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Kocuria tytonicola, new bacteria from the preen glands of American barn owls (*Tyto furcata*)

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ABSTRACT

Although birds are hosts to a large number of microorganisms, microbes have rarely been found in avian oil glands. Here, we report on two strains of a new bacterial species from the preen oil of American barn owls (*Tyto furcata*). Phenotypic as well as genotypic methods placed the isolates to the genus *Kocuria*. Strains are non-fastidious, non-lipophilic Gram-positive cocci and can be unambiguously discriminated from their closest relative *Kocuria rhizophila* DSM 11926^T. In phylogenetic trees, the owl bacteria formed a distinct cluster which was clearly separated from all other known *Kocuria* species. The same conclusion was drawn from MALDI-TOF MS analyses. Once again, the new bacterial strains were very similar to one another, but exhibited substantial differences when compared to the most closely related species. Besides, the results of the biochemical tests, optimum growth conditions and pigmentation differed from closely related *Kocuria* spp. Finally, ANIb values of less than 87% provided striking evidence that the isolates recovered from American barn owls represent a hitherto undescribed species, for which we propose the name *Kocuria tytonicola* sp. nov. The type strain is 489^T (DSM 104133^T = LMG 29945^T, taxonumber TA00340).

Introduction

The oil gland (preen gland or uropygial gland) is the largest avian gland and present in the majority of birds. In American barn owls (*Tyto furcata*), as in most other bird species, it is organized in two lobes holding the preen oil, and the papilla which is used to transport the preen oil to the body surface [10]. During preening, birds use their bills to squeeze the oil gland, channel the preen oil to the body exterior and apply it to the plumage. Taking into account the significant amount of time birds spend for preening, the oil gland and its preen oil are thought to serve critical fitness-related functions [12]. Possible roles of preen oils are diverse and range from maintaining feather integrity, inhibiting feather-degrading bacteria to repelling predators [27].

To date, data on bacteria in preen oil and uropygial glands of birds are still scarce. However, bacteria have been isolated from the oil glands of hoopoes (*Upupa epops*) [21], green woodhoopoes (*Phoeniculus purpureus*) [18], turkeys (*Meleagris gallopavo*) [4], Egyptian geese [5] and great spotted woodpeckers (*Dendrocopos major*) [6]. Moreover, bacteria could be detected in the preen glands of American barn owls very recently [3]. While hoopoes and green woodhoopoes house *Enterococcus* spp., the actinobacteria *Kocuria uropygioeca* and *Kocuria uropygialis* have been recovered from the oil glands of woodpeckers, *Corynebacterium uropygiale* has been detected in the uropygial glands of turkeys and *Corynebacterium heidelbergense* has been reported as a part of the normal preen gland microbiome of geese. While oil gland-associated *Enterococcus* spp. have been shown to engage in mutualistic relationships with their hosts [21,18], data on symbiosis concerning the remaining bacteria is lacking or inconclusive [7].

The genus *Kocuria* was described in 1995, when *Micrococcus* was divided into *Dermacoccus* gen. nov., *Kocuria* gen. nov., *Kytococcus* gen. nov., *Nesterenkonia* gen. nov., and *Micrococcus* gen. emend. [32]. Representatives are mesophilic Gram-positive, non-spore forming, non-encapsulated, chemo-organotrophic cocci and mostly obligate aerobes. Major menaquinones are MK-7(H_2), MK-8 and MK-9(H_2) and polar lipids contain diphosphatidylglycerol, phosphatidylglycerol and phosphatidylinositol. The predominant

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cellular fatty acid is anteiso-$C_{15:0}$ and G + C content accounts to 60–75 mol% [31]. *Kocuria* consists of <30 species with validly published names (http://www.bacterio.net) and occurs in various surroundings such as air [39], skin [11], feathers [37] and preen oils [4].

