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Corynebacterium uropygiale sp. nov., isolated from the preen gland of Turkeys (*Meleagris gallopavo*)

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ABSTRACT

A novel species of fastidious, lipophilic, club-shaped, Gram-positive bacteria was recovered from the preen glands of healthy Turkeys (*Meleagris gallopavo*) from two different locations. Phylogenetic analysis of the 16S rRNA gene showed highest similarity to *Corynebacterium spheniscorum* DSM 44757^T (96.8%) with a 3.2 kb stretch of *rpoB* sharing 82.4% sequence similarity to the same species. DNA fingerprinting by ERIC-PCR and polar lipid profiles clearly differentiated the Turkey isolates from the most closely related Corynebacteria, as did MALDI-TOF MS analysis. Chemotaxonomic tests revealed the presence of corynemycolic acids with $C_{16:0}$, $C_{18:0}$, $C_{18:1}\omega9c$ and tuberculostearic acid as the major cellular fatty acids. The G+C content of the type strain was 60.7 mol%. The species was susceptible to ampicillin, kanamycin A, streptomycin, amikacin, polymyxin B and vancomycin. From our results, it becomes evident that the isolated organisms represent a new species, for which the name *Corynebacterium uropygiale* sp. nov. is proposed. The type strain is Iso10^T (=DSM 46817^T = LMG 28616^T).

Introduction

A large number of microorganisms have been found to co-exist with birds [5,6,15,16,31]. Corynebacteria have been isolated from healthy avian tissues of different body parts such as cloacae of Magellanic Penguins (*Spheniscus magellanicus*) [12,13], trachea of Black Storks (*Ciconia nigra*) [9] and choanae of Golden Eagles (*Aquila chrysaetos*) [8]. However, no coryneform bacteria have to date been isolated from preen glands of birds. Reports on bacteria in preen glands are confined to two species of birds within the order Bucerotiformes, namely the European Hoopoe (*Upupa epops*) [22] and the Red-billed Woodhoopoe (*Phoeniculus purpureus*). In both cases, the microorganisms belong to the genus *Enterococcus*.

Corynebacterium spp. are Gram-positive, non-spore forming organisms and members of the class *Actinobacteria*. They constitute a diverse genus containing species of pathological relevance to men and animals [11], but also represent a part of the normal microbiome [9,14]. Since the emergence of molecular tools, the genus has seen a significant increase in newly described species [12,9]. It now includes more than 90 members and is one of the largest taxa within coryneform organisms [17,1]. Typical characteristics are pleomorphic or club shaped rods, which assemble in V-arrangements or palisade-like patterns [2]. The genetic information is encoded in G+C rich DNA with a guanine and cytosine content accounting to as much as 46–74 mol% [2]. The cell wall contains corynemycolic acids (C_{22}–C_{30}), $C_{16:0}$, $C_{18:0}$ and $C_{18:1}\omega9c$ being the main components of their cellular fatty acid profile [10]. When compared to other genera, the 16S rRNA gene is particularly conserved among different *Corynebacterium* species and sometimes the sequence divergence is less than 1% between them [17]. Here, we describe a hitherto unknown *Corynebacterium* species from the preen gland of Turkeys by means of a polyphasic approach. The bacterium was isolated while assessing the functional importance of preen gland secretions for birds. As a name for the novel species we propose *Corynebacterium uropygiale*. This is the first report on coryneform bacteria isolated from preen glands of healthy birds and the first publication on preen gland dwelling microorganisms outside the order Bucerotiformes.

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Materials and methods

Isolation and culture conditions

Samples were obtained from captive Turkeys kept at two different localities near Heidelberg, Germany. Secretions from the preen gland of Turkeys dedicated to human consumption were collected after excising the preen glands from freshly slaughtered animals at Annerose Ziegler GbR, Bammental, Germany. The glands were surface sterilized in 70% ethanol for 10 min to avoid contamination from surrounding tissues and excised using sterile scalpels and forceps. Uropygial gland secretions from Turkeys originating from Heidelberg Zoo were taken from live animals by gently squeezing the base of the preen glands. The secretions were collected using a micropipette and the autoclaved tips were transferred into sterile screw-capped tubes. The preen wax was streaked on Luria–Bertani (LB) agar, LB agar supplemented with 0.3% Tween-80, Columbia agar supplemented with 5% sheep blood, Tryptic soy agar (TSA) and Mueller–Hinton–Agar (MHA) and was incubated at 37 °C with and without 5% CO_2. The different conditions were used to heighten the probability that fastidious organisms can be cultured. Single colonies were randomly picked and organisms were sub-cultured three times on new agar plates. Thereafter, the pure cultures were stored at −80 °C in 30% glycerol. The reference strains *C. sphenisicorum* DSM 44757^T, *C. falsenii* DSM 44353^T, *C. testudinoris* DSM 44614^T and *C. jeikeium* DSM 7171^T were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and tested simultaneously with the preen wax isolates using identical culture conditions and LB medium supplemented with 0.3% Tween-80.

