***Agrococcus citreus* sp. nov., isolated from a medieval wall painting of the chapel of Castle Herberstein (Austria)**

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A bacterial strain, D-1/1a^T, isolated from a medieval wall painting of the chapel of Herberstein (Styria, Austria) was characterized by a polyphasic approach. Strain D-1/1a^T shared 98.1% 16S rRNA sequence similarity to *Agrococcus jenensis*. The chemotaxonomic characteristics including polar lipid pattern, whole cell sugars, quinone system, polyamine pattern, cell wall composition and fatty acid profile were in good agreement with those of *Agrococcus jenensis*. The G+C content of the DNA was determined to be 74 mol%. The value of 47% DNA reassociation obtained after DNA–DNA hybridization between DNA of *Agrococcus jenensis* and strain D-1/1a^T as well as differences in the amino acid composition of the peptidoglycan and in physiological characteristics demonstrate that the isolate represents a new species of the genus *Agrococcus*. The name *Agrococcus citreus* sp. nov. is proposed for the new species harbouring isolate D-1/1a^T. The type strain is DSM 12453^T.

Keywords: *Agrococcus citreus* sp. nov., 16S rDNA sequence analysis, chemotaxonomy, physiology, DNA reassociation

INTRODUCTION

Many unheated buildings of historic and artistic value with mural paintings, such as old churches, provide favourable conditions for the growth of certain microorganisms (Karpovich-Tate & Rebrikova, 1990). Members of the actinomycetes are known to inhabit wall paintings of such places (Karpovich-Tate & Rebrikova, 1990; Sorlini *et al.*, 1987; Weirich, 1988; Petushkova *et al.*, 1990; Altenburger *et al.*, 1996).

During the course of isolation and classification of heterotrophic bacteria assumed to be responsible for biodecay from wall paintings in the chapel of the castle of Herberstein, a yellow pigmented coryneform bacterium was isolated. Here we describe the characterization of this strain, which could be clearly allocated to the genus *Agrococcus*. The name *Agro-*

coccus citreus sp. nov. is proposed. The type strain has been deposited in the German Collection of Microorganisms and Cell Cultures as strain DSM 12453^T.

METHODS

Bacterial strains and cultural conditions. Strain D-1/1a^T was isolated from the medieval wall painting of the chapel of the castle Herberstein. Surface material was scraped off to a depth of 3–10 mm from areas showing extensive damage. Samples were collected in a sterile tube, suspended in sterile saline, shaken for 1 h and dilutions were transferred on PYES-agar plates [3 g peptone (from casein) l⁻¹, 3 g yeast extract l⁻¹, 2.3 g Na₂-succinate l⁻¹ (all from Merck), pH 7.2]. Incubation was performed at room temperature.

Morphological and physiological characteristics. Cell morphology was determined by using phase-contrast microscopy of cultures of different ages. Colony morphology was studied with a stereo microscope. Acid production from carbohydrates was tested using the method of Hugh & Leifson (1953) modified by Gledhill & Casida (1969). Utilization of organic acids was studied in the medium described by Cowan & Steel (1965) by adding the sodium salts of organic acids to a final concentration of 0.2%.

Abbreviation: DAB, diaminobutyric acid.

The EMBL accession number for the 16S rDNA sequence of strain D-1/1a^T is AJ012826.

Table 1. Physiological properties of *Agrococcus citreus* and *Agrococcus jenensis*

All three strains were negative for decomposition of adenine, gelatin, hippurate, hypoxanthine, Tween 80, urea and xanthine, and acid production from cellobiose, dextrin, galactose, glucose, inulin, lactose, maltose, raffinose, salicin, sucrose, potato starch and trehalose, and utilization of benzoate, citrate, formate and DL-tartrate, and nitrate reduction. All three strains were negative in the Voges-Proskauer, methyl red, oxidase and indole tests. All three strains were positive for decomposition of aesculin, potato starch and tyrosine, and acid production from L-arabinose, fructose, mannitol, L-rhamnose, ribose and D-sorbitol, and utilization of acetate, and production of H₂S and catalase. All three strains were weakly positive for acid production from glycerol, mannose and D-xylose. All three strains grew in the presence of 2 or 4%, but not 10%, NaCl. All three strains grew at 28 or 37 °C, but not at 50 °C. All three strains were susceptible to the following antibacterial agents: ampicillin, 10 µg; chloramphenicol, 30 µg; erythromycin, 15 µg; gentamicin, 10 µg; kanamycin, 30 µg; lincomycin, 2 µg; neomycin, 30 µg; oxacillin, 5 µg; tetracycline, 30 µg; penicillin G, 10 IU; rifampicin, 30 µg; and streptomycin, 10 µg. All three strains were weakly positive for susceptibility to 300 IU polymyxin B and 200 µg sulfonamide. w, Weakly positive.

