APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 2000, p. 1183–1189 Vol. 66, No. 3
0099-2240/00/$04.00+0

21

Survival and Epiphytic Fitness of a Nonpathogenic Mutant of *Xanthomonas campestris* pv. Glycines

YAYA RUKAYADI,[1] ANTONIUS SUWANTO,[1]* BUDI TJAHJONO,[2] AND ROB HARLING[3]

Department of Biology, Faculty of Science and Mathematics, and IUC Biotechnology,[1] and Department of Plant Pathology, Faculty of Agriculture,[2] Bogor Agricultural University, Bogor, Indonesia, and Scottish Agricultural College/University of Edinburgh, Edinburgh, Scotland[3]

Received 12 July 1999/Accepted 1 November 1999

***Xanthomonas campestris* pv. glycines is the causal agent of bacterial pustule disease of soybeans. The objective of this work was to construct a nonpathogenic mutant derived from the pathogenic wild-type strain YR32 and to evaluate its effectiveness in preventing growth of its parent on the soybean phyllosphere. A mini-Tn*5*-derived transposon was used to generate nonpathogenic mutants. Southern hybridization and pulsed-field gel electrophoresis confirmed the presence of a single transposon in each of the nonpathogenic mutants. One of the nonpathogenic mutants, M715, failed to induce a hypersensitive response in tomato leaves. An ice nucleation gene (*inaZ*) carried in pJL1703 was introduced into strain YR32 as a reporter gene to demonstrate that the presence of M715 could reduce colonization of the soybean phyllosphere by YR32. de Wit serial replacement analysis showed that M715 competed equally with its wild-type parental strain, YR32. Epiphytic fitness analysis of YR32 in the greenhouse indicated that the population dynamics of strains YR32, YR32(pJL1703), and M715 were similar, although the density of the mutant was slightly less than that of its parent. The M715 mutant was able to survive for 16 days after inoculation on soybean leaves and maintained population densities of approximately 10^4 to 10^5 cells g (fresh weight) of leaf^{-1}. Therefore, M715 shows promise as an effective biocontrol agent for bacterial pustule disease in soybeans.**

Bacterial diseases are among the most serious problems in soybean production since they reduce the total production of this important protein-producing legume. *Xanthomonas campestris* pv. glycines causes bacterial pustule disease on soybeans worldwide (17, 22). Although this bacterium is widely known as *X. campestris* pv. glycines, following DNA-DNA hybridization analysis, Vauterin et al. (28) have reclassified it as *X. axonopodis* pv. glycines.

Various soybean production technologies such as specific planting, soil and nutrient management strategies, and the use of pathogen-free soil and seeds have resulted in a reduction in pests and diseases. Disease-resistant plants have also been introduced. However, our recent finding that *X. campestris* pv. glycines strains exhibit very diverse genotypes as shown by DNA fingerprinting (18) may complicate efforts to construct or select soybean plants resistant to all strains of *X. campestris* pv. glycines because breeding may need to take account of this diversity if resistance is to be achieved against all strains. In addition, the inappropriate use of antibacterial pesticides can cause health and environmental problems because of antibiotic residues in the environment, or it can cause the emergence of bacterial resistance in plant and human pathogens. Therefore, a reliable biocontrol agent may be a promising alternative for control of bacterial pustule disease in soybeans.

Certain groups of bacteria, such as *Pseudomonas*, *Xanthomonas*, and *Erwinia* spp., are present normally on leaves. Nonpathogenic members of these groups have been used as potential biocontrol agents (8). Lindow (11) reported that two different strains of bacteria located on the same leaf surface could compete for the same nutrient sources or habitats. He also reported that the competition for nutrients or space among microorganisms may play an important role in determining the population density of phyllosphere bacteria. However, the use of randomly selected antagonistic bacteria may result in a loss of biocontrol when environmental conditions change, as different bacterial strains or species may respond differently to these changing conditions. An alternative approach would be to use nonpathogenic, isogenic mutants of the wild type, as these would be expected to behave similarly to the parent strain, provided the loss of virulence did not compromise fitness ability. An isogenic mutant would be expected to reduce the population of its parent in the same habitat through competition under a range of conditions. There has been relatively little work published to test this concept, although isogenic mutant strains of *Pseudomonas syringae* which lack a certain factor were reported to reduce the proliferation of their wild-type strains on the phylloplane (2, 3, 6, 14). The construction of isogenic, nonpathogenic strains of *X. campestris* pv. glycines would also be a mechanism to study factors affecting survival and epiphytic fitness on the phylloplane, as well as offering the potential use of the mutant strains to suppress bacterial pustule disease on soybean plants.

In this study, we used transposon mutagenesis to generate a nonpathogenic mutant of *X. campestris* pv. glycines and then analyzed its competitive ability and epiphytic fitness in planta, using an ice nucleation gene (*inaZ*) as a reporter molecule.

MATERIALS AND METHODS

Bacterial strains and plasmids. Bacterial strains and plasmids used in this work and their relevant characteristics are described in Table 1.

Growth conditions and media. *X. campestris* pv. glycines YR32, YR32(Ice+), and the nonpathogenic mutant M715 (Table 1) were grown routinely in Luria-Bertani broth (LB) at pH 7.0 or on YDCA (10 g of yeast extract, 5 g of dextrose 20 g of $CaCO_3$, and 15 g of agar per liter) at 32°C. *Escherichia coli* strains were cultured at 37°C in LB. The *Pseudomonas fluorescens* strain was grown at 26°C in nutrient agar (NA; 10 g of Lab-Lemco powder, 10 g of peptone, 5 g of sodium chloride, and 15 g of agar per liter). Antibiotics were supplemented when appropriate at concentrations of 30 (chloramphenicol), 25 (kanamycin), and 100 (rifampin and trimethoprim) μg ml^{-1}.

* Corresponding author. Mailing address: Department of Biology, Faculty Science and Mathematics, Bogor Agricultural University, Jl. Raya Padjadjaran, Bogor 16144, Indonesia. Phone: (62) 251 625965. Fax: (62) 251 315107. E-mail: asuwanto@indo.net.id.

