Swaminathania salitolerans gen. nov., sp. nov., a salt-tolerant, nitrogen-fixing and phosphate-solubilizing bacterium from wild rice (*Porteresia coarctata* Tateoka)

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A novel species, *Swaminathania salitolerans* gen. nov., sp. nov., was isolated from the rhizosphere, roots and stems of salt-tolerant, mangrove-associated wild rice (*Porteresia coarctata* Tateoka) using nitrogen-free, semi-solid LGI medium at pH 5·5. Strains were Gram-negative, rod-shaped and motile with peritrichous flagella. The strains grew well in the presence of 0·35 % acetic acid, 3 % NaCl and 1 % KNO_3, and produced acid from L-arabinose, D-glucose, glycerol, ethanol, D-mannose, D-galactose and sorbitol. They oxidized ethanol and grew well on mannitol and glutamate agar. The fatty acids 18 : 1ω7*c*/ω9*t*/ω12*t* and 19 : 0cyclo ω8*c* constituted 30·41 and 11·80 % total fatty acids, respectively, whereas 13 : 1 AT 12–13 was found at 0·53 %. DNA G+C content was 57·6–59·9 mol% and the major quinone was Q-10. Phylogenetic analysis based on 16S rRNA gene sequences showed that these strains were related to the genera *Acidomonas*, *Asaia*, *Acetobacter*, *Gluconacetobacter*, *Gluconobacter* and *Kozakia* in the *Acetobacteraceae*. Isolates were able to fix nitrogen and solubilized phosphate in the presence of NaCl. Based on overall analysis of the tests and comparison with the characteristics of members of the *Acetobacteraceae*, a novel genus and species is proposed for these isolates, *Swaminathania salitolerans* gen. nov., sp. nov. The type strain is PA51^T (=LMG 21291^T=MTCC 3852^T).

Screening for acetic acid bacteria from wild rice sources resulted in isolation of a number of strains, which were tentatively identified as *Gluconacetobacter*, but closer examination and comparison with other strains in the family revealed that they belonged to a novel taxon. This paper describes the phenotypic, biochemical and genotypic characterizations of these novel strains, which were isolated from mangrove-associated wild rice in Pichavaram, Tamil Nadu, India. Based on comparative analysis with other related genera/species (Table 1), a novel species in a new genus within the family *Acetobacteraceae*, *Swaminathania salitolerans* gen. nov., sp. nov., is proposed.

Samples of mangrove-associated wild rice (*Porteresia coarctata* Tateoka) were collected from three different sites along coastal Tamil Nadu. Root, stem and leaf samples were washed in sterile water and surface-sterilized with sodium hypochlorite (4 %) for 5 min, washed several times using sterile water and macerated in a blender. The supernatants were serially diluted and aliquots of 100 µl from stem, root or leaf macerates were inoculated into 30 ml test tubes containing 10 ml N-free, semi-solid LGI medium (Cavalcante & Dobereiner, 1988) supplemented with 250 mM NaCl. Vials were also inoculated with 100 µl serially diluted rhizosphere soil and non-rhizosphere soil suspensions. These were incubated at 30 °C for 5 days. Acid-producing and nitrogenase-positive vials with a yellow surface pellicle were streaked onto LGI agar plates and incubated at 30 °C. Pure cultures were obtained from individual colonies. In total, 41 strains were isolated and, after biochemical characterization, two isolates (PA12 and PA51^T) were selected for further study.

The genomic DNA was extracted from isolates as described by Ausubel *et al.* (1987). A large fragment of the 16S rRNA gene was amplified using primers fD1 [5′-AGAGTTTG-ATCCTGGCTCAG-3′; positions 7–26 in *Escherichia coli* (Brosius *et al.*, 1981)] and rP2 (5′-ACGGCTACCTTGTTA-CGACTT-3′; positions 1513–1494) as described previously (Weisburg *et al.*, 1991; Loganathan, 2002). Amplification products were separated on a 1·5 % agarose gel in 1 × TBE buffer. Products were purified using a Sephaglas BandPrep

The GenBank accession numbers for the 16S rRNA gene sequences of strains PA51^T and PA12 are AF459454 and AF459455, respectively.