Materials and methods

Isolation and culture conditions

Preen oil samples were obtained from live American barn owls, which were kept at RWTH Aachen University, Germany. Oil glands were gently squeezed and preen oils were collected using sterilized equipment [5]. Samples were streaked on Columbia Blood Agar (CBA) and Luria-Bertani Agar (LBA) plates and subsequently transferred to a 35 °C incubator. After incubation, random colonies were selected and sub-cultured three times on LBA or CBA. Type strains of the most closely related species (hereinafter referred to as reference strains) *K. rhizophila* DSM 11926^T, *K. varians* DSM 20033^T, *K. salsicia* DSM 24776^T, *K. marina* DSM 16420^T and *Kocuria indica* DSM 25126^T were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ)^T and maintained using the same conditions when compared to the bird isolates.

Morphology

Colony morphology of all strains of the study was determined after 48 h of incubation at 35 °C on CBA and Müller-Hinton Agar (MHA). Gram-staining and KOH lysis tests were performed according to standard methods [15]. The hanging-drop approach and semi-solid agar were used to evaluate the motility of the isolates [36].

Temperature, sodium chloride and pH tolerance

Optimal growth conditions were determined in LB broth after an incubation of 24 h in ambient atmosphere (no additional CO_2). Temperatures were 4 °C, 37 °C and 10–45 °C and was raised in increments of 5 °C. Proliferation in the presence and absence of sodium chloride was quantified at 0, 1, 2, 5, 7.5, 10 and 15% (w/v) NaCl, while pH tolerance was assessed at between pH 2 and pH 10 at 35 °C. Growth was measured in McFarland units using a densitometer (Biosan DEN-1) as a function of turbidity [30].

Biochemical characteristics

Oxidase test strips (Fluka Analytical), API Coryne, API ZYM and API 20 NE test kits (bioMérieux) were used for the biochemical characterization of *Kocuria tytonicola* sp. nov. strain 473, *Kocuria tytonicola* sp. nov. strain 489^T and the reference strains. Since preen oil is mainly composed of lipids and *Corynebacterium uropygiale* from the uropygial glands of turkeys was proven obligate lipophilic [4], lipophilism was tested for according to Riegel et al. by adding 0.3% Tween-80 to the media formulation [26]. The relation to oxygen was determined by means of thioglycollate broth with oxygen gradient (Fluka Analytical) and anaerobic atmosphere generation bags (Sigma–Aldrich).

Antimicrobial susceptibility

Broth microdilutions were conducted in order to evaluate the susceptibilities of the bird isolates against ten antibiotics (amoxicillin, ampicillin, oxacillin, amikacin, kanamycin A, streptomycin, bacitracin, ciprofloxacin, doxycycline and vancomycin). Antimicrobial breakpoints were recorded as minimum inhibitory concentrations (MICs) according to Clinical & Laboratory Standards Institute [8]. Briefly, microtiter plates were filled with antibiotics in the range of 0.03–64 μg/mL. Bacteria were added and plates were incubated at 35 °C for 20 h. Minimum concentrations completely inhibiting visible growth (MIC) were determined. All tests were performed three times, with *Escherichia coli* ATCC 25922 serving as a quality control.

Chemotaxonomic analysis

Fatty acid methyl esters (FAMEs) of cellular fatty acids of both strains of the new species were prepared after cultivation for 48 h at 37 °C on TSA by saponification of the cells in sodium hydroxide in 50% methanol. Derivatization was achieved by boron trichloride and fatty acid methyl esters (FAMEs) were extracted using hexane: methyl tert-butyl ether. Cellular fatty acids were identified using the Sherlock Microbial Identification System and the TSBA6 library [17,28].

Bacterial biomass for polar lipid analysis was produced in TSB at 37 °C and harvested by centrifugation. Polar lipids were obtained from the fresh bacterial cell mass by chloroform: methanol extraction. The chloroform phase was evaporated to dryness, resuspended and subjected to 2D-thin-layer chromatography (TLC) on silica gel 60 plates (Merck). Chloroform:methanol:water (first dimension) and chloroform:acetic acid:methanol:water (second dimension) were used as mobile phases and lipids were identified using common spray reagents, as well as standards [9].

