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# Value of Acid Metabolic Products in Identification of Certain Corynebacteria†

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Acid metabolic products of 23 strains of human and animal pathogenic corynebacteria, representing eight different species, were determined by gas chromatography. The results showed that the species examined were metabolically heterogeneous and could be presumptively identified based on the acid products produced. *Corynebacterium equi* did not produce any acids; *C. renale* produced lactate; and *C. pyogenes* produced major amounts of lactate, variable amounts of acetate, and minor amounts of succinate and pyruvate. *C. kutscheri* produced propionate and lactate as major products and pyruvate and oxalacetate as minor products. *C. diphtheriae* and *C. pseudotuberculosis* produced major amounts of propionate, acetate, and formate. In addition, *C. pseudotuberculosis* produced major amounts of pyruvate and minor amounts of succinate, lactate, and oxalacetate, whereas *C. diphtheriae* strains produced minor but variable amounts of lactate, succinate, fumarate, pyruvate, and oxalacetate. *C. bovis* produced acid products similar to those of *C. pyogenes* but was readily distinguishable from the latter by the lack of hemolysis on blood agar, colony morphology, catalase reaction, and biochemicals. *C. suis* characteristically produced major amounts of ethanol, acetate, and formate and minor amounts of lactate and succinate but no propionate.

It has been well established that metabolic products represent a firm basis upon which to differentiate certain bacterial species, particularly anaerobes (28, 38, 39, 43). The metabolic products are known to be similar among strains within each species, are reproducible from culture to culture within a strain, and are rapidly determined by gas chromatography (28, 39).

Previous studies showed that human pathogen *Corynebacterium acnes* and related anaerobic coryneforms produce major amounts of propionic and acetic acids from carbohydrate fermentation (16, 40, 41). Hence, these organisms were excluded from the genus *Corynebacterium* (48) and were reclassified as propionibacteria (41). In contrast, the metabolic products produced by *C. suis* (52), an animal pathogenic anaerobic coryneform, are not known, and its relatedness to *Propionibacterium acnes* and related anaerobic coryneforms is not clear. Also, there have been no systematic studies in the past on the acid metabolic products produced by aerobic to facultatively anaerobic human and animal pathogenic corynebacteria. Fujita and Kodama (18), by qualitative analysis, demonstrated that washed cell suspensions of *C. diphtheriae* produce formate, acetate, lactate, and succinate and no detectable propionic acid. Tasman and Branwijk (55), on the other hand, reported that the Tomosik strain of *C. diphtheriae* produced major amounts of propionate, acetate, and formate; ethanol, when produced, was only a minor product. In contrast, the Bandoeng strain of *C. diphtheriae* produced large amounts of formate, acetate, lactate, and succinate, small amounts of propionate, and traces of ethanol (55). There has been little or no further information in the last 40 years on the metabolic products produced by *C. diphtheriae*. In this paper, we report the acid metabolic products produced by strains of *C. diphtheriae, C. pseudotuberculosis, C. kutscheri, C. renale, C. bovis, C. pyogenes, C. equi,* and *C. suis* and their value in a rapid presumptive identification of the above species.

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## MATERIALS AND METHODS

**Bacteria.** Reference strains (48) of *C. renale* (ATCC 19412), *C. pseudotuberculosis* (ATCC 19410, NCTC 3450), *C. kutscheri* (ATCC 15677), *C. equi* (ATCC 6939, NCTC 1621), and *C. bovis* (ATCC 7715, NCTC 3224, NIRD 61), as well as nonreference strains 10146, 7699, and 7698 and *C. equi,* were all obtained from the American Type Culture Collection, Rockville, Md. Strains SS7-74, BT331-76, BT343-76 and

BT400-76 of *C. pyogenes*, BT435-76 of *C. bovis*, CE565-73 of *C. renale*, MM120-71 of *C. equi* were obtained from G. R. Carter of this department. *C. pyogenes* strain 5 and *C. diphtheriae* strains 1, 2, and 3 were from the culture collection of our department. A strain of *C. suis* was obtained from M. A. Soltys, Ontario Veterinary College, Guelph, Ontario. We experienced considerable difficulty in obtaining larger numbers of authentic cultures of some species, and in such cases the use of only one or two strains was unavoidable.