The fatty acid composition of isolates and type strains is available as supplementary material in IJSEM Online.

Table 1. Bacterial strains used in this study

Abbreviations: LMG, Laboratorium voor Microbiologie, Universiteit Gent (RUG); NRIC, Nodai Research Institute Culture Collection, Tokyo University of Agriculture, Japan; CFN, cataloguing number of Centro de Investigacion sobre Fijacion de Nitrogeno, Mexico.

Strain	Source of isolate
S. salitolerans PA12	*P. coarctata* rhizosphere (this study)
S. salitolerans PA51^T	*P. coarctata* root (this study)
Gluconacetobacter diazotrophicus LMG 7603^T	LMG
Gluconacetobacter azotocaptans CFN-Ca54^T	Coffee (obtained from J. Caballero-Mellado*)
Gluconacetobacter johannae CFN-Cf55^T	Coffee (obtained from J. Caballero-Mellado*)
Gluconacetobacter sacchari LMG 19747^T	LMG
Gluconacetobacter liquefaciens LMG 1382^T	LMG
Asaia bogorensis NRIC 0311^T	NRIC
Asaia siamensis NRIC 0323^T	NRIC
Acetobacter aceti LMG 1504^T	LMG
Acidomonas methanolica LMG 1668^T	LMG
K. baliensis NRIC 0488^T	NRIC

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kit (Amersham Pharmacia Biotech) and the fragment was cloned using a Fermentas InsT/A clone PCR Product cloning kit according to the manufacturer's instructions. Clones were sequenced using the ABI PRISM Dye Terminator Ready Reaction kit (Applied Biosystems) according to the manufacturer's instructions. For sequencing, the following primers were used: M13 forward and reverse, PC513F (5′-CCCGGCTACTTCGTGC-3′; positions 513–518), PC513R (5′-GCACGAAGTAGCCGGG-3′; positions 518–513), PC970F (5′-CGCGCAGAACCTTACCAG-3′; positions 970–987) and PC970R (5′-CTGGTAAGGTTCTGCGCG-3′; positions 987–970). The BLAST algorithm (Altschul *et al.*, 1997) was used to search nucleotide databases for similar sequences. Gene sequences were manually aligned with each other and multiple alignments of the sequences were carried out with the program CLUSTAL_W version 1.6 (Thompson *et al.*, 1994). Distance matrices for the aligned sequences were determined using the two-parameter method of Kimura (1980). The neighbour-joining method was used to construct a phylogenetic tree (Saitou & Nei, 1987). The sequence data obtained were compared using 1450 bases. The robustness of individual branches was estimated by bootstrapping with 1000 replicates (Felsenstein, 1985).

A phylogenetic tree (Fig. 1) was constructed using 20 strains: including isolates PA12 and PA51^T, and the type strains of *Acetobacter aceti*, *Acidomonas methanolica*, *Asaia siamensis*, *Asaia bogorensis*, *Gluconacetobacter johannae*, *Gluconacetobacter azotocaptans*, *Gluconacetobacter diazotrophicus*, *Gluconacetobacter sacchari*, *Gluconacetobacter liquefaciens*, *Gluconacetobacter intermedius*, *Gluconacetobacter oboediens*, *Gluconacetobacter europaeus*, *Gluconacetobacter xylinus*, *Gluconobacter frateurii*, *Gluconobacter cerinus*, *Gluconobacter oxydans*, *Kozakia baliensis* and *Rhodospirillum rubrum*. Isolates PA12 and PA51^T constituted a separate branch within the lineage including species of the genus *Asaia* and distinct from the lineage containing species of the genera *Acetobacter*, *Acidomonas*, *Gluconobacter*, *Gluconacetobacter* and *Kozakia*. 16S rDNA sequences of isolates PA12 and PA51^T were 99·9 % similar to each other. The sequence similarities of isolate PA51^T with the type strains of *Gluconobacter frateurii*, *Acidomonas methanolica*, *Acetobacter aceti*, *Gluconacetobacter xylinus*, *Gluconacetobacter johannae*, *Gluconacetobacter sacchari*, *K. baliensis* and *Asaia siamensis* were 92·9, 93·2, 93·8, 94·1, 94·6, 95·1, 96·5 and 98·6 %, respectively.

