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Reclassification of Subspecies of *Acidovorax avenae* as *A. Avenae* (Manns 1905) emend., *A. cattleyae* (Pavarino, 1911)comb.nov., *A. citrulli* Schaad et al.,1978)comb.nov., and proposal of *A. oryzae* sp. nov.

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Reclassification of subspecies of *Acidovorax avenae* as *A. Avenae* (Manns 1905) emend., *A. cattleyae* (Pavarino, 1911) comb. nov., *A. citrulli* Schaad et al., 1978) comb. nov., and proposal of *A. oryzae* sp. nov.

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Abstract

The bacterium *Acidovorax avenae* causes disease in a wide range of economically important monocotyledonous and dicotyledonous plants, including corn, rice, watermelon, anthurium, and orchids. Genotypic and phenotypic relatedness among strains of phytopathogenic *A. avenae* subsp. *avenae*, *A. avenae* subsp. *citrulli*, *A. avenae* subsp. *cattleyae* and *A. konjaci*, as well as all other *Acidovorax* species, including *A. facilis*, the type strain of *Acidovorax*, was determined. The 16s rDNA sequencing confirmed previous studies showing the environmental species to be very distant from the phytopathogenic species. DNA/DNA reassociation assays on the different strains of *A. avenae* revealed four (A, B, C, and D) distinct genotypes. Taxon A included six *A. avenae* subsp. *avenae* strains from corn that had a mean reciprocal similarity of 81%; taxon B included six *A. avenae* subsp. *avenae* strains from rice that had a mean reciprocal similarity of 97%; taxon C contained 11 *A. avenae* subsp. *citrulli* strains from cucurbits (cantaloupe, watermelon, and pumpkin) that had a mean reciprocal similarity of 88%, and taxon D contained four *A. avenae* subsp. *cattleyae* strains from orchids that had a mean similarity of 98%. The mean reciprocal relatedness between taxa A, B, C, and D was less than 70%. Sequence analysis of 16S rDNA and the 16S–23S rDNA internally transcribed spacer region, as well as AFLP analysis, revealed the same four taxa. All four were easily differentiated phenotypically from each other and from all other recognized *Acidovorax* species. Strains of *A. avenae* did not contain 3-hydroxyoctanoic acid, which was found in all other species. On the basis of these and previous genetic and phenotypic results, we propose an emendation of the species *A. avenae*. *A. avenae* subsp. *citrulli* (C strains) and *A. avenae* subsp. *cattleyae* (D strains) should be elevated to species rank as *A. citrulli* and *A. cattleyae*, respectively. We further propose a new taxon for the B strains, *A. oryzae* sp. nov. with FC-143^T = ICPB 30003^T = ICMP 3960^T = ATCC 19882^T as the type strain.
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Keywords: *Acidovorax*; Taxonomy; 16S and ITS sequencing; AFLP; Phenotypic tests; DNA/DNA reassociation; Emendation

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Introduction

Acidovorax avenae subsp. *avenae* (*Pseudomonas avenae*) and *A. avenae* subsp. *citrulli* (synonymous with *P. pseudoalcaligenes* subsp. *citrulli*) have emerged worldwide as serious pathogens on corn (*Zea mays* L.) [40] and watermelon (*Citrullus lanatus* (Thunb.) Matsumura and Nakai) [42], respectively. *Pseudomonas avenae*, originally described in 1909 as the causal agent of leaf blight of oats (*Avenae sativa* L.) [31], causes disease under conditions of high rainfall and high temperatures in numerous species of the family *Graminaceae*, including wheat (*Triticum aestivum* L.), finger millet (*Eleusine coracana* (L.) Gaertn.), Italian millet (*Setaria italica* (L.) Beauv.), pearl millet (*Pennisetum glaucum* (L.) R. Br.), and proso millet (*Panicum miliaceum* L.) [4,9,32,33]. Additional hosts include tea (*Thea sinensis* L.), barley (*Hordeum vulgare* L.), mountain brome (*Bromus carinatus* Hook. & Arn.), rescue grass (*B. catharticus* Vahl), vasey grass (*Paspalum urvillei* Steud.), teosinte (*Zea mexicana* (Schrad.) Kuntze), and sugarcane (*Saccharum officinarum* L.) [2,13,17,49,50]. *P. alboprecipitans*, a pathogen of foxtail (*Chaetochloa lutescens* (Weigel) Stuntz) [36], was later shown to be synonymous with the oats and corn pathogen, *P. avenae* [40]. Additionally, the red stripe disease of sugarcane caused by *P. rubrilineans* [17] was shown to be synonymous with *P. avenae* [35]. More recently *A. avenae* subsp. *avenae* has emerged as a serious pathogen of rice (*Oryzae sativa* L.) [16,24,26,27,34]. The causal agent of bacterial stripe of rice also has been referred to as "[*P.*] *setariae*" [14] and *P. panici* [34], however, neither epithet has been validly published [56]. A pathogen of orchids (*Cattleya*, *Dendrobium*, *Oncidium,* and *Phalaenopsis*), *P. cattleyae*, was described in 1946 [1]. A pathogen of konjac (*Amorphophallus konjac* K. Koch.), *A. konjaci,* was described in 1983 [15]. All of these phytopathogenic bacteria were described previously as a heterogenous group of non-fluorescent, oxidase positive pseudomonads, which accumulated poly-β-hydroxybutyrate (PHB) and utilized D-arabinose [38].

Schaad et al. [41] described a non-fluorescent pseudomonad as the causal agent of a seedling blight of watermelon. The bacterium differed phenotypically from other known plant pathogenic pseudomonads and was classified as *P. pseudoalcaligenes* subsp. *citrulli* [41]. Later, Wall and Santos determined that the same organism caused a disease referred to as watermelon fruit blotch of mature watermelon fruit [42]. Additional hosts of *A. avenae* subsp. *citrulli* include melon/cantaloupe (*Cucumis melo* L. subsp. *melo* var. *cantalupensis* Naudin), cucumber (*Cucumis sativus* L.) [41], and pumpkin (*Cucurbita pepo* L.) [28]. The above non-fluorescent phytopathogenic pseudomonads together with *P. pseudoalcaligenes* subsp. *cattleyae* [1] and *P. pseudoalcaligenes* subsp. *konjaci* [15] were all shown to be part of the "acidovorans" DNA–rRNA homology group [7].

