

Differentiation of *Corynebacterium uropygiale* strains from Northern Bobwhite (*Colinus virginianus*) and Turkeys (*Meleagris gallopavo*)

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Received date: September 22, 2017, Accepted date: November 09, 2017, Published date: November 16, 2017

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Abstract

Genetic and biochemical characterizations were performed on the bacterial isolates from the intestinal contents of the Northern Bobwhite (*Colinus virginianus*) in western Texas, USA. The bobwhite bacterial isolates were Gram-stain-positive, non-acid fast, catalase-positive, oxidase-negative and non-spore forming rods. The 16S rRNA gene sequences of the bobwhite isolates were identical and showed the highest sequence similarity to *Corynebacterium uropygiale* (99%) isolated from the wild captive turkey (*Meleagris gallopavo*) in Germany.

Phylogenetic analysis based on the 16S rRNA gene sequences and the rpoB gene sequences suggested that bobwhite strains belong to *Corynebacterium uropygiale*. However, DNA-DNA hybridization showed 76.5% relatedness between bobwhite DSM 101879T and *C. uropygiale* DSM 46817T, indicating that Bobwhite and Turkey strains may represent different subspecies of *C. uropygiale*. The genetic separation is supported by the distinct biochemical properties of bobwhite and turkey strains. Compared to turkey strains, bobwhite strains have no pyrazinamidase and alkaline phosphatase activities, do not ferment mannitol, and have lower content of fatty acids C_{18:1} ω_{9c} (27.8%) and 18:0 10-methyl, TBSA (1.0%). To reflect the origin of isolation, the name *Corynebacterium uropygiale* subsp. colini subsp. nov. is proposed for bobwhite isolates. The type strain is DSM 101879T (=KCTC 49003T).

Keywords: *Corynebacterium uropygiale*; *Colinus virginianus*; Northern Bobwhite; Biochemical characteristics; Fatty acids profile; Phylogeny

Introduction

The GenBank accession number for 16S rRNA gene sequence and rpoB gene sequence are KY490590 and KY490591, respectively.

The genus *Corynebacterium* was first described by Lehmann and Neumann [1] and currently comprises more than 130 species. Members of *Corynebacterium* are Gram-stain positive, non-spore-forming, catalase positive, rod- or irregularly-shaped bacteria [2]. *Corynebacterium spp.* are widely distributed in nature and possess certain chemotaxonomic features such as the presence of tuberculostearic acid and short chain mycolic acids in cellular fatty acids, menaquinone (MK-8H₂) or isoprenoid quinones (MK-9H₂) and meso-diaminopimelic acid as components of the cell wall peptidoglycan [3,4]. The organisms are routinely isolated as commensals or pathogens of mammals and birds [5].

The most well characterized species include *C. diphtheriae*, the etiologic agent of diphtheria [6]; *C. pseudotuberculosis*, the causative agent of caseous lymphadenitis in sheep and goats and lymphadenitis in horses [7,8] and *C. renale* group that causes bovine cystitis and pyelonephritis [9]. Over the past two decades, many new species have been isolated from wild birds such as *C. kroppenstedtii* from a lovebird, *C. falsenii* and *C. aquilae* from eagles, *C. sphenisci* from wild

penguins, *C. spheniscorum*, *C. ciconiae* from black storks, *C. pelargi* from white stork nestlings and *C. trachiae* from a white stork [8, 10-15]. In late 2015, *C. uropygiale spp. nov.* was cultured from the preen gland of captive turkeys in Heidelberg, Germany [16].

Methodology

The Northern Bobwhite (*Colinus virginianus*) belongs to the order Galliformes which consists of ground-feeding birds, such as chicken and turkey. In a previous study, we characterized the intestinal and respiratory microbiota of wild-caught bobwhite and collected a number of isolates that exhibited morphological and biochemical characteristics of genus *Corynebacterium* [17].