Genomic DNA samples were analysed for their G+C content by HPLC (Shimadzu SPE 10A, 10AD) as described by Ezaki *et al.* (1990) and Xu *et al.* (2000). DNA relatedness was assessed based on relative levels of reassociation to ^{32}P-labelled total DNA, using the *red*iprime DNA-labelling kit (Amersham). Labelled DNA in independent experiments was from isolates and type strains. DNA–DNA reassociation was carried out for 16 h at 65 °C and the nylon filters were washed once in 1× SSC at room temperature for 15 min and once in 1× SSC for 5 min at 65 °C. Autoradiography was performed for 2 h, filter lanes were cut out and the radioactivity was estimated with a Beckman multi-purpose scintillation counter (LS 6500). The percentage reassociation was calculated for each strain tested in relation to the homologous control (Fuentes-Ramirez *et al.*, 2001). Ubiquinone homologues were analysed quantitatively by HPLC with a C18 column. Standard preparations of Q-10 and Q-9 were prepared from cells of *Gluconacetobacter diazotrophicus* LMG 7603^T and *Acetobacter aceti* LMG 1504^T, respectively (Franke *et al.*, 1999; Lisdiyanti *et al.*, 2002).

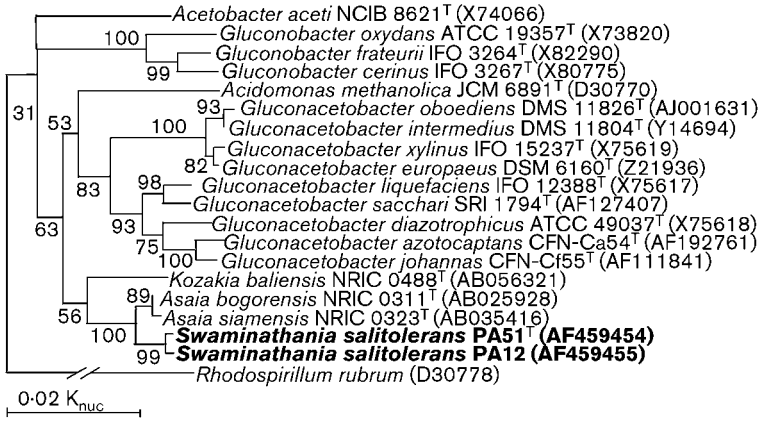

Fig. 1. Phylogenetic relationship between isolates PA12, PA51^T and other species of the family *Acetobacteraceae* determined from 16S rRNA gene sequence similarities.

Isolates PA12 and PA51^T had a G+C content of 57·6–59·9 mol%, which was lower than that of the type strains of *Asaia bogorensis* and *Asaia siamensis* (59–61 mol%) and higher than that of *K. baliensis* (56–57 mol%) (Lisdiyanti *et al.*, 2002). The isolates had high DNA–DNA similarity values (84–100 % between strains PA12 and PA51^T) indicating that they belonged to the same species. Low similarity values (12–30 %) were observed with the type strains of *Asaia bogorensis*, *Asaia siamensis* and *K. baliensis*. The major quinone of the two isolates was Q-10.

The type of flagellation and the cell dimensions of strains PA12 and PA51^T were determined using cells negatively stained with 2 % (v/v) aqueous uranyl acetate at pH 3·5 by TEM (model JEM 1200 EX11 JEO2). Five replications were used for each characterization in this study. Colony morphology was examined on LGI medium (Cavalcante & Dobereiner, 1988). Bacterial motility was tested by growth in semi-solid medium 0·3 % WL nutrient agar. Oxidase and catalase tests were determined using commercially available discs (Himedia). The production of water-soluble brown pigment was determined using GYC agar medium as described by Swings *et al.* (1992) and overoxidation of glucose and ethanol, lactate and acetate was conducted as described by De Ley *et al.* (1984) and Asai *et al.* (1964). Tolerance to NaCl was also tested in SM medium (De Ley & Swings, 1984). Pigmentation, growth on glutamate agar, ketogenic activity and acid production were tested using the methods of Asai *et al.* (1964).

