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Isolation and Characterization of Transposon ΩKm-Mediated Nonpathogenic Mutants of *Xanthomonas campestris* pv. *vesicatoria*

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고추 세균성 반점병균의 비병원성 돌연변이체 분리 및 생리적 특성

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ABSTRACT : Transposon mutation of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) was induced by using transposon omegon (Ω)-Km (Tn ΩKm), which was confirmed by resistance to kanamycin (Km^r), and nonpathogenic mutants were selected through the inoculation test on pepper plants. The mutagenesis frequency was about 6×10^{-8}, and 53 out of 2,000 Km^r bacterial colonies tested were nonpathogenic to the pepper cultivar Cheung-Hong. Optimum conditions for the Tn ΩKm mutagenesis of *Xcv* were Luria Bertani (LB) broth medium for culture of *Xcv*, yeast extract-dextrose-$CaCO_3$ (YDC) agar medium for selection of Tn ΩKm-mediated mutants, and over 1 to 2 in the ratio of the donor (*Escherichia coli* S17-1 with the plasmid pJFF350 ΩKm) and the recipient (*Xcv*) in the culture for the mutagenesis. One of the 4 nonpathogenic mutants (WNP1, WNP3, WNP4 and WNP5), which had been reconfirmed through the inoculation on pepper cv. Dabokgun, showed no differences in the production of exoenzymes such as protease and polygalacturonase and extracellular polysaccharides *in vitro* and the bacterial growth rate from those of the wild type of *Xcv*.

***Key words* : *Xanthomonas campestris* pv. *vesicatoria*, transposon ΩKm, nonpathogenic mutants.**

Bacterial leaf spot, caused by *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) (Doidge) Dye is an important bacterial disease in pepper (*Capsicum annuum* L.) and tomato (*Lycopercicon esculentum*) (7, 12). One of control methods for this disease is use of nonpathogenic mutants of the bacterial pathogen as biological control agents. Nonpathogenic bacterial mutants also can be used for the study on the characterization of genetics and physiology related to the pathogenicity, which gives basic information for the development of genetic control methods of the bacterial disease.

N-methyl-N-nitro-N-nitrosoguanidine (NTG) has been widely used to induce mutation of *X. campestris* pathovars by the experimental protocols similar to those employed for *Escherichia coli* (3, 20). On the other hand, transposon mutation (mutation mediated by transposon) has not been used widely, but has some advantageous features in working with transposon-mediated mutants, which include selective marker(s), low reversion rate, etc. However, the mutation frequency of the transposon mutagenesis in *X. campestris* pathovars is substantially low, compared with other mutagenesis methods (2, 9, 10). Recently, low frequency of transposon mutagenesis in non-enteric bacteria has been overcome by use of artificial transposon Omegon (Ω) Km (Tn ΩKm). Tn ΩKm is carried on the plasmid pJFF350 which can be conjugally mobilized into a broad range of Gram-negative bacteria, and has characteristics of high transposition frequency and no secondary transposition (5, 6, 15).

In this study, nonpathogenic mutants were induced by Tn ΩKm-mediated mutagenesis. Optimal culture conditions of *Xcv* for the mutagenesis and pathogenesis-related physiological characteristics of the Tn ΩKm-

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mediated mutants were examined.

MATERIALS AND METHODS

Bacterial strains and plasmids. *Xcv* strain Euijeungbu, which was isolated from pepper and is resistant to rifampicin (13), was used as a wild type of the bacterium and subjected to Tn ΩKm-mediated mutation. *E. coli* S17-1 (*recA thi pro hsdR*$^-$M$^+$ < RP4:2-Tc:Mu:Km:Tn7 > Tr^r, Sm^r) (16) and SM10 (C600 *thi thr leu recA* Muc$^+$) (15) were kindly offered by Dr. I. G. Hwang. Plasmid pJFF350 containing ΩKm fragment and pSup1011 (RP-2-Tc::Mu) was harbored in *E. coli* S17-1 and SM10, respectively (5, 15). The *Xcv* strain was cultured routinely on peptone sucrose agar (PSA) at 30°C, and *E. coli* strains were grown in Luria-Bertani (LB) medium at 37°C (13).

