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Occurrence of Bacterial Brown Stripe of Creeping Bentgrass on Golf Course Green in Kyushu

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A bacterial disease of creeping bentgrass (*Agrostis stolonifera* L.) was observed in golf course in Fukuoka Prefecture, Kyushu, Japan in 2000. It was characterized by brown stripes of the leaf sheaths. A non–fluorescent, aerobic, Gram–negative bacterium was consistently isolated from the infected plants. Infiltration of ten of the isolates into tobacco leaves resulted in a typical hypersensitive reaction. When creeping bentgrass plants were inoculated with the suspect isolates, typical bacterial brown stripe symptoms similar to those after natural infections were reproduced. The pathogen was reisolated from infected leaves two weeks after inoculation, thereby fulfilling the Koch's postulates. A sequence analysis of the genes coding for 16S rDNA revealed that the bacterium closely related to *Acidovorax* sp. On the basis of biochemical and physiological tests, the bacterium was identified as *A. avenae* subsp. *avenae*. The bacterium was pathogenic to *Sorghum sudanense, S. vulgare, Zea mays, Avena sativa, Lolium multiflorum, Tricum aestivum*, and *Hordeum vulgare*. It was only weakly pathogenic to *Oryzae sativa* and *Panicum maximum*. It was not pathogenic to *P. coloratum*, *Astragalus sinicus, Desmodium intortum, Medicago sativa* and *Lotus cornicolatus*. This is the first report of bacterial brown strip of creeping bentgrass caused by *A. avenae* subsp. *avenae* occurred in Kyusyu.

INTRODUCTION

Creeping bentgrass (*Agrostis stolonifera* L.) is a commonly used turfgrass species on golf course putting greens throughout Japan because of its ability to withstand low mowing heights and intense cultural practices, and because it provides a high–quality putting surface. Creeping bentgrass requires frequent watering, mowing, aerating, and dethatching, and high levels of fertilizer. In green golf–fields, creeping bentglasses are densely planted under warm temperature and high humidity. These conditions provide a favorable environment for the incidence of various types of bacterial diseases and the diseases make serious damages for creeping bentglasses in every place. In Kyushu golf–fields, the bacterial diseases outbreak constantly, but the pathogens have not been identified exactly. A bacterial disease of creeping bentgrass was found in Fukuoka Prefecture, Japan in 2000. The symptoms consisted of leaf streak and brown stripe, which extended into the sheaths. This study was designed to identify the causal agent of bacterial brown stripe of creeping bentgrass based on the pathological and bacteriological characteristics.

MATERIALS AND METHODS

Isolation of causal bacteria

The bacterial strains used in this study are listed in Table 1. The creeping bentgrass strains were isolated from diseased plants collected at Koga country Club in Koga, Fukuoka Prefecture. Leaf tissues (3–5 mm^2 tissue pieces) of the infected plants were macerated or crushed by sterilized dissecting needle in Eppendorf tube contained 0.1 ml of sterilized distilled water. The resulting suspentions were streaked on yeast extract–peptone–dextrose agar medium (YPDA : yeast extract 3 g, dextrose 3 g, peptone 0.6 g, agar 15 g, distilled water 1000 ml, pH 7.2) plates. Culture plates were incubated at 37 °C for 3 days. Ten individual colonies were picked from the initial plates of the various suspentions and were streaked on YPDA. At least three successive single–colony isolations were made for each isolate to ensure purity. For routine work, the isolates were stored in sterilized distilled water. For long–term preservation, each cultured bacterial strain was taken from agar surface, suspended in cryo–protectant mixture of skim milk and sodium glutamate, followed by froze, and lyophilized.

Inoculation test

Ten present strains of the suspect bacterium were tested for pathogenicity to creeping bentgrass as a original host plant. Inocula were prepared from cultures grown on YPDA medium at 30 °C for 2 days. Each bacterial suspension was prepared with sterilized distilled water to adjust its concentration to ca. 10^9 cfu/ml. Potted creeping bentgrass was inoculated by clipping the tip (about 5 mm) of the leaf with scissors that had been dipped into the bacterial suspension (ca. 10^9 cfu/ml). Sterilized distilled water was used as a control. The inoculated plants were incubated at 30 °C for 48 hr in a moist chamber (RH 100%), then transferred to an air–conditioned greenhouse bench at 25–30 °C. Recovery

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of the causal bacterium from inoculated plants was accomplished by placing ca. 5 mm^2 tissue pieces in 0.1 ml of sterilized distilled water as described above. The resulting suspension was streaked onto YPDA, and incubated at 30 °C.

