Bavariococcus seileri gen. nov., sp. nov., isolated from the surface and smear water of German red smear soft cheese

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The phylogenetic position and physiological characters of six hitherto-unknown lactic acid bacterial isolates, which form part of the surface microbiota of German red smear soft cheese, are reported. The coccoid cells are aerotolerant, Gram-positive, catalase-negative and non-motile. The cell-wall peptidoglycan contains alanine, glutamic acid, lysine and aspartic acid and is of the A4 α type (L-Lys–D-Asp). The sequences of the 16S rRNA, *groEL* and *rpoB* genes of the six isolates are identical and reveal that these isolates represent an independent lineage within the radiation of the family *Enterococcaceae* in the phylum *Firmicutes*. Their closest phylogenetic neighbour is the lactic acid bacterium *Atopobacter phocae* M1590/94/2^T, with which they share 94.9% 16S rRNA gene sequence similarity; representatives of other genera such as *Granulicatella*, *Carnobacterium* and *Trichococcus* are more distantly related. DNA–DNA hybridization studies reveal that the six isolates are members of a single species, and this is confirmed by similarities in biochemical characteristics. The six isolates were assigned four different groups by Fourier-transform infrared and randomly amplified polymorphic DNA typing. Therefore, it is formally proposed that these isolates should be classified in a single novel species of a novel genus and be named *Bavariococcus seileri* gen. nov., sp. nov. The type strain of *Bavariococcus seileri* is WCC 4188^T (=DSM 19936^T =CCUG 55508^T).

Red smear cheeses are mainly produced in Austria, Germany, France, Scandinavia and Switzerland. For the ripening process, the microbial consortia of mature cheeses are washed off the surfaces with a brine solution, which is then used for inoculation of freshly produced cheeses. By virtue of the traditional manufacturing process, a variety of different smear cheeses exist which often comprise quite complex, species-rich microbial consortia (Brennan *et al.*, 2004; Feurer *et al.*, 2004; Maoz *et al.*, 2003; Mounier *et al.*,

2005; Wenning *et al.*, 2006). Some recently described species have been isolated from these consortia, such as *Agrococcus casei* (Bora *et al.*, 2007), *Arthrobacter bergerei* and *Arthrobacter arilaitensis* (Irlinger *et al.*, 2005), *Brevibacterium aurantiacum* (Gavrish *et al.*, 2004), *Corynebacterium casei* (Brennan *et al.*, 2001) and *Microbacterium gubbeenense* (Brennan *et al.*, 2001). All of these are members of the class *Actinobacteria*.

We have studied the species composition of South German red smear microbial consortia by aerobic cultivation on PC agar supplemented with 3% NaCl (Maoz *et al.*, 2003) and quantitative analysis of the species composition by Fourier-transform infrared (FT-IR) spectroscopy. FT-IR sample preparation, spectrum recording and data evaluation were performed as described by Kümmerle *et al.* (1998) using an IFS-28B spectrometer and OPUS version 5.5 (Bruker). To reduce the difficulties arising from unavoidable baseline shifts and to improve the resolution of complex bands, the first derivation of the digitized original spectra was used for data evaluation. Isolates were identified using spectral

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Abbreviation: FT-IR, Fourier-transform infrared; RAPD, randomly amplified polymorphic DNA.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene and *groEL* gene sequences of strain WCC 4188^T are FM177901 and FM177902, respectively.

Tables showing cellular fatty acid compositions and DNA–DNA relatedness values and figures showing FT-IR-based similarity dendrograms and RAPD fingerprints for the novel strains are available as supplementary material with the online version of this paper.

reference libraries of coryneform bacteria (Oberreuter *et al.*, 2002) and lactic acid bacteria (M. Wenning and others, unpublished data). As expected, the majority of bacteria forming the consortia belonged to the class *Actinobacteria*. However, besides this majority, a number of isolates displayed FT-IR spectra that did not match any reference in our database. These coccus-shaped, hitherto-undescribed bacteria originated from two samples of smear water and two surfaces of mature red smear soft cheese, which had been produced by the same manufacturer in South Germany and were collected at the beginning (consortium I) and the end (consortium II) of a 6-month period (Table 1). The novel bacteria were found in considerable numbers in their cheese habitats, constituting 14 and 8% of the total cell counts in the smear water samples relating to consortia I and II, respectively, and 9 and 1% of the cultivable cells of the surface consortia I and II, respectively.

