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Differentiation of *Alcaligenes*-Like Bacteria of Avian Origin and Comparison with *Alcaligenes* spp. Reference Strains

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Although standard biochemical tests used for the identification of *Alcaligenes* spp. revealed only minor differences, the oxidative low-peptone technique clearly differentiated between *Alcaligenes*-like bacteria of avian origin and *Alcaligenes* spp. reference strains. Based on their colonial morphology, biochemical profiles, and hemagglutination, the *Alcaligenes*-like bacteria of avian origin were further divided into two subgroups, C1-T1 and C2-T2. Colonies of subgroup C1-T1 were nondescript, round, raised, glistening, translucent, greyish, and about 2 mm in diameter. Colonies of subgroup C2-T2 were off-white, flat, dry and wrinkled, generally round, and resembled tiny lily pads. Biochemical profiles by the oxidative low-peptone method showed the C1-T1 subgroup alkalinizing only three substrates (citrate, acetate, and succinate), whereas the C2-T2 subgroup alkalinized eight substrates (citrate, acetate, butyrate, itaconate, malonate, saccharate, succinate, and *M*-tartrate). Subgroup C1-T1 agglutinated human, chicken, and turkey erythrocytes, whereas subgroup C2-T2 did not. The recognition of these two subgroups within the *Alcaligenes*-like bacteria of avian origin is important, since it may explain the differences seen in pathogenicity among isolates.

Due to the lack of reactivity of carbohydrate-inert gram-negative rods on commonly used culture media (Sellers, oxidation-fermentation, urea, triple sugar iron, and others), identification of *Alcaligenes* spp. has been based mainly on negative results. Otto and Pickett's development of the oxidative alkalinization method of carbon substrate utilization tests (8) represented a substantial improvement over standard methods and has been used to distinguish human isolates within the *Alcaligenes* group (10). In veterinary medicine, gram-negative bacteria similar to *Alcaligenes* spp. cause an economically important respiratory disease in turkeys (*Alcaligenes* rhinotracheitis) (12). On the basis of biochemical (oxidative alkalinization) and physiological tests (11), turkey isolates have been separated into two distinct groups. Furthermore, an association among biochemical characteristics, hemagglutination, and pathogenicity of field isolates has been shown to exist for turkey strains (11). Recently, *Alcaligenes*-like bacteria have also been found in the respiratory tracts of chickens experiencing respiratory disease (1, 2). The need for a reliable identification scheme, as well as a taxonomic designation for the avian pathogens, is obvious. This report compares avian isolates of *Alcaligenes*-like bacteria with *Alcaligenes* spp. reference strains and identifies characteristics of subgroups within the avian isolates.

MATERIALS AND METHODS

Bacterial strains. Chicken isolates of *Alcaligenes*-like bacteria were obtained from the trachea. Turkey strains were isolated from trachea and lungs. Primary isolation was made on MacConkey agar incubated aerobically at 37°C for 18 to 24 h. Preliminary identification of *Alcaligenes* spp. was based on the following tests: oxidase, catalase, triple sugar iron, Simmons citrate, urea, motility, nitrate-nitrite reduction, and colonial morphology on blood agar plates. Reference strains of *Alcaligenes* spp. were obtained from the sources cited in Table 1. Cultures were maintained at 4°C on brain heart infusion agar slants covered with sterile mineral oil.

Colonial and cellular morphology. One passage through 5- to 7-day-old chicken embryos, inoculated via the yolk sac and harvested after 24 h of incubation, was done routinely with the bacterial isolates before determining colonial morphology on Trypticase soy (BBL Microbiology Systems, Cockeysville, Md.) blood agar plates. Plates were streaked with a loopful of infected yolk material as inoculum and incubated aerobically for 24 h. Presence and arrangement of flagella was demonstrated by the Forbes staining technique (4).

