***Agrococcus baldri* sp. nov., isolated from the air in the 'Virgilkapelle' in Vienna**

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Five coccoid, Gram-positive strains were isolated from the air of the 'Virgilkapelle' in Vienna. A representative of these five strains, V-108^T, shared 99.0 and 98.4% 16S rDNA sequence similarity, respectively, with *Agrococcus jenensis* DSM 9580^T and *Agrococcus citreus* DSM 12453^T. Colonies of the five strains were white when grown in the dark and turned yellow in the light. The strains displayed highly similar biochemical and physiological characteristics and showed only small differences in their protein patterns obtained after SDS-PAGE. Based on Fourier-transform infra-red (FT-IR) spectra, the five strains were grouped together and separated from the other members of the genus, *A. jenensis* and *A. citreus*. Chemotaxonomic characteristics analysed from selected members of the five isolates, including polar lipids, quinone systems, polyamine patterns, cell wall composition and fatty acid profiles, were in good agreement with those of the two species of the genus *Agrococcus* described to date. The G+C content of the genomic DNA was determined to be within the narrow range of 73.8–74.9 mol%. The results of DNA–DNA hybridization with *A. citreus* DSM 12453^T and *A. jenensis* DSM 9580^T, as well as differences in biochemical/physiological characteristics, peptidoglycan composition, fatty acids, polar lipid profiles and FT-IR spectra, demonstrated that the five isolates represent a novel species of the genus *Agrococcus*. The name *Agrococcus baldri* sp. nov. is proposed for the novel species, of which strain V-108^T (= DSM 14215^T = CCM 4953^T) is the type strain.

Keywords: *Agrococcus baldri* sp. nov., 16S rDNA sequence, chemotaxonomy, biochemical/physiological traits

INTRODUCTION

In a project concerning bacterial colonization and biodiversity, two experimental wall paintings were exposed in the chapels 'Virgilkapelle' in Vienna and the 'Katharinenkapelle' in the castle Herberstein (Styria, Austria). The wall paintings had been constructed using the same materials and had been treated equally. In order to study the influence of airborne bacteria on the colonization of the wall paintings, the biodiversity among airborne bacteria collected at the two locations was studied.

Among the airborne strains collected in the 'Virgilkapelle' underneath the Stephansplatz in

Vienna, five coccoid, yellow-pigmented, airborne bacteria were isolated that showed photochromogenic behaviour. Characterization of these strains demonstrated that they are members of the genus *Agrococcus*, which so far consists of two species, *Agrococcus jenensis* and *Agrococcus citreus*. Strains of *A. jenensis* have been isolated from compost soil and sandstone (Groth *et al.*, 1996), whereas *A. citreus* was isolated from a medieval wall painting in the 'Katharinenkapelle' inside the castle Herberstein (Wieser *et al.*, 1999). Here, we present evidence that the five airborne strains represent a single novel species of the genus *Agrococcus*.

METHODS

Bacterial strains and cultural conditions. The five strains V-108^T, V-50, V-64, V-98 and V-109 were isolated from the air of the 'Virgilkapelle' in Vienna using an air sampler (Biotest

Abbreviation: FT-IR, Fourier-transform infra-red.

The GenBank accession number of the 16S rDNA sequence of *Agrococcus baldri* V-108^T is AJ309928.

RCS Plus). The strains were collected on CasMM agar (Altenburger *et al.*, 1996). CasMM strips were incubated at room temperature. After 4 days, single colonies were transferred to PYES agar [3 g peptone from casein l⁻¹, 3 g yeast extract l⁻¹, 2.3 g disodium succinate l⁻¹, 15 g agar l⁻¹ (all from Merck), pH 7.2] and incubated at room temperature.

Morphological, biochemical and physiological characteristics. Cell morphology was determined by phase-contrast microscopy of cultures of different ages. Colony morphology was studied with a stereo microscope. Gram-staining and the KOH test were performed as described previously (Moaledji, 1986). Utilization of organic acids and carbohydrates was studied in the medium described by Kämpfer *et al.* (1991). Acid production from carbohydrates was tested using Phenolred-Bouillon (Merck) with the carbohydrates added to a concentration of 7.5 g l⁻¹. Nitrate reduction was tested with nitrate broth (Merck), urease activity with urea-indole medium (bioMérieux), indole production, motility and hydrogen sulfide production with SIM agar (Oxoid) and methyl red and Voges-Proskauer reactions with VP-MR agar (Oxoid). Oxidase activity was tested with Bactident oxidase strips (Merck). All tests were carried out according to the instructions of the manufacturers. Catalase production was tested by the method of Gledhill & Casida (1969). Hydrolysis of casein, hippurate, Tween 20 and Tween 80 was tested as described previously (Gordon *et al.*, 1973; Isenberg, 1992; Cowan & Steel, 1974). Hydrolysis of starch and tyrosine was tested by adding double-concentrated nutrient agar (Gordon *et al.*, 1973) to equivalent and separately autoclaved amounts of 10% potato starch solution and 1% tyrosine solution in water, respectively. Growth at 4, 28, 37 and 42 °C was tested on PYES agar. NaCl tolerance was investigated on PYES agar supplemented with 2, 4, 6.5 and 10% NaCl. Microaerobic growth was studied on tryptic soy agar (TSA) in an anaerobic jar using an atmosphere consisting of 5% H₂, 5% CO₂ and 90% N₂, as recommended by Fox *et al.* (1994).

