

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

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**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<sub>2</sub>. 16S rRNA sequence analysis revealed a high similarity with sequences of *Azoarcus evansii* and *Azoarcus tolulyticus*. Strain LuFRes1<sup>T</sup> (= DSM 12081<sup>T</sup>) 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<sup>T</sup>, 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<sup>T</sup> (DSM 12081<sup>T</sup>) 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<sup>T</sup> 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<sub>2</sub>/CO<sub>2</sub> (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<sup>T</sup> 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<sup>T</sup> could be transferred in ammonium-free medium for more than ten generations; however, this was only in the presence of

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

**Table 1.** Overall 16S rRNA sequence similarities of strain LuFRes1<sup>T</sup> and selected relatives of the  $\beta$ -subclass of the *Proteobacteria*. The range of sequence similarity is given for strain LuFRes1<sup>T</sup> and various strains of *A. evansii* and *A. toluolyticus*. In all other cases, the mean similarity value is shown for strain LuFRes1<sup>T</sup> and the reference species. The following species were studied (accession nos given in parentheses): 1, *A. evansii/A. toluolyticus* (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 <sup>T</sup>	Y14701	96.1–97.1	95.0	93.8	93.8	94.2	92.7	90.7
<i>A. evansii/A. toluolyticus</i>	L33687–L33692, U44853, X77679		95.3	93.7	93.6	94.0	92.7	90.7
<i>A. indigens</i>	L15531			95.0	94.2	94.6	92.4	90.1
OS-ac-16	U46748				93.4	94.3	91.2	90.2
<i>Thauera selenatis</i>	X68491					97.7	91.7	90.8
<i>Thauera aromatica</i>	X77118						92.7	90.4
<i>Zoogloea ramigera</i> ATCC 19544	X74913							91.3

nitrate. No acetylene-reducing activity was found in these cultures. Cultures grown with N<sub>2</sub>O 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<sup>T</sup> 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 toluolyticus* (17). However, strain LuFRes1<sup>T</sup> is clearly separated from this cluster as indicated by remarkably higher intra-cluster values (98.1% and higher). Strain LuFRes1<sup>T</sup>, together with the other *Azoarcus* and *Thauera* species as well as the isolate OS-ac-16, represents a phylogenetic subgroup of the  $\beta$ -subclass of the *Proteobacteria*.

According to the 16S rRNA sequence data, strain LuFRes1<sup>T</sup> 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. toluolyticus* (17), which are represented by 16S rRNA sequence data in public databases. Strains of *A. evansii* and *A. toluolyticus* 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

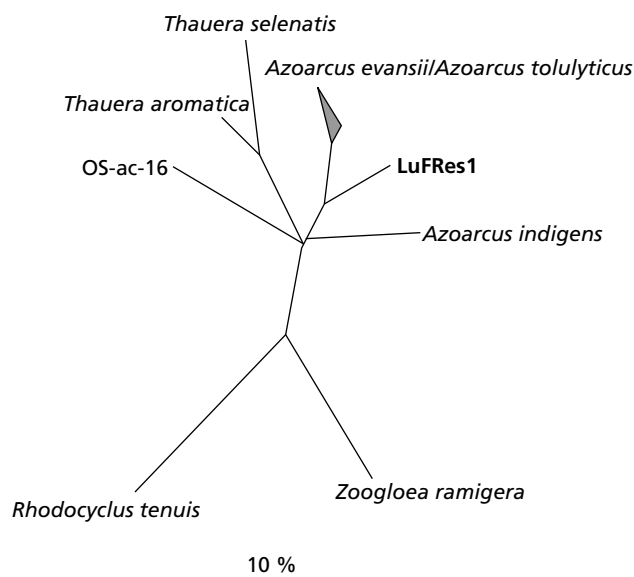
(4). The similarities of these partial sequences and the homologous parts of the 16S rRNA primary structures of the other *Azoarcus* species are in the range 95.1–95.4% which do not indicate a close relationship between *A. communis* and any other species.

The closest relatives of strain LuFRes1<sup>T</sup> are *A. evansii* and *A. toluolyticus* (Fig. 1). The separate status of strain LuFRes1<sup>T</sup> is indicated by 16S rRNA sequence similarity values of 96.1–97.1% (Table 1) with the members of the *A. evansii/A. toluolyticus* 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<sup>T</sup> and the *A. evansii/A. toluolyticus* 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  $\mu$ m in size, motile, Gram-negative, catalase-negative, superoxide-



**Fig. 1.** 16S rRNA-based tree reflecting the relationships of strain LuFRes1<sup>T</sup> and a selection of its closest relatives among members of the  $\beta$ -subclass of the *Proteobacteria*. Strain OS-ac-16 is a  $\beta$ -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  $\beta$ -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-( $\beta$ -hydroxy)butyrate is accumulated. Metabolism strictly oxidative, nitrate used as the only electron acceptor, which is quantitatively reduced to N<sub>2</sub> gas. Nitrite accumulates intermediately, N<sub>2</sub>O was not detected. Sulfate, thio-sulfate, sulfite, sulfur, trimethylamine *N*-oxide, DMSO, Fe(OH)<sub>3</sub>, K<sub>3</sub>[Fe(CN)<sub>6</sub>], 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<sub>2</sub>. 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<sup>T</sup> (= DSM 12081<sup>T</sup>).

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