All isolates grew well on common commercial media for lactic acid bacteria such as APT (Merck) and TSA (Oxoid) but, to achieve the fastest growth, organisms were cultivated in M17 broth (Merck) with 2% glucose without shaking, and M17 was the medium used for phenotypic tests. The ability to grow under anaerobic conditions was determined in an anaerobic jar containing the anaerobic catalyst Anaerocult A (Merck), which was prepared according to the manufacturer's instructions. No catalase activity was detected when cells were treated with 3% hydrogen peroxide. Morphological examination of the Gram-staining behaviour was positive (Gregersen, 1978). Phase-contrast microscopy visualized non-spore-forming cocci with a cell diameter of 0.9–1.2 µm after anaerobic cultivation on APT agar. The effects of pH (pH 5.0–10, at intervals of 0.5 pH units) and NaCl concentration (6.5 and 7–13%, w/v, at intervals of 1%) were assessed at 30 °C. The temperature range for growth was determined from 5 to 10 and 37 to 41 °C in increments of 1 °C. Growth within 14 days was indicated by visible turbidity. Additionally, all isolates were characterized biochemically by using the systems API 50CH with the API 50 CHB inoculation medium and Rapid ID 32 STREP and API 20 Strep (bioMérieux). Results are listed in the species description.

The phylogenetic positions of the six isolates were determined by sequence analyses of the 16S rRNA, *groEL* and *rpoB* genes. Complete 16S rRNA gene sequences were

amplified by PCR (Büchl *et al.*, 2008) using universal primers and cycling conditions specified by Oberreuter *et al.* (2002). Amplification products were purified with QIAquick PCR Purification Kit (Qiagen) and cycle sequencing PCR was performed by Sequiserve (Vaterstetten, Germany). Amplification of *groEL* gene sequences was performed as described previously (Goh *et al.*, 1996) with a modified thermal program of 5 min at 95 °C, 35 cycles of 20 s at 95 °C, 40 s at 55 °C and 1 min at 72 °C and a final extension step of 7 min at 72 °C. Sequencing of the 600 bp PCR products was done by 4base lab (Reutlingen, Germany). Primer sequences (StreptoF, StreptoR) for amplification of *rpoB* sequences were obtained from Drancourt *et al.* (2004) and cycle conditions were the same as described for *groEL* sequences but with an annealing temperature of 40 °C. Sequencing of the 700 bp PCR products was performed by GATC (Konstanz, Germany). In addition to the six isolates, sequences of the 16S rRNA, *groEL* and *rpoB* genes of the closest known relatives were either obtained as described above or, if available, retrieved from database searches (NCBI; <http://www.ncbi.nlm.nih.gov/blast/Blast>). The sequences obtained were aligned with CLUSTAL X version 1.8 (Thompson *et al.*, 1997). Distance matrices of the resulting multiple sequence alignments were calculated using TREECON (Van de Peer & De Wachter, 1997). Rooted phylogenetic trees were constructed with the neighbour-joining method. The stability of clusters was tested by regenerating trees using UPGMA (data not shown). The sequences of the 16S rRNA (Fig. 1), *groEL* (Fig. 2) and *rpoB* (data not shown) genes of the six isolates were identical and revealed a distinct lineage with respect to other established taxa. The 16S rRNA gene sequence similarity of the isolates compared with the closest neighbours *Atopobacter phocae* M1590/94/2^T and *Granulicatella* strains was 94.9 and 94.3–94.9%, respectively, supporting their affiliation to a novel genus (Ludwig *et al.*, 1998). Comparing the isolates with *Atopobacter phocae* CCUG 42358^T and *Granulicatella* strains, the *groEL* gene sequence similarity was 81.3 and 78.7–80.2%, respectively, and the *rpoB* gene sequence similarity was 75.3 and 76.7–80.3%, respectively.

FT-IR typing shows that novel strains and the phylogenetically closely related genera *Atopobacter*, *Carnobacterium*, *Enterococcus*, *Trichococcus* and *Vagococcus* form different groups (Supplementary Fig. S1, available in IJSEM Online).

Table 1. Bacterial isolates included in this study

Strain	Source
WCC 4185	Smear water, consortium I
WCC 4186	Smear water, consortium I
WCC 4187	Cheese surface, consortium I, isolated at expiry date after ripening at 16 and then 12 °C
WCC 4188 ^T	Cheese surface, consortium I, isolated at expiry date after ripening at 16 and then 12 °C
WCC 4189	Smear water, consortium II
WCC 4190	Cheese surface, consortium II, isolated at expiry date after ripening at 13 °C

Fig. 1. Nucleotide sequence similarity tree based on comparison of 16S rRNA gene sequences from the novel strains and related taxa, constructed with the neighbour-joining method and based on a comparison of approximately 1360 nucleotides. Bootstrap values (>50%) based on 500 replications are shown at branch nodes. The same tree topology was recovered with the UPGMA algorithm (not shown). The sequence of *Staphylococcus aureus* ATCC 12600^T was used as the outgroup. Bar, 5% sequence divergence.

