Curtobacterium ammoniigenes sp. nov., an ammonia-producing bacterium isolated from plants inhabiting acidic swamps in actual acid sulfate soil areas of Vietnam

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The ammonia-producing bacteria B55^T, CA73, SA69 and SA72 were isolated from the waterweeds *Ludwigia adscendens* (B55^T) and *Eleocharis dulcis* (CA73, SA69 and SA72) grown in highly acidic swamps (pH 2–4) in actual acid sulfate soil areas of Vietnam. The isolates were Gram-positive, irregular rod-shaped, non-spore-forming bacteria. On the basis of 16S rRNA gene sequence similarity, strain B55^T was shown to belong to the genus *Curtobacterium* of the class *Actinobacteria*. Chemotaxonomic data (MK-9 as major isoprenoid quinone, D-ornithine as cell-wall diamino acid, acetyl as the acyl type of peptidoglycan) supported the affiliation of all four strains to this genus. Although their 16S rRNA gene sequence similarity was 99 % to species with validly published names within the genus, they formed a group that was distinct in the phylogenetic tree, and DNA–DNA relatedness values to these established species were less than 10 %. The results of physiological and biochemical tests and major fatty acids (cyclohexyl-C_{17:0}, anteiso-C_{17:0} and cyclohexyl-C_{19:0}) allowed phenotypic differentiation of these strains from the species of *Curtobacterium* with validly published names. Therefore, strains B55^T, CA73, SA69 and SA72 represent a novel species, for which the name *Curtobacterium ammoniigenes* sp. nov. is proposed. The type strain is B55^T (=NBRC 101786^T = VTCC D6-11^T = JCM 14609^T).

Abbreviation: AASS, actual acid sulfate soils.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequences of strains B55^T, CA73, SA69 and SA72 are AB266597–AB266600, respectively.

A scanning electron micrograph of cells of strain B55^T and details of the fatty acid compositions of the novel strains and related type strains are available as supplementary material with the online version of this paper.

The genus *Curtobacterium* was first described by Yamada & Komagata (1972) and, at present, the genus comprises six recognized species, isolated from rice (*Curtobacterium citreum*, the type species; Komagata & Iizuka, 1964; Yamada & Komagata, 1972), common bean (*Curtobacterium flaccumfaciens* pv. *flaccumfaciens*; Collins & Jones, 1983), the litter layer (*Curtobacterium herbarum*; Behrendt *et al.*, 2002), Chinese rice paddies [*Curtobacterium luteum* (Komagata & Iizuka, 1964; Yamada & Komagata, 1972) and *Curtobacterium albidum* (Komagata & Iizuka, 1964;

Yamada & Komagata, 1972)] and oil brines (*Curtobacterium pusillum*; Iizuka & Komagata, 1965; Yamada & Komagata, 1972). The status of a seventh species, *Curtobacterium plantarum*, as a member of the genus is debatable. During the course of a study to develop bioremediation measures for actual acid sulfate soils (AASS), we isolated a number of ammonia-producing bacteria from plants adapted to AASS in South-east Asia. In this study, we characterized four ammonia-producing bacterial strains isolated from the waterweeds *Ludwigia adscendens* and *Eleocharis dulcis* in highly acidic swamps (pH 2–4) at AASS areas of Can Tho in Vietnam. By phenotypic, genotypic, chemotaxonomic and phylogenetic analyses, the strains have been affiliated to the genus *Curtobacterium*. The data obtained also suggest that the isolates represent a novel species.

Strains were grown on one-tenth-strength tryptic soy (1/10 TS) agar plates [3.0 g tryptic soy broth l⁻¹ solidified with 15.0 g agar l⁻¹ (Difco), pH 4.1] at 28 °C under aerobic conditions followed by selection on the basis of neutralization of 1/10 TS liquid medium (pH 4) determined by measuring the pH of culture supernatants after a 3 day cultivation period. Strain B55^T was obtained from a stem of *L. adscendens*, and strains CA73, SA69 and SA72 were isolated from stems of *E. dulcis*. All strains showed growth between pH 3.5 and 8.0, with an optimum at pH 4–5. On 1/10 TS liquid medium (pH 4.1), the strains alkalinized the medium to pH 7.2 and the concentration of ammonium ions in the medium was increased from 0.14 to 0.61 mM. This increase seemed to be responsible for the neutralization since, by the addition of ammonium to fresh acidic medium to a final concentration of 0.61 mM, the pH increased from pH 4.1 to 7.2. No or little alkalinization or increase in the ammonium ion concentration was observed on 1/10 TS liquid medium of pH 7 or above. All strains showed good growth on 1/10 TS (pH 4.1) at 25–37 °C with an optimum at 30 °C, but did not grow at 4 or 45 °C. On 1/10 TS agar (pH 4.1), the organisms formed smooth, round, convex, pale-yellow colonies. The strains were Gram-positive (Ryu, 1938), non-endospore-forming and aerobic. Motility was not observed.