Tn ΩKm mutagenesis. The wild *Xcv* strain and *E. coli* S17-1 with pJFF350 ΩKm were grown to late-exponential and early-exponential phases, respectively. For transposon mutagenesis, the membrane filter mating method was employed. Tn ΩKm, which shows kanamycin resistance, was transferred into the wild *Xcv* strain by using the delivery vector of pJFF350 as follows. Both bacterial suspensions (1 ml for each) were suspended in saline solution and mixed together, and then filtered on (0.45 μm pore size, ϕ 25 mm) filter membrane. The filtrated membrane was placed on LB agar medium at 30°C for 16 hrs. The bacteria on the membrane were resuspended in sterilized water and plated onto YDC (1% yeast extract, 2% calcium carbonate, and 2% glucose) agar containing 100 μg/ml of rifampicin and 50 μg/ml of kanamycin and incubated at 30°C for the selection of Tn ΩKm-mediated mutants (5). Bacterial colonies formed on the agar medium were regarded as Tn ΩKm-mediated mutant bacterial colonies.

Isolation of nonpathogenic mutants. Kanamycin-resistant transconjugants obtained from the above culture medium for Tn ΩKm mutagenesis were transferred onto PSA medium containing rifampicin and kanamycin at the same concentrations as above, cultured at 30°C for 2 days, and used for pathogenicity test. For mass selection of nonpathogenic mutants, pathogenicity of the Tn ΩKm-mediated mutants was tested on pepper cv. Cheung-Hong. The pepper cv. Cheung-Hong was grown in a greenhouse at 28±2°C for 30 days. The fully expanded pepper leaves were inoculated with the kanamycin-resistant mutants of *Xcv* by using needles and cotton swabs. After inoculation, the peppers were incubated in a moistened chamber at 30°C. Production of bacterial leaf spot lesions was examined for more than 2 weeks. The bacterial isolates that produced no lesion on the pepper leaves were selected, and the inoculation test was replicated three times to confirm their nonpathogenicity. Fifty three putative nonpathogenic isolates selected in the above preliminary test were further tested for pathogenicity on the pepper cv. Dabokgun by the same method described above to select definite nonpathogenic mutants.

Optimal culture conditions for transposon mutagenesis of *Xcv*. Three culture media of *Xcv* (peptone sucrose broth (PSB), LB and nutrient agar (NA)), three agar media (WF-P< Wakimodo ferrous sulfate media >, YDC and LB agar) for selection of mutants, incubation temperature and duration in the dual culture of the donor (*E. coli*) and the recipient (*Xcv*) for transconjugation, and the ratios of the donor and recipient bacterial inoculum density were tested for efficiency of transposon mutation by following the mutagenesis procedures mentioned above. The frequencies of Tn5 and Tn ΩKm mutagenesis in the same culture conditions, for which plasmids pSup1011 and pJFF350 were used, respectively, were also compared.

Characteristics of Tn ΩKm-mediated nonpathogenic mutants. In this study, 4 nonpathogenic mutant strains, WNP1, WNP3, WPN4 and WPN5, were selected via the preliminary and final pathogenicity tests. Because there was no difference in growth rate among the selected mutants, and because no differences in physiological traits were noted during our experiment, WPN1 was selected and used for comparison of some pathogenesis-related characteristics with the wild type strain (Euijeungbu) of *Xcv*.

Growth rate of the nonpathogenic mutant was compared with that of the wild type strain of *Xcv*. Each strain was incubated at 30°C at 150 rpm in LB broth medium. UV absorbance (at 600 nm) of the bacterial culture was examined every hour to measure the bacterial population density.

For the production of extracellular polysaccharides (EPS), bacterial strains grown in flasks with YT (1.6% Bacto-peptone, 0.5% sodium chloride and 1% yeast extract) broth medium at 28°C overnight were inoculated into XG004 medium (20 g glucose, 5 g Bacto-tryptone, 2.5 g yeast extract, 0.1 g $CaCl_2$, 6.8 g KH_2PO_4, 0.2 g $MgSO_4 \cdot 7H_2O$, 2.2 g sodium glutamate, 2 g citric acid, 2.25 mg $FeCl_3 \cdot 6H_2O$, 0.141 mg $MnSO_4$

· H_2O, 0.22 mg $ZnSO_4 \cdot 7H_2O$, 0.025 mg $CuSO_4 \cdot 5H_2O$, 0.04 mg $CoCl_2 \cdot 6H_2O$, 0.026 mg $Na_2MoO_4 \cdot 2H_2O$, 0.04 mg H_3BO_3 and 0.006 mg KI per liter of medium, pH 7.0) with the concentration of about 10^7 cells/flask and cultured on a rotary shaker (150 rpm) at 28°C. At the time of every sampling, the number of bacterial cells was determined by measuring UV absorbance at 600 nm, and two volumes of ethanol containing 10% (v/v) saturated-KCl solution as an electrolyte was added to each flask to precipitate EPS. The precipitate was collected on Gelman Supor-450 filter (ϕ25 mm), dried in an oven and then weighed (8).