16S rDNA sequencing

Bacterial 16S ribosomal DNA (16S rDNA) was amplified from the extracted genomic DNA by using the following universal bacterial 16S rDNA primers (Thorn and Tsuneda, 1996); forward primer pA(=8F) (5'–AGAGTTTGATCMTGGCTCAG–3') and reverse primer pH (5'–AAGGAGGTGATCCANCCRCA–3'). One loopful of bacterial cells was suspended in 1 ml of sterilized distilled water and held at 95 °C for 5 min and centrifuged at 10,000 rpm for 3 min. This supernatant was used directly as a template for the PCR. The PCR amplifications were performed in a 50 μl reaction mixture containing 2 μl of DNA solution, 1.5 mM $MgCl_2$, 1 μM of each primer, 1 mM dNTPs, 2 unit of *Tth* DNA polymerase (TOYOBO Biochemicals, Co., Japan) and reaction buffer (1 mM Tris–HCL pH8.9, 8 mM KCl, 0.15 mM $MgCl_2$, 0.01% Triton X–100). After the initial denaturation for 4.5 min at 95 °C, there were 40 cycles consisting of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, and extension at 72 °C for 2 min and then a final extension step consisting of 5 min at 72 °C. Negative controls using all reagents of the reaction except template were always included. The amplified products were purified from the gel slice by using Wizard SV Gel and PCR Clean–up System (Promega). PCR products were cloned using a pGEM–T easy kit (Promega). Plasmids containing the amplified fragments were extracted and sequencing reactions were done with an ABI Prism BigDye terminator cyclesequencing kit (Applied Biosystems) using both forward and reverse M13 primers. The sequence was read with an ABI 310 automatic sequencer (Applied Biosystem). All sequencing procedures were repeated at least twice.

Identification of bacteria

As shown in Table 1, *Acidovorax avenae* subsp. *avenae* (ATCC 19860^T), *A. avenae* subsp. *cattleyae* (MAFF 301576), *A. avenae* subsp. unidentified (MAFF 301027, MAFF 301141, MAFF 301504) were used as standards in identification tests and compared with the 10 pathogenic present strains isolated from the infected plants. Cells and flagella were stained with 2% phosphotungstenic and observed with a transmission electron microscope (JEOL, JEM–100 CX IIK). API 20NE stripes (Analytab Products, Plainview, USA) were used according to the manufacturer's instructions. The following tests were carried out according to the methods described in the references (Goto and Takikawa, 1984a–d; Schaad, 1988) to characterize the 10 present strains ; Gram reaction by 3% KOH, fluorescein production on King's B agar medium (20 g proteose peptone No. 3 (Difco), 15 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7H_2O$, 10 ml glycerol and 15 g agar/l of distilled water, pH 7.2), levan production, nitrate reduction, denitrification, hypersensitive reaction on *Nicotiana tabacum* L.(cv. Xanthi nc), potato soft rot. Nutritional tests were also conducted. All carbon sources were filter–sterilized and added to Ayer's basal agar medium (Ayers *et al*., 1939) to give a final concentration of 1.0% (w/v). A suspension of bacterial cells grown on a YPDA slant was streaked onto duplicate slants of each test medium, incubated at 30 °C, and examined periodically for 21 days. All tests were done at least twice.

