Identification of Two Pathogenic Avian Mycoplasmas as Strains of *Mycoplasma pullorum*

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Two mycoplasma strains were isolated from dead turkey embryos. Growth properties, biochemical and serological characteristics, and protein profiles indicated that these strains were closely related to *Mycoplasma pullorum* CKK^T (T = type strain). This was confirmed by 16S rRNA sequence analysis, and the phylogenetic position of *M. pullorum* was established. Pathogenicity studies showed that the two strains, as well as *M. pullorum* CKK^T, induced a statistically significant level of mortality after inoculation into chicken embryos.

Mycoplasma gallisepticum, *Mycoplasma synoviae*, *Mycoplasma meleagridis*, and *Mycoplasma iowae* are known to be pathogenic for turkeys (13, 34). Other species, such as *Mycoplasma cloacale*, *Mycoplasma gallinaceum*, *Mycoplasma gallopavonis*, *Mycoplasma iners*, and *Mycoplasma lipofaciens*, may be isolated from turkeys but have not been clearly associated with pathogenicity. Recently, we isolated two mycoplasma strains, one from a dead turkey embryo found in a specific-pathogen-free (SPF) flock (strain 94254) and one from a dead turkey embryo which occurred in a field flock (strain 95098GC). In these two flocks, the hatchability percentages (average, 87.7%) were lower than the expected values (more than 95%). Preliminary experiments indicated that the mycoplasma strains did not seem to be related to any of the species mentioned above but resembled *Mycoplasma pullorum*, a species not known to be pathogenic for turkeys (14). In order to determine if the two newly isolated strains belonged to the species *M. pullorum*, we analyzed their cultural, biochemical, and serologic properties and compared them to the type strain of *M. pullorum*, strain CKK. We also evaluated the pathogenicities of the three strains for chicken and turkey embryos. To confirm our results and because the phylogenetic position of *M. pullorum* has not been established previously, we analyzed the 16S rRNA sequences of the three strains.

MATERIALS AND METHODS

Mycoplasma strains. The following strains were used: *Mycoplasma anatis* 1340^T (= ATCC 25524^T) (T = type strain), *Mycoplasma columbinum* MMP-1^T (= ATCC 29257^T), *M. gallinaceum* DD^T (= ATCC 33350^T), *Mycoplasma gallinarum* PG16^T (= ATCC 19708^T), *M. gallopavonis* WR1^T (= ATCC 33551^T), *M. gallisepticum* ATCC 15302, *Mycoplasma glycophilum* 486^T (= ATCC 35277^T), *M. iowae* 695^T (= ATCC 33552^T), *M. pullorum* CKK^T (= ATCC 33553^T), *M. synoviae* WVU 1853^T (= ATCC 25204^T), and *M. meleagridis* 17529^T (= ATCC 25294^T). One additional mycoplasma field strain, strain 90000, which is serologically related to *M. pullorum* and was isolated from pheasant trachea (17), was included. Mycoplasmas were grown in FM4 medium supplemented with yeast extract and pig serum (11).

Isolation procedure. Strain 94254 was isolated from 12 dead turkey embryos found in an SPF flock. SPF flocks are repeatedly tested (every 6 weeks) for the presence of any specific pathogens, including viruses and bacteria. The vitellus from each dead embryo was isolated and placed in FM4 medium. After 24 h of incubation, cultures were plated onto FM4 solid medium, and 1 day later medium-size colonies that did not have the typical fried-egg appearance (rounded without a central point) were observed. For each embryo, two colonies were

cloned three times with filtration through 450-nm-pore-size membrane filters between platings, as recommended by the Subcommittee on the Taxonomy of *Mollicutes* (12). One of the clones obtained was named strain 94254. Strain 95098GC was isolated from dead turkey embryos from a field flock by the same procedure.

Biochemical tests. Biochemical tests were performed as previously described (23). These tests included tests for digitonin sensitivity, glucose fermentation, arginine hydrolysis, tetrazolium salt reduction, phosphatase activity, film and spot production, and growth in the absence of NAD and L-cysteine.

Serologic tests. Dot immunoblotting was performed as previously described (26) by using Millititer filtration plates containing Durapore membranes (Millipore Corp., Milford, Mass.). The hyperimmune specific antisera were prepared by the procedure of Senterfit (28). Growth inhibition tests were performed with antiserum-saturated discs placed on FM4 plates inoculated by the running drop method (6).

SDS-PAGE of cellular proteins. Cells from 50-ml cultures of strains 94254, 95098GC, and 90000, *M. pullorum* CKK^T, *M. gallinarum* PG16^T, *M. gallinaceum* DD^T, and *M. gallisepticum* ATCC 15302 were pelleted by centrifugation at 16,000 × g for 30 min at 4°C, washed with 25 ml of 0.1 M Na₂PO₄–0.33 M NaCl (pH 7.0), pelleted again, and suspended in 1 ml of distilled water. The protein concentration was determined by the method of Lowry et al. (22) and was adjusted to 2 mg/ml. One volume of a solution containing 2% sodium dodecyl sulfate (SDS), 15% glycerol, 5% β-mercaptoethanol, and 0.1% bromophenol blue was added. Samples were boiled for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) for 18 h at 40 V in 12.5% polyacrylamide gels as described by Laemmli (20).