Character	<i>A. citreus</i> D-1/1a ^T	<i>A. jenensis</i> DSM 9580 ^T	<i>A. jenensis</i> DSM 9996
Decomposition of:			
Casein	—	+	—
Utilization of:			
Aconitate	+	—	—
Malate	—	+	+
Succinate	—	+	+
Antibiotic susceptibility:			
Ciprofloxacin, 5 µg	w	w	+
Nitrofurantoin, 200 µg	—	+	+

Nitrate reduction, urease activity, indole production, hydrogen sulfide production, hydrolysis of Tween 80 and gelatin, methyl red and Voges-Proskauer reactions were tested as described by Lanyi (1987). Catalase production and hydrolysis of casein and starch were tested by the methods of Gledhill & Casida (1969). Decomposition of adenine, hypoxanthine, xanthine and tyrosine was determined as recommended by Gordon *et al.* (1974). Oxidase activity was tested by the oxidation of 1% tetramethyl-*p*-phenylenediamine solution on discs of filter paper (Cowan & Steel, 1965). Hydrolysis of hippurate was carried out on hippurate agar (Cowan & Steel, 1965). Growth was tested at 28 and 37 °C, NaCl tolerance was checked at concentrations between 2 and 10% on R-medium. Susceptibility to antibiotics was examined by placing antibiotic discs (bioMérieux) on R-medium agar plates (Yamada & Komagata, 1972) seeded with a suspension of the test strains. Oxygen requirement was tested with Generbag anaer- and microaer-incubation systems (bioMérieux).

16S rDNA sequence analysis. The 16S rRNA gene from strain D-1/1a^T was amplified by the polymerase chain reaction (PCR) using the universal primers 27f and 1492r (Lane, 1991). Amplification products were purified as described by Muyzer *et al.* (1995) or by precipitation with PEG. For PEG precipitation, equal volumes of PCR products and 3M NaCl solution containing 24% PEG 6000 were mixed, incubated at 37 °C for 10 min and recovered by centrifugation at 16500 r.p.m. for 1 h at 4 °C. Purified PCR products were directly sequenced at the Service Department at the Vienna Biocentre (MIG-BASE) on a LI-COR 4000 L, as outlined by

Middendorf *et al.* (1992). The following fluorescently labelled primers were used: 27f, 926f, 530f and 1100r (Lane, 1991). The derived sequence was aligned and compared with those of other bacterial 16S rDNA sequences available in the EMBL database using the GCG program (Wisconsin Package, 1995).

DNA isolation and characterization. Isolation of DNA from acetone prewashed biomass and DNA-DNA hybridization (De Ley *et al.*, 1970; Escara & Hutton, 1980), as well as determination of renaturation rates (Huß *et al.*, 1983; Jahnke, 1992), were performed as described previously. The G+C content was determined as described previously (Groth *et al.*, 1996).

Chemotaxonomic investigations. Menaquinones were extracted and analysed as described previously (Tindall, 1990). Polar lipids were extracted and analysed by TLC according to the methods of Ventosa *et al.* (1993). Cellular fatty acid methylesters were analysed according to Groth *et al.* (1996). Detection of the diagnostic cell-wall diamino acid was performed by the method of Schleifer (1985). Analysis of the cell-wall amino acids was done as described by Groth *et al.* (1997). Whole-cell sugars were determined by the method of Schaal (1985). Extraction and detection of polyamine pattern were performed as described by Altenburger *et al.* (1997). The HPLC system (Waters) was equipped with two model 510 HPLC pumps, an U6K injector, a reversed phase column (Hypersil ODS RP 18, 250 × 4.6 cm, 5 µm particles) and with a Jasco model 821-FP fluorescence detector.

RESULTS

Morphological and cultural characteristics

Colonies of the isolate D-1/1a^T are yellow, opaque and convex with glistening surfaces in a range of 2–4 mm in diameter, colony margins are entire on nutrient agar. Cells of the isolate D-1/1a^T are Gram-positive and have irregular coccoid to rod-shaped forms of 0.7–1.0 × 1.1–1.7 µm. Cells occur singly, in pairs or occasionally in irregular clusters. A mycelial growth phase was never observed. Strain D-1/1a^T grew well on complex organic media at 28 °C. At 37 °C growth was observed, but not at 50 °C. No growth occurred under anaerobic conditions or in the presence of 10 % NaCl.

