Haemophilus avium, a New Species from Chickens

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Twelve Haemophilus strains (previously designated group II strains by Hinz) from chickens and of uncertain taxonomic position and 29 Haemophilus paragallinarum strains were investigated. The findings indicate the existence of a hitherto unknown species, for which the name Haemophilus avium sp. nov. is proposed. The main characters which differentiate H. avium from H. paragallinarum are as follows: all of the H. avium but none of the H. paragallinarum strains tested produce catalase, alkaline phosphatase, and α-glucosidase, acidify galactose and trehalose, and do not require serum for optimal growth. Most of the H. avium strains produce yellow pigment, grow aerobically, and do not require CO₂ for optimal growth. In further contrast to H. paragallinarum, none of the H. avium strains are able to cause infectious coryza of chickens. The type strain of H. avium is IPDH 2654 (= ATCC 29546).

MATERIALS AND METHODS

Bacterial strains. The designations and sources of the strains used in this study are listed in Table 1. Cultures of the strains were stored in the freeze-dried state until needed.

Morphological, physiological, and biochemical tests. The morphologies of cells and colonies and the staining reactions (Gram and capsule staining) were determined as described by Hinz (9).

The morphologies and the requirement for V and X factors were determined by use of the following basic media: (i) proteose peptone agar consisted of 2.0% (wt/vol) proteose peptone no. 3 (Difco), 0.6% (wt/vol) NaCl, 0.5% (wt/vol) glucose, and 1.0% (wt/vol) Noble agar; the final pH was 7.2 to 7.3; (ii) brain heart infusion agar (BHIA) (Difco); the final pH was 7.2 to 7.3; (iii) brain heart infusion agar (BHA) (Difco); the final pH was 7.2 to 7.3.

The media were adjusted to pH 7.6 with 1 M NaOH and were then autoclaved for 10 min. X factor and serum (X, S), or X and V factors (X, V), or V factor (V) or V factor and serum (V, S) were added to each medium. A filter-sterilized solution of β-nicotinamide adenine dinucleotide (NAD) (Serva) and cysteine-hydrochloride (Merck) was added to give a final concentration of 100 µg/ml for both substances. Hemin (Roth) dissolved in triethanolamine (Kodak) was added as a filtered solution to give a final concentration of 10 µg/ml of medium. The chicken sera added to the media were free of agglutinating antibodies against the Haemophilus strains used. Inoculated media were incubated aerobically, microaerophically (90% air and 10% CO₂ [vol/vol]), and anaerobically (BBL-Gas Pak 110 System) for 24 and 48 h at 37°C. The results were evaluated as described by Zinnemann et al. (31). Haemophilus influenzae strain TS 43 was used as a control.

The ability to synthesize porphyrin from δ-amino-levulinic acid was determined as described by Kilian (15).

For satellite growth and hemolysis, 24- to 48-h-old
growth was examined on blood agar containing Columbia agar base (Oxoid) and 7% (wt/vol) defibrinated ox blood. A _Staphylococcus epidermidis_ strain (TS 49) was used as a V-factor feeder.

Oxidase was tested for by Kovács's method (18). Catalase activity was determined using tryptose agar-(X, V, S) and BHIA-(X, V, S) cultures which were incubated under microaerophilic and/or aerobic conditions. In testing for catalase, a few colonies were removed with a cover slip and mixed in a drop of 3% (vol/vol) H₂O₂. The production of bubbles was regarded as a positive test for catalase.

Indole production was demonstrated, using Kovács's reagent (4), in tryptose broth (Difco) with 50 μg of NAD per ml and 1% chicken serum after 1 and 3 days of growth.

Reduction of nitrate and nitrite was tested as described by Cowan (4) by use of the same tryptose broth as above but with 0.1% KNO₃.

The production of urease was determined as described by Lautrop (19).

Alkaline phosphatase was determined by the method of Kersters and DeLey (14).

