Azonexus hydrophilus sp. nov., a *nifH* gene-harbouring bacterium isolated from freshwater

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Three Gram-negative, non-pigmented, rod-shaped, facultatively aerobic bacterial strains, designated d8-1[T], d8-2 and IMCC1716, were isolated from a freshwater spring sample and a eutrophic freshwater pond. Based on characterization using a polyphasic approach, the three strains showed highly similar phenotypic, physiological and genetic characteristics. All of the strains harboured the nitrogenase gene *nifH*, but nitrogen-fixing activities could not be detected in nitrogen-free culture media. The three strains shared 99.6–99.7 % 16S rRNA gene sequence similarity and showed 89–100 % DNA–DNA relatedness, suggesting that they represent a single genomic species. Phylogenetic analysis based on 16S rRNA gene sequences showed that strains d8-1[T], d8-2 and IMCC1716 formed a monophyletic branch in the periphery of the evolutionary radiation occupied by the genus *Azonexus*. Their closest neighbours were *Azonexus caeni* Slu-05[T] (96.7–96.8 % similarity) and *Azonexus fungiphilus* BS5-8[T] (96.3–96.6 %). The DNA–DNA relatedness of the novel strains to these two species of the genus *Azonexus* was less than 70 %. The isolates could also be differentiated from recognized members of the genus *Azonexus* on the basis of phenotypic and biochemical characteristics. It is evident, therefore, that the three strains represent a novel species of the genus *Azonexus*, for which the name *Azonexus hydrophilus* sp. nov. is proposed. The type strain is d8-1[T] (=LMG 24005[T]=BCRC 17657[T]).

The genus *Azonexus*, proposed by Reinhold-Hurek & Hurek (2000), currently comprises two bacterial species with validly published names, *Azonexus fungiphilus* and *Azonexus caeni*. The genus *Azonexus* is a member of the family *Rhodocyclaceae* in the class *Betaproteobacteria* and encompasses Gram-negative, non-spore-forming, highly motile and slightly curved rods. *A. fungiphilus*, formerly named *Azoarcus* sp. group E, was isolated from the resting stage of a plant-associated basidiomycete in the rhizosphere soil (Hurek *et al.*, 1997) and *A. caeni* is a denitrifying bacterium isolated from sludge of a wastewater treatment plant (Quan *et al.*, 2006). This study focuses on the taxonomic study of three *Azonexus*-like strains isolated from two different freshwater environments. Based on a polyphasic taxonomic investigation, the three strains represent a novel species in the genus *Azonexus*.

Two bacterial strains (d8-1[T] and d8-2) were isolated from a water sample collected from a freshwater spring located in Hsinchu County, Taiwan, on tryptic soy agar (BD Difco) at 35 °C. Strain IMCC1716 was isolated from a eutrophic freshwater pond located at Inha University, Korea, on R2A agar (BD Difco) at 25 °C. The isolates were routinely maintained on tryptic soy broth (BD Difco) or R2A agar at 35 °C. *A. fungiphilus* BS5-8[T] and *A. caeni* Slu-05[T], obtained from the Korean Collection for Type Cultures, Yusong, Taejon, Republic of Korea, were used as reference strains. The morphology of bacterial cells was observed during the lag, exponential and stationary phases of growth under a phase-contrast microscope (DM2000; Leica) and by