Exozyme activities, proteolytic and polygalacturonate lyase activities, were assayed. Proteolytic activity was measured by the degradation of milk proteins in skimmed milk-supplemented (1%, w/v) NYGA (Bacto-peptone, 5 g; yeast extract, 3 g; glycerol, 20 g; agar, 15 g) medium which was seeded with the bacterial strains and incubated at 30°C for 1 day (19). For polygalacturonate lyase assay, neutralized polygalacturonic acid (PGA) was added to a final concentration of 0.25% (w/v) in NYGA medium seeded with the bacterial strains. After incubation for 2 days at 30°C, the bacterial culture media in plates were flooded with saturated copper acetate solution (4). Bacterial clones producing the above exoenzymes will show the halo around the colony.

RESULTS

Isolation of Tn ΩKm-mediated nonpathogenic mutants. Transposon mutagenesis was performed between *E. coli* S17-1 pJFF350 and *Xcv* Euijeungbu. Tn ΩKm-mediated mutants were selected by the drug resistance to both rifampicin and kanamycin. Approximately 2,000 bacterial colonies of Tn mutants were selected in our study, which was estimated to be 5.17×10^{-8} as mutation frequency. These Tn ΩKm-mediated mutants were assayed for pathogenicity on

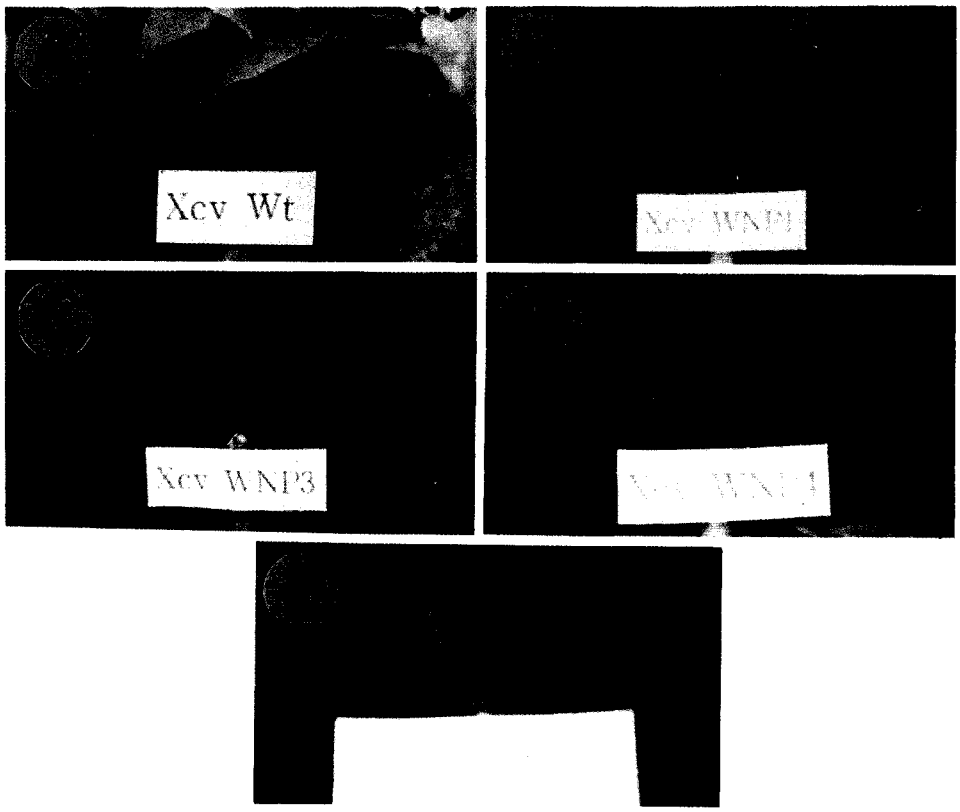

Fig. 1. Pathogenicity test of the wild type strain Euijeungbu (A) and Tn ΩKm-mediated mutants (WNP1(B), WNP3(C), WNP4(D) and WNP5(E)), showing chlorotic spots on pepper (cv. Dabokgun) 15 days after inoculation by the wild strain (A) but no lesion formation by the mutants (B-D).