Media and biochemical tests. The inocula for all tests were 24-h Trypticase soy blood agar cultures. The oxidase test was conducted on Whatman no. 1 filter paper with 0.5% *N,N,N',N'*-tetramethyl-*p*-phenylene diamine dihydrochloride reagent (Fisher Scientific Co., Fair Lawn, N.J.). A slide catalase test was done with 3% H_2O_2. MacConkey and Trypticase soy agar plates were prepared from dehydrated media (BBL). Prepared triple sugar iron and Simmons citrate slants, as well as dehydrated cetrimide and salmonella-shigella agar, were obtained from Granite Diagnostics, Burlington, N.C. DNase, urea, and glucose oxidation were determined with the Uni-N/F-Tek plate (Flow Laboratories, McLean, Va.). Nitrate and nitrite reduction broths were prepared according to the *Manual of Clinical Microbiology* (6). Broth cultures were incubated at 30°C for 3 days; gas evolution was detected in inverted Durham tubes.

OLP carbon substrate utilization tests. The ability of isolates to alkalinize substrates (organic salts, acids, and amides) was tested with our modification of Greenwood's oxidative low peptone (OLP) media as described by Pickett (9). A concentrated stock solution was prepared which contained (g/100 ml): $(NH_4)_2HPO_4$, 2.0; KCl, 0.4; yeast

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TABLE 1. Sources of isolates

Isolate	Source[a]
C1 (28 isolates)	Chicken trachea, NCSU
C2 (25 isolates)	Chicken trachea, NCSU
T1 (8 isolates)	Turkey trachea, lungs, NCSU
T2 (4 isolates)	Turkey trachea, lungs, NCSU
Alcaligenes faecalis type 1 K1251	M. J. Pickett (human)
Alcaligenes faecalis 4604G	G. L. Gilardi (human)
Alcaligenes faecalis type 2 K1813	M. J. Pickett (human)
Alcaligenes faecalis type 2 K1835	M. J. Pickett (human)
Alcaligenes faecalis ATCC 15173 (*A. denitrificans*)	ATCC
Alcaligenes denitrificans K1159	M. J. Pickett (cooked shrimp)
Alcaligenes faecalis ATCC 15554 (*A. odorans*)	ATCC
Alcaligenes faecalis ATCC 8750 (*A. odorans*)	ATCC
Alcaligenes odorans K1571	M. J. Pickett (human)

[a]NCSU, North Carolina State University; ATCC, American Type Culture Collection; M. J. Pickett, affiliation; G. L. Gilardi, affiliation.

extract (BBL), 1.0; Trypticase peptone (BBL), 0.75; phenol red (Difco Laboratories, Detroit, Mich.), 0.04. Samples of this concentrate were diluted 1/10 with deionized water. The desired substrate (final concentration, 0.4%), $MgSO_4$ (0.04%), and glucose (0.04%) were added. The solution was adjusted to pH 6.5 and filter sterilized. This sterile broth was mixed with an equal volume of sterile molten 3% agar and pipetted into designated wells in a 24-well tissue culture plate (Flow Laboratories). Two sets of 12 substrates each were tested per plate. Inoculum for the test plates was 1 drop per well of a slightly turbid normal saline suspension of the test organism grown for 24 h on a Trypticase soy blood agar plate. Reactions on the OLP media were recorded after 24 h of incubation at 37°C. Only a dark fuchsia color was rated as positive. Yellow or orange-red was rated as negative.

Hemagglutination. Bacterial isolates to be tested for their ability to agglutinate erythrocytes of human and avian species were streaked on brain heart infusion agar, brucella agar, and colonization factor antigen agar plates (3). After incubation for 18 to 24 h at 37°C, confluent growth on each plate was harvested with 2 ml of 0.3% formalinized phosphate-buffered saline. Fresh (less than 1-week-old) erythrocytes of human, chicken, and turkey origin were used in the tests. Blood was collected aseptically with heparin as anticoagulant and stored at 4°C. The erythrocytes were separated, washed, and suspended in phosphate-buffered saline to a 10% concentration before use. One drop of the bacterial suspension was mixed with one drop of erythrocytes on a slide, rocked back and forth for 2 min, and observed for agglutination against a white background. A negative control (formalinized phosphate-buffered saline plus erythrocyte suspension) and a positive control (*Bordetella bronchiseptica*, swine origin) accompanied each test.