TABLE 1. Bacterial strains and plasmids used[a]

Strain or plasmid	Relevant characteristics	Reference(s) or source
Bacterial strains		
E. coli		
DH5α	F^- *lacZ*ΔM15 *recA hsdR17 gyrA thi*	20
HB101	Res^- Mod^- *recA13* Sm^r	20
S17-1λ*pir*	Pro^- Res^- Mod^+ *recA*, integrated plasmid RP4-Tc::Mu-Km::Tn7, lysogenized with λ*pir* phage	21
P. fluorescens 5064	Wild type	4
X. campestris pv. glycines		
YR32	Wild type, Rif^r	18, 30
8ra	Wild type	7
M715	Rif^r Km^r	This study
YR32(Ice+)	YR32 containing pJL1703; Rif^r Km^r *inaZ*	This study
X. campestris pv. campestris 33913	Wild type	ATCC
R. sphaeroides 2.4.1	Wild type	24
Plasmids		
pJL1703	Km^r, *inaZ* with its own promoter	15
pYR103	pUTmini-Tn5Km^r-Tp^r	19
pRK2013	ColE1 replicon, Tra^+ of RK2, Km^r	5

[a] Abbreviations: Rif, rifampin; Km, kanamycin; Sm, streptomycin; Tp, trimethoprim; ATCC, American Type Culture Collection.

Transposon mutagenesis. pYR103 is a suicide plasmid harboring mini-Tn5-Km^r, modified to carry *Pac*I and *Pme*I sites, as well as the gene for trimethoprim resistance. *Pac*I and *Pme*I are rare-cutting restriction endonucleases important in our effort to physically map the genome of *X. campestris* pv. glycines while the trimethoprim resistance gene is necessary to tag certain strains of *X. campestris* pv. glycines which are resistant to other antibiotics. In addition, there is a unique *Ase*I site within Kan^r gene (from Tn*903*) to facilitate localization of the transposon insertion using *Ase*I schizotyping, i.e., digestion of intact genomic DNA with *Ase*I, one of the rare-cutting restriction endonucleases for *Xanthomonas*, followed by separation of the resulting DNA fragments by pulsed-field gel electrophoresis (26).

Transposon Tn*5* derivatives (pUTmini-Tn5Km^r-Tp^r) (19) were thus introduced into *X. campestris* pv. glycines YR32 (18) by biparental conjugal mating (25). The donor strain, *E. coli* S17-1λ*pir* (pUTmini-Tn5Km^r-Tp^r), was grown in LB medium containing kanamycin and trimethoprim. The recipient strain, *X. campestris* pv. glycines YR32, was grown at 28°C in LB containing rifampin. Bacterial cells in logarithmic phase (10^8 cells ml^{-1}) were washed twice in LB and resuspended to a concentration of about 10^9 ml^{-1} in LB; 250 μl of the donor and 1,000 μl of the recipient cells were mixed, and 50 μl of the mixture was placed on sterile nitrocellulose filters on solidified LB agar (LA). The cells were allowed to conjugate on the filter at 30°C overnight. The filters were then transferred into 500 μl of LB in a microcentrifuge tube and vortexed well, and 100 μl of the cell suspension was spread onto LA plates supplemented with rifampin, kanamycin, and trimethoprim. Colonies appearing after 2 to 3 days were restreaked several times onto YDCA supplemented with rifampin and kanamycin (YDCA-RK) before use in pathogenicity assays.

Pathogenicity assay. Pathogenicity of the mutants was determined in two separate experiments by soybean cotyledon bioassay and leaf bioassay.

(i) Cotyledon bioassay. A soybean cotyledon bioassay as reported previously (7, 16) was used as follows. Each mutant was tested for pathogenicity on 10 cotyledons, and each trial was repeated four times. Detached soybean cotyledons from 7-day-old seedlings grown in the greenhouse were surface sterilized with 0.5% sodium hypochlorite for 5 min and washed with sterile distilled water for 5 min. The centers of the cotyledons were wounded with multiple pins attached to the end of a iron stick (five pins spaced evenly in an area 6 mm in diameter). Onto the wounds was gently dropped 20 μl (10^7 cells ml^{-1}) of a suspension of mutant and wild-type strains of *X. campestris* pv. glycines. Inoculated cotyledons were placed in moist trays and kept in a lighted incubator (16-h photoperiod) at 30°C. A standard curve of turbidity against CFU was used to obtain the number of cells in suspension. The nonpathogenicity of the mutants was identified by the absence of chlorotic symptoms around the inoculation site within 3 to 4 days after inoculation. The bacterial inocula were prepared by growing strains at 30°C for 48 h in YDCA-RK. Wild-type (YR32) and strain 8ra positive controls and negative controls using *X. campestris* pv. campestris 33913 and *E. coli* DH5α were included in each trial.

(ii) Leaf bioassay. A quantity of 250 ml of the nonpathogenic mutant (M715) (10^8 cells ml^{-1}) was sprayed over 10 plants, each ca. 20 cm high, contained in 10-cm pots. A standard curve of turbidity against CFU was used to obtain the number of cells in suspension. The inoculated plants were incubated in a lighted incubator (16-h photoperiod) at 30°C. The pathogenicity of the mutant on the inoculated plants was observed 3 to 4 days after inoculation. The bacterial inocula were prepared by culturing strains at 30°C for 48 h in YDCA-RK. The wild-type strain (YR32) and potassium phosphate (KP) buffer as a control (10 mM, pH 7.0) were included in each trial.

HR. The hypersensitive response (HR) of a nonpathogenic mutant (M715) was tested on tomato (*Lycopersicon esculentum* var. Lukullus) leaves. The tomato leaves were injected into the mesophyll with 10 μl of bacterial cells (10^8 ml^{-1}) in suspension, using a 1-ml hypodermic syringe without a needle. A standard curve of turbidity against CFU was used to obtain the number of cells in suspension. Detached injected leaves were placed on moist trays and kept at room temperature. The HR (water-soaking of injected tissue) was observed 18 h after inoculation. The bacterial inocula were prepared by growing strains of *X. campestris* pv. glycines at 30°C for 48 h in YDCA-RK and *P. fluorescens* at 25°C for 24 h in NA. Wild-type (YR32) and negative controls using a phyllosphere strain of *P. fluorescens* (5064) and KP buffer were included in each trial.