Respiratory quinones were analyzed as described by Tindall [34,35]. Briefly, lipoquinones were extracted with methanol:hexane (2:1 v/v) and menaquinones were separated from ubiquinones by TLC in methyl *tert*-butyl ether:hexane. High-performance liquid chromatography (HPLC) (LDC Analytical, Thermo Separation Products) was carried out using a reverse phase column (Macherey-Nagel, 2 mm × 125 mm, 3 μm, RP18) and methanol:heptane as the mobile phase. Respiratory lipoquinones were detected at a wavelength of 269 nm.

MALDI-TOF mass spectrometry

Colonies were homogenized in water, ethanol was added and proteins were extracted using 70% formic acid: acetonitrile [29]. Matrix-assisted linear desorption/ionization-time-of-flight mass spectrometry (MALDI) was carried out with alpha-cyano-4-hydroxycinnamic acid (HCCA) as a matrix. Strains were applied on 8 positions of the template and measured three times using standard methods [1]. Main-spectrum (MSP) dendrograms were generated using MALDI Biotyper 3.0 software (Bruker Daltonics), as described in the MALDI Biotyper MSP creation method.

Gene sequencing and phylogenetic analysis

Phenol-chloroform extraction of total DNA was in line with Rainey et al. [24]. 16S rRNA gene loci were amplified by polymerase chain reactions (PCR) using the primers 27f (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1541R (5′-AAG GAG GTG ATC CAG CCG CA-3′) [24,23]. Sequencing was carried out by StarSeq (Mainz, Germany) on an ABI 3730 automated capillary sequencer in combination with an ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). Chimera test was performed using Bellerophon [13] and DNA sequences were deposited in the GenBank. Phylogenetic analyses were based on the best nucleotide substitution model identified by MrModeltest 2.3 [22] which was executed in PAUP* 4.0 [33]. Alignments were created in ARB 6.02 [19] and manually curated with regard to the secondary structure of the 16S rRNA gene. MEGA7 was used to reconstruct phylogenetic trees [16].

Table 1
Biochemical characteristics differentiating *Kocuria tytonicola* sp. nov. and its most closely related species. (+) positive; (−) negative; (w) weakly positive. All data were generated simultaneously in this study using API ZYM, API 20 NE and API Coryne kits.

Characteristic	*K. tytonicola*		*K. rhizophila*	*K. indica*	*K. varians*	*K. marina*	*K. salsicia*
	489^T	473	DSM 11926^T	DSM 25126^T	DSM 20033^T	DSM 16420^T	DSM 24776^T
Alkaline phosphatase	w	w	+	+	–	–	+
Esterase	+	+	+	+	–	w	–
Esterase lipase	+	+	+	+	w	+	+
Valine arylamidase	–	–	–	–	–	w	–
Cystine arylamidase	–	–	–	+	–	+	–
β-Galactosidase	–	–	–	–	+	+	–
α-Glucosidase	–	–	w	+	–	+	+
β-Glucosidase	+	–	–	–	–	–	–
Pyrrolidonyl arylamidase	+	+	+	+	+	+	–
Nitrate reduction	–	–	–	+	–	+	+
Urease	+	+	–	+	+	+	+
Esculin hydrolysis	+	–	–	–	–	–	–
Gelatin hydrolysis	+	+	–	–	–	+	–
Assimilation of:							
Phenylacetic acid	+	+	+	–	+	–	+
Production of acid from:							
Ribose	+	–	+	w	+	+	+
Xylose	+	–	+	–	+	–	+
Maltose	+	+	+	+	–	+	+
Sucrose	–	–	+	+	–	+	–
Pigment color	Pale orange	Pale orange	Yellow	Yellow	Yellow	Orange	Yellow

Table 2
Average nucleotide identities (%) between the type strain of *K. tytonicola* sp. nov. and the most closely related species.