Based on DNA/DNA and DNA–rRNA homologies [5] and phenotypic assays, Willems et al. [55] proposed that the above "generically misnamed phytopathogenic pseudomonads" be transferred to the newly proposed genus *Acidovorax* [54]. Strains of *P. avenae* [*P. rubrilineans*] from corn, sugar cane, Indian shot (*Canna indica*), teosinte, finger millet, and "*P. setariae*" from rice were all classified as *A. avenae* subsp. *avenae*. Strains of *P. pseudoalcaligenes* subsp. *citrulli* from *Cucurbitaceae* hosts were classified as *A. avenae* subsp. *citrulli*; strains of *P. cattleyae* from orchids were renamed as *A. avenae* subsp. *cattleyae*; and strains of *P. pseudoalcaligenes* subsp. *konjaci* [15] from konjac were classified as *A. konjaci* [55]. Recently two new phytopathogenic species of *Acidovorax*, *A. anthurii* on anthurium (*Anthurium palmatum* (L.) G. Don) [11] and *A. valerianellae* on lambs' lettuce (*Valerianella locusta* (L.) Lat.) [12], as well as a denitrifying species, *A. caeni* from activated sludge [18], have been described.

The purpose of this study was to re-evaluate the phylogenetic relatedness among the above phytopathogenic *A. avenae* strains and between *A. avenae* and the phytopathogenic *A. konjaci*, *A. anthurii*, and *A. valerianellae*. A polyphasic taxonomic study revealed considerable differences among the different subspecies of *A. avenae* and between the other species of *Acidovorax*. Therefore, we propose a new species, *A. oryzae* for the rice strains of *A. avenae.* We also propose *A. avenae* subsp. *citrulli* and *A. avenae* subsp. *cattleyae* be elevated to species rank as *A. citrulli* comb. nov. and *A. cattleyae* comb. nov.

Materials and methods

Source of strains and confirmation of identity

All the strains used in this study were obtained from the International Collection of Phytopathogenic Bacteria (ICPB) maintained at the USDA, ARS Foreign Disease-Weed Science Research Unit (FDWSRU), Fort Detrick, MD or from other recognized culture collections (Table S-1). Each culture was streaked onto yeast extract-dextrose $CaCO_3$ (YDC) agar [23] and beige-tan colored, transparent, round, non-mucoid, convex colonies were retained. Cultures were maintained on YDC slants at room temperature and also archived at −80 °C [38].

Pathogenicity

Pathogenicity was determined for all *A. avenae* strains by inoculating 2–3 plants each of corn (cv. 'Iochief'),

rice (cv. 'Nortai'), watermelon (cv. 'Charleston Grey'), or orchid (*Phalaenopsis* sp., *Dendrobium* sp., and *Cattleya* sp.). Additionally, wheat (cv. 'Anza') was included in pathogenicity tests for *A. avenae* strains. Strains of *A. konjaci*, *A. anthurii*, and *A. valerianellae* were not tested. Bacteria were grown overnight in 5 ml of liquid nutrient broth (Difco, Detroit, MI) or nutrient broth yeast extract (NBY) medium [52] on a rotary shaker and the optical density was adjusted to 0.1 at 600 nm. After diluting the suspension 1:100 in water, the growing points of 2–3 leaf-stage sweet corn and rice seedlings were injected using a 26-gauge needle and syringe. For watermelon, cotyledons of seedlings were infiltrated with the same suspension using the blunt end of a 2 ml disposable syringe. Sterile water was included as a negative control. All seedlings were incubated in a lighted dew chamber (Percival Scientific, Inc. model I-60 DLM, Perry, IA 50220) at 30 °C/25 °C day/night (12 h) and results were recorded after 10 days. For orchids, three to four sites of two new fully expanded leaves of flowering plants were infiltrated with inoculum using a 26-gauge needle and syringe and placed into a dew chamber at 27 °C/28 °C day/night for 21 days.

DNA preparation and DNA/DNA reassociation assays

DNA was extracted by a modified lysozyme, phenol/chloroform/ isoamyl alcohol method [43] and sheared using a French pressure cell (Spectronic Unicam, Rochester, NY), as described previously [43]. The purity and concentration of DNA was determined by measuring the 260/280 ratio using a "SmartSpec 3000" (Bio Rad, Richmond, CA) and only preparations with 260/280 ratios of 1.8 or greater were included. All samples were adjusted to 200 ng/μl and stored at −20 °C. The DNA/DNA reassociation assays were carried out using the S1 nuclease protocol [22], as described previously [43,44]. The probe and target DNA were reassociated at 71°C for 23 h in the presence of 22.7% formamide. For every one percent formamide, the temperature can be decreased by 0.6 °C [22]. Using 22.7% formamide equated to reassociation at 84–85 °C in the absence of formamide ($T_m = 98.6-15\,°C = 83.6\,°C$) for *A. avenae* DNA with an average mol% G + C value of 72.2% [40]. Each reaction was repeated 3–5 times. The following phytopathogenic strains of *Acidovorax* from corn, FC-320^T and 371; rice, FC-143^T; tea, FC-501; watermelon, FC-247^T and 513; konjac, FC-321^T; orchid, FC-502; anthurium, CFBP 3232^T; lambs' lettuce, CFBP 4730^T; finger millet, FC-500; and non-phytopathogenic *A. facilis* FC-208^T, were labeled for DNA/DNA reassociation assays. Data for *A. temperans*, *A. delafieldii*, and *A. defluvii* were taken from Schulze et al. [45].

AFLP

The AFLP procedure was carried out as described previously [44]. Briefly, an AFLP template was prepared for PCR using a combination of *Mse*I and *Eco*RI restriction endonucleases. Selective amplification was performed with *Mse*I+*C* (5′-GAT GAG TCC TGA GTA AC-3′) and *Eco*RI + 0 (5′-GAC TGC GTA CCA ATT C-3′) primers. The *Eco*RI + 0 primer had an infrared fluorescent dye IRDye^{TM} 700 (Li-Cor Inc., Lincoln, NE). The separation of amplified products was performed on a 6.5% polyacrylamide gel using a LI-COR Long ReadIR^{TM} DNA Sequencer (LI-COR model 4200) and electrophoresis data were automatically collected and simultaneously recorded during the run. The data were analyzed with GelCompar (v. 4.2) software (Applied Maths, Kortrijk, Belgium) and dendrograms were generated using the unweighted pair group method with averages (UPGMA). Strains of *A. anthurii* and *A. valerianellae* were not included in the AFLP analysis.

