Detection of a Protein Specific to the Strain of *Pseudomonas avenae* Manns 1909 Pathogenic to Rice

Ikuo KADOTA*, Akifumi MIZUNO** and Koushi NISHIYAMA*

Key words: *Pseudomonas avenae*, bacterial brown stripe, rice, pathogenicity, bacterial protein.

Pseudomonas avenae Manns 1909¹¹⁾ is a pathogen of barley (*Hordeum vulgare* L.)⁵⁾, finger millet (*Eleusine coracana* Gaertn.)^{1,13)} italian millet (*Setaria italica* Beauv.)^{6,14)}, maize (*Zea mays* L.)¹⁶⁾, mountain brome (*Bromus marginatus* Nees ex Steud.)¹⁷⁾, oat (*Avena sativa* L.)¹¹⁾, dallisgrass (*Paspalum dilatatum* Poir.)¹⁸⁾, pearl millet (*Pennisetum americanum* (L.) Leeke)²⁾, phalaenopsis (*Phalaenopsis* spp.)⁹⁾, proso millet (*Panicum miliaceum* L.)³⁾, rescue grass (*Bromus catharticus* Vahl)¹⁷⁾, rice (*Oryza sativa* L.)⁴⁾, tea (*Camellia sinensis* (L.) Kuntze)¹⁵⁾, teosinte (*Euchlaena mexicana* Schrad.)¹⁹⁾ and wheatgrass (*Agropyron* spp.)¹⁷⁾. As the host range of the pathogen is wide, it is thought to be polyxenic.

According to the inoculation tests by Okabe¹⁴⁾, Tomi-naga²⁰⁾ and Nishiyama *et al.*¹³⁾, isolates from finger millet, italian millet, maize, rescue grass, teosinte and wheatgrass were not pathogenic to rice. Therefore, it is thought that the isolates from rice might be different from the isolates from the other plants on the pathogenicity to rice. Here, the isolates from several plants including rice were compared on pathogenicity to rice and on components of proteins derived from them.

Bacterial strains. Five isolates of *P. avenae* from rice (rice strain) and 7 isolates from the other plants (non-rice strain) were used in this study. These bacterial isolates were incubated on PPGA medium¹²⁾ at 28°C for 24 hr and used for the tests. Details for each isolate such as source plant, locality, serovar designation and pathogenicity to rice are given in Table 1.

Inoculation tests. The bacteria were suspended in sterilized distilled water and their concentration adjusted to about 10⁸ cfu/ml. These suspensions were used to inoculate rice seedlings (cultivar: Niigata-wase) at the 4th leaf stage by a single-needle inoculation method. The needles were soaked in each bacterial suspension for a few seconds and pricked into the sheath at a point 2 cm above the ground. The seedlings were maintained in the greenhouse. One week after inoculation, the pathogenicity of each isolate was determined by assessing the development of brown stripe around the inoculation site. Pathogenicity of the isolates from maize and finger millet was confirmed by inoculating their respective source plants and assessing disease development after 10

days. All five isolates from rice formed clear symptoms on rice, but seven isolates from the other plants did not (Table 1). Accordingly, pathogenicity to rice differs between the rice and non-rice strains of the bacteria.

Extraction of bacterial proteins. Bacterial proteins were extracted by the method of Yakrus and Schaad²⁴⁾ as follows: one ml of the bacterial suspension (about 10⁸ cfu/ml) was inoculated to 200 ml of modified medium 523⁷⁾ (10 g glucose, 8 g casein hydrolysate, 4 g yeast extract, 2 g K₂HPO₄, 0.3 g MgSO₄·7H₂O in 1000 ml distilled water) in Sakaguchi-flasks and incubated on a rotary shaker at 28°C for 48 hr. The bacterial suspensions were centrifuged at 5000 × *g* for 10 min and 1 g wet weight of the bacterial cells were suspended into 10 ml of 0.2 M LiCl solution. The suspensions were agitated with 1 g of glass beads (710-1,180 microns, acid-washed, SIGMA, U.S.A.) in flasks on a rotary shaker for 2.5 hr at 45°C to extract the proteins. The suspensions were centrifuged at 5000 × *g* for 20 min, and the supernatants were centrifuged at 30,000 × *g* for 40 min at 4°C to remove large membranous debris. The resulting supernatants were centrifuged at 100,000 × *g* for 2 hr at 4°C. The pellets were suspended in a solution of 0.02% NaN₃ and stored at -20°C.