	Strain		1	2	3	4	5	6	7
1	*K. tytonicola* strain 489	DSM 104133^T	*	98.79	85.40	84.05	83.93	82.37	82.24
2	*K. tytonicola* strain 473	–	98.73	*	85.32	84.04	83.93	82.34	82.34
3	*K. rhizophila*	DSM 11926^T	85.45	85.30	*	84.21	83.36	82.42	82.39
4	*K. salsicia*	DSM 24776^T	83.77	83.77	84.03	*	86.40	82.51	82.56
5	*K. varians*	DSM 20033^T	83.69	83.60	82.93	86.29	*	82.15	82.33
6	*K. marina*	DSM 16420^T	81.94	81.91	82.22	82.16	82.08	*	95.51
7	*K. indica*	DSM 25126^T	82.08	81.98	82.23	82.49	82.27	95.41	*

G + C content

DNA was dissolved in sodium citrate buffer and slowly heated until complete denaturation of double strands was achieved (Jasco V-630 UV–Vis Spectrophotometer). G + C contents were inferred from the melting points according to Marmur and Doty [20]. Additionally, G + C contents were calculated from their genomic data.

Genome comparison

Genome sequencing was accomplished on the Illumina MiSeq platform with 2 × 250 bp paired-end reads (*K. tytonicola* strain 489^T and *K. rhizophila*) and the Illumina HiSeq platform with 2 × 125 bp paired-end reads (*K. tytonicola* strain 473 and *K. indica*). Reads were clipped and trimmed using Sickle 1.33 [14] (q > 30, minimum length > 45 bp) and assembled in SPAdes 3.10 [2]. The remaining genomes were taken from public databases (see Supplementary Table 1 for all accession numbers). Average nucleotide identities (ANIb) were calculated with JSpecies [25].

DNA fingerprinting

PCR was carried out using BOX1AR primers (5′-CTACGGCAAGGCGACGCTGACG-3′) and the conditions stated in Versalovic et al. [38]. Amplicons were separated on a 1.5% agarose gel containing ethidium bromide for 19 h at 90 V. Images were captured using a GelDoc-It Imager (UVP).

Results and discussion

Two strains of the proposed species *Kocuria tytonicola* sp. nov. were isolated from the oil glands of laboratory animals (American barn owls) kept at RWTH Aachen University. The isolates stained Gram-positive, were oxidase negative, catalase positive, non-motile; the non-sporulating cocci occurred in packets of six and more. Reduced growth was observed in the absence of oxygen when compared to the ambient atmosphere. In spite of its occurrence in lipid-rich preen oil, *K. tytonicola* sp. nov. did not exhibit fondness for lipophilic environments, but grew equally well in non-lipid-supplemented media, such as LBA and MHA. After 48 h of incubation at 35 °C on MHA and CBA, colonies of both strains exhibited a circular shape and pale orange color. They were non-viscous and 1–2 mm in diameter. On MHA, elevation was convex, while flat colonies occurred on CBA. Under the same culture conditions, the most closely related *K. rhizophila* DSM 11926^T differed in terms of increased colony diameter on CBA (2–3 mm) and an intense yellow pigmentation, regardless of whether cells were grown on MHA or CBA. Optimum growth conditions of *K. tytonicola* sp. nov. strains 473 and 489^T were met between 0 to 2% NaCl, a pH of 7, but growth was also detected between pH 6 and pH 9. Temperatures between 20 and 37 °C could be tolerated, while *K. rhizophila* also grows at 40 °C. Besides, sodium chloride tolerance differed from *K. rhizophila* DSM 11926^T and ranged from 0% to 7.5% (w/v) in the oil gland isolates.

Moreover, API test systems revealed clear differences between *K. tytonicola* sp. nov. and their most closely related species. For example, the type strain 489^T, in contrast to *K. rhizophila*, expressed

Table 3
Digital Protologue table of *Kocuria tytonicola* sp. nov. strain 489^T DSM 104133^T.