Phenotypic characters

Cells were grown overnight in liquid NBY shake cultures unless stated otherwise. Arginine dihydrolase activity [19] was determined at 28 and 37 °C. Lipase activity, starch hydrolysis, and PHB accumulation were determined as described previously [23]. For the oxidase test, filter paper impregnated with a 1% (w/v) solution of *p*-aminodimethylaniline oxalate (Difco, Detroit, MI) was used [38]. Gelatin hydrolysis and reduction of nitrate to nitrite was tested as described previously [8]. Degradation of pectate was tested using Hildebrand's media at three pH levels [19]. Growth at 4 and 41 °C was determined by liquid NBY shake cultures in a New Brunswick Scientific (Edison, NJ) Innova refrigerated incubator shaker with a temperature variance of ±0.1 °C. The cultures were observed for growth after 3 and 10 days. Action on litmus milk was determined using reconstituted powdered skim milk, as described previously [8]. Acid production and utilization of carbohydrates were determined on Dye's medium C containing bromthymol blue and a 1% final carbon source concentration [38], modified as follows: an overnight culture was adjusted to 0.1 OD_{600} then serially diluted ten-fold to 10^{-4} and 10 μl was spotted into individual wells of a 24 well tissue culture plate containing 2 ml of the carbon source medium. All tests were repeated twice and read after 7 and 14 days, unless stated otherwise. The type strain and several additional strains (Table S-1) of each recognized phytopathogenic species and subspecies of *Acidovorax* along with the type strain of the genus, *A. facilis*, were included. Results for the phylogenetically distant environmental species

A. temperans, *A. defluvii*, and *A. caeni* were from Willems et al. [55], Schulze et al. [45], and Heylen et al. [18], respectively.

Fatty acid analysis

The procedures used to prepare, extract, and differentiate fatty acids by gas–liquid chromatography have been described previously [39]. The fatty acid profiles of *A. facilis* strains, and each phytopathogen, including the newly described *A. anthurii* and *A. valerianellae*, were compared with those in the Sherlock® Microbial Identification System MIDI database (MIDI, Inc., Newark, DE) and were used to determine the Euclidian distance to *Acidovorax* spp. Results for *A. caeni* were from Heylen et al. [18].

16S rDNA and 16S–23S rDNA internal transcribed spacer (ITS) region sequencing

Direct sequencing of 16S rDNAs was performed with the following primers: 27f-AGA GTT TGA TCA TGG CTC AG and 1488r-CGG TTA CCT TGT TAC GAC TTC ACC [3]. The 16S rDNAs of the following strains were sequenced: phytopathogenic *A. avenae* subsp. *avenae* from corn (FC-320^T), rice (FC-143^T and 192), tea (FC-501), finger millet (FC-500), *A. avenae* subsp. *citrulli* from watermelon (FC-247^T), *A. anthurii* from anthurium (CFBP 3232^T), *A. valerianellae* from lambs' lettuce (CFBP 4730^T), and non-phytopathogenic strains of *A. facilis* (FC-208^T) and *Comamonas testosteroni* (FC-418). Sequences of *A. avenae* subsp. *cattleyae*, *A. temperans*, *A. konjaci*, *A. defluvii*, *A. delafieldii,* and *A. caeni* were obtained from GenBank.

Sequencing of the ITS region was performed with the following primers: 1493f-AGT CGT AAC AAG GTA GCC GT and 23r-GTG CCA AGG CAT CCA CC, as previously described [29]. The following strains were used for ITS comparisons: *A. avenae* FC-320^T, FC-371, FB-966, FC-179 (corn); FC-501, and FC-506 (tea); FC-143^T, FC-155, and FC-192 (rice); FC-247^T (watermelon), FC-356, and FC-526 (melon), FC-528 (pumpkin); FC-500 (finger millet); FC-502, and FC-509 (orchids); *A. konjaci* FC-321^T (konjac); *A. anthurii* CFBP 3232^T (anthurium); *A. valerianellae* 4730^T (lambs' lettuce); and *A. facilis* FC-208^T (soil). The PCR fragments of both regions were amplified, from DNA prepared as above for DNA/DNA reassociation assays, in a 9700 Sequence Detection System (Applied Biosystems Inc., Foster City, CA) with an initial denaturation at 94 °C for 2 min, followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 30 s, and extension at 72 °C for 45 s. Final extension was performed at 72 °C for 10 min. Aliquots of amplified samples were analyzed on 2.0% agarose gels. The fragments were purified using a "Wizard DNA Clean-Up System" (Catalog # A7280, Promega, Madison, WI), according to the manufacturer's protocol, and sequenced with an Applied Biosystems model 310 sequencer and Big Dye Terminator cycle sequencing kit.

Sequences were analyzed with the Sequence Navigator 1.01 program (Applied Biosystems) and compared using the Gene Inspector 1.5 *f* program (Textco, Inc., Research Triangle Park, NC). Alignments were verified manually.

Results

Pathogenicity

All strains originating from rice were pathogenic to both rice and corn (Table S-2). In contrast, strains from corn were pathogenic to corn but not rice (Table S-2). Strain FB-965, originating from corn, was not pathogenic to corn, wheat, or rice. Corn strains differed by their reaction on wheat (data not shown): strains FC-179, FC-320, FB-966, and FC-371 were pathogenic, whereas strains FC-358, FC-369 were not. Rice strains were not pathogenic to wheat. All *A. avenae* subsp. *citrulli* strains were pathogenic to watermelon, but not to corn. Orchid strains FC-502, 507, and 509, were pathogenic to *Phalaenopsis* and *Cattleya*, but not *Dendrobium*. Water-soaked lesions were visible after 7–10 days and the tissue became black after 14–21 days. Strain FC-503 was weakly virulent on *Phalaenopsis* and *Cattleya*. Control plants injected with water did not develop any symptoms.

