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Molecular Approaches to Determine the Character of *Serratia marcescens* Associated with the Insect Pathogenicity to Brown Planthopper

*Serratia marcescens*의 곤충 병원성 관련형질 탐색을 위한 분자생물학적 연구

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ABSTRACT A bacterium, pathogenic to *Nilaparvata lugens* Stal. causing high mortality in 3~5 days, were selected and identified as *Serratia marcescens* biotype A2a which is not a nosocomially infective strain. In order to determine the characters of *Serratia marcescens* associated with insect pathogenicity, Tn5 mutagenesis was carried out by conjugating with *E. coli* pJB4J1. Transconjugants were plate-assayed for missing chitinase, protease and DNase activity. A protease negative mutant was selected for missing insect pathogenicity. SEM and TEM revealed the presence of bacterial cells in the epithelial tissue of inner abdomal tissue of the hypodermic layer of abdomen. Such a colonization was limmited to the subjacent tissue inside the intact cuticular epidermis. These observation supported our result of pathogenicity tests of transconjugants.

KEY WORDS *Serratia marcescens*, Tn mutagenesis, insect pathogenicity related character, *Nilaparvata lugens*.

초 록 벼멸구에 강한 병원성이 있는 *Serratia marcescens*, biotype A2a를 분리, 동정하였다. 벼 유묘에 분무한 후 성충-계절풍을 따라 비래하는 형태-을 공시하고 병원성을 조사하여 3~5일만에 강한 살충력을 발견하였다. 따라서, 본 세균의 곤충병원성 관련 형질 탐색을 하기 위하여 Tn5로써 돌연변이를 시도한 후, Chitinase, Protease, DNase indicator media에서 돌연변이 계통을 분리하였다. 이들을 공시충에 병원성을 검정한 결과, Pro-Strain중에서 병원력이 현저히 떨어지는 현상을 관찰하였다. 공시충을 전자현미경(SEM, TEM)으로 관찰하여, abdomen의 전장부위와 표피사이에 다수의 세균이 증식하였음을 발견하였다. 곤충복부표피조직 중 cuticle층은 intact한 상태였다. 따라서, 이에 관련된 유전자를 분리하기 이해 genomic library실험을 진행하고 있다.

검 색 어 벼멸구 병원성세균, *Serratia marcescens*, 병원성관련형질

Rice Brown planthopper(BPH) (*Nilaparvata lugens* Stal.) is one of the most important insect pests of rice, causing "hopper burn", which has been the subject of the intensive control practice in rice paddies in Korea as well as elsewhere. Moreover, this BPH highly variable so that very low efficacies of agrochemical treatment are obtainable recently, either through "the develop-

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ment of tolerance" to chemical insecticides within a short period of time from their release or "the resurgence", unexpected multiplication beyond the threshold population to epidemic level by destroying natural enemies. So, the varietal resistance of rice has been the major concern for the rice breeders to get over such problems. Unfortunately, however, new biotypes appeared again, within a short period of time after resistance-gene-exploitation, so that the resistance of new cultivar was succumbed to bph.

It is a migratory insect pest through the air currents, which was confirmed not to overwinter in Korea. It's feeding site is at the sheaths of rice plant just above the irrigation water level, where high humidities are readily available for microbial colonization. This same ecological niche is the primary infection site for rice sheath blight fungus, an endemic pathogen in rice paddies and no germ-plasm sources are available for resistance gene exploitation.

Therefore, we have attempted to isolate the microorganisms that are potentially pathogenic on rice brown plant hopper, and to determine the characters associated with insect pathogenicity through the molecular biological approaches.

MATERIALS AND METHODS

Isolation and identification of insect pathogenic microorganisms from the ecological niche

Periodically collected samples of bph were dilution-plated on Nutrient agar and incubated at 25±2℃ for 3days. Only virulent isolates were selected for further standard identification procedure (Collins & Lyne 1984, Kado 1973, Krieg 1984) and biotyping (Grimont & Grimont 1978b) together with repressantative species of *Serratia*.