16S rDNA sequence analysis. Two loops of biomass were scraped off the agar plates, suspended in 100 µl sterile water and lysed by repeated cycles of freezing in liquid nitrogen and thawing at 50 °C. The DNA contained in 15 µl of the supernatant after centrifugation was used as the template for PCR amplification. 16S rDNA was amplified by the PCR using the universal primers 27f and 1492r (Lane, 1991). Amplification products were purified by precipitation with polyethylene glycol (Wieser *et al.*, 1999). Purified PCR products were sequenced directly at the Service Department of the Vienna Biocenter (MIG-BASE) on a LI-COR 4000 L sequencer, as outlined by Middendorf *et al.* (1992). Fluorescently labelled primers 27f and 1492r were used (Lane, 1991). The derived sequence was aligned and compared with other bacterial 16S rDNA sequences available in the EMBL database using the Wisconsin package (GCG, 1995).

DNA isolation and characterization. Isolation of DNA from acetone-pre-washed biomass and DNA-DNA hybridization were performed as described previously (Lind & Ursing, 1986; Ziemke *et al.*, 1998). The G+C content was determined as described previously (Cerdà-Cuéllar *et al.*, 1997).

Chemotaxonomic investigations. Menaquinones were extracted and analysed as described previously (Tindall, 1990). Polar lipids were extracted and analysed by TLC according to Ventosa *et al.* (1993). Cellular fatty acid methyl esters were extracted and analysed according to Osterhout *et al.* (1991) and Wieser & Busse (2000).

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). Extraction of polyamines and detection of their patterns were performed as described by Altenburger *et al.* (1997). For Fourier-transform infra-red (FT-IR) analysis, strains were grown on PYES agar at 28 °C for 48 h. One loopful of cells was resuspended in 80 µl deionized water and an aliquot of 35 µl was applied to a pre-defined sample area on a zinc selenite optical plate. The samples were dried under reduced pressure to give a homogeneous film. The IR spectra were recorded in transmission mode between wave numbers 3000 and 699 cm⁻¹ in an FT-IR spectrometer type IFS 28/B (Bruker) as described by Helm *et al.* (1991a, b). Data acquisition and analysis were carried out using the OPUS 3.0 software for bacterial identification from the same manufacturer. Mean spectra were calculated from six independent measurements. First derivatives of the spectra were compared with the hierarchical cluster analysis methods implemented in OPUS 3.0.

SDS-PAGE. The protein patterns of strains V-108^T, V-50, V-64, V-98 and V-109 were investigated by whole-cell-extract SDS-PAGE (Altenburger *et al.*, 1996).

RESULTS AND DISCUSSION

Morphological, physiological and biochemical characteristics

Colonies of the five isolates were light yellow when grown in the light and white when grown in the dark. They were opaque and convex with glistening surfaces, 2–7 mm in diameter. Colony margins were entire on nutrient agar. Cells of the isolates were irregular coccoid to rod-shaped, 0.7–1.0 µm wide by 1.1–1.7 µm long. Cells occurred singly, in pairs or occasionally in irregular clusters. They were non-motile. A mycelial growth phase was not observed. The five isolates showed Gram-positive behaviour in staining and KOH test. They grew best on complex organic media such as TSA at temperatures ranging from 18 to 28 °C and in the presence of 4% NaCl. Weak growth was observed at 4 and 37 °C. No growth occurred at 42 °C or in the presence of 10% NaCl or under microaerobic conditions. Biochemical and physiological properties of the five isolates are summarized in Table 1. Among 108 biochemical and physiological characteristics tested, these five strains showed variable results in 17 traits.