Determination of the enzymes α-fucosidase and α-
glucosidase was performed by the method of Kilian and Bulow (17) and Kilian (16), using p-nitrophenyl-α-L-fucopyranoside and p-nitrophenyl-α-D-glucopyranoside (Serva) as substrates. *H. parainfluenzae* strain TS 44 and *Escherichia coli* strain TS 47 were used as positive controls for the α-glucosidase test. One *Yersinia pseudotuberculosis* strain (TS 48), *H. parasuis* TS 46, and strain TS 45, listed as *H. suis*, acted as positive controls for the production of α-fucosidase.

Acid production from carbohydrates (Merck) was determined in phenol red broth base (Difco).

The base medium together with the carbohydrate (Table 2) and 1% (vol/vol) chicken serum was filter-sterilized through 0.2-μm-pore-size filters. The different test media were dispensed in 5-ml volumes in test tubes, and Durham tubes were inserted in the glucose medium for the detection of gas. The bacterial inoculum was added simultaneously with neutralized NAD.

### Table 2. Characteristics of 13 previously unidentified strains of haemophilus (includes ATCC 14385) and of 29 *Haemophilus paragallinarum* strains from chickens

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Previously unidentified strains</th>
<th><em>H. paragallinarum</em> strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-factor requirement</td>
<td>TS13 TS3 TS4 TS1 TS2 TS5 TS6 TS7 TS8 TS9 TS10 TS11 TS12 TS14-TS42</td>
<td></td>
</tr>
<tr>
<td>δ-Aminolevulinic utilization</td>
<td>+ + + + + + + + + + + + + (29)</td>
<td></td>
</tr>
<tr>
<td>V-factor requirement</td>
<td>+ + + + + + + + + + + + + (29)</td>
<td></td>
</tr>
<tr>
<td>Aerobic growth</td>
<td>+ + + + + + + + + + + + + (29)</td>
<td></td>
</tr>
<tr>
<td>Anaerobic growth</td>
<td>+ + + + + + + + + + + + + (29)</td>
<td></td>
</tr>
<tr>
<td>CO2 requirement</td>
<td>- - - - - - - - - - - - - (0)</td>
<td></td>
</tr>
<tr>
<td>Serum requirement</td>
<td>- - - - - - - - - - - - - (0)</td>
<td></td>
</tr>
<tr>
<td>Serum improves growth</td>
<td>- - - - - - - - - - - - - (0)</td>
<td></td>
</tr>
<tr>
<td>Hemolysis</td>
<td>- - - - - - - - - - - - - (0)</td>
<td></td>
</tr>
<tr>
<td>Yellow-pigment production</td>
<td>+ + + + + + + + + + + + + (0)</td>
<td></td>
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<tr>
<td>Nitrate reduction</td>
<td>- - - - - - - - - - - - - (0)</td>
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</tr>
<tr>
<td>Indole production</td>
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<tr>
<td>Urease</td>
<td>- - - - - - - - - - - - - (0)</td>
<td></td>
</tr>
<tr>
<td>Oxidase</td>
<td>- - - - - - - - - - - - - (0)</td>
<td></td>
</tr>
<tr>
<td>Catalase</td>
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<td></td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>+ + + + + + + + + + + + + (0)</td>
<td></td>
</tr>
<tr>
<td>ONPGβ-D-galactosidase</td>
<td>+ - - - - - - - - - - - - (0)</td>
<td></td>
</tr>
<tr>
<td>α-Fucosidase</td>
<td>- - - - - - - - - - - - - (0)</td>
<td></td>
</tr>
<tr>
<td>PNPGα-glucosidase</td>
<td>+ + + + + + + + + + + + + (3)</td>
<td></td>
</tr>
<tr>
<td>Pathogenicity</td>
<td>- - - - - - - - - - - - - (0)</td>
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<tr>
<td>Acid from:</td>
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<tr>
<td>Maltose</td>
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<tr>
<td>Fructose</td>
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<td>Galactose</td>
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<td></td>
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<tr>
<td>Trehalose</td>
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</tr>
<tr>
<td>Mannitol</td>
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</tr>
<tr>
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</tr>
<tr>
<td>Dextrin</td>
<td>+ + + + + + + + + + + + + (29)</td>
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<tr>
<td>Xylose</td>
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<tr>
<td>Lactose</td>
<td>+ + + + + + + + + + + + + (29)</td>
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</tr>
<tr>
<td>Cellobiose</td>
<td>+ + + + + + + + + + + + + (29)</td>
<td></td>
</tr>
<tr>
<td>Arabinose</td>
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<td></td>
</tr>
<tr>
<td>Salicin</td>
<td>+ + + + + + + + + + + + + (29)</td>
<td></td>
</tr>
<tr>
<td>Inulin</td>
<td>+ + + + + + + + + + + + + (29)</td>
<td></td>
</tr>
<tr>
<td>Deoxyribose</td>
<td>+ + + + + + + + + + + + + (29)</td>
<td></td>
</tr>
<tr>
<td>Glucose, gas</td>
<td>+ + + + + + + + + + + + + (29)</td>
<td></td>
</tr>
<tr>
<td>Glucose, acid</td>
<td>+ + + + + + + + + + + + + (29)</td>
<td></td>
</tr>
</tbody>
</table>