DNA preparation and DNA/DNA reassociation assays

Results of DNA/DNA reassociation assays revealed four (A, B, C, and D) distinct genotypes among the *A. avenae* strains (Table 1), based upon 70% or greater similarity [37,48,53]. Taxon A included six *A. avenae* subsp. *avenae* strains from corn that had a mean reciprocal similarity of 81%; taxon B included six *A. avenae* subsp. *avenae* strains from rice that had a mean reciprocal similarity of 97%; taxon C contained 11 *A. avenae* subsp. *citrulli* strains from cucurbits that had a mean reciprocal similarity of 88%; and taxon D contained four *A. avenae* subsp. *cattleyae* strains from orchids that had a similarity of 98%. The mean reciprocal relatedness between taxa A, B, C, and D was less than 70% (Table 1). The two tea strains were 100% similar to each other but shared less than 70% relatedness to any of the four taxa above. The single strains from millet and finger millet and the six strains of *A. avenae* were moderately related but all showed less

Table 1. Percentage DNA relatedness between strains of phytopathogenic *Acidovorax avenae* subsp. *avenae* (Aaa) from corn, finger millet, millet, tea, vasey grass and rice. *A. avenae* subsp. *citrulli* (Aac) from curcurbits, *A. avenae* subsp. *cattleyae* (Aaca) from orchid, *A. konjaci* (Ak) from konjac, *Acidovorax anthurii* (Aan) from anthurium, *Acidovorax valerianellae* (Av) from lamb's lettuce and between *A. facilis* (Af), *A. temperans* (At), *A. delafieldii* (Adf), and *A. defluvii* (Adv), as determined by the S1 nuclease method using single-stranded target DNAs and ^{33}P-labeled single-stranded probe DNAs at T_m−15 °C

^{33}P-labelled probe DNAs, percentage aanealing																
Tester DNA	Strain	320^T	371	500	501	143^T	247^T	513	502^T	208^T	7169^T	411^T	5943^T	321^T	3232^T CFBP	4730^T CFBP
Taxon A, mean internal relatedness: 181%[a]																
Aaa	FC 320^T	**100**[b]	85[c]	63	65	53	43	41	27	4	18[e]	4[e]	17[e]	1	8	3
	FC 135	91	79	63	67	53	40	34	–	7	–	–	–	–	–	–
	FC 371	90	**100**	65	–	58	–	–	23	7	–	–	–	7	4	4
	FC 965	87	75	58	–	48	40	35	30	6	–	–	–	8	–	–
	FC 966	–[d]	70	–	66	52	–	–	–	–	–	–	–	–	–	–
	FC 369	–	72	68	61	53	–	–	–	10	–	–	–	–	–	–
Taxon B, mean internal relatedness; 97%																
Aaa	FC 143^T	43	52	48	68	**100**	45	43	–	6	–	–	–	–	8	5
	FC 499	49	46	50	57	99	43	40	–	6	–	–	–	–	–	–
	FC 192	62	49	56	63	90	–	–	22	5	–	–	–	6	5	8
	FC00	62	42	43	58	98	–	–	23	6	–	–	–	–	–	–
	FC 504	44	48	44	56	98	–	–	30	6	–	–	–	8	9	3
	FC 155	53	46	39	61	100	–	–	–	5	–	–	–	–	–	–
Taxon C, mean internal relatedness; 88%																
Aac	FC47^T	30	35	25	38	40	**100**	79	21	6	20[e]	8[e]	19[e]	6	5	6
	FC 183	–	28	35	35	47	92	92	–	5	–	–	–	–	–	–
	FC 374	–	30	27	35	44	100	83	30	9	–	–	–	6	5	6
	FC 376	30	–	–	–	–	94	93	–	–	–	–	–	–	–	–
	FC 379	–	–	–	–	–	97	92	–	–	–	–	–	–	–	–
	FC 440	–	30	26	38	39	96	83	25	6	–	–	–	7	6	6
	FC 464	–	–	–	–	–	91	84	–	–	–	–	–	–	–	–
	FC 513	30	32	31	38	42	82	**100**	–	6	–	–	–	–	7	4
	FC 526	–	30	–	37	45	85	–	–	3	–	–	–	–	–	–
	FC 528	26	36	31	36	42	80	–	–	0	–	–	–	–	–	–
	FC 356	–	29	25	39	37	80	–	–	8	–	–	–	–	–	–
Taxon D, mean internal relatedness; 98%																
Aaca	FC 502^T	34	37	38	44	40	42	35	**100**	9	18[e]	12[e]	22[e]	7	19	17
	FC 503	–	42	27	44	47	–	–	96	10	–	–	–	10	–	–
	FC 507	35	32	39	40	47	–	–	99	6	–	–	–	9	7	–
	FC 509	30	37	–	42	46	–	–	100	7	–	–	–	5	–	17
Aaa	FC 185	60	64	60	67	46	44	38	31	**3**	–	–	–	5	3	3
	FC 500	55	57	**100**	66	53	41	39	22	**2**	–	–	–	–	–	6
	FC 501	63	66	58	**100**	55	39	36	41	**5**	–	–	–	–	7	4
	FC 506	–	59	54	100	55	–	–	28	**8**	–	–	–	14	–	–
	FC 180	54	59	53	64	44	39	35	23	**2**	–	–	–	5	5	4

Af	FC08	3	3	8	5	3	3	3	0	**100**	45[e]	17[e]	56[e]	3	5	10
At	LMG 7169^T	15[e]	–	–	–	–	–	–	–	–	**100**[e]	22[e]	23[e]	10e	–	–
Adv	BSB 411^T	14[e]	–	–	–	–	–	–	–	–	33[e]	**100**[e]	25[e]	16e	–	–
Adf	LMG 5943^T	25[e]	–	–	–	–	–	–	–	–	28[e]	38[e]	**100**[e]	25e	–	–
Ak	FC 321^T	9	11	0	13	12	15	6	3	7	15[e]	20[e]	15[e]	**100**	4	1
	FC 505	–	8	2	13	10	–	–	6	6	–	–	–	100	11	10
Aan	CFBP 4344	–	–	–	–	–	–	–	–	–	–	–	–	–	100	–
	CFBP 3242	–	–	–	–	–	–	–	–	–	–	–	–	–	100	–
	CFBP 3232^T	8	7	–	7	9	5	4	1	2	–	–	–	10	**100**	8
Av	CFBP 4730^T	5	2	–	2	3	5	1	1	2	–	–	–	12	20	**100**
	CFBP 6487	–	–	–	–	–	–	–	–	–	–	–	–	–	–	100

Taxon A:B relatedness, 51%[f].
Taxon B:C relatedness, 42%.
Taxon A:C relatedness, 32%.
Taxon A:D relatedness, 33%.
Taxon B:D relatedness, 35%.
Taxon C:D relatedness, 31%.

[a]Mean internal % DNA relatedness: mean calculated from heterologous pairwise tests (non-bolded figures) within the taxon (boxed figures) but excluding 100% homologous values.