At the time, these isolates could not be identified to a species level based on phenotypic characteristics, biochemical profiles and 16S rRNA gene sequenced deposited to public databases. In the present study, further biochemical and molecular analyses were carried out which lead to the classification of these isolates to the newly described *C. uropygiale* [16].

During initial bacterial culture and biochemical characterizations, *Corynebacterium jeikeium* (ATCC 43734T) was used as a control. *C. jeikeium* and the bobwhite isolates (n=5) under investigation were stored in 20% glycerol Luria broth (LB) at -80°C. Frozen cultures were inoculated onto Columbia blood agar with 5% sheep blood (Thermo Fisher Scientific Remel Products).

Inoculated agar plates were incubated at 37°C in a 5% CO₂ atmosphere for 48 hours. To ensure purity, bacterial cultures were passaged a second time on agar plates. Pure cultures on agar were used for biochemical and molecular analysis. The colonies of the isolates on Columbia agar with 5% sheep blood were small (approx. 1-1.5 mm in diameter), circular, creamy, opaque with entire edge.

The isolates were Gram-stain positive, rod-shaped, acid-fast stain negative, catalase weak positive and none-spore forming. Phase contrast microscopic examination of 10 h BHI cultures indicated that the organisms were non-motile. Matrix-assisted laser desorption/ionization (MALDI) failed to assign the isolates to any known bacterial species.

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Carbohydrate fermentation patterns and enzymatic activities of five isolates were examined using API Coryne strip and API ZYM test strips (BioMerieux, Germany), respectively. In brief, API Coryne and API ZYM kits were inoculated with isolated colonies of a pure culture of the control and each isolate was grown for 24 hours according to the manufacturer's instructions (BioMerieux, Germany).

The inoculated biochemistry strips were incubated for 24 h at 37°C in an aerobic, non-carbon dioxide environment. After the addition of appropriate reagents provided in the kit, biochemical reactions were evaluated and the results were recorded. Catalase was weak positive.

The isolates were positive for esterase (C4), esterase lipase (C8), lipase (C14), leucine arylamidase, acid phosphatase, α -glucosidase and β -glucosidase. The isolates reduced nitrate and hydrolyzed aesculin, but not urea or gelatin.

Acid was produced from glucose, ribose, maltose and sucrose, but not from xylose, mannitol, lactose and glycogen. Two of the five isolates had weak pyrazinamidase activity. The isolates showed no activity of pyrrolidonyl arylamidase, alkaline phosphatase, β -glucuronidase, β -galactosidase, N-acetyl-B glucosaminidase, valine arylamidase and cystine arylamidase.

The cellular fatty acids profile of the isolates was determined by Gas Chromatography using the Agilent ChemStation and Sherlock software (Microbial ID, Inc. Newark, DE 19713).

The results showed that the major cellular fatty acids were C_{16:0} (25.5%), C_{18:0} (20.1%) and C_{18:1} ω 9c (27.8%). Small amounts of other

fatty acids were also present such as C_{14:0} (1.3%), C_{17:0} (3.6%), C_{17:0} cyclo (2.1%), C_{17:1} ω 8c (1.7%), 10-methyl C_{18:0} (Tuberculostearic acid, 1.0%) and summed feature 3 (C_{16:1} ω 7c/C_{16:1} ω 6c, 2.1%), summed feature 5 (C_{18:0} ante/C_{18:2} ω 6,9c, 9.4%) and summed feature 8 (C_{18:1} ω 7c/C_{18:2} ω 6c, 4.7%).

Results

The cell wall peptidoglycan structure was determined by DSMZ (Braunschweig, Germany) according to a previously published method [16]. The cross linkage type was meso-Dpm-direct (A1 γ) and meso-diaminopimelic acid was the diagnostic diamino acid of the peptidoglycan.

The DNA G+C content was analyzed by HPLC at the DSMZ (Braunschweig, Germany) as described previously [18,19]. In brief, DNA was hydrolyzed with P1 nuclease and nucleotides dephosphorylated as described previously [19]. The resulting deoxyribonucleosides were analyzed by HPLC and the G+C value was calculated according to a previously published method [19]. The G+C content of C110598 was 58.3 %.

