***Kytococcus schroeteri* sp. nov., a novel Gram-positive actinobacterium isolated from a human clinical source**

¹ Institute of Medical Microbiology, University of Münster, Hospital and Clinics, 48149 Münster, Germany

² DSMZ – German Collection of Microorganisms and Cell Cultures, 38124 Braunschweig, Germany

³ Labor Centrum Nordhorn, 48531 Nordhorn, Germany

Karsten Becker,¹ Peter Schumann,² Jörg Wüllenweber,¹ Martina Schulte,¹ Hans-Peter Weil,³ Erko Stackebrandt,² Georg Peters¹ and Christof von Eiff¹

Author for correspondence: Karsten Becker. Tel: +49 251 8355360. Fax: +49 251 8355350. e-mail: kbecker@uni-muenster.de

A strain of a Gram-positive, coccoid, yellow-pigmented bacterium was isolated from human blood. The bacterium was aerobic, non-encapsulated and non-motile. Phenotypically, the bacterium closely resembled *Kytococcus sedentarius*, but could be distinguished from this species by physiological tests and chemotaxonomic investigations. The peptidoglycan type is L-Lys-Glu₂, variation A4₂. The predominant menaquinones are MK-8 and MK-7. The major cellular fatty acids are iso-C_{17:1}, iso-C_{17:0}, iso-C_{15:0} and anteiso-C_{17:0}. The strain contains catalase and does not produce acid from carbohydrates. The ability to hydrolyse Tween 80 and the lack of α -glucosidase activity are the most characteristic features. The results of comparative 16S rDNA analysis revealed that the strain represents a novel species within the genus *Kytococcus*, for which the name *Kytococcus schroeteri* sp. nov. is proposed. The type strain is strain Muenster 2000^T (= DSM 13884^T = CCM 4918^T).

Keywords: *Kytococcus schroeteri*, *Micrococcineae*, taxonomy, phylogeny, 16S rDNA

INTRODUCTION

One of the first systematic descriptions of the pigmentation of bacteria and of the taxonomic ranks of pigmented micro-organisms was published by the German microbiologist J. Schroeter, based on his culture experiments in the years 1868–70 at the Institute of Physiology of Plants in Breslau (now Wrocław, Poland). He proposed '*Bacteridium luteum*' (Schroeter, 1875), which was included by Cohn (1875) as *Micrococcus luteus* (Schroeter) Cohn 1872 in the characterization of the genus *Micrococcus*. Since this first description of 'micrococci' [in the present study, the authors used the term 'micrococci' in quotes to indicate members of the genus *Micrococcus* Cohn 1872 as understood before the emendation by Stackebrandt *et al.* (1995)], the taxon has been revised several times and various novel species, including *Micrococcus sedentarius* (ZoBell & Upham, 1944), have been added

(Kocur, 1986). In 1995, the genus *Micrococcus* Cohn 1872 was taxonomically dissected into five genera, which have been reclassified into the new hierarchic classification system of *Actinobacteria* classis nov. (Stackebrandt *et al.*, 1995, 1997). In this context, the genus *Kytococcus* was established with the type species *Kytococcus sedentarius* and had been classified as a member of the family *Dermatophilaceae*. Both the *Micrococcaceae* and the *Dermatophilaceae* are included in the suborder *Micrococcineae* (Stackebrandt *et al.*, 1997). Presently, *Kytococcus sedentarius* (ZoBell & Upham 1944) Stackebrandt *et al.* 1995 is the only recognized species of this genus, which is characterized by Gram-positive, coccoid, non-encapsulated, non-motile, aerobic and catalase-positive cells. This species differs from 'micrococci' by being resistant to penicillin and methicillin and exhibiting arginine dihydrolase activity. Colonies of *Kytococcus sedentarius* are deep buttercup yellow or cream-white and they grow more slowly than those of other 'micrococci' (Kloos & Bannerman, 1999). *Kytococcus sedentarius* is usually regarded as part of the human skin flora (Kloos & Musselwhite, 1975). Nevertheless, the bacterium has been documented to be a causative organism in various infections (Greene *et al.*, 1980; Nordstrom *et al.*, 1987;

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Abbreviation: AP-PCR, arbitrarily primed-PCR.

The GenBank/EMBL accession number for the 16S rDNA sequence of *Kytococcus schroeteri* DSM 13884^T is AJ297722.