16S rRNA sequence determination. The 16S rRNA genes were enzymatically amplified from strains 94254 and 95098GC and *M. pullorum* CKK^T. The PCR assays were performed in final volumes of 50 µl, and each assay mixture contained 16.6 mM (NH₄)₂SO₄, 67 mM Tris-HCl (pH 8.0), 2 mM MgCl₂, 0.001% anionic detergent, 4.4 µg of bovine serum albumin per ml, 200 µM dATP, 200 µM dTTP, 200 µM dCTP, 200 µM dGTP, each primer at a concentration of 1 µM, 0.5 U of *Tth* DNA polymerase (MBI Fermentas, Vilnius, Lithuania), and 5 ng of genomic DNA. The PCR primers were the fD1 and rP1 oligonucleotides (30), which can be used to amplify the 16S rRNA genes of most eubacteria. PCR were performed in a programmable heating block (Perkin-Elmer Cetus Corp., Norwalk, Conn.) by using 40 cycles. Each cycle consisted of denaturation at 92°C for 20 s, annealing of primers at 62°C for 20 s, and extension at 72°C for 40 s. The PCR products of the expected size (1.5 kb) were cloned into plasmid vector pGEM-T (Promega Biotech, Madison, Wis.) according to the manufacturer's instructions and were propagated in *Escherichia coli* XL1-Blue (Stratagene, La Jolla, Calif.) by using electroporation (9). For each mycoplasma strain, three recombinant plasmids were sequenced by using a T7 sequencing kit (Pharmacia, Uppsala, Sweden) and universal forward and reverse sequencing primers, as well as three 16S rRNA gene internal universal primers as described previously (21).

Phylogenetic analysis. A search for similarity to the 16S rRNA gene sequence of *M. pullorum* CKK^T was performed by using the SIMILARITY-RANK tool of the Ribosomal Databank Project (24) and the BLAST (2) and FASTA (32) software with data from the GenBank and EMBL databases. Alignment with the most similar sequences was performed by using the Pileup program of the Genetics Computer Group package (7), edited manually, and 1,331 nonambiguous positions were used to establish pairwise evolutionary distances as described by Jukes and Cantor (15). The DNADIST and FITCH programs from the PHYLIP 3.5 package (10) were used for dendrogram construction. The *M. gallisepticum* 16S rRNA sequence was used as an outgroup.

Pathogenicity studies. Pathogenicity studies were conducted with SPF chicken eggs or SPF turkey eggs. Twenty 7-day-old embryonated eggs were inoculated via

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FIG. 1. SDS-PAGE of cellular proteins. Lane 1, *M. pullorum* CKK^T; lane 2, strain 94254; lane 3, strain 95098GC; lane 4, strain 90000; lane 5, *M. gallinaceum* DD^T; lane 6, *M. gallinarum* PG16^T; lane 7, *M. gallisepticum* ATCC 15302; lanes M, premixed protein molecular weight marker (Boehringer, Mannheim, Germany). The numbers on the left are molecular masses (in kilodaltons).

the yolk sac with 0.1-ml portions of cultures of *M. pullorum* CKK^T (SPF chicken and turkey eggs), strain 94254 (SPF chicken and turkey eggs), and strain 95098GC (SPF chicken eggs). For each strain, three dilutions were tested (approximately 10^1 , 10^3 , and 10^5 color-changing units [CCU]/ml). A total of 40 7-day-old embryonated eggs were inoculated with sterile medium and 20 other eggs were not inoculated as controls. The eggs were candled daily until the day 13 of incubation for chicken embryos or day 26 for turkey embryos. All of the dead and surviving embryos were examined for lesions and cultured for mycoplasmas.

Nucleotide sequence accession number. The 16S rRNA gene sequence of *M. pullorum* CKK^T has been assigned GenBank accession number U58504.

RESULTS AND DISCUSSION

Cultural and biochemical properties. Strains 94254, and 95098GC and *M. pullorum* CKK^T exhibited identical cultural and biochemical properties. They grew in the absence of NAD and L-cysteine in culture medium. They were sensitive to 1.5% digitonin, fermented glucose, did not hydrolyze arginine, did not display phosphatase activity, did not reduce 2,3,5-tetrazolium chloride, and did not produce films and spots. These results were consistent with the results of previous studies describing *M. pullorum* CKK^T (14), but these properties are shared by *M. gallinaceum*.

