**Turicibacter bilis** sp. nov., a novel bacterium isolated from the chicken eggshell and swine ileum

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**INTRODUCTION**

The genus *Turicibacter* comprises obligately anaerobic, Gram-positive bacteria belonging to the phylum *Firmicutes*, class *Erysipelotrichia*, order *Erysipelotrichales* and family *Turicibacteraceae* (formerly considered a member of the family *Erysipelotrichaceae*) [1]. Currently, there is only one designated species within the genus *Turicibacter*, *Turicibacter sanguinis*, which was isolated from a blood culture of a febrile adult human [2]. Strains of *T. sanguinis* have been isolated from other animals, and *T. sanguinis* DNA sequences have been identified in culture-independent studies in many species, including turkeys, swine and even termites [3–7]. However, few strains within the genus have been isolated. Here, we describe novel *Turicibacter* strains MMM721$^T$, ISU324 and PIG517, which were isolated from the eggshell surface of two distinct flocks of chickens and the ileum of a healthy pig, respectively. We propose that these strains represent a novel species within the genus and propose the name *Turicibacter bilis* sp. nov., with strain MMM721$^T$ as the type strain.

Chicken egg washes in 1×PBS and ileal contents were treated 1:1 with 70% ethanol and incubated overnight to enrich for spores by killing vegetative cells, as described previously [8]. Spore preparations were plated on brain–heart infusion (BHI) agar (Difco), supplemented with 0.1% (v/v) whole bile isolated from healthy white leghorn chickens, 0.05% (w/v) l-cysteine hydrochloride and 0.0001% resazurin, and incubated at 42°C under anaerobic conditions (chamber inflated with 85% N$_2$, 5% CO$_2$, 10% H$_2$) in a Coy anaerobic chamber to isolate spore-forming anaerobes. Bacterial colonies were randomly selected for 16S rRNA gene amplification PCR and Sanger sequencing after 72h with the previously described bacterial primers 27F-YM and 1492R, using previously described methods [9, 10]. A total of 192 chicken isolates and 32 pig isolates were screened. The
generated 16S rRNA gene fragments were 1392, 1397 and 1393 bp long for MMM721 T, ISU324, and PIG517, respectively (accession numbers MT500785, MT500789 and MT500788). These sequences were nearly identical (>99%) to one another. When these sequences were assigned taxonomic identification using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (blast), they all grouped closely (99% 16S rRNA gene identity) to *T. sanguinis* MOL361 T, the type strain and only described species within the genus *Turicibacter* [2, 11]. A 16S rRNA gene phylogeny was reconstructed with sequences from publicly available *Turicibacter* isolates downloaded from the Ribosomal Database Project (http://rdp.cme.msu.edu/). The resulting phylogenetic tree showed the three *T. bilis* isolates forming a clade separate from the *T. sanguinis* strains (Fig. S1, available in the online version of this article). All three strains were maintained on a modified BHI medium (BHIGL) supplemented with 1.0% (v/v) glycerol, 1.1% (w/v) sodium dl-lactate, 0.05% (w/v) l-cysteine hydrochloride and 0.0001% resazurin throughout the course of the study.

*Turicibacter sanguinis* MOL361 T was obtained from DSMZ for use as a reference strain for comparison purposes. *Turicibacter sanguinis* MOL361 T was capable of growth on BHIGL at 37°C, so this medium was used for strain maintenance throughout the course of this study.