Tn5 mutagenesis of *S. marcescens* with *E. coli* pJB4JI

The bacteria strains used and their relevant characteristics are shown in Table 1. The *E. coli* pJB4JI and the *S. marcescens*(Rif^+) were grown overnight in 5ml LB broth with Kanamycin(100 μl/ml) and Rifampicin(50 μl/ml) at 28℃, from which 0.05ml of the suspension were inoculated in 5 ml LB containing antibiotics respectively. these were grown to the log-phase and cells were harvested. The cells pellets were suspended in 1ml of 0.85% NaCl and pelleted again respectively. Both bacterial cells were mixed by suspending in the 0.2ml of 0.85% MaCl and transferred to membrane filter on LB without antibiotics and incubated at 28℃ overnight. Bacterial cells on the filterpaper was washed with distilled water in test tube. The 0.2ml solution was transferred to LB with both antibiotics and spread with sterile glass rod. Those transconjugants appeared in 12hours, were picked up with sterile tooth pick and streaked on the counter-selective media. Only those actively growing colonies on counter-selectivie medium containing both Kanamycin and Rifampicin but not on Streptomycin and Rifampicin were selected and stored for futher study.

Screening of *S. marcescens* and transconjugant by plate assay

S. marcescens and several thousand transconjugatant were screened by replica plating on the indicator media for chitinase, protease, nuclease. Indicator media were: chitinase test agar [Dehydrated nutrient broth, 5g; swollen chitin, 25ml; Bactoagar, 8g; distilled water, 1 ℓ], Protease test agar [Bactopeptone, 5g;

Table 1. Bacterial strains transposons, and plasmids

Strain, phage, or plasmid Relevent characteristics	Sourse
Serratia marcescens R. wild type	H.K. Kim
Serratia marcescens No. 1 wild type	〃
Serratia marcescens No. 2 wild type	〃
E. coli	
HB101 F⁻ *hsd*S20(rb⁻mb⁻)*rec*A13 *ara*-14 *pro*A2 *lac*Y1 *gal*K2 *rps*20 (Str^r) *xyl*-5 *mtl*-1 *sup*E44λ^- *thi leu*	N. Panopoulos
DH5 α F⁻ *end*A1 *hsd*R17(^{r}K⁻mk⁺)*sup*E44 *thi*-1 *rec*A1 *gyr*A96 *rel*A1 Φ80d*lac*ZM15	M. Chong
Plasmids	
pLAFR3 pLAFR1 containing *Hae*Ⅲ fragment of pUC8	B. Staskawicz
pRK2013 IncP, Km^r Tra Rk2⁺ *rep*RK2 *rep*El	N. Panopoulos
pUW964 Km^r(Tn5), Sm^r(Tn7)	M. Schroth
pB329 Ap^r, Tc^r, Cm^r	M. Schroth
pJB4JI Km^r(Tn5), Sm^r(Tn7)	E. Ely
pUC18 & pUC19 Cloning vector	Messing et al.

Skim milk power, 10g; Bactoagar 15g; distilled weter, 1 ℓ) and DNase test agar (DNase agar, 42g; methyl green, 0.07g; distilled weter, 1 ℓ). Replica plates were observed in 48~72 hours for protease or DNase activity and in 96~120 hours for chitinase activity at 28℃.

Growth of plant and maintenance of hopper population

Rice plants(*Oryza sativa* L. cv. Chuchung byeo) were grown for two weeks and three plants of two week old seedlings were transplanted into pot(10cm in diameter and 12cm in height) in a growth chamber for two weeks and then the plants were covered with a mylar cages. Brown planthopper populations were rountinely maintained in rearing cages feeded with rice seedlings in laboratory. Development stages of bphs were synchronized by intermittent isolation of nymph of same stage from the heterogenous mixture population and maintained in the individual rearing cages. An adult BPH populations that are just molted from 4th instar nymphs was subjected to bioassay of bacterial strains for insect mortality.