Table 1. Conditions for transposon mutagenesis of *Xanthomonas campestris* pv. *vesocatoria*

Culture conditions		Vectors containing the transposon	
		pSUP1011(Tn5)	pJFF350(Omegon Km)
Culture medium of *Xcv*	Peptone sucrose broth	–[a]	–
	Luria-Bertani (LB) broth	–	+
	Nutrient broth	–	–
Selection medium of Tn mutants	Wakimodo-$FeSO_4$ agar	–	–
	Yeast extract-dextrose-$CaCO_3$	–	+
	LBA	–	–
Incubation time in LBA (on filter membrane)	5 hrs	–	–
	16~24 hrs	–	+
Incubation temperature (on filter membrane)	4°C for 7 days	–	–
	30°C for 24 hrs	–	+
Ratio of donor (*E. coli* containing Tn vector) to recipient (*Xcv*)	1 : 1	–	–
	above 1 : 2	–	+

[a] – : No Tn mutans occurred. + : Tn mutants occurred.

pepper leaves. In the preliminery pathogenicity test for mass selection of nonpathogenic mutants, which was carried out on the pepper cv. Cheung-Hong, 53 out of the 2,000 mutants tested were nonpathogenic to the pepper cultivar. The 53 mutants were further tested for pathogenicity on another pepper cv. Dabokgun, and 4 mutants were nonpathogenic, forming no bacterial spot lesion on the leaves, while the *Xcv* wild type strain was pathogenic as indicated by the formation of chlorotic spots on the pepper leaves (Fig. 1). The pepper cultivars, Cheung-Hong and Dabokgun, showed similar symptoms by the infection of *Xcv* Euijeungbu, but bacterial spots appeared more rapidly in Cheung-Hong than in Dabokgun.

Optimal conditions for Tn ΩKm mutagenesis. Transposon mutagenesis using Tn5 was not successful in any conditions we tested in this study. The Tn ΩKm mutation of *Xcv* occurred only in case of LB broth as culture medium of *Xcv*, YDC agar medium for selection of Tn ΩKm-mediated mutants, and over 1 to 2 in the ratio of *E. coli* S17-1 pJFF350 ΩKm (donor) to *Xcv* (recipient) in the bacterial inoculum density (Table 1). The optimum incubation temperature and incubation time were 30°C and 16~24 hrs, respectively. *Xcv* and *E. coli* S17-1 pJFF350 cultures grown to the late and early exponential phases, respectively, were optimum for the transposon mutagenesis.

Characteristics of Tn ΩKm-mediated nonpathogenic mutants. Absorbance of both bacterial cultures

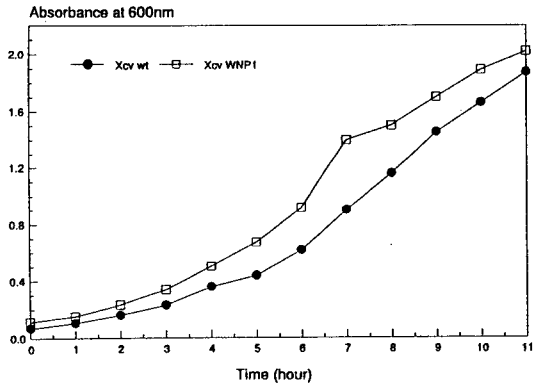

Fig. 2. Measurements of growth rates of *Xanthomonas campestris* pv. *vesicatoria*in LB broth by UV absorbance at 600 nm of the wild type (*Xcv* wild type, ●—●) and the nonpathogenic mutant (*Xcv* WNP1, □—□).

increased continuously, indicating that the bacterial populations increased up to 11 hrs after inoculation (Fig. 2). The growth rate of the nonpathogenic mutant strain was similar to that of the wild type strain, and the population densities in the early and late phases of incubation were not significantly different between the two strains, suggesting that reproductivity was not significantly altered by the Tn ΩKm-mediated mutation.

The accumulation of EPS in the cultures of both the wild type strain and the nonpathogenic mutant of *Xcv* increased with time, and the final EPS concentration

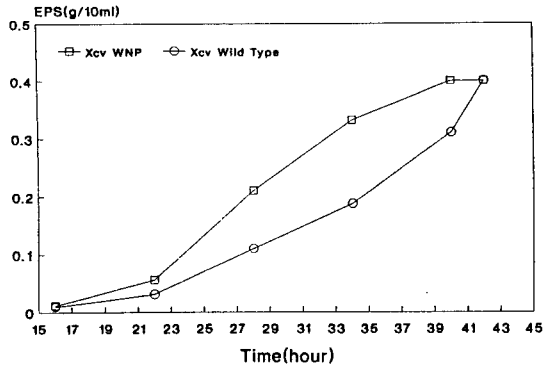

Fig. 3. Time course of accumulation of extracellular polysaccharides (EPS) by the wild type (*Xcv* Wild Type) and the nonpathogenic mutant (*Xcv* WNP) of *Xanthomonas campestris* pv. *vesicatoria*. Each time 10 ml bacterial cultures were taken, and EPS accumulation was measured.