Growth conditions were determined for MMM721 T on BHIGL agar plates (Table 1). Determinations of growth rate and terminal OD600 were determined in BHIGL broth inoculated with an agar plug of a single colony of MMM721 T grown for 48 h on BHIGL. Cellular motility, catalase activity and sporulation activity were determined as described previously [8, 12–14]. Strain MMM721 T

### Table 1. Comparison of strain MMM721 T and *Turicibacter sanguinis* MOL361 T

| Characteristic | *T. sanguinis* MOL361 T | *T. bilis* MMM721 T |
|----------------|------------------------|---------------------|
| Morphology     | Gram+ bacilli chains    | Variable: Gram+ bacilli chains and Gram+ coccoid clusters |
| Cell size (µm) | 0.5–2.0×0.7–7.0         | Rods: 0.8–8.2×0.5–2.0; Cocci: 1.0–2.0 |
| Temperature range for growth (°C) | 25–46 | 30–45 |
| pH range for growth (pH) | 6.5–8.0 | 6.5–8.5 |
| Motility       | –                       | –                   |
| Catalase       | –                       | –                   |
| Biochemical reactivity: | | |
| α-D-Glucoside  | +                       | –                   |
| α-D-Galactoside| +                       | –                   |
| Glycine        | +                       | –                   |
| Arginine       | –                       | +                   |
| Serine         | +                       | –                   |
| Gelatin        | +                       | +                   |
| Aesculin       | ±                       | ±                   |
| Maltose        | +                       | –                   |
| Potassium 5-ketogluconate | w | – |
| Susceptibility to (50 µg ml⁻¹): | | |
| Penicillin     | S                       | S                   |
| Kanamycin      | S                       | S                   |
| Vancomycin     | S                       | S                   |
| Colistin       | R                       | R                   |
| SCFAs          | Ac, Lac                 | Ac, But, Lac, Val, Cap, Phen |
| DNA G+C content (mol%) | 34.2 | 34.4 |
Fig. 1. (a) Transmission electron microscope image of MMM721^{T} cells. Bar, 1 μm. (b) Scanning electron microscope image of MMM721^{T} cells. Bar, 2 μm. Cells were cultured in BHIGL for 24 h at 42 °C prior to fixation and visualization.
was strictly anaerobic, as exemplified by the absence of growth on aerobically incubated plates after 72 h. Strain MMM721<sup>T</sup> was capable of growth between 30 and 45 °C, with optimal growth achieved at 42 °C (Table 1). The pH range of MMM721<sup>T</sup> growth was between pH 6.5–8.5, with an optimal pH of 7.5 (Table 1). Resistance to 70 % ethanol and survival under aerobic conditions suggested all strains were capable of forming spores. Strain MMM721<sup>T</sup> was negative for catalase activity (Table 1). The doubling time of MMM721<sup>T</sup> was estimated to be approximately 30 min. The terminal OD<sub>600</sub> was 0.600, corresponding to 3.75×10<sup>6</sup> c.f.u. ml<sup>−1</sup>. Strain MMM721<sup>T</sup> was grown for 3 days on BHIGL and BHIGL agar +0.1 % whole chicken bile to determine colony and cellular morphology. On BHIGL, colonies appeared as small to medium-sized irregularly shaped, umbonate colonies with undulating edges. The raised centre of the colony was an opaque white that transitioned to a translucent tan or grey when moving toward the colony’s outer edge. On BHIGL +0.1 % whole chicken bile, colonies appeared larger, with an opaque white, almost-filamentous interior that was raised with translucent, mucoid margins that were undulating to lobate in nature. Cells stained Gram-positive (Table 1). Cellular morphology was assessed with both transmission electron microscopy (TEM) and scanning electron microscopy (SEM). For TEM, the bacteriological samples were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. They were post-fixed with 2% osmium and placed in 2% agar. Then the samples were processed through graded alcohols, propylene oxide