Bioassay of Tn5 mutants against brown planthopper

The each of protease, chitinase and DNAase negative strains were grown in 10ml LB media overnight. Bacterial cells were pelleted and resuspended in 0.1M $MgSO_4$ solution. Inoculum concentration was adjusted to 4×10^6 per milliliter with Spectrophotometer(*Spectronic 20*, MILTONROY CO.). The suspension of 3ml were sprayed on the rice plants per pot, first. Then, the fifteen adult bphs from routinely maintaining synchronized popultion just molted from 4th instar stage nymph were introduced in each caged pots with three replicates. Insect mortali-

ty was rated in 5 days.

Electron microscopy

Scanning electron microscopy of healthy vs. infected insect tissue: The healthy and the infected adults were longitudinally sectioned with a razor blade in 90 hours after inoculation (10^9 cells/ml of bacteria), prefixed in 10 volume of 2.5% glutaraldehyde(pH 7.0) for 2~3 hours and washed three times with 0.1M sodium phosphate buffer(pH 7.4)(PBS) for 1 hour. The specimens of longitudinal sections then were postfixed with 1% OsO_4(Osmium tetraoxide) for 1~2 hours, washed with PBS and subjected to a series of dehydration with ethanol from 60, 70, 80, 90, to absolute absolute ethanol and acetone. The specimens then were dryed and subjected with the gold-coating and observed at 8,000 X under the scanning electron microscope (DS-130).

Transmission electron microscopy of infected insect tissue: The head, thorax and abdomen of the infected and/or healthy brown planthoppers were carefully sectioned with a razor blade, fixed in 2.5% glutaraldehyde (pH 7.0) for 2 hours at 4℃, and washed in 0.2M sodium phosphate buffer(SPB)(pH7.2) for 2 hours. The specimens were then dehydrated sequentially with ethanol from 50, 70, 80, 90, 95 to 100% and embedded in Epon 812. The specimen blocks trimmed to an appropriate shapes were semithin-sectioned to 0.35㎛ thick with Ultratome(Reichert Jung. ULTRA CUT), stained with Toluidine blue and previewed with a light microscope at 800X magnification to screen the most suitable specimen block.

For the transmission electron microscopy, the infected tissue in specimen blocks were the ultra-thin sectioned to 70nm thick, followed by staining with a uranyl acetate and a lead citrate, and observed at 8,000X~10,000X under transmission electron microscope(Hitachi, H-600).

RESULTS AND DISCUSSION

Identification of insect pathogenic bacteria

Isolates were characterized to be gram negative, straight rod, oxidase negative and catalase positive, which are differentiating traits of Enterobacteriaceae from the other families of facultatively anaerobic, gram negative rods. From other traits of red color pigment, gelatin liquefaction, and the ability of utilizing citrate, this bacterium was classified as genus *Serratia*. So far, 7 species of *Serratia* are described. It was identified as *Serratia marcescens* from the characteristics of methyl red negative, positive for sucrose, negative for arabinose and cellobiose(Table 2). *Serratia marcescens* is known to occur widespread in nature. This bacterium has been isolated from 19 plant species of wide diversity, representing 50% of all *Serratia* spp. in water and playing a mineralization role in soil. No phytopathogenic *Serratia* has ever been reported but, interestingly enough, inoculation of *Serratia marcescens* to tobacco and bean leaves also produces a typical hypersensitivity reaction(Grimont & Grimont 1978a). This implies that this bacterium might be associated, if not a pathogen, with plants more closely than many other microbes merely existing as epiphytes at the surface of plants. Sher et al. (1988) reported the colonization of soy bean root by *Serratia* species. Ordentlich et al. (1988) also observed the biocontrol effect of *Serratia marcescens* on *Sclerotium rolfii*.

Serratia marcescens has also been responsible for many diseases of many insects in the field as well as in insect rearing laboratory. Biotypes

of *Serratia marcescens* are essentially pigmented, representing 74.2% of all strains, and non pigmented biotype A4 and 3A are also ubiquitous, representing 19.3% and 3% respectively. Non pigmented biotypes A8 and TCT are the ones that are limited to hospitalized human patient (Grimont & Grimont 1978a). We have identified the biotype of our bph isolate as A2a, apparently different from those nosocomially infective strains according to the method of Grimont & Grimont(1978b). Therefore, the use of red pigmented *Serratia marcescens* for microbial control of insect injurious to crops should not be discouraged. A *Serratia* strain later identified as a new species, *Serratia entomophila* (Grimont et al. 1998) was found as frequent natural pathogen of amber disease of grass grups (Scarabidae) in New Zealand pastures.