Preparation of intact genomic DNA and restriction digests. Bacterial suspensions were prepared as follows. One loopful from a single colony was inoculated into 10 ml of LB (pH 7.0) before incubation at 30°C for 24 h. Bacterial cells were harvested by centrifugation and resuspended in PIV buffer (10 ml of Tris-HCl [pH 7.5], 1 M NaCl) to a final concentration of approximately 2 × 10^9 cell ml^{-1}. The gel plugs (10 by 5 by 1 mm) were prepared as described by Smith and Cantor (23). Restriction endonuclease digests were performed as described previously (24), with 8 to 15 U of enzyme for each digest. Digestion of DNA in the agarose plugs was performed at 37°C for 12 h. All restriction buffers were prepared, and reaction conditions were as recommended by the supplier. *Ase*I and *Spe*I were obtained from New England Biolabs (through Research Biolabs, Pte, Ltd., Singapore, Republic of Singapore). Low-melting-point agarose for gel plugs was obtained from Bio-Rad (through P. T. Diastika Biotekindo, Jakarta, Indonesia).

DNA fragment separation. For separation of *Ase*I and *Spe*I fragments, electrophoresis was carried out at 3.6 V cm^{-1} for 22 h with 5- to 40-s pulse times in a CHEF DR-II (Bio-Rad, Richmond, Calif.) apparatus. *Ase*I-digested *Rhodobacter sphaeroides* 2.4.1 genomic DNA (24) was routinely used as a molecular size standard.

Plasmid isolation and Southern hybridization analysis. Plasmid isolation and digestion were carried out as described by Sambrook et al. (20). Southern hybridization analysis was performed by vacuum transfer of DNA from the agarose gel onto a nylon membrane (Photogene), using a Vacu Gene XL (Pharmacia Biotech) for 4 h, followed by membrane fixation using a GS Gene Binder UV chamber (Bio-Rad) at a wavelength of 280 nm twice for 150 s each time. DNA for nonradioactive probing was prepared from a 2.8-kb *Eco*RI DNA fragment containing trimethoprim and kanamycin resistance genes (Table 1). Probe labeling and hybridization detection were conducted using the ECL (enhanced chemiluminescence direct nucleic acid labeling and detection system (Amersham Life Science) and exposed on X-ray Hyperfilm ECL (high-performance ECL film; Amersham Life Science).

Introduction of the ice nucleation gene. pJL1703 containing the ice nucleation gene (*inaZ*) from *P. syringae* (15) was introduced into *X. campestris* pv. glycines YR32 by triparental mating as follows. *E. coli* DH5α(pJL1703) as a donor and *E. coli* HB101(pRK2013) as a helper strain were grown at 37°C overnight in LB containing kanamycin. *X. campestris* pv. glycines YR32 was grown at 30°C for 24 h in LB containing rifampin. Bacterial cells (10^8 ml^{-1}) were washed twice in KP buffer and resuspended in LB at a concentration of about 10^9 ml^{-1}. Then 100 μl each of the donor and helper cell suspensions and 1,000 μl of the recipient cell suspension were mixed, and 50 μl of the mixture was placed on a sterile nitrocellulose filter on a solidified LA. The cells were allowed to conjugate on the filter at 30°C overnight. The filters were then transferred into 200 μl of LB in microcentrifuge tubes and vortexed well, and the cell suspensions were spread onto LA plates supplemented with kanamycin and rifampin. Colonies appearing after 2 to 3 days were restreaked several times onto YDCA-RK before being subjected to an ice nucleation activity assay (11, 13). The cumulative number of ice nuclei was calculated by the method of Vali (27).

Competition analysis. Competition analysis between *X. campestris* pv. glycines M715 and YR32 was conducted in planta on 2-week-old soybean plants (*Glycine max* Merrill var. Willis) inside a greenhouse as follows.

(i) Tube nucleation assay. *X. campestris* pv. glycines strains were cultured in YDCA-RK at 30°C for 48 h. Bacterial cells were removed from the agar surface with a glass spreader and suspended in KP buffer. The concentrations of the cell suspensions were determined turbidimetrically after adjustment by dilution in KP buffer. A standard curve of turbidity against CFU was used to obtain the number of cells in suspension. The concentration of the suspension was adjusted to 10^4 cells ml^{-1} for each bacterial inoculum. Fifty milliliters of M715 suspension was used to spray four pots each containing 10 soybean plants, making a total of 40 plants. Two days later, the same plants were sprayed with 50 ml of YR32(Ice+) suspension. Individual pots were then covered with a transparent plastic bag to maintain humidity and placed in a completely randomized block design on a bench in a greenhouse at 30 to 32°C and 82 to 87% relative humidity. After 3 days, all of the pots were moved to a 18°C room overnight; 20 leaves were

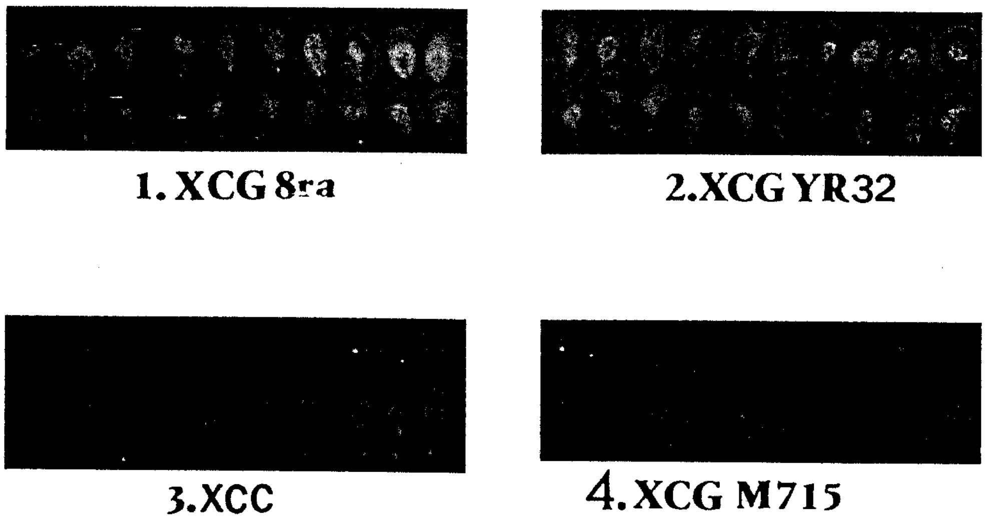

FIG. 1. Pathogenicity test using a cotyledon bioassay. *X. campestris* pv. glycines (XCG) M715 is the nonpathogenic mutant; *X. campestris* pv. glycines 8ra is the positive control; *X. campestris* pv. campestris is the negative control; *X. campestris* pv. glycines YR32 is the wild type. The loss of pathogenicity by strain M715 is shown by the absence of water-soaking or yellow chlorosis symptoms around the lesions on the cotyledon.

then taken randomly from the 10 plants in each pot, making a total of 80 leaves; each leaf was considered a replicate, and therefore *n* was equal to 80. The leaves were put individually into 20-ml tubes containing 5 ml of KP buffer and then analyzed for ice nucleation activity after incubation at −4.5°C for 10 min. Competition capability (Nf) was quantified based on the number of frozen tubes divided by the total number of tubes to give a ratio of frozen to unfrozen tubes (11); a ratio of <0.25 indicated that the mutant had good competition capability.

