Pseudomonas pseudoalcaligenes subsp. *konjaci* subsp. nov., the Causal Agent of Bacterial Leaf Blight of Konjac (*Amorphophalus konjac* Koch.)

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A bacterial disease characterized by the development of rectangular lesions on the leaves of konjac (*Amorphophalus konjac* Koch.) is described. The size of the lesions increased rapidly under humid conditions, resulting in leaf blight. Leaves with severe blight drooped and finally decayed. The disease was identified from the symptoms as bacterial leaf blight on the basis of the descriptions made by Uyeda in 1910. The causal agent was, however, different from *Xanthomonas conjaci* (Uyeda) Burkholder in many respects and was found to be a new subspecies of *Pseudomonas pseudoalcaligenes* based on the results of a direct comparison of pathological and biochemical properties. The name *Pseudomonas pseudoalcaligenes* subsp. *konjaci* subsp. nov. is proposed for the new subspecies, and strain K2 (= ATCC 33996 = PDDCC 7733) is designated the type strain.

A bacterial disease that is prevalent on the foliage of the konjac plant (*Amorphophalus konjac*), whose root tubers are processed for the Japanese food product called konjac, has been described in textbooks of plant pathology as a bacterial leaf blight caused by *Xanthomonas conjaci* (Uyeda) Burkholder. However, until now no information about this disease except the original report of Uyeda (17) has been available. No cultures of the causal agent have been maintained in any collection of plant-pathogenic bacteria. Therefore, the causal agent was designated species incertae sedis in *Bergey's Manual of Determinative Bacteriology*, 8th ed., and was not listed on the Approved Lists of Bacterial Names (12) or the Pathovar List of Plant Pathogenic Bacteria (5). Thus, the pathogen of the disease of konjac has been left without an acceptable name despite its economic importance to konjac growers.

In a preliminary study in 1978 the konjac bacterium was compared with several pathovars of *Pseudomonas syringae* and *Pseudomonas andropogonis*. The konjac strains appeared to be distinctly different from these other bacteria in their biochemical and pathological characteristics. In contrast, some of the descriptions in the literature suggest a relationship between the konjac bacterium and the nonfluorescent organism *Pseudomonas pseudoalcaligenes* with regard to important biochemical properties, especially the ability to utilize glucose as a sole source of carbon. Therefore, I compared cultures of the konjac bacterium with cultures of *Pseudomonas alcaligenes*, *P. pseudoalcali-*

genes, and *P. pseudoalcaligenes* subsp. *citrulli*, a newly described plant pathogen (10).

In this paper I present the results of a study of these four bacteria and give a revised description of the bacterium responsible for bacterial leaf blight of konjac.

MATERIALS AND METHODS

Bacterial strains. The pseudomonads included in this study are listed in Table 1. A total of 17 strains of the konjac bacterium were isolated from small, water-soaked lesions of diseased leaves; these strains included 7 strains isolated in Shizuoka Prefecture and 10 strains isolated in Gumma Prefecture. The strains of *P. alcaligenes*, *P. pseudoalcaligenes*, and *P. pseudoalcaligenes* subsp. *citrulli* used as reference strains were provided by the Culture Collection of the Institute for Fermentation, Osaka, Japan, and the Plant Diseases Division Culture Collection, Auckland, New Zealand. All strains were grown on yeast extract-peptone (YP) agar slants (0.5% yeast extract, 1.0% peptone, 1.5% agar) and were maintained at 4°C for routine work.

To isolate the konjac bacterium, leaves with newly formed lesions were collected, and the diseased tissues with a water-soaked appearance were cut into pieces approximately 5 mm². The surface of each piece was sterilized by dipping the piece into 70% alcohol for 1 s. After the tissues were washed once in sterile water, they were crushed with a glass rod in a test tube containing 2 ml of 1% peptone water. A loopful of the resulting suspension was streaked onto YP agar plates. After 2 to 3 days at 28°C, white, round colonies were transferred to YP agar slants.

