Characterization of the Dominant Aerobic Microorganism in Cattle Feedlot Waste

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The dominant aerobic microorganism in cattle feedlot waste (FLW) is a corynebacterium. It is ubiquitous to FLW except on sites where antibiotics are a constant part of the animals’ diet. The organism requires Dl-aspartic acid as its nitrogen source for growth, and individual strains also require or are stimulated by L-tyrosine; acetate serves as the carbon source. Amylolytic activity is weak; protease, lipase, and cellulase activities are nil. Despite the abundance of the organism, it probably does not decompose the waste appreciably.

The extent of the animal waste problem and the pollution hazard inherent in the annual production of 1.4 billion tons of cattle feedlot waste (FLW) is well documented (2, 11). As part of the research at the Northern Regional Research Laboratory on the utilization of FLW, we are studying its microflora as a basis for potential solutions of both utilization and pollution control. We have enumerated and categorized the microflora of FLW and associated sites throughout a year (14) and have examined the enterobacteria more closely (9). One organism, a corynebacterium, was constantly present in FLW. It represented 2 to 70% of the total viable aerobic population. It was readily discernible on plates and was present in greater numbers than the enterobacterial population. It decreased both in numbers and in percentage of the total population as FLW was diluted in runoff to a field ditch and after application of FLW to cropland or during “composting.” The organism was also isolated in large numbers from FLW samples taken throughout a year at five of seven other feedlots in seven major beef-producing states. Antibiotics were routinely incorporated in the feed at the two sites where the corynebacterium was not recovered. Since coryneform bacteria are present in the flora of both the rumen (5) and the intestinal tract (12) of cattle, only the large number of this corynebacterium in the FLW was surprising. Because of the organism’s predominance in the aerobic flora of FLW, its physiological and biochemical characterization was necessary. The 52 isolates examined herein appear to be one species.

MATERIALS AND METHODS

Isolates. Of the 52 isolates, 34 were obtained from FLW from 6 feedlots, including 1 isolate from a freshly excreted sample. The remaining 18 were obtained from the following related sites at 1 feedlot: 12 were from runoff from the pens and 4 were from dirt in the pen; 1 was from a field on which the FLW had been recently spread, and 1 was from a drainage ditch which received both runoff and field drainage. At this feedlot, the organism was not isolated from the feed, from the same fields 6 months later after corn had been grown, or from an FLW stockpile which had undergone stabilization for over a year.

Isolates were obtained from total viable aerobic count plates on Eugonagar (BBL). At times, multiple colonies were selected when color intensity of colonies varied. Isolates were kept on Eugonagar slants at 4°C after a 3-day incubation period at 28°C. Test media were either inoculated directly from Eugonagar slants or from Eugon broth cultures. Tests were incubated at 28°C.

Antibiotic sensitivities. All isolates were tested for sensitivity to the antibiotics reportedly included in the feed. Filter paper disks containing 10 U of penicillin G, 10 μg of dihydrostreptomycin sulfate, or 30 μg of aureomycin were applied to Eugonagar plates immediately after the surface was inoculated with each isolate. Sensitivities were recorded after incubation for 24 and 48 h at 28°C.

Stains. Procedures given in Manual of Microbiological Methods (16) were followed for the Gram reaction (Hucker modification) and for metachromatic granules using Loeffler’s alkaline methylene blue stain. The cells were from 2-day-old colonies on Eugonagar. The Ziehl-Neelsen acid-fast stain procedures outlined in the Manual of Microbiological Methods (16) and by Gordon and Smith (7) were both employed. Cells were examined after 5-, 14-, and
20-day incubation on Eugonagar slants, after 10-day incubation on starch agar plates (see below), and after 30-day incubation on Loeffler's serum slants.

**Physiological tests.** Tellurite inhibition was tested by adding potassium tellurite to Eugonagar to give 1 and 5% concentrations. Blood agar base, nutrient agar, and Loeffler's serum slants were Difco products. Maximum temperature for growth was determined in Eugon broth with a loopful of pellicle growth as the inoculum. Complete pellicle formation in each serial transfer through three tubes was considered a positive result.