Table 2. Identification of insect pathogenic microbes

Characteristics	SMN.01	SMN.02	SMR	*S. marcesens*
Gram-reaction	−	−	−	−
O/F test	O/F	O/F	O/F	O/F
Motility	+	+	+	+
Pigment	Pink/Red	P/R	P/R	P/R
Oxidase test	−	−	−	−
Citrate test	+	+	+	+
Gelatin liquefaction	+	+	+	+
Catalase test	+	+	+	+
MR test	−	−	−	[−]
Utilization of				
Arabinose	−	−	−	−
Cellobiose	−	−	−(+)	−
Sucrose	+	+	+	+

Screening of transconjugants by plate assay

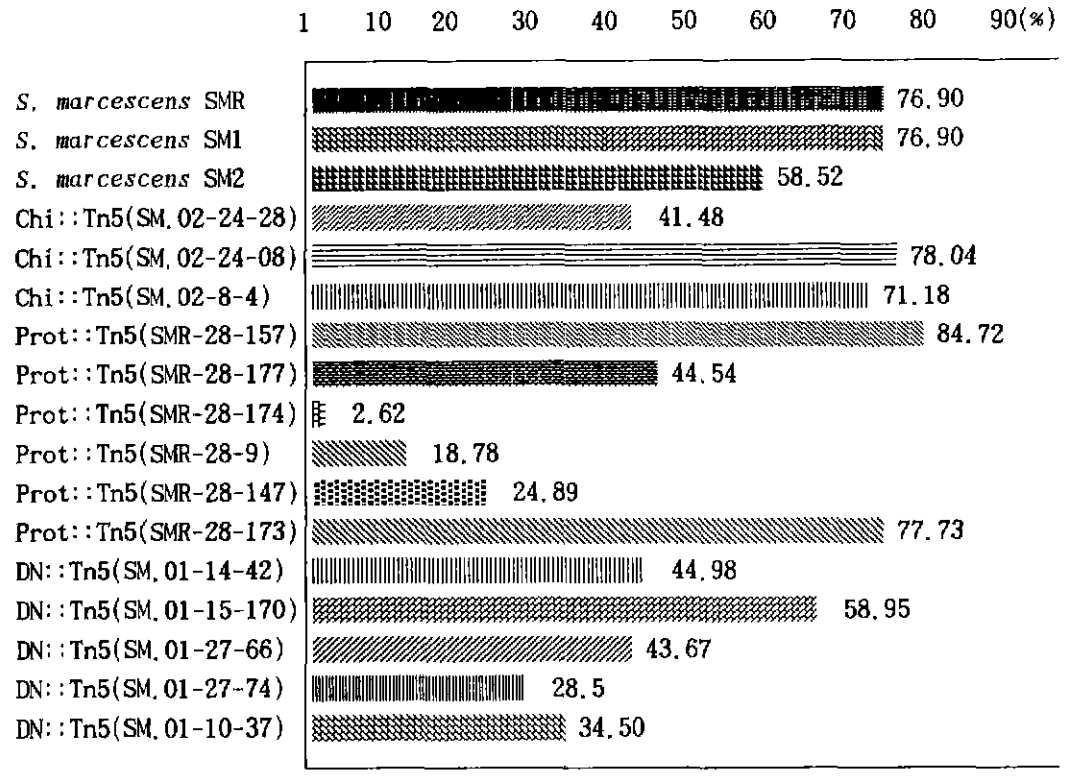

Fig. 1. Mortalities of *Nilaparvata lugens* Stal. by *Serratia marcescens* and transconjugatants

Grass grubs, ingested with *Serratia entomophila*, stop feeding very quickly, but do not die for several weeks. However, this bacterium, *Serratia marcescens*, is very aggressive, causing heavy mortality of host rice brown plant hopper in 3~5 days.