Pathogenicity. (i) **Konjac.** Tubers of *A. konjac* cv. native aoguki were planted in pots (diameter, 15 cm) and grown in a greenhouse at 20 to 35°C. When the leaves were fully expanded (in July), bacterial suspensions were sprayed onto the plants either before or

after the leaf surfaces were wounded with a needle by puncturing. Bacterial suspensions were prepared by washing cells from 24- to 48-h YP agar slants into distilled water and adjusting the concentration to about 10^8 cells per ml, as determined by optical density. The inoculated plants were maintained in a mist chamber for 24 h and then transferred to a greenhouse, where disease development was monitored.

(ii) **Cucurbitaceous plants.** Watermelon (*Citrullus lanatus* cv. kinkisuika), cucumber (*Cucumis sativus* cv. okinmegami), muskmelon (*Cucumis melo* cv. purinumeron), and pumpkin (*Cucurbita maxima* cv. ebisu) were used. Seedlings were grown in pots (diameter, 15 cm), and plants were inoculated as described above. After inoculation the procedures described above for the konjac plant study were followed.

Bacteriological properties. Morphological measurements were made with a light microscope after staining with Ziehl carbolfuchsin (13). Poly- β -hydroxybutyrate was detected by staining 3-day-old YP-fructose agar cultures with sudan black B (9). The modified Yamanaka silver staining method (11) was used for flagellar staining.

Tests for catalase, amino acid decarboxylase, amylases, lipase (Tween 80), urease and oxidase, reduction of KNO_3 , production of acetylmethylcarbinol, 2-ketogluconate, indole, and H_2S were performed as described by Cowan and Steel (3). Phenylalanine deaminase was tested (3) by using 2-day-old phenylalanine agar slant cultures. Tests for the litmus milk reaction, gelatin liquefaction, methyl red, and growth inhibition by KCN, as well as the Hugh-Leifson test and the esculin hydrolysis test, were also performed as described by Cowan and Steel (3). The methods described by Dye (4) were used to test for the utilization of asparagine as a sole source of carbon and nitrogen and tolerance to NaCl. The production of reducing substances from sucrose, arginine dihydrolase, and levan formation were tested by methods described previously (9). Utilization of carbon sources was tested in the basal media developed by Ayers et al. (9) and

by Starr (15). Denitrification was tested by the method of Stanier et al. (14). Fluorescence was tested on King medium B (9).

Guanine-plus-cytosine content of deoxyribonucleic acid. Deoxyribonucleic acid was isolated and purified by the method of Marmur (7). The guanine-plus-cytosine content was determined spectrometrically by the method of Ulitzur (16).

RESULTS

Bacteriological properties. The morphological, physiological, and biochemical characteristics of the konjac bacterium are summarized in Tables 2 and 3, where they are compared with the characteristics of the other pseudomonads included in this study.

The konjac bacterium was rod shaped with a single polar flagellum (Fig. 1) and gram negative. Growth of this bacterium on YP agar plates was relatively slow, and its colonies closely resembled those of *P. alcaligenes*, *P. pseudoalcaligenes*, and *P. pseudoalcaligenes* subsp. *citrulli* in being white, round, transparent, smooth, and slightly convex. The colonies were 0.8 to 1.0 mm in diameter within 48 h and 2 to 3 mm in diameter within 5 days at 28°C. The konjac bacterium was characterized by a positive urease reaction and the production of a water-soluble brown pigment. A strong urease reaction was observed from 24 to 48 h. A mild urease reaction was also detected in *P. pseudoalcaligenes* subsp. *citrulli*, but no urease reaction was detected in *P. alcaligenes* and *P. pseudoalcaligenes*. The konjac strains turned various agar media (King medium B, YP agar, potato-glucose agar) deep brown in 3 to 5 days. The growth of the konjac bacterium in YP broth at 41°C was relatively poor compared with the other bacte-