Near complete 16S rRNA gene and a 310 bp fragment of a housekeeping gene (rpoB) were amplified by PCR as described previously [20,21]. The 16s rRNA gene sequences of the isolates were identical and shared 99% identity with the 16s rRNA gene sequence of *C. uropygiale*. Multiple alignments of the 16S rRNA gene sequences (1,500 bp) and rpoB sequence (310 bp) were conducted using Clustal W method [9]. Phylogenetic trees were constructed using the Neighbor-Joining (NJ), maximum-parsimony (MP) and maximum-likelihood (ML) algorithms each with 1000 randomly selected bootstrap replications [22-24].

Comparable tree topology was produced by all methods in which the bobwhite isolates formed one cluster that was closely related to *C. uropygiale*. The phylogenetic relationship of 16S rRNA gene (Figure 1A) was supported by the rpoB gene tree (Figure 1B). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches.

Although greater than 99% of similarity has been used as the cutoff value for bacterial species identification, poor discriminatory power for some genera has been reported [25]. To determine the relatedness of bobwhite isolates and *C. uropygiale* type strain, DNA-DNA hybridization (DDH) between a representative isolate that had been deposited to DSMZ Bacteria Collection (DSM 101879T) and *C. uropygiale* (DSM 46817T) was carried out by DSMZ (Braunschweig, Germany). Genomic DNA was isolated as described previously [18].

DDH was performed as described previously [25,26]. The result showed 76.5% \pm 3.6 similarity between DSM 101879 T and DSM 46817 T, indicating that the turkey and quail isolates may represent separate subspecies of *C. uropygiale* as DDH values of 79-80% are considered the threshold for defining subspecies [22,27].

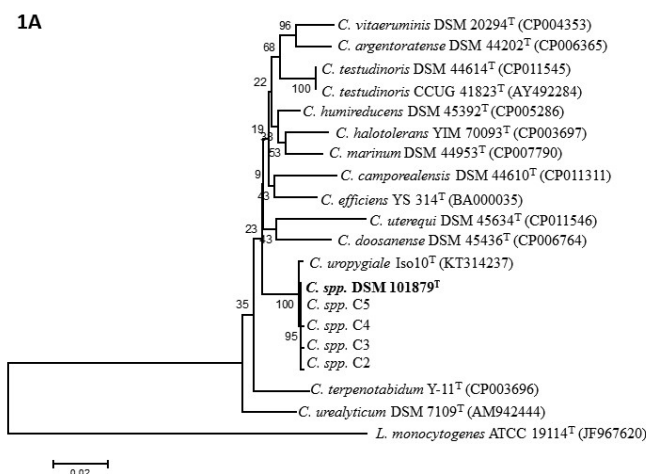


Figure 1A: The phylogenetic relationship of 16S rRNA gene.

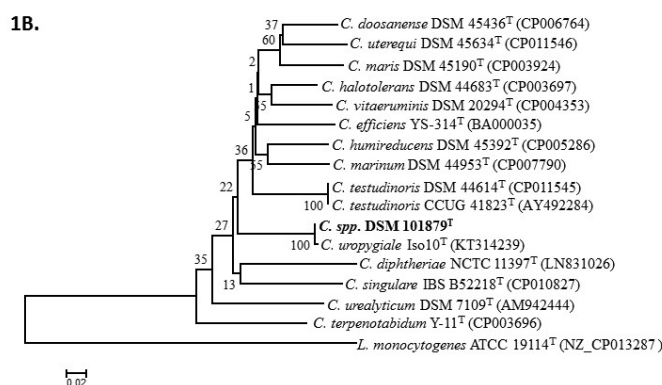


Figure 1B: The phylogenetic relationship of 16S rRNA gene was supported by the rpoB gene tree.