(ii) de Wit analysis. For de Wit serial replacement analysis, cultures of M715 and YR32(Ice+), each prepared as described above to give a concentration around 5×10^6 cells ml^{-1}, were mixed in proportions of 0:10, 1:9, 2;8, 3:7, 4:6, 5:5, 6:4, 7:3, 8:2, 9:10, and 10:0; 50 ml of each mixture was then used to spray four pots containing 10 soybean plants, making a total of 40 plants. Each treatment was again replicated four times. After incubation in the greenhouse at 30 to 32°C for 72 h and 82 to 87% relative humidity, 5 leaves were taken randomly from the 10 plants in each pot, making a total of 20 leaves per mixture. Individual leaves were then placed into 20-ml tubes containing 5 ml of KP buffer and sonicated for 7 min in a mini Braun waterbath sonicator. Leaves were subsequently agitated vigorously using a vortex mixer to detach the phyllosphere bacteria. Appropriate dilutions of the suspension were plated on YDCA-R to estimate bacterial cell numbers. Each leaf was weighed to allow the bacterial population size to be normalized to the fresh weight of leaf tissue. To differentiate colonies of strains YR32(Ice+) and M715 on YDCA-R plates, 30 to 100 colonies were placed using a toothpick on aluminum foil smeared with a thin layer of margarine and covered with a drop of 20 μl of KP buffer. The foil was then placed at −4.5°C for 10 min and subsequently analyzed for ice nucleation activity.

Epiphytic fitness analysis. Simultaneous inoculation of M715 and YR32 (Ice+) on soybean phyllosphere was conducted to examine the relative fitness of M715 in comparison to that of YR32. Plant inoculation was performed in two ways: (i) individual inoculation and (ii) coinoculation with a mixture of M715 and YR32(Ice+) at a 1:1 ratio. Bacterial cultures were prepared as described above to a final concentration of 4.5×10^5 cells ml^{-1}. Then 50 ml of bacterial suspension was used to spray four pots containing 10 soybean plants, making a total of 40 plants. The plants were transferred to the greenhouse at 30 to 32°C for 16 days and at 82 to 87% relative humidity. Three leaves were taken randomly from the 10 plants in each pot, making a total of 12 leaves per day. Estimation of bacterial numbers and the relative proportions of mutant to wild type were made as described above for the de Wit analysis. The experiment was repeated four times.

Statistical methods. Statistical calculations were performed using SAS Graph (version 6.12; SAS Institute Inc., Cary, N.C.) for graphics and Minitab 12 for calculation of standard deviation.

RESULTS

Transposon mutagenesis and mutant analysis. Transposon mutagenesis of *X. campestris* pv. glycines YR32 employing *E. coli* S17-1λ*pir*(pYR103) as the donor strain generated *X. campestris* pv. glycines mutants which were resistant to kanamycin. The average frequency of transposition was 8.3×10^{-6} per recipient, which is higher than the frequency of transposition obtained from other transposons (19).

A total of 2,187 mutants were analyzed for pathogenicity. By cotyledon bioassay, we identified six mutants which apparently lacked pathogenicity, as revealed by the absence of disease

TABLE 2. Pathogenicity of transposon-generated mutants of strain YR32 determined by cotyledon bioassay

Population	No. of chlorotic cotyledons/no. tested
Mutants[a]	
$M715_1$	1/40
$M715_2$	3/40
$M715_3$	1/40
$M715_4$	1/40
$M715_5$	3/40
$M715_6$	0/40
Proportions of M715 and YR32(Ice+)[b]	
0:10	39/40
1:9	36/40
2:8	36/40
3:7	28/40
4:6	21/40
5:5	16/40
6:4	16/40
7:3	11/40
8:2	8/40
9:1	3/40
10:0	3/40
Positive controls	
YR32 (wild type)	39/40
8ra (wild type)	40/40
Negative controls	
X. campestris pv. campestris 33913	2/40
E. coli DH5α	0/40

[a] Total cell concentration is approximately 10^7 ml^{-1}.
[b] Total cell concentration is approximately 5×10^6 ml^{-1}.

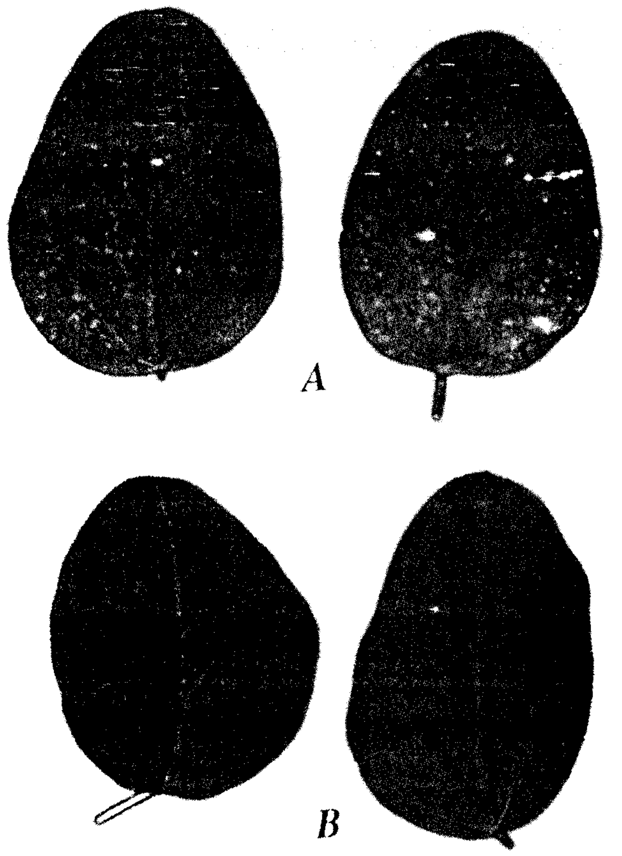

FIG. 2. Pathogenicity test using the leaf bioassay. *X. campestris* pv. glycines YR32 is the wild type (A); the nonpathogenicity of M715 (B) is shown by the absence of symptoms on the leaves.

