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AWARD

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Molecular biological studies of *Ralstonia solanacearum* and related plant pathogenic bacteria

Ralstonia solanacearum is the causal organism of bacterial wilt in more than 200 species and 50 families of plants in tropical, subtropical, and warm regions in the world. This bacterium is a heterogeneous species with phenotypic and genetic variability. For more than four decades, *R. solanacearum* strains have been classified according to race (based on host range) and biovars (based on utilization of carbohydrates).

Recent changes in agricultural systematics and the global trade of seeds and saplings have unexpectedly brought new host plants and invasive strains of pathogens. Detection and identification methods need to be improved through the elucidation of genetic diversity of foreign strains and indigenous *R. solanacearum* strains to help solve both taxonomic and plant quarantine problems.

The public demand for sustainable agriculture has helped drive research on the biological control of plant diseases and the practical use of antagonistic microorganisms. To assess the risk posed by biocontrol agents introduced into the agroenvironment, effective discrimination and monitoring methods of strains are also necessary.

Recent advanced molecular techniques have been effective in the analysis of genetic diversity and relationships among certain plant pathogens as well as among plant-associated bacteria. Under these circumstances, molecular biological studies have been conducted to characterize *R. solanacearum* strains and plant-associated bacteria from diverse origins.

Molecular biological studies of the strains of *R. solanacearum*. In Japan, bacterial wilt disease caused by *R. solanacearum* has been reported on more than 40 species and 20 families of plants. However, the genetic background and the systematic relationship among strains have been poorly investigated.

Phenotypic characteristics and pathogenicity of Japanese strains. Based on pathogenicity tests, the Japanese strains examined were divided into four pathogenic groups. Three groups (I–III) were pathogenic to many solanaceous plants such as tomato and eggplant, which corresponded to race 1. Group IV was pathogenic to potato and weakly pathogenic or nonpathogenic to tomato; it corresponded to race 3. Race 1 strains were isolated from various plants in most parts of Japan, whereas race 3 strains were found only in cultivated potato fields in limited regions.

Using biovar determination tests, strains were divided into three biovars (N2, 3, 4). Biovars 3 and 4 were most common. Japanese N2 strains were distinct from foreign biovars 2 and N2 strains in several of their phenotypic traits.

Genetic diversity of Japanese and foreign strains. A comparison of 16S rDNA sequences separated the Japanese strains into two groups: group 1 with strains of biovars N2, 3, and 4 (belonging to race 1) and group 2 with strains of biovar N2 (corresponding to race 3). Group 1 strains all had identical sequences, and strains representing the three biovars within the group did not differ from each other. Group 2 strains had characteristic nucleotides that differed at seven positions from group 1 strains. In a comparative analysis of Japanese and foreign strains based on 16S rDNA sequences, Japanese group 1 was closely related to Asian and Australian biovars 3, 4, and 5 and belonged to division 1. Japanese group 2 was homogeneous with Indonesian biovars 2 and N2 in subdivision 2b, suggesting a close relationship between them.

A dendrogram was constructed based on the repetitive polymerase chain reaction (rep-PCR) genomic fingerprints of Japanese strains. The three primer sets (REP, ERIC, BOX) defined 35 fingerprint types at the 95% similarity level. Each strain that differed by race or biovar represented a distinct fingerprint type. The strains were separated into two main groups: one with all race 1 and the other with only race 3. Race 1 strains were further subdivided into

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six groups at 80% similarity. Within race 1, each biovar (N2 and 4) separated into a single group, with high similarity within each biovar. On the other hand, biovar 3 strains were divided into five groups, with low average similarity among the strains.

Comparative analysis of the rep-PCR fingerprints of *R. solanacearum* strains, including six biovars from Japan and various countries, revealed two main clusters. Cluster 1 comprised all strains of biovars 3, 4, and 5 (races 1, 4, and 5) from Asia and Australia and strains of biovars 1 and N2 (race 1) from Reunion and Japan. Cluster 2 included most strains of biovars 1, 2, and N2 (races 1, 2, and 3) from 13 countries. In cluster 2, the average similarities within biovar 2 from eight countries and biovar N2 strains from Brazil were 94% and 65%, respectively, whereas the average similarity between biovar N2 strains from Brazil and Japan was 21%.