Host range test

The strains of the suspect bacterium and *A. avenae* subsp. *avenae* (ATCC 19860^T), *A. avenae* subsp. *cattleyae* (MAFF 301576) and *A. avenae* subsp. unidentified (MAFF 301027, MAFF 301141, MAFF 301504) were tested for pathogenicity to *A. stolonifera* L. cv. Pencross, *Sorghum sudanense* cvs. Hay sudan, Umaka roll and Piper, *S. vulgare* cv. BMR sweet, *Zea mays* L. cv. Snow dent, *A. sativa* cv. New almighty, *Lolium multiflorum* Lam. cvs. Ace, Mammoth B, Tachimasari and Hanamiwase, *Triticum aestivum* cv. Nourin 61 gou, *Hordeum vulgare* cv. Haruna nijyou, *Oryza sativa* L. cv. Nipponbare, *Panicum maximum* Jacq. cv. Natsukaze, *P. coloratum* cv. Tamidori, *Astragalus*

Table 1. Bacterial strains used in this study

Species and/or isolate	Host	Locality	Source [a)]
Present strains			
K1~K10	*Agrostis stolonifera*	Fukuoka, Japan	This study
Reference strain			
Acidovorax avenae subsp. *avenae*			
ATCC 19860^T	*Zea mays*	USA	ATCC
Acidovorax avenae subsp. *cattleyae*			
MAFF 301576	*Phalaenopsis sp.*	Tochigi, Japan	MAFF
Acidovorax avenae subsp. unidentified			
MAFF 301027	*Agropyron trichophorum*	Chiba, Japan	MAFF
MAFF 301141	*Eleusine coracana*	Tochigi, Japan	MAFF
MAFF 301504	*Oryza sativa*	Niigata, Japan	MAFF

[a)] Abbreviations for culture collections : ATCC, American Type Culture Collection ; MAFF, Ministry of Agriculture, Forestry and Fisheries Gene Bank

sinicus L, *Desmodium intortum, Medicago savita* and *Lotus cornicolatus*. Inocula were prepared from cultures grown on YPDA medium at 30 °C for 2 days. Each bacterial suspension was prepared with sterilized water to adjust its concentration to about 10^9cfu/ml. Potted young plants were inoculated by the pricking methods. Sterilized distilled water was used as a control. The inoculated plants were incubated at 30 °C for 24 hr in a moist chamber (RH 100%), then transferred to a greenhouse bench at 25–30 °C, and observed for disease development during 6 weeks. The experiment was repeated twice.

RESULTS AND DISCUSSION

A single type of bacterial colony was isolated from the diseased plants. Ten strains were selected from the isolated colonies. Colonies, cultured for 4 to 5 days on King's B, were 1 to 2 mm in diameter, circular, whitish–grey, raised, entire edges. This appearance was very similar to that of *A. avenae*. Creeping bentgrass inoculated with the present strains in the greenhouse at 25–30 °C showed brown stripes (Fig. 1), and the bacteria were recovered from the inoculated plants. The symptoms on the inoculated plants were identical to the natural symptoms. Based on the initial tests, preliminary results on API 20NE strips and descriptions of Kobayashi *et al*. (2004), Kadota *et al*. (1996) and *Bergey's Manual of Systematic Bacteriology* (Krieg and Holt, 1984), the isolated strains of the suspect bacteria were identified as members of the genus *Acidovorax*.

The bacteriological characteristics of the present strains are summarized in Table. 2, where they are compared with the characteristics of the *Acidovorax* sp. included in this study. All the present strains were Gram negative, positive for the tobacco hypersensitive reaction and negative for arginine dihydrolase reaction. Cells were non–sporing, straight rods with round ends, motile with a single flagellum and 0.6–1.0×1.6–2.0 μm in size (Fig. 2). Oxidase reaction was positive. They could not produce a fluorescent pigment on King's B medium. All the present strains utilized xylose, dextrose, sorbitol, galactose, mannitol, L–arabinose. Several amino acids and organic acids were also utilized. The kinds of substrates utilized were generally analogous to the kinds of substrates utilized by the member of the *A. avenae* group. As shown in Fig. 3, the 16S rDNA sequence of the isolate was most similar to the sequence of *A. avenae* subsp. *avenae*. (level of similarity, 97.8%).