scanning electron microscopy (S-3500N; Hitachi). Flagellar staining was performed using the Spot Test Flagella stain (BD Difco). The Gram reaction was performed using the Gram Stain set (BD Difco) and the Ryu non-staining KOH method (Powers, 1995). Accumulation of poly-β-hydroxy-butyrate granules was observed by light microscopy after staining cells with Sudan black (Gerhardt *et al.*, 1994). Colony morphology was examined using a stereoscopic microscope (SMZ800; Nikon) after incubating the cells on R2A agar at 30 °C for 2 days. The optimum pH range for growth was examined by measuring OD_{595} of the cultures grown in nutrient broth (BD Difco) adjusted to various pH values (pH 4–10 at intervals of 1.0 pH unit) using appropriate biological buffers (Chung *et al.*, 1995). The NaCl tolerance range of the strains was tested in nutrient broth adjusted to various NaCl concentrations (0, 0.5 and 1.0–4.0 %, w/v, at 1.0 % intervals). The temperature range for growth (4, 10, 15, 20, 25, 30, 35, 40 and 45 °C) was examined in nutrient broth. Anaerobic cultivation was performed on R2A agar using the Oxoid AnaeroGen system. For the nitrogenase activity test, the three strains were inoculated in 33 ml rubber-septa-sealed vials containing 10 ml semi-solid nitrogen-free medium [DSMZ medium no. 3 or nitrogen-free SM medium (Reinhold *et al.*, 1986)] and incubated in the dark at 35 °C. After 48–240 h of incubation, 10–50 % (v/v) of the air phase was replaced by acetylene. Nitrogen-fixing ability was determined using the acetylene reduction assay as described by Elliott *et al.* (2007). Catalase, oxidase, DNase, arginine dihydrolase, urease and lipase activities, nitrate reduction and hydrolysis of starch, casein and Tweens 20, 40, 60 and 80 were determined using standard methods (Gerhardt *et al.*, 1994; Lanyi, 1987; MacFaddin, 2000). Additional biochemical tests were performed using the API 20NE (bioMérieux), API ZYM (bioMérieux) and Biolog GN2 (Biolog) microtest systems according to the methods outlined by the manufacturers. Eleven different kinds of antimicrobial agent (listed in Table 1) were tested by the

Table 1. Differential biochemical characteristics of *Azonexus* species

All data were acquired in the present study, except for the DNA G + C content of *A. caeni* (Quan *et al.*, 2006). +, Positive; −, negative; R, resistant; S, sensitive; V, variable; ND, no data available. Data in parentheses for *A. hydrophilus* are for the type strain (d8-1^T). All strains have the following features: positive for the oxidation of Tween 40, Tween 80, β-hydroxybutyric acid, DL-lactic acid, propionic acid, succinic acid and L-glutamic acid; negative for the oxidation of α-cyclodextrin, dextrin, glycogen, *N*-acetyl-D-galactosamine, *N*-acetyl-D-glucosamine, adonitol, L-arabinose, D-arabitol, cellobiose, i-erythritol, D-fructose, L-fucose, D-galactose, gentiobiose, α-D-glucose, *myo*-inositol, α-D-lactose, lactulose, maltose, D-mannitol, D-mannose, melibiose, methyl β-D-glucoside, D-psicose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose, turanose, xylitol, succinic acid monomethyl ester, acetic acid, citric acid, formic acid, D-galacturonic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxyphenylacetic acid, α-ketobutyric acid, α-ketovaleric acid, malonic acid, quinic acid, D-saccharic acid, sebacic acid, L-alaninamide, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, D-serine, L-serine, L-threonine, γ-aminobutyric acid, urocanic acid, inosine, uridine, thymidine, phenylethylamine, putrescine, 2-aminoethanol, 2,3-butanediol, glycerol, DL-α-glycerol phosphate, α-D-glucose 1-phosphate, D-glucose 6-phosphate, glucuronamide, D-alanine, L-alanine, L-alanyl glycine, D-galactonic acid lactone, DL-carnitine, L-phenylalanine, glycyl-L-aspartic acid, and glucosidase and galactosidase activities. All strains are sensitive to ampicillin (10 µg), chloramphenicol (30 µg), erythromycin (15 µg), gentamicin (10 µg), kanamycin (30 µg), nalidixic acid (30 µg), novobiocin (30 µg), penicillin G (10 U), streptomycin (10 µg) and tetracycline (30 µg).