New bacterial wilt diseases of Zingiberaceae plants. New bacterial wilt diseases of Zingiberaceae plants (curcuma, ginger, and mioga) have been appearing in Kochi Prefecture since 1995. All isolates from diseased plants were identified as *R. solanacearum* biovar 4. In host range tests, ginger and potato wilted severely, whereas tomato and tobacco did not wilt and HR was induced in tobacco. These isolates were designated race 4, a race previously unknown in Japan. In rep-PCR analysis of the strains, two types (I and II) of DNA fingerprint patterns were obtained. None of the DNA patterns of indigenous races or biovars was identical to any of the types of I and II. The DNA pattern of one group was identical to that of strains from Australia and China, and that of another group was identical to Thai strains. These two pathogenic strains may therefore have been introduced independently through contaminated seed materials and spread to an epidemic scale by separate routes.

Serological and molecular characterization of the phytopathogenic bacteria. The monospecificity of monoclonal antibodies (MABs) is particularly useful as a molecular probe not only for specific detection but also for characterization of differences among epitopes (or antigenic determinants) of the bacterial cells. Production and application of MABs were tested in combination with an enzyme-linked immunosorbent assay (ELISA) for rapid and sensitive diagnosis of diseased plants.

Xanthomonas campestris pv. *vesicatoria* (*Xcv*), the causal agent of bacterial spot disease of tomato and pepper, was classified into two races based on its ability to hydrolyze starch (Amy^-, Amy^+). MABs were produced that specifically differentiated these two races. By assaying a Japanese collection of strains against these MABs, Amy^+ strains were discriminated from Amy^- strains, and their serological relationships with worldwide strains were demonstrated. Using these MABs in a competitive ELISA, a bacterial population as small as 5×10^3 cfu/ml can be detected. We can also use the MABs to follow bacterial growth in inoculated plants. Immunoblotting showed that the epitopes of the MABs were lipopolysaccharides (LPSs) of 41–80 kDa.

Bacterial black spot disease of mango, caused by *X. c.* pv. *mangiferaeindicae* (*Xcm*) has become serious in subtropical regions of Japan, Okinawa, and the Ogasawara Islands. *Xcm* strains consist of two genotypically and phenotypically distinct groups. In a test using MABs that specifically distinguish groups I and II of the *Xcm* strains, all the Japanese strains reacted equally with one MAB, which is the most specific for worldwide group I strains. Bacteria were effectively detected at about 10^3 cfu/ml before symptoms appeared when infected leaf materials were sonicated prior to the ELISA assay.

Among selected polyclonal and MABs against *R. solanacearum*, 13 MABs [all immunoglobulin M (IgM)] were divided into groups based on their specific reactivity to strains from tobacco and eggplant (race 1), potato (race 3), or ginger (race 4).

Collections of strains of the soft rot pathogen *Erwinia carotovora* ssp. *carotovora* (Ecc) isolated from various host plants in Japan, Korea, and Thailand were classified into four groups based on pathogenicity and into two groups based on phenotypic characteristics. In the latter, group A contained typical Ecc strains, to which all Thai strains and most of the Korean strains belonged, whereas group B, which is similar to *E. c.* ssp. *atroseptica* (Eca), contained strains isolated in Japan and Korea. By polymerase chain reaction – restriction fragment length polymorphism (PCR-RFLP) analysis of 16S rDNA, Ecc strains were differentiated into two groups. Most strains from Korea and Japan were found to belong to the same group. In the RFLP analysis of 16S–23S rDNA intergenic spacer regions (ISRs), all Thai strains showed the same pattern. Bacterial strains isolated from mulberry were unique, consisting of two types, type 1 and type 2; type 1 was similar to Ecc. Based on a polyphasic study that included a serological assay, a specific PCR assay for Eca, PCR-RFLP of a pectate lyase (*pel*) gene, random amplification of polymorphic DNA (RAPD)-PCR, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the strains, type 2 strains was discriminated clearly from other soft rot *Erwinia* species and may belong to a different subspecies.

Practical detection and molecular characterization of biocontrol agents. Of the candidate microbial biocontrol agents recently explored, fluorescent or nonfluorescent pseudomonads predominated. To assess beneficial traits and potential risks, bacteriological, serological, and molecular biological properties were characterized.

