First publ. in: International Journal of Systematic Bacteriology 48 (1998), pp. 953-956

Azoarcus anaerobius sp. nov., a resorcinol-degrading, strictly anaerobic, denitrifying bacterium

Nina Springer,[1] Wolfgang Ludwig,[1] Bodo Philipp[2] and Bernhard Schink[2]

Author for correspondence: Bernhard Schink. Tel: +49 7531 88 2140. Fax: +49 7531 88 2966.
e-mail: bernhard.schink@uni-konstanz.de

[1] Lehrstuhl für Mikrobiologie der Technischen Universität München, Arcisstr. 16, D-80290 München, Germany

[2] Fakultät für Biologie, Universität Konstanz, Postfach 5560, D-78434 Konstanz, Germany

A strictly anaerobic, nitrate-reducing bacterium, strain LuFRes1, was isolated using resorcinol as sole source of carbon and energy. The strain reduced nitrate to dinitrogen gas and was not able to use oxygen as an alternative electron acceptor. Cells were catalase-negative but superoxide-dismutase-positive. Resorcinol was completely oxidized to CO_2. 16S rRNA sequence analysis revealed a high similarity with sequences of *Azoarcus evansii* and *Azoarcus tolulyticus*. Strain LuFRes1^T (= DSM 12081^T) is described as a new species of the genus *Azoarcus*, *Azoarcus anaerobius*.

Keywords: *Azoarcus anaerobius* sp. nov., resorcinol-degrading bacterium

INTRODUCTION

Degradation of aromatic compounds has been studied in much detail in the recent past. Whereas most aromatic compounds are degraded by the benzoyl-CoA pathway (2), other aromatics are degraded via resorcinol (1,3-dihydroxybenzene) or phloroglucinol (1,3,5-trihydroxybenzene) as key intermediates (8). Fermenting bacteria reduce resorcinol to dihydroresorcinol (1,3-dioxocyclohexane) before the ring is cleaved hydrolytically (6, 13). Nitrate-reducing bacteria use a different pathway for resorcinol degradation which does not include primary reduction (3, 6).

In experiments for enrichment of resorcinol-degrading, nitrate-reducing bacteria, a strictly anaerobic, denitrifying bacterium was isolated which was unable to use oxygen as an alternative electron acceptor (3). In the present study, this organism, strain LuFRes1^T, is described as the type strain of a new species, *Azoarcus anaerobius*, on the basis of 16S rRNA sequence comparisons.

METHODS

A pure culture of strain LuFRes1^T (DSM 12081^T) was taken from the laboratory collection. The strain has been deposited with DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) under the reference number indicated. Strain LuFRes1^T was originally isolated from anoxic sewage sludge under strictly anoxic conditions (3).

The strain was cultivated in an oxygen-free, bicarbonate-buffered medium (9, 15) which contained trace element solution SL10 (16), selenite tungstate solution (16) and seven-vitamin solution (15) under a N_2/CO_2 (80/20) atmosphere. Details of cultivation and characterization are given in the original description (3). Ammonium-free medium was prepared without addition of ammonium chloride. This medium contained less than 0·05 mM ammonium ions.

In vitro amplification and direct sequencing of 16S rRNA encoding DNA fragments was done as previously described (10). The new 16S rRNA sequence was fitted into an alignment of about 8000 homologous full and partial primary structures available in public databases (7) using the respective automated tools of the ARB software package (12). Distance-matrix, maximum-parsimony and maximum-likelihood methods were applied as implemented in the ARB software package. Different data sets that varied with respect to included outgroup reference organisms (sequences) as well as alignment positions were analysed.

RESULTS AND DISCUSSION

The physiological properties of strain LuFRes1^T have been documented in detail before (3); the taxonomically relevant points are summarized in the species description at the end of this section. Since nitrogen fixation is an important property of members of the genus *Azoarcus*, this ability was also studied in the present strain. Strain LuFRes1^T could be transferred in ammonium-free medium for more than ten generations; however, this was only in the presence of

The EMBL accession number for the sequence reported in this paper is Y14701.