| Peak       | T. bilis MMM721<sup>T</sup> | T. sanguinis MOL361<sup>T</sup> |
|------------|-----------------------------|---------------------------------|
| 10:0       | 0.29                        | 0.12                            |
| 11:0       | 1.08                        | 0.56                            |
| 12:0       | 2.84                        | 1.88                            |
| 13:0       | 0.86                        | 2.65                            |
| 14:0       | 1.41                        | 1.33                            |
| 15:0       | 54.1                        | 42.39                           |
| C<sub>16:1</sub> ω<sub>9c</sub> | 1.35                      | 1.66                            |
| C<sub>16:1</sub> ω<sub>7c</sub> | 0.07                      | 0.07                            |
| C<sub>16:1</sub> ω<sub>5c</sub> | 1.08                      | 0.56                            |
| 17:0       | 0.41                        | 1.52                            |
| C<sub>16:0</sub> 3OH       | 1.8                         | 3.8                             |
| C<sub>18:2</sub> ω<sub>6,9c</sub> | 0.75                      | 0.75                            |
| C<sub>18:2</sub> ω<sub>9c</sub> | 5.78                      | 5.78                            |
| C<sub>18:2</sub> ω<sub>7c</sub> | 16.28                     | 23.46                           |
| C<sub>18:1</sub> ω<sub>5c</sub> | 1                         | 1.65                            |
| 18:0       | 11.76                       | 8.92                            |
| C<sub>20:1</sub> ω10c     | 0.66                        | 0.66                            |
| 20:0       | 1.04                        | 0.75                            |

*Summed features are fatty acids that cannot be resolved reliably from another fatty acid using the chromatographic conditions chosen. The MIDI system groups these fatty acids together as one feature with a single percentage of the total.
†Unknown fatty acids are not listed in the MIDI system library; values are the equivalent chain lengths.

Table 2. Fatty acid methyl esters of MMM721<sup>T</sup> and *Turicibacter sanguinis* MOL361<sup>T</sup>, grown on BHIGL media
Table 3. Pairwise comparison of ANIb values between MMM721T, ISU324, PIG517 and all publicly available genomes on the PATRIC genome database.

Strains: 1. *T. sanguinis* MGYG-HGUT-00143; 2. *Turicibacter* sp. H121; 3. *T. sanguinis* PC909; 4. *Turicibacter* sp. UBA1159; 5. *T. sanguinis* am_0171; 6. *Turicibacter* sp. HGF1; 7. *Turicibacter* sp. UBA7094; 8. *T. sanguinis* MGYG-HGUT-00037; 9. *T. sanguinis* MOL361T; 10. *Turicibacter* sp. Lab28B1bin27; 11. *Turicibacter* sp. Nc150P1bin9; 12. *T. bilis* ISU324; 13. *T. bilis* PIG517; 14. *T. bilis* MMM721T. Bolded values exceed the 95–96% threshold for species assignment. The * indicates 100% similarity when genomes were compared against themselves.