a Symbols: +, positive result (with respect to acid production from carbohydrates, + indicates a final pH of <6.5); −, negative result.
b Numbers in parentheses are numbers of strains positive for the characters indicated.
c These strains required CO2 on primary isolation.
d ONPG, o-nitrophenyl-β-D-galactopyranoside.
e PNPG, p-nitrophenyl-β-D-glucoside.
f Ability to produce infectious coryza in chickens.
solution to give a final concentration of 50 μg of NAD per ml of medium. Each of the tubes was inoculated with 0.05 ml (>10^3 colony-forming units per tube) of a bacterial suspension in 0.15 M NaCl made from 16- to 24-h-old BHIA-(V, S) cultures. Unincubated tubes incubated under the same condition as described above served as controls. The final reactions were measured with a pH meter after 24 and 48 h of incubation.

The pathogenicity tests were performed as described by Hinz (11). Eight-week-old chickens free of avian encephalomyelitis virus, adenovirus, infectious bronchitis virus, Newcastle disease virus, respiratory enteric orphan virus, Marek disease herpesvirus, laryngotracheitis virus, Rouss sarcoma virus, infectious bursal disease virus, influenza virus A, fowlpox virus, Mycoplasma gallisepticum, Mycoplasma synoviae, Salmonella gallinarum, and S. pullorum were used.

Haemophilus cells used for inoculation of the chickens were obtained from 8- to 10-h-old BHIA-(V, S) cultures and were suspended in phosphate-buffered saline (pH 7.2) with 1% chicken serum and 10 μg of NAD per ml. Haemophilus cells used for inoculation were not washed. To test for the ability to cause infectious coryza, more than 5 x 10^8 colony-forming units in 0.05 ml of inoculum was instilled into nostrils and on the mucous membranes of the birds. Each of the Haemophilus strains was tested in five birds. A suspension of each Haemophilus strain inactivated at 100°C for 10 min was inoculated into two control chickens housed separately from the infected groups. Inoculated chickens were examined for signs every day for 14 days. Necropsied birds were then examined for gross pathological changes, and samples from the respiratory tract were cultured for haemophilus.

### RESULTS

The characteristics of the Haemophilus strains studied are presented in Tables 2 and 3. On the basis of their characteristics, the strains studied fell into two groups: one group contains all of the strains of H. paragallinarum; the other contains the strains TS1 to TS12 inclusive and ATCC 14385 (TS13) which could not be assigned to one of the known Haemophilus species.