Old & McNeill, 1979). Here, we report the characterization of a novel muddy yellow-pigmented, Gram-positive coccus that was isolated from four different blood cultures of a patient with endocarditis. Based on the results of phylogenetic and phenotypic studies, we propose a second species of the genus *Kytococcus*, *Kytococcus schroeteri* sp. nov.

METHODS

Bacterial strain. In 1999, three isolates designated K8356^T, K8473 and K8645 were cultivated in the diagnostic laboratory of the Institute of Medical Microbiology, University of Münster, Germany, and an isolate designated 28997 was cultivated in the Labor Centrum Nordhorn, Germany. They were isolated in pure culture from four different blood specimens (Bactec 9240 System, Becton Dickinson) obtained from a 34-year-old woman with endocarditis. After genotyping, the isolates were recognized as a single strain, named Muenster 2000^T.

Morphological and phenotypic analyses. The morphological and phenotypic analyses described in this paper were performed on the four unidentified isolates. Isolates were grown at 37 °C on Columbia agar (Becton Dickinson) supplemented with 5 % sheep blood. Nutrient broth (Oxoid) was used as liquid or semi-solid medium. Biomass for menaquinone and cell wall analyses was obtained by cultivation in Trypticase soy/yeast extract medium no. 92 (DSMZ, 2001) and biomass for analysis of cellular fatty acids was obtained by cultivation on Trypticase soy broth (BBL Microbiology Systems) with agar at 28 °C for 48 h. Pigmentation and morphology were observed after 24–48 h growth. All physiological tests were performed at 37 °C except for studies concerning the temperature range (27, 30, 37, 43, 50 °C). Catalase production was demonstrated on slides by the formation of bubbles after mixing a suspension of culture material with a drop of 3 % (v/v) hydrogen peroxide solution. Oxidase activity was tested by oxidase reagent (Biotest) based on tetramethyl *p*-phenylenediamine (Tarrand & Groschel, 1982). Lysostaphin and lysozyme lysis susceptibility were assessed as described previously (Geary & Stevens, 1986; Kloos *et al.*, 1974). Urease activity, nitrate reduction, aesculin hydrolysis and hydrolysis of Tween 80 were tested according to published methods (Christensen, 1946; Cowan & Steel, 1974). Hydrolysis of starch was determined on inorganic salts/starch agar (Difco) after flooding the agar surface with Lugol's solution containing 0.1 % (w/v) iodine and 0.2 % (w/v) potassium iodide. Decomposition of an emulsion of UV-sterilized cellophane strips incubated in phosphate buffer was used to verify gelatin hydrolysis. Carbohydrate degradation and acid production from carbohydrates and alcohols were determined via the OF test as described by Hugh & Leifson (1953). In addition, physiological reactions were examined using the substrate panels of the API ID 32 STAPH, API CORYNE and API ZYM test strips according to the manufacturer's instructions (bioMérieux). NaCl tolerance was tested by adding this salt to nutrient broth at final concentrations of 2, 5, 7, 10, 12, 15 and 17 % (w/v). Susceptibility to antibiotics was evaluated after 48 h by measuring diameters of inhibition zones on Mueller–Hinton agar plates (Mast Diagnostics) containing antibiotic disks (Oxoid).

Chemotaxonomic characterization. Cell wall analysis was performed as described previously (Groth *et al.*, 1999). For GC analysis of cellular fatty acids, 40 mg cells was saponi-

fied, methylated, extracted and analysed using the Microbial Identification System described by Miller (1982). Menaquinones were extracted as described by Collins *et al.* (1977) and were analysed by HPLC as described previously in detail (Stackebrandt *et al.*, 1995).

PCR fingerprinting. The clonality of the clinical isolates was examined in arbitrarily primed (AP)-PCR with prolonged ramp times, applying random primer AP 90-1 (Becker *et al.*, 2000; Ellinghaus *et al.*, 1999). The resulting banding patterns were displayed on a 2.0 % agarose gel, visualized by ethidium bromide staining.