|    | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  | 11  | 12  | 13  | 14  |
|----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| 1  | *   | 76.15 | 99.43 | 73.73 | 99.38 | 99.4 | 73.96 | 76.36 | 99.38 | 65.87 | 67.07 | 76.34 | 76.27 | 76.45 |
| 2  | 76.96 | *   | 76.59 | 76.5 | 76.66 | 76.15 | 76.87 | 98.34 | 76.62 | 66.06 | 67.2 | 98.11 | 98.66 | 98.1 |
| 3  | 99.55 | 76.15 | *   | 73.8 | 99.58 | 99.36 | 74.03 | 76.36 | 99.73 | 65.93 | 67.07 | 76.45 | 76.24 | 76.4 |
| 4  | 73.89 | 76.35 | 73.86 | *   | 73.85 | 73.89 | 81.88 | 76.4 | 73.81 | 65.96 | 67.02 | 76.4 | 76.36 | 76.35 |
| 5  | 99.44 | 76.02 | 99.58 | 73.55 | *   | 99.4 | 73.83 | 76.2 | 99.51 | 65.86 | 67.19 | 76.12 | 76.05 | 76.21 |
| 6  | 99.31 | 75.86 | 99.25 | 73.72 | *   | 99.26 | 73.89 | 76.02 | 99.2 | 65.86 | 66.78 | 76.08 | 75.98 | 75.99 |
| 7  | 74.33 | 77.18 | 74.33 | 82.13 | 74.25 | 74.36 | *   | 77.3 | 74.3 | 66.06 | 67.04 | 77.18 | 77.29 | 77.21 |
| 8  | 76.46 | 98.04 | 76.25 | 76.38 | 76.3 | 76.2 | 77.22 | *   | 76.3 | 66.29 | 67.62 | 98.14 | 98.04 | 98.04 |
| 9  | 99.46 | 76.18 | 99.68 | 73.83 | 99.47 | 99.33 | 74.04 | 76.42 | *   | 65.89 | 67.22 | 76.57 | 76.24 | 76.6 |
| 10 | 66   | 66.25 | 66.08 | 66.03 | 65.96 | 65.99 | 65.86 | 66.18 | 66.06 | *   | 65.4 | 66.31 | 66.25 | 66.31 |
| 11 | 67.02 | 67.18 | 67.02 | 67.04 | 67.06 | 67.02 | 66.89 | 67.27 | 67.03 | 65.49 | *   | 67.22 | 67.24 | 67.18 |
| 12 | 76.39 | 97.79 | 76.44 | 76.23 | 76.23 | 76.21 | 76.72 | 98.04 | 76.44 | 66.14 | 67.55 | *   | 97.97 | 98.76 |
| 13 | 76.36 | 98.5 | 76.17 | 76.54 | 76.06 | 76.06 | 76.84 | 98.25 | 76.14 | 66.08 | 67.31 | 98.17 | *   | 97.93 |
| 14 | 76.51 | 97.27 | 76.45 | 76.27 | 76.17 | 76.23 | 76.92 | 97.98 | 76.43 | 65.92 | 67.32 | 98.76 | 97.71 | *   |
and Eponate 12 resin followed by a 48 h polymerization. An uranyl acetate and Reynold’s lead stain were performed on the thin section before being examined with a ThermoFisher FEI Tecnai G2 BioTWIN electron microscope (FEI) and images were taken with Nanosprint12 camera (AMT) [15]. For SEM, bacterial cells were put through a 0.22 µm Swinney filter and the filters were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer. The samples were further processed through sequential exposure to osmium and thiocarbohydrazide [16]. Samples were dehydrated through graded alcohols and chemically dried with hexamethyldisilizane [17]. Samples were decorated with a thin coating of gold–palladium mixture and viewed on a TM3030Plus scanning electron microscope (Hitachi). Under magnification, strain MMM721T was pleomorphic in presentation, composed of long chains of rods (0.8–8.2×0.5–2.0 µm) as well as clusters of coccoid-like cells (1.0–2.3 µm) after 24 h of incubation (Fig. 1a, b, Table 1). Despite the appearance of multiple cellular morphologies, 16S rRNA gene sequencing of cells from the broth cultures pre- and post- microscopy sample collection confirmed the culture was free from contamination. Similar cellular pleomorphy was previously observed within the order Erysipelotrichales, most notably the animal pathogen Erysipelothrix rhusiopathiae [18].

The cellular fatty acid profile of strain MMM721T was compared to T. sanguinis MOL361T to assess the relatedness between the species. Both isolates were grown in BHIGL broth inoculated with an agar plug of a single colony and pelleted via centrifugation prior to submission to Microbial ID for cellular fatty acids analysis, using the MIDI Sherlock Microbial Identification System [19]. Abundant cellular fatty acids for MMM721T were similar to T. sanguinis MOL361T, though the proportions of those cellular fatty acids varied between the two species. The main cellular fatty acids were C_{16:0}, C_{18:1}^{ω7c} and C_{18:0} (Table 2).
The biochemical characteristic of the novel isolates MMM721T, ISU324 and PIG517 were assessed using the RapID Ana II (Remel), API 20A (bioMérieux) and API 50CHL (bioMérieux) systems according to the manufacturer’s instructions (Tables 1 and S1). All tests were conducted in duplicate. The RapID Ana II system and the API 20A systems are used for the identification of anaerobic bacteria while the API50 CHL system is used for the identification of Lactobacillus and other related genera. Strain MMM721T was only positive for arginine and gelatin biochemical reactivity, with variable aesculin hydrolysis activity (Table 1). Strain ISU324 was positive for gelatin and aesculin biochemical reactivity while strain PIG517 was capable of biochemical reactivity to α-glucoside, arginine, gelatin and aesculin, with weak reactivity to serine, methyl β-D-xlyopyranoside, D-glucose, arbutin, salicin, cellobiose and maltose (Table S1). Together, the novel strains presented biochemical reactivity profiles that differentiated them from T. sanguinis MOL361T (Tables 1 and S1).