[b]Controls: homologous reassociations (bolded figures) between the probe and tester single-stranded DNAs from the same strain were set to 100% DNA relatedness; heterologous reassociations between the probe and salmon sperm single-stranded DNAs (not shown) were set to 0% DNA relatedness.

[c]Heterologous pairwise reassociations (non-bolded figures) between ^{33}P-labeled and tester single-stranded DNAs; average of at least two determinations presented.

[d]Pair-wise test not performed.

[e]Data from Schulze et al., [45] using T_m −25 °C.

[f]Mean reciprocal % DNA relatedness calculated from pair-wise, heterologous tests between two taxa.

than 70% relatedness to the other taxa. The strains from konjac and strains of *A. anthurii* and *A. valerianellae* had 19% or less similarity with strains of *A. avenae*. All other strains of *Acidovorax* shared a similarity of 25% or less with *A. avenae* (Table 1).

AFLP analysis

Similarities between the non-phytopathogenic strains (*A. facilis*, *C. testosteroni*, and *P. pseudoalcaligenes*) and the phytopathogenic strains (except *A. konjaci*) were less than 18% (Fig. 1). These AFLP data correlated highly with DNA/DNA homology groupings: strains that fell into the same DNA/DNA homology group also clustered closely with AFLP. Phylogenetic analysis of AFLP patterns revealed four major host-based clusters among the phytopathogenic strains: corn (with minimal internal linkage of 68%), rice (44%), cucurbits (58%), and orchids (68%). Strains from konjac clustered into a distinct group separately from the other phytopathogens at a similarity coefficient of less than 20%.

Phenotypic characteristics

Results with *A. avenae* (Table S-3) were in good agreement with the previous data of Willems et al. [55] and Schaad et al. [41]. Strains of *A. avenae* were easily distinguished from each other and from all other described species *A. konjaci, A. delafieldii*, *A. temperans*, *A. defluvii*, *A. anthurii*, *A. valerianellae*, *A. caeni*, and *A. facilis* (Table S-3).

Fatty acids

None of the phytopathogenic strains, except *A. valerianellae*, *A. anthurii* and *A. konjaci*, contained the 3-hydroxyoctanoic acid (8:0 3-OH). In contrast, fatty acid 8:0 3-OH was present in *A. facilis* (Table S-4) and the other non-phytopathogenic species [18].

16S rDNA and 16S–23S rDNA internal transcribed spacer (ITS) region sequencing

The phytopathogenic *A. avenae* strains from rice, tea, corn, watermelon, and orchid, were highly related by 16S rDNA sequencing forming a tight cluster with three lineages (Fig. 2). The percentage similarity values among the *A. avenae* strains ranged from 99.7% to 100% (1–5 nucleotides different). The strains from corn and rice differed by two nucleotides (nts). *A. konjaci, A. anthurii* and *A. valerianellae* were 98.1% (28 nts), 98.3% (25 nts), and 98.0% (30 nts) similar to *A. avenae* FC-320^T, respectively. All were clearly distinguishable from the non-phytopathogenic *A. temperans*, *A. facilis*, *A. defluvii*, *A. caeni*, and *A. delafieldii* (Fig. 2) with similarities of 96.7–98.4% (24–49 nts different). The newly described plant pathogenic *A. anthurii* formed a separate

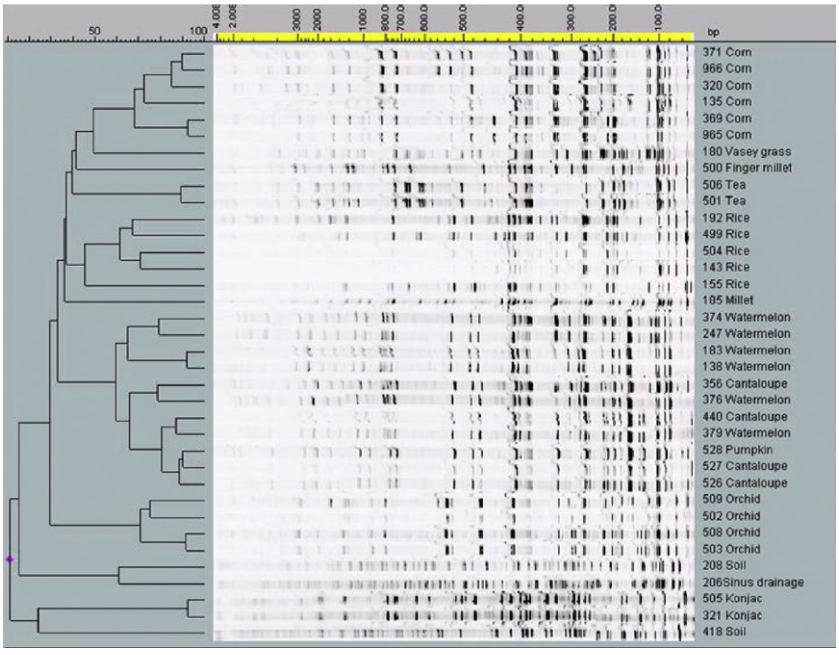

Fig. 1. AFLP patterns of genomic DNA for phytopathogenic *Acidovorax avenae* strains from corn, rice, finger millet, vasey grass, tea, orchid, watermelon, cantaloupe, and pumpkin, *A. konjaci* from konjac; and non-phytopathogenic strains of *A. facilis* (208), *Pseudomonas pseudoalcaligenes* (206), and *Comamonas testosteroni* (418). The dendrogram was constructed by using the unweighted pair group method with averages (UPGMA). Strain codes are the same as presented in Table S-1.