TAXONUMBER	TA00340
DATE OF THE ENTRY	2018-08-21
DRAFT NUMBER/DATE	005
VERSION	Draft
SPECIES	*Kocuria tytonicola*
GENUS NAME	*Kocuria*
SPECIFIC EPITHET	*tytonicola*
SPECIES STATUS	sp. nov.
SPECIES ETYMOLOGY	*tytonicola* (ty.to.ni'co.la. N.L. fem. n. *Tyto* a genus of owls; N.L. suff. -*cola* (from L. masc. or fem. n. *incola*) an inhabitant, dweller; N.L. fem. n. *tytonicola* an inhabitant of owls)
AUTHORS	Braun MS, Wang E, Zimmermann S, Wagner H, Boutin S, Wink M
TITLE	*Kocuria tytonicola* sp. nov., new bacteria from the preen glands of American barn owls (*Tyto furcata*)
JOURNAL	Systematic and Applied Microbiology
CORRESPONDING AUTHOR	Markus Santhosh Braun
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SUBMITTER	MARKUS SANTHOSH BRAUN
E-MAIL OF THE SUBMITTER	m.braun@uni-heidelberg.de
HAS THE TAXON BEEN SUBJECTED TO EMENDATION?	- NO
DESIGNATION OF THE TYPE STRAIN	489
STRAIN COLLECTION NUMBERS	DSM 104133, LMG 29945
16S rRNA GENE ACCESSION NUMBER	MG547560
GC mol%	71.0
DATA ON THE ORIGIN OF THE SAMPLE FROM WHICH THE STRAIN HAD BEEN ISOLATED	
COUNTRY OF ORIGIN	DEU
REGION OF ORIGIN	Baden-Württemberg
DATE OF ISOLATION UNKNOWN (< yyyy)	2014/06/27
SOURCE OF ISOLATION	Preen gland of *Tyto furcata*
SAMPLING DATE	2014-06-27
GEOGRAPHIC LOCATION	RWTH Aachen University, Aachen, Germany
LATITUDE	N50°46′38.06"
LONGITUDE	E6°2′51.623"
NUMBER OF STRAINS IN STUDY	2
SOURCE OF ISOLATION OF NON-TYPE STRAINS	Preen gland of *Tyto furcata*
GROWTH MEDIUM, INCUBATION CONDITIONS [Temperature, pH, and further information] USED FOR STANDARD CULTIVATION	48 h, 35 °C, pH 7 on LB MHA or Columbia Agar supplemented with 5% sheep blood
ALTERNATIVE MEDIUM 1	Luria-Bertani
ALTERNATIVE MEDIUM 2	Müller Hinton
ALTERNATIVE MEDIUM 3	Columbia Agar supplemented with 5% sheep blood
CONDITIONS OF PRESERVATION	−80 °C in 15% glycerol or freeze-dried
GRAM STAIN	POSITIVE
CELL SHAPE	coccus
MOTILITY	nonmotile
SPORULATION (resting cells)	none
COLONY MORPHOLOGY	on MHA: circular pale orange non-viscous 1–2 mm in diameter convex On Columbia sheep blood Agar: circular pale orange non-viscous 1–2 mm in diameter flat
TEMPERATURE RANGE	20–37
LOWEST TEMPERATURE FOR GROWTH	20
HIGHEST TEMPERATURE FOR GROWTH	37
TEMPERATURE OPTIMUM	30
LOWEST pH FOR GROWTH	6
HIGHEST pH FOR GROWTH	9
pH OPTIMUM	7
pH CATEGORY	neutrophile
LOWEST NaCl CONCENTRATION FOR GROWTH	0
HIGHEST NaCl CONCENTRATION FOR GROWTH	7.5
SALINITY OPTIMUM	0–2%
RELATIONSHIP TO O2	facultative aerobe
Positive tests with API	API 20 NE: URE, ESC, GEL, GLU, NAG, MAL, GNT, ADI, PAC API ZYM: Esterase (C4), Esterase Lipase (C8), leucine arylamidase, β-glucosidase

Table 3 (*Continued*)

Negative tests with API	API 20 NE: NO3, TRP, GLU, ADH, PNPG, ARA, MAN, CAP, MLT, CIT OX API ZYM: Alkaline phosphatase, Lipase (C14), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, naphtolphosphohydrolase, α-galactosidase, β-galactosidase, β-glucoronidase, α-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase, α-fucosidase
OXIDASE	negative
CATALASE	positive
QUINONE TYPE	MK-7(H2) (82%)
MAJOR FATTY ACIDS	anteiso-C15:0, anteiso-C17:0
PHOSPOLIPID PATTERN OR DIAGNOSTIC PHOSPOLIPID	Diphosphatidylglycerol, phosphatidylglycerol, unknown phospholipid, unknown aminophospholipid, unknown glycolipids
HABITAT	Preen gland of *Tyto furcata*