DNA–DNA hybridization, determination of 16S rDNA sequences and phylogenetic analyses. DNA–DNA hybridization analysis was carried out as described previously (Martin *et al.*, 1997). For 16S rDNA sequence determination, genomic DNA extracts were prepared from an 18-h brain/heart infusion broth culture by using the QIAamp tissue kit (Qiagen) following the manufacturer's recommendations. The 16S rDNA was amplified using universal prokaryotic 16S rDNA primers 27f, 357f and 907r (Lane, 1991) and primer 16S-4 (5'-GGACTACCMGGGTATCTAATCC-3'). The PCR products were purified using Centri-Sep columns (Princeton Separations) and sequenced directly by using the Taq Dye Deoxy terminator cycle sequencing kit (Applied Biosystems) and the ABI PRISM 310 Genetic Analyzer automated sequencing system (Applied Biosystems). The sequence determined was aligned with the 16S rDNA sequences of reference organisms of the genera *Arthrobacter*, *Dermacoccus*, *Dermatophilus*, *Janibacter*, *Kocuria*, *Kytococcus*, *Microbacterium*, *Micrococcus*, *Nesterenkonia* and *Terrabacter* obtained from the EMBL database by using OMIGA software (Oxford Molecular) and the program CLUSTAL W (Thompson *et al.*, 1994). The resulting multiple sequence alignment was corrected manually. Levels of sequence similarity for approximately 1400 nucleotides were determined with the program package DAMBE (Xia, 2000). A phylogenetic tree was constructed according to the neighbour-joining method (Saitou & Nei, 1987), the stability of the groupings was estimated by bootstrap analysis (500 replications) and branch lengths were evaluated by using a program based on the method of Fitch & Margoliash (1967).

Nucleotide accession numbers. The accession numbers of the reference strains used in the phylogenetic analysis are shown in Fig. 2.

RESULTS AND DISCUSSION

Bacterial isolates

The isolates were obtained within a period of 5 days from four different blood specimens of a patient with endocarditis, suggesting a possible disease association. They grew in pure culture after 24 h incubation. A direct stain of blood smears was positive for Gram-positive cocci. All isolates were clonally related, representing one strain (named Muenster 2000^T), as tested by AP-PCR fingerprinting (Fig. 1).

Morphological characteristics and phenotypic analysis

The unidentified isolates consisted of Gram-positive or Gram-variable cells. In accordance with the genus description of *Kytococcus* (Stackebrandt *et al.*, 1995), the cells were non-motile and non-encapsulated. Endo-

Fig. 1. Agarose gel electrophoresis patterns of AP-PCR-amplified products of *Kytococcus* strains. Lanes: M, DNA molecular size marker (combined 100 bp/1 kb ladder; New England Biolabs); 1, amplicon of *Kytococcus sedentarius* DSM 20547^T; 2–4, amplicons of *Kytococcus schroeteri* sp. nov. isolates 28997, K8356^T (= DSM 13884^T), K8473 and K8645 of strain Muenster 2000^T.

spores were not detected. Gram stains of the culture showed spherical cells (1.1–1.5 µm in diameter), predominantly occurring singly, in pairs, in tetrads and occasionally in clusters.

After 24 h incubation on Columbia agar supplemented with 5% sheep blood under aerobic conditions, colonies were tiny, increasing in size to about 1.5–2.5 mm in diameter after 48 h. The colonies were circular, entire, convex, smooth and muddy-yellow without a haemolytic reaction. The temperature range for growth on Columbia blood agar was between 27 and 43 °C. The optimal growth temperature was 37 °C. Growth did not occur at 50 °C. The isolates grew well in up to 12% NaCl and sparsely at 15% NaCl, but no growth was detected above 15% NaCl. Growth did not occur in Simmons citrate agar and growth was not observed under anaerobic conditions. The bacterium was oxidase-negative, as are most *Kytococcus sedentarius* strains. The isolates were resistant to lysostaphin lysis, whereas they were susceptible to lysozyme, indicating an affiliation to the ‘micrococci’ but not to the staphylococci (Jeffries, 1968; Klesius & Schuhardt,

Table 1. Characteristics that differentiate yellowish-pigmented ‘micrococci’

Data for reference taxa were obtained from Kocur *et al.* (1972), Kloos *et al.* (1974), Schleifer *et al.* (1981) and Stackebrandt *et al.* (1995) unless otherwise indicated. Characters are scored as: +, positive; ±, weakly positive; –, negative; > denotes ‘a frequency greater than’; commas between symbols denote nearly equal frequency. VP, Voges–Proskauer.

