Dechlorosoma suillum Achenbach *et al.* 2001 is a later subjective synonym of *Azospira oryzae* Reinhold-Hurek and Hurek 2000

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In order to clarify the taxonomic position of *Dechlorosoma suillum*, which shares 99.9% 16S rDNA sequence identity (1433 of 1435 bp) with *Azospira oryzae*, we compared the two species in a polyphasic taxonomic approach. Results of 122 physiological and biochemical tests for *D. suillum* DSM 13638^T and *Azospira oryzae* 6a3^T were identical, except for the lack of growth of *Azospira oryzae* 6a3^T with perchlorate as the terminal electron acceptor. Presence of a *nifH* gene and nitrogenase activity, a key feature of *Azospira*, were also detected in *D. suillum* by Southern hybridization and by the acetylene reduction assay, respectively. Whole-cell SDS-PAGE profiles of SDS-soluble proteins of strains DSM 13638^T and 6a3^T were almost identical. DNA–DNA hybridization studies showed more than 90% binding between *D. suillum* and two strains of *Azospira oryzae*. These data provide evidence that the two bacteria belong to the same species and that *D. suillum* is a later subjective synonym of *Azospira oryzae*.

The genera *Azoarcus*, *Azospira*, *Azovibrio* and *Azonexus* form phylogenetically distinct branches within the *Rhodocyclus* rDNA cluster of the β -subclass of the *Proteobacteria* (Reinhold-Hurek & Hurek, 2000). *Azospira oryzae* consists of nitrogen-fixing bacteria that occur as endophytes of grass roots, especially rice (Engelhard *et al.*, 2000), and in association with fungi (Reinhold-Hurek & Hurek, 2000). In the original description, they were assigned to the genus *Azoarcus sensu lato* (group D) (Reinhold-Hurek *et al.*, 1993). Partial and almost complete sequences of their 16S rRNA genes were respectively published by Hurek & Reinhold-Hurek (1995) and Hurek *et al.* (1997a). Cells of *Azospira oryzae* are motile rods that grow mostly on salts of organic acids and have a strictly respiratory metabolism with oxygen or nitrate as terminal electron acceptors.

Recently, bacteria that are capable of dissimilatory (per)chlorate reduction have been isolated from a wide range of environmental samples (Coates *et al.*, 1999; Michaelidou *et al.*, 2000). For four closely related isolates with 16S rDNA phylogenetic distances ranging from 0 to 0.12%, a new genus, *Dechlorosoma* gen. nov., was proposed that was reported to be phylogenetically most closely related to *Rhodocyclus* (Achenbach *et al.*, 2001). Strain PS^T was described as the type strain of *Dechlorosoma suillum* sp. nov. (Achenbach *et al.*, 2001). Bacteria of this species have a strictly respiratory metabolism with oxygen, nitrate or (per)chlorate as terminal electron acceptors.

In the phylogenetic trees published by Achenbach *et al.* (2001) and Coates *et al.* (2001), *Azospira* was not included; however, 16S rDNA sequences of *D. suillum* show highest

similarity to *Azospira oryzae*. In order to clarify the taxonomic relationships between *Azospira oryzae* and *D. suillum*, we carried out a polyphasic taxonomic analysis that suggested that these bacteria belong to the same species, *D. suillum* being a later subjective synonym of *Azospira oryzae*.

The type strain of *D. suillum*, DSM 13638^T, was received from the DSMZ. *Azospira oryzae* strains 6a3^T (=LMG 9096^T) and OM8A-5 and *Azovibrio restrictus* OSB2-4 were revived from stocks stored in liquid nitrogen in our laboratory. Unless stated otherwise, all strains were grown at 37 °C on VM ethanol medium (Reinhold-Hurek & Hurek, 2000).

Biotype 100 strips and API 20E systems with medium 2 (bioMérieux) were used to determine substrate utilization patterns and enzyme profiles. Both strips were inoculated according to the manufacturer's instructions. Growth was recorded after 1, 2, 4 and 7 days incubation at 30 °C (Reinhold-Hurek & Hurek, 2000). Data recorded at day 4 are given; they did not differ from recordings at day 7. Perchlorate reduction was tested by anaerobic culturing techniques as described previously (Bruce *et al.*, 1999; Coates *et al.*, 1999). For the medium, solutions A (1000 ml), B (10 ml) and C (10 ml) (<http://www.dsmz.de/media/med908.htm>) were autoclaved separately and anaerobically under N₂/CO₂ (90 : 10). Dissolved O₂ was removed from the medium by boiling. Vitamin solution (5 ml) and trace element solution SL-10 (1 ml) (<http://www.dsmz.de/media/med908.htm>), as well as sodium salts of acetate and chlorate (10 mM each) as the electron donor and acceptor (Coates *et al.*, 1999), were added.