symptoms. In some cases, minor chlorosis was noted around the inoculation site, but this also occurred on negative controls (Fig. 1). There was no evidence of water-soaking or intracellular growth of the mutants. The cotyledon bioassay data for mutants and biocontrol pathogenicity are shown in Table 2. The nonpathogenic mutant (M715) showed no symptoms (pustule formation and chlorosis) on either detached cotyledons or leaves (Fig. 2). The mutant showed no water-soaking on tomato leaves, unlike the wild type (Fig. 3). Buffer and *P. fluorescens* controls also showed no symptoms.

*Ase*I-schizotyping analysis, in conjunction with Southern hybridization analysis of nonpathogenic (*pat*) mutant genomes using a 2.8-kb *Eco*RI fragment (Km^r-Tp^r fragment) derived from pYR103 as a probe, revealed that the transposon was located in the 185-kb *Ase*I fragment such that the fragment was split into 105- and 80-kb *Ase*I fragments. Therefore the genome of every mutant derived from the transposon mutagenesis in this study carried one additional *Ase*I site. All of the six nonpathogenic mutants had an insertion in the same 185-kb fragment. We picked one of the mutants (designated M715) for further characterization.

Competition assay. M715 and its wild-type parent strain (YR32) showed identical colony morphologies and other observable phenotypic characters, such as pigmentation and mucoidy. Therefore, it was necessary to label one of these strains before we could perform competition analysis in planta. The *inaZ* gene from *P. syringae* was successfully used as a molecular marker for YR32. YR32(pJL1703), designated YR32(Ice+), expressed ice nucleation at −4.5°C with an average of one to three ice nuclei per 10^6 cells.

M715, YR32, and YR32(Ice+) showed indistinguishable colony morphologies and growth profiles in LB medium. In addition, *Ase*I and *Spe*I schizotyping demonstrated that YR32 and YR32(Ice+) were genetically very similar. YR32(Ice+) differs from its wild-type counterpart (YR32) only by the acquisition of pJL1703. Unfortunately, this information does not reveal the identity of the disrupted gene, which would require further sequence data.

Competition analysis employing the test tube nucleation assay showed that M715 was able to repress the growth of the YR32(Ice+) population. The average Nf value obtained was 0.24, which indicated that up to 75% of the YR32(Ice+) population may have been replaced by M715. de Wit serial replacement analysis (Fig. 4) showed that the population density for each combination of YR32(Ice+) and M715 was close to that predicted, assuming that all strains competed equally for limiting resources and hence replaced each other equally when present in mixtures (Fig. 4). This result showed that the transposon insertion in the M715 mutant did not affect its compet-

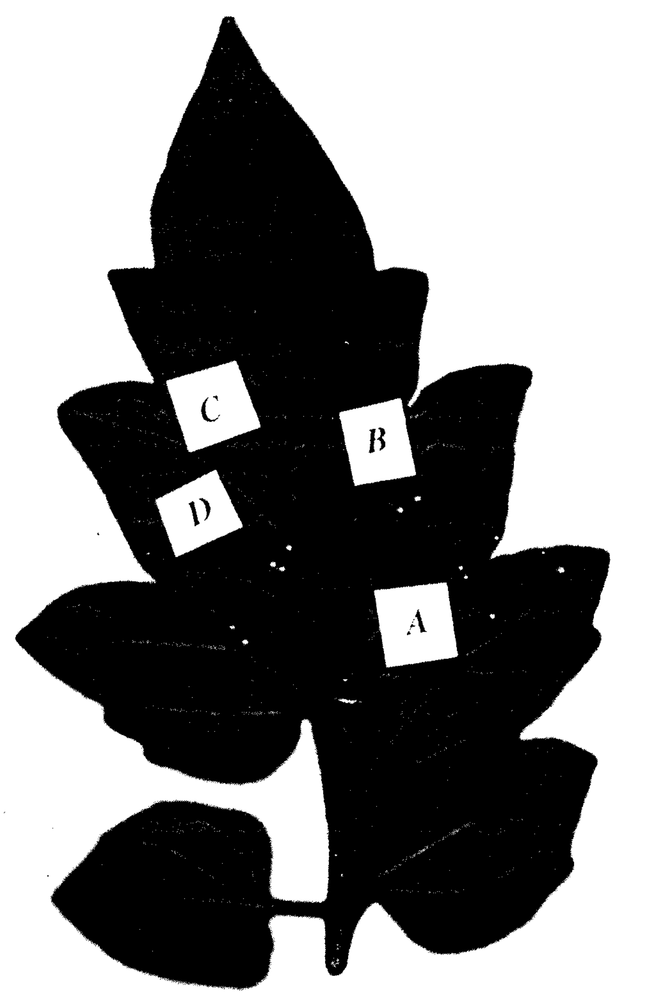

FIG. 3. HR using tomato leaves. *X. campestris* pv. glycines YR32 is the wild type (A); M715 is the nonpathogenic mutant (B); *P. fluorescens* 5064 (C) and KP buffer (D) serve as the negative controls.

