Azonexus caeni sp. nov., a denitrifying bacterium isolated from sludge of a wastewater treatment plant

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A polyphasic taxonomic study was carried out to determine the taxonomic position of a newly isolated denitrifying bacterium, designated Slu-05^T, which had been isolated from sludge from the main aerobic treatment tanks of a municipal sewage treatment plant. Phylogenetic analysis based on comparative 16S rRNA gene sequencing indicated that strain Slu-05^T was closely related to *Azonexus fungiphilus* LMG 19178^T (96.4 % sequence similarity), the sole species in the genus *Azonexus*. Strain Slu-05^T comprised Gram-negative, motile, non-spore-forming and slightly curved rods. The predominant respiratory lipoquinone was Q-8. The major fatty acids were C_{16:1}ω7c, C_{16:0}, C_{18:1} isomers and C_{10:0} 3-OH. The G + C content of the genomic DNA was 65.6 mol%. The results of DNA–DNA hybridization (15.6 %) together with phenotypic determination showed that strain Slu-5^T could be distinguished from *A. fungiphilus*. Moreover, some phenotypic properties concerning enzyme activity, the substrates utilized as carbon sources and growth conditions distinguish strain Slu-5^T from *A. fungiphilus*. On the basis of the results obtained in this study, Slu-05^T (=DSM 17719^T=KCTC 12530^T=CCBAU 10199^T) is the type strain of a novel species of *Azonexus*, for which the name *Azonexus caeni* sp. nov. is proposed.

The genus *Azonexus* was proposed by Reinhold-Hurek & Hurek (2000). The only species in the genus is *Azonexus fungiphilus*, formerly *Azoarcus* sp. group E. This species was isolated from the resting stages of a plant-associated basidiomycete in rhizosphere soil of rice growing in Pakistan (Hurek *et al.*, 1997). The genus *Azonexus* accommodates Gram-negative, non-spore-forming, highly motile, slightly curved rods.

In the course of screening micro-organisms from sludge of the main aerobic treatment tanks of municipal sewage treatment plants located in Daejeon, South Korea, a Gram-negative and denitrifying bacterial strain (Slu-05^T) was isolated. On the basis of 16S rRNA gene sequence analysis, strain Slu-05^T was considered to be an *Azonexus*-like strain. To determine its exact taxonomic position, strain Slu-05^T was subjected to a detailed polyphasic taxonomic investigation, including genotypic, chemotaxonomic and classical phenotypic analyses. These results indicate that strain

Slu-05^T should be placed in a novel species in the genus *Azonexus*.

Strain Slu-05^T was isolated from sludge from the main aerobic treatment tanks of municipal sewage treatment plants located in Daejeon, South Korea. The suspension was spread on R2A agar plates (Difco) after being serially diluted with 50 mM phosphate buffer (pH 7.0). The plates were incubated at 30 °C for 2 weeks. Single colonies on the plates were purified by transferring them onto new plates and incubating them once again under the same conditions. The isolate was routinely cultured on R2A agar at 30 °C and maintained as a glycerol suspension (20 %, w/v) at –70 °C. *Azonexus fungiphilus* LMG 19178^T, obtained from BCCM/LMG (the Belgian Co-ordinated Collections of Microorganisms/Laboratorium voor Microbiologie, Ghent University, Ghent, Belgium), was used as a reference strain and its phenotypic properties were determined along with those of strain Slu-05^T.

Extraction of the genomic DNA was done using a commercial genomic DNA-extraction kit (Core Biosystem). PCR-mediated amplification of the 16S rRNA gene and sequencing of purified PCR products were carried out according to Kim *et al.* (2005). The full 16S rRNA gene sequence was compiled using SeqMan software (DNASTAR).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence of strain Slu-05^T and the partial *nifH* gene sequences of strain Slu-05^T and *Azonexus fungiphilus* LMG 19178^T are AB166882, DQ029203 and DQ029204, respectively.