We have assayed several thousand Tn5 mutants for missing protease, chitinase and DNase and they were subjected to the bioassay to determine the characters associated with the insect pathogenicity (Fig. 1).

Hines et al.(1988) demonstrated the capability of *Serratia marcescens* to secrete extracellular proteases, chitinases, a nuclease and lipase and isolated many independent mutations affecting the extracellular enzyme after chemical and transposon mutagenesis. Grimont et al.(1977) implicated the taxonomic significance of protease pattern by investigating that *Serratia marcescens* produced one to four cathodic proteinases and patterns were in good accord with biotyping. However, none of the previous results are available as to the relation or association of these characters to insect virulence of *Sarratia marcescens*.

Pathogenicity of Transconjugants

As shown in figure 1, protease negative mutants, if not all of them, are the ones that virulence is affected considerably to variable degrees. So far, chitinase activity of microbial agent have been the subject of many previous works (Cho et al. 1990, Fuchs et al. 1986, Lesenko 1974, Ordentlich et al. 1988, Sunheim et al. 1988). In our system of bacteria -bph interaction, it is suggested that chitinase activity may not be as important as have one expected. Rather, protease or DNase may be important traits that are contributing to the insect pathogenicity. This requires vertification, however, through the complementation of mutant strains negative for given traits and bioassay of complemented strains for bph mortality.

Currently, genomic libraries of *S. marcescens* are being constructed in pLAFR3 according to the strategy of Staskawicz et al. (1987) with promising results.

Electron microscopy of healthy vs. infected tissue of insect

Preliminary observation by SEM of intact and/or longitudinal sections of morbid bph revealed that no bacterial cells were present at the surface of intact bph or head of half-cut specimen. Numerous bacteria were apparently

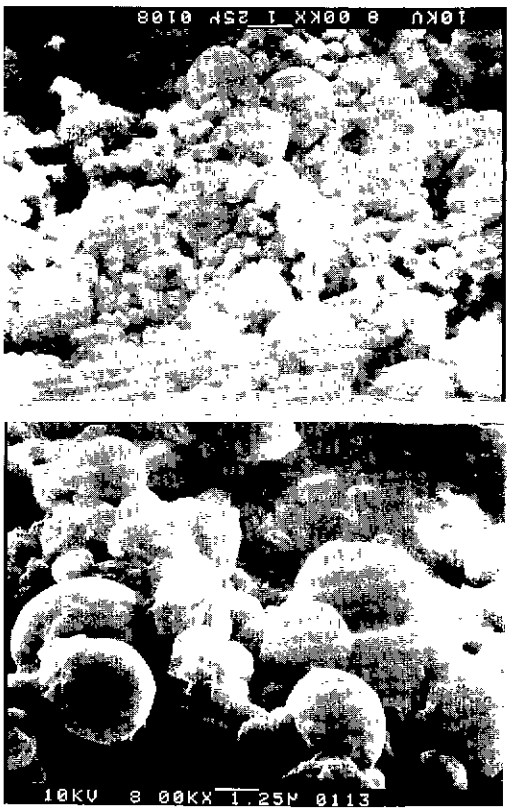

Fig. 2. Morphological differences between healthy and infected exoepithelial tissue of inner abdomal tissue of the hypodermic layer of abdomen of *Nilaparvata lugens* Stal. by Scanning Electron Microscopy.
Top, infected; Bottom, healthy.

colonized inside the thorax and abdomen. Figure 2. shows distinct morphological difference between the infected and the healthy ones at the exoepithelial tissue of the inner abdomal tissue of hypodermic layer of abdomen. Figure 3. illustrated the presence of bacterial cells in the subjacent tissues of abdomen and thorax epidermis of infected *N. lugens*, episcopically on the upper row. Each corresponding tissues were cross-sectioned to reveal that bacterial colonization were limited to the subjacent tissues other than cuticle layer. Peritrophic membrane in the mid-gut is a thin acellular membrane of chitin(ca. 10%) -protein(ca. 40%) complex, which surrounds the food bolus and lined with the gut epithelial cells and the gut contents, thereby protecting the gut epithelial cells. This membrane is known to function as mechanical barrier to microbial infection via intestinal tract(Lysenko, 1981). Bacterial amber disease of grass grub in New Zealand pasture described by Grimont et al. (1988) would fall on this case of mechanism. However, it would be possible that the microbial enzyme may damage the peritrophic membrane and assist the penetration of *Serratia marcescens* A2a, since heavy mortality results in 3~5 days. Due to the little information on the anatomy and ultrastruture of brown planthopper cur-