Characteristic	*A. hydrophilus* sp. nov. (*n*=3)	*A. caeni* Slu-05^T	*A. fungiphilus* BS5-8^T
Nitrate reduction	+	+	−
Arginine dihydrolase	−	+	−
Urease	V (+)	+	+
Naphthol-AS-BI-phosphohydrolase	+	+	−
Growth in 2 % NaCl	−	−	+
Susceptibility to rifampicin (5 µg)	S	R	S
Oxidation of:			
α-Ketoglutaric acid	+	+	−
Pyruvic acid methyl ester	+	+	−
Bromosuccinic acid	−	+	−
L-Pyroglutamic acid	−	−	+
Succinamic acid	+	+	−
Itaconate	V (+)	−	−
cis-Aconitic acid	V (+)	+	−
L-Proline	−	−	+
L-Asparagine	V (−)	−	−
L-Aspartic acid	V (−)	−	−
DNA G + C content (mol%)	64.4–66.0	65.6	ND

diffusion method on Mueller–Hinton agar (BD Difco). The effect of antimicrobial agents on cellular growth was assessed after 3 days of incubation and susceptibility was scored based on the distance between the edges of the clear zone and the disc.

Detailed morphological, physiological and biochemical characteristics of strains d8-1[T], d8-2 and IMCC1716 are provided in the species description, Table 1 and Supplementary Fig. S1 (available with the online version of this paper). Cells were Gram-negative, facultatively aerobic, non-spore-forming, slightly curved rods that were motile by a single polar flagellum. A comparison of biochemical characteristics of the three novel strains and those of the type strains of species of the genus *Azonexus* is presented in Table 1. Strains d8-1[T], d8-2 and IMCC1716 did not grow in nitrogen-free DSMZ medium no. 3 supplemented with glucose or mannitol as sole carbon sources. In contrast, the three strains grew well in nitrogen-free SM medium supplemented with malate as sole carbon source. However, nitrogen-fixing activity was not observed in the acetylene reduction assay.

Extraction of genomic DNA, PCR amplification and sequencing of the 16S rRNA gene were carried out as described by Chen *et al.* (2001). The potential for nitrogen fixation was examined by amplifying the *nifH* gene using the nifH3 and nifH4 primer set, as described by Zani *et al.* (2000). The resultant 16S rRNA gene and *nifH* gene sequences were compared with sequences available from GenBank. Multiple sequence alignments of the genes of strains d8-1[T], d8-2, IMCC1716 and their closest relatives were performed using BIOEDIT (Hall, 1999) and MEGA version 3.1 (Kumar *et al.*, 2004) software. Phylogenetic trees were inferred using the maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) algorithms. Evolutionary distance matrixes were generated for the neighbour-joining tree using the Kimura two-parameter distance model (Kimura, 1983) and bootstrap analysis was performed based on 1000 resamplings.