The 16S rRNA gene sequences of related taxa were obtained from the GenBank database. Multiple alignments were performed by the CLUSTAL_X program (Thompson *et al.*, 1997). Gaps were edited using the BioEdit program (Hall, 1999). Evolutionary distances were calculated using the Kimura two-parameter model (Kimura, 1983). Phylogenetic trees were constructed by using the neighbour-joining method (Saitou & Nei, 1987) and the maximum-parsimony method (Fitch, 1971) using the MEGA 3 program (Kumar *et al.*, 2004) with bootstrap values based on 1000 replications (Felsenstein, 1985).

A nearly complete 16S rRNA gene sequence of strain Slu-05^T (1449 bp) was obtained. Preliminary sequence comparison against 16S rRNA gene sequences deposited in the GenBank database indicated that strain Slu-05^T belonged to the family *Rhodocyclaceae* of the *Betaproteobacteria*. On the basis of 16S rRNA gene sequence similarity, the closest cultured relative was *A. fungiphilus* LMG 19178^T (96.4%), and the phylogenetic distances from other species of the *Rhodocyclaceae* with validly published names were greater than 5% (i.e. less than 95% similarity). This relationship between strain Slu-05^T and other members of the family *Rhodocyclaceae* was also evident in the phylogenetic tree (Fig. 1). Strain Slu-05^T and *A. fungiphilus* LMG 19178^T formed a monophyletic clade with a high bootstrap value (97%) supported by the two separate tree-making methods employed in this study.

The Gram reaction was determined using the non-staining method, as described by Buck (1982). Cell morphology was examined by using light microscopy (Nikon) and transmission electron microscopy (EM912Q; Leo Zeiss) after negative staining with 2% (w/v) uranyl acetate. Catalase activity was determined by bubble production in 3% (v/v) H₂O₂;

Fig. 1. Neighbour-joining tree, based on 16S rRNA gene sequences, showing the phylogenetic positions of strain Slu-05^T and its nearest neighbours. Dots indicate generic branches that were also recovered by using maximum-parsimony algorithms. Bootstrap values (expressed as percentages of 1000 replications) greater than 70% are shown at branch points. Bar, 1 substitution per 100 nucleotide positions.

oxidase activity was determined using 1% (w/v) tetramethyl *p*-phenylenediamine. The utilization of different substrates as sole carbon sources, along with the determination of some physiological characteristics, was assessed using API 32GN and API 20NE galleries according to the instructions of the manufacturer (bioMérieux). Tests for anaerobic growth were performed in a serum bottle containing R2A broth supplemented with thioglycolate (1 g l⁻¹) under N₂. Nitrate- and nitrite-reduction tests were performed in serum bottles containing R2A broth supplemented with KNO₃ (10 mM) and NaNO₂ (10 mM), respectively, and the reduction of nitrate and nitrite was monitored by an ion chromatograph (model 790 personal IC; Metrohm) equipped with a conductivity detector and an anion-exchange column (Metrosep Anion Supp 4; Metrohm). The degradation of DNA (using DNA agar from Difco, supplemented with 0.01% toluidine blue from Merck), chitin, CM-cellulose, starch (Atlas, 1993), lipid (Kouker & Jaeger, 1987) and xylan (Ten *et al.*, 2004) was also investigated; reactions were read after 5 days. Growth at different temperatures was assessed after 5 days incubation. Nitrogen-fixing ability was determined using growth in 50 ml nitrogen-free medium (DSMZ medium no. 3) and nitrogen-free SM medium (Reinhold *et al.*, 1986), including a carbon source which can be used for growth, contained in a 500 ml Erlenmeyer flask. Primer system PolF–PolR (Poly *et al.*, 2001) was used to amplify the *nifH* gene, as described by Im *et al.* (2004).