Serologic tests. Dot immunobinding tests were performed with strains 94254 and 95098GC by using hyperimmune antisera prepared against the following avian mycoplasmas: *M. pullorum*, *M. gallinaceum*, *M. anatis*, *M. glycyphilum*, *M. gallopavonis*, *M. meleagridis*, and *M. iowae*. A positive reaction was obtained only with anti-*M. pullorum* CKK^T serum. Growth inhibition tests performed with antisera against *M. pullorum*, *M. gallinaceum*, *M. gallisepticum*, *M. gallopavonis*, *M. glycyphilum*, *M. columbinum*, *M. anatis*, and *M. synoviae* gave similar results. The inhibition zone was 12 mm in diameter with anti-*M. pullorum* serum, and a similar value was obtained with antiserum to *M. pullorum* CKK^T.

Cellular protein analysis. The cellular protein patterns exhibited by *M. pullorum* CKK^T and strains 94254, 95098GC, 95068, and 90000 were almost identical to each other but clearly distinct from those of *M. gallisepticum* ATCC 15302, *M. gallinarum* PG16^T, and *M. meleagridis* 17529^T (Fig. 1). How-

ever, they seemed to share some bands with the pattern of *M. gallinaceum* DD^T, a member of the *Mycoplasma hominis* phylogenetic group.

16S rRNA gene analysis and phylogenetic position of *M. pullorum* CKK^T. The nucleotide sequences of the 16S rRNA genes of strains 94254 and 95098GC and *M. pullorum* CKK^T were identical. Phylogenetic analyses indicated that *M. pullorum* belongs to the *M. hominis* phylogenetic group (Fig. 2), which is clearly distinct from the *Mycoplasma pneumoniae* cluster containing *M. gallisepticum* and *M. iowae* (31). Within the *M. hominis* cluster, *M. pullorum* is part of a group containing four avian and two feline mycoplasmas (*Mycoplasma corogypsi*, *Mycoplasma leocaptivus*, *Mycoplasma felis*, *Mycoplasma sturnidae*, *M. gallinaceum*, and *M. synoviae*). Our results are consistent with those obtained by other workers (4, 5, 25).

Pathogenicity studies. The kinetics of chicken embryo mortality after inoculation with strains 94254 and 95098GC and *M. pullorum* CKK^T are shown in Fig. 3. The mortality of noninoculated eggs or eggs inoculated with sterile medium was less than 15% until day 13 of incubation (data not shown). The mortality values obtained with *M. pullorum* CKK^T ranged from 30 to 65% depending on the number of CCU inoculated. With strain 94254, the mortality reached 50% after 8 days regardless of the number of CCU inoculated. With strain 95098GC, 80 to 100% mortality was obtained after 6 days. The levels of mortality were statistically significant; on the last day of incubation, the χ^2 values were 4.43 ($P = 0.04\%$) for eggs inoculated with strain 94254, 30.6 ($P = 0.00\%$) for eggs inoculated with strain 95098GC, and 12.5 ($P = 0.00\%$) for eggs inoculated with *M. pullorum* CKK^T. The dead embryos from inoculated eggs were

FIG. 2. Phylogenetic tree of the *M. hominis* phylogenetic group, including *M. pullorum*.

FIG. 3. Chicken embryo mortality following inoculation with strain 94254 (A), *M. pullorum* CKK^T (B), and strain 95098GC (C). Symbols: open triangles, 10 CCU/egg; light squares, 10³ CCU/egg; dark squares, 10⁵ CCU/egg.

dwarfed. With turkey eggs, 30 and 70% mortality with *M. pullorum* CKK^T and strain 94254, respectively, was observed on day 19 of incubation (data not shown). Because it was not possible to hatch injected eggs, we decided to stop the incubation on day 26. This may have reduced the level of mortality that would have been obtained if eggs had been incubated until they hatched, as under field conditions. Indeed, in *M. pullorum*-infected flocks, embryo mortality is usually observed during the last days of incubation. Mycoplasmas that had biochemical properties of *M. pullorum* could be cultured from dead embryo vitellus.

Conclusions. The results presented here demonstrate that mycoplasma strains 94254 and 95098GC, which were isolated from SPF and field turkey flocks, respectively, belong to the species *M. pullorum*. Until now, *M. pullorum* was not identified

as pathogenic and had never been isolated from dead turkey embryos. Avian serotype C and O mycoplasmas, later named *M. pullorum* (14), have been isolated from chickens, quails, partridges, and pheasants (1, 3, 17, 19, 27, 29, 33, 35). Previous pathogenicity experiments did not clearly show if *M. pullorum* was pathogenic (8, 18, 35). From our data it seems that the embryo mortality observed is significant. Other mycoplasma strains having biochemical and serologic characteristics of *M. pullorum* have recently been isolated in France from adult turkeys and dead turkey embryos (16). Thus, the incidence of infection by *M. pullorum* and the pathogenicities of the different strains need to be studied in more detail.

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