RESULTS

Colonial morphology was a valuable distinguishing characteristic between *Alcaligenes* reference strains and avian *Alcaligenes*-like isolates. Figure 1 illustrates three general colony types observed among the isolates. Figure 1A shows the morphology demonstrated by *A. faecalis* type 2. *A. faecalis* type 2 was a maximum of 6 mm in diameter, slightly raised, and granular with a definitely spreading periphery. This type was quite similar to the colonies of *Alcaligenes odorans*. *A. odorans* produced, in addition, a characteristic green discoloration of the blood agar plate and a fruity odor. Colonies of *Alcaligenes faecalis* type 1, *Alcaligenes denitrificans*, and avian C1 (C = chicken) and T1 (T = turkey) isolates (Fig. 1B) were very similar to each other, producing round, raised, glistening, translucent, greyish colonies about 2 mm in diameter. A startlingly unique colony type was produced by the avian C2 and T2 isolates (Fig. 1C). These colonies were off-white, flat, dry and wrinkled, generally round, and resembled tiny lily pads. Where well separated, they reached a diameter of up to 12 mm. Although generally observed in fresh isolates, this colony type was not stable. Isolates maintained in the laboratory on slants lost this characteristic and reverted to a C1-type morphology. The original colony could be regained by passage through embryonated hen eggs.

All isolates and reference strains were gram-negative rods with four to eight peritrichous flagella. Table 2 lists the results obtained when *Alcaligenes*-like avian isolates and *Alcaligenes* spp. reference strains were examined by standard laboratory tests. Only minor differences between avian isolates and *Alcaligenes* reference strains were found: (i) most C1 and T1 isolates were unable to utilize Simmons citrate, and (ii) avian isolates did not reduce nitrate or nitrite. This last characteristic, the inability to reduce nitrate or nitrite, was also shared by *A. faecalis* type 2 reference strains.

Table 3 shows the reactions of the bacteria in 12 substrates tested in OLP media. With this method, major differences between *Alcaligenes*-like bacteria of avian origin and *Alcaligenes* reference strains were found. Avian isolates alkalinized fewer substrates than the type 1 human reference strains. *A. faecalis* type 2 reference strains alkalinized only four substrates. C1 and T1 isolates were biochemically identical, consistently alkalinizing only three substrates, whereas C2 and T2, also biochemically identical, alkalinized eight substrates.

The C1 and T1 isolates tested were positive in hemagglutination tests (Table 4). C2 or T2 isolates did not agglutinate erythrocytes. All *A. odorans* and *A. faecalis* type 2 strains tested were also negative in the hemagglutination test. Results with the other reference strains were variable. Hemagglutination results were reproducible regardless of the erythrocytes used (human, chicken, or turkey). The type of growth medium, however, greatly influenced hemagglutination test results. Reproducible results were obtained only with colonization factor antigen agar and could not be obtained when the bacteria were grown on other media commonly used for antigen preparation (brain heart infusion agar and brucella agar).

DISCUSSION

These results show that avian isolates, originally classified as *A. faecalis* (12), have characteristics in common with some *Alcaligenes* spp. reference strains but that they also differ from them in many respects. Colonial morphology

TABLE 2. Standard biochemical and screening tests

Test	C1	C2	T1	T2	*A. faecalis* type 1	*A. faecalis* type 2	*A. denitrificans*	*A. odorans*
Triple Sugar iron	K/NC[a]	K/NC	K/NC	K/NC	K/NC	K/NC	K/NC	K/NC
Growth on agar:								
MacConkey	+	+	+	+	+	+	+	+
Centrimide	+	+	+	+	+	+	+	+
Salmonella-shigella	+	+	+	+	+	+	+	+
Simmons citrate	V[b]	+	V	+	+	+	+	+
Esculin	−	−	−	−	−	−	−	−
Urea	−	−	−	−	−	−	−	−
DNase	−	−	−	−	−	−	−	−
Glucose, oxidative								
Utilization	−	−	−	−	−	−	−	−
$NO_3^- \rightarrow NO_2^-$	−	−	−	−	+	−	+gas[c]	−
NO_2^- reduction	−	−	−	−	−	−	+	+gas
Oxidase	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Growth at 42°C	+	+	+	+	+	+	+	+
Gelatin liquefaction	−	−	−	−	−	−	−	−

[a] K, Alkaline slant; NC, no change in butt.
[b] Variable results, usually negative.
[c] Gas production detected in Durham tube.

within the *Alcaligenes* genus and among the avian subgroups was quite diverse. Colonies of C1 and T1 isolates were morphologically identical to *A. faecalis* type 1 and *A. denitrificans*. The colonial morphology seen in C2 and T2 isolates was completely unique compared with the other strains tested. This characteristic of C2 and T2, always expressed in fresh isolates, will aid in identification of this subgroup.

