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BACTERIAL AND PHYTOPLASMA DISEASES

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Burkholderia gladioli associated with symptoms of bacterial grain rot and leaf-sheath browning of rice plants

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Abstract Rice plants with bacterial leaf-sheath browning and grain rot were observed in Fukuoka Prefecture in Japan during the autumn seasons of 1995 and 1996. *Burkholderia* spp. were consistently isolated from the infected leaf sheaths and grains. These isolates were pathogenic and induced symptoms of seedling rot, grain rot, and leaf-sheath browning in rice plants, as well as in some orchidaceous plants (cymbidium, dendrobium, and oncidium leaves), gladiolus leaves, and onion bulbs. On the basis of morphological, physiological and pathological tests, and species-specific polymerase chain reaction, the isolates were identified as belonging to either *Burkholderia glumae* or *Burkholderia gladioli*. *B. gladioli*, as well as *B. glumae*, attacked rice plants after artificial inoculation and reproduced the symptoms similar to those after natural infections. We confirmed that rice is an additional natural host of *B. gladioli.* It is clarified that bacterial grain rot of rice is caused not only by *B. glumae* but also by *B. gladioli.*

Key words *Burkholderia gladioli* · *Burkholderia glumae* · Leaf-sheath browning · Rice

Introduction

During the autumn seasons of 1995 and 1996, bacterial leaf-sheath browning of rice plants was observed in paddy fields in Fukuoka Prefecture in Japan. As shown in Fig. 1, symptoms initially appeared on flag-leaf sheaths as water-soaked, brown, irregular spots that enlarged to dark to grayish brown blotches with no definite margins. Young panicles in diseased sheaths were also infected. On the panicle, infected grains were shrunken and pale green, which changed to dirty yellow and then brown before they dried up. The infected portions of the lemma and palea had purplish brown to dark brown lesions, which were similar to those reportedly caused by *Burkholderia glumae* (Kurita and Tabei 1967) Urakami, Ito-Yoshida, Araki, Kijima, Suzuki, and Komagata 1994 (Furuya et al. 2002; Kurita and Tabei 1967; Saddler 1994c; Yasunaga et al. 1986). Although *B. glumae* was constantly isolated from some lesions, *Burkholderia gladioli* (Severini 1913) Yabuuchi, Kosako, Oyaizu, Yano, Hotta, Hashimoto, Ezaki, and Arakawa 1993 was also isolated from several other lesions (Ura et al. 1996). Eight pathogens have been documented as the causal agents of bacterial rice diseases in Japan (Phytopathological Society of Japan 2000) – *Acidovorax avenae* subsp. *avenae* (Manns 1909) Willems, Goor, Thielemans, Gillis, Kersters, and De Ley 1992 (bacterial brown stripe); *B. glumae* (bacterial grain rot, bacterial seedling rot); *Burkholderia plantarii* (Azegami et al. 1987) Urakami, Ito-Yoshida, Araki, Kijima, Suzuki, and Komagata 1994 (bacterial seedling blight); *Erwinia chrysanthemi* pv. *zeae* (Sabet 1954) Victoria, Arboleda, and Muñoz 1975 (bacterial foot rot); *Erwinia ananas* Serrano 1928 (bacterial palea browning); *Pseudomonas fuscovaginae* Miyajima, Tanii, and Akita 1983 (sheath brown rot); *Pseudomonas syringae* pv. *oryzae* (ex Kuwata 1985) Young, Bradbury, Davis, Dicky, Ercolani, Hayward, and Vidaver 1991 (halo blight), and *Xanthomonas oryzae* pv. *oryzae* (Ishiyama 1922) Swings, Van den Mooter, Vauterin, Hoste, Gillis, Mew, and Kerters 1990 (bacterial leaf blight). Thus, the bacterial disease of rice plants caused by *B. gladioli* has not yet been reported (Saddler 1994a, b). This study was designed to detect and identify the causal agent(s) of leaf-sheath browning of rice using pathological and bacteriological characteristics.

