

Gas-Liquid Chromatography Technique for Detection of Hippurate Hydrolysis and Conversion of Fumarate to Succinate by Microorganisms

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Received 2 June 1982/Accepted 27 July 1982

A gas-liquid chromatography technique which allows simultaneous detection of hippuric acid (*N*-benzoylglycine) hydrolysis and conversion of fumaric acid to succinic acid by microorganisms uses a new medium, hippurate-formate-fumarate broth, and a gas chromatograph equipped with a thermal conductivity detector. This technique gave more reproducible results than other tests used in the study for detecting hippurate hydrolysis and also gave consistent results in detecting succinic acid produced from utilization of fumaric acid

Hydrolysis of hippurate has been shown to be a useful characteristic for characterizing and identifying various microorganisms (1, 4-7, 9, 12). In work with *Campylobacter*, it has been found that the results obtained with conventional biochemical tests for hippurate hydrolysis (6, 7, 9) were difficult to interpret in some cases. For this reason we developed an alternative procedure which uses a special medium, hippurate-formate-fumarate (HFF) broth, and a gas-liquid chromatograph equipped with a thermal conductivity detector for detecting hippurate hydrolysis. This technique is less laborious to perform than that described by Ziegler and Kutzner (12).

Bacterial strains used in developing the procedure were *Campylobacter jejuni* (Centers for Disease Control [CDC] Anaerobe Laboratory A3309; Collection of Institute Pasteur CIP702); *Campylobacter coli* (CDC Anaerobe Laboratory A3315 [CIP7080]) from Robert Weaver, CDC; and *Clostridium sordellii* (CDC Anaerobe Laboratory 14337) and a group B *Streptococcus* strain, *Streptococcus agalactiae* (CDC Anaerobe Laboratory 18434), from Richard Facklam, CDC.

The bacterial strains were tested in the following media: (i) hippurate broth (HB), Lombard-Dowell broth (3) supplemented with 5 mg of hippuric acid sodium salt per ml (ICN Pharmaceuticals, Cleveland, Ohio); (ii) hippurate formate (HFO) broth, hippurate broth with 3 mg of sodium formate per ml; (iii) hippurate fumarate broth (HFU), hippurate broth with 5 mg of

sodium fumarate per ml; and (iv) HFF broth, hippurate broth with 3 mg of sodium formate and 5 mg of sodium fumarate per ml (final concentrations).

The bacteria were tested for their ability to hydrolyze hippurate as follows. A tube of each medium (HB, HFO, HFU, HFF) was inoculated with 0.2 ml of a 48-h chopped meat-glucose broth (4) culture and incubated in an appropriate atmosphere (5% O₂, 10% CO₂, 85% N₂ for *Campylobacter* strains; 5% CO₂, 10% H₂, 85% N₂ for *C. sordellii*; air for *S. agalactiae*) for 48 h at 35°C. The broth cultures were then analyzed for nonvolatile acid products as described previously (2), except a Gow-Mac Series 550P gas chromatograph and a Gow-Mac model 70-700 recorder were used instead of an Ana Bac instrument.

The quantities of benzoic acid produced by *C. jejuni* varied greatly in the four media. By far the best production of benzoic acid occurred in HFF, followed by decreasing amounts in HFO, HFU, and HB medium, in that order. It took 4 to 6 days of incubation for adequate hydrolysis of hippurate by *C. jejuni* in HFF broth because it grew rather slowly as compared to the other microorganisms used in the study. However, the growth of *C. jejuni* and *C. coli* was stimulated by the presence of formate and fumarate. Much better growth of these microorganisms occurred in HFF broth than in HB. The time required to detect hippuric acid hydrolysis by *C. jejuni* can be decreased to 24 h by using a larger inoculum of cells, e.g., 0.1 ml of a cell suspension equivalent to the turbidity of a no. 3 McFarland nephelometer standard from growth on anaerobe blood agar (3) (G. L. Lombard, unpublished data).