OLP tests were especially helpful in distinguishing between the two avian subgroups. The OLP tests also enabled us to establish many differences between the avian isolates and the *Alcaligenes* spp. reference strains. The OLP results were reproducible and agreed closely with reports characterizing *Alcaligenes* species using the oxidative alkalinization technique (10) and OLP technique (M. J. Pickett, personal communication). In our hands, the OLP method yielded clear results within 24 h as compared with 3 to 4 days needed for the oxidative alkalinization method.

Although OLP tests allow differentiation of *Alcaligenes*-like bacteria found in the respiratory tracts of chickens and turkeys into two subgroups, these biochemical tests do not clarify the taxonomic classification of these organisms. The two avian subgroups identified are likely to be the same strains as those studied by other research groups. With minor discrepancies, our C1 and T1 organisms may be the same as those referred to as *A. faecalis* group 1 (11) and as *Bordetella*-like (5). Our C2 and T2 groups may be the same organism as the avian isolates classified as *A. faecalis* exhibiting a flat, wrinkled colony type (12). Colonial morphology reversion and different carbon substrate utilization testing techniques, as well as the choice of substrates for testing, makes a comparison of our C2 and T2 subgroups to isolates of other researchers difficult at this time.

When we examined the results of characterization studies of gram-negative nonfermentative bacteria published by Martin et al. (7), we concluded that our C1-T1 subgroup also differs biochemically from IVc-2 (Centers for Disease Control, Atlanta, Ga.), a member of the *Alcaligenes-Bordetella* complex.

Although organisms of the C1 subgroup seem to be more

TABLE 3. Substrate alkalinization on OLP media

Strain(s)	Citrate	Acetamide	Acetate	Adipate	Butyrate	Histidine	Itaconate	Malonate	Nicotinamide	Saccharate	Succinate	*M*-Tartrate
C1, T1	+	−	+	−	−	−	−	−	−	−	+	−
C2, T2	+	−	+	−	+	−	+	+	−	+	+	+
A. faecalis type 1												
K1251	+	+	+	+	+	+	+	+	−	+	+	+
4604G	+	+	+	+	+	+	+	+	−	+	+	+
A. faecalis type 2												
K1813	+	−	+	−	+	−	−	−	−	−	+	−
K1835	+	−	+	−	+	−	−	−	−	−	+	−
A. denitrificans												
ATCC 15173	+	+	+	+	+	+	+	+	−	+	+	+
K1159	+	+	+	+	+	+	+	+	−	+	+	+
A. odorans												
ATCC 15554	+	+	+	−	+	+	−	+	+	−	+	−
ATCC 8750	+	+	+	−	+	+	−	+	+	−	+	−
K1571	+	+	+	−	+	+	−	+	+	−	+	−

TABLE 4. Hemagglutination by avian isolates and reference strains

Strain(s)	Hemagglutination with erythrocytes from:		
	Chickens	Turkeys	Humans
C1, T1	+	+	+
C2, T2	−	−	−
A. faecalis type 1	V[a]	V	V
A. faecalis type 2	−	−	−
A. odorans	−	−	−
A. denitrificans	V	V	V
B. bronchiseptica	+	+	+

[a] V, Variable results: some strains consistently positive, some strains consistently negative.