Cells of strain Slu-05^T were Gram-negative, non-spore-forming, slightly curved, short rods that were highly motile by means of single polar flagella. The rods were 0.7–1.1 µm in diameter and 1.6–2.3 µm in length (Fig. 2). Strain Slu-05^T grew well at 25–30 °C and also grew at 40 °C. Strain Slu-05^T has oxidase, catalase, urease and arginine dihydrolase activities but a negative result was obtained for indole production and for glucosidase, protease and galactosidase activities. Strain Slu-05^T assimilated malate, propionate, valerate, 3-hydroxybutyrate, L-proline, acetate, DL-lactate and malonate but not most other carbon sources (Table 1). Strain Slu-05^T cannot acidify glucose and cannot assimilate most polymers, such as DNA, chitin, lipid, CM-cellulose and xylan. Strain Slu-05^T reduced nitrate to N₂ in R2A broth, unlike *A. fungiphilus* LMG 19178^T. Strain Slu-05^T did not grow on nitrogen-free medium (DSMZ medium no. 3 or nitrogen-free SM medium), but it has a nitrogenase gene, and the *nifH* gene sequence of strain Slu-05^T (DQ029203) showed 94% similarity to that of *A. fungiphilus* LMG 19178^T (DQ029204).

For quantitative analysis of cellular fatty acid compositions, a loop of cell mass was harvested and the cellular fatty acids were saponified, methylated and extracted according to the protocol of the Sherlock Microbial Identification System (MIDI). The fatty acids were analysed in a gas chromatograph (Hewlett Packard 6890) and in a gas chromatograph/mass spectrometer (Hewlett Packard 5972) and identified using the Microbial Identification software package (Sasser, 1990). Isoprenoid quinones were extracted with chloroform/

Fig. 2. Morphological features of strain Slu-05^T, observed with a transmission electron microscope.

methanol (2:1, v/v), purified via TLC and subsequently analysed by HPLC, as described previously (Collins & Jones, 1981; Shin *et al.*, 1996). For the determination of G+C content, DNA was degraded enzymically into nucleosides and analysed by reversed-phase HPLC as described by Mesbah *et al.* (1989). *Escherichia coli* DNA (Sigma) was used as the calibration reference. DNA–DNA hybridization was performed fluorometrically, according to the method developed by Ezaki *et al.* (1989), using photobiotin-labelled DNA probes and microdilution wells. Hybridization was conducted with five replications for each sample. The highest and lowest values obtained for each sample were excluded and the DNA relatedness values quoted are the means of the remaining three values.

The cellular fatty acid profile of strain Slu-05^T was characterized by the predominance of C_{16:1ω7c}, C_{16:0}, C_{18:1} isomers and C_{10:0} 3-OH, and was similar to that of *A. fungiphilus* LMG 19178^T (Table 2). Both strain Slu-05^T and *A. fungiphilus* LMG 19178^T contained Q-8 as the major respiratory lipoquinone. The G+C content of the genomic DNA of strain Slu-05^T was 65.6 mol%. Strain Slu-05^T exhibited a relatively low level of DNA–DNA relatedness with respect to *A. fungiphilus* LMG 19178^T (15.6 %).

Table 1. Phenotypic characteristics of strain Slu-05^T and *A. fungiphilus* LMG 19178^T

Both strains have the following features: positive for utilization of malate, propionate, valerate, 3-hydroxybutyrate, L-proline, acetate and DL-lactate; negative for the assimilation of mannitol, N-acetylglucosamine, maltose, glucose, caprate, adipate, phenylacetate, salicin, D-melibiose, L-fucose, D-sorbitol, L-arabinose, histidine, 2-ketogluconate, rhamnose, D-ribose, inositol, D-sucrose, itaconate, suberate, L-alanine, 5-ketogluconate, glycogen, 3-hydroxybenzoate and L-serine; positive for urease activity, but negative for glucosidase, protease and galactosidase activities.