Granulicatella and *Abiotrophia* were not included in this examination, as recording of spectra under aerobic conditions on TSA could not be performed due to the fastidious growth of these genera. FT-IR spectra, therefore, reveal a phenotype that clearly differentiates the novel strains from the most closely related genera.

The G + C content of genomic DNA was determined by the Identification Service of the DSMZ (Braunschweig,

Germany) using HPLC (Mesbah *et al.*, 1989) for isolates WCC 4186 (39 mol%), WCC 4188^T (38 mol%) and WCC 4189 (38 mol%).

Analysis of the cell-wall peptidoglycan was performed by the DSMZ according to Schleifer & Kandler (1972), Schleifer (1985), MacKenzie (1987) and Groth *et al.* (1996). Isolates WCC 4186, WCC 4188^T and WCC 4189 contained the amino acids alanine, glutamic acid, lysine

Fig. 2. Nucleotide sequence similarity tree based on comparison of *groEL* gene sequences from the novel strains and related taxa, constructed with the neighbour-joining method and based on a comparison of approximately 470 nucleotides. Bootstrap values (>50%) based on 500 replications are shown at branch nodes. The same tree topology was recovered with the UPGMA algorithm (not shown). The sequence of *Staphylococcus aureus* ATCC 12600^T was used as the outgroup. Bar, 10% sequence divergence.

and aspartic acid, indicating the peptidoglycan type A4 α (L-Lys-D-Asp). In contrast, the nearest relatives *Atopobacter phocae* and *Granulicatella* species have been reported to exhibit different peptidoglycan types (Collins & Lawson, 2000; Lawson *et al.*, 2000).

Quinones, which were examined as described by Altenburger *et al.* (1996), could not be detected. Polar lipid profiles were studied according to Altenburger *et al.* (1996) and Tindall (1990a, b) from biomass grown in M17 broth with 2 % glucose and the presence of cholesterol was examined as described recently (Worliczek *et al.*, 2007). The six isolates exhibited rather similar polar lipid profiles, differing only in the presence of minor components or slightly varying amounts of certain lipids. The polar lipid profiles included a predominant but unidentified glycolipid (GL1), phosphatidylglycerol and diphosphatidylglycerol, moderate amounts of an unknown polar lipid (L2) and an unknown glycolipid (GL2) and minor amounts of phosphatidylethanolamine and several unknown aminolipids, phospholipids and polar lipids (Fig. 3a). The nearest relative of the isolates, *Atopobacter phocae* CIP 106392^T, exhibited significant amounts of cholesterol but no unknown aminolipids (Fig. 3b).

Fatty acids were extracted and analysed by the DSMZ as described previously (Verborg *et al.*, 2008). Fatty acid profiles consisted almost exclusively of unbranched saturated and unsaturated acids. Major compounds were C_{16:0} (16–30 %) and C_{18:1 ω 9c} (35–73 %); C_{18:0} (4–22 %) was detected in minor to moderate amounts. Other fatty acids are listed in Supplementary Table S1.

Taken together, the 16S rRNA, *groEL* and *rpoB* gene sequence analyses revealed that these isolates represent an independent lineage within the radiation of the family *Enterococcaceae* in the phylum *Firmicutes*. The six coccoid isolates can be clearly distinguished from the rod-shaped monospecific genus *Atopobacter* and from the genera *Granulicatella* and *Trichococcus* by phenotypic characteristics such as the peptidoglycan type, polar lipid profiles (Table 2) and FT-IR spectra.