Characteristic	<i>M. luteus</i>	<i>Kocuria varians</i>	<i>Kytococcus sedentarius</i>	<i>Kytococcus schroeteri</i> sp. nov. DSM 13884 ^T
Pigmentation of colonies	Yellow or yellowish green	Dark yellow	Cream white/deep buttercup yellow	Muddy yellow
Growth on Simmons citrate agar	–	+ > ±, –	–	–
Oxidase	+ > ±	– > ±	–*	–
Aerobic acid production from:				
D-Glucose	– > ±	+ > ±	– > ±	–
D-Fructose	–	+ > ±	–	–
D-Xylose	–	+, –	–	–
Activity of:				
Alkaline phosphatase	–	–	+	+
Arginine dihydrolase	–	–	+	+
α-D-Glucosidase	+ †	– †	+	–
Urease	+, –	+	–	–
Hydrolysis of:				
Gelatin	+	+, –	+	+
Starch	– ‡	+, –	–	–
Tween 80	– §	–	–	+
Production of acetoin (VP test)	–	±, –	–	–
Resistance to:				
Penicillin	–	–	+	+
Methicillin	–	–	+	+

* Most strains are negative in the oxidase test (Stackebrandt *et al.*, 1995).

† Data from this study (*M. luteus* DSM 20030^T and *Kocuria varians* DSM 20022^T tested).

‡ Most strains negative, few strains weakly positive (Kloos *et al.*, 1974).

§ Strains of biotype II show variable hydrolysis of Tween 80 (Wieser *et al.*, 2002).

Table 2. Cellular fatty acid compositions of species of *Kytococcus*

Values are percentages of total fatty acids; values less than 1% are not shown for *Kytococcus sedentarius* (data taken from Stackebrandt *et al.*, 1995).

Fatty acid	<i>Kytococcus sedentarius</i>	<i>Kytococcus schroeteri</i> sp. nov. DSM 13884 ^T
C _{15:0}	10.6	
C _{15:1}	2.6	
C _{16:0}	2.8	
C _{16:1}	3.1	1.1
C _{17:0}	22.2	
C _{17:1}		0.7
iso-C _{15:0}	2.7	17.9
iso-C _{15:1}		0.7
iso-C _{16:0}	2.0	3.2
iso-C _{17:0}	8.1	21.1
iso-C _{17:1}	10.6	32.5
anteiso-C _{15:0}	1.2	3.5
anteiso-C _{17:0}	29.8	15.3
anteiso-C _{17:1}		4.0
Total	95.7	100.0

1968; Schleifer & Kloos, 1975). Nitrate reduction did not occur. They shared the following physiological properties with the type species of the genus *Kytococcus*, *Kytococcus sedentarius*, as investigated in comparison to the type strain, DSM 20547^T: growth under aerobic conditions; alkaline phosphatase, arginine dihydrolase, pyrazinamidase and catalase were positive; lecithinase, β -galactosidase and urease were negative; acid production from L-arabinose, D-galactose, D-glucose, maltose, D-mannose, D-melibiose, raffinose, L-rhamnose, D-ribose, sucrose, D-xylose, adonitol, glycerol and D-mannitol was negative; utilization of D-fructose, D-glucose, lactose, D-mannose, D-

galactose, L-rhamnose, sucrose, D-xylose, glycerol, D-mannitol, D-pantothenate, DL-arginine, L-leucine, L-lysine and L-valine was negative; hydrolysis of gelatin was positive; hydrolysis of aesculin and starch was negative; acetoin was not produced. The isolates differed from *Kytococcus sedentarius* in hydrolysis of Tween 80 and lack of α -glucosidase activity (Table 1).

Tests of susceptibility to antibiotics showed the following results: all four unknown isolates were susceptible to chloramphenicol, ciprofloxacin, gentamicin, tetracycline, vancomycin and teicoplanin; all isolates were resistant to penicillin, oxacillin and erythromycin. In addition, the isolates were susceptible to bacitracin and resistant to nitrofurantoin, as typically described for 'micrococci', in contrast to staphylococci, which are noted for the opposite pattern of susceptibility (Baker, 1984; Baker *et al.*, 1986).

Chemotaxonomy

The peptidoglycan type was L-Lys-Glu₂, variation A4 α (A11.43), as described for the genus *Kytococcus* (Stackebrandt *et al.*, 1995). The isoprenoid quinones of the unknown bacterium were represented by MK-8, MK-7 and MK-9 at a peak area ratio of 43:36:1 whereas, in *Kytococcus sedentarius*, menaquinones MK-8, MK-9 and MK-10 predominate. The fatty acid profile was dominated by iso-C_{17:1}, with smaller amounts of iso-C_{15:0}, iso-C_{17:0} and anteiso-C_{17:0} (Table 2). In contrast to these findings, the type species of the genus, *Kytococcus sedentarius*, shows, in addition to iso-C_{17:1} and anteiso-C_{17:0}, predominantly the straight-chain saturated acids C_{15:0} and C_{17:0}, but only minor amounts of iso-C_{15:0}.