TABLE 1. *Pseudomonas* strains included in this study

Taxon	Strain(s)	History ^a	Habitat
Konjac bacterium	K1, K2 ^T , K3, K4, K5, K6, K7 ^b	Goto	Konjac
Konjac bacterium	7901, 7904, 7907, 7910, 7913, 7916, 7919, 7923, 7925, 7928 ^c	Hayashi	Konjac
<i>P. alcaligenes</i>	IFO 4 ^T (= ATCC 14909 ^T)	ATCC → Yabuuchi → IFO	Water
<i>P. pseudoalcaligenes</i>	IFO 5 ^T (= ATCC 17440 ^T)	ATCC → Yabuuchi → IFO	Sinus drainage
<i>P. pseudoalcaligenes</i> subsp. <i>citrulli</i>	PDDCC 6521 ^d	Schaad → PDDCC	Watermelon
<i>P. pseudoalcaligenes</i> subsp. <i>citrulli</i>	PDDCC 6522	Schaad → PDDCC	Watermelon

^a Goto, M. Goto, Shizuoka University, Shizuoka, Japan; Hayashi, N. Hayashi, Gumma Agricultural Experiment Station, Gumma, Japan; ATCC, American Type Culture Collection, Rockville, Md.; Yabuuchi, E. Yabuuchi, Kansai Medical College, Osaka, Japan; IFO, Institute for Fermentation, Osaka, Japan; Schaad, N. W. Schaad, University of Georgia, Athens; PDDCC, Plant Diseases Division Culture Collection, Auckland, New Zealand. The arrows indicate route of culture.

^b Isolated by M. Goto.

^c Isolated by N. Hayashi.

^d Isolated by N. W. Schaad.

TABLE 2. Phenotypic characteristics of the konjac bacterium, *P. alcaligenes*, *P. pseudoalcaligenes*, and *P. pseudoalcaligenes* subsp. *citrulli*

Character	Konjac bacterium (n = 17)	<i>P. alcaligenes</i> (n = 1)	<i>P. pseudoalcaligenes</i> (n = 1)	<i>P. pseudoalcaligenes</i> subsp. <i>citrulli</i> (n = 2)
Gram stain	— ^a	—	—	—
Motility	+	+	+	+
Flagellum ^b	Polar	Polar	Polar	Polar
Poly-β-hydroxybutyrate accumulation	+	—	—	+
Aerobic growth	+	+	+	+
Catalase reaction	+	+	+	+
Kovacs oxidase	+	+	+	+
Oxidation/fermentation test	0	0	0	0
Growth at 41°C	+	+	+	+
Growth at 4°C	—	—	—	—
Growth factor required	—	—	—	—
Utilization of arginine as C source	—	—	+	—
Utilization of betaine as C source	—	—	+	—
Utilization of asparagine as C and N sources	+	—	—	—
Pigmentation on King medium B	Brown	—	—	—
Nitrate reduction	+	+	+	+
Denitrification	—	—	—	—
Levan formation	—	—	—	—
Arginine dihydrolase	—	+	+	—
Gelatin liquefaction	—	±	—	±
Starch hydrolysis	—	—	—	—
Litmus milk	Alk	Alk	Alk	Alk
Tween 80 hydrolysis	+	+	—	+
H ₂ S production	+	+	+	+
Oxidation of gluconate	+	—	—	+
Reducing substance from sucrose	+	+	+	+
Urease	+	—	—	+
Arginine decarboxylase	+	+	+	+
Lysine decarboxylase	—	—	—	—
Ornithine decarboxylase	—	—	—	—
Phenylalanine deaminase	+	+	+	—
Growth in 3% NaCl	+	+	+	+
Growth in 4% NaCl	±	±	±	±
KCN inhibition	—	+	+	+
Indole production	—	—	—	—
Methyl red test	—	—	—	—
Acetoin production	—	—	—	—
Guanine-plus-cytosine content of deoxyribonucleic acid	66–68 mol%	66 mol%	64 mol%	65–67 mol%
Tobacco hypersensitivity reaction	+	—	—	+
Potato rot	—	—	—	—
Pathogenicity on konjac	+	—	—	—
Pathogenicity on watermelon	— (HR)	—	—	+

^a +, Positive; —, negative; ±, slow; (d), delayed reaction; (w), weak reaction; (HR), hypersensitive reaction; Alk, alkaline; 0, oxidative.