16S rDNA sequencing and DNA-DNA relatedness

Analysis of the 16S rDNA of strain V-108^T resulted in a fragment of 1400 bases (positions 50–1469, *Escherichia coli* numbering; Brosius *et al.*, 1978). Sequence comparison with corresponding sequences (positions 158–1392, *E. coli* numbering) showed that strain V-108^T shared the highest similarities with *A. jenensis* DSM 9580^T (99.0%) and *A. citreus* DSM 12453^T (98.4%). The genus *Agrococcus* constitutes a separate lineage within the family *Microbacteriaceae* (Park *et al.*, 1993; Rainey *et al.*, 1994; Groth *et al.*, 1996; Stackebrandt *et al.*, 1997; Suzuki *et al.*, 1997;

Table 1. Physiological and biochemical properties of *Agrococcus baldri* sp. nov., *A. citreus* and *A. jenensis*

+, Positive; —, negative; d, some strains positive. Symbols indicate results that are not in line with those reported previously for *A. jenensis* (Groth *et al.*, 1996) (†) and/or *A. citreus* (Wieser *et al.*, 1999) (‡). All eight strains were negative for decomposition of casein, † gelatin, Tween 80 and urea, acid production from D-glucose, D-trehalose, D-galactose, D-mannose, ‡ D-ribose, ‡ D-lactose, D-melibiose, D-inulin, † D-xylose, ‡ D-raffinose, *myo*-inositol, dulcitol and sorbitol, utilization of *p*-arbutin, D-melibiose, salicin, adonitol, *i*-inositol, maltitol, acetate, ‡ propionate, adipate, azelate, citrate, glutarate, itaconate, mesaconate, pyruvate, suberate, L-alanine, β -alanine, L-aspartate, L-histidine, L-leucine, L-ornithine, L-phenylalanine, L-serine, L-tryptophan, 3-hydroxybenzoate, 4-hydroxybenzoate and phenylacetate and hydrolysis of *p*-nitrophenyl (pNP) β -D-galactopyranoside, pNP β -D-glucuronide, 2-deoxythymidine-5'-pNP phosphate, L-alanine *p*-nitroanilide (pNA), L-glutamate- γ -3-carboxy pNA and L-proline pNA. All eight strains were negative in the Voges–Proskauer, methyl red, oxidase and indole tests, as well as in production of H₂S†‡ and nitrate reduction. All eight strains were positive for decomposition of potato starch and hippurate, †‡ acid production from D-sucrose, †‡ L-rhamnose and D-mannitol, hydrolysis of pNP α -D-glucopyranoside and pNP phenylphosphonate and production of catalase. All eight strains grew in the presence of 2 and 4 % NaCl, but not 10 % NaCl. All eight strains grew well at 28 °C and weakly at 4 and 37 °C, but not at 42 °C.

Characteristic	<i>A. baldri</i> (n = 5)	<i>A. citreus</i> (n = 1)	<i>A. jenensis</i> (n = 2)
Utilization of:			
N-Acetyl D-galactosamine	+	—	—
L-Arabinose	—	+	—
D-Cellobiose	+	+	—
D-Fructose	+	+	—
D-Galactose	d (4/5) ^{a*}	+	—
Gluconate	+	+	—
D-Glucose	+	+	—
D-Mannose	+	+	—
D-Maltose	+	+	—
L-Rhamnose	+	+	—
D-Ribose	+	+	—
Sucrose	d (4/5) ^b	+	—
D-Trehalose	+	+	—
D-Xylose	—	+	—
D-Mannitol	d (1/5) ^c	—	—
D-Sorbitol	d (1/5) ^d	—	—
Putrescine	d (4/5) ^a	—	—
<i>cis</i> -Aconitate	d (1/5) ^d	—	—
<i>trans</i> -Aconitate	d (1/5) ^c	—	—
4-Aminobutyrate	+	—	—
Fumarate	d (4/5) ^e	—	—
DL-3-Hydroxybutyrate	d (2/5) ^f	+	—
DL-Lactate	d (4/5) ^e	+	—
L-Malate	d (4/5) ^e	—	—†
Oxoglutarate	d (3/5) ^g	+	—
L-Proline	+	+	—
Hydrolysis of:			
pNP β -D-glucopyranoside	—	+	+
bis-pNP phosphate	d (2/5) ^f	+	+
pNP phosphorylcholine	—	—	d ^h
Acid production from:			
L-Arabinose	—	+	+
Maltose	d (4/5) ⁱ	—	—
Glycerol	d (4/5) ⁱ	—‡	—†

Table 1 (cont.)