SDS-PAGE. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in vertical slab gels on an electrophoresis apparatus (ATTO, Japan) with the discontinuous buffer system of Laemmli¹⁰⁾. Separating and stacking gels contained 10% and 3% acrylamide, respectively. For molecular weight (M.W.) determination, bacterial proteins and standard proteins (electrophoresis calibration kit, Pharmacia, Sweden) were dissolved in Laemmli's sample buffer for 4 min at 100°C. Samples were applied to each sample well and electrophoresis was performed. The proteins were stained with Coomassie Brilliant Blue R250 (CBB). All isolates of rice strain tested had a common band with a molecular weight of about 50,000 (Fig. 1). Except for this band, however, protein profiles of the rice strain and non-rice strain did not differ significantly.

Cross-absorption of antiserum and purification of IgG. Antiserum⁸⁾ to *P. avenae* H8502 isolated from rice was cross-absorbed with MAFF 301141 isolated

* National Institute of Agro-Environmental Sciences, Tsukuba 305, Japan 農業環境技術研究所

** Yokohama Plant Protection Station, Yokohama 231, Japan 横浜植物防疫所

Table 1. *Pseudomonas avenae* Isolates used in this study

Isolate No. ^{a)}	Host	Locality	Serovar ^{b)}	Pathogenicity to rice
MAFF 301502	Rice	Niigata Pref.	A	+
MAFF 301503	Rice	Toyama Pref.	D	+
MAFF 301505	Rice	Fukui Pref.	B	+
MAFF 311049	Rice	Niigata Pref.	A	+
MAFF 311050	Rice	Niigata Pref.	C	+
MAFF 301024	Maize	Saitama Pref.	D	—
MAFF 301027	Wheatgrass	Chiba Pref.	E	—
MAFF 301031	Rescue grass	Chiba Pref.	E	—
MAFF 301036	Teosinte	Tochigi Pref.	D	—
MAFF 301141	Finger millet	Tochigi Pref.	N.A. ^{c)}	—
MAFF 301576	Phalaenopsis	Tochigi Pref.	N.A.	—
MAFF 301609	Dallisgrass	Kumamoto Pref.	N.A.	—

a) The isolates are maintained under Ministry of Agriculture, Forestry and Fisheries of Japan.

b) The serovars were determined by Kadota *et al.* (1991)⁸⁾.

c) N.A. means that the isolate could not be assigned to serovars A through E.

Fig. 1. SDS-PAGE of extracted bacterial proteins of *P. avenae*. Lanes 1 : MAFF 301502 (rice), 2 : MAFF 311049 (rice), 3 : MAFF 311050 (rice), 4 : MAFF 301503 (rice), 5 : MAFF 301505 (rice), 6 : MAFF 301024 (maize), 7 : MAFF 301027 (wheatgrass), 8 : MAFF 301031 (rescue grass), 9 : MAFF 301141 (finger millet), 10 : MAFF 301576 (phalaenopsis), M : molecular weight markers. Arrows on the right side indicate the molecular weights of markers, the arrow on the left side indicates the band specific to rice strain.

from finger millet at 37°C for 2 hr to prepare specific antibodies to rice strain. Cross-absorbed and non-absorbed antisera were purified to the IgG with an affinity device kit by protein A (Membrane Affinity Separation System, NYGENE, U.S.A.).

Western transfers and immunoblotting. The proteins separated by SDS-PAGE were electroeluted onto Polyvinylidene difluoride membranes (ATTO, Japan). The membranes were blocked by incubation at 37°C for 2 hr in TTBS (0.1 M Tris-HCl pH 7.5, 0.05% Tween 20, 50 mM NaCl) containing 3% skim milk. The proteins were reacted with absorbed or non-absorbed antibody to *P. avenae* H 8502 (1 : 4000 dilution) for 1 hr at room temperature and washed three times for 10 min in TTBS. The membranes were incubated in secondary antibody (goat anti-rabbit IgG(H+L) conjugated to biotin, VECTOR LABORATORIES, U.S.A.) at a dilution of 1 : 400 in TTBS for 1 hr at room temperature and washed three times for 10 min in TTBS. The membranes were incubated in VECTASTAIN ABC-AP KIT

solution (VECTOR LABORATORIES, U.S.A.) at room temperature for 1 hr, washed three times for 10 min in TTBS, and twice for 10 min in substrate buffer (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 0.05 M MgCl₂). The membranes were placed in color-substrate solution (Nitro blue tetrazolium (NBT) 0.33 mg/ml, 5-bromo-4-chloro-3-indolylphosphate (BCIP) 0.175 mg/ml in the substrate buffer) for a minute. When using the non-absorbed antiserum for detection, several bands, including a band specific to rice strain, were detected (Fig. 2). On the other hand, using the absorbed antiserum, only the rice strain-specific band at 50,000 in M.W. was strongly detected. MAFF 301503, MAFF 301024, MAFF 301036 and MAFF 301576 had another band larger than 94,000 in M.W. The three isolates of MAFF 301503, MAFF 301024 and MAFF 301036 belong to the serovar D, as does the isolate H 8502, which was used for antigen to prepare the antiserum⁸⁾. So, the band larger than 94,000 is thought to be serovar specific. In our previous study⁸⁾, MAFF 301576 could not be assigned to any serovars, but

