Differentiation of *Escherichia, Enterobacter,* and *Klebsiella* by Thin-Layer Chromatography (37633)

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Recent publications from the Anaerobe Laboratory at Virginia Polytechnic Institute (1, 2) have shown that many genera and species of anaerobic bacteria produce a characteristic array of fermentation acids thus greatly simplifying and accerelating identification. In an effort to devise rapid and simple means for detection and identification of bacterial pathogens, possible differentiation of enteric bacteria by analysis of fermentation acids has been investigated. Cells from a single colony of bacteria were allowed to grow in a small amount of liquid medium and the organic acids, succinic, fumaric, and pyruvic acids, arising from the fermentation permitted differentiation of the genera, *Escherichia, Enterobacter,* and *Klebsiella.*

Materials and Methods. Bacterial strains. All wild-type strains of the three genera investigated were isolated from clinical specimens and identified by conventional methods in the clinical laboratory at this Institute. Authentic cultures were obtained from The American Type Culture Collection (ATCC), Rockville, Maryland. Stock cultures of all strains were grown on trypticase soy agar and stored at 4°.

Growth of cultures from single colonies. Cell suspensions of the various strains were streaked on eosinmethylene blue agar and incubated at 37° for 24 hr. From such plates the entire cell mass from a single colony, 1–2 mm in diameter, was tamped into a 5 μl disposable pipette and then expelled into 0.25 ml of liquid medium in a 12 ml centrifuge tube. The centrifuge tube was tightly closed with a neoprene stopper and the cell mass was evenly dispersed by agitation. These "mini cultures" were incubated in a 38° water bath for the desired length of time. After incubation, the tubes were cooled to room temperature and 0.05 ml of 5% sulfuric acid was added and mixed thoroughly. The sulfuric acid reduced the pH to approximately 1.5 which both stopped further growth and metabolism and permitted extraction of the organic acids arising from fermentation.

Chromatographic procedure. To the acidified culture, 0.25 ml of diethyl ether was added and the tube again tightly stoppered. Fermentation acids were extracted into the ether by manual inversion of the tubes 25 times. The tubes were then subjected to light centrifugation to break possible emulsions. Thirty μl of ether extract (10 μl per application) were spotted on Gelman ITLC sheets, type SG, 8 × 8 in. Chromatography was conducted according to Gelman recommendations in a Gelman chromatography chamber employing a solvent composed of chloroform-methanol-formic acid in an 80:1:0.75 ratio. Development was allowed to proceed until the solvent had advanced 11 cm beyond the application point; this required 10 min. The sheets were then removed from the tank, air dried for 10 min, dried an additional 20 min in a stream of warm air, and then rehumidified in a humid (50% relative humidity) 37° incubator for 10–20 min. Subsequently, the sheets were sprayed with an aqueous indicator solution which contained 40 mg bromcresol purple and 0.94 ml of 0.1 *N* NaOH/100 ml of solution. The organic acids appeared as yellow spots on a bluish purple background. Before the contrast faded, the nearly rectangular spots were accurately outlined with a soft pencil. Spot dimensions were measured with a mm ruler and the approximate area in mm^2 was calculated. When the area of the spot was plotted against

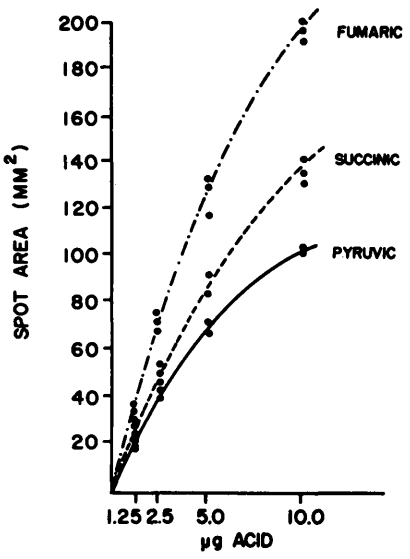

FIG. 1. Standard curves for succinic, fumaric, and pyruvic acids as determined by TLC.

μg of acid spotted, standard curves were established (Fig. 1). Many repetitions of this procedure with solutions of known acids have shown that the greatest error for this procedure is ± 15% of the mean. Identification of the acids produced by fermentation was made by chromatography of ether extracts along with known compounds in two solvent systems as described by Bleiweis *et al.* (3). Acetic and formic acids are not detected by this procedure due to their volatility during processing of the chromatograms.

Medium composition. The liquid medium for the single colony cultures had the following composition per 100 ml: 40 ml of 5% acid hydrolyzed casein; 20 ml of 10% yeast extract; 10 ml of 10% glucose; 10 ml of 10 m*M* dithiothreitol; 20 ml of sterile water; and 0.8 ml of a salts solution containing the following salts per liter: $CaCl_2$, 1.0 g; $MgSO_4$, 1.0 g; K_2HPO_4, 5.0 g; KH_2PO_4, 5.0 g; $NaHCO_3$, 50 g; and NaCl, 10 g. All ingredients were sterilized separately and combined aseptically to make the complete medium.