Characteristic	Slu-05 ^T	<i>A. fungiphilus</i> LMG 19178 ^T
Denitrification	+	–
Arginine dihydrolase	+	–
Utilization of:		
Citrate	–	+
4-Hydroxybenzoate	–	+
Malonate	+	–

The results of the chemotaxonomic and phylogenetic analyses allow strain Slu-05^T to be reliably assigned to the genus *Azonexus*. Accordingly, in view of the combined morphological, phenotypic, chemotaxonomic and phylogenetic data, strain Slu-05^T should be classified as a member of this genus, but it can be differentiated from *A. fungiphilus* on the basis of some physiological characteristics, such as denitrification, arginine dihydrolase activity and assimilation of malonate. The level of 16S rRNA gene sequence similarity (96.4 %) between strain Slu-05^T and *A. fungiphilus* LMG

Table 2. Cellular fatty acid profiles of strain Slu-05^T and *A. fungiphilus* LMG 19178^T

Values are percentages of total fatty acid content. Fatty acids representing less than 1.0 % in both strains were omitted; –, not detected.

Fatty acid	Slu-05 ^T	<i>A. fungiphilus</i> LMG 19178 ^T
Straight-chain fatty acids		
C _{12:0}	2.9	2.7
C _{14:0}	1.6	2.1
C _{15:0}	1.2	2.5
C _{16:0}	19.3	16.7
C _{18:0}	1.1	3.5
Unsaturated fatty acids		
C _{16:1ω7c}	48.0	40.2
C _{17:1ω6c}	–	1.0
C _{18:1} isomers	18.8	18.0
Hydroxy fatty acids		
C _{10:0} 3-OH	5.4	4.1
C _{12:0} 3-OH	1.1	0.7

19178^T is sufficiently low to exclude the possibility of assigning strain Slu-05^T to *A. fungiphilus* (Stackebrandt & Goebel, 1994). In addition, the DNA–DNA relatedness value between strain Slu-05^T and *A. fungiphilus* LMG 19178^T (15.6%) was far below the threshold value suggested for species delineation (Wayne *et al.*, 1987). Therefore, on the basis of the phenotypic, phylogenetic and genomic evidence, strain Slu-05^T should be placed in the genus *Azonexus* as a novel species, for which we propose the name *Azonexus caeni* sp. nov.

Description of *Azonexus caeni* sp. nov.

Azonexus caeni (ca.e'ni. L. gen. neut. n. *caeni* of sludge).

Aerobic, Gram-negative, non-spore-forming, slightly curved, short rods (0.7–1.1 × 1.6–2.3 µm), highly motile by means of single polar flagella. Can reduce nitrate to N₂ in R2A broth. Cells grow at 20–40 °C, with an optimum at 25–30 °C. Positive for urease and arginine dihydrolase activities, but negative for indole production and for glucosidase, protease and galactosidase activities. Carbon sources used include malate, propionate, valerate, 3-hydroxybutyrate, L-proline, acetate, DL-lactate and malonate; most other carbon sources are not utilized. The predominant respiratory lipoquinone is Q-8. The major fatty acids are C_{16:1}ω7c, C_{16:0}, C_{18:1} isomers and C_{10:0} 3-OH. The G + C content of the genomic DNA is 65.6 mol%.

The type strain, Slu-05^T (=DSM 17719^T=KCTC 12530^T=CCBAU 10199^T), was isolated from sludge from a wastewater treatment plant in Daejeon, South Korea.