The classification of subspecies is supported by the substantial differences in fatty acids composition and biochemical properties between bobwhite and turkey isolates. For instance, bobwhite isolates have much less $C_{18:1} \omega 9c$ (27.8%) and $C_{18:0}$ 10-methyl, TBSA (1.0%)

than turkey isolates ($C_{18:1} \omega 9c$, 37.4% and $C_{18:0}$ 10-methyl, 10.8%). Bobwhite isolates also have small amounts of $C_{14:0}$ (1.3%), $C_{17:0}$ (3.6%), $C_{17:0}$ 2-OH (3.2%) and $C_{17:1} \omega 8c$ (1.7%) which were not described for *C. uropygiale* turkey stains [16]. Contrary to turkey strains, bobwhite

isolates lack pyrazinamidase (PYZ) and alkaline phosphatase (PAL) activities and do not produce acid from mannitol. The comparative characteristics of *Corynebacterium uropygiale* strains are provided in Table 1 [28-30].

Fatty Acids composition*	1	2
C _{14:0}	1.3	N/A
C _{16:0}	25.5	21
C _{17:0}	3.6	N/A
C _{17:0} cyclo	2.1	N/A
C _{17:1} ω8c	1.7	N/A
C _{18:0}	20.1	18.5
C _{18:1} ω9c	27.8	37.4
C _{18:0} 10-methyl, TBSA	1	10.8
Summed feature 3	2.1	N/A
Summed feature 5	9.4	N/A
Summed feature 8	4.7	N/A
Biochemical reactions		
Nitrate reduction (NIT)	+	+
Urease	-	+/-
Aesculin (ESC)	+	+
Pyrazinamidase (PYZ)	-	w
Alkaline Phosphatase (PAL)	-	+
Production of acid from		
Mannitol	-	+
Maltose (MAL)	+	+
Sucrose	+	+
Glucose	+	+
Ribose	+	+
*Fatty acids with <1% are not shown. Summed feature 3: C _{16:1} ω7c/C _{16:1} ω6c. Summed feature 5: C _{18:0} ante/C _{18:2} ω6,9c. Summed feature 8: C _{18:1} ω7c/C _{18:2} ω6c. Column 1: Bobwhite <i>C. uropygiale</i> isolate (DSM101879T). Column 2: Turkey <i>C. uropygiale</i> isolate (DSM46817T). (+) positive; (-) negative; w: weakly positive; and N/A: not available.		

Table 1: Comparative characteristics of *Corynebacterium uropygiale* strains isolated from bobwhite and turkey.

Conclusion

In conclusion, the collective results of 16S rRNA gene and rpoB sequencing, phylogenetic study, DDH and phenotypic characterizations suggest that bobwhite isolates belong to species *C. uropygiale*. While it is interesting to see that bobwhites in Texas, USA and turkeys in Germany share the same *Corynebacterium* species, the bacterial strains can be differentiated based on the following characteristics: bobwhite strains are negative for pyrazinamidase (PYZ), negative for alkaline phosphatase (PAL), and no acid production from mannitol, as well as low amounts of C_{18:1} ω9c and C_{18:0} 10-methyl (TBSA) and the summed features 3, 5 and 8. To reflect the differences in host origin, geographic location and

phenotypic characteristics, we propose that bobwhite and turkey strains represent two subspecies of *C. uropygiale*. The name *C. uropygiale* subsp. colini (co.li'ni. N.L. gen. n. colini of *Colinus*, scientific name of bobwhites) may be considered for bobwhite strains. The type strain of *C. uropygiale* subsp. colini. is DSM 101879T (=KCTC49003T). The NCBI GenBank accession number for the 16S rRNA gene sequence is KY490590 and rpoB gene sequence is KY490591.

Acknowledgements

This work was financially supported by the Rolling Plains Quail Research Foundation. The authors thank Dr. Steve Presley, Dr. Anna

Gibson, and Dr. Kristan Urban with the Texas Institute of Environmental and Human Health at Texas Tech University for their assistance with sample procurement. The authors thank Ms. Christine Y. Zhang for editing the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

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