FT-IR spectra differentiated between the six isolates WCC 14185, WCC 4186, WCC 4187, WCC 4188^T, WCC 4189 and WCC 4190, as shown in Supplementary Fig. S2. Isolates WCC 4185, WCC 4186 and WCC 4188^T show high phenotypic similarity, indicated by low spectral distances, whereas isolates WCC 4187, WCC 4189 and WCC 4190 are more distantly related to the latter as well as to each other. Hence, WCC 4185, WCC 4186 and WCC 4188^T might be clonally related, whereas isolates WCC 4187, WCC 4189 and WCC 4190 may represent three different groups. In order to validate the FT-IR analysis, a randomly amplified polymorphic DNA (RAPD) analysis was performed. DNA was extracted as described previously (Büchl *et al.*, 2008) with an additional 5 min at 95 °C after cell lysis. Approximately 100 ng extracted DNA was subjected to PCR amplification in a total volume of 50 μ l following the protocol described by Büchl *et al.* (2008). Thermal cycling

Fig. 3. Polar lipid profiles of strain WCC 4188^T (a) and *Atopobacter phocae* CIP 106392^T (b) after two-dimensional TLC and detection with molybdatophosphoric acid. Chol, Cholesterol; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; DPG, diphosphatidylglycerol; PL1–4, unknown phospholipids; AL1–3, unknown aminolipids; L–7, unknown polar lipids; GL1–5, unknown glycolipids.

was performed on a T3000 Thermocycler (Biometra) with the following conditions: 6 min at 95 °C; 35 cycles of 1 min at 94 °C, 1 min at 38 °C and 2 min at 72 °C; and 10 min at 72 °C. Amplification products (6 μ l) were separated on a 1.5 % agarose gel in TBE buffer and the gels were stained with ethidium bromide, visualized with a UV lamp and photographed with ImageMaster VDS-CL (Amersham Biosciences). RAPD analysis using the M13 primer (5'-GAGGGTGGCGTTCT-3') (Gräser *et al.*, 1993) revealed the same four groups as FT-IR spectroscopy (Supplementary Fig. S2). Therefore, the applicability of

Table 2. Differential characteristics of the novel strains (*Bavariicoccus seileri* gen. nov., sp. nov.) and the type species of phylogenetically related genera

Data were taken from Collins & Lawson (2000), Liu *et al.* (2002), Lawson *et al.* (2000) and this study. +, Positive; –, negative; ND, no data available.

Characteristic	<i>Bavariicoccus seileri</i>	<i>Atopobacter phocae</i>	<i>Granulicatella adiacens</i>	<i>Trichococcus flocculiformis</i>
Cell morphology	Cocci	Rods	Cocci	Spherical to ovoid, sometimes olive-shaped
Aerobic growth	+	+	–	+
Polar lipids				
Cholesterol	–	+	ND	ND
Unknown glycolipids (<i>n</i>)	2	5	5*	2†
Unknown GL1 predominant	+	+	+	–†
Unknown aminolipids (<i>n</i>)	3	0	–	2†
Major cellular fatty acids	C _{16:0} , C _{18:1} ω9 <i>c</i>	C _{16:0} , C _{18:1} ω9 <i>c</i>	C _{16:0} , C _{18:1} ω9 <i>c</i>	C _{14:0} , C _{16:0} , C _{16:1} , C _{18:1} ω9 <i>c</i>
Murein type(s)	A4 <i>z</i>	A4β	A3 <i>z</i>	A4 <i>z</i>
Nutritional requirements	Non-fastidious	Non-fastidious	Complex	Non-fastidious

*The type strains of the three established species of the genus *Granulicatella*, *G. adiacens*, *G. balaenopterae* and *G. elegans*, were studied in the course of this study. *G. adiacens* DSM 9848^T and *G. elegans* DSM 11693^T exhibited almost-identical polar lipid profiles, each with five unknown glycolipids and GL1 predominant. In contrast, *G. balaenopterae* DSM 15827^T exhibited only two unknown glycolipids, GL1, which was not a predominant compound, and a second glycolipid that was clearly distinguishable from GL2 of the novel strains by two-dimensional TLC (Fig. 3), thus having a different chemical structure.

†Data for *T. flocculiformis* DSM 2094^T obtained in this study.

FT-IR spectroscopy for typing these lactic acid bacteria below the species level is demonstrated by our data, as has already been done for coryneform bacteria by Goerges *et al.* (2008).

In order to see whether these four groups belong to the same species, the six isolates were studied with DNA–DNA hybridization analyses based on renaturation curves, performed by the Identification Service of the DSMZ. DNA was isolated using a French pressure cell (Thermo Spectronic) and purified by chromatography on hydroxyapatite as described by Cashion *et al.* (1977). Hybridizations were carried out as described by De Ley *et al.* (1970) with the modifications described by Huß *et al.* (1983) using a Cary 100 Bio UV-Vis spectrophotometer equipped with a Peltier-thermostatted 6 × 6 multicell changer and temperature controller with *in-situ* temperature probe (Varian).