The results of host range test were summarized in Table 3. *A. avenae* subsp. *avenae* isolated from creeping bentgrass leaves was strongly pathogenic on *A. stolonifera* L. cv. Pencross, *S. sudanense* (cv. Hay sudan, Umaka roll and Piper), *S. vulgare* cv. BMR sweet, *Z. mays* L. cv. Snow dent, *A. sativa* cv. New almighty, *L. multiflorum* Lam. (cv. Ace, Mammoth B, Tachimasari and Hanamiwase), *T. aestivum* cv. Nourin 61 gou and *H. vulgare* cv. Haruna nijyou. The bacterium was weakly pathogenic to *O. sativa* L. cv. Nipponbare and *P. maximum* Jacq. cv. Natsukaze but was nonpathogenic to *P. coloratum* cv. Tamidori, *A. sinicus* L., *D. intortum, M. savita* and *L. cornicolatus*. In *Z. mays* L. cv. Snow dent, three strain (MAFF 301027 from *A. trichophorum*, MAFF 301141 from *E. coracana* and MAFF 301504 from *O. sativa* L) and *A. avenae* subsp. *cattleyae* (MAFF 301576 from *Phalaenopsis* sp.) did

Fig. 1. Symptoms on artificially inoculated leaves of *Agrostis stolonifera* L. by clipping inoculation.

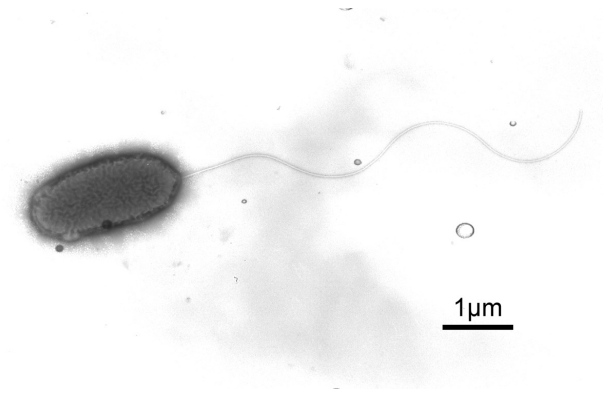

Fig. 2. Transmission electron micrograph of a bacterial cell of the present strain.

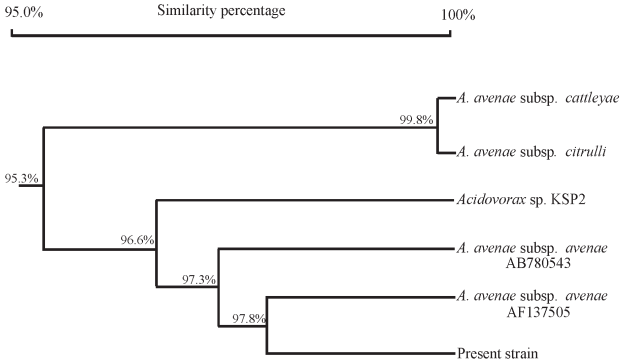

Fig. 3. Identification of the present strain based on 16S rDNA analysis.

Table 2. Comparison of physiological and biochemical properties of present strains isolated from *Agrostis stolonifera* with reference strains