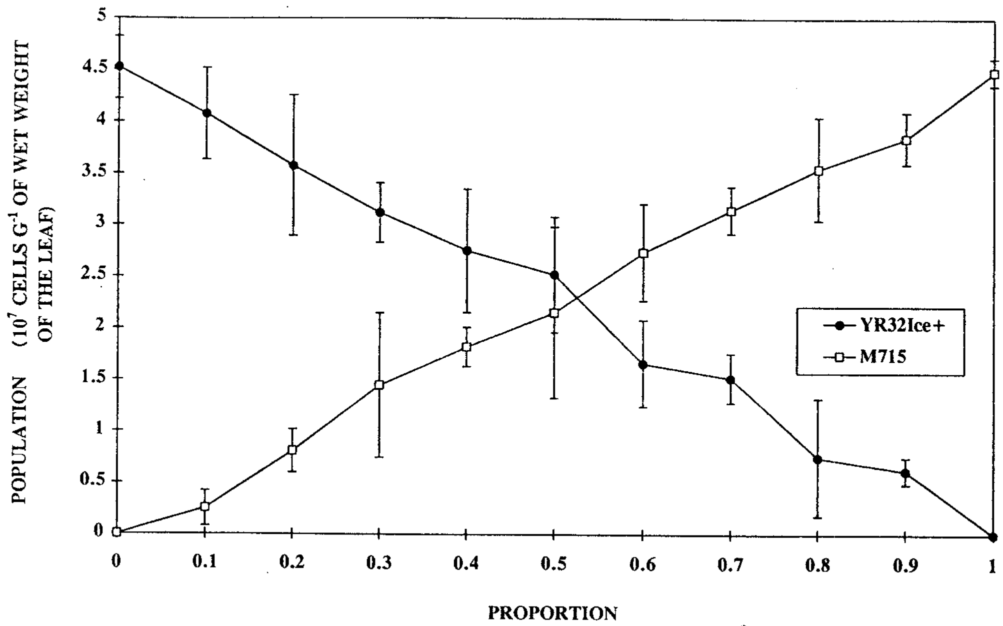

FIG. 4. de Wit serial replacement analysis between YR32(Ice+) and M715. Each strain was applied at a concentration of 5×10^6 cells ml^{-1}. The ratios of YR32(Ice+) to M715 used in this experiment were 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9, and 0:10. Error bars show ±1.4 standard errors of the mean derived from four replicate experiments.

itive ability on the soybean leaves and that M715 might still colonize the same ecological niche as YR32.

Epiphytic fitness analysis of M715. Individual inoculation of either strain resulted in similar dynamics (Fig. 5) except that the population size of YR32(Ice+) was always higher than that of M715. This indicated that the insertion of mini-Tn5, leading to the *pat* phenotype of M715, may have reduced epiphytic fitness. Nevertheless, in coinoculation experiments, the YR32 (Ice+) population size decreased such that the two population dynamic plots became similar (Fig. 6). This showed that M715 was able to repress the growth of YR32(Ice+) despite the slightly lower epiphytic fitness.

DISCUSSION

Biocontrol usually requires an antagonist bacterial population to be present in high numbers before the arrival of a pathogen (10). Thus, M715 was sprayed on the soybean leaves 2 days before inoculation by YR32(Ice+). Competition analysis between M715 and YR32(Ice+) by a tube nucleation assay

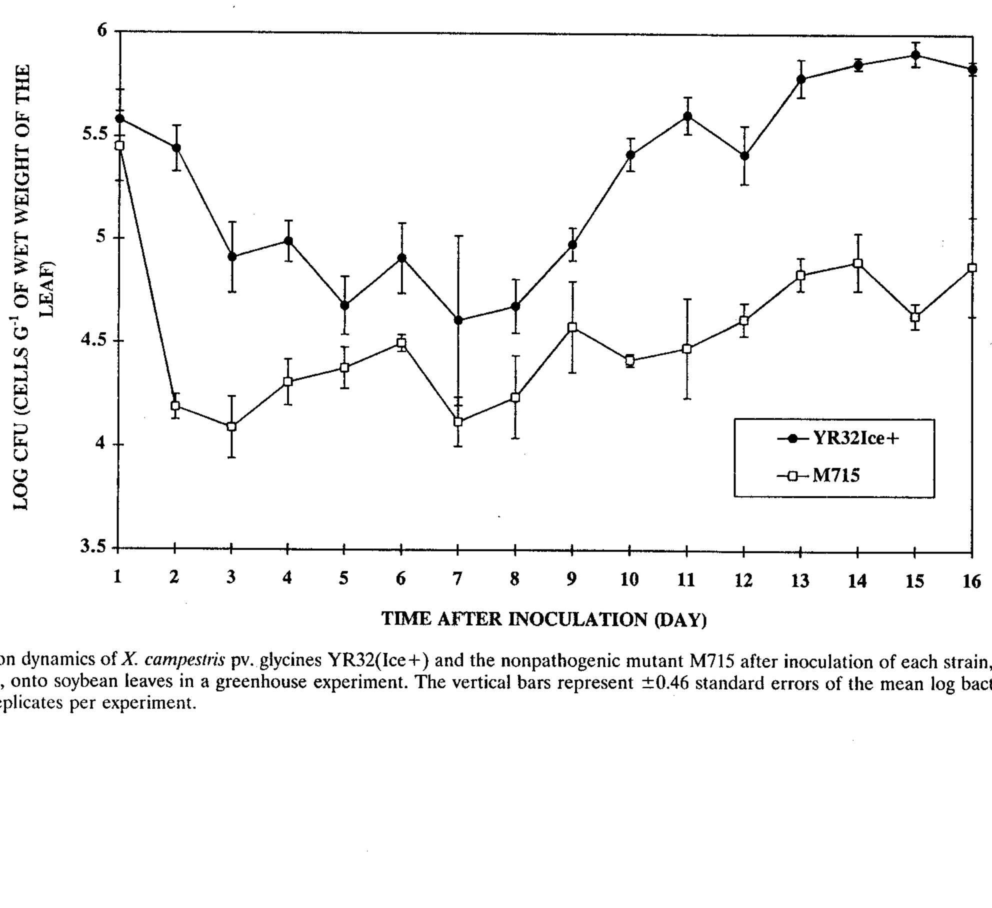

FIG. 5. Population dynamics of *X. campestris* pv. glycines YR32(Ice+) and the nonpathogenic mutant M715 after inoculation of each strain, at a concentration of 4.5×10^5 cells ml^{-1}, onto soybean leaves in a greenhouse experiment. The vertical bars represent ±0.46 standard errors of the mean log bacterial population sizes derived from four replicates per experiment.

