frequently infest tomatoes and eggplants to cause serious damage leading to yield loss. We developed a system for continuously rearing and maintaining these insects on tomato leaves in the laboratory (Otsu *et al*., 2003a,b). Third- and fourth-instar larvae were especially suitable for the treatments in the present study because of their active, synchronous feeding of leaves.

Obtained from the leaf surface of tomato, the entomopathogenic strain (KPM-018P) of *P. fluorescens* here was easily detected by the pale-yellow colony turning red when the incubation temperature was lowered from 37 to 25°C. Its pathogenicity on larvae of ladybird beetles was stable even after the bacteria were continuously subcultured on a minimal medium for several months. Although the pathogenicity factor for KPM-018P was not obvious, its stable pathogenicity, even after the bacteria were sprayed onto leaves, could be a prominent feature in its success as a biocontrol agent.

Micropipette application of bacteria into the larvae effectively introduced a known volume of suspension, which was useful in synchronizing the progress of the disease. Many bacteria injected were excreted from the larvae in fecal pellets at the early stage after injection. In the introduction of bacterial suspension at the highest density (10^7 cells/larva), between 10^4 and 10^5 bacterial cells/larva remained in the larvae, suggesting that these levels were essential to initiate disease in the larvae. After inoculation and subsequent discoloration of the infected larvae, multiplication of the bacteria was prominent and the insects then ceased moving. The enzymatic activities (chitinase and protease) increased in parallel with the multiplication of bacteria, causing the softening of the larval body. This activity may lead to gushing of the multiplied bacteria from the larvae.

We previously entrapped a leaf-inhabiting bacterium (*A. paradoxus*) in alginate beads to control the insect pests, because of lower leaf-surface colonization (Otsu *et al*., 2003a). In contrast, our new strain could stably colonize tomato leaf surface when it was directly applied to leaves as a cell suspension. In general, the successful colonization of bacteria on foliage is dependent upon the ability of bacteria to survive under drought or UV irradiation stress (Andrews, 1992; Beattie & Lindow, 1995). KPM-018P was frequently recovered from bacteria-sprayed leaves, even 7 days after spraying. The present study proposed data for possible functions to ensure stable colonization of the bacteria on leaf surface.

Using *gfp*-marked KPM-018P, we could directly observe GFP-fluorescence in the bacteria on the leaf surface and localizing their sites of colonization. During the experimental period, the GFP-fluorescence was consistently detected in the junctions of epidermal cells of bacteria-sprayed leaves. These junctions are likely to be depressions in which KPM-018P can survive and colonize the leaf surface.

Chemical aspects of our bacterial strain also contributed to its stable habitation of the leaf surface. Its production of biosurfactants may be useful during its attachment to the leaf surface, assisting its colonization of the phyllosphere by controlling the balance of hydrophobic and hydrophilic substances between bacterial cells and leaf surface (Bunster *et al*., 1989) and conferring wettability to the cells attached to the hydrophobic cuticle (Bunster *et al*., 1989). In fact, chemically induced mutants of KPM-018P that lack biosurfactants were recovered at a lowered rate from the leaves than were those with the biosurfactants (unpublished data), supporting the involvement of these substances in the leaf surface colonization by bacteria. Desai and Banat (1997) reported some strains of *P. fluorescens* produce lipopeptides or carbohydrorate–protein–lipid biosurfactants. Identification of biosurfactants produced by KPM-018P is underway in our laboratory.

In addition, the extracellular polysaccharides produced by bacteria may form biofilms that protect the bacteria from detrimental environmental factors that hinder colonization (Allison

et al., 1998; Morris *et al*., 1998). Biofilm formation by KPM-018P on sprayed leaves is of practical interest to us in its support of sustainable colonization of bacteria in field applications. Because strain KPM-018P is a sustainable colonizer of tomato leaves and its pathogenicity is stably expressed, spraying bacterial suspensions on leaves of tomato seedlings at 1-week intervals for 2 months may be of practical use on unsprayed tomato seedlings with few leaves that can be severely damaged by larvae feeding on leaves in the growth box. An application of KPM-018P may completely protect the seedlings by killing larvae and suppressing the emergence of the adults in the growth box. After planting, tomatoes with flourishing leaves are not seriously affected when attacked by adults or larvae of ladybird beetles. Therefore, control measures are necessary only to protect tomato at the seedling stage.

ACKNOWLEDGEMENTS

This work was supported partly by a grant (No. 99L01205) from the 'Research for the Future' program of the Japan Society for the Promotion of Science. We would like to acknowledge Dr. Beth E. Hazen for critically reading the manuscript.

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