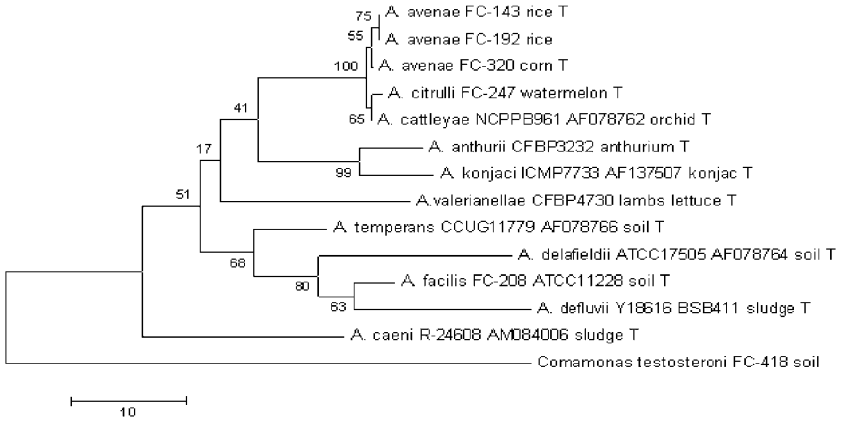

Fig. 2. 16S rDNA neighbor-joining consensus tree of strains of *Acidovorax avenae* subsp. *avenae* (*A. avenae*), *A. avenae* subsp. *cattleyae* (*A. cattleyae*), *A. avenae* subsp. *citrulli* (*A. citrulli*), *A. konjaci*, *A. anthurii*, *A. valerianellae*, *A. temperans*, *A. delafieldii*, *A. defluvii*, *A. caeni*, *Comamonas testosteroni*, and *A. facilis*. Distance between strains was measured as a number of nucleotide differences. Data for strains designated AF and Y were obtained from GenBank. Strains FC-143 and 192 are proposed to be named *A. oryzae*.

group more closely related to *A. konjaci*, whereas *A. valerianellae* was more distant (Fig. 2).

The ITS similarities among strains of *A. avenae* from corn, rice, cucurbits, and orchids ranged from 97.3 (orchid vs. cucurbit strains) to 99.0% (corn vs. rice strains) (Table 2) (16 and 6 nts), respectively. Tea strains, FC-501 and FC-506 and finger millet strain FC-500 shared 99.5% (3 nts) and 99.7% similarity (2 nts), respectively, with the type strain of *A. avenae* subsp. *avenae*. The other three plant pathogenic species, *A. konjaci*, *A. anthurii* and *A. valerianellae* shared similarities of only 81.8 (109 nts)–87.8% (73 nts) with the *A. avenae* strains from corn, rice, cucurbits, and orchids.

The following16S rDNA and ITS sequences were deposited in GenBank: 16S rDNA sequences included DQ360414 (FC-143, ATCC19882, rice), DQ360415 (FC-501, Supp86, tea) and DQ360416 (FC-500, Supp150, finger millet). ITS sequences included EU368726 (FC-320, corn), DQ360417 (FC-502, Supp 364, orchid), DQ360418 (FC-247, ATCC 29625, watermelon), DQ360420 (FC-143, rice), DQ360422 (FC-500, finger millet), DQ360423 (FC-501, tea), DQ360425 (FC-208; ATCC11228, soil).

Discussion

The results support the elevation of two subspecies of *Acidovorax* to species rank, and the naming of a new species. In this regard, the results agree with some of the groupings previously reported by Willems et al. [55], who observed two main DNA/DNA similarity groups at the species level within the phytopathogenic strains. Group one, related at a 54–100% similarity, contained strains of [*P.*] *avenae*, [*P.*] *rubrilineans*, "[*P.*] *setariae*", [*P.*] *cattleyae*, and [*P.*] *pseudoalcaligenes* subsp. *citrulli*. Within this group, three subgroups were delineated; subgroup one contained strains of [*P.*] *avenae*, [*P.*] *rubrilineans*, and "[*P.*] *setariae*" which were 75–100% similar; subgroup two contained strains of [*P.*] *cattleyae* which were 95% similar; and subgroup three contained strains of [*P.*] *pseudoalcaligenes* subsp. *citrulli*, which were 93–100% similar. Group two contained [*P.*] *konjaci*. Willems et al. [55] proposed combining the corn, sugarcane, and rice strains of group one into *Acidovorax* as a single subspecies, *A. avenae* subsp. *avenae*, based on a DNA/DNA similarity range of 74–100%. However, our DNA/DNA reassociation assays showed the corn and rice strains were not the same species, since they shared a mean reciprocal similarity of only 51% (range 43–68%). These DNA data agree with previous results showing differences between corn and rice strains in cell protein profiles [25,51] serology [10,25], and carbohydrate utilization [41]. Therefore, we agree that the corn and rice strains should be considered as separate species [50]. Although not cited by Willems et al. [55], rice strains had been shown earlier to differ phenotypically from [*P.*] *avenae* [16]. Goto and Ohata suggested the rice organism be called "*P. setariae* until more conclusive evidence will be established" [17]. Discrepancies between the DNA/DNA reassociation data of Willems et al. [55] and our findings are most likely due to differences in protocols used for DNA/DNA reassociation. The much less robust spectrophotometric method used by Willems et al. [55] is based on an initial renaturation rate technique [5], which does not allow reciprocal tests [6] and uses an equal amount of labeled and unlabeled DNA. Since no S1 nuclease is included, mismatched fragments and loops are not degraded and results often

Table 2. Summary of characters useful for differentiating between species of *Acidovorax*, *A. avenae* (Aa), *A. oryzae* (Ao), *A. citrulli* (Ac), and *A. cattleyae* (Aca)

Character	Species			
	Aa (6)[a]	Ao (6)	Ac (11)	Aca (4)
DNA/DNA relatedness to[b]				
A. avenae	81	53	39	27
A. oryzae	46	97	43	25
A. citrulli	47	43	88	25
A. cattleyae	35	45	39	98
ITS similarity to[c]				
A. avenae	100	99.0	98.0	98.3
A. oryzae	99.0	100	97.8	98.0
A. citrulli	98.0	97.8	100	97.3
A. cattleyae	98.3	98.0	97.3	100
Utilization of				
D-arabitol	−[d]	−	V^+	+
Sodium citrate	+	−	+	+
Maltose	+	−	−	−
D-fucose	V^+	+	$(V^+)^3$	−[2]
D-mannitol	+	+	−	+
Ethanol	−	+	+	+
Lipase production	+	+	−	+
Gelatin liquefaction	(+)	(+)	(+)	−
Nitrate reduction	+	+	−	+
Litmus milk	Alk	Alk	Alk (P)	Alk
Reaction to PCR[e]				
Aaaf5, Aaaf3/Aaar2	−	+	−	−
Aacf2/Aacr2	−	−	+	−
Hosts	Corn	Rice	Cucurbits	Orchid

[a]Number in parenthesis represents the number of strains tested.

[b]DNA/DNA reassociations were carried out at T_m-15; figures are a mean percentage.

[c]ITS, 16S–23S internally transcribed spacer region; figures are a percentage.