Salt tolerance was determined in Eugon broth with sodium chloride added in 1% increments to give 5 to 15% salt concentrations. A loopful of pellicle growth was the inoculum. Serial transfer through three tubes of medium after complete pellicle formation in each tube was a positive result.

Media and procedures in Ewing and Davis (6) were used for the following tests: acetate, citrate (Simmons?), methyl red, Voges-Proskauer (Barritt), urease (Stuart and Christensen), nitrate reduction, motility (SIM), hydrogen sulfide (SIM), indol (Kovac's) on SIM, gelatin liquefaction (Thiogel), esculin hydrolysis, phenylalanine deaminase, lysine and ornithine decarboxylases, and arginine dihydrolase. All tests were incubated at 28 C.

The tests for sodium hippurate hydrolysis and tyrosine decomposition followed the methods of Baird-Parker (3) and Gordon and Smith (8), respectively.

The basal medium for zein and starch hydrolysis, lipase production, and cellulose digestion was 0.7% tryptone, 0.5% sodium chloride, 0.3% yeast extract, and 2% agar (all Difco). For zein hydrolysis, a solution of 2.5% zein (Sigma Chemical Co., St. Louis, Mo.) in 80% ethanol was rapidly pipetted into hot basal agar to give a 0.1% zein concentration, and the medium was then autoclaved for 10 min. Plates were streaked and incubated for 10 days at 28 C. Hydrolysis was indicated by a clearing of insoluble zein in the medium surrounding the growth.

For starch hydrolysis, 1% soluble starch (Difco) was added to the basal medium. It was incorporated directly and also tested separately as a thin layer over a basal layer. The method in Skerman (15) was also employed, substituting 1% for 5% potato starch in thin layer over the same basal medium. Plates were incubated for 20 days at 28 C before flooding with iodine to detect hydrolysis.

For cellulose digestion, a strip of Whatman no. 1 filter paper was added to both the basal medium without agar and to the medium specified in Skerman (15). Powdered cellulose was also added to the latter medium in a separate test. After inoculation, the tubes were incubated for 30 days at 28 C.

A modification of Alford and Steinle's (1) double-layer plate method was used to test lipase production. An emulsion layer containing 1.25% corn oil and 10% of a 1:1,500 solution of Victoria blue B was used under the basal nutrient layer.

Oxidase and catalase activities were checked by the method of Skerman (15).

Acid and gas production from individual carbohydrates were done with 12 selected isolates. Carbohydrates were autoclaved separately and added to phenol red broth base (BBL) supplemented with 0.3% yeast extract (Difco) to give a final concentration of 1%. For the remaining 40 strains, those carbohydrates not converted to acid by the 12 indicator strains were tested in combinations of three at 0.5% concentration of each carbohydrate. Strains showing positive reactions to combined carbohydrates were retested for acid production from individual carbohydrates at 1% concentration. Observations were made daily for 5 days and periodically thereafter. Negative tests were discarded after 20 days.

Ability to utilize carbon and nitrogen sources was tested in the yeast nitrogen base of Wickerham (17) without amino acids and ammonium sulfate (Difco). Carbon and most nitrogen sources were added at 1% and 0.5% concentrations, respectively. D-Amino acids were tested at 1% levels, and the tyrosine supplement was used at 0.1%. A loopful of the pellicle growth was the inoculum. Serial transfer through three tubes of medium after complete pellicle formation in each tube was a positive result.

Vitamin requirements were determined in a basal medium containing 0.7% vitamin-free Casamino Acids (Difco), 0.1% L-aspartic acid, 0.1% D-aspartic acid, 0.1% L-tyrosine, 0.5% sodium acetate, and the minerals in Wickerham's yeast nitrogen base (17). The medium was adjusted to pH 7.0. Requirements for eight vitamins were determined for two isolates. All isolates were tested by combining the three vitamins required by the indicator isolates and by deleting each singly from the eight vitamins tested. A loopful of pellicle growth was the inoculum for serial transfer through three tubes of medium after complete pellicle formation in each tube. Growth through the combined series and no growth in each single deletion series established the vitamin requirement.