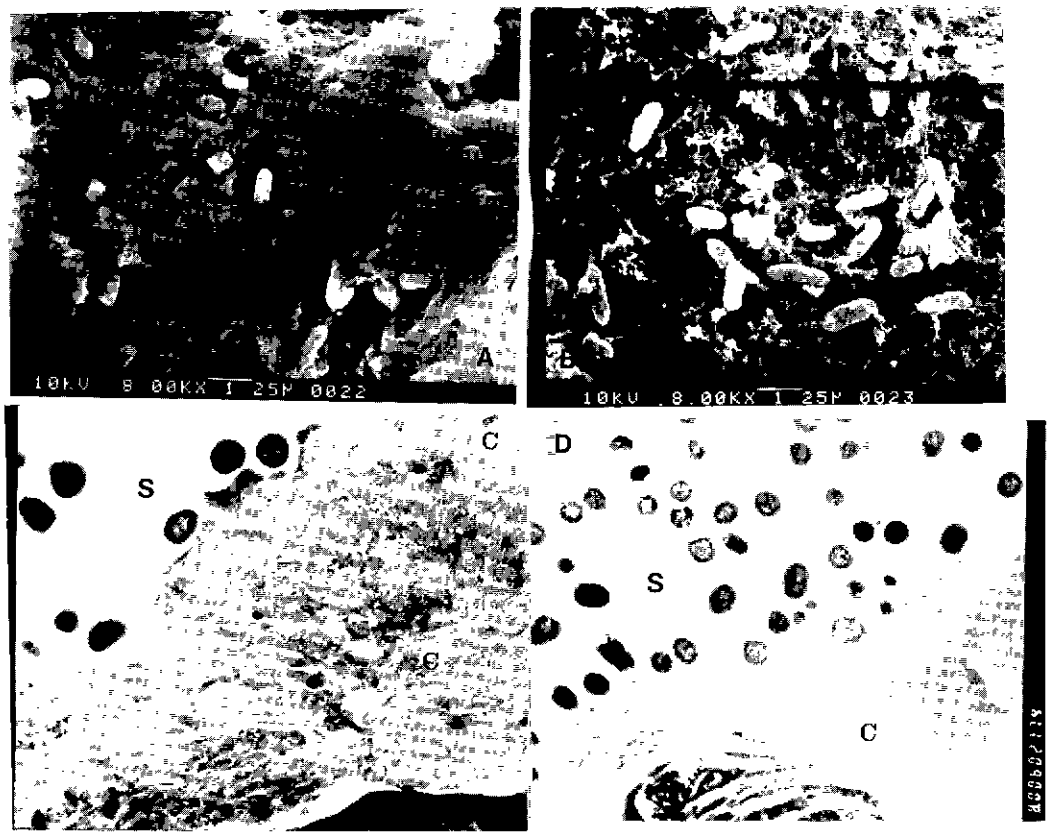

Fig. 3. Morphology of subjacent tissues and cuticular tissues of abdomen and thorax of infected *Nilaparvata lugens* stal.
Plate A and B, scanning electron micrographs of inner tissue subjacent to epidermis of Abdomen and Thorax, respectively (8,000X).
Plate C and D, Transmission electron micrographs of cross-sections of Abdomen and Thorax epidermal(C) and subjacent(S) tissues corresponding with plate A and B, respectively (17,000X).

rently available, we have not been able to work out the intestinal tract in detail at the earlier stage of infection unfortunately. Instead, we have shown the bacterial penetration and multiplication at the later stages. We would like to mention that our result supports the primary role of protease on the pathogenesis of *N. lugens*.

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