Nearly complete 16S rRNA gene sequences were obtained for strains d8-1[T], d8-2 and IMCC1716 (1454, 1464 and 1488 nt, respectively). The 16S rRNA gene sequence similarities between the strains were 99.6–99.7 %. The three strains were most closely related to *A. caeni* Slu-05[T] (96.7–96.8 % similarity) and *A. fungiphilus* BS5-8[T] (96.3–96.6 % similarity). Phylogenetic analysis based on 16S rRNA gene sequences indicated that the strains belonged to the family *Rhodocyclaceae* of the class *Betaproteobacteria* and fell within the evolutionary radiation of the genus *Azonexus* (Fig. 1). No other species in the class *Betaproteobacteria* with validly published names showed more than 96 % 16S rRNA gene sequence similarity. The overall tree topologies obtained from neighbour-joining, maximum-parsimony and maximum-likelihood methods were similar (data not shown). Because the *nifH* gene phylogeny of diazotrophic bacteria has been reported to generally correlate well with the 16S rRNA gene phylogeny
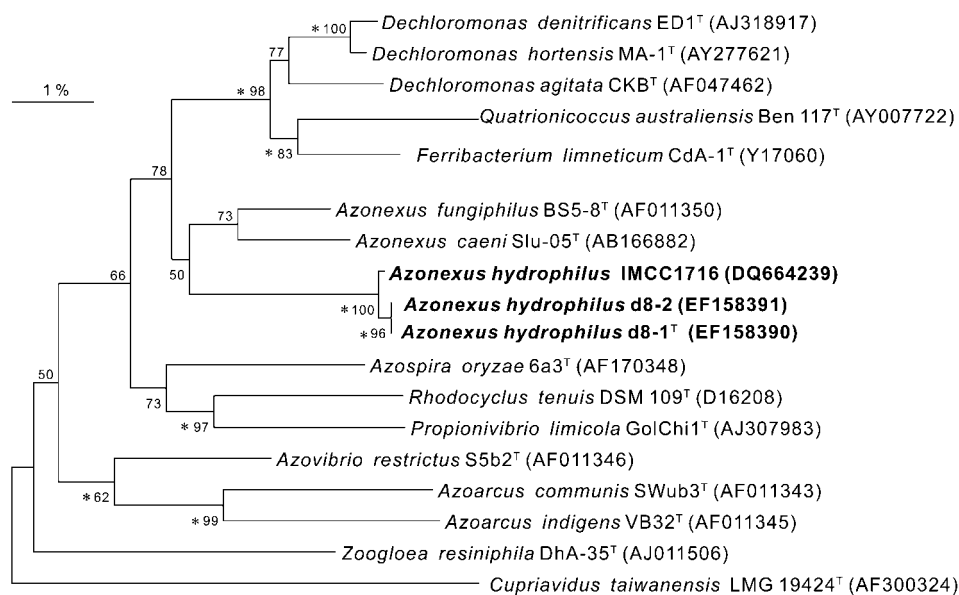

Fig. 1. Phylogenetic tree based on 16S rRNA gene sequences showing the relationships between strains d8-1[T], d8-2 and IMCC1716 and related taxa in the class *Betaproteobacteria*. The neighbour-joining method was employed with the Kimura two-parameter model. Numbers at nodes are percentages of bootstrap values based on 1000 resamplings; only values above 50 % are shown. Asterisks indicate branches that were recovered using the maximum-parsimony algorithm. Bar, 1 % sequence dissimilarity. The sequence of *Cupriavidus taiwanensis* LMG 19424[T] was used as an outgroup.

(Chen *et al.*, 2003; Moulin *et al.*, 2001; Rosado *et al.*, 1998; Xie & Yokota, 2004; Young, 1992), the *nifH* gene was selected as another marker to elucidate phylogenetic relationships for the three novel strains and related taxa. The *nifH* gene sequence similarities between strains d8-1[T], d8-2 and IMCC1716 were 99.0–100 %. The strains showed 95.7–96.1 % and 92.2 % *nifH* gene sequence similarities to *A. fungiphilus* and *A. caeni*, respectively. A phylogenetic tree based on *nifH* gene sequences also showed the monophyletic relationship between the three novel freshwater isolates and related species of the genus *Azonexus* (see Supplementary Fig. S2 in the online version of this paper).

Whole genome DNA–DNA hybridization experiments were performed with photobiotin-labelled probes as described by Ezaki *et al.* (1989). The degree of DNA–DNA relatedness was calculated from triplicate experiments. The DNA–DNA relatedness values between strains d8-1[T], d8-2 and IMCC1716 were 89–100 %, indicating that the three strains are members of the same genomic species (Wayne *et al.*, 1987). Strain d8-1[T] showed 45 ± 7 % and 22 ± 10 % DNA–DNA relatedness with *A. caeni* Slu-05[T] and *A. fungiphilus* BS5-8[T], respectively.