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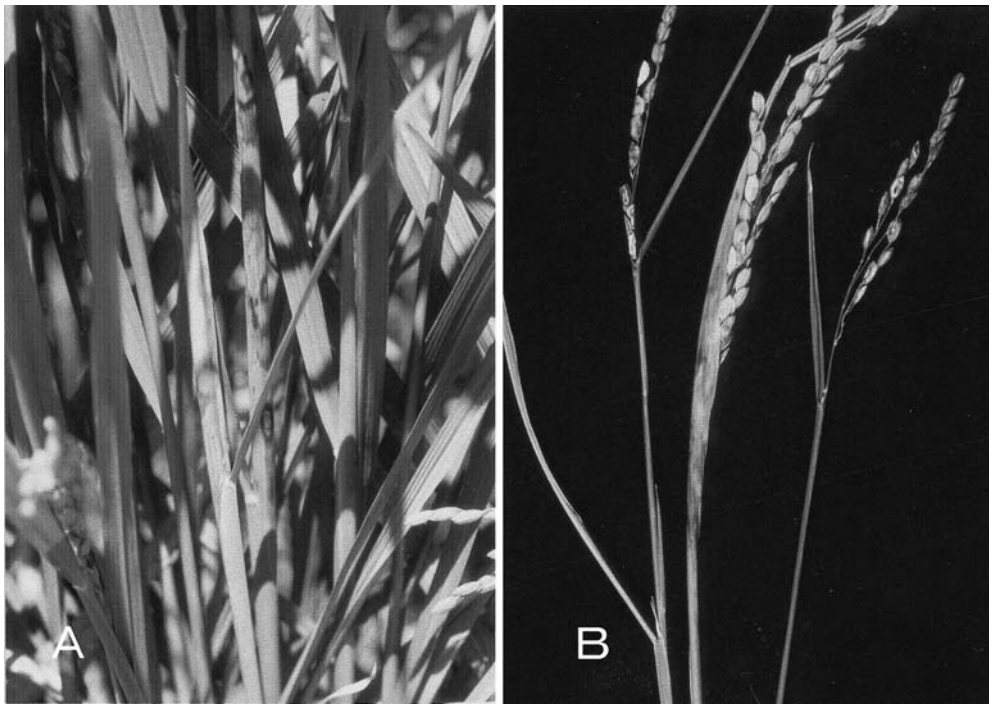

Fig. 1A,B. Rice plants with disease symptoms caused by natural infection in a paddy field. **A** Leaf-sheath browning, **B** leaf-sheath browning and grain rot

Materials and methods

Isolation of the pathogen in 1995

Bacterial leaf-sheath browning of rice plants was observed in paddy fields at Tsuyazaki in Fukuoka Prefecture in Japan. To isolate the pathogen, the infected leaf sheaths and grains were cut off, surface-disinfected in 70% ethanol for a few seconds followed by 3% sodium hypochlorite solution for 1 min, rinsed in sterilized distilled water, and crushed in 5 ml of sterilized distilled water with a sterilized knife. The resulting suspension was streaked onto yeast peptone dextrose agar medium (YPDA; yeast extract 3.0 g, peptone 0.6 g, dextrose 3.0 g, agar 15.0 g, distilled water 1000 ml, pH 7.2). The isolates from the leaf-sheath and grains of different rice plants in a paddy field were designated as T1 and T2, respectively. Serological relationships of these isolates with *Burkholderia gladioli*, *Burkholderia glumae*, and *Burkholderia cepacia* were investigated by immunodiffusion tests (Wakimoto et al. 1987; Tsuchiya et al. 1986).

Isolation of the pathogen in 1996

In 1996, 11 rice plants with bacterial leaf-sheath browning or grain rot were collected from two sites (Tsuyazaki and Hiikawa) in Fukuoka Prefecture. Suspension of bacteria that were isolated as described, were plated on YPDA and S-PG medium (Tsushima et al. 1986). After incubation, the colonies were observed for morphological features and pigment production. In addition, species-specific polymerase chain reaction (PCR) was carried out as reported before (Takeuchi et al. 1997; Furuya et al. 2002). To ascertain the identity of the amplicon, restriction analysis of the amplicon using *Hha*I and *Sau*3AI was also carried out (Ura et al. 1998).