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References

- Atlas, R. M. (1993). *Handbook of Microbiological Media*. Edited by L. C. Parks. Boca Raton, FL: CRC Press.
- Buck, J. D. (1982). Nonstaining (KOH) method for determination of Gram reactions of marine bacteria. *Appl Environ Microbiol* **44**, 992–993.
- Collins, M. D. & Jones, D. (1981). Distribution of isoprenoid quinone structural types in bacteria and their taxonomic implications. *Microbiol Rev* **45**, 316–354.
- Ezaki, T., Hashimoto, Y. & Yabuuchi, E. (1989). Fluorometric deoxyribonucleic acid–deoxyribonucleic acid hybridization in micro-dilution wells as an alternative to membrane filter hybridization in which radioisotopes are used to determine genetic relatedness among bacterial strains. *Int J Syst Bacteriol* **39**, 224–229.
- Felsenstein, J. (1985). Confidence limit on phylogenies: an approach using the bootstrap. *Evolution* **39**, 783–791.
- Fitch, W. M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Syst Zool* **20**, 406–416.
- Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
- Hurek, T., Wagner, B. & Reinhold-Hurek, B. (1997). Identification of N₂-fixing plant- and fungus-associated *Azoarcus* species by PCR-based genomic fingerprints. *Appl Environ Microbiol* **63**, 4331–4339.
- Im, W.-T., Bae, H.-S., Yokota, A. & Lee, S. T. (2004). *Herbaspirillum chlorophenicum* sp. nov., a 4-chlorophenol-degrading bacterium. *Int J Syst Evol Microbiol* **54**, 851–855.
- Kim, M. K., Im, W.-T., Ohta, H., Lee, M. & Lee, S.-T. (2005). *Sphingopyxis granuli* sp. nov., a β-glucosidase-producing bacterium in the family Sphingomonadaceae in α-4 subclass of the Proteobacteria. *J Microbiol* **43**, 152–157.
- Kimura, M. (1983). *The Neutral Theory of Molecular Evolution*. Cambridge: Cambridge University Press.
- Kouker, G. & Jaeger, K.-E. (1987). Specific and sensitive plate assay for bacterial lipase. *Appl Environ Microbiol* **53**, 211–213.
- Kumar, S., Tamura, K. & Nei, M. (2004). MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief Bioinform* **5**, 150–163.
- Mesbah, M., Premachandran, U. & Whitman, W. B. (1989). Precise measurement of the G + C content of deoxyribonucleic acid by high-performance liquid chromatography. *Int J Syst Bacteriol* **39**, 159–167.
- Poly, F., Monrozier, L. J. & Bally, R. (2001). Improvement in the RFLP procedure for studying the diversity of *nifH* genes in communities of nitrogen fixers in soil. *Res Microbiol* **152**, 95–103.
- Reinhold, B., Hurek, T., Niemann, E.-G. & Fendrik, I. (1986). Close association of *Azospirillum* and diazotrophic rods with different root zones of Kallar grass. *Appl Environ Microbiol* **52**, 520–526.
- Reinhold-Hurek, B. & Hurek, T. (2000). Reassessment of the taxonomic structure of the diazotrophic genus *Azoarcus sensu lato* and description of three new genera and new species, *Azovibrio restrictus* gen. nov., sp. nov., *Azospira oryzae* gen. nov., sp. nov. and *Azonexus fungiphilus* gen. nov., sp. nov. *Int J Syst Evol Microbiol* **50**, 649–660.
- Saitou, N. & Nei, M. (1987). The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.
- Sasser, M. (1990). *Identification of bacteria by gas chromatography of cellular fatty acids*. Technical Note no. 101. Newark, DE: MIDI.
- Shin, Y. K., Lee, J.-S., Chun, C. O., Kim, H.-J. & Park, Y.-H. (1996). Isoprenoid quinone profiles of the *Leclercia adecarboxylata* KCTC 1036^T. *J Microbiol Biotechnol* **6**, 68–69.
- Stackebrandt, E. & Goebel, B. M. (1994). Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.
- Ten, L. N., Im, W.-T., Kim, M.-K., Kang, M.-S. & Lee, S.-T. (2004). Development of a plate technique for screening of polysaccharide-degrading microorganisms by using a mixture of insoluble chromogenic substrates. *J Microbiol Methods* **56**, 375–382.
- Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.
- Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987). International Committee on Bacterial Systematics. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.