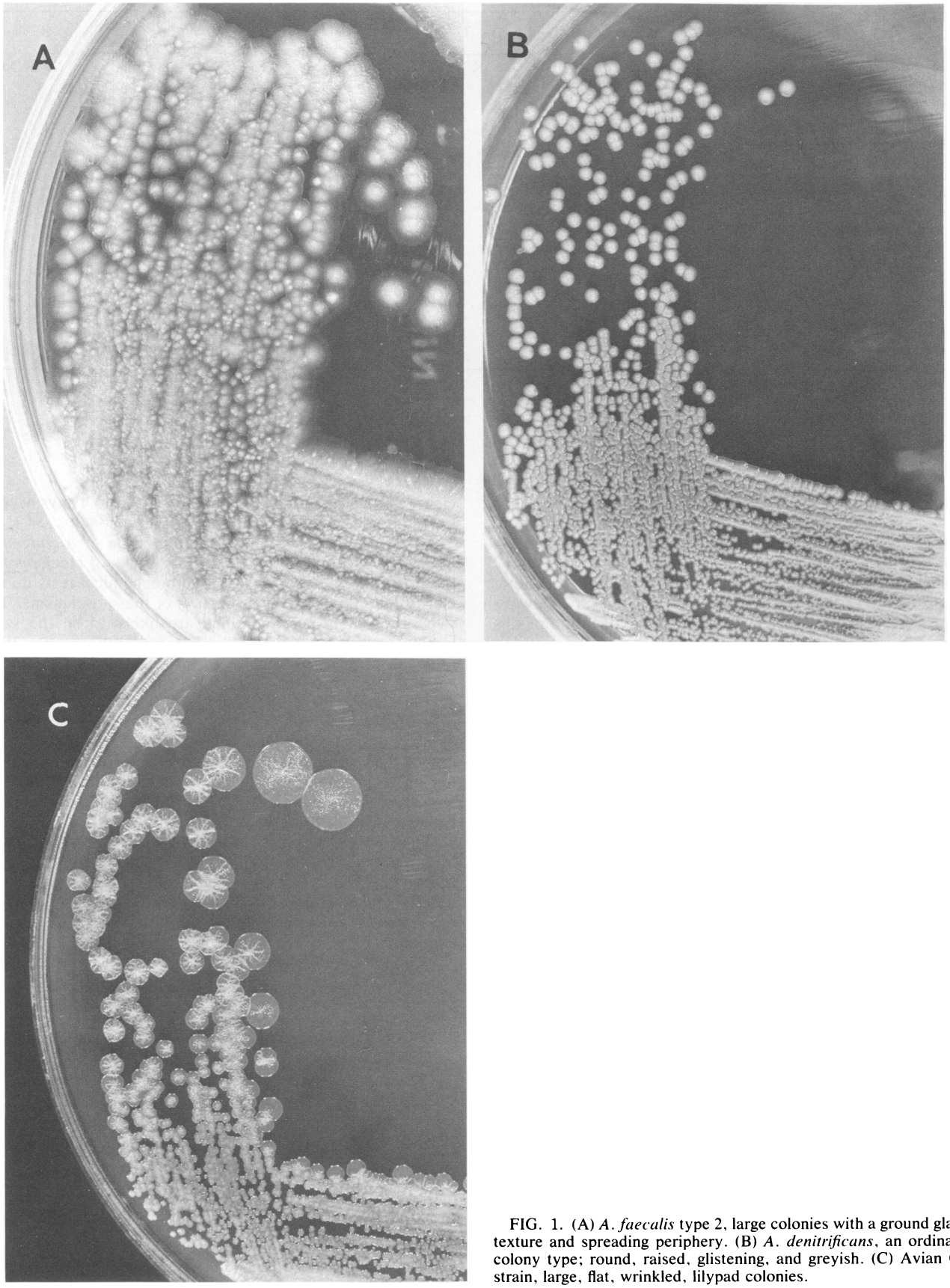

FIG. 1. (A) *A. faecalis* type 2, large colonies with a ground glass texture and spreading periphery. (B) *A. denitrificans*, an ordinary colony type; round, raised, glistening, and greyish. (C) Avian C2 strain, large, flat, wrinkled, lilypad colonies.

pathogenic (1, 2), a pathogenic role for *Alcaligenes*-like bacteria in chickens has not been clearly established. Both avian subgroups, C1 and C2, occupy the same ecological niche and both have been isolated, simultaneously, from the same flock of chickens. In one instance, both were recovered from the trachea of a single bird. The possibility also exists that subgroups C1-T1 and C2-T2 are different phenotypic expressions of the same organism.

In this research, characterization of *Alcaligenes*-like avian respiratory pathogens was based on colonial morphology, biochemical profiles utilizing the OLP method, and hemagglutination. By comparing outer membrane protein profiles of *Alcaligenes* and *Bordetella* reference strains, a recent study (5) has taken a first step toward a taxonomic classification of the turkey rhinotracheitis organism. Results of taxonomic studies determining DNA homologies will no doubt delineate a genus classification for these avian pathogens. To verify the existence of the avian subgroups in isolates of *Alcaligenes*-like or *Bordetella*-like organisms from other geographical areas, further studies will need to be done.

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