Characteristic	<i>A. baldri</i> (n = 5)	<i>A. citreus</i> (n = 1)	<i>A. jenensis</i> (n = 2)
Decomposition of:			
Aesculin	—	+	+
Tyrosine	d (4/5) ⁱ	+	d ^h †
Tween 20	—	+	+
Growth in the presence of 6.5 % NaCl	d (2/5) ^k	—	—

* Strain-dependent results are indicated as: a, V-50 negative; b, V-108^T negative; c, V-108^T positive; d, V-109 positive; e, V-109 negative; f, V-108^T and V-64 positive; g, V-98 and V-109 negative; h, *A. jenensis* DSM 9996 positive; i, V-98 negative; j, *A. jenensis* DSM 9580^T positive; k, V-64 and V-109 positive.

† Data not in line with Groth *et al.* (1996).

‡ Data not in line with Wieser *et al.* (1999).

Wieser *et al.*, 1999) of the *Actinobacteria*. The sequence similarities of V-108^T to other species of the family *Microbacteriaceae* were in the range 91.0–94.0%. These data demonstrate clearly that strain V-108^T belongs to the genus *Agrococcus*.

Almost identical protein patterns obtained after SDS-PAGE (results not shown) indicated that the five strains are members of a single species. DNA reassociation values of V-108^T with the other four strains were in the range 95.0–100%, confirming that they are members of a single species. A relatively close relationship of the five isolates to *A. jenensis* DSM 9580^T was indicated from DNA relatedness values between 61 and 69%, whereas values of only 30–40% were detected with *A. citreus* DSM 12453^T. These data indicate that the five strains are separate from *A. citreus* but closely related to *A. jenensis*. The G+C content of the genomic DNA of isolate V-108^T DNA was determined to be 74.8 mol%. The other four strains were shown to have genomic DNA G+C contents of 73.8–74.9 mol%.

Chemotaxonomic characteristics

Comparison of FT-IR spectra revealed a high degree of similarity between strains V-108^T, V-50, V-64, V-98 and V-109, as indicated from their clustering and separation from *A. jenensis* DSM 9580^T, *A. jenensis* DSM 9996 and *A. citreus* DSM 12453^T (Fig. 1). These data confirm not only the results from DNA relatedness and protein patterns, which demonstrate that the five isolates are members of a single species, but also the suitability of FT-IR spectra for species affiliation within this group of bacteria. The major menaquinones detected in strain V-108^T were MK-11 (41%) and MK-12 (47%). In addition, small amounts of MK-10 (9%) and MK-9 (3%) were found. A similar menaquinone system was also detected for strain V-64. The characteristic cell wall diamino acid was diaminobutyric acid. In addition, alanine, glycine and aspartic and glutamic acids were detected.

Fig. 1. Average linkage dendrogram based on the distances of first derivatives of FT-IR spectra (frequency ranges 3000–2800 cm⁻¹; 1201–900 cm⁻¹; 901–699 cm⁻¹).

Aspartic acid was found to be the amino-terminal amino acid of the interpeptide bridge. The presence of diaminobutyric acid as a diagnostic cell wall diamino acid is found in several taxa within the family *Microbacteriaceae*, including the genera *Clavibacter*, *Rathayibacter*, *Cryobacterium*, *Agromyces*, *Agrococcus*, *Leucobacter* and *Leifsonia*, and also in the misnamed species '*Brevibacterium helvolum*' (Collins & Jones, 1980; Davis *et al.*, 1984; Döpfer *et al.*, 1982; Evtushenko *et al.*, 2000; Groth *et al.*, 1996; Sasaki *et al.*, 1998; Suzuki *et al.*, 1997; Takeuchi *et al.*, 1996; Zgurskaya *et al.*, 1992, 1993). Like *A. citreus* DSM 12453^T, the peptidoglycan of isolate V-108^T contained no threonine, while this amino acid was reported to be present in the cell wall of *A. jenensis* DSM 9580^T (Groth *et al.*, 1997). Strain V-108^T showed a polyamine pattern with the single major compound spermine [0.34 µmol (g dry weight)⁻¹]. This polyamine pattern has been reported for *A. jenensis* DSM 9580^T and *A. citreus* DSM 12453^T only (Altenburger *et al.*, 1997; Wieser *et al.*, 1999).

Diphosphatidylglycerol and phosphatidylglycerol were found to be predominant in polar lipid extracts of strains V-108^T, V-64 and V-98. In addition, two

Table 2. Fatty acid composition of *A. baldri*, *A. citreus* and *A. jenensis*

Strains: 1–5, *A. baldri* strains V-108^T (1), V-50 (2), V-64 (3), V-98 (4) and V-109 (5); 6, *A. jenensis* DSM 9580^T (data from Groth *et al.*, 1996); 7, *A. citreus* DSM 12453^T (data from Wieser *et al.*, 1999).