The 16S rRNA gene of each strain was amplified by PCR using universal primers (Tamura & Hatano, 2001) and nearly complete 16S rRNA gene nucleotide sequences

(>1400 bp) were determined for the four strains. Their sequences were almost identical and showed high similarities to species of the genus *Curtobacterium*. The similarities among strain B55^T and *C. flaccumfaciens* pv. *flaccumfaciens* LMG 3645^T, *C. luteum* DSM 20542^T, *C. albidum* NBRC 15078^T, *C. pusillum* DSM 20527^T, *C. citreum* DSM 20528^T and *C. herbarum* DSM 14013^T were between 98.2 and 99.4 %. Phylogenetic relationships with closely related species were determined by using MEGA version 3.1 (Kumar *et al.*, 2004) and the PHYLIP package version 3.65 (Felsenstein, 2005) after multiple alignment of data performed by CLUSTAL X (Thompson *et al.*, 1997). Evolutionary distances were computed as described previously (Jukes & Cantor, 1969). Phylogenetic trees were constructed using the maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) methods. The reliability of the tree topologies was evaluated by bootstrap analysis with 1000 replicates (Felsenstein, 1985). The phylogenetic trees constructed by the two methods were topologically similar and showed that all four isolates belonged to the genus *Curtobacterium*. Although these strains showed high degrees of 16S rRNA gene sequence similarity to the established species of the genus, they formed a separate line of descent in the phylogenetic cluster of the genus (Fig. 1; maximum-parsimony tree not shown). Stackebrandt & Goebel (1994) pointed out that a high degree of 16S rRNA gene sequence similarity ($\geq 97\%$) is of limited value for differentiating species and that DNA–DNA hybridization studies need to be performed to determine species affiliation under these circumstances; we therefore performed DNA–DNA hybridization studies by using the microplate hybridization method (Ezaki *et al.*, 1988, 1989; Tamura *et al.*, 1999) between strain B55^T and its neighbours on the phylogenetic tree. The DNA–DNA relatedness values between strain B55^T and *C. luteum* JCM 1480^T, *C. albidum* NBRC 15078^T, *C. pusillum* JCM 1350^T, *C. citreum* JCM 1345^T, *C. flaccumfaciens* pv. *flaccumfaciens* JCM 9670^T and strains CA73, SA69 and SA72 were respectively 7.9, 8.9, 6.2, 9.2, 7.8, 91.2, 80.7 and 89.9 %. The DNA–DNA relatedness values of B55^T with respect to the type strains of closely related species were far lower than 70 %, which is the recommended threshold value for the delineation of genomic species (Wayne *et al.*, 1987), whereas the values with respect to the other three strains were higher than 70 %.

Fig. 1. Neighbour-joining tree, based on nearly complete 16S rRNA gene sequences (positions 52–1476 of the *Escherichia coli* 16S rRNA gene), showing the positions of strains B55^T, SA69, CA73 and SA72 among their phylogenetic neighbours. Numbers at branch nodes are values based on 1000 bootstrap resamplings; only values over 500 are given. The sequence of *Clavibacter michiganensis* DSM 46364^T was used as an out-group. Bar, 0.01 substitutions per nucleotide position.

Table 1. Physiological characteristics of *C. ammoniigenes* sp. nov. and closely related type strains of *Curtobacterium* species

Strains: 1, *C. ammoniigenes* sp. nov. strains B55^T, CA73, SA69 and SA72; 2, *C. citreum* JCM 1345^T; 3, *C. pusillum* JCM 1350^T; 4, *C. luteum* JCM 1480^T; 5, *C. albidum* NBRC 15078^T; 6, *C. flaccumfaciens* pv. *flaccumfaciens* JCM 9670^T. +, Positive; –, negative; (+), weakly positive. Results for hydrolysis were obtained as described by Smibert & Krieg (1994), results for acid production were obtained by use of the API 50 CH system, results for enzyme activities were obtained by use of the API Coryne system and results for assimilation were obtained by use of Biolog GP2 MicroPlates. All strains were negative for hydrolysis of starch and casein. All strains were positive for acid production from glycerol, L-arabinose, D-xylose, D-galactose, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, aesculin, salicin, D-cellobiose, D-maltose, D-lactose, D-melibiose, sucrose, gentiobiose and D-turanose. All strains had the following characteristics: negative for acid production from L-xylose, dulcitol and D-fucose, positive for catalase, pyrazinamidase and β -glucosidase activities, negative for oxidase, nitrate reduction and urease activities, positive for assimilation of dextrin, D-fructose, D-galactose, α -D-glucose, D-mannose, D-melibiose, sucrose and pyruvic acid methyl ester and negative for assimilation of L-rhamnose, acetic acid, *cis*-aconitic acid, citric acid, D-galactonic acid lactone, D-galacturonic acid, D-glucosaminic acid, α -hydroxybutyric acid, γ -hydroxybutyric acid, *p*-hydroxyphenylacetic acid, itaconic acid, α -ketoglutaric acid, α -ketovaleric acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinamic acid, glucuronamide, L-aspartic acid, glycyl L-aspartic acid, L-histidine, L-leucine, L-ornithine, L-pyroglutamic acid, D-serine, DL-carnitine, γ -aminobutyric acid, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, DL- α -glycerol phosphate, α -D-glucose 1-phosphate and D-glucose 6-phosphate.