^b Each organism had one flagellum.

ria. All bacteria reduced nitrate to nitrite, but did not exhibit denitrification. All bacteria were weakly positive for arginine decarboxylase; i.e., the medium under oil turned purple within 5 to 7 days. Except for *P. pseudoalcaligenes* subsp. *citrulli*, the bacteria tested were positive for the phenylalanine deaminase reaction.

The konjac bacterium utilized D-ribose, D-fructose, ethanol, glycerol, and D-mannitol as sole sources of carbon, with production of acid.

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 members of the *P. alcaligenes* group; an exception was the utilization of D-mannitol by the konjac bacterium, which distinguished this organism from the other bacteria. *P. pseudoalcaligenes* subsp. *citrulli* utilized L-arabinose and D-galactose, and this characteristic enabled me to differentiate *P. pseudoalcaligenes*

TABLE 3. Utilization of substrates by the konjac bacterium, *P. alcaligenes*, *P. pseudoalcaligenes*, and *P. pseudoalcaligenes* subsp. *citrulli*

Substrate	Konjac bacterium (n = 17)	<i>P. alcaligenes</i> (n = 1)	<i>P. pseudoalcaligenes</i> (n = 1)	<i>P. pseudoalcaligenes</i> subsp. <i>citrulli</i> (n = 2)
L-Arabinose	— ^a	—	—	+
D-Ribose	+	—	—	+
D-Galactose	—	—	—	+
D-Fructose	+	—	+	+
Ethanol	+	—	+	+
Glycerol	+	—	+	+
Propanol	+	—	+	+
D-Mannitol	+	—	—	—
Acetate	+	—	—	+
Lactate	+	+	+	+
Maleate	+	+	+	+
D-Malate	+	+	+	+
Fumarate	+	+	+	+
Succinate	+	+	+	+
Citrate	+	+	+	+
Malonate	+	—	—	+
Propionate	+	+	—	+
n-Caprate	+	+	+	+
DL-Tartrate	+	—	—	+
β-Alanine	+	+	+	+
L-Serine	+	+	+	+
L-Leucine	+	—	—	+
L-Isoleucine	+	—	—	+
γ-Aminobutyric acid	+	+	+	+

^a +, Positive; —, negative; (d), delayed. The following compounds were not utilized by any of the bacteria: D-arabinose, D-xylose, D-glucose, D-mannose, L-rhamnose, cellobiose, maltose, lactose, trehalose, sucrose, melibiose, raffinose, melezitose, *meso*-erythritol, *myo*-inositol, ribitol, galactitol, glucitol, xylan, inulin, glycogen, starch, carboxymethyl cellulose, salicin, α-methyl-D-glucoside, esculin, formate, oxalate, gluconate, glycolate, n-butyrate, sebacate, and hippurate.

subsp. *citrulli* from the other organisms in this study.

The strains of *P. pseudoalcaligenes* subsp. *citrulli* tested differed from the original description of this taxon in being positive for nitrate reduction and the tobacco hypersensitivity reaction.

Symptoms. The disease developed on leaf blades and leaf petioles in summer, causing severe leaf blight. The lesions in the early stage of disease development were water-soaked spots that were a few millimeters in diameter. These spots quickly enlarged to form lesions with an average size of 15 to 25 mm long by 5 to 10 mm wide. The lesions showed distinctive characteristics, such as a rectangular shape limited by veins, a dark green to brownish green

discoloration, and evidence of water congestion. The lesions turned dark brown in several days, and their marginal areas were surrounded by yellow halos a few millimeters wide. When dried, the lesions became thin, transparent films with black veins. The disease was relatively mild under dry weather conditions, so that lesions limited by veins were individually scattered on the leaves. Under humid conditions, however, the size of the lesions rapidly increased, and frequently several lesions coalesced with each other, covering an entire leaf. Such leaves often rotted and dropped off, leaving naked stalks (Fig. 2).