Hybridizations between isolates WCC 4187, WCC 4189 and WCC 4190 revealed reassociation values between 74 and 96 % (Supplementary Table S2). Pairing of WCC 4188^T with WCC 4187 and WCC 4190 revealed reassociation values between 95 and 98 %. All of these values are above the 70 % cut-off point for species delineation recommended by Wayne *et al.* (1987). However, DNA–DNA hybridization between WCC 4188^T and WCC 4189 revealed only 61 % relatedness (mean), a value that falls below the threshold of relatedness at the species level. Four independent DNA–DNA hybridization experiments

between WCC 4188^T and WCC 4189, with two independent DNA preparations, resulted in hybridization values within the range of 54–64 %. Yet, since all hybridization results of these two isolates with WCC 4187 and WCC 4190 were clearly above the threshold value of 70 %, we do not conclude that WCC 4188^T and WCC 4189 should be assigned to separate species. This conclusion is supported by identical 16S rRNA, *groEL* and *rpoB* gene sequences and almost-identical physiological and biochemical characteristics. Hence, we suggest that the six strains represent a novel genus and species, for which the name *Bavariicoccus seileri* gen. nov., sp. nov. is proposed.

Description of *Bavariicoccus* gen. nov.

Bavariicoccus (Ba.va.ri.i.coc'cus. L. fem. *Bavaria* Bavaria, Germany; N.L. masc. n. *coccus* coccus from Gr. masc. n. *kokkos* berry; N.L. masc. n. *Bavariicoccus* a coccoid-shaped bacterium isolated in Bavaria).

Cells stain Gram-positive and are non-spore-forming, aerotolerant and catalase-negative. Polar lipid profiles are dominated by an unknown glycolipid and moderate amounts of diphosphatidylglycerol and phosphatidylglycerol. Cholesterol is absent. Fatty acid profiles are predominantly composed of unbranched saturated and unsaturated fatty acids (>90 %). Major fatty acids are C_{16:0} and C_{18:1}ω9*c*. Quinones are not detectable. The cell-wall peptidoglycan contains the amino acids alanine, glutamic acid, lysine and aspartic acid (A4*z* type). The

DNA G + C content of the type species is 38–39 mol%. The type species is *Bavariicoccus seileri*.

Description of *Bavariicoccus seileri* sp. nov.

Bavariicoccus seileri (sei'le.ri. N.L. gen. masc. n. *seileri* named in honour of Herbert Seiler, former microbiologist of the Technical University of Munich with great merit in FT-IR spectroscopic identification of micro-organisms).

The characteristics are the same as those given in the description of the genus with the following additions. Small, smooth colonies up to 1 mm in diameter are formed aerobically on TSA at 30 °C and up to 2 mm in diameter on APT agar under anaerobic conditions at 34 °C. Cell diameter is 0.9–1.2 µm. Growth occurs at 10 and 40 °C, at pH 5.5 and with 11 % (w/v) NaCl. Produces DL-lactic acid, ethanol and acetic acid from D-glucose. Utilizes galactose, D-glucose, D-fructose, amygdalin, arbutin, salicin, cellobiose, maltose, β-gentiobiose, trehalose, lactose (except isolates WCC 4189 and WCC 4190) and pyruvate as the sole sources of carbon and energy, but not glycerol, erythritol, D- or L-arabinose, ribose, D- or L-xylose, adonitol, methyl β-D-xyloside, L-sorbose, rhamnose, dulcitol, inositol, mannitol, sorbitol, methyl α-D-mannoside, inulin, melezitose, raffinose, glycogen, xylitol, melibiose, sucrose, turanose, D-lyxose, D-tagatose, D-fucose, D- or L-arabitol, gluconate, 2- or 5-ketogluconate, pullulan or starch. Positive for β-glucosidase, pyrrolidonyl arylamidase and leucine arylamidase, but negative for α-galactosidase, β-galactosidase (except isolate WCC 4187), β-glucuronidase and alkaline phosphatase. Hippurate is not hydrolysed. In addition to the lipids listed in the genus description, several unknown lipids (polar lipids, glyco-, phospho- and aminolipids) are found in the polar lipid profile. The DNA G + C content of the type strain is 38 mol%.

The type strain is WCC 4188^T (=DSM 19936^T =CCUG 55508^T). The type strain and reference strains WCC 4187 (=CCUG 55507) and WCC 4189 (=CCUG 55509) were isolated from the surface and smear water of German smear-ripened soft cheeses.

Acknowledgements

We are grateful to Dr Jean P. Euzéby for his expert suggestions concerning nomenclature.

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