Table 1. Overall 16S rRNA sequence similarities of strain LuFRes1^T and selected relatives of the β-subclass of the *Proteobacteria*. The range of sequence similarity is given for strain LuFRes1^T and various strains of *A. evansii* and *A. tolulyticus*. In all other cases, the mean similarity value is shown for strain LuFRes1^T and the reference species. The following species were studied (accession nos given in parentheses): 1, *A. evansii/A. tolulyticus* (L33687–L33692, U44853 and X77679); 2, *A. indigens* (L15531); 3, OS-ac-16 (U46748); 4, *Thauera selenatis* (X68491); 5, *Thauera aromatica* (X77118); 6, *Zoogloea ramigera* ATCC 19544 (X74913); and 7, *Rhodocyclus tenuis* (D16209).

Organism	Accession no.	Similarity (%)						
		1	2	3	4	5	6	7
LuFRes1^T	Y14701	96·1–97·1	95·0	93·8	93·8	94·2	92·7	90·7
A. evansii/A. tolulyticus	L33687–L33692, U44853, X77679		95·3	93·7	93·6	94·0	92·7	90·7
A. indigens	L15531			95·0	94·2	94·6	92·4	90·1
OS-ac-16	U46748				93·4	94·3	91·2	90·2
Thauera selenatis	X68491					97·7	91·7	90·8
Thauera aromatica	X77118						92·7	90·4
Zoogloea ramigera ATCC 19544	X74913							91·3

nitrate. No acetylene-reducing activity was found in these cultures. Cultures grown with N_2O as electron acceptor required the addition of ammonium chloride as nitrogen source. It is concluded that this strain does not express nitrogenase activity but can use nitrate as a nitrogen source through assimilatory nitrate reduction.

16S rRNA encoding DNA from strain LuFRes1^T was amplified *in vitro* and directly sequenced. A comparative database analysis revealed highest sequence similarity (96·1–97·1%; Table 1) with strains of *Azoarcus evansii* (1) and *Azoarcus tolulyticus* (17). However, strain LuFRes1^T is clearly separated from this cluster as indicated by remarkably higher intracluster values (98·1% and higher). Strain LuFRes1^T, together with the other *Azoarcus* and *Thauera* species as well as the isolate OS-ac-16, represents a phylogenetic subgroup of the β-subclass of the *Proteobacteria*.

According to the 16S rRNA sequence data, strain LuFRes1^T should be classified as a new species of the genus *Azoarcus*. So far, the genus comprises four validly described species: *Azoarcus communis* (4), *A. evansii* (1), *Azoarcus indigens* (5) and *A. tolulyticus* (17), which are represented by 16S rRNA sequence data in public databases. Strains of *A. evansii* and *A. tolulyticus* are closely related. Overall 16S rRNA sequence similarity among these strains is 98·1% and higher. The sequence data do not provide sufficient information for unambiguous assignment of the strains to two different species and their current taxonomy needs revision. In some cases, the overall 16S rRNA similarity is higher for strains of different species than for strains of the same species. *A. indigens* is separated from this cluster by sequence similarities of 95·1–95·4%. Unfortunately, in the case of *A. communis*, only partial sequences are available so far (4). The similarities of these partial sequences and the homologous parts of the 16S rRNA primary structures of the other *Azoarcus* species are in the range 95·1–95·4% which do not indicate a close relationship between *A. communis* and any other species.

The closest relatives of strain LuFRes1^T are *A. evansii* and *A. tolulyticus* (Fig. 1). The separate status of strain LuFRes1^T is indicated by 16S rRNA sequence similarity values of 96·1–97·1% (Table 1) with the members of the *A. evansii/A. tolulyticus* group, which are lower than the corresponding intragroup values (98·1% and higher; Table 1). Currently, the definition of a bacterial species is mainly based on the 70% criterion of genomic DNA similarity obtained by DNA–DNA hybridization (14). It is well-known that comparative 16S rRNA sequence analysis usually does not provide differentiating information at the strain level and often cannot be used even for species differentiation. The correlation of genomic DNA and rRNA similarities has been evaluated by Stackebrandt & Goebel (11). These authors described a level of rRNA sequence similarity of 97% and higher, at or above which DNA–DNA reassociation studies are a superior method. In the case of strain LuFRes1^T and the *A. evansii/A. tolulyticus* cluster, rRNA similarity values at or below this level justify the rRNA-based description of a new species of the genus *Azoarcus* although this strain does not fix nitrogen as most other members of this genus do.

Description of *Azoarcus anaerobius* sp. nov.

Azoarcus anaerobius (an.a.e.ro'bi.us. Gr. pref. *an* not; Gr. n. *aer* air; Gr. n. *bios* life; N.L. adj. *anaerobius* not living in air, anaerobic).