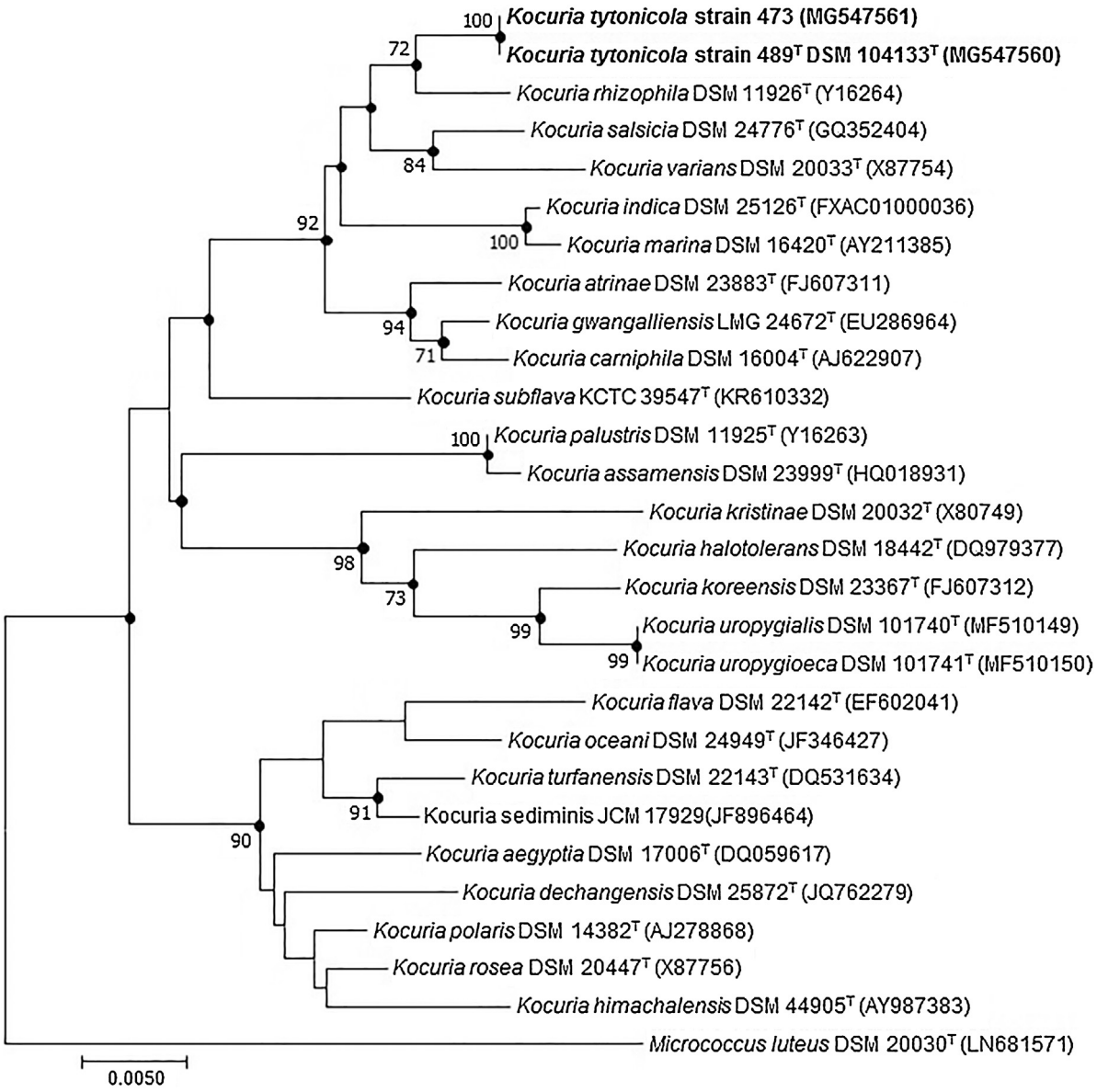

Fig. 1. Neighbor joining tree of the 16S rRNA gene illustrating the phylogenetic position of *K. tytonicola* sp. nov. strains 489^{T}DSM 104133^T and 473 in the genus *Kocuria*. Bootstrap values >70 are indicated and based on 1000 replication.

urease, gelatinase and β-glucosidase, but tests for α-glucosidase were negative (Table 1).