Properties	Present strains (n=10)	Reference strains of *Acidovorax avenae* subsp.						*Acidovorax konjaci*[c) d)]
		avenae	*cattleyae*	*citrulli*[a) b) c)]	unidentified			
		ATCC 19860^T	MAFF 301576	ATCC 29625	MAFF 301027	MAFF 301141	MAFF 301504	
Gram reaction	−	−	−	−	−	−	−	−
Fluorescence on KBA	−	−	−	−	−	−	−	−
Growth at 40 °C	+	+	+	+	+	+	+	+
Levan production	−	−	−	−	−	−	−	−
Oxidase activity	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Arginine dihydrolase	−	−	−	−	−	−	−	−
Nitrate reduction	+	+	+	+	+	+	+	+
Denitrification	−	−	−		−	−	−	
Urease	+	+	+	+	+	+	+	+
Hydrolysis of starch	+	+	+	−	+	+	+	−
Hydrolysis of esculin	−	−	−	−	−	−	−	
Liquefaction of gelatin	−	−	−	+	−	−	−	−
Tobacco HR	+	+	+	+	+	+	+	+
Potato soft rot	−	−	−	−	−	−	−	−
Utilization of :								
Sucrose	−	−	−	−	−	−	−	−
Inositol	−	−	−	−	−	−	−	
Xylose	+	+	+	+	+	+	+	−
Dextrose	+	+	+		+	+	+	
Lactose	−	−	−	−	−	−	−	
Maltose	−	−	−	−	−	−	−	
Trehalose	−	−	−	+	−	−	−	
Sorbitol	+	+	+	−	+	+	+	
Galactose	+	+	+	+	+	+	+	
Mannitol	+	+	+	−	+	+	+	
Mannose	−	−	−		−	−	−	
L–Arabinose	+	+	−	+	−	+	+	−
D–Arabinose	−	−	−		−	−	−	
Adonitol	−	−	−	−	−	−	−	−
Raffinose	−	−	−		−	−	−	
D–Cellobiose	−	−	−		−	−	−	
Threonine	−	−	−		−	−	−	
L–leucine	+	+	+	+	+	+	+	+
Adipic acid	+	+	+		+	+	+	
Sodium citrate	−	−	−		−	−	−	
Phenyl acetate	−	−	−		−	−	−	
Potassium gluconate	+	+	+		+	+	+	
N–Acetyl–D–glucosamine	−	−	−		−	−	−	
L–Cystein	−	−	−		−	−	−	
Salicin	−	−	−	−	−	−	−	−
DL–Alanine	+	+	+		−	+	+	
Propionic acid	−	−	+		−	−	−	+
Benzoic acid	−	−	−	−	−	−	−	−
L–Tryptophan	+	+	−		−	−	−	
Pectic acid	−	−	−		−	−	−	
Phthalic acid	−	−	−		−	+	+	
n–Decanoic acid	−	+	−		−	−	−	+

Plus sign, positive; minus sign, negative

[a)] Cited from Shirakawa *et al*., 2000. b) Cited from Rane *et al*., 1992. c) Cited from Hu *et al*., 2001. d) Cited from Goto *et al*., 1983.

not cause disease. On the other hand, the symptoms on the plants caused by present strains were similar to that by *A. avenae* susp. *avenae* ATCC19860^T from *Z. mays* L.

A similar bacterial disease was reported from Hiroshima, Mie, Shizuoka, Aichi, Nagano and Hokkaidou in 2004, and the causal agent was identified as *A. avenae* (Kobayashi *et al*., 2004). We determined the subspecies of the present strains of *A. avenae*. Our results indicated clearly that the pathogen of the bacterial brown stripe of creeping bentgrass is *A. avenae* subsp. *avenae*.

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Table 3. Pathogenicity of the present strains and reference strains of *Acidovorax avenae* to various plants

Plant tested	Present strains (n=10)	Reference strains of *Acidovorax avenae* subsp.				
		avenae	*cattleyae*	unidentified		
		ATCC 19860T	MAFF 301576	MAFF 301027	MAFF 301141	MAFF 301504
Agrostis stolonifera L. cv. Pencross	+	+	w	+	+	+
Sorghum sudanense cv.						
Hay sudan	+	+	+	+	+	+
Umaka roll	+	+	+	+	+	+
Piper	+	+	+	+	+	+
Sorgum vulgare cv. BMR sweet	+	+	+	+	+	+
Zea mays L. cv. Snow dent	+	+	–	–	–	–
Avena sativa cv. New almighty	+	w	+	+	+	+
Lolium multiflorum Lam. cv.						
Ace	+	+	+	+	+	+
Mammoth B	+	+	+	+	+	+
Tachimasari	+	+	+	+	+	+
Hanamiwase	+	+	+	+	+	+
Triticum aestivum cv. Nourin 61gou	+	+	+	+	+	+
Hordeum vulgare cv. Haruna nijyou	+	+	w	w	w	w
Oryza sativa L. cv. Nipponbare	w	+	w	+	w	+
Panicum maximum Jacq. cv. Natsukaze	w	–	–	–	–	w
Panicum coloratum cv. Tamidori	–	–	–	–	–	–
Astragalus sinicus L.	–	–	–	–	–	–
Desmodium intortum	–	–	–	–	–	–
Medicago savita	–	–	–	–	–	–
Lotus cornicolatus	–	–	–	–	–	–