Next, short chain fatty acid (SCFA) production was determined for MMM721T, ISU324 and PIG517 in response to the substrates streaked on plates containing each of the four antibiotics in duplicate and was incubated for 48 h at 42 °C, with lack of growth being interpreted as susceptibility. After 48 h, only plates supplemented with colistin displayed growth, suggesting MMM721T was resistant or insensitive to colistin but susceptible to kanamycin, vancomycin, and penicillin G (Table 1).

Antibiotic susceptibility was measured using BHIGL agar plates supplemented with 50 µg ml⁻¹ of either kanamycin, colistin, vancomycin or penicillin G, as these antibiotics were previously assessed for T. sanguinis MOL361T [2]. Strain MMM721T was streaked on plates containing each of the four antibiotics in duplicate and was incubated for 48 h at 42°C, with lack of growth being interpreted as susceptibility. After 48 h, only plates supplemented with colistin displayed growth, suggesting MMM721T was resistant or insensitive to colistin but susceptible to kanamycin, vancomycin, and penicillin G (Table 1).

Genomic DNA was extracted from MMM721T, ISU324 and PIG517 using the Purelink genomic DNA extraction minikit (Invitrogen) according to the manufacturer’s instructions, and DNA quality was determined using a NanoDrop (Thermo Fisher Scientific), Qubit fluorimeter (dsDNA Broad Range kit; Life Technologies) and gel electrophoresis. A Nextera Flex barcoding kit (Illumina) was used to prepare 2x250 bp paired-end read genomic libraries for sequencing on an Illumina MiSeq instrument according to the manufacturer’s instructions. Each isolate was sequenced twice on separate MiSeq runs. The raw Illumina reads are available on NCBI’s sequence read archive (accession numbers: SRR11784102, SRR11784103, SRR11825064, SRR11825065, SRR11825066 and SRR11825067). Raw Illumina reads were uploaded to the PathoSystems Resource Integration Centre (patric) web resource [22, 23]. For each strain, the raw reads for the two MiSeq runs were pooled and assembled using patric’s online genome assembly tool, selecting the ‘SPAdes’ assembly strategy and using default settings [24]. Draft assemblies were annotated through patric’s RASTtk-enabled Genome Annotation Service [22, 25]. The MMM721T draft assembly was composed of 94 contigs, with an assembly length of 2717947 bp and a G+C content of 34.4mol%. The draft assembly for ISU324 had 118 contigs, with an assembly length of 2751587 bp and 34.2mol% G+C content. The PIG517 draft assembly had contigs and totalled 2585443 bp in size, with a G+C content of 34.3mol%. The three isolate assemblies had similar G+C content to the draft assembly of T. sanguinis MOL361T available through the patric genome database, though the genome length for MOL361T was 2946097 bp, nearly 200 kb higher than ISU324, which possessed the longest genome length of the three isolates (Table S1).