Inoculation tests. (i) **Konjac.** The konjac bacterium produced characteristic symptoms when the spray inoculation method or the wound inoculation method or both were used. The first symptoms appeared 48 to 72 h after inoculation as small, water-soaked spots at the wound sites. The development of scattered spots followed 5 to 7 days after inoculation of a nonwounded leaf area. These spots enlarged to form rectangular lesions that were 10 to 20 by 2 to 4 mm in 1 week. The lesions were limited by veins and soon turned light brown to dark brown with yellow halos. The disease continued to progress, causing typical leaf blight. Soft rot of the diseased leaves was induced outdoors under rainy conditions in August to September but not in the greenhouse. No strain of *P. alcaligenes*, *P. pseudoalcaligenes*, or *P. pseudoalcaligenes* subsp. *citrulli* was pathogenic on konjac plants.

(ii) **Cucurbitaceous plants.** Strains of *P. alcaligenes* and *P. pseudoalcaligenes* failed to infect seedlings of watermelon, cucumber, muskmelon, and pumpkin. The konjac strains produced white lesions on the cotyledons 2 to 3 days after inoculation, but not on true leaves when the plants were inoculated by the pricking method.

FIG. 1. Polar flagellation of strain K2^T. ×2,000.

FIG. 2. Symptoms of bacterial leaf blight of konjac. (A) Leaf spot phase at the early stages. (B) Leaf blight phase at the later stages.

Such lesions never became water soaked. Therefore, the white areas of dead tissue were considered to be due to a hypersensitive reaction and not to a pathogenic reaction (6). Two strains of *P. pseudoalcaligenes* subsp. *citrulli* produced water-soaked lesions on leaves, cotyledons, and hypocotyls of cucurbitaceous plants after a few days. The symptoms were identical to those described by Schaad et al. (10).

(iii) **Hypersensitive reactions and potato rot.** Strains of the konjac bacterium and *P. pseudoalcaligenes* subsp. *citrulli* produced positive hypersensitive reactions on tobacco leaves (*Nicotiana tabacum* cv. Bright Yellow) within 12 h. No strain tested caused soft rot of potato slices.

DISCUSSION

Uyeda (17) described the symptoms of bacterial leaf blight of konjac as follows: "the leaves turn brown from the top and black brown later. The lesions enlarge to leaf lamina, which results in wilting and/or drooping within 2 to 3 weeks. In severe cases petioles are attacked, the leaves fall and the tuber shrivels or rots. The whole plant finally falls down on the ground." No mention was made of the presence of rectangular leaf spots limited by veins, which are the characteristic symptoms at the beginning of the disease. However, the description of Uyeda generally

agrees with the description of the severe form of the disease which I studied. Under humid conditions associated with frequent rain and high temperature, konjac plants become heavily infected, showing symptoms similar to those described by Uyeda. Moreover, no other bacterial disease has been detected on konjac plants, except soft rot caused by *Erwinia carotovora* subsp. *carotovora*. Therefore, I identified the disease which I observed as the bacterial leaf blight described by Uyeda in 1910.

However, the bacterium that causes this disease appears to be entirely different from *X. conjaci* (Uyeda) Burkholder (1), although the properties of the latter were poorly defined. According to the original description of Uyeda, this bacterium showed the following characteristics: "gram-positive rods with 1 to 4 polar flagella; colonies white and then light yellow; gelatin liquefied; milk coagulated and peptonized; indole produced; KNO_3 reduced; H_2S produced; gas from glucose; optimum temperature, 24°C ." It can be assumed from the symptoms which Uyeda described that isolation of the pathogen from plants had been attempted at the advanced stage of the disease. Therefore, it is possible that *X. conjaci* might have been a saprophytic bacterium which contaminated such old lesions.

The bacterium which I studied shares with the *P. alcaligenes* group the following phenotypic

TABLE 4. Phenotypic characteristics that differentiate *P. alcaligenes*, *P. pseudoalcaligenes*, *P. pseudoalcaligenes* subsp. *citrulli*, and the konjac bacterium