For chemotaxonomic analyses, all of the strains employed for the analyses were grown on R2A agar for 3 days at 30 °C. Fatty acid methyl esters were prepared, separated and identified according to the instructions of the Microbial Identification system (Microbial ID; Sasser, 1990). Polar lipids were extracted and analysed by two-dimensional TLC according to Embley & Wait (1994). The DNA G+C content was determined in triplicate by HPLC as described by Mesbah *et al.* (1989). Preparation of whole-cell proteins and SDS-PAGE were performed as described by Pot *et al.* (1994). Electrophoretic protein patterns were recorded on an Astra 1220S scanner (Umax Systems). Bands with migration differences of less than 3 % were considered to be the same band. Normalization of the protein profiles and clustering by UPGMA were performed with Universal Software 1D Advanced (Advanced American Biotechnology and Imaging).

The major cellular fatty acid constituents of strain d8-1[T] were $C_{16:0}$ (28.2 %), $C_{16:1}\omega 7c$ (48.1 %) and $C_{18:1}$ isomers (13.0 %) (Table 2). The overall cellular fatty acid profiles of strain d8-1[T] were generally in accordance with those of the two recognized species of the genus *Azonexus*. However, strain d8-1[T] could be differentiated from the type strains of *A. fungiphilus* and *A. caeni* by the absence of $C_{12:0}$ 3-OH, $C_{15:0}$ and $C_{17:1}\omega 6c$ and the presence of $C_{19:1}\omega 6c$ (Table 2). The predominant polar lipids of strain d8-1[T] were phosphatidylethanolamine, phosphatidylglycerol and diphosphatidylglycerol. The DNA G+C contents of strains d8-1[T], d8-2 and IMCC1716 were 66.0 ± 0.5, 66.0 ± 1.0 and 64.4 ± 1.0 mol%, respectively; these were similar to the DNA G+C content of *A. caeni* (65.6 mol%; Table 1). When the whole-cell protein profiles of the three freshwater isolates were compared with those of the type strains of *A. fungiphilus* and *A. caeni*, the three novel strains formed a single cluster with similarities of >93 %, but showed less than 83 % similarity to the two *Azonexus* species (Fig. 2). These results further confirm the separate taxonomic position of strains d8-1[T], d8-2 and IMCC1716 within the genus *Azonexus*.

Based on 16S rRNA and *nifH* gene sequence comparisons, strains d8-1[T], d8-2 and IMCC1716 occupied a separate phylogenetic position in the genus *Azonexus*. This was

Table 2. Fatty acid content (%) of *Azonexus hydrophilus* sp. nov. and the type strains of two *Azonexus* species

Data for *A. caeni* Slu-05[T] and *A. fungiphilus* BS5-8[T] are from Quan *et al.* (2006). Strains d8-1[T], d8-2 and IMCC1716 were cultured on R2A agar (BD Difco) at 30 °C, the same culture conditions as reported for *A. fungiphilus* BS5-8[T] and *A. caeni* Slu-05[T]. −, Not detected. Data in parentheses for *A. hydrophilus* are for the type strain (d8-1[T]).

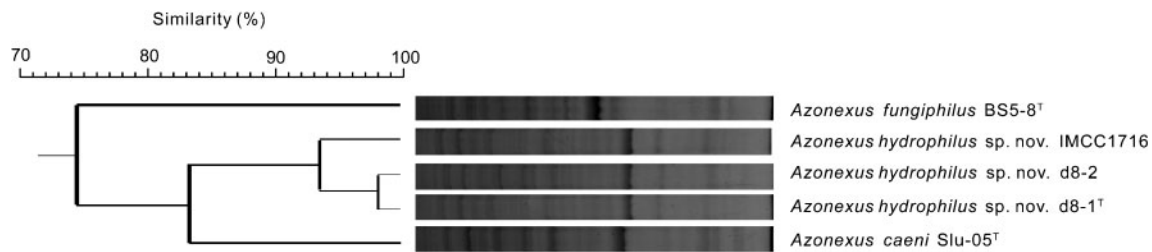| Fatty acid	*A. hydrophilus* sp. nov. (*n*=3)	*A. caeni* Slu-05[T]	*A. fungiphilus* BS5-8[T]
Straight-chain fatty acids:			
12:0	2.0–3.0 (3.0)	2.9	2.7
14:0	1.0–1.8 (1.0)	1.6	2.1
15:0	− (−)	1.2	2.5
16:0	25.2–29.4 (28.2)	19.3	16.7
18:0	0.3–1.0 (0.7)	1.1	3.5
Unsaturated fatty acids:			
16:1$\omega 7c$	44.4–48.1 (48.1)	48.0	40.5
17:1$\omega 6c$	− (−)	−	1.0
18:1 isomers	11.3–17.2 (13.0)	18.8	18.0
19:1$\omega 6c$	0.5–1.8 (1.7)	−	−
Hydroxy fatty acids:			
10:0 3-OH	4.4–5.4 (4.6)	5.4	4.1
12:0 3-OH	− (−)	1.1	0.7