Determination of 16S rDNA sequence, genomic DNA-DNA hybridization and phylogenetic analyses

In order to ascertain the phylogenetic position of the unknown bacterium, its almost complete 16S rDNA sequence was determined and subjected to compara-

Fig. 2. 16S rDNA-based phylogenetic dendrogram showing the phylogenetic position of *Kytococcus schroeteri* sp. nov. Numbers at the nodes are bootstrap values. Bar, 1% sequence divergence.

tive analysis. The highest sequence similarity was shown to *Kytococcus sedentarius* (97.9%). Somewhat lower levels of relatedness were found to other members of the suborder *Micrococcineae* (*Dermacoccus nishinomiyaensis*, 94.5%; *Janibacter limosus*, 94.2%). Even lower sequence similarity (approx. 92–93%) was found to other members of this suborder, including recent as well as former members of the genus *Micrococcus*, such as the species of the genera *Kocuria* and *Nesterenkonia* (data not shown).

The level of DNA–DNA relatedness between the unknown bacterium (strain DSM 13884^T) and the type strain of *Kytococcus sedentarius*, DSM 20547^T, was 45.5%, which is clearly below the threshold value for the delineation of genospecies (Wayne *et al.*, 1987).

A tree constructed by the neighbour-joining method, showing the phylogenetic position of the unknown human bacterium with respect to some related Gram-positive taxa, is shown in Fig. 2. The treeing analysis clearly demonstrated that the unknown micro-organism represents a novel subline within the *Kytococcus* clade. Bootstrap resampling showed that the relationship to *Kytococcus sedentarius* was statistically significant.

The morphological and biochemical properties of the unknown Gram-positive coccus were found to be consistent with its assignment to the genus *Kytococcus*, although it did not correspond to the hitherto-established species of this genus, *Kytococcus sedentarius*. Furthermore, the results of DNA–DNA hybridization experiments showed that the yellow-pigmented bacterium merits classification as a distinct species. Thus, based on phylogenetic and phenotypic evidence, we consider that the unidentified micro-organism from human blood should be assigned to the genus *Kytococcus* as *Kytococcus schroeteri* sp. nov.

Description of *Kytococcus schroeteri* sp. nov.

Kytococcus schroeteri (schroe'ter.i. N.L. gen. n. *schroeteri* of Schroeter, honouring Joseph Schroeter, a German microbiologist).

The description of the physiological and morphological characteristics is based on results of studies of one strain. In addition to the properties given in the genus description (Stackebrandt *et al.*, 1995), the species has the following properties. Cells are spherical (diameter 1.1–1.5 µm) and occur predominantly in pairs or tetrads. Colonies are up to 2.5 mm in diameter (after 48 h incubation), circular, entire, convex and smooth and develop rather slowly. Colonies are muddy-yellow. Grows well in the presence of NaCl concentrations up to 12%. No growth above 15% NaCl. Growth does not occur in Simmons citrate agar. Negative in the oxidase test. Positive for alkaline phosphatase, arginine dihydrolase and pyrazinamidase. Negative for lecithinase, β-galactosidase and urease. Nitrate is not reduced. Hydrolyses gelatin and Tween 80, but does not hydrolyse aesculin or starch. Acetoin is not produced. Metabolically inert for acid

production from carbohydrates. No acid is produced from L-arabinose, D-galactose, D-glucose, maltose, D-mannose, D-melibiose, raffinose, L-rhamnose, D-ribose, sucrose, D-xylose, adonitol, glycerol or D-mannitol. The optimum growth temperature is 37 °C. Non-haemolytic. Resistant to penicillin G, oxacillin and erythromycin and susceptible to ampicillin, chloramphenicol, ciprofloxacin, gentamicin, tetracycline, vancomycin and teicoplanin. Menaquinones with eight and seven completely unsaturated isoprene units (MK-8, MK-7) predominate. The major cellular fatty acids are iso-C_{17:1}, iso-C_{17:0}, iso-C_{15:0} and anteiso-C_{17:0}. The type strain is strain Muenster 2000^T (= DSM 13884^T = CCM 4918^T).

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