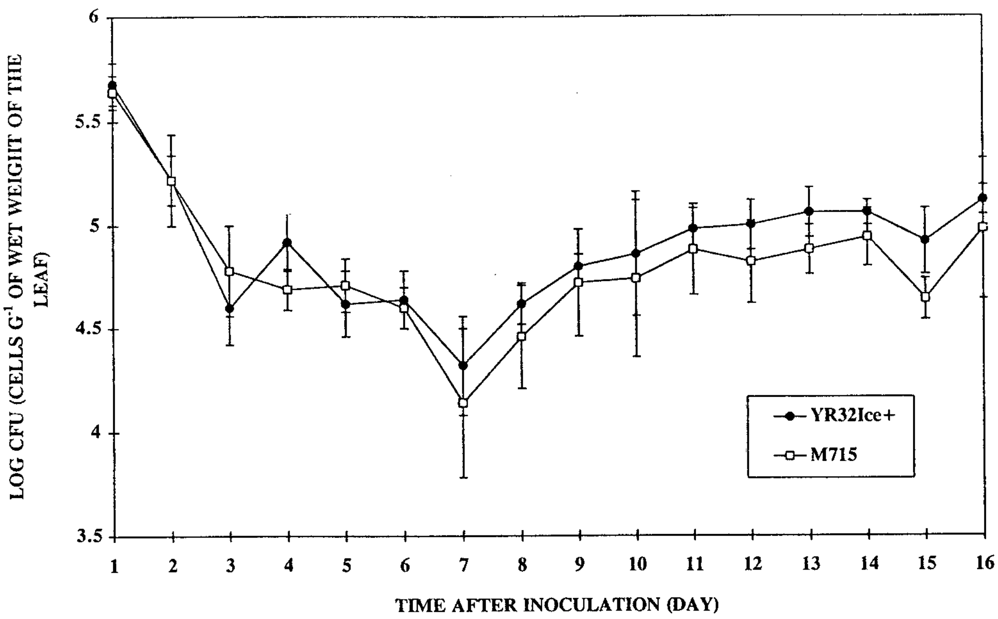

FIG. 6. Population dynamics of a mixture of *X. campestris* pv. glycines YR32(Ice+) and the nonpathogenic mutant M7+5 after coinoculation in a proportion of 1:1 onto soybean leaves in the greenhouse experiment. Each strain was present at a concentration of 4.5×10^5 cells ml^{-1} in the inoculum mixture. The vertical bars represent ±0.51 standard errors of the mean log bacterial population sizes derived from four replicates per experiment.

showed that the population of M715 could repress the growth of YR32(Ice+). M715 failed to induce a hypersensitive response in tomato leaf, in contrast to its wild-type strain that is HR$^+$. This result suggested that M715 might be an *hrp* mutant. Therefore, the low value of Nf in competition analysis suggested that the presence of M715 made the soybean phyllosphere not favorable for the growth of its wild-type parental strain. By lacking a functional *hrp* system, M715 was acting to induce host resistance to the parental strain since it no longer could inhibit the plant defense and thus sensitized the plant to the present of the parental strain. Lindemann (9) reported that the competition between pathogen and biocontrol agent based on the ecological niche monopoly is a reliable method to detect biocontrol effectiveness. Bacteria such as M715 might not be able to monopolize the soybean phyllosphere habitat, but M715 could be considered a potential biocontrol agent due to its ability to induce the host defense system.

de Wit serial replacement analysis showed that the mutant and its parent had similar competitive abilities, suggesting that the mutation did not influence competitive ability. We also evaluated the fitness of M715 in the phyllosphere. The wild type maintained a population density higher than that of M715, which suggested that the transposon reduced the epiphytic fitness of the mutant bacterium. Mutant M715 appears to have lost basic pathogenicity because of the lack of symptoms on both cotyledons and leaves and the inability to induce the HR on a nonhost. Loss of pathogenicity factor in the mutant M715 appears to have affected initial establishment on the leaf, because of the greater initial drop in population size in the mutant than in the wild type. However, in coinoculation experiments it still could reduce the wild-type pathogen population. This result suggested that M715 could induce a host defense since it no longer could suppress the host defense, and the wild-type strain was trapped in the defense reaction. Similar findings were also reported by Hirano et al. (6), who studied *lemA*, the gene required for in regulation of brown spot lesion formation and for the production of syringomycin and extracellular proteases in snap beans. These products contribute to the epiphytic fitness of *P. syringae* pv. syringae in the field. The *lemA* mutant survived and also significantly reduced the population of wild-type *P. syringae* pv. syringae in the field when coinoculated with the wild-type strain at a 1:1 ratio. The *lemA* mutant and wild type achieved similar large population sizes. However, population sizes of the wild type in the coinoculation treatment were much lower than those when it was inoculated alone. Inactivation of the *lemA* gene appeared to have rendered the mutant suppressive to the wild type (6).

The relatively high survival rate of M715 could be due to its natural ability to colonize the soybean phyllosphere. Wilson and Lindow (29) reported that the success of certain biocontrol agents was not only dependent on their ability to be isolated, identified, or engineered but also influenced by their fitness and survival in the natural habitat. Moreover, Beattie and Lindow (1) reported that the leaf surface is an extremely competitive area for microorganisms because of the limited area to support the growth of bacterial populations [average of 10^7 cells g (wet weight) of the leaf^{-1} or about 10^6 cells (cm^2)$^{-1}$] in comparison to other habitats such as soil or plant roots (10). The ability of epyphitic microorganisms to grow in a dense population depends on their fitness in that microenvironment (11). If two different bacterial strains occupy the same niche on the leaf surface, then the antagonism between them can occur only when they use the same nutrient source and occupy the same niche. M715 is an isogenic nonpathogenic strain derived from YR32; thus, it can assumed that M715 has the same ecological niche as YR32 and must compete for the same nutrient source. In summary, the results strongly suggest that M715 can be developed as a biocontrol agent to prevent bacterial pustule disease in soybeans. We are also in the process of characterizing the gene inactivated by transposon insertion leading to the M715 phenotype.

ACKNOWLEDGMENTS

We thank Steve Lindow and Maria Brandl for the training and suggestions on the ice nucleation assay used in this study and Peter Jeffries for critical suggestions to improve the manuscript.

This work was supported by a team grant (Hibah Tim-URGE) from the Indonesian Directorate General of Higher Education, Ministry of Education and Culture (029/HTPP-II/URGE/1996-1999).