Test	1	2	3	4	5	6
Optimal growth pH	4	7	7	7	7	7
Motility	–	+	+	+	–	–
Ammonia production	+	–	–	–	–	–
Hydrolysis of:						
Gelatin	–	–	+	–	+	+
Aesculin	+	+	+	–	+	+
Tween 80	–	–	–	+	+	–
Acid production from:						
D-Arabinose	+	+	+	–	+	–
D-Ribose	+	+	+	+	–	–
L-Rhamnose	+	–	+	+	+	+
D-Sorbitol	+	–	–	–	–	–
N-Acetyl-D-glucosamine	–	–	+	+	–	(+)
Amygdalin	–	–	+	+	–	–
D-Trehalose	–	+	+	+	(+)	+
D-Melezitose	–	+	+	+	+	+
D-Raffinose	–	–	+	–	(+)	+
Starch	–	–	+	–	–	–
D-Lyxose	+	–	–	–	–	–
L-Arabitol	+	+	+	+	–	–
Potassium 2-ketogluconate	+	–	–	–	–	–
Activity of:						
Pyrrolidonyl arylamidase	–	–	–	+	–	+
β -Galactosidase	+	+	–	+	–	+
α -Glucosidase	+	+	+	+	–	+
Assimilation of:						
α -Cyclodextrin	–	+	–	+	–	–
Glycogen	–	+	–	+	–	(+)
Tween 40	–	(+)	–	(+)	+	(+)
Tween 80	–	(+)	–	(+)	+	–
N-Acetyl-D-galactosamine	–	–	–	–	–	+
N-Acetyl-D-glucosamine	–	+	–	+	(+)	+
Adonitol	–	–	(+)	–	–	+
L-Arabinose	–	–	–	–	–	+
D-Arabitol	–	–	(+)	–	–	+
D-Cellobiose	–	+	–	+	+	+
i-Erythritol	–	–	(+)	–	–	+
L-Fucose	–	+	–	+	–	(+)
Gentiobiose	–	–	+	–	+	+

Table 1. cont.

Test	1	2	3	4	5	6
<i>myo</i> -Inositol	—	—	—	—	—	+
α -D-Lactose	+	+	—	+	+	+
Lactulose	—	+	—	+	+	+
D-Mannitol	+	—	+	—	(+)	+
D-Melibiose	+	+	—	+	(+)	+
Methyl β -D-glucoside	—	—	+	—	(+)	+
D-Raffinose	—	—	—	—	+	+
D-Sorbitol	+	—	+	—	+	+
Turanose	+	—	+	—	+	+
Xylitol	—	—	+	—	+	+
Succinic acid monomethyl ester	+	—	—	—	—	(+)
D-Gluconic acid	+	—	+	—	+	+
D-Glucuronic acid	—	—	—	—	—	+
α -Ketobutyric acid	—	(+)	—	+	—	—
DL-Lactic acid	+	(+)	—	(+)	—	+
Succinic acid	—	(+)	—	(+)	—	+
Bromosuccinic acid	—	(+)	—	—	—	+
L-Alaninamide	(+)	—	—	—	+	(+)
D-Alanine	—	—	—	—	(+)	+
L-Alanine	—	—	—	—	(+)	+
L-Alanyl glycine	—	—	—	—	(+)	+
L-Glutamic acid	—	+	—	—	—	+
Hydroxy-L-proline	—	+	—	+	—	—
L-Proline	—	—	—	—	—	+
Urocanic acid	—	—	+	—	—	—
Uridine	—	—	—	—	(+)	+

On the basis of these results, these strains represent a single novel species of the genus *Curtobacterium*.