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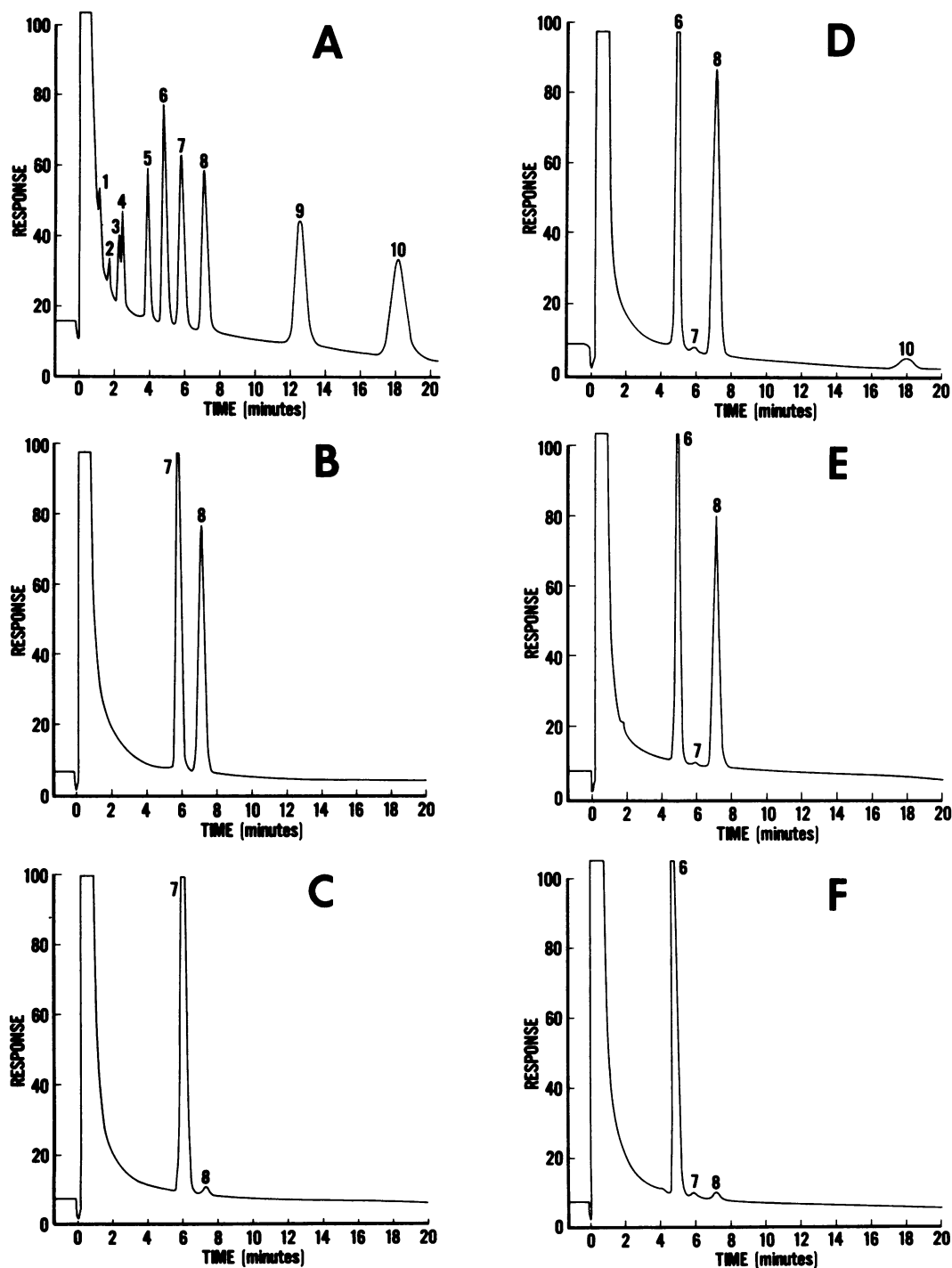


FIG. 1. Chromatograms of gas-liquid chromatography of (A) nonvolatile acid standards (peak 1, pyruvic; 2, lactic; 3, oxalacetic; 4, oxalic; 5, malonic; 6, fumaric; 7, succinic; 8, benzoic; 9, phenylacetic; 10, hydrocinnamic); (B) *C. jejuni* in HFF broth, 6 days (peak 7, succinic acid; 8, benzoic acid); (C) *C. coli* in HFF broth, 6 days (peak 7, succinic acid; 8, trace of benzoic acid); (D) *C. sordellii* in HFF broth, 4 days (peak 6, fumaric acid; 7, trace of succinic acid; 8, benzoic acid; 10, hydrocinnamic acid); (E) *S. agalactiae* in HFF broth, 4 days (peak 6, fumaric acid; 7, trace of succinic acid; 8, benzoic acid); (F) uninoculated HFF broth control held 6 days at 35°C (peak 6, fumaric acid; 7, trace of succinic acid; 8, trace of benzoic acid).

TABLE 1. Summary of reactions in HFF broth cultures detected by GLC and stimulation of growth by formate and fumarate

Microorganism	Hippurate hydrolyzed	Fumarate converted to succinate	Growth stimulated by formate and fumarate ^b
<i>C. jejuni</i> ^a	+	+	+
<i>C. coli</i> ^a	-	+	+
<i>C. sordellii</i>	+	-	-
<i>S. agalactiae</i>	+	-	-

^a Type strains.

^b Heavier growth in HFF broth than in HB without formate or fumarate.

Hippurate hydrolysis by *C. sordellii* and *S. agalactiae* was not affected by the presence of formate or fumarate in the media. These microorganisms grew rapidly in all of the media and produced sufficient benzoic acid for detection of hippurate hydrolysis in 2 to 4 days. The *C. coli* strain was negative for hippurate hydrolysis.

Smibert and Holdeman (10) in their study of *Vibrio succinogenes* (*Wolinella succinogenes*) (11), *Bacteroides corrodens* (*Bacteroides ureolyticus*) (8), and related gram-negative bacteria found that some of the microorganisms when grown in a peptone-yeast extract-glucose broth supplemented with formate (0.3%) and fumarate (0.5%) were able to convert fumarate to succinate as revealed by gas-liquid chromatographic analysis. Both formate and fumarate were demonstrated in the uninoculated medium, and only succinic acid was present in 5-day cultures.

In our study we found that HFF broth allowed determination of the ability of the microorganism to convert fumarate to succinate as well as to hydrolyze hippurate (Fig. 1, Table 1). The chromatograms from the analysis of the bacterial strains grown in HFF broth, uninoculated HFF broth, and the mixed nonvolatile acid standard are shown in Fig. 1. These clearly show that the *C. jejuni* and *C. coli* strains were able to convert fumarate to succinate, and the *C. sordellii* and group B *Streptococcus* strains tested were not. A summary of the reactions for hippurate hydrolysis and conversion of fumarate to succinate exhibited by the microorganisms tested is given in Table 1. This table also shows that the growth of *C. jejuni* and *C. coli* was stimulated by formate and fumarate, as described for

certain other microorganisms (10), but these supplements had no effect on the growth of *C. sordellii* and the group B *Streptococcus* strains, which did not convert fumarate to succinate.

Since gas chromatographs equipped with thermal conductivity detectors, as used in this study, are now commonly used in reference and clinical laboratories for analysis of volatile and nonvolatile acid products of bacteria, the technique described should prove to be useful for characterization and identification of various microorganisms.

H. K. is a guest researcher in the Anaerobic Bacterial Diseases Branch.

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