**Culture maintenance.** All organisms, except *C. suis*, were maintained on stock culture agar (Difco) slants. *C. suis* was maintained on prereduced anaerobically sterilized peptone-yeast extract-maltose (PRAS-PYM) medium as described by Holdeman and Moore (28), using anaerobic techniques also described by these investigators; the carbon dioxide gas phase was used in all cases. All media were sterilized by autoclaving at 115°C for 15 min. Slants of sterile stock culture agar or PRAS-PYM slants were inoculated with a loopful of lyophile material of a given strain suspended in 0.5 ml of brain heart infusion (BHI; BBL) broth, incubated at 37°C for 24 to 48 h, and stored at 4°C. Subcultures were made once every 3 weeks to slants of maintenance medium.

The purity of each culture was checked routinely at each transfer by examination of Gram-stained smears, examination of wet mounts under a phase-contrast microscope, and streaking on a blood agar plate (BHI agar plus 5% sheep blood for all cultures, except *C. suis*, and PRAS-supplemented BHI-blood agar (28) for *C. suis*) for examination of characteristic colonial features (9, 30, 48, 52).

**Preparation of inocula.** In the case of aerobic to facultatively anaerobic cultures, inocula for biochemical test media were prepared by transferring fresh stock cultures with a sterile loop to 3 ml of sterile BHI broth contained in foam-plugged tubes (12 by 75 mm) and incubated at 37°C for 24 to 48 h. A drop of this culture was transferred to a tube of fresh BHI broth and incubated as above. After two further transfers, a drop or loopful of these cultures was used as the inoculum for various liquid and solid biochemical test media, respectively.

*C. suis* inoculum was prepared by three serial transfers of the culture in PRAS-PYM broth, and the third transfer culture was used for inoculating various biochemicals.

**Biochemical testing and gas chromatographic analyses.** Procedures described previously (28) were used for the biochemical testing of *C. suis*. For all other cultures, the procedures described by Blair et al. (6) were used. For sugar fermentation tests, sterile Trypticase soy broth (BBL) was supplemented with enough 10% (wt/vol) filter-sterilized solution of a given sugar to give a final concentration of 1% in the medium.

For gas chromatographic analyses of acid metabolic products, all aerobic to facultatively anaerobic cultures, except *C. pyogenes*, were grown in BHI broth supplemented with 0.3% (wt/vol) dextrose. The medium for *C. pyogenes* additionally contained 0.0002% (wt/vol) hemin. The media were dispensed in 10-ml amounts in foam-plugged tubes (18 by 150 mm). *C. suis* was grown in 10 ml of PYM broth medium (28) contained in rubber-stoppered tubes (18 by 150 mm). All tubes were incubated at 37°C for 72 h, by which time all the strains attained maximum growth. In all cases, growth was estimated by measuring the absorbance at 600 nm with a Bausch & Lomb Spectronic 20 colorimeter.

Acidification of the cultures, extraction of the volatile and nonvolatile products, and gas chromatographic procedures were as described by Holdeman and Moore (28). The kinds and amounts of acids produced were determined by comparing their retention times and peak heights to retention times and peak heights of a standard concentration of known acids under the same experimental conditions. Strains within a given species generally produced similar metabolic products, unless mentioned otherwise. Results on gas chromatographic analyses and biochemical testings represent an average of at least two determinations with each strain.

## RESULTS AND DISCUSSION

The volatile and nonvolatile acid metabolic products of the corynebacterial species studied and their key biochemical characteristics are presented in Table 1. The results showing the ability of different corynebacterial species to grow anaerobically on BHI-blood agar in GasPak anaerobic jars (BBL), using GasPak disposable $CO_2$ + $H_2$ envelopes (BBL), are also presented in Table 1. The results showed that the *Corynebacterium* species examined are metabolically heterogeneous and that each species produces distinctive acid metabolic products.