Practical detection and characterization of antibiotic-producing fluorescent Pseudomonas *spp.* By combining and ELISA using species-specific antisera with selective media, certain species of pseudomonads, including *P. fluorescens* and *P. putida,* were efficiently detected and isolated from rhizosphere soil. Bacterial strains thus isolated were assayed for antibiotic activity against important plant pathogens such as *R. solanacearum* and *R. solani*. They were also tested for disease suppressiveness or growth promotion of plants by seed or root bacterization.

Through these experiments, many antagonistic pseudomonads were evaluated. Antibiotic substances were identified using various techniques, including thin-layer chromatography, high-performance liquid chromatogra-

phy, and so on, or with molecular techniques such as PCR assays using DNA primers specific to the synthetic genes. Based on these analyses, certain siderophores, 2,4-diacetylphloroglucinol, and HCN were identified from highly antagonistic strains of *P. fluorescens*. Additional substances including pyrrolnitrin and pyoluteorin were also identified. These strains also suppressed target diseases such as wheat take-all and damping-off of radish. The Tn5 mutants, which either lost or decreased antibiotic productivity, were less antagonistic and were also less suppressive of disease, suggesting critical roles in the antagonism. A comparison of genetic relatedness by RAPD analysis showed much diversity among representative antagonistic strains such as Q2–87 and CHA0 from foreign countries; and the DNA pattern of Japanese strains differed from all the others.

Benefits and risk assessment of the Burkholderia cepacia *complex as a biocontrol agent.* The *B.cepacia* complex (Bcc) can control certain plant diseases, although it is also a plant pathogen and an opportunistic human pathogen.

Development of detection methods is important not only for risk assessment of microbe behavior in the soil ecosystem but also to evaluate biocontrol efficacy of the Bcc strains. In a comparison of six ELISA procedures consisting of direct and indirect methods, a convenient, highly sensitive, discriminating method was developed to detect the Bcc in rhizosphere soil. By combining a simple direct ELISA using a highly specific alkaline phosphatase conjugate, which was prepared from a certain glutaraldehyde-fixed Bc strain with a two-step incubation method (first at 40°C for 4 days, followed by 28°C for 3 days) on a selective medium, Bcc was specifically detected at 10^3–10^6 cfu/g from various rhizosphere soils in the field as well as from artificially infested soils. Heating the samples increased the reaction sensitivity, and Bcc in the soil was detected at about 10^2 cfu/g. The avidin-biotin peroxidase complex (ABC)-ELISA was also effective for discriminating serotypes (A–J) among Bcc strains with diverse origins.

Several MABs that were specific to five epitopes (lipopolysaccharide, protein) were also established. The specificity of MABs differed among Bcc strains, one of which was specific to serovar group A, which encompasses most of the natural strains from the environment. By combining two MABs that differed in reactivity, each Bcc strain could be detected selectively from soil artificially inoculated with mixed strains, indicating the practical applicability of the method to study population dynamics in the field.

Burkholderia cepacia complex strains were also characterized by their in vitro antagonistic activity against bacterial and fungal plant pathogens. The antibiotic activity of strains derived from natural sources was higher than that from clinical sources.

Genomovar (Gv.) identification and characterization of the Bcc strains recovered from clinical and environmental sources were investigated. Strains derived from clinical sources were assigned to Gv. I, Gv. III, Gv. IV, and Gv. V. On the other hand, most Bcc strains from environmental sources belonged to Gv. I, and the rest belonged to Gv. III. The *B. cepacia* epidemic strain marker encoded by *emsR* and the pyrrolnitrin biosynthetic locus encoded by *prnC* were present in 18% and 74% from all sources, respectively. All *emsR*-positive strains belonged to Gv. III, whereas most *prnC*-positive strains belonged to Gv. I.

Conclusions. Recent developments in molecular biological techniques have made possible complete sequencing of the genomes of certain plant pathogenic bacteria, including *R. solanacearum*. Such information will contribute to our development of an effective system to differentiate complex genetic relationships through studying the genetic diversity between and among domestic and worldwide strains. This differentiation and knowledge can help us discriminate and assess emerging pathogens or biocontrol agents that have been introduced into the environment. It will also be useful in the development of molecular methods for practical diagnosis and establishing new strategies for disease control.

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