Physiological characteristics

The physiological properties of the isolate D-1/1a^T and those of the two *Agrococcus jenensis* strains DSM 9580^T and DSM 9996 are summarized in Table 1. In the recent study, the results of acid production from some sugars and of utilization of some organic compounds were found to be different from results reported previously for the type strain of the species *Agrococcus jenensis* (Groth *et al.*, 1996). In contrast to the previous study, *Agrococcus jenensis* DSM 9580^T produced acid from ribose, mannose and xylose but not from inulin, acetate was utilized and tyrosin decomposed. So far, this observation cannot be explained. Thus, these features should be considered as variable in the species description.

Chemotaxonomic characteristics

The major menaquinones detected in strain D-1/1a^T were MK-11 and MK-12. The characteristic cell wall diamino acid was diaminobutyric acid (DAB). In addition, alanine, glycine, aspartic and glutamic acid were detected. The cell wall Ala-Gly-DAB-Asp-Glu ratio was 3.6:2.1:0.8:0.9:1.0. L-Asp was found to be the amino-terminal amino acid of the interpeptide bridge. Predominant fatty acids were ai-C_{15:0}, ai-C_{17:0} and i-C_{16:0}, whilst i-C_{15:0}, i-C_{15:1}, i-C_{17:0} and C_{16:0} were present in minor amounts (Table 2). The poly-

Fig. 1. Two-dimensional TLC of a polar lipid extract obtained from strain D-1/1a^T and stained with molybdotetraphosphoric acid. DPG, diphosphatidylglycerol; PG, phosphatidylglycerol; GL1 and GL2, unknown glycolipids; L1–4, unknown polar lipids; P, pigments.

amine pattern contained mainly spermine. Diphosphatidylglycerol and phosphatidylglycerol were found to be predominant in the polar lipid extracts of the isolate D-1/1a^T. In addition, two unknown glycolipids and four non-characterized lipids were detected (Fig. 1). The same polar lipid profile was detected in *Agrococcus jenensis* DSM 9580^T (results not shown). The whole-cell hydrolysate contained glucose, rhamnose, mannose, ribose, galactose and tyvelose.

16S rDNA sequencing

The analyses of the 16S rRNA coding gene of strain D-1/1a^T resulted in a fragment of 1212 bases (positions 158–1392, *Escherichia coli* numbering; Brosius *et al.*, 1978). Sequence comparison with homologous sequences showed that strain D-1/1a^T shared the highest similarity with *Agrococcus jenensis* (98.1%). The sequence similarities to other species of the family *Microbacteriaceae* were in the range of 89.9% (*Rathayibacter rathayi*) and 93.8% (*Aureobacterium testaceum* and *Microbacterium aurum*).

Table 2. Fatty acid composition of *Agrococcus jenensis* DSM 9580^T and strain D-1/1a^T

The abbreviations for fatty acids are illustrated by the following examples: C_{16:0}, hexadecanoic acid; C_{18:1}, octadecenoic acid; i-C_{15:0}, 13-methyltetradecanoic acid; ai-C_{15:0}, 12-methyltetradecanoic acid.

	i-C _{14:0}	C _{14:0}	i-C _{15:0}	i-C _{15:1}	ai-C _{15:0}	C _{15:0}	i-C _{16:0}	C _{16:0}	C _{16:1}	i-C _{17:0}	C _{17:1}	ai-C _{17:0}	C _{18:0}	C _{18:1}
<i>A. jenensis</i> DSM 9580 ^T	0.6	0.3	12.2	1.9	57.8		12.6	2.0		1.9		9.3	0.1	0.1
D-1/1a ^T	0.6	0.3	10.0	5.6	53.1	0.1	12.0	1.7	0.2	1.7	0.1	13.2	0.2	0.1

G + C content and DNA–DNA hybridization

The G + C content of the isolate D-1/1a^T DNA was determined to be 74 mol %. Strain D-1/1a^T exhibited a DNA reassociation value of 47% with *Agrococcus jenensis* DSM 9580^T.

DISCUSSION

Group B murein-containing bacteria are located in a separate branch within the class *Actinobacteria* and belong to the family *Microbacteriaceae* (Park *et al.*, 1993; Rainey *et al.*, 1994; Stackebrandt *et al.*, 1997). The presence of diaminobutyric acid as diagnostic cell wall diamino acid is quite rare and only found in some taxa comprising the genera *Clavibacter*, *Rathayibacter*, *Cryobacterium*, *Agromyces*, *Agrococcus*, *Leucobacter* and in the misnamed species '*Brevibacterium helvolum*' and '*Corynebacterium aquaticum*' (Collins & Jones, 1980; Davis *et al.*, 1984; Döpfer *et al.*, 1982; Groth *et al.*, 1996; Sasaki *et al.*, 1998; Suzuki *et al.*, 1997; Takeuchi *et al.*, 1996; Zgurskaya *et al.*, 1992, 1993).