*C. equi*, an important cause of pneumonia in foals (see references 7 and 10), was first described by Magnusson (37) and was later shown to cause a number of infections in several other species of domestic animals (7, 29, 37) and in humans (20, 27). This is the only corynebacterium pathogenic for animals that did not produce acid from any of the carbohydrates tested (Table 1; 23, 31, 48), although it grew very well in these tubes. It failed to grow anaerobically on BHI-blood agar plates (Table 1), but gave excellent growth on the same medium under aerobic conditions. Furthermore, none of the six strains of *C. equi* used in this study produced detectable acid end products and in this respect were different from the others (Table 1). Previous studies also showed that *C. equi* is different from *C. diphtheriae* and related animal pathogens (48) in mycolic acids, in producing a salmon pink pigment, in biochemical reactions, and in producing large mucoid colonies on blood agar (7, 9, 11, 22, 23, 32, 48). Several numerical taxonomic studies also clearly established that *C. equi* is distinctly different from typical members of the genus *Corynebacterium* (15, 26, 31, 53), is related to mycobacteria and nocardia, and

TABLE 1. *Acid metabolic products and selected biochemical characteristics of animal pathogenic corynebacteria and C. diphtheriae*

| Organism[a] | Acid metabolic products[b] | | Biochemical characteristics[c] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Volatile | Nonvolatile | Catalase | Hemolysis | Glucose | Maltose | Sucrose | Lactose | Litmus milk | $NO_3$ reduction | Gelatin | Indole | Esculin hydrolysis | Urease | Anaerobic growth[d] |
| *C. equi* | | | + | – | – | – | – | – | – | + | – | – | – | + | – |
| *C. pyogenes* | a or A | Lspy | – | + | + | + | + | + | ACP | – | + | – | – | – | ++ |
| *C. bovis* | a or A | Lspyoa | + | – | + | + | – | – | – | – | – | – | – | – | ++ |
| *C. renale* | a(p) | L(pys) | + | ± | + | – | – | – | KP | – | – | – | + | + | + |
| *C. kutscheri* | Pa | Lpyoa (s) | + | – | + | + | + | – | – | + | – | – | + | + | ++ |
| *C. pseudotuberculosis* | AFP[e] | Pysloa | + | + | + | W | – | – | – | + | – | – | – | + | + |
| *C. diphtheriae* 1 | AFP | Ls(oa) | | | | | | | | | | | | | |
| *C. diphtheriae* 2 | AFP | s | + | – | + | W | – | – | – | + | – | – | – | – | ++ |
| *C. diphtheriae* 3 | AFP | fupyoa | | | | | | | | | | | | | |
| *C. suis* | AFEt | Ls | – | ± | – | + | – | – | – | – | – | – | – | + | ++ |

[a] Six strains of *C. equi*, five strains of *C. pyogenes*, two strains each of *C. bovis*, *C. renale*, *C. kutscheri*, and *C. pseudotuberculosis*, one strain of *C. suis*, and three strains (1, 2, and 3) of *C. diphtheriae* have been included in this study.

[b] A, Acetic acid; Et, ethanol; Fu, fumaric acid; F, formic acid; L, lactic acid; OA, oxalacetic acid; P, propionic acid; Py, pyruvic acid; S, succinic acid. Uppercase letters represent major products (1 meq or greater/100 ml); lowercase letters represent minor products (<1 meq/100 ml). Products in parentheses are not produced by all strains.

[c] +, Positive for acid from sugars; ±, variable; W, weak reaction; –, negative; A, acid; K, alkaline; C, clot; P, peptonization.

[d] In the anaerobic growth column, ++ = good growth, + = fair growth, and – = no growth.

[e] An unidentified three-carbon alcohol was produced by *C. pseudotuberculosis* strain 19410 in significant amounts.

should be placed in the "rhodochrous" group (31). Goodfellow and Alderson recently proposed that *C. equi* belongs in the newly created genus *Rhodococcus* (21).