Rod-shaped bacterium, 2·7–3·3 × 1·5 μm in size, motile, Gram-negative, catalase-negative, superoxide-

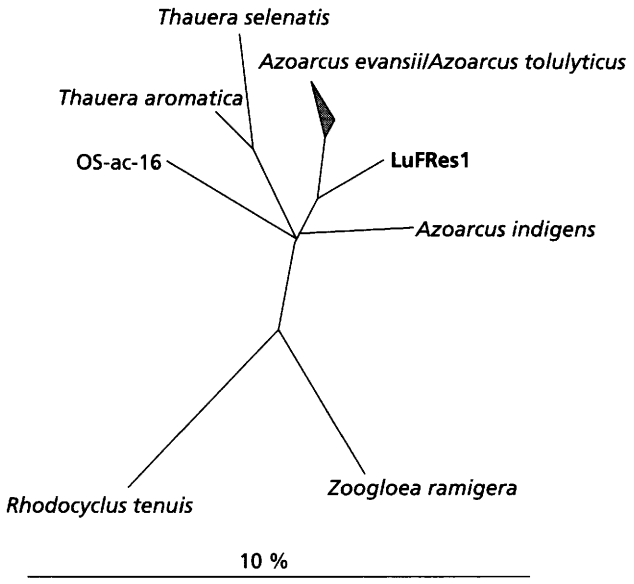

Fig. 1. 16S rRNA-based tree reflecting the relationships of strain LuFRes1^T and a selection of its closest relatives among members of the β-subclass of the *Proteobacteria*. Strain OS-ac-16 is a β-proteobacterium isolated from a hot spring microbial mat. The topology of the tree is based on the results of distance-matrix analysis. Only sequence positions which share identical residues among 50% of all available complete 16S rRNA sequences from the β-subclass *Proteobacteria* were included for tree construction. Multifurcations indicate that a common relative branching order was not supported by the results obtained performing different treeing methods. The bar indicates 10% estimated sequence divergence.

dismutase-positive. Poly-(β-hydroxy)butyrate is accumulated. Metabolism strictly oxidative, nitrate used as the only electron acceptor, which is quantitatively reduced to N_2 gas. Nitrite accumulates intermediately, N_2O was not detected. Sulfate, thiosulfate, sulfite, sulfur, trimethylamine *N*-oxide, DMSO, $Fe(OH)_3$, $K_3[Fe(CN)_6]$, or fumarate not reduced, oxygen not reduced, not even at low pressures in gradient cultures.

Acetate, propionate, butyrate, valerate, ethanol, propanol, lactate, pyruvate, fumarate, succinate, cyclohexanecarboxylate, phenol, resorcinol, benzoate, 3-hydroxybenzoate, 4-hydroxybenzoate, phenylacetate, *p*-cresol, phenylalanine, tyrosine used as electron donors. No growth with D,L-malate, adipate, D(+)-xylose, L(−)-arabinose, D(+)-glucose, D(−)-fructose, formate, 5-oxocaproate, pimelate, catechol, hydroquinone, 2-hydroxybenzoate, *o*-cresol, *m*-cresol. Growth only chemo-organoheterotrophic. No autotrophic growth with hydrogen or thiosulfate. Substrates completely oxidized to CO_2. No nitrogenase activity but assimilatory utilization of nitrate. Doubling time of growth with resorcinol plus nitrate 4·8–7·4 h. pH range 6·5–8·2; optimum pH 7·2. Temperature range 20–32 °C; optimum temperature 28 °C. Growth optimal in freshwater medium; enhanced salt concentrations impair growth. DNA G+C ratio 66·5 ± 0·5 mol%. Habitat is sewage sludge. The type strain is *Azoarcus anaerobius* LuFRes1^T (= DSM 12081^T).

ACKNOWLEDGEMENTS

This study was supported by a grant of the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg, in its special research program on biochemistry of anaerobic bacteria. The authors thank Norbert Gorny for his initial work on characterization of this strain. The technical assistance of Ingrid Pomper is highly acknowledged.