Cellular fatty acid composition of strains PA12 and PA51^T, and the type strains of *Asaia bogorensis*, *Asaia siamensis* and *K. baliensis* were estimated in cells grown on trypticase soy agar (without NaCl) for 48 h at 30 °C. Methyl esters of cellular fatty acids were prepared and identified following the instructions of the Microbial Identification system (MIDI; Hewlett Packard).

A summary of biochemical and physiological characteristics of strains PA12 and PA51^T is presented in Table 2. Water-soluble brown pigment was produced on D-glucose- and $CaCO_3$-containing agar plates. Catalase was positive and oxidase was negative. Brown pigmentation was observed on yeast extract-, D-glucose- and $CaCO_3$-containing medium. The strains did not produce gelatinase. Acetate and lactate were oxidized to carbon dioxide and water, but the activity was weak. The isolates produced acetic acid from ethanol and grew in the presence of 0·35 % (v/v) acetic acid at pH 3·5 and 3 % NaCl using 1 % KNO_3 as a nitrogen source. They also grew on mannitol and glutamate agar and did not utilize methanol as a sole source of carbon on Hoyer–Frateur medium. Acid was produced from L-arabinose, D-glucose, D-galactose, D-mannose, glycerol, sorbitol and ethanol, but not from L-rhamnose or D-mannitol.

The predominant fatty acid found in all the acetic acid bacteria tested, including the isolates and type strains, was the straight-chain unsaturated 18 : 1ω7*c*/ω9*t*/ω12*t*, which accounted for 30·41 % total fatty acid content in PA51^T, compared with 43·76 % in *Asaia bogorensis*, 54·29 % in *Asaia siamensis* and 37·00 % in *K. baliensis*. Fatty acids common to these species included 14 : 0, 14 : 0 2-OH, 15 : 0, 16 : 0, 16 : 0 2-OH, 16 : 0 3-OH, 17 : 0, 18 : 0, 19 : 0cyclo ω8*c* and 20 : 3ω6,9,12*c*. However, strain PA51^T contained 11·8 % 19 : 0cyclo ω8*c* compared with $<$1 % in *Asaia bogorensis* and *Asaia siamensis*, and 5·6 % in *K. baliensis*. The fatty acid composition of isolates and type strains is available as supplementary material in IJSEM Online.

The acetylene reduction assay (ARA) was used to test the isolates grown on semi-solid LGI medium (Cavalcante & Dobereiner, 1988) for potential nitrogen fixation. The amount of ethylene produced was measured using 10 % (v/v) acetylene according to the method of Li & MacRae (1992) using a Hewlett Packard 4890 GC equipped with a Poropack N column. Strains PA51^T and PA12 were able to reduce acetylene to ethylene. The strains were subjected to a *nifD*-specific PCR amplification using the primers of Ueda *et al.* (1995) and the expected 450 bp amplification product was observed in both isolates. The ARA and *nifD* amplification results confirmed that the isolates were nitrogen fixers.

Isolates PA12 and PA51^T were also tested for their mineral phosphate solubilization activity. Individual strains were grown in LGI medium and 10 µl grown cells were spotted

Table 2. Characteristics differentiating *S. salitolerans* gen. nov., sp nov. from other members of the family *Acetobacteraceae*

Genera: 1, *Swaminathania*; 2, *Asaia*; 3, *Kozakia*; 4, *Acetobacter*; 5, *Gluconacetobacter*; 6, *Gluconobacter*; 7, *Acidomonas*. With the exception of *Swaminathania*, data are consolidated from our experiments and from Lisdiyanti *et al.* (2002) and De Ley & Swings (1984). +, Positive; −, negative; V, positive or negative; W, weak.