Pathogenicity tests

The 13 present strains, as well as the reference strains of *B. glumae* and *B. gladioli*, were tested for their pathogenicity on rice plants (*Oryza sativa*, cv. Asominori), gladiolus (*Gladiolus gandavensis*), cymbidium (*Cymbidium* sp., cv. Marilyn Monroe), dendrobium (*Dendrobium* sp., cv. Yukidaruma Queen), oncidium (*Oncidium* sp.), and onion (*Allium cepa*).

Inoculation of rice seeds

Twenty chemically sterilized rice seeds were placed on 20 ml of 0.5% plain agar in a 100-ml Erlenmeyer flask. The seeds were inoculated by addition of 0.4 ml of bacterial suspension (ca. 10^8 cfu/ml), followed by incubation in a plant growth chamber maintained at 30°C with continuous illumination. Pathogenicity was determined 10 days after inoculation.

Inoculation of rice leaf sheath

Each bacterial suspension (0.7 ml of ca. 10^8 cfu/ml) was injected into a rice leaf sheath at the boot stage. The inoculated plants were placed in an inoculation chamber

Table 1. Bacterial strains used in this study

Species and/or isolate	Host	Locality and/or year	Source
Present strain			
T-1	Leaf sheath of rice	Tsuyazaki, Fukuoka, Japan, 1995	This study
T-2	Grain of rice	Tsuyazaki, Fukuoka, Japan, 1995	This study
T-3	Grain of rice	Tsuyazaki, Fukuoka, Japan, 1996	This study
T-4	Leaf sheath of rice	Tsuyazaki, Fukuoka, Japan, 1996	This study
T-5	Leaf sheath of rice	Tsuyazaki, Fukuoka, Japan, 1996	This study
T-6	Leaf sheath of rice	Tsuyazaki, Fukuoka, Japan, 1996	This study
T-7	Leaf sheath of rice	Tsuyazaki, Fukuoka, Japan, 1996	This study
T-8	Grain of rice	Tsuyazaki, Fukuoka, Japan, 1996	This study
T-9	Grain of rice	Tsuyazaki, Fukuoka, Japan, 1996	This study
T-10	Grain of rice	Tsuyazaki, Fukuoka, Japan, 1996	This study
T-11	Leaf sheath of rice	Tsuyazaki, Fukuoka, Japan, 1996	This study
H-1	Grain of rice	Hiikawa, Fukuoka, Japan, 1996	This study
H-2	Grain of rice	Hiikawa, Fukuoka, Japan, 1996	This study
Reference strain			
Burkholderia gladioli			
ATCC 10248^T	Gladiolus	USA	ATCC
MAFF 302537	Onion	Japan, 1985	MAFF
NIAS 1065	Freesia	Japan, 1962	NIAS
E-14	Rice	Fukuoka, Japan, 1980	KU
Burkholderia glumae			
MAFF 301169^T	Rice	Ehime, Japan, 1967	MAFF
N7401	Rice	Fukushima, Japan, 1974	NIAS
N7504	Rice	Okayama, Japan, 1975	NIAS
2	Rice	Japan	NIAS

ATCC, American Type Culture Collection; MAFF, Ministry of Agriculture, Forestry, and Fisheries Gene Bank; NIAS, National Institute of Agricultural Sciences (present affiliation: National Institute for Agro-Environmental Sciences); KU, Kyushu University

maintained at 30°C with high humidity in the dark for 2 days. They were transferred to a greenhouse and observed daily for 21 days for symptom development.

Inoculation of rice panicles

The rice panicles at the flowering stage were dipped in 350 ml of inoculum suspension (ca. 10^8 cfu/ml) for 3 min. The inoculated plants were maintained as described for rice seeds.