The characteristics of these unidentified strains (TS1 to TS13, inclusive) are as follows: Gram-negative, cocoid to pleomorphic, motile, nonsporforming rods (0.4 to 0.5 by 0.9 to 3.0 μm), which occur singly, in pairs, and in filamentous forms. Strains TS1 to TS6, inclusive, and TS9 to TS12, inclusive, formed smooth, convex, grayish-white or yellowish opaque colonies with entire edges on BHIA-(V, S). Colonies of strains TS7 and TS8 were wrinkled and gave lumpy suspensions in a 0.15 M NaCl solution. TS13 formed rough colonies with a granular surface. All of the strains except TS13 were encapsulated and showed iridescence on transparent solid media in oblique transmitted light throughout the first 8 to 14 h of incubation. Iridescence disappeared completely after 36 h of incubation. Most of the strains did not require CO₂ for luxuriant growth in serial passages on solid media. However, strains TS10 and TS11 required increased CO₂ tension on primary isolation. Subcultures made from single colonies of those isolates on BHIA-(V, S) gave aerobic growth of a few colonies which did not require increased CO₂ tension in continued passages. It is probable that this adaptation was caused by dissociation. Since the properties of TS10 and TS11 were otherwise similar to those of the other strains, they were retained in the group of unidentified strains. All of the strains showed satellite phenomenon after microaerobic or aerobic incubation on ox blood agar streaked with a strain of Staphylococcus aureus as a V-factor feeder. They required the V factor but not the X factor or serum for growth. X-factor independence was confirmed by the ability of these strains to carry out biosynthesis of porphyrin from δ-aminolevulinic acid. In phenol red base, acid was produced from carbohydrates in an amount sufficient to give a clear-cut change of the indicator (pH < 6.5) after 1 to 2 days of incubation. The decrease of pH in carbohydrate- and serum-free phenol red broth produced by some of the unidentified strains reached only 0.6 U. All strains were catalase and phoshatase positive and produced acid from trehalose and galactose; some of the strains also produced acid from lactose, cellobiose, and arabinose. Four of the 13 strains produced β-galactosidase. In tryptose peptone broth, used as the basal medium for sugar fermentation in a previous study by Hinz (9), fewer strains produced acid from mal-

### Table 3. Characteristics useful in differentiating Haemophilus avium sp. nov. from H. paragallinarum

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>H. avium</th>
<th>H. paragallinarum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic growth</td>
<td>+/−</td>
<td>−</td>
</tr>
<tr>
<td>CO₂ requirement</td>
<td>−/+</td>
<td>+</td>
</tr>
<tr>
<td>Serum improves growth</td>
<td>−</td>
<td>+</td>
</tr>
<tr>
<td>Yellow-pigment production</td>
<td>+/−</td>
<td>−</td>
</tr>
<tr>
<td>Catalase</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Acid from:</td>
<td>−</td>
<td>+/−</td>
</tr>
<tr>
<td>Trehalose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>Galactose</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>α-Glucosidase (PNPG)</td>
<td>+</td>
<td>−/−</td>
</tr>
<tr>
<td>Pathogenicity</td>
<td>−</td>
<td>+</td>
</tr>
</tbody>
</table>

* Symbols: +/−, most strains are positive; −/+ or −, most strains are negative; +, positive result; −, negative result.

^ Produces infectious coryza in chickens.
tose, dextrin, and xylose than in phenol red basal medium. None of the strains produced clinical or pathological signs in chickens. Infectious coryza could not be produced with any of these strains.

**DISCUSSION**

At present there is general agreement that the genus *Haemophilus* should be restricted to gram-negative, nonmotile, nonsporeforming rods with a requirement for hemin or other porphyrins (X factor) and/or for NAD (V factor) or other definable coenzyme-like substances (3, 29, 30). Results obtained in this study indicate the existence of a new species of the genus *Haemophilus* for which the name *Haemophilus avium* (a'vi.um. L.n. avis a bird; L. gen. pl. n. avium of birds) is proposed. This species differs from *H. gallinarum* and *H. paragallinarum* in its physiological, biochemical, and serological features and by its inability to cause infectious coryza of chickens (4, 9-12, 21, 23, 24, 28, 30). The characteristics which distinguish *H. avium* from *H. paragallinarum* are presented in Table 3. Some properties of *H. avium* are identical with those of *H. parasuis* and *H. parainfluenzae* but not with those of other known *Haemophilus* species. However, the data presented by Kilian (16) show that *H. parasuis* differs from *H. avium* in its α-fucosidase activity and by its weak or lack of fermentation of carbohydrates and from the existence of a new species of the genus *Haemophilus* with the proposal of a new species.

**REPRINT REQUESTS**

Address reprint requests to: Dr. K.-H. Hinz, Institute for Poultry Disease, The School of Veterinary Medicine, Hannover, Federal Republic of Germany.

**LITERATURE CITED**

24. Roberts, D. H., B. S. Hanson, and L. Timms. 1964. Observations of the incidence and significance of *Hae-