Characteristic	1	2	3	4	5	6	7
Pigmentation	+	V	−	−	−	+	−
Production of water-soluble brown pigments	+	−	−	−	V	V	−
Overoxidation of:							
Ethanol	+	−	+	+	+	+	+
Acetate	W	W	W	+	+	−	+
Lactate	W	W	W	+	+	−	−
Growth on:							
Mannitol agar	+	+	+	V	V	+	−
Glutamate agar	+	+	−	V	V	−	−
Methanol	−	−	−	−*	−	−	+
Production of acetic acid from ethanol	+	−	+	+	+	+	+
Growth in the presence of:							
0·35 % acetic acid	+	−	+	+	+	+	+
3 % NaCl	+	−	−	−	−	−	−
1 % KNO_3	+	−	−	−	−	−	+
Ketogenesis from glycerol	+	V	+	V	V	+	−
Acid production from:							
L-Arabinose	+	+	+	V	V	+	−
D-Mannitol	−	V	−	−	V	+	−
D-Glucose	+	+	+	V	+	+	+
Dulcitol	V	+	−	−	−	+	−
Fructose	V	+	−	−	+	+	−
Glycerol	+	+	+	−	+	+	−
Rhamnose	−	V	−	−	−	−	−
D-Mannose	+	+	+	V	V	+	−
D-Xylose	V	+	+	V	V	+	−
Ethanol	+	−	+	+	+	+	+
D-Galactose	+	+	+	+	+	+	−
Sorbitol	+	V	−	+	+	+	−
Major ubiquinone	Q-10	Q-10	Q-10	Q-9	Q-10	Q-10	Q-10
G+C content (mol%)	57·6–59·9	59–61	56–57	52–60	55–66	52–60	63–66

**Acetobacter pomorum* was reported to assimilate methanol weakly (Sokollek *et al.*, 1998).

onto Pikovskaya's medium (Pikovskaya, 1948) and the zonal clearing generated was measured. Isolates PA12 and PA51^T showed clearing zones on the medium, indicating that these strains were capable of solubilizing the tri-calcium phosphates contained in the medium.

The polyphasic study of acetic acid bacteria isolated from *Porteresia coarctata* has revealed the following characteristics. Cells of all isolates were Gram-negative, rod-shaped, approximately 0·7–0·9 × 1·9–3·1 μm and motile with peritrichous flagella. Strains were aerobic and able to fix atmospheric nitrogen micro-aerophilically. Colonies were initially yellowish, becoming dark orange later on, smooth and raised with an entire margin on LGI medium. The isolates were capable of acid formation from glucose and overoxidation of ethanol, produced water-soluble brown pigments in GYC medium, and were oxidase-negative and catalase-positive. It is well known that Gram-negative, rod-shaped, aerobic bacteria that oxidize ethanol to acetic acid in neutral and acidic media are candidates for members of the family *Acetobacteraceae* (Swings *et al.*, 1992). Additionally, the family *Acetobacteraceae* can be distinguished from other α-*Proteobacteria* by two internal *Sph*I sites and one *Nco*I restriction site in their 16S rDNA genes (Jimenez-Salgado *et al.*, 1997). Analysis of the 16S rDNA nucleotide sequence of the majority of the acetic acid bacterial strains reported in GenBank revealed that only a few strains lack the *Nco*I restriction site (nt 110) (Jimenez-Salgado *et al.*, 1997). These restriction sites are also present in isolates PA12 and PA51^T, thereby distinguishing them from the

other α-*Proteobacteria*. The BLAST search of rDNA sequences also showed that the isolates had a high similarity to members of the family *Acetobacteraceae*. On the basis of the phenotypic and biochemical characteristics described and restriction sites in the 16S rDNA, it can be concluded that the nitrogen-fixing, salt-tolerant strains belong to the family *Acetobacteraceae*.