Fig. 2. Dendrogram based on numerical analysis of the whole-cell protein profiles of strains d8-1[T], d8-2 and IMCC1716 and the type strains of two closely related *Azonexus* species.

confirmed by their unique combination of chemotaxonomic characteristics (Tables 1 and 2; Fig. 2) and biochemical traits (Table 1). It is clear from the genotypic and phenotypic data that strains d8-1[T], d8-2 and IMCC1716 represent a novel species in the genus *Azonexus* for which the name *Azonexus hydrophilus* sp. nov. is proposed.

Description of *Azonexus hydrophilus* sp. nov.

Azonexus hydrophilus [hy.dro′phi.lus. Gr. n. *hudor* (or *hydor*) water; Gr. adj. *philos* loving; N.L. masc. adj. *hydrophilus* water-loving, referring to its source of isolation].

Cells are Gram-negative, non-spore-forming, slightly curved rods (0.3–0.5 µm in diameter and 1.1–2.2 µm in length), motile by means of a single polar flagellum. Colonies on R2A agar are round with umbonate elevation and semi-transparent with irregular edges. Colonies are approximately 0.3–0.6 mm in diameter on R2A agar after 48 h incubation at 35 °C. Optimum growth occurs at 35–40 °C, 0–1 % NaCl and pH 7.0–8.0. Cells grow under anaerobic conditions, but growth is weaker than under aerobic conditions. Positive for catalase, cytochrome oxidase, and hydrolysis of starch (weak), Tween 40 and Tween 60. Negative for DNase, lipase (corn oil) and hydrolysis of skimmed milk and Tween 20. In API 20NE tests, positive for nitrate reduction and malate assimilation and negative for indole production, arginine dihydrolase, aesculin hydrolysis, gelatinase, β-galactosidase and assim-ilation of glucose, arabinose, mannose, mannitol, *N*-acetylglucosamine, maltose, caprate, adipate, citrate and phenylacetate. Urease and gluconate assimilation are strain-dependent. Positive (API ZYM) for alkaline phos-phatase, C4 esterase, C8 lipase, leucine arylamidase and naphthol-AS-BI-phosphohydrolase, but negative for C14 lipase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, α-galactosidase, β-galac-tosidase, α-glucosidase, β-glucosidase, β-glucuronidase, *N*-acetyl-β-glucosaminidase, α-mannosidase and α-fucosi-dase. Acid phosphatase activity is strain-dependent. The results for carbon source oxidation and antimicrobial susceptibility tests are presented in Table 1. Oxidation of the following compounds is strain-dependent: itaconic acid, *cis*-aconitic acid, L-asparagine and L-aspartic acid. The

major cellular fatty acids are $C_{16:0}$, $C_{16:1}\omega 7c$ and $C_{18:1}$ isomers. The predominant polar lipids are phosphatidyl-ethanolamine, phosphatidylglycerol and diphosphatidyl-glycerol. The DNA G + C content is 64–66 mol%.

The type strain, d8-1[T] (=LMG 24005[T]=BCRC 17657[T]), was isolated from a water sample collected from a spring located in Nature Valley, Hsinchu County, Taiwan.

Acknowledgements

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