To determine divergence of the three Turicibacter isolates to other Turicibacter genomes, pairwise average nucleotide identity (ANI) and digital DNA-DNA hybridization (dDDH) calculations were conducted with MMM721T, ISU324, PIG517 and all publicly available Turicibacter species genomes on the patric genome database (Tables 3, S3 and S4). Both ANI and dDDH provide an in silico method to delineate boundaries between species [26–28]. Pairwise ANI calculations were conducted using both BLASTN+ (ANB) and MUMmer (ANIm) algorithms through the JSpecies Web server (http://jspecies.ribohost.com/jspeciesws/) [29]. The dDDH calculations were made through the Genome-to-Genome Distance Calculator (version 2.1) using the recommended settings (http://ggdc.dsmz.de/) [28]. Typically, ANI values >95% and dDDH values >70% are considered to be the same species. Comparisons between MMM721T, ISU324 and PIG517 produced ANI values >97% (both ANIb/ANIm) and dDDH values >84%, suggesting all strains belonged to the same species. Compared to T. sanguinis MOL361T, MMM721T, ISU324 and PIG517 had ANIb values of 76.43, 76.44 and 76.14%, and ANIm values of 85.97, 86.04 and 85.79%, respectively, suggesting these isolates represent a distinct species from T. sanguinis (Tables 3 and S3). The ANI results were corroborated by the dDDH results, with values of 22.5, 22.5 and 22.1% obtained for MMM721T, ISU324 and PIG517 when compared to T. sanguinis MOL361T (Table S4). Interestingly, the ANI and dDDH results provided additional taxonomic information for several of the previously isolated and sequenced Turicibacter strains. Strains H121 and T. sanguinis MOL361T, open reading frames (ORFs) from all four genomes were uploaded to eggNOG-mapper (http://eggnog-mapper.embl.de) for functional annotation and classification [30, 31]. A quadruple venn diagram of shared and unique ORFs was constructed using the ‘venn()’ function in the
The genus *Turicibacter* MOL361\(^{\text{T}}\), further evidencing these three strains constitute a novel species within *Turicibacter*.

A phylogenetic tree of publicly available *Turicibacter* genomes was reconstructed by using PATRIC’s Phylogenetic Tree Building Service using the ‘Codon Tree’ method on 100 single-copy genes allowing for up to five deletions/duplications (Fig. 2) [33]. This programme utilizes both DNA and amino acid sequences to build both gene and protein alignments, generating a phylogenetic tree from those alignments. *Eggerthia catenaformis* OT569 was used as an outgroup. Similar to the ANI and dDDH results, the codon tree had MMM721\(^{\text{T}}\), ISU324 and PIG517 together in a clade (including strains H121 and *T. sanguinis* MGYG-HGUT-00037), distinct from the clade containing *T. sanguinis* MOL361\(^{\text{T}}\), further evidencing these three strains constitute a novel species within the genus *Turicibacter* (Fig. 2).

**DESCRIPTION OF TURICIBACTER BILIS SP. NOV.**

*Turicibacter bilis* (bi’lis. L. gen. n. bilis of bile).

Strictly anaerobic, Gram-positive, catalase-negative, non-motile, spore-forming, pleomorphic micro-organisms. Supplementation of media with 0.1% (v/v) chicken bile induces spore germination. Growth is poor in broth alone, but improves significantly in biphasic media or broth with an agar plug. Cells primarily exist as long chains of irregular rods (0.8–8.2×0.5–2.0 μm) or as individual or clusters of coccolid cells (1.0–2.3 μm). The organism is capable of growth between 30–45°C and at pH 6.5–8.5, with optimums of 42°C and pH 7.5, respectively. Colonies are visible on BHIGL agar after 1–2 days and appear as small to medium-sized irregularly shaped, umbonate colonies with undulating margins, with the raised centre an opaque white that transitions to a translucent tan or grey at the margins. Colonies are 2.5–5.0 mm after 3 days at 42°C. The primary fermentation end products are acetate, butyrate and lactate. The organism is positive for arginine and gelatin biochemical reactivity.

The type strain is MMM721\(^{\text{T}}\) and was isolated from the surface of a chicken eggshell (=ATCC TSD-238\(^{\text{T}}\)=CCUG 74757\(^{\text{T}}\)). The genome is 2.7 Mb with a G+C content of 34.4 mol%.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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