Members of the family *Acetobacteraceae* are recognized for their unique ability to oxidize ethanol to acetic acid in neutral and acidic media (Swings, 1992). This family consists of the genera *Acetobacter*, *Asaia*, *Gluconobacter*, *Gluconacetobacter*, *Acidomonas* and the recently described *Kozakia* (Yamada *et al.*, 2000, Lisdiyanti *et al.*, 2002). The isolates described in this study could be distinguished from the six genera of acetic acid bacteria at the generic level. The representative isolates showed 16S rDNA similarity to *Asaia* (98·6 %) followed by *Kozakia* (96·5 %). Clustering on the basis of the neighbour-joining algorithm showed that isolates PA12 and PA51^T formed a sublineage with type genus *Asaia* at a similarity level of 98·6 %. However, based on biochemical characteristics, these isolates differed from the type strains of the genus *Asaia* in production of acetic acid from ethanol, growth in the presence of 0·35 % acetic acid at pH 3·5, acid production from ethanol and growth in the presence of 3 % NaCl. The fatty acid 19 : 0cyclo $\omega 8c$ comprised 11·80 % total fatty acids in isolate PA51^T, compared to 0·62–0·74 % in the genus *Asaia*. Furthermore, the fatty acids 10 : 0, 10 : 0 3-OH, 12 : 0, 17 : 1$\omega 6c$, 19 : 0 10-methyl, 20 : 0, 20 : 3$\omega 6,9,12c$,15, 20 : 4$\omega 6,9,12,15c$ and summed feature 4 (16 : 1$\omega 7c$/15 iso 2-OH) were not found in PA51^T, whereas they were present in *Asaia bogorensis*, and 13 : 1 AT 12–13 was present in PA51^T, but not in *Asaia bogorensis*. All these data indicate that the isolates can be distinguished from members of the genus *Asaia*.

The isolates could be differentiated from the genus *Acetobacter* on the basis of the quinone system, as the genus *Acetobacter* has Q-9 and the isolates and the rest of the genus have Q-10 (Yamada *et al.*, 1997). Although the isolates oxidized acetate and lactate to carbon dioxide and water like *Gluconacetobacter* and *Acetobacter*, they differed phylogenetically from these genera and from the genus *Gluconobacter*, and were most closely related to the genus *Asaia*. The isolates also differed from the genus *Acidomonas*, since they did not utilize methanol as a sole source of carbon.

The isolates were able to grow well in the presence of 1 % KNO_3. These data indicate that the isolates may be distinguished biochemically from the genera *Asaia*, *Acetobacter*, *Gluconobacter*, *Gluconacetobacter* and *Kozakia*. Interestingly, the isolates were able to grow well in the presence of 3 % NaCl in SM medium, which differentiates them from the other genera. Based on the above description, it is proposed that the isolates belong to a new genus and novel species for which the name *Swaminathania salitolerans* gen. nov., sp. nov. is proposed.

Description of *Swaminathania* gen. nov.

Swaminathania (swa.mi.na.tha′ni.a. N.L. fem. n. *Swaminathania* after Swaminathan, Indian biologist, the father of the Green Revolution in India).

Gram-negative, straight rods with round ends, approximately 0·7–0·9 × 1·9–3·1 μm, possesses peritrichous flagella, oxidase-negative and catalase-positive. Capable of oxidizing ethanol to acetic acid in neutral and acid conditions, glucose to acetic acid, and oxidized acetate and lactate to CO_2 and water. Able to produce water-soluble brown pigments on GYC agar medium. Does not hydrolyse gelatin and starch. Grows well in the presence of 0·35 % acetic acid, 3 % NaCl and 1 % KNO_3. Produces acid from L-arabinose, D-glucose, glycerol, ethanol, D-mannose, D-galactose and sorbitol. Contains 18 : 1$\omega 7c$/$\omega 9t$/$\omega 12t$ (30·41 %), 13 : 1 AT 12–13 (0·53 %) and 19 : 0cyclo $\omega 8c$ (11·84 %). Able to fix nitrogen and solubilize phosphate. DNA G+C content is 57·6–59·9 mol% and the major quinone is Q-10. The type species is *Swaminathania salitolerans*.

Description of *Swaminathania salitolerans* sp. nov.

Swaminathania salitolerans (sa.li.to′le.rans. L. n. *sal* salt; L. part. adj. *tolerans* tolerating; N.L. part. adj. *salitolerans* salt tolerating).

Characteristics are the same as those described for the genus. The type strain is PA51^T (=LMG 21291^T=MTCC 3852^T), isolated from mangrove-associated wild rice in Pichavaram, Tamil Nadu, India.

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