Inoculation of gladiolus, orchidaceous plants, and onion

The pathogenicity of each bacterial strain on detached leaves of gladiolus and orchidaceous plants (cymbidium, dendrobium, and oncidium) was tested by puncture inoculation with a needle dipped in the inoculum. The leaves were placed on filter paper impregnated with sterilized water in petri dishes to keep a high level of humidity and incubated at 30°C for 3 days. Pathogenicity on scaly bulbs of onion was similarly tested.

Characterization of the pathogenic bacteria

To characterize the 13 isolates from diseased rice plants, the following tests were conducted according to reported methods (Goto and Takikawa 1984a, b): Gram reaction by 3% KOH, glucose metabolism in Hugh-Leifson's medium, growth at 40ºC, organic growth factor requirement, oxidase reaction, catalase reaction, gelatin liquefaction, Tween 80 hydrolysis, esculin hydrolysis, gluconate oxidation, arginine dihydrolase, lecithinase, tyrosinase, and nitrate reduction. Nutritional studies were also performed. All carbon sources were added in mineral salts medium (Ayers et al. 1919) for a final concentration of 1% (w/v). Bacterial cells grown on YPDA slants were added to each test broth, incubated at 30°C without shaking, and evaluated periodically for 14 days. Toxoflavin production on YPDA slant was examined as reported previously (Iiyama et al. 1995). The reference bacteria in Table 1 were also used as standard.

Results

Isolation of the causal bacteria in 1995

Two strains (T-1 and T-2) were isolated from two rice plants showing leaf-sheath browning and grain rotting in a field. These isolates produced a yellowish, chloroform-soluble pigment on YPDA medium. This pigment was identified as toxoflavin by methods described before (Iiyama et al. 1994). In serological tests, T-1 and T-2 were antigenically related to both *Burkholderia gladioli* and *Burkholderia glumae* but not to *Burkholderia cepacia* (data not shown). The results of morphological and physiological tests demonstrated that T-1 and T-2 belong to *B. glumae* and *B. gladioli*, respectively (detail described below).

Isolation of the causal bacteria in 1996

The bacteria isolated from diseased plants were cultured on YPDA and S-PG media. The colonies from a single lesion were morphologically uniform on both media. A yellowish pigment, toxoflavin, was detected from all YPDA cultures. When more than ten colonies from a single lesion were tested with a species-specific PCR, the *B. gladioli*- or *B. glumae*- specific amplicons were detected. In addition, restriction analysis of the amplicon resulted in uniform *Hha*I or *Sau3*AI restriction patterns of all isolates from a lesion, indicating their clonal identity (data not shown). One isolate from each lesion was selected, and they were designated as T-3~T-11 (Tsuyazaki isolates) and H-1~H-2 (Hiikawa isolates). In 1996, 67% of the isolates were *B. gladioli* and 33% were *B. glumae* from leaf sheaths and grain, respectively, of diseased rice plants in Tsuyazaki. On the other hand, only *B. gladioli* was isolated from the samples of Hiikawa. These isolates were used in further experiments.

Pathogenicity of the suspect bacteria

Thirteen toxoflavin-productive strains of present bacteria (Table 1) were isolated from infected grains and leaf sheaths. As shown in Table 2, all the present strains were pathogenic on the rice leaf sheath. Dark brown spots with water-soaked halos appeared on the inoculated leaf sheath 5–10 days after inoculation. Moreover, all the present strains with the exception of H-1 and H-2 caused grain and seedling rot of rice and were pathogenic on gladiolus. Although H-1 and H-2 isolated from diseased grains were pathogenic to grains, no noticeable lesions on seedling was observed. Some orchid plants such as dendrobium, cymbidium, and oncidium were infected by all the present strains. Furthermore, the present strains caused rotting of inoculated onion bulbs held at 30°C for 3 days. These symptoms were quite similar to those caused by *B. glumae* or *B. gladioli*. All toxoflavin-productive strains, except for the type culture of *B. gladioli* (ATCC 10248^T), were pathogenic on leaf sheaths and seedlings of rice, whereas nontoxoflavin-productive strains (*B. glumae* N7504 and *B. gladioli* pv. *gladioli* NIAS 1065) were not pathogenic to the test plants. *Burkholderia gladioli* is a heterogeneous species with phenotypic and genetic variability (Saddler 1994a, b).