Morphology

Colony morphology of the isolates was observed after cultivation on LB agar enriched with 0.3% Tween-80 and Columbia agar supplemented with 5% sheep blood. Microscopic evaluation was done after Gram-, Ziehl–Neelsen- and spore stains. In addition to Gram-stain, KOH lysis test was carried out. Motility was assessed using wet mount technique as well as semi-solid agar [35]. For electron microscopy, samples were fixed and dehydrated following the method of Piroeva et al. [28]. Subsequently, they were covered with a carbon layer, using a Leica EM ACE200 (Leica Microsystems). Images were taken by means of a LEO 1530 field emission scanning electron microscope (FE-SEM) with a Schottky cathode (LEO GmbH). The chamber pressure during the imaging process was $<5 \times 10^{-6}$ mbar, the accelerating voltage was 3 kV.

Biochemical characteristics

The biochemical properties of the strains Iso10^T, C4 and the reference strains were investigated in parallel using API Coryne (bioMérieux) and Oxidase Strips (Fluka Analytical) according to the manufacturer's instruction but were incubated for 48 h for analytical profile indexes [1]. Lipophilic requirements were tested following Riegel et al. [30]. The Christie–Atkins–Munch–Petersen (CAMP) test was conducted with *Staphylococcus aureus* ATCC 25923 [10]. Oxygen requirements were evaluated by growing the isolates in an aerobic and anaerobic environment (anaerobic atmosphere generation bags) and in thioglycollate broth (Fluka Analytical).

Antimicrobial susceptibility

Minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of ampicillin, vancomycin, kanamycin A, polymyxin B, streptomycin and amikacin were investigated using the microdilution approach following the method of CLSI [4], which was slightly modified in order to allow growth of lipophilic organisms. Briefly, the cell number of the vital strains Iso10^T and C4 was adjusted to approximately 5×10^5 cfu/mL in Mueller–Hinton broth supplemented with 0.3% Tween-80. Antibiotics were added to the 96-well plates at concentrations between 64 μg/mL and 31.25 ng/mL and incubated for 48 h at 35 °C. The MIC was taken as the minimum concentration completely inhibiting visual bacterial growth. For the determination of the MBC, 3 μL of each well higher or equal the MIC were spread on agar plates and incubated for 48 h. The lowest concentration killing ≥99.9% of the initial inoculum was regarded as MBC. All tests were conducted in triplicate per microtiter plate and repeated three times.

Lipid analysis

Analysis of cellular fatty acids of the representative strain Iso10^T was performed by the DSMZ identification service using the Sherlock Microbial Identification System (MIDI) and standard methods [19,23]. Methanolysis was conducted to extract lipids and the presence of mycolic acids was demonstrated by thin-layer-chromatography (TLC) [24]. Extraction and 2D-TLC of polar lipids of Iso10^T, C4 and all reference strains were in compliance with Minnikin et al. [25].

MALDI-TOF mass spectrometry

Ethanol/formic acid extraction was done according to Bruker Daltonics [3] and matrix-assisted linear desorption/ionization-time-of-flight mass spectrometry (MALDI) was performed using a Microflex LT (Bruker Daltonics) following the method of Aravena-Roman et al. [1]. The main-spectrum (MSP) dendrogram was constructed in MALDI Biotyper 3.0 according to the MALDI Biotyper MSP creation method (Bruker Daltonics). An overlay of the calibrated spectra as well as peak annotation was generated in mMass 5.5.0 [26].

Gene sequencing and phylogenetic analysis

Genomic DNA was isolated following the procedure of Rainey et al. [29]. Almost the complete 16S rRNA gene was amplified by PCR using the forward primer 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′), the reverse oligo 1541R (5′-AAG GAG GTG ATC CAG CCG CA-3′) [27] and standard conditions [29]. Sequencing was accomplished by an ABI 3730 automated capillary sequencer and the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Bellerophon was used to check for chimeric sequences and the sequences were submitted to the GenBank. The appropriate nucleotide substitution model was identified by MrModeltest 2.3 in PAUP* 4.0. Multiple alignments were created, manually inspected and phylogenetic analyses were conducted using the ARB software environment 6.0.2 [20]. Additionally, a large fragment of the β subunit of RNA polymerase (*rpoB*) of Iso10^T and C4 was amplified using the primers C35F (5′-GGA AGG ACC CAT CTT GGC AGT-3′), C630F (5′-GAC CGC AAG CGY CGC CAG-3′), C1295F (5′-CAG TTY MTG GAC CAG AAC AAC-3′), C1800f (5′-ATG GGY GCS AAC ATG CAG-3′), C2350F (5′-ACA TCC TGG TCG GTA AGG TCA C-3′) and C3490R (5′-CAC GGG ACA GGT TGA TGC C-3′) and conditions mentioned in Khamis et al. [17]. Analyses of *rpoB* were done as stated above for the 16S rRNA gene except for multiple alignments with sequences from GenBank being performed using the MUSCLE algorithm in MEGA 6.06 [34].

ERIC fingerprinting

ERIC fingerprints were obtained using PCR primers and conditions described by De Bruijn [7]. Amplicons were separated on a