was not different between the two bacterial cultures (Fig. 3). This aspect indicates that the mutant induced via Tn ΩKm mutagenesis had no difference from the wild type strain in EPS production which was reported to be one of major factors related to pathogenesis in *X. campestris* pathovars or *Pseudomonas* species (1, 18).

For exozyme activities, both the wild type strain and the nonpathogenic mutant showed no proteolytic and polygalacturonase activities on NYGA medium supplemented with skimmed milk and polygalacturonic acid, respectively (Table 2).

DISCUSSION

Recently mutation of phytopathogenic bacteria by drug-resistance transposable elements has been adopted in the area of plant pathology. Delivery vectors such as pSUP1011, pJBJ1, pSUP2011, pRK210, and pUW944 have been applied to many phytopathogenic bacteria including *Agrobacterium*, *Erwinia*, and *Pseudomonas* species. However, in many cases lack of proper delivery systems which introduce transposable elements into target bacterial species has been main obstacles for the general use of this method. For example, transposon mutagenesis in many xanthomonads has been unsuccessful because of lack of vector systems.

A new transposon pJFF350 ΩKm which has been constructed by Falley *et al.* (5) showed high transpositional frequency in many Gram-negative bacteria. In *X. campestris* pv. *glycines*, transpositional frequency of Km was in the range of 10^{-7} to 10^{-8} in our previous experiment (unpublished), which is similar to that in *Xcv* of this study. On the contrary, in the case of *X. oryzae* pv. *oryzae*, Tn ΩKm did not induce the Tn mutants in any conditions (unpublished data). The difference in transpositional frequency might be derived from the states of either the delivery vector pJFF350 or the bacterial species and strains or both in the transposon mutagenesis.

Table 2. Comparison of exoenzyme activities of the wild strain and the Tn Ωkm mutant strain of *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*)

Bacterial strain	Enzyme activity	
	Protease[a]	Polygalacturonase[b]
Xcv Euijeungbu (wild type)	–[c]	–
Xcv WNP (mutant)	–	–

[a] Proteolytic activity was measured by degradation of milk proteins in skimmed milk-supplemented (1%, v/v) NYGA medium.

[b] For polygalacturonate lyase assay, neutralized polygalacturonic acid (PGA) was added to a final concentration of 0.25% (w/v) in NYGA medium.

[c] – : No enzyme activity was detected.

Genetic and structural characterizations of avirulence gene have been a main topic to elucidate disease resistance in molecular mechanisms (11, 16, 17). In our study, nonpathogenic mutants with cultural and physiological characteristic similar to the pathogenic wild type *Xcv* strain were induced with relatively high frequency by Tn ΩKm mutagenesis. EPS production and polygalacturonase and protease activities of the nonpathogenic mutant were not significantly different from those of the wild pathogenic strain, suggesting that at least those factors may not be related to avirulence gene of *Xcv*. Also bacterial growth rate was similar between the wild and mutant strains, and this suggests that bacterial reproductivity may not be directly related to the pathogenicity. Other enzymes such as pectic enzyme and other substances should be tested for the characterization of avirulence gene of *Xcv* by using nonpathogenic mutants obtained in this study. Also further studies on the stability of nonpathogenic mutants are needed to select appropriate mutants useful as biological control agents.

요 약

Transposon ΩKm을 이용하여 고추 세균성 반점병

균의 비병원성 돌연변이체가 선발되었으며 돌연변이체 발현 빈도는 6×10^{-8}이었다. Transposon ΩKm에 의해 유도된 변이체는 kanamycin에 대한 저항성 획득으로 확인되었으며, 돌연변이 유도의 최적조건은 배양배지, *Xcv*와 *E. coli* S17-1의 균수의 비, 배양시간 및 배양온도에 따라 결정되었다. 고춧잎에 접종하여 최종적으로 4개의 돌연변이체가 선발되었다(WNP1, WNP3, WNP4, WNP5). 비병원성 돌연변이체는 protease나 polygalacturonase와 같은 exoenzyme의 발현에 영향을 받지 않았으며, 변이체는 EPS 생성능력과 생장속도에 있어 모균주와 차이가 없었다.

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