**RESULTS**

**Antibiotic sensitivity.** Aureomycin was routinely incorporated in the feed at two feedlots and Combiotic was incorporated at a third. At a fourth, aureomycin was added to the feed only for the first 30 days that an animal was there. Therefore, the sensitivity of all isolates to aureomycin, streptomycin, and penicillin was checked. All strains were sensitive to aureomycin and to penicillin; 49 of 52 isolates were also sensitive to streptomycin (three resistant isolates were all from one site). The two feedlots from which the organism was not isolated used antibiotics continuously in feed; one used aureomycin and the other used Combiotic.

**Physiological and biochemical characteristics.** Rods: 0.5 to 1.0 x 1.0 to 3.0 µm, ellipsoidal cells to swollen and clubbed forms with granules (Fig. 1). Nonmotile. Gram positive to variable. Not acid fast.

Gelatin: No liquefaction.

Eugonagar colonies: 2 to 4 mm in diameter,
flat, dry with roughened surface, cream to cadmium yellow in 2 to 4 days.

Tellurite agar: Black colonies (tellurite reduction) on 1% potassium tellurite agar; growth inhibited on 5% potassium tellurite agar.

No hemolysis on human blood agar.

Eugonagar slant: Flat, dry, rough, cream to cadmium yellow.

Nutrient agar slant: Similar to Eugonagar slant but growth sparse.

Leoffler's serum: Similar to Eugonagar, growth develops tan coloration after 15 to 20 days; serum not digested.

Broth: Very faint turbidity on first day, pellicle forms after 1 to 2 days, flocculent sediment after 4 to 7 days. Pellicle noticeably thinner for 11 of the 52 isolates.

Optimum temperature: 28 to 30°C; maximum temperature: 38 to 40°C.

Usually grow in 10% salt. Although all isolates initially grew in 11% salt, 5 isolates grew only in 8 or 9% salt through 3 serial transfers; 44 isolates were serially transferable in 10 or 11% salt and 3 isolates grew in 12 or 13% salt through serial transfer.

Catalase: Positive.

Oxidase: Negative.

Litmus milk: Unchanged.

Zein not hydrolyzed.

Tyrosine not decomposed.

Nitrites are produced from nitrates by 39 of 52 isolates.

Indol not formed.

No hydrogen sulfide produced.

Methyl red test negative.

Acetyl methyl carbinol not produced.

No growth in standard test media for citrate (Simmons'), acetate, phenylalanine deaminase, lysine and ornithine decarboxylases, and arginine dihydrolase.

Urease produced by 5 of 52 isolates.

Sodium hippurate hydrolyzed by 15 of 52 isolates.

Esculin: Not hydrolyzed.

Starch hydrolysis: Weakly positive on thin layer of 1% potato starch; negative on thin layer of 1% soluble starch.

Cellulose digestion: Negative for both cellulose strip and powdered cellulose.

Not lipolytic on corn oil.

Acid and gas production: Acid only from maltose, trehalose, sucrose, and ribose (all isolates); inulin (45 of 52 isolates); glucose, fructose, galactose, mannose, and soluble starch (47 of 52 isolates). Growth on ribose is minimal, and acid production from mannose is delayed and weak. No acid from arabinose, xylose, rhamnose, glycerol, adonitol, sorbitol, mannitol, dulcitol, inositol, lactose, cellobiose, melibiose, methyl-α-D-glucoside, salicin, esculin, raffinose, cellulose, potassium gluconate, sodium glucuronate, or potassium saccharate; dextrin (1 positive of 52 isolates). Acid was demonstrable within 2 to 5 days. At about the same time, a flocculent sediment appeared, and the upper quarter of test media became distinctly alkaline. When bromocresol purple was substituted for phenol red in the glucose fermentation, visible detection of acid formation was delayed 2 to 3 days.

Utilization of sole carbon sources: Acetate (52 isolates); glucose, maltose, or sucrose (47 of 52 isolates); malate (11 of 52 isolates); succinate and citrate not used.

