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Kocuria uropygioeca sp. nov. and *Kocuria uropygialis* sp. nov., isolated from the preen glands of Great Spotted Woodpeckers (*Dendrocopos major*)

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ABSTRACT

Two new species of Gram-positive cocci were isolated from the uropygial glands of wild woodpeckers (*Dendrocopos major*) originating from different locations in Germany. A polyphasic approach confirmed the affiliation of the isolates to the genus *Kocuria*. Phylogenetic analysis based on the 16S rRNA gene showed high degree of similarity to *Kocuria koreensis* DSM 23367^T (99.0% for both isolates). However, low ANIb values of <80% unequivocally separated the new species from *K. koreensis*. This finding was further corroborated by DNA fingerprinting and analysis of polar lipid profiles. Furthermore, growth characteristics, biochemical tests, MALDI-TOF MS analysis, and G + C contents clearly differentiated the isolates from their known relatives. Besides, the woodpecker isolates significantly differed from each other in their whole-cell protein profiles, DNA fingerprints, and ANIb values. In conclusion, the isolated microorganisms constitute members of two new species, for which the names *Kocuria uropygioeca* sp. nov. and *Kocuria uropygialis* sp. nov. are proposed. The type strains are 36^T (DSM 101740^T = LMG 29265^T) and 257^T (=DSM 101741^T = LMG 29266^T) for *K. uropygialis* sp. nov. and *K. uropygioeca* sp. nov., respectively.

Introduction

Members of the genus *Kocuria* represent coccoid Gram-positive, non-motile, non-spore forming bacteria of strictly aerobic metabolism and are members of the phylum *Actinobacteria* [31]. The genus is relatively young and was separated from *Micrococcus* following extensive phylogenetic investigations in 1995 [32]. To date, the genus *Kocuria* comprises around 20 species and is commonly encountered as commensal bacteria on human skin [8]. Besides, *Kocuria* have been isolated from various environments such as meat [35], fermented sea food [20,21,37], rhizoplanes [12], sediments [6], and air [38]. G + C contents typically range from 60 to 75 mol% [1]. The 16S rRNA gene shows a high degree of conservation and the commonly used 97% sequence similarity for species delineation does not apply to *Kocuria* [31].

Healthy birds are frequently colonized by bacteria of various genera [29]. Also, members of *Kocuria* have been shown to be associated with birds. For instance, *Kocuria rhizophila* has been isolated from the feathers of Spectacled Thrushes (*Turdus nudigenis*) in Venezuela [36] and *Kocuria* spp. were recovered from House Finches (*Haemorhous mexicanus*) in North America [28,30]. However, so far no *Kocuria* have been described in the preen glands of birds.

The uropygial gland (preen gland or oil gland) is a holocrine gland located at the base of the tale and exists in most of the birds. It serves a critical role in the suppleness of the plumage and confers water-repellent properties to the feathers [10]. Preen gland bacteria have rarely been detected and to date, reports focus on *Enterococcus* sp. and *Corynebacterium* sp. In case of the Red-billed Woodhoopoe (*Phoeniculus purpureus*), the bacteria discovered in oil glands led to the description of *Enterococcus phoeniculicola* as did *Corynebacterium uropygialis* which has been recovered from preen glands of Turkeys (*Meleagris gallopavo*) [3]. In the present work, we describe two hitherto unknown species of bacteria within the genus *Kocuria* originating from preen glands of Great Spotted Woodpeckers (*Dendrocopos major*). We thereby show that oil gland bacteria are not confined to the orders Bucerotiformes and Galliformes, but also occur in Piciformes.

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Materials and methods

Isolation and culture conditions

Wild birds were captured with mist-nets in the wildlife sanctuary Eich/Gimbsheimer Altrhein, Germany (coordinates 49.751803, 8.376903) and in a forest in Bad Homburg vor der Höhe, Germany (coordinates 50.236999, 8.577812). Preen gland secretions of Great Spotted Woodpeckers were collected from live animals and screened for the presence of bacteria following the method of Braun et al. [3]. Briefly, the secretions were channeled to the body surface by gently rubbing the oil glands. They were streaked on Columbia Agar supplemented with 5% sheep blood, Luria–Bertani Agar (LBA), Tryptic Soy Agar (TSA) and Müller–Hinton Agar (MHA), and incubated at 35 °C. Colonies were randomly selected, sub-cultured three times on fresh agar plates, and cultures were subsequently transferred to −80 °C in 20% glycerol for long-term storage. The type strains of the most closely related species *Kocuria koreensis* DSM 23367^T, *Kocuria halotolerans* DSM 18442^T, *Kocuria kristinae* DSM 20032^T and *Kocuria carniphila* DSM 16004^T were provided by the German Collection of Microorganisms and Cell Cultures (DSMZ) and processed in parallel with the woodpecker isolates using identical methods.