For protein patterns, cells were grown on VM ethanol plates for 36 h at 37 °C. Extraction of SDS-soluble proteins and electrophoresis conditions were described before (Reinhold-Hurek & Hurek, 2000).

For Southern hybridization, genomic DNA was extracted (Hurek *et al.*, 1993) from *D. suillum* DSM 13638^T and *Azospira oryzae* 6a3^T and digested separately with restriction enzymes *Eco*RI and *Pst*I. Fragments were separated by agarose gel electrophoresis, transferred onto nylon filter membranes and hybridized to a DNA probe labelled with digoxigenin (Roche). The *nifH* gene probe was generated by PCR with primer pair TH25/TH26 from a plasmid carrying *nifH* of strain BH72 (Hurek *et al.*, 1997b). Hybridization was carried out at medium stringency as described by Hurek *et al.* (1997b).

For gas-chromatographic detection of nitrogenase activity, strains were grown on semi-solid nitrogen-free SM medium (Reinhold *et al.*, 1986) at 28 °C for 4 days. The acetylene reduction assay was carried out with 1% (v/v) acetylene and the ethylene formed was quantified by using a gas chromatograph with flame-ionization detector (Karg & Reinhold-Hurek, 1996).

For DNA–DNA hybridization, DNAs were extracted and purified according to the method of Marmur (1961). Levels of DNA relatedness were determined by the initial renaturation rate method (De Ley *et al.*, 1970) as described previously (Reinhold-Hurek *et al.*, 1993).

16S rDNA sequences obtained from the GenBank database were aligned by RDP (Ribosomal Database Project) (Maidak *et al.*, 1999). Distances of the aligned sequences (corresponding to positions 56–1492 of the *Escherichia coli* 16S rRNA gene) were calculated by the method of Jukes & Cantor (1969). The tree topology was inferred by the neighbour-joining method (Saitou & Nei, 1987) and the phylogenetic tree was constructed by using the TREECON software package (Van de Peer & de Wachter, 1994).

As the description of *D. suillum* did not include a detailed analysis of carbon sources for growth or enzymic activities (Achenbach *et al.*, 2001), we subjected *D. suillum* DSM 13638^T and *Azospira oryzae* strains 6a3^T and OM8A-5 to a comparative analysis of 122 physiological and biochemical tests. All three strains showed identical characteristics. They used D(–)-tartrate, L(–)-malate, DL-lactate, succinate, fumarate, DL-β-hydroxybutyrate (3-hydroxybutyrate), L-aspartate, L-glutamate, propionate, 2-oxoglutarate, butyrate, acetate and ethanol as sole carbon sources. Except for these 13 positive reactions (butyrate, acetate and ethanol tests were not included in Biotype 100 strip), all strains tested could not use the other 89 carbon sources supplied in the Biotype 100 strip. These physiological features are in agreement with previous observations for *Azospira oryzae* (Reinhold-Hurek & Hurek, 2000). All tested strains showed catalase, cytochrome *c* oxidase and denitrification activity, but no gelatinase, tryptophan deaminase, urease, ornithine

decarboxylase, lysine decarboxylase, arginine dihydrolase or β-galactosidase activity. No fermentation or oxidation of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin or arabinose occurred. These data demonstrated that *D. suillum* and *Azospira oryzae* have many physiological and biochemical characteristics in common. However, perchlorate reduction was only observed in *D. suillum*. In contrast to strain DSM 13638^T, *Azospira oryzae* strains 6a3^T and OM8A-5 did not show significant growth within 4 days in the presence of perchlorate.

For comparison of profiles of SDS-soluble proteins, three strains (*D. suillum* DSM 13638^T, *Azospira oryzae* 6a3^T and *Azovibrio restructus* OSB2-4) were used (Fig. 1a). *D. suillum* DSM 13638^T and *Azospira oryzae* 6a3^T had almost identical

Fig. 1. (a) SDS-PAGE of SDS-soluble whole-cell proteins of *D. suillum*, *Azospira oryzae* and *Azovibrio restructus* strains. Lanes: 1, molecular-mass standards; 2, *Azovibrio restructus* OSB2-4; 3, *D. suillum* DSM 13638^T; 4, *Azospira oryzae* 6a3^T. (b) Detection of a *nifH* gene homologue by Southern blot hybridization. Total DNA of bacteria digested with *Eco*RI (lanes 1 and 3) or *Pst*I (lanes 2 and 4) was hybridized with a digoxigenin-labelled *nifH* gene probe from *Azoarcus* sp. strain BH72 at medium stringency. Lanes 1 and 2, *D. suillum* DSM 13638^T; lanes 3 and 4, *Azospira oryzae* 6a3^T.

protein patterns. As a negative control, extracts of cells of a different diazotrophic member of the *Rhodocyclus*-group, *Azovibrio restrictus* OSB2-4, were significantly different.