With acetate as the carbon source, dl-aspartic acid plus L-tyrosine were utilized as the sole sources of nitrogen by all isolates; L-tyrosine was required by 11 isolates and was stimulatory to the remainder growing on dl-aspartate. Dl-asparagine can replace dl-aspartic acid. In the presence of L-tyrosine, the following substrates did not serve as nitrogen sources: L-aspartic acid, L-aspartic acid plus D-alanine, D-aspartic acid plus L-alanine, L-glutamic acid plus D-alanine, DL-glutamic acid, DL-alanine, or ammonium sulfate.

With glucose as the carbon source, the following compounds were not utilized as the sole source of nitrogen: L-glutamic acid, L-glutamine, DL-alanine, L-tyrosine, choline, uric acid, urea, ammonium sulfate, sodium nitrite, or potassium nitrate.

All isolates required thiamin, biotin, and pyridoxine for growth.

A grouping of these isolates according to their variable characteristics is shown in Table 1. The majority belong to group I on the basis of pellicle formation, tyrosine requirement, and
malate utilization. This distinction is blurred by the results of nitrite production and hippurate hydrolysis. Group I organisms were isolated from all sites although they were not obtained from every sample. The ability of a few isolates (group Ia) to produce urease and the inability of another small group (group Ic) to ferment hexoses represent extremes within the group. Of the 11 isolates designated in group II, 5 were FLW samples from 2 sites; the remaining 6 were from samples of runoff and pen dirt. However, isolates classified in group I were obtained from both of these sites and were isolated at the same time as the group II isolates in four of seven samples from these sites.

Representative cultures are deposited in the Agricultural Research Service Culture Collection. The group designation (Table 1) for each isolate and its ability to hydrolyze hippurate are shown in parentheses. Type culture: B-4168 (Ib, +); others: B-4169 (Ia, +), B-4170 (Ia, −), B-4171 (Ib, −), B-4172 (Ib, −), B-4173 (Ic, +), B-4174 (Ic, −), B-4175 (Iib, +), B-4176 (Iib, −), B-4177 (Iib, +), B-4178 (Iib, −), and B-4179 (Iic, −).

**DISCUSSION**

The FLW isolates form a single closely clustered group based on their morphology and staining properties, growth rate, and specific requirement for DL-aspartic acid or DL-asparagine as the basic sole carbon source, as well as by identical reactions in 26 carbohydrate fermentations and 27 other biochemical tests. They were not divisible on a geographic basis and were ubiquitous in cattle FLW except where antibiotics were constantly incorporated in the feed. Morphologically the FLW isolates belong in one of the “coryneform” genera since they are nonmotile, gram-positive to gram-variable, non-acid-fast, irregularly shaped rods containing metachromatic granules (10). The flat, dry, rough, yellow colonies which form on agar and the hydrophobic pellicle formation in broth with almost no turbidity are very similar to the growth of the saprophytic mycobacteria (4, 7, 8). However, the FLW isolates are not acid fast, require amino acid nitrogen, and grow more rapidly than do mycobacteria (4, 15). Growth of the FLW isolates on agar and in broth is not like the soft, butyrous, or smooth colonies and turbid growth in broth typical of Arthrobacter, Cellulomonas, Microbacterium, corynebacteria from plant sources, or the Corynebacterium acnes–Propionibacterium group. Cellulose is not digested as by Cellulomonas, and cystines are not formed as in Arthrobacter. Proteolysis, common in Arthrobacter, Cellulomonas, and the C. acnes–Propionibacterium group, is not present in the FLW isolates. Inorganic sources of nitrogen are not used by the FLW isolates as they often are by corynebacteria from plant sources and Arthrobacter; the FLW isolates do not produce acid from polyhydroxy alcohols as do most plant corynebacteria and the C. acnes–Propionibacterium group. However, the FLW isolates are similar to the corynebacteria from animal sources in many respects: requirement for amino acids as a nitrogen source, lack of proteolytic capabilities, and ability to ferment some carbohydrates, but not polyhydroxy alcohols, with minimal acidity and no gas. In addition, the colonial appearance and growth pattern in broth of FLW isolates are similar to those of many corynebacteria from animal sources (4, 10, 13, 15).

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