[d]+,80% or more of all strains positive; (+), delayed positive; −,80% or more of all strains negative; V^+, 50–79% positive; V^-, 19–49% negative.

[e]Based on the polymerase chain reaction (PCR) using the same strains for DNA/DNA reassociations.

lead to artificially higher DNA similarity values [21]. The S1 nuclease method used in our study has been shown to lower similarity values by as much as 15–20% [22,21] when compared to the spectrophotometric method. Also, Willems et al. [54] used a less stringent renaturation temperature of 82 °C, whereas we used 84 + 1 °C (71 + 1 °C in the presence of 22.7% formamide). This discrepancy in reassociation temperature occurred because of the different mol% G + C content used to determine the renaturation temperature. Willems et al. [55] used a mol% G + C content of 69.8% in determining their reassociation temperature for *A. avenae*, rather than the previously reported 72.2% [40]. Use of such a reduced temperature would result in a lower stringency and therefore a higher percentage similarity [5]. Schulze et al. [45] reported 70% and 77% similarity between *A. avenae* subsp. *avenae* and *cattleyae* and between *A. avenae* subsp. *avenae* and *citrulli*, respectively. In contrast, our results showed a mean similarity of 33% and 32%, respectively. They used a single strain and reported observing differences of up to 21%, and suggested the variation was due to differences in DNA quality. Also, they used a stringency of 65 °C whereas we used 71 °C.

Our AFLP results agreed with the DNA/DNA similarity results and clearly showed each of the four taxa including the corn and rice strains were easily differentiated from each other and from the non-phytopathogens, as well as *A. konjaci*. These results support the use of AFLP assays for identification of some phytopathogenic bacteria at the species level [30].

The 16S and ITS sequencing results clearly differentiated between *A. avenae* strains, as well as between *A. avenae* and all other species of *Acidovorax*. Our results agree with previous results showing most phytopathogenic *A. avenae* subsp. *avenae*, subsp. *citrulli*, and subsp. *cattleyae* strains could be differentiated easily from each other by phenotypic traits [4,15,20,40,41,55], cellular protein profiles [25], and serology [24]. *A. valerianellae* was the only pathogen that failed to grow at 41 °C [12, this study]. *A. anthurii* and *A. valerianellae* were easily separated from *A. avenae*. Unlike *A. avenae*, *A. anthurii* did not utilize L-tryptophan [11] and *A. valerianellae* failed to utilize D-sorbitol, adipate, ethanolamine, reduce nitrate, liquefy gelatin, or produce lipase [this study, 12]. Neither *A. anthurii* nor *A. valerianellae* utilized D-xylose, or produced alkaline from litmus milk, whereas *A. avenae* did (Table S-3). Both contained the fatty acid 3-hydroxyoctanoic acid (8:0 3-OH). All *A. avenae* species utilized D-glucose and adipate, whereas *A. caeni* did not. *A. avenae* did not contain the fatty acid 8:0 3-OH, whereas *A. caeni* did [18]. Strains of *A. delafieldii*, *A. temperans*, and *A. defluvii* were very different from *A. avenae* (Table S-3). *A. defluvii* failed to grow at 37 °C [45], did not utilize D-glucose, D-sorbitol or ethanolamine, and nor did it liquefy gelatin (Table S-3), but it contained the fatty acid 8:0 3-OH [18,45]. Our results do not agree that all strains of recognized *Acidovorax* species contain the fatty acid 8:03-OH [18] unless one places the plant species into another genera.

These genetic (16S, ITS sequencing, DNA/DNA reassociation assays, AFLP analysis) and phenotypic data, including fatty acid profiles, support an emendation of the species *A. avenae*. Since *A. avenae* Group A corn strains and Group B rice strains have less than 70% DNA/DNA similarity, differ serologically [24], have distinct protein patterns [25], and can be differentiated phenotypically, we propose a new species, *A. oryzae*, for Group B strains from rice. We propose Group C strains from cucurbits and Group D strains from orchids be elevated to species rank as *A. citrulli* and *A. cattleyae*,

respectively. Although strains from tea, vasey grass, finger millet, and millet shared a moderate DNA/DNA similarity of 54% to 68% with *A. avenae*, suggesting separate species, we would suggest that these strains be classified as *Acidovorax* spp. until additional strains are tested.

Summary of characters

Table 2 summarizes some of the most important characters for distinguishing between *Acidovorax avenae*, *A. oryzae*, *A. citrulli*, and *A. cattleyae*.

Protologues

Abbreviations for culture collections and depositories of type strains are:

ATCC = American Type Culture Collection, Manassas, VA, USA;
CFBP = Collection Francaise de Bacteries Phytopathogenes, Angers, France;
ICMP = International Collection of Microorganisms from Plants, Auckland, New Zealand;
ICPB = International Collection of Phytopathogenic Bacteria, USDA, Ft. Detrick, MD;
LMG = Laboratorium Microbiologie Gent, Belgium;
NCPPB = National Collection of Plant Pathogenic Bacteria, England;
IBSBF = Instituto Biologico, Seçao de Bacteriologia Fitopatologica, Campinas, SP, Brazil.

Acidovorax avenae (Manns 1909) Willems et al., 1992

Acidovorax avenae (a.ve'na. e. N.L. gen. n. *avenae*, of *Avena*, a genus of oat plants).

Basonym: *Pseudomonas avenae* Manns 1909 (Approved Lists 1980).

Other homotypic synonyms: *Pseudomonas avenae* subsp. *avenae* Manns 1909, *Acidovorax avenae* subsp. *avenae* (Manns 1909) Willems et al., 1992. Another heterotypic synonym: *Pseudomons rubrilineans* (Lee et al., 1925) Strap 1928.

The species is pathogenic to corn and oats, whereas pathogenicity to wheat is variable.

Acidovorax avenae can be distinguished from *A. oryzae, A. citrulli*, and *A. cattleyae* by DNA/DNA reassociation assays (Table 1), PCR, AFLP analysis (Fig. 1), and phenotypic traits (Tables 2 and S-3) [this paper, 15,20,40,54,55]. Nitrate is reduced and an alkaline reaction is produced in litmus milk. Arginine is not dihydrolyzed. Gelatin is weakly liquefied. *A. avenae* utilizes D-glucose, D-xylose, D-mannitol, adipate, D-frucose, D-mannose, citraconate, D-sorbitol, maltose [this paper], β-alanine [41], L-leucine [38], L-arabinose, D-galactose, isobutyrate, isovalerate, pimelate, L-threonine, L-histidine, L-tryptophan, 2-ketoglutarate, and malonate [55]. D-arabitol and ethanol are not utilized (Table S-3). Starch hydrolysis is negative and ammonia is not produced. [40]. *Acidovorax avenae* utilizes sodium citrate and maltose, whereas *A. oryzae* does not (Tables 2 and S-3).