*C. pyogenes* (48) is reported to be the most frequently isolated bacterium from pyogenic conditions in domestic animals (7, 44) and is also known to cause serious infections in humans (1, 8, 17, 35, 36, 56). Our results (Table 1) showed that *C. pyogenes* is different from the other species, except *C. bovis,* in producing major amounts of lactate, minor amounts of acetate, and trace amounts of succinate and pyruvate; only one of the five strains examined produced major amounts of acetate. Reddy and Cornell (Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, I51, p. 163) recently studied 19 strains of *C. pyogenes,* including the 5 used in this study, and showed that all the strains produce major amounts of formate, acetate, and succinate and variable amounts of lactate in media supplemented with bicarbonate. In contrast, major amounts of lactate and minor amounts of acetate were produced when bicarbonate was deleted from their medium. Reddy and Cornell also showed that this organism grows well either aerobically or under strict anaerobic conditions in their medium.

*C. pyogenes* was the only species included in this study that was catalase negative, liquefied gelatin, and produced acid coagulation and peptonization in litmus milk (Table 1; 9, 30, 47–49). It also produces characteristic pinpoint, whitish, beta-hemolytic colonies on blood agar; the zone of hemolysis is at least twice the diameter of the colony. Previous studies also showed that *C. pyogenes* was distinct from *C. diphtheriae* and related animal pathogens in cell wall composition and serology and in not containing mycolic acids (13, 14, 22). Furthermore, several numerical taxonomic studies showed that *C. pyogenes* strains are quite homogeneous as a group (46), but are distinctly different from other human and animal pathogenic corynebacteria (26, 31). In confirmation of our previous results (45), we showed that hemin is highly stimulatory for the growth of this organism but not for the other species. As proposed previously (31), the degree of dissimilarities between *C. pyogenes* and the other species appears large enough to exclude this organism from the genus *Corynebacterium.*

*C. pyogenes* appears to be very similar to actinomyces in being gram positive, nonsporing, non-acid-fast, facultatively anaerobic, and nonmotile; in morphology, in being negative for indole and urease, in being catalase negative (*Actinomyces viscosus* is an exception); in containing a *b*-type cytochrome; in having the

sugars rhamnose and glucose, but not arabinose, and the diamino acid lysine in its cell walls; in producing acetic, formic, lactic, and succinic acids, but not propionic acid, from carbohydrate fermentation; and in serological relationships (7, 9, 41, 47–50, 54). Slack and Gerencser (50) have also noted the close relationship between *C. pyogenes* and *Actinomyces* species.

At the present time, there is general, if not universal, agreement that among the animal pathogenic corynebacteria, only *C. renale, C. bovis, C. kutscheri,* and *C. pseudotuberculosis,* which are closely related to the type species *C. diphtheriae,* should remain in the genus *Corynebacterium* and that *C. equi* and *C. pyogenes,* which are quite different from the other species (as discussed above), should be excluded from the genus (2, 21, 23, 31, 48). Several numerical taxonomic studies have shown that *C. diphtheriae* and the four animal pathogenic species mentioned above are very similar and form a close cluster (15, 26, 31, 53). Cummins and Harris (14) and Cummins (13) showed that *C. diphtheriae, C. pseudotuberculosis, C. renale, C. kutscheri,* and *C. bovis* are similar in containing arabinose and galactose as the major sugar components, *meso*-diaminopimelic acid as the major diamino acid, and a common polysaccharide antigen in their cell walls. *C. pseudotuberculosis, C. renale,* and *C. kutscheri* were also similar to *C. diphtheriae* in containing dihydromenaquinones having eight isoprene units, whereas *C. bovis* was an exception in containing dihydromenaquinone with nine isoprene units (11). These five species were also shown to be similar in their mycolic acid content (22, 32). In view of the many similarities between these five species, it was interesting to observe their metabolic heterogeneity as revealed by the acid metabolic products produced (Table 1).

*C. bovis* (48) is generally regarded as a commensal on cow's udder but may occasionally cause clinical mastitis (10). Although this organism was readily distinguishable from *C. pyogenes* on the basis of biochemical characteristics, colony morphology, and in being nonhemolytic, it was similar to *C. pyogenes* in producing major amounts of lactate and minor amounts of acetate, succinate, and pyruvate (Table 1); strain BT 435-76 was different from ATCC 7715 in producing major amounts of acetic acid. This relatively minor strain variation is not surprising since Jayne-Williams and Skerman (30) previously demonstrated variation in biochemical characteristics among strains of *C. bovis.*

*C. renale* causes specific cystitis and pyelonephritis in cattle, but other animals are also affected (7, 12). This is the only corynebacterium pathogenic to animals that produced alkaline coagulation and peptonization in litmus milk (Table 1; 7, 9, 12, 48). Our results also show that it is distinct from others in producing lactic acid as the sole major product (Table 1); one of the two strains tested (ATCC 19412) produced trace amounts of acetate, propionate, pyruvate, and succinate.