Fig. 2. Immunodetection of extracted bacterial proteins of *P. avenae*. Lanes 1 to 10 are the same isolates to Fig. 1. M: molecular weight markers. A: Antiserum to H 8502 was used for the first antibody for detection. B: Antiserum to H 8502 absorbed with MAFF 301141 was used. Arrows on the left side indicate the band specific to rice strain.

isolates H 8502 and MAFF 301576 were considered to have the same protein as detected by serological reaction.

As described above, the non-rice strain was pathogenic to several gramineous plants but not to rice^{13,14,20}. On the other hand, Goto and Ohata⁴ examined the host range of the rice strain and clarified that the strain was pathogenic to barley, barnyard millet, italian millet, maize, oats, proso millet and sorghum. Tsuchiya *et al.*²¹ also reported that the rice strain was pathogenic to barley, barnyard millet, italian millet, maize, oats, proso millet, rye and wheat. Our inoculation tests also supported these results (unpublished data). From these facts, we conclude that the rice and non-rice strains have a common host range, but they differ in their pathogenicity to rice.

The five isolates of the rice strain used in this study have the protein identified as about 50,000 in M.W. and detected by the antiserum to rice strain absorbed with

non-rice strain. As the serovars and the localities of each isolate are different, the protein (50,000 in M.W.) appears to be common to the rice strain. A similar phenomenon was investigated in *Erwinia chrysanthemi* by Uesugi, *et al.*^{22,23} They analyzed the membrane proteins of *E. chrysanthemi* isolated from various host plants by SDS-PAGE; patterns of the membrane proteins showed close correlation between the host of origin and its pathovars. Therefore, the protein specific to rice strain of *P. avenae* may play an important role in host-parasite interactions. Further study is necessary to elucidate the function of the protein specific to rice strain in recognition of host plants.

This work was supported in part by Grants-in-Aid from the Ministry of Agriculture, Forestry and Fisheries [Biocosmos Program 96-I-C-(2)].

Literature cited

1. Billimoria, K.N. and Hegde, R.K. (1971). A new bacterial disease of ragi, *Eleusine coracana* (Linn.) Gaertn., in Mysore state. *Curr. Sci.* 40: 611-612.
2. Claffin, L.E., Ramundo, B. A., Leach, J.E. and Erinle, I.D. (1989). *Pseudomonas avenae*, causal agent of bacterial leaf stripe of pearl millet. *Plant Dis.* 73: 1010-1014.
3. Elliott, C. (1923). A bacterial stripe disease of proso millet. *J. Agr. Res.* 26: 151-159.
4. Goto, K. and Ohata, K. (1961). Bacterial stripe of rice. *Nat'l Taiwan Univ. Spec. Pub.* 10: 49-59.
5. Goto, M. (1964). Nomenclature of the bacteria causing bacterial leaf streak and bacterial stripe of rice. *Rep. Fac. Agric. Shizuoka Univ.* 14: 3-10.
6. Goto, M. and Okabe, N. (1952). Studies on the causal organisms of the bacterial stripe disease of millet and the brown stripe disease of italian millet. *Rep. Fac. Agric. Shizuoka Univ.* 2: 15-24.
7. Kado, C.I. and Heskett, M.G. (1970). Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. *Phytopathology* 60: 969-976.
8. Kadota, I., Ohuchi, A. and Nishiyama, K. (1991). Serological properties and specificity of *Pseudomonas avenae* Manns 1909, the causal agent of bacterial brown stripe of rice. *Ann. Phytopathol. Soc. Jpn.* 57: 268-273.
9. Kijima, T. and Minegishi, N. (1983). New diseases of cymbidium and other orchids. *Bull. Tochigi Agric. Exp. Stn.* 29: 75-86.
10. Laemmli, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680-685.
11. Manns, T.F. (1909). The blade blight of oat—a bacterial disease. *Ohio Agric. Exp. Stn. Bull.* No. 210: 91-167.
12. Nishiyama, K. (1978). Proposed method for rapid identification of plant pathogenic bacteria. *Plant Prot. (Shokubutsu Boeki)* 32: 283-288 (in Japanese).
13. Nishiyama, K., Nishihara, N. and Ezuka, A. (1979). Bacterial brown stripe of ragi caused by *Pseudomonas alboprecipitans*. *Ann. Phytopathol. Soc. Jpn.* 45: 25-31.
14. Okabe, N. (1934). Bacterial diseases of plants occur-