Character	Konjac bacterium	<i>P. alcaligenes</i>	<i>P. pseudoalcaligenes</i>	<i>P. pseudoalcaligenes</i> subsp. <i>citrulli</i>
Poly- β -hydroxybutyrate	+	—	—	+
Pigmentation	Brown	—	—	—
Gelatin liquefaction	—	$\pm$	—	$\pm$
Tween 80 hydrolysis	+	+	—	+
Urease	+	—	—	+
Phenylalanine deaminase	+	+	+	—
KCN inhibition	—	+	+	+
Utilization of:				
L-Arabinose	—	—	—	+
D-Ribose	+	—	—	+
D-Fructose	+	—	+	+
Ethanol	+	—	+	+
Glycerol	+	—	+	+
D-Mannitol	+	—	—	—
Malonate	+	—	—	+
Propionate	+	+	—	+
DL-Tartrate	+	—	—	+
L-Leucine	+	—	—	+
L-Isoleucine	+	—	—	+
Propanol	+	—	+	+

^a +, Positive; —, negative; $\pm$, slow; (w), weak.

characteristics, which distinguish it from most other members of the genus *Pseudomonas*: single polar flagellum, positive oxidase reaction, no requirement for organic growth factors, no hydrolysis of starch, growth at 41°C but not 4°C, utilization of lactate, caprate, and γ -aminobutyrate, but no utilization of D-glucose, cellobiose, sebacate, or gluconate (8). The differences among the four bacteria included in this study are summarized in Table 4. The differences listed in Table 4 may have resulted from the limited number of strains used in this study, because it is known that both *P. alcaligenes* and *P. pseudoalcaligenes* are heterogeneous in many of their biochemical properties and are closely related to each other (8). The only character which distinguishes *P. alcaligenes* and *P. pseudoalcaligenes* is the utilization of fructose as a sole source of carbon. Moreover, some of the properties listed in Table 4 have not been tested and described previously.

Despite the diversity in the biochemical properties of *P. alcaligenes* and *P. pseudoalcaligenes*, I believe that the konjac bacterium should be included in *P. pseudoalcaligenes* because of its ability to utilize D-fructose as a sole source of carbon. However, the konjac strains

differ from *P. pseudoalcaligenes* in plant pathogenicity, as well as in some biochemical properties, such as urease reaction, utilization of D-ribose, D-mannitol, DL-tartrate, and malonate, brown pigmentation, and Tween 80 hydrolysis.

The konjac bacterium differs from the plant-pathogenic organism *P. pseudoalcaligenes* subsp. *citrulli* in host range and other characters, such as utilization of L-arabinose, D-mannitol, and D-galactose, growth inhibition by KCN, pigmentation, gelatin liquefaction, and phenylalanine deaminase.

On the basis of the differences mentioned above, I concluded that the konjac bacterium should be recognized as a separate subspecies of *P. pseudoalcaligenes* and should be named *Pseudomonas pseudoalcaligenes* subsp. *konjaci* subsp. nov.

A condensed description of *P. pseudoalcaligenes* subsp. *konjaci* is given below.

***Pseudomonas pseudoalcaligenes* subsp. *konjaci* subsp. nov.** *P. pseudoalcaligenes* subsp. *konjaci* (kon.ja'ci. *konjaci* name of host species; L. n. *konjaci* of konjac) cells are gram-negative rods 0.5 μ m wide by 1.8 to 2.3 μ m long, are motile by means of a single polar flagellum, and accumulate poly- β -hydroxybutyrate. Colonies on YP agar are white, round, transparent, smooth, slightly convex, and 2 to 3 mm in diameter after incubation for 5 days at 28°C. A water-soluble brown pigment is produced on King medium B. Aerobic. Positive for nitrate reduction but negative for denitrification. Growth occurs at 41°C but not at 4°C. Oxidase positive. Utilizes D-fructose, D-ribose, D-mannitol, glycerol, ethanol, several amino acids, and organic acids as sole carbon sources. No growth occurs with the other sugars and polysaccharides tested (Table 3). Tobacco hypersensitivity positive. Pathogenic on konjac (*A. konjac*). The guanine-plus-cytosine content of the deoxyribonucleic acid is 66 to 68 mol%. Additional characteristics are listed in Table 2. Strain K2 is the type strain and has been deposited with the American Type Culture Collection as ATCC 33996 and with the Plant Diseases Division Culture Collection as PDDCC 7733.

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