Strains of *Azospira oryzae* were originally isolated from roots of Kallar grass and several species of rice under conditions of nitrogen fixation. Diazotrophy is a main feature of this genus and species. However, this feature had not been tested for *D. suillum* (Achenbach *et al.*, 2001). Southern hybridization of *D. suillum* DSM 13638^T with a *nifH* gene probe (Fig. 1b) indicated that this strain also harbours a *nifH*-homologous gene, albeit with a slight restriction fragment length polymorphism in comparison with *Azospira oryzae* 6a3^T. Reduction of acetylene (C₂H₂) to ethylene (C₂H₄) was detected by gas chromatography after 2–4 days of growth of strain DSM 13638^T in semi-solid N-free medium. This suggests that the *nifH* gene is functional and nitrogenase is present in *D. suillum*.

A phylogenetic analysis of the 16S rRNA genes of *Azospira oryzae*, *D. suillum* and related genera showed that *Azospira oryzae* and *D. suillum* were located in the same branch and formed a lineage distinct from others, supported by bootstrap values of 100 or 61 % (Fig. 2). The similarity of the 16S rDNA sequences of *Azospira oryzae* and *D. suillum* was 99.9 %, with only 2 bp different in 1435 bp. These data suggested that *Dechlorosoma* is highly related to *Azospira*.

The similarity level of DNA–DNA hybridization is crucial for assessment of bacterial species identity (Stackebrandt & Goebel, 1994). DNA–DNA hybridizations were performed between DNA of strains DSM 13638^T, 6a3^T and OM8A-5. Respective similarity levels of 90 and 92 % were recorded between strains DSM 13638^T and OM8A-5 and strains DSM 13638^T and 6a3^T. The high DNA relatedness, of more

than 70 %, strongly suggested that *D. suillum* and *Azospira oryzae* belong to the same species.

In conclusion, the following observations, based on the analysis of type strains, demonstrate that *D. suillum* and *Azospira oryzae* belong to the same species: (i) the 16S rDNA shows 99.9 % similarity; (ii) the SDS-soluble proteins show almost identical patterns in electrophoregrams, which indicates a high degree of genomic similarity (Kerstens, 1985); (iii) the two bacteria share most of the phenotypic characteristics tested, such as carbon source utilization, enzymic activities and diazotrophy, a key feature of the genus *Azospira* [the difference in a single characteristic, dissimilatory (per)chloride reduction, would not justify separation into two species or genera]; and (iv) the DNA–DNA binding level of 90–92 % is well above the suggested limit for species identity.

The name *Azospira oryzae* was validly published in 2000 (Reinhold-Hurek & Hurek, 2000), the taxon being first described as *Azoarcus sensu lato* group D in 1993 (Reinhold-Hurek *et al.*, 1993), and 16S rDNA sequences were available in GenBank and published as a fragment in 1995 (Hurek & Reinhold-Hurek, 1995) and as almost complete sequences in 1997 (Hurek *et al.*, 1997a). However, neither *Azoarcus* sp. group D nor *Azospira oryzae* was included in phylogenetic analyses of *D. suillum* (Achenbach *et al.*, 2001; Coates *et al.*, 1999, 2001). Our data, however, provide evidence that *D. suillum* (Achenbach *et al.*, 2001) is a later subjective synonym of *Azospira oryzae* (Reinhold-Hurek & Hurek, 2000) within the β -subclass of the *Proteobacteria*. This extends the known habitats for *Azospira oryzae*, which was previously thought to harbour exclusively plant- and fungus-associated strains.

Emended description of *Azospira oryzae* Reinhold-Hurek and Hurek 2000

Azospira oryzae (o.ry'zae. N.L. fem. n. *Oryza* genus name of rice; N.L. gen. n. *oryzae* from rice, referring to its frequent occurrence in association with rice roots).

The following description is based on the data of Reinhold-Hurek *et al.* (1993), Reinhold-Hurek & Hurek (2000) and Achenbach *et al.* (2001) and the data presented in this paper. On VM ethanol medium, colonies are translucent, pinkish to salmon-coloured, smooth, convex with an entire margin. Growth optimum at 37 °C. Sources of isolates are surface-sterilized roots of Gramineae such as Kallar grass [*Leptochloa fusca* (L.) Kunth] or rice (*Oryza*) species, grown in the Punjab of Pakistan or Nepal, Philippines and Italy, respectively, resting stages (sclerotia) of basidiomycetes (Pakistan) and (per)chlorate-reducing enrichments from samples collected from a primary treatment lagoon of swine waste in the USA.

The type strain is strain 6a3^T (=LMG 9096^T), which has a G+C content of 65.2 mol% and was isolated from Kallar grass.

Fig. 2. Phylogenetic tree of *Azospira oryzae*, *D. suillum* and related genera. The tree was constructed by the neighbour-joining method from 16S rDNA sequences. Bootstrap support percentages from 1000 replications are indicated for each node. GenBank accession numbers are shown in parentheses.

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