The partial analysis of the 16S rRNA sequence of strain D-1/1a^T and comparison to other sequences indicated that this isolate belongs to the genus *Agrococcus*. The only species, *Agrococcus jenensis* is phylogenetically clearly separated from other members of the family *Microbacteriaceae* (Groth *et al.*, 1996; Suzuki *et al.*, 1997). Chemotaxonomic characteristics such as cell wall composition, polyamine pattern, menaquinone, whole cell sugars and fatty acid profile were in accordance with the characteristics of *Agrococcus jenensis* DSM 9580^T and different from the other DAB-containing taxa (Groth *et al.*, 1996; Altenburger *et al.*, 1997). The polar lipid profile of strain D-1/1a^T was different from that reported for *Agrococcus jenensis* DSM 9580^T (Groth *et al.*, 1996). It consisted not only of phosphatidylglycerol, diphosphatidylglycerol and two unknown glycolipids, but also contained four non-characterized polar lipids. Reinvestigation of the polar lipid profile of *Agrococcus jenensis* DSM 9580^T displayed the same constituents as found in strain D-1/1a^T (results not shown).

In respect to physiological properties, strain D-1/1a^T could be distinguished from the two strains of *Agrococcus jenensis* based on the utilization of three organic acids and susceptibility to an antibiotic. In contrast to *Agrococcus jenensis* strains, isolate D-1/1a^T utilized aconitate, but not malate and succinate and it was resistant to nitrofurantoin.

DNA–DNA hybridization studies and cell wall analysis clearly indicate that strain D-1/1a^T and *Agrococcus jenensis* do not belong to the same species. Strain D-1/1a^T exhibited a low level of DNA reassociation with *Agrococcus jenensis* (47%). Furthermore the cell wall of isolate D-1/1a^T contained no threonine and is therefore clearly distinguishable from that of *Agrococcus jenensis*.

On the basis of our results it is demonstrated that

strain D-1/1a^T represents a new species of the genus *Agrococcus* and we propose the name *Agrococcus citreus* sp. nov. for this strain.

Description of *Agrococcus citreus* sp. nov.

Agrococcus citreus (cit.re.us. M.L. adj. *citreus* describing the lemon-yellow pigmentation).

Cells are irregular spherical, ovoid or rodshaped. The cell sizes vary in a range of 0.7–1.0 × 1.1–1.7 µm. Cells occur singly, in pairs or in small irregular clusters. They are Gram-positive and non-motile. Endospores are not formed, colonies are slight convex and smooth. Colony diameters are in a range of 2–4 mm. The colour of the colonies on nutrient agar is yellow. Aerobic and microaerophilic. Catalase is produced. Oxidase-negative. Nitrate is not reduced to nitrite. Acids are produced from arabinose, fructose, mannitol, rhamnose, ribose and sorbitol. A weak acid production is observed from mannose, glycerol and xylose. No acid production was observed from cellobiose, dextrin, galactose, glucose, glycerol, inulin, lactose, maltose, raffinose, salicin, sucrose, starch or trehalose. Acetate and aconitate are utilized as carbon sources. Benzoate, citrate, formate, malate, succinate and tartrate are not utilized. Starch, aesculin and tyrosine are decomposed. Adenine, casein, gelatin, hippurate, xanthine, hypoxanthine, urea and tween 80 are not decomposed. Cells are susceptible to ampicillin, chloramphenicol, ciprofloxacin, erythromycin, gentamicin, kanamycin, lincomycin, neomycin, oxacillin, tetracycline, penicillin G, polymyxin B, rifampicin and streptomycin. Isolate D-1/1a^T is resistant to nitrofurantoin. The cell wall diamino acid is diaminobutyric acid (DAB), additionally alanine, glycine, glutamic acid, and aspartic acid are found. No threonine is present. The whole cell sugars are glucose, rhamnose, mannose, ribose, galactose and tyvelose. The polar lipid profile is composed of diphosphatidylglycerol, phosphatidylglycerol, two unknown glycolipids and four uncharacterized polar lipids. The fatty acid profile consists of the predominant compounds ai-C_{15:0}, ai-C_{17:0} and i-C_{16:0}, whereas i-C_{15:0}, i-C_{15:1}, i-C_{17:0} and C_{16:0} were present in minor amounts. The main menaquinones are MK-11 and MK-12. Spermine is the predominant compound in the polyamine pattern. The G + C content is 74 mol %. Strain D-1/1a^T has been isolated from a medieval wall painting in the church of castle Herberstein in Styria, Austria. The type strain, D-1/1a^T, has been deposited in the DSMZ–German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany, as strain DSM 12453^T.

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