Fatty acid	1	2	3	4	5	6	7
i-C _{14:0}						0.6	0.6
C _{14:0}						0.3	0.3
i-C _{15:0}	5.7	5.5	4.7	6.8	5.8	12.2	10.0
i-C _{15:1}						1.9	5.6
ai-C _{15:0}	44.9	42.3	42.3	48.3	49.0	57.8	53.1
C _{15:0}							0.1
i-C _{16:0}	7.1	6.3	7.0	8.8	8.1	12.6	12.0
C _{16:0}	3.0	3.2	2.8	3.0	2.7	2.0	1.7
C _{16:1}							0.2
i-C _{17:0}	1.5	1.8	1.2	1.7	1.7	1.9	1.7
C _{17:1}							0.1
ai-C _{17:0}	24.3	23.7	24.6	26.0	28.0	9.3	13.2
C _{18:0}						0.1	0.2
C _{18:1}						0.1	0.1

unknown glycolipids and one unknown polar lipid were detected. One of these unknown glycolipids was found neither in *A. jenensis* DSM 9580^T nor in *A. citreus* DSM 12453^T. Predominant fatty acids were ai-C_{15:0} and ai-C_{17:0}, and i-C_{16:0} and i-C_{15:0} were present in significant amounts (Table 2). These fatty acid profiles differed significantly from those of *A. jenensis* DSM 9580^T in the contents of i-C_{15:0}, i-C_{16:0} and ai-C_{17:0}.

These chemotaxonomic characteristics of strain V-108^T and selected strains from the group of isolates were in good agreement with the characteristics reported for other members of the genus *Agrococcus* and are useful for distinction from other diamino-butyric acid-containing taxa (Groth *et al.*, 1996; Altenburger *et al.*, 1997; Wieser *et al.*, 1999), but they also suggested that the isolates belong to a separate species.

Taxonomic considerations

Whereas DNA relatedness clearly demonstrated that the five isolates do not belong to the species *A. citreus*, the values of DNA–DNA hybridization with *A. jenensis* DSM 9580^T were only slightly below the threshold value of 70% DNA relatedness considered for species delineation (Wayne *et al.*, 1987). However, the five strains could be distinguished easily from *A. jenensis* DSM 9580^T on the basis of several characteristics. They differed from *A. jenensis* DSM 9580^T in 16 biochemical properties, peptidoglycan composition, polar lipid pattern and fatty acid profiles. Inclusion of the five isolates into an emended species *A. jenensis* appears not to be justified, since this would create a species indistinguishable from *A. citreus*. Thus,

it becomes evident that the five strains V-108^T, V-50, V-64, V-98 and V-109 represent a novel species of the genus *Agrococcus*, which can be distinguished from the other two species of the genus. Here, we propose the name *Agrococcus baldri* sp. nov. for the novel species.

Description of *Agrococcus baldri* sp. nov.

Agrococcus baldri (bal'dri. N.L. gen. n. *baldri* of Baldr, ancient German god of light, referring to the photochromogenic behaviour).

Cells are irregular spherical, ovoid or rod-shaped. The cells vary in size from 0.7 to 1.0 µm wide by 1.1 to 1.7 µm long. Cells occur singly, in pairs or in small, irregular clusters. They are Gram-positive and non-motile. Endospores are not observed. Colonies are slightly convex and smooth. Colony diameters on TSA are 2–7 mm. The colour of the colonies on nutrient agar is light yellow when grown in the light and white when grown in the dark. Aerobic. Catalase is produced. Oxidase-negative. Nitrate is not reduced to nitrite. Other biochemical characteristics are given in Table 1. The cell wall diamino acid is diaminobutyric acid; additionally, alanine, glycine and glutamic and aspartic acids are found. No threonine is present. The polar lipid profile is composed of diphosphatidylglycerol, phosphatidylglycerol, two unknown glycolipids and one unknown polar lipid. Fatty acid profile consists of the predominant compounds ai-C_{15:0} and ai-C_{17:0}, and i-C_{16:0} and i-C_{15:0} are present in significant amounts. The quinone system consists of the major menaquinones MK-11 and MK-12. Spermine is the predominant compound in the polyamine pattern. The G+C content is 73.8–74.9 mol% (HPLC).

The type strain, V-108^T (= DSM 14215^T = CCM 4953^T), was isolated from the air in the 'Virgilkapelle' in Vienna, Austria. Other strains of this species are V-64 (= DSM 14216 = CCM 4954), V-50, V-98 and V-109.

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