Type strain: FC-320^T = ICPB 30071 (PA117)^T = ATCC 19860^T = NCPPB 1011^T = LMG 2117^T = ICMP 3183^T = CFBP 2425^T = IBSBF 193^T.

Acidovorax oryzae sp. nov.

Acidovorax oryzae [o.ry'za. e. L. gen. n. *oryzae*, of rice, of *Oryza* (a genus of rice plants)].

The species infects rice. *Acidovorax oryzae* can be distinguished from *A. avenae, A. citrulli*, and *A. cattleyae* by DNA/DNA reassociation assays (Table 1), AFLP analysis (Fig. 1), and several phenotypic traits (Tables 2 and S-3) [14,16]. Gelatin is weakly liquefied. D-mannose and D-fructose are utilized by *A. oryzae*, whereas utilization by *A. avenae* and *A. citrulli* is variable. *Acidovorax oryzae* utilizes ethanol, whereas *A. avenae* does not. *A. oryzae* does not utilize sodium citrate or maltose, whereas *A. avenae* does. *Acidovorax oryzae* utilizes D-mannitol, reduces nitrate and hydrolyzes lipid, whereas *A. citrulli* does not. *A. oryzae* does not utilize D-arabitol, whereas *A. cattleyae* does. *Acidovorax oryzae* utilizes D-fucose whereas *A. avenae* and *A. citrulli* are variable, and *A. cattleyae* is negative. Arginine is not dihydrolyzed. All strains react with PCR primers Aaaf5 or Aaaf3/Aaar2, whereas *A. avenae, A. citrulli,* and *A. cattleyae* do not (Table 2) [47].

Type strain: FC-143^T = ICPB 30003 (PS 177)^T = ATCC 19882^T = NCPPB 1392^T = ICMP 3960^T.

Acidovorax citrulli (Schaad et al. 1978) comb. nov.

Acidovorax citrulli (ci.trul'li. N.L. gen. n. *citrulli*, of *Citrullus*, a genus of melon plants).

Basonym: *Pseudomonas pseudoalcaligenes* subsp. *citrulli* Schaad et al. 1978 (Approved Lists 1980).

Other homotypic synonyms: *Pseudomonas avenae* subsp. *citrulli* (Schaad et al. 1978) Hu et al. 1991, *Acidovorax avenae* subsp. *citrulli* (Schaad et al. 1978) Willems et al. 1992.

The bacterium infects many plants in the *Cucurbitaceae*. *Acidovorax citrulli* can be distinguished from *A. avenae, A. oryzae*, and *A. cattleyae* by DNA/DNA reassociation assays (Table 1), AFLP analysis (Fig. 1), and several phenotypic traits (Tables 2 and S-3) [19,41,55]. Gelatin is weakly liquefied and delayed (Tables 2 and S-3) [41]. The bacterium does not reduce nitrate, or produce lipase, whereas *A. avenae* and

A. oryzae do. Alkali is produced in litmus milk and a precipitate forms (Tables 2 and S-3); no precipitate is produced by *A. avenae, A. oryzae*, or *A. cattleyae. Acidovorax citrulli* does not utilize D-mannitol whereas *A. oryzae, A. avenae* and *A. cattleyae* do (Tables 2 and S-3). The organism utilizes sodium citrate, ethanol, ethanolamine, citraconate, adipate, and D-sorbitol (Tables 2 and S-3), β-alanine, L-arabinose, D-galactose, D-ribose, DL-tartrate [15]; 2-ketoglutrate [55] but not L-histidine, and L-threonine [55]. Arginine is not dihydrolyzed. *Acidovorax citrulli* reacts with PCR primers Aacf2/Aacr2, whereas *A. avenae, A. oryzae,* and *A. cattleyae* do not (Table 2) [46].

Type strain: FC-247^T = ICPB 30064^T = ATCC 29625^T = ICMP 7500^T = IBSBF 1851^T.

Acidovorax cattleyae (Pavarino 1911) comb. nov.

Acidovorax cattleyae (cat.tle'ya. e. N.L. gen. n. *cattleyae*, of *Cattleya*, a genus of orchid plants).

Basonym: *Pseudomonas cattleyae* (Pavarino 1911) Savulescu 1947 (Approved Lists 1980).

Other homotypic synonym: *Acidovorax avenae* subsp. *cattleyae* (Pavarino 1911) Willems et al. 1992.

The organism naturally infects *Cattleya, Dendrobium, Phalaenopsis*, and their hybrids. *Acidovorax cattleyae* can be distinguished from *A. avenae*, *A. citrulli*, and *A. oryzae* by DNA/DNA reassociation assays (Table 1), AFLP analysis (Fig. 1), and several phenotypic traits (Tables 2 and S-3) [1,20,55]. Nitrate is reduced. *A. cattleyae* utilizes D-glucose, D-xylose, D-mannitol, D-sorbitol, sodium citrate, adipate, D-mannose, citraconate, D-arabitol, ethanolamine, ethanol (Tables 2 and S-3), L-arabinose, dulcitol, galactose, glycerol, lactose, sucrose [1] and does not utilize D-fucose. The bacterium utilizes sodium citrate, whereas *A. oryzae* does not, and D-mannitol, whereas *A. citrulli* does not (Table 2). *A. cattleyae* utilizes D-arabitol, whereas *A. avenae* and *A. oryzae* do not. *A. cattleyae* does not utilize maltose, whereas *A. avenae* does. It does not liquefy gelatin, whereas *A. avenae, A. oryzae,* and *A. citrulli* do.

Type strain: FC-507^T = ICPB 30134 (PC21)^T = ATCC 33619^T = NCPPB 961^T = LMG 2364^T = ICMP 2826^T = CFBP 2423^T = IBSBF 209^T.

All strains are available in the International Collection of Phytobacteriology (ICPB) maintained at the USDA ARS, FDWSRU, Fort Detrick, MD (N.W. Schaad and A. Sechler, Curators).

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.syapm.2008.09.003.

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