In terms of their polar lipid profiles, strains 473 and 489^T could not be reliably be distinguished. Both contained phosphatidylglycerol, diphosphatidylglycerol and unidentified aminoglycolipids, glycolipids and phospholipids. However, they differed from the most closely related species in the absence of an aminophospholipid and unidentified lipids (Supplementary Fig. 1). Cellular fatty acid distribution in both strains of *Kocuria tytonicola* sp. nov. was nearly identical, mainly consisted of anteiso-$C_{15:0}$ and anteiso-$C_{17:0}$

(Supplementary Table 2) and thereby perfectly coincides with the genus *Kocuria* [7]. Menaquinone composition in *K. tytonicola* strain 489^T was dominated by MK-7(H_2) (82%) and in line with patterns reported for other *Kocuria* spp. [7].

More than 1400 nucleotides of the 16S rRNA gene were sequenced from *K. tytonicola* sp. nov. strain 489^T (16S rRNA gene accession number MG547560, genome accession number PHOA00000000) and *K. tytonicola* sp. nov. strain 473 (16S rRNA gene accession number MG547561, genome accession number RDEX00000000) revealing 100% sequence similarity between each other. Sequence similarities of *K. tytonicola* sp. nov. to the most closely related species were 99.9% (*K. rhizophila* DSM 11926^T), 98.9% (*K. salsicia* DSM 24776^T), 98.4% (*Kocuria indica* DSM 25126^T), 98.5% (*K. varians* DSM 20033^T) and 98.3% (*K. marina* DSM 16420^T).

Phylogenetic analysis of the 16S rRNA gene clearly separated the new species from all members of *Kocuria* sp., confirmed the identity of *Kocuria rhizophila* DSM 11926^T as the closest relative and thereby perfectly mirrored sequence similarities. Moreover, the phylogenetic tree exhibited high statistical support in nearly all nodes, which in turn were recovered by all tree reconstruction methods (Fig. 1).

While G + C content derived from thermal denaturation was 70.4 mol% for 489^T, it amounted to 70.6 mol% in strain 473. G + C content derived from NGS data was 71.0 mol% in the type strain of *K. tytonicola* and 71.1 mol% in strain 473, as opposed to 70.8 mol% in the most closely related species *K. rhizophila*. Further genome features can be found in Supplementary Table 3. Our proposal of *K. tytonicola* as a new species was further corroborated by ANI values substantially below the level suggested for species delineation (Table 2). In DNA fingerprints, the two strains of *K. tytonicola* sp. nov. exhibited similar band patterns, but unambiguously showed that strains 489^T and 473 are not clonal varieties of each other (Supplementary Fig. 2).

Similarly, MALDI-TOF MS analysis placed the owl isolates into a well separated cluster within *Kocuria* sp. (Supplementary Fig. 3). The two strains of *Kocuria tytonicola* could be discriminated by minor deviations, namely the inability of 473 to hydrolyze esculin (Table 1) and slightly different band patterns in DNA fingerprint analysis (Supplementary Fig. 2). On the other hand, they could not be distinguished using 16S rRNA gene sequences (Fig. 1), polar lipid profiles (Supplementary Fig. 1), MALDI-TOF MS (Supplementary Fig. 3), colony and cell morphology or growth characteristics. While both strains are susceptible to most the antibiotics tested, 8 μg/mL and 32 μg/mL were needed to inhibit bacterial growth using kanamycin and polymyxin B, respectively (Supplementary Table 4).

In conclusion, the data presented in this manuscript provide strong evidence that the preen gland isolates are new members of the genus *Kocuria*. We therefore propose the name *Kocuria tytonicola* for the microorganisms which have been recovered from the uropygial glands of American barn owls. Typical characteristics of the type strain of the species are depicted in the Digital Protologue table (Table 3).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.syapm.2018.11.004.

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