Plus sign, expanded symptoms (necrosis); w sign, necrosis around inoculation site (weak); minus sign, no symptoms

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REFERENCES

Ayers, T. T., C. C. Lefebvre and H. W. Johnson 1939 Bacterial wilt of lespedeza. *U.S.D.A. Tech. Bull.*, **704**: 1–22

Goto, M. and Y. Takikawa 1984a Methods for identification of plant pathogenic bacteria (1). *Plant Protection*, **38**: 339–344 (in Japanese)

Goto, M. and Y. Takikawa 1984b Methods for identification of plant pathogenic bacteria (2). *Plant Protection*, **38**: 385–389 (in Japanese)

Goto, M. and Y. Takikawa 1984c Methods for identification of plant pathogenic bacteria (3). *Plant Protection*, **38**: 432–437 (in Japanese)

Goto, M. and Y. Takikawa 1984d Methods for identification of plant pathogenic bacteria (4). *Plant Protection*, **38**: 479–484 (in Japanese)

Goto, M. 1983 *Pseudomonas pseudoalcaligenes* subsp. *konjaci* subsp. nov., the causal agent of bacterial leaf blight of konjac (*Amorphophalus konjac* Koch.). *Int. J. Syst. Bacteriol.*, **33**: 539–545

Hayashida, M., Y. Takikawa, M. Kobayashi, T. Hayakawa and S. Yaguchi 2002 *Pseudomonas fuscovaginae* causing sheath brown rot of turgrasses. *Ann. Phytopathol. Soc. Jpn.*, **68**: 256 (abstr. in Japanese)

Hu, F. P., J. M. Young, C. M. Triggs, D. C. Park and D. J. Saul 2001 Relationships within the Proteobacteria of plant pathogenic *Acidovorax* species and subspecies, *Burkholderia* species, and *Herbaspirillum rubisubalbicans* by sequence analysis of 16S rDNA, numerical analysis and determinative test. *Antonie van Leeuwenhoek*, **80**: 201–214

Kadota, I. 1996 Studies on the pathogen of bacterial brown stripe of rice and its ecology. *Bull. Hokuriku Natl. Agric. Exp. Stn.*, **38**: 113–171

Klement, Z. 1968 Rapid detection of the pathogenicity of phytopathogenic pseudomonads. *Nature*, **199**: 299–300

Kobayashi, M., T. Hayakawa, S. Yaguchi and Y. Takikawa 1992 A new bacterial disease of creeping bentgrass on golf course greens caused by *Acidovorax avenae* (Manns 1909) Willems, Goor, Thielemans, Gillis, Kersters and De Ley. *Ann. Phytopathol. Soc. Jpn.*, **70**: 76 (abstr. in Japanses)

Kobayashi, M., T. Hayakawa, S. Yaguchi, M. Hayashida and Y. Takikawa 2004 Occurrence of bacterial sheath brown rot and bacterial leaf blight of cool–season turfgrasses. *Ann. Phytopathol. Soc. Jpn.*, **68**: 256 (abstr. in Japanese)

Palleroni, N. J. 1984 Family I. Pseudomonadaceae Winslow, Broadhurst, Buchanan, Krumwiede, Rogers and Smith, 1917, 555. In *Bergey's Manual of Systematic Bacteriology* Krieg, N. R. and Holt, J. G., eds.). pp. 141–213, Williams & Wilkins, Baltimore

Rane, K. K. and R. X. Latin 1992 Bacterial fruit blotch of watermelon association of the pathogen with seed. *Plant Disease*, **76**: 509–512

Schaad, N. W., ed. 1988 *Laboratory Guide for Identification of Plant Pathogenic Bacteria*. American Phytopathological Society, St. Paul, MN

Shirakawa, T., S. Kikuchi, T. Kato, K. Abiko and A. Kawai 2001 Occurrence of watermelon bacterial fruit blotch in Japan. *Ann. Phytopathol. Soc. Jpn.*, **66**: 223–231

Thorn G. and A. Tsuneda 1996 Molecular genetic characterization of bacterial isolates causing brown bloch on cultivated mushrooms in Japan. *Mycoscience*, **37**: 409–416