Characterization of the pathogenic bacteria

Preliminary tests including toxoflavin production and a species-specific PCR assay suggested that the strains isolated in this study were *B. glumae* or *B. gladioli*, which are shown as group 1 (T-2, T-3, and T-7~T-11) and group 2 (T-1, T-4 ~ T-6, H-1, and H-2) in Table 3. The strains were compared with *B. glumae* and *B. gladioli* in morphological and physiological properties (Table 3) and found to be Gram-negative, rod-shaped, and motile with single or multitrichous polar flagellation. On S-PG agar medium, the colonies were reddish brown to reddish purple, while on YPDA they were creamy colored. The strains of group 1 could utilize raffinose, but not L-tartrate, mesaconate, nicotinate, and citraconate as sole carbon source, whereas the reactions of group 2 in these tests differed. From these results, groups 1 and 2 were identified as *B. glumae* and *B. gladioli*, respectively (Azegami 1994). *Burkholderia gladioli* has been divided into three pathovars (pv. *gladioli*, pv. *alliicola*, and pv. *agaricicola*) based on their host ranges. We could not determine the pathovar of the present strains of *B. gladioli*, because there was no difference in their pathogenicity on rice, gladiolus, and onion (Table 2).

Discussion

Thirteen bacterial strains from infected leaf sheaths and grains of rice, which were isolated from 13 lesions on samples collected in Fukuoka Prefecture in Japan, were pathogenic to rice plants. On the basis of bacterial properties, seven isolates were identified as *Burkholderia glumae* and six isolates as *Burkholderia gladioli*. *B. glumae* is known to attack seedlings, leaf sheaths, and grains of rice (Goto

Table 2. Pathogenicity of the present strains and reference strains of *Burkholderia gladioli* and *Burkholderia glumae*

Pathogenicity to	Present strains		Reference strains					
	Tsuyazaki isolates (T-1~T-11)	Hiikawa isolates (H-1~H-2)	*B. gladioli*			*B. glumae*		
			ATCC 10248^T	MAFF 302537	NIAS 1065	MAFF 301169^T	N7401	N7504
Rice leaf sheath	+	+	±	+	−	+	+	−
Rice seedling	+	±	±	+	−	+	+	−
Rice grain	+	+	+	+	−	+	+	−
Gladiolus	+	+	+	+	−	+	+	−
Dendrobium	+	+	+	+	−	+	+	−
Cymbidium	+	+	+	+	−	+	+	−
Oncidium	+	+	+	+	−	+	+	−
Onion bulb	+	+	+	+	−	+	+	−

Plus sign, positive; plus/minus sign, doubtful; minus sign, negative

Table 3. Bacteriological properties of present strains and reference strains of *Burkholderia gladioli* and *Burkholderia glumae*