- ring in Formosa. IV. Bacterial brown stripe of Italian millet. J. Soc. Trop. Agric. Formosa 4: 54-63.
15. Takikawa, Y., Ando, Y., Hamaya, E., Tsuyumu, S. and Goto, M. (1988). Identification of the pathogens responsible for bacteriosis of tea plant occurred in 1983. Ann. Phytopathol. Soc. Jpn. 54: 224-228.
 16. Tominaga, T. (1968). Bacterial brown stripe of maize. Ann. Phytopathol. Soc. Jpn. 34: 350-351 (Abstract).
 17. Tominaga, T. (1968). Brown stripe of bromegrasses and wheatgrasses caused by *Pseudomonas setariae* (Okabe) Savulescu. Jpn. J. Bacteriol. 23: 176-183.
 18. Tominaga, T. (1971). Bacterial brown stripe of dallisgrass caused by *Pseudomonas alboprecipitans*. Ann. Phytopathol. Soc. Jpn. 37: 368 (Abstract).
 19. Tominaga, T. (1971). Studies on the diseases of forage crops in Japan. I. Kinds, developmental process, and damage of diseases of forage crops in the Kanto-Tosan districts. Bull. Nat. Inst. Agric. Sci., Ser. C25: 189-204.
 20. Tominaga, T. (1971). Studies on the diseases of forage crops in Japan. II. Etiological studies on the bacterial diseases of forage crops in Japan. Bull. Nat. Inst. Agric. Sci., Ser. C25: 205-306.
 21. Tsuchiya, Y., Date, H., Shirata, A., Matsuda, I. and Sato, Z. (1983). Bending symptoms of young rice seedlings grown in nursery flat caused by *Pseudomonas avenae*, the causal agent of bacterial stripe of rice. II. Identification of the causal bacteria. Bull. Nat. Inst. Agric. Sci., Ser. C38: 149-159.
 22. Uesugi, C. H., Tsuchiya, K., Tsuno, K., Matsuyama, N. and Wakimoto, S. (1990). SDS-polyacrylamide gel electrophoretic profiles of membrane proteins associated with host of origin in *Erwinia chrysanthemi* strains. Ann. Phytopathol. Soc. Jpn. 56: 597-604.
 23. Uesugi, C. H., Tsuno, K. and Wakimoto, S. (1991). Detection of antigen determinants and peptide mapping of the membrane proteins of *Erwinia chrysanthemi* pathovars. Ann. Phytopathol. Soc. Jpn. 57: 377-386.
 24. Yakrus, M. and Schaad, N.W. (1979). Serological relationships among strains of *Erwinia chrysanthemi*. Phytopathology 69: 517-522.

和 文 摘 要

門田育生・水野明文・西山幸司：イネに病原性を示す *Pseudomonas avenae* Manns 1909 に特異的に存在するタンパク質の検出

イネ褐条病細菌(イネ系統)とイネ以外の作物から分離した同種の細菌(非イネ系統)を供試し、第4葉期イネの地上約2cmの葉鞘部位に細菌懸濁液を単針接種して病原性を調査した。その結果、イネ系統は接種部位を中心に数cmにわたって明瞭な褐色の条斑を形成したが、非イネ系統はイネに病斑を形成しなかった。つぎに、各菌株の菌体タンパク質を抽出し、SDS-PAGEで分析したところ、分子量約50,000のバンドがイネ系統に共通して認められたが、その他の泳動パターンにおいてイネ系統と非イネ系統との間で明瞭な差異はなかった。泳動後のタンパク質をフッ化ビニリデン膜に転写し、これに非イネ系統の生菌体で吸収したイネ褐条病細菌 H 8502 株抗血清を反応させたところ、SDS-PAGEで分子量約50,000に認められたタンパク質が強く認識され、そのタンパク質はイネ系統に共通していた。よって、イネ褐条病細菌は各種植物から分離される褐条病細菌の中でイネに病原性を有する系統であり、本系統は供試抗血清によって認識される菌系特異的なタンパク質を共有することが示唆された。

(Received January 11, 1996; Accepted May 2, 1996)