Results and Discussion. In single colony cultures, both growth, as judged by optical density, and acid production, as determined by chromatography, attained maximum values in approximately 3.5 hr (Fig. 2). The results, for a strain of wild type *Escherichia coli,* show that the mass doubling time during growth was 30 min and that there was an 8- to 10-fold increase in optical density indicating that slightly more than 3 generations occurred. All other strains of *E. coli, Klebsiella,* and *Enterobacter* species tested for growth kinetics (3 of each) exhibited nearly identical curves for optical density and for acid production. Therefore, a 3.5-hr incubation period was adopted for surveying a number of wild type isolates and authentic

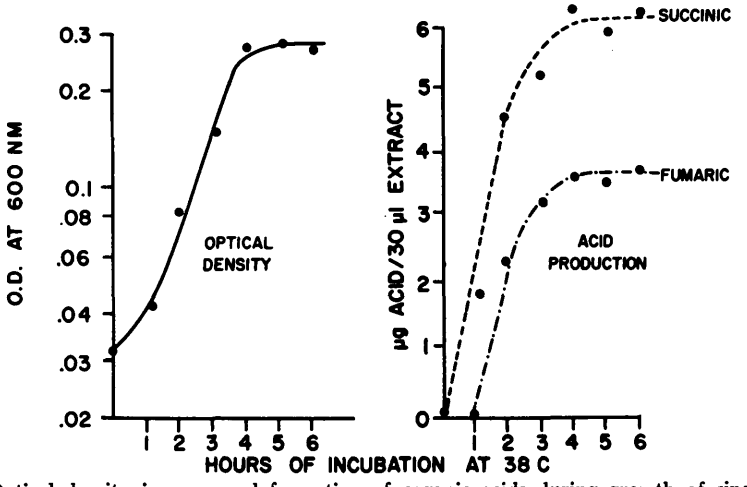

FIG. 2. Optical density increase and formation of organic acids during growth of single colonies of a wild type strain of *Escherichia coli* in hydrolyzed casein medium at 38°. Twenty representative colonies cultured in 5 ml amounts of medium.

TABLE I. Fermentation Acid Production by Wild Type and Authentic Strains of *Escherichia*, *Enterobacter*, and *Klebsiella* spp.[a]

Organism	No. of strains	Organic acid in μg/30 μliter ether extract		
		Succinic	Fumaric	Pyruvic
E. coli	12	5.7–8.2	1.8–2.7	0
Enterobacter spp.	5	1.7–2.1	1.0–1.2	0
Klebsiella spp.	8	1.4–2.0	1.0–2.2	0.7–1.3
Ent. cloacae, Bact-Chek	1	2.1	1.2	0
Ent. aerogenes ATCC #13048	1	3.7	1.1	0
Ent. liquefaciens ATCC #14460	1	1.4	0.2	0
Ent. hafnia ATCC #11604	1	2.9	1.1	0
K. pneumoniae ATCC #13883	1	3.1	1.4	0.9

[a] Single colonies incubated in hydrolyzed casein medium for 3.5 hr at 38°, acidified, and ether extracted; 30 μliter of ether extract analyzed by TLC. Data for wild type strains are presented as the full range of values.

cultures.

In Table I are presented the results of analysis for organic acids produced by various species and strains of the three genera investigated. The data are given as the full range of values encountered with the majority of values for individual strains clustering around the middle of the range. *E. coli* strains produced about 3 times as much succinic acid and twice as much fumaric acid as members of the other two genera. Representatives of *Klebsiella* and *Enterobacter* produced comparably lower amounts of succinic and fumaric acids; but *Klebsiella* also showed distinct and measurable amounts of pyruvic acid and thus could be differentiated from *Enterobacter* on this basis. Included in Table I are the results with authentic specimens from The American Type Culture Collection. These data demonstrate that the authentic strains behave in an identical manner to the wild types isolated from clinical specimens. Although some ATCC cultures showed slightly higher amounts of succinic acid than wild types, none of them could be confused with *E. coli*. *Enterobacter liquefaciens* grew rather poorly in the medium employed thus yielding amounts of acids somewhat less than for other species of the genus. The ability of this method to differentiate decisively between members of the three genera studied is clear. No other detectable acids are produced during fermentation by these three genera with the exception of trace amounts of propionic acid common to all three.

Although the study of fermentation acids from anaerobic bacteria (1, 2) employed gas chromatography (GLC), such instrumentation was deferred in favor of thin layer chromatography (TLC) for several reasons: the minor cost of TLC compared to GLC; in GLC, samples must be analyzed in sequence whereas in TLC many samples can be analyzed nearly simultaneously; the need to convert some organic acids, notably succinic, fumaric, and pyruvic, to volatile methyl esters before using GLC, thus adding to the time required for analysis. An effort was made to maintain simplicity and ease of performance, points which heavily favored TLC.

The procedure described permits the identification of three genera, *Escherichia, Enterobacter,* and *Klebsiella,* in 4.5 hr, 3.5 hr for incubation and roughly 1 hr for chromatographic analysis. In an effort to accomplish more rapid identification, up to 4 colonies were inoculated per 0.25 ml of medium. This procedure allowed identification in 3 hr which appears to be the minimum time

required.

Summary. A thin layer chromatographic method has been developed for differentiation of the genera *Escherichia, Enterobacter,* and *Klebsiella* based on the amounts of succinic, fumaric, and pyruvic acids formed during fermentative growth. The method employs only a single colony of bacterial cells and can be completed in 4.5 hr.

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Received June 22, 1973. P.S.E.B.M., 1973, Vol. 144.