Morphology

Colony morphology of the isolates and reference strains was assessed on Columbia Agar supplemented with 5% sheep blood and on Müller–Hinton Agar after 48 h of incubation at 35 °C. Microscopic evaluation was carried out following Gram-stains and wet mounts. Besides, KOH lysis tests and spore stains were performed. Motility was evaluated by means of semi-solid agar and the hanging drop technique [34].

Temperature, NaCl and pH tolerance

Temperature, NaCl and pH tolerance were determined after an incubation period of 24 h in LB. Temperatures were set at 4 °C and 10–45 °C, and raised at increments of 5 °C. Salt tolerance was assessed at 0, 2, 5, 7.5, 10, and 15% NaCl and pH tolerance at pH 2–11 at increments of pH 1 at 35 °C. Growth was monitored as function of turbidity [23].

Biochemical characteristics

Biochemistry of *Kocuria uropygioeca* sp. nov. strain 257^T and *Kocuria uropygialis* sp. nov. strain 36^T, as well as the most closely related species were recorded simultaneously by means of Oxidase test strips (Fluka Analytical), API ZYM, and API 20 NE kits (bioMérieux) following the instructions given by the manufacturer. Lipophilism was tested according to Riegel et al. using identical media with and without the addition of Tween-80 [25]. Oxygen requirements were assessed by culturing the bacteria under aerobic and anaerobic conditions in anaerobic atmosphere generation bags (Sigma–Aldrich) and in thioglycollate broth (Fluka Analytical).

Antimicrobial susceptibility

Ten antibiotics (amoxicillin, ampicillin, oxacillin, amikacin, kanamycin A, streptomycin, bacitracin, ciprofloxacin, doxycycline, and vancomycin) were tested in 96-well plates for their minimum inhibitory concentrations (MICs) using broth microdilution assays [4]. Antibiotics were applied in a range of 0.03 to 64 μg/mL by serial dilution. After the addition of bacteria, plates were incubated at 35 °C for 20 h. The minimum concentration showing no visible growth was regarded as the MIC. Minimum bactericidal concentrations (MBCs) were determined by streaking bacterial dilutions higher or equal the MICs on agar plates. After incubation, colonies were counted and the least concentration yielding a reduction of ≥99.9% of the initial inoculum was taken as MBC [27]. *Escherichia coli* ATCC 25922 was included as quality control strain. All tests were conducted in triplicate per 96-well microtiter plate and repeated twice.

Lipid analysis

Identification of cellular fatty acids was performed using standard methodology after cultivation of *Kocuria uropygioeca* sp. nov. strain 257^T and *Kocuria uropygialis* sp. nov. strain 36^T on TSA for one day at 28 °C. Briefly, 40 mg of cells were scraped from agar plates and saponification was conducted using sodium hydroxide in 50% methanol. The samples were acidified with hydrochloric acid and derivatized into fatty acid methyl esters (FAMEs) by the addition of boron trichloride. FAMEs were extracted with hexane/methyl *tert*-butyl ether, washed with 0.3 M NaOH and injected into the gas chromatograph. FAME separation and identification were carried out using the Sherlock Microbial Identification System [13,16]. The extraction of polar lipids of *Kocuria uropygioeca* sp. nov., *Kocuria uropygialis* sp. nov., and all reference strains, as well as 2D-TLC were in compliance with Minnikin et al. [17].

MALDI-TOF mass spectrometry

Samples were prepared for ethanol/formic acid extraction by homogenizing cells in deionized water. Ethanol was added, the samples were vortexed, centrifuged, and the ethanol was removed. Samples were extracted with 70% formic acid/acetonitrile and subjected to matrix-assisted linear desorption/ionization-time-of-flight mass spectrometry (MALDI) [26]. MALDI was performed by transferring 1 μL of the extract to the target plate of a Microflex LT (Bruker Daltonics). The sample was overlaid with 1 μL alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix solution and allowed to air dry. Each strain was applied on 8 different spots and measured in triplicate as mentioned in Aravena-Roman et al. [1]. MALDI Biotyper 3.0 software (Bruker Daltonics) was used for the construction of main-spectrum (MSP) dendrograms as outlined in the MALDI Biotyper MSP creation method.

Gene sequencing and phylogenetic analysis

DNA was extracted according to Rainey et al. [22]. PCR was carried out using the universal 16S rRNA gene primers 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1541R (5′-AAG GAG GTG ATC CAG CCG CA-3′) [21,19]. Sequencing was accomplished by StarSeq (Mainz, Germany) using an ABI 3730 automated capillary sequencer and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Chimera test was done using Bellerophon before sequences were submitted to GenBank [9]. MrModeltest 2.3 [18], executed in PAUP* 4.0 [33], was used to determine the best nucleotide substitution model. Sequences were aligned, manually curated with respect to secondary structure and phylogenetic analyses were performed using the ARB software environment 6.0.2 [14].

G + C content

The G + C contents of *Kocuria uropygioeca* sp. nov. and *Kocuria uropygialis* sp. nov. were deduced from their genomic data. In addition, they were derived from the melting temperature of bacterial DNA in saline-sodium citrate buffer by thermal denaturation [15].

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