Property	Present strain		Reference strain			
	Group 1[a] (n = 7)	Group 2[b] (n = 6)	*B. glumae* MAFF 301169^T	*B. glumae* 2	*B. gladioli* ATCC 10248^T	*B. gladioli* E-14
Gram reaction	–	–	–	–	–	–
Cell diameter (μm)	0.5–0.7	0.5–0.8	0.5–0.7	0.5–0.7	0.5–0.8	0.5–0.8
Cell length (μm)	1.5–2.5	1.5–2.5	1.5–2.5	1.5–2.5	1.5–2.5	1.5–2.5
Number of flagella	1–3	1–3	1–4	1–3	1–3	1–2
O-F test (glucose)	O	O	O	O	O	O
Growth at 40°C	+	+	+	+	+	+
Organic growth factor requirement	–	–	–	–	–	–
Oxidase reaction	d (+1/–7)	d (+5/–6)	–	–	+	+
Catalase reaction	+	+	+	+	+	+
Gelatin liquefaction	+	+	+	+	+	+
Hydrolysis of Tween 80	+	+	+	+	+	+
Hydrolysis of esculin	–	–	–	–	–	–
Oxidation of gluconate	–	–	–	–	–	–
Arginine dihydrolase	–	–	–	–	–	–
Lecithinase	+	+	+	+	+	+
Tyrosinase	–	–	–	–	–	–
Nitrate reduction	+	d (4/6)	+	+	–	–
Toxoflavin production	+	+	+	+	+	–
Utilization of						
D-Arabinose	+	+	+	+	+	+
D-Cellobiose	+	+	+	+	+	+
Lactose	+	+	+	+	+	+
Maltose	–	–	–	–	–	–
D-Mannose	+	+	+	+	+	+
Raffinose	+	–	+	+	–	–
D-Ribose	+	+	+	+	+	+
Salicin	d (6/7)	+	+	+	+	–
Starch	–	–	–	–	–	–
Sucrose	–	–	–	–	–	–
L-Tartrate	–	+	–	–	+	+
D-Trehalose	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+
α-Methyl D-Glucoside	–	–	–	–	–	–
β-Methyl D-Glucoside	d (5/7)	–	+	+	–	–
Glycerol	+	+	+	+	+	+
β-Alanine	+	+	+	+	+	+
n-Amylamine	–	–	–	–	–	–
Benzoic acid	–	–	–	–	–	–
Citraconate	–	+	–	–	+	+
m-Hydroxybenzoic acid	–	–	–	–	–	–
Levulinate	–	–	–	–	+	–
Mesaconate	–	+	–	–	+	+
Nicotinate	–	+	–	–	+	+
D-Tartrate	+	+	+	+	+	+
m-Tartrate	d (6/7)	+	+	–	+	+
L-Valine	+	+	+	+	+	+

Plus sign, positive; minus sign, negative; d, 11%–89% of strains are positive
[a] Seven strains of group 1 include T-2, T-3, and T-7~T-11
[b] Six strains of group 2 include T-1, T-4~T-6, H-1, and H-2

and Ohata 1956; Saddler 1994c; Uematsu et al. 1976a, b; Yasunaga et al. 1986). In addition to *B. glumae*, Azegami et al. (1987, 1994) showed that some strains of *B. gladioli* were pathogenic to seedlings and grains after artificial inoculation. Moreover, several strains of *B. gladiloli* were isolated from healthy rice plants (Hirayae et al. 1987) and rice nursery soil (Azegami et al. 1994). In this experiment, *B. gladioli* were isolated from naturally infected leaf sheaths and grains of rice plants. Although both *B. gladioli* and *B. glumae* were isolated separately from samples in a field in Tsuyazaki, these pathogens were not coisolated from a single lesion, indicating that *B. gladioli* could induce leaf-sheath browning by itself. This is the first report of sheath browning and grain rot of rice caused naturally by *B. gladioli*. In this study, *B. gladioli* and *B. glumae* are clarified as causal agents of bacterial grain rot disease of rice plants. Further studies are needed to detail the damage and distribution of this disease caused by *B. gladioli*.

Pseudomonas fuscovaginae also induces similar symptoms on rice; necrotic lesions of the flag leaf sheath and reduced peduncle elongation result in the inhibition of inflorescence emergence and sterility of the panicle. Species-

specific PCR (Takeuchi et al. 1997, Furuya et al. 2002) and S-PG (Tsushima et al. 1986) will be effective tools for diagnosis of the disease.

Previously, we demonstrated the role of toxoflavin in virulence of *B. glumae* or *B. gladioli* to rice seedling and grain (Iiyama et al. 1994, 1995, 1998). In this study, we showed that six strains of *B. gladioli* (group 2) also produce toxoflavin and were pathogenic to rice leaf sheath. We now need to investigate the relationship between toxoflavin productivity and leaf-sheath browning of rice.

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