REFERENCES

1. **Anders, H. J., Kaetzke, A., Kaempfer, P., Ludwig, W. & Fuchs, G. (1995).** Taxonomic position of aromatic-degrading denitrifying pseudomonad strains K 172 and KB 740 and their description as new members of the genera *Thauera*, as *Thauera aromatica* sp. nov., and *Azoarcus*, as *Azoarcus evansii* sp. nov. *Int J Syst Bacteriol* **45**, 327–333.
2. **Fuchs, G., Mohamed, M., Altenschmidt, U., Koch, J., Lack, A., Brackmann, R., Lochmeyer, C. & Oswald, B. (1994).** Biochemistry of anaerobic biodegradation of aromatic compounds. In *Biochemistry of Microbial Degradation*, pp. 513–553. Edited by C. Ratledge. Dordrecht: Kluwer.
3. **Gorny, N., Wahl, G., Brune, A. & Schink, B. (1992).** A strictly anaerobic nitrate-reducing bacterium growing with resorcinol and other aromatic compounds. *Arch Microbiol* **158**, 48–53.
4. **Hurek, T. & Reinhold-Hurek, B. (1995).** Identification of grass-associated and toluene-degrading diazotrophs, *Azoarcus* spp., by analyses of partial 16S ribosomal DNA sequences. *Appl Environ Microbiol* **61**, 2257–2261.
5. **Hurek, T., Burggraf, S., Woese, C. R. & Reinhold-Hurek, B. (1993).** 16S rRNA-targeted polymerase chain reaction and oligonucleotide hybridization to screen for *Azoarcus* spp., grass-associated diazotrophs. *Appl Environ Microbiol* **59**, 3816–3824.
6. **Kluge, C., Tschech, A. & Fuchs, G. (1990).** Anaerobic metabolism of resorcylic acids (*m*-dihydroxybenzoic acids) and resorcinol (1,3-benzenediol) in a fermenting and in a denitrifying bacterium. *Arch Microbiol* **155**, 68–74.
7. **Ludwig, W. (1995).** Sequence databases. In *Molecular Microbial Ecology Manual*, chapter 3.3.5, pp. 1–22. Edited by A. D. L. Akkermans. Dordrecht: Kluwer.
8. **Schink, B., Brune, A. & Schnell, S. (1992).** Anaerobic degradation of aromatic compounds. In *Microbial Degradation of Natural Products*, pp. 219–242. Edited by G. Winkelmann. Weinheim: VCH.
9. **Schink, B. & Pfennig, N. (1982).** Fermentation of trihydroxybenzenes by *Pelobacter acidigallici* gen. nov. sp. nov., a new strictly anaerobic non-sporeforming bacterium. *Arch Microbiol* **133**, 195–201.
10. **Springer, N., Ludwig, W., Drozanski, V., Amann, R. & Schleifer, K. H. (1992).** The phylogenetic status of *Sarcobium lyticum*, an obligate intracellular parasite of small amoebae. *FEMS Microbiol Lett* **96**, 199–202.
11. **Stackebrandt, E. & Goebel, B. M. (1994).** Taxonomic note: a place for DNA–DNA reassociation and 16S rRNA se-

quence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* **44**, 846–849.

12. **Strunk, O. & Ludwig, W. (1995).** ARB- a software environment for sequence data. Department of Microbiology, University of Munich, Munich, Germany (e-mail arb@mikro.-biologie.tu-muenchen.de).
13. **Tschech, A. & Schink, B. (1985).** Fermentative degradation of resorcinol and resorcylic acids. *Arch Microbiol* **143**, 52–59.
14. **Wayne, L. G., Brenner, D. J., Colwell, R. R. & 9 other authors (1987).** International Committee on Systematic Bacteriology. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. *Int J Syst Bacteriol* **37**, 463–464.
15. **Widdel, F. & Pfennig, N. (1981).** Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. I. Isolation of new sulfate-reducing bacteria enriched with acetate from saline environments. Description of *Desulfobacter postgatei* gen. nov., sp. nov. *Arch Microbiol* **129**, 395–400.
16. **Widdel, F., Kohring, G. W. & Mayer, F. (1983).** Studies on dissimilatory sulfate-reducing bacteria that decompose fatty acids. 3: Characterisation of the filamentous gliding *Desulfonema limicola* gen. nov. sp. nov., and *Desulfonema magnum* sp. nov. *Arch Microbiol* **134**, 286–294.
17. **Zhou, J., Fries, M. R., Chee-Sanford, J. C. & Tiedje, J. M. (1995).** Phylogenetic analyses of a new group of denitrifiers capable of anaerobic growth on toluene and description of *Azoarcus tolulyticus* sp. nov. *Int J Syst Bacteriol* **45**, 500–506.