Additionally, the isolates could be distinguished from the five phylogenetically closely related species of *Curtobacterium* on the basis of physiological characteristics (Table 1). The isolates are ammonia-producing acidophiles, whereas other species are neutrophiles producing no or little ammonia. Standard physiological tests were carried out according to methods described previously (Gordon *et al.*, 1974, Smibert & Krieg, 1994). Acid production from carbon sources and enzyme activities were assessed by using the API 50 CH and API Coryne systems (bioMérieux), respectively, according to the manufacturer's instructions (incubation times of up to 7 days). The utilization of various substrates as sole carbon sources was tested by using Biolog GP2 MicroPlates in accordance with the manufacturer's instructions.

Morphology was observed by scanning electron microscopy. After critical-point drying, specimens were sputter-coated with gold palladium. After growth on 1/10 TS agar (pH 4.1) at 28 °C for 3 days, cells were irregular rods, 0.4–0.5 μ m wide and 0.5–1.0 μ m long (see Supplementary Fig. S1 in IJSEM Online).

Analyses of cell-wall amino acids, cell-wall sugar pattern, the acyl type of peptidoglycan, menaquinones, DNA G+C

content and cellular fatty acids were performed as described previously (Uchida *et al.*, 1999; Nishiuchi *et al.*, 1999; Tamura *et al.*, 1994). The amino acids in the cell wall of strain B55^T were D-ornithine, D-glutamic acid, D-alanine, glycine and L-homoserine, indicating murein type B2 β according to Schleifer & Kandler (1972). The major isoprenoid quinone of the isolates was MK-9. The DNA G+C content of strain B55^T was 68.8 mol%. These data also supported our contention that the isolates belong to the genus *Curtobacterium*. The acyl type of peptidoglycan was acetyl. Mannose, glucose, fucose and rhamnose were found in the cell-wall hydrolysate of strain B55^T. The strains were grown on both acidic (pH 4) and neutral (pH 7) 1/10 TS agar media and their cellular fatty acid compositions were determined (Supplementary Table S1). Six fatty acids were detected from all isolates under both culture conditions, and the major fatty acid was 11-cyclohexyl undecanoic acid (ch-C_{17:0}), making up 80.9–88.4% of fatty acids in cells grown on neutral medium and 54.6–75.4% of those grown on acidic medium. Relatively large amounts of anteiso-C_{17:0} (11.6–30.6%) were detected from cells grown on acidic medium, whereas small amounts (1.4–2.9%) were found in cells grown on neutral medium. Similar differences in the content of anteiso-C_{15:0}, C_{16:0} and iso-C_{16:0} were detected between cells grown on the acidic and neutral media. Small amounts of 13-cyclohexyl tridecanoic acid (ch-C_{19:0}) were

also detected from cells grown on both media. These profiles, with a large proportion of ω -cyclohexyl acids, are similar to those of *C. pusillum* (Suzuki *et al.*, 1981) and *C. flaccumfaciens* pv. *flaccumfaciens* (Supplementary Table S1). Mycolic acids were not detected.

Therefore, based on the physiological, biochemical, chemotaxonomic and molecular genetic results described above, strains B55^T, CA73, SA69 and SA72 represent a novel species, for which the name *Curtobacterium ammoniigenes* sp. nov. is proposed.

Description of *Curtobacterium ammoniigenes* sp. nov.

Curtobacterium ammoniigenes (am.mo.ni.i'ge.nes. N.L. n. *ammonia* -ae ammonia; Gr. v. *gennao* to produce; N.L. part. adj. *ammoniigenes* ammonia-producing).

The morphological, physiological and chemical description of the species is based on the four strains described above. Gram-positive, strictly aerobic, non-spore-forming, non-motile rods of irregular shape. Colonies are pale yellow, smooth, convex and round with entire margins. Growth occurs between 15 and 37 °C but not at 4 or 45 °C. The pH range for growth is 3.5–8.0, with optimum growth at pH 4. Produces ammonia on 1/10 TS medium (pH 4.1) and neutralizes the medium to pH 7. Physiological characteristics are summarized in Table 1. The diagnostic diamino acid of the peptidoglycan is D-ornithine; peptidoglycan is of the B2 β type with acetyl residues. The major menaquinone is MK-9, as described for the genus. The predominant cellular fatty acid is 11-cyclohexyl undecanoic acid (ch-C_{17:0}). The DNA G + C content of the type strain is 68.8 mol%.

The type strain, B55^T (=NBRC 101786^T=VTCC D6-11^T=JCM 14609^T), was isolated from a stem of *Ludwigia adscendens* inhabiting acidic swamps in actual acid sulfate soil areas in Vietnam. Strains CA73 (=NBRC 101790=VTCC D6-15=JCM 14610), SA69 (=NBRC 101788=VTCC D6-13=JCM 14611) and SA72 (=NBRC 101789=VTCC D6-14=JCM 14612), isolated from stems of *Eleocharis dulcis* in the same area, are also assigned to this species.

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