REFERENCES

1. **Beattie, G. A., and S. E. Lindow.** 1994. Epiphytic fitness of phytopathogenic bacteria. *In* J. L. Lang (ed.), Bacterial pathogenesis of plant and animals: molecular and cellular mechanisms—1994. Springer-Verlag, Berlin, Germany.
2. **Brandl, M. T., and S. E. Lindow.** 1998. Contribution of indole-3-acetic acid production to the epiphitic fitness of *Erwinia herbicola*. Appl. Environ. Microbiol. **64:**3256–3263.
3. **Cooksey, D. A.** 1988. Reduction of infection by *Pseudomonas syringae* pv. *tomato* using a nonpathogenic, copper resistant strain combined with a copper bactericide. Plant Dis. **78:**601–603.
4. **Darling, D., R. Harling, R. A. Simpson, N. McRoberts, and E. A. Hunter.** Eur. J. Plant Pathol., in press.
5. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for Gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
6. **Hirano, S. S., E. M. Ostertag, S. A. Savage, L. S. Baker, D. K. Willis, and C. D. Upper.** 1997. Contribution of the regulatory gene *lemA* to field fitness of *Pseudomonas syringae* pv. syringae. Appl. Environ. Microbiol. **63:**4304–4312.
7. **Hwang, I., S. M. Lim, and P. D. Shaw.** 1992. Use of detached soybean cotyledons for testing pathogenicity of *Xanthomonas campestris* pv. *glycines*. Plant. Dis. **76:**182–183.
8. **Knudsen, G. R., and H. W. Spurr, Jr.** 1988. Management of bacterial population for foliar disease biocontrol, p. 83–92. *In* K. G. Mukerji and K. L. Garg (ed.), Biological control of plant diseases, vol. 1. CRC Press, Boca Raton, Fla.
9. **Lindemann, J.** 1985. Genetic manipulation of microorganisms for biological control, p. 116–130. *In* C. E. Windels and S. E. Lindow (ed.), Biological control on the phylloplane. APS Press, St. Paul, Minn.
10. **Lindow, S. E.** 1985. Strategies and practice of biological control of ice nucleation active bacterial on plants, p. 293–311. *In* N. Fokkema (ed.), Microbiology of the phyllosphere. Cambridge University Press, Cambridge, England.
11. **Lindow, S. E.** 1987. Competitive exclusion of epiphytic bacteria by ice mutants of *Pseudomonas syringae*. Appl. Environ. Microbiol. **53:**2520–2627.
12. **Lindow, S. E.** 1993. Novel method for identifying bacterial mutant with reduced epiphytic fitness. Appl. Environ. Microbiol. **59:**1586–1592.
13. **Lindow, S. E., D. C. Arny, and C. D. Upper.** 1978. *Erwinia herbicola*: an active ice nucleus incites frost damage to maize. Phytopathology **68:**523–527.
14. **Lindow, S. E., D. K. Willis, and N. J. Panopoulus.** 1987. Biological control of bacterial brown spot diseases of bean with Tn5-induced avirulent mutants of the pathogen. Phythopathology **77:**1768.
15. **Loper, J. E., and S. E. Lindow.** 1994. A biological sensor for iron available to bacteria in their habitats on plant surfaces. Appl. Environ. Microbiol. **60:** 1934–1941.
16. **Mesak, F. M., A. Suwanto, B. Tjahjono, and E. Guhardja.** 1994. Bioassay to test the pathogenicity of *Xanthomonas campestris* pv. *glycines* and the transposition of transposable elements. J.Il. Pert. Indon. **4:**77–82.
17. **Moffet, M. L., and B. J. Croft.** 1983. *Xanthomonas*, p. 189–228. *In* P. C. Fahy and G. L. Persley (ed.), Plant bacterial disease: a diagnostic guide. Academic Press, New York, N.Y.
18. **Rukayadi, Y.** 1995. DNA genome profiling analysis of several isolates of *Xanthomonas campestris* pv. *glycines* employing pulsed field gel electrophoresis. M.S. thesis. Bogor Agricultural University, Bogor, Indonesia.
19. **Rukayadi, Y., A. Suwanto, and B. Tjahjono.** 1998. Plasmid construction containing *Pac*I and *Pme*I sites for transposon mutagenesis in *Xanthomonas campestris*. Hayati **5:**79–85.
20. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, New York, N.Y.
21. **Simon, R., V. Priefer, and A. Puhler.** 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in Gram-negative bacteria. Bio/Technology **1:**784–791.
22. **Sinclair, J. B., and C. R. Cantor.** 1987. Compendium of soybean diseases, p. 1–9. APS Press, St. Paul, Minn.
23. **Smith, C. L., and C. R. Cantor.** 1987. Purification, specific fragmentation and separation of large DNA molecules. Methods Enzymol. **155:**449–465.
24. **Suwanto, A., and S. Kaplan.** 1989. Physical and genetic mapping of *Rhodobacter sphaeroides* 2.4.1. genome: genome size, fragment identification, and gene localization. J. Bacteriol. **171:**5840–5849.
25. **Suwanto, A., and S. Kaplan.** 1992. A self-transmissible narrow host range endogenous plasmid of *Rhodobacter sphaeroides* 2.4.1: physical structure, incompatibility determinants, origin of replication, and transfer function. J. Bacteriol. **174:**1124–1134.
26. **Suwanto, A., and S. Kaplan.** 1992. Chromosome transfer in *Rhodobacter sphaeroides*: Hfr formation and genetic evidence for two unique circular chromosomes. J. Bacteriol. **174:**1135–1145.
27. **Vali, G.** 1971. Quantitative evaluation of experimental results on the heterogenous freezing nucleation of supercooled liquids. J. Atmos. Sci. **28:**402–406.
28. **Vauterin, L., B. Hoste, K. Kerters, and J. Swings.** 1995. Reclassification of *Xanthomonas*. Int. J. Syst. Bacteriol. **45:**472–489.
29. **Wilson, M., and S. E. Lindow.** 1994. Ecological similarity and coexistence of epiphitic ice-nucleating (Ice^+) *Pseudomonas syringae* strains and a non-ice-nucleating (Ice^-) biological control agent. Appl. Environ. Microbiol. **60:** 3128–3137.
30. **Widjaja, R., A. Suwanto, and B. Tjahjono.** 1999. Genome size and macrorestriction map of *Xanthomonas campestris* pv. glycines YR32 chromosome. FEMS Microbiol. Lett. **175:**59–68.