*C. kutscheri* (syn., *C. murium;* 48), first described by Kutscher in 1894 (33), was known to cause pulmonary infections, sometimes in epidemic proportions, in mice (5) and rats (19). It appeared distinct from the other organisms in producing major amounts of propionate and lactate and minor amounts of pyruvate and oxalacetate and in not producing acetate or formate (Table 1). It is more closely related to *C. diphtheriae* and *C. pseudotuberculosis* than to others, but is easily distinguishable from these two species in fermenting sucrose and hydrolyzing esculin (Table 1). It is further distinguishable from *C. pseudotuberculosis* in being nonhemolytic and from *C. diphtheriae* in producing urease (Table 1).

*C. pseudotuberculosis* (syn., *C. ovis;* 48) is an important animal pathogen and is known to be the causative agent of ulcerative lymphangitis in horses and caseous lymphadenitis in sheep (4, 7). It also causes infections in other animals and in humans (3, 4, 7, 25, 36). As discussed above, it is closely related to *C. diphtheriae* in cell wall composition and serology, in being lysogenized by *C. diphtheriae* phages and synthesizing diphtherial toxin, in containing similar mycolic acids and menaquinones, and on the basis of numerical taxonomic studies (2, 15, 22, 23, 31, 48, 53). However, the present results (Table 1) show that it is metabolically different from *C. diphtheriae* in producing major amounts of pyruvate and considerable amounts of succinate (0.8 meq/100 ml). Although both *C. pseudotuberculosis* and *C. diphtheriae* produced major amounts of formic, acetic, and propionic acids, the former species produced only about half the amounts of acetic and propionic acids as those produced by *C. diphtheriae.* Furthermore, *C. pseudotuberculosis* is distinguishable from the latter by its ability to produce beta hemolysis on blood agar and hydrolyze urea (Table 1; 9, 48). It should also be noted that *C. pseudotuberculosis* is readily distinguishable from all the other animal pathogenic corynebacteria studied on the basis of acid end products (Table 1).

*C. diphtheriae,* the well-known causative agent of human diphtheria (2, 48), infrequently causes infections in animals (24). *C. diphtheriae* strains examined were similar in producing major amounts of volatile products, acetate, for-

mate, and propionate, but showed considerable heterogeneity in the nonvolatile acids produced. Strain 1 produced minor amounts of lactate and succinate and traces of oxalacetate; strain 2 produced minor amounts of succinate only; and strain 3 produced major amounts of fumarate, pyruvate, and oxalacetate but no succinate or lactate (Table 1). Our results are similar to those of Fujita and Kodama (18) and Tasman and Branwijk (55) in showing differences among strains of *C. diphtheriae* in acid products. It should also be noted that *C. diphtheriae* and *C. pseudotuberculosis* are similar to propionibacteria (41) in producing major amounts of acetate and propionate and minor amounts of lactate and succinate. However, propionibacteria apparently either do not produce formate or produce only minor amounts of this acid (41), whereas *C. diphtheriae* and *C. pseudotuberculosis* produce major amounts of formate (Table 1).

*C. suis,* a catalase-negative, nonsporing, obligately anaerobic coryneform, was originally isolated by Soltys and Spratling (52) from cases of cystitis and pyelonephritis in pigs. Several other cases of urinary infections by *C. suis* have since been reported (34, 42, 51). It is quite different from the other corynebacterial species examined in producing major amounts of acetate, formate, and ethanol and trace amounts of lactate and succinate. Unlike other anaerobic coryneforms, now reclassified as propionibacteria (41), it did not produce major amounts or propionic acid. These results and several other lines of evidence (J. Wegienk and C. A. Reddy, unpublished data) indicate that *C. suis* does not belong either in the genus *Corynebacterium* (48) or in the genus *Propionibacterium* (41).

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