Effects of Time and Growth Media on Short-Chain Fatty Acid Production by Bacteroides fragilis

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Gas-liquid chromatography was used to monitor the evolution of short chain fatty acids by Bacteroides fragilis in five media. Acetic and succinic acids, the prominent end products encountered, were readily detected within 24 h. Propionic, isobutyric, butyric, isovaleric, and lactic acids were usually recorded in more limited quantities. Maximum rates of bacterial multiplication, glucose catabolism, and end-product production coincided with the first 24 h in carbohydrate-supplemented media. Extended incubation (672 h) favored substantial succinate increases in three of five media. These observations suggest that incubation time and composition of the medium are important determinants in short chain fatty acid production by B. fragilis.

Analysis of short chain fatty acid (SCFA) end products of fermentation and putrefaction in bacteriological media is a useful tool in the taxonomy of anaerobic bacteria (4). Fatty acid end products, i.e., C_1 to C_4 acids including lactic and succinic acids, have also been detected in clinical specimens. The detection in pus of four-carbon acids (isobutyric, butyric, and succinic) has been suggested to correlate with the presence of putrefactive anaerobes (3). It is generally recognized that microbial end products may vary with phase of growth, medium composition, gaseous environment, pH, and other conditions. The purpose of this study was to monitor the production of SCFA by five strains of Bacteroides fragilis, isolated from clinical material, in five different broth media. Since taxonomic judgments of anaerobes are often based on SCFA chromatograms, it is important to clarify the effect of medium composition and time on the chromatographic results.

MATERIALS AND METHODS

Microorganisms. Five strains of B. fragilis (four subspp. fragilis; one subspp. vulgatus) isolated from clinical specimens and identified in our laboratory were used.

Culture media. Approximately 0.1 ml of an 18-h peptone yeast glucose (PYG) culture was inoculated in an anaerobic chamber into 10-ml aliquots of five broth media: sterile asctic fluid (ASC); chopped meat glucose (CMG); ascitic fluid-chopped meat glucose (1:1) (ASC-CMG); peptone yeast (PY); and PYG (4). The tubes were chromatographically assayed for SCFA and were allowed to equilibrate in the anaerobic chamber for 24 h prior to inoculation in an atmosphere of 5% CO_2, 10% H_2, and 85% N_2. At specific time intervals, 0.5-ml aliquots were removed from each tube for gas-liquid analysis. An additional 0.1 ml was withdrawn for determination of viable counts.

Viable cell count. Aliquots of broth media were diluted in VPI diluent (4) and 0.1-ml portions were surface plated in the chamber on Brucella Broth (BioQuest) containing menadione and blood. Colony counts were made after 72 h of anaerobic incubation at 37 C.

Glucose. Glucose levels were determined on duplicate 10-ml PYG culture menstruum aliquots with the Beckman glucose analyzer (glucose oxidase reaction). The data were recorded as milligram percent glucose.

Volatile fatty acid extraction. A 0.5-ml aliquot of culture menstruum was supplemented with a small amount of NaCl and acidified with 0.5 ml of 7% aqueous H_2SO_4. After chilling in an ice bath, 1.0 ml of diethyl ether was added. The complete specimen was agitated and centrifuged at 5 C, and etheral aliquots were withdrawn for gas-liquid chromatography of the volatile fatty acids in triplicate. Run time to n-hexanoic acid was 15 min.

Methylation procedure. After gentle evaporation (35 C, slow N_2 stream) of the diethyl ether phase, the aqueous residue was supplemented with 1.0 ml of boron trifluoride methanol. Parallel analyses with known acid concentrations revealed uniform lactic and succinic recoveries from identical aliquots which were either methylated directly or shaken with ether, subjected to gentle organic phase evaporation, and then methylated. The stoppered tube was heated at 55 C for 30 min and extracted with 0.5 ml of chloroform. Chromatography of the chloroform phase found lactyl monomethyl ester and succinyl dimethyl ester retained for 2 and 7 min, respectively.

Chromatography instrumentation. A Packard
419 dual column gas chromatograph equipped with flame ionization detectors was employed. Chromatographic deflections were visualized on a Honeywell 1.0-mV span recorder, and peak areas were interpreted as microvolt × seconds on a Shimadzu ITG-2A electronic digital integrator. Samples of 2.0 μl were injected with a 5-μl Hamilton syringe directly onto coiled, silanized pyrex columns, (6 feet long [ca. 183 cm] by 2 mm internal diameter). The analytical columns contained 6% carbowax 20M-terephthalic acid on 80/100 mesh Gas-Chrom Q (AW-DMCS; Applied Science, State College, Pa.). Peak identity was verified by chromatography on an alternate phase (10% carbowax 20M, unconjugated) at elevated temperature and carrier flow (150 C and 30 ml/min). The specimens were supplemented with n-hexanoate (volatile analysis) and malonyldimethyl ester (derivatized analysis) as relative retention time standards for both phases. Standard analysis conditions included: injection and detector blocks, 200 C; oven isothermal at 125 C; nitrogen, hydrogen, and air flows, respectively, at 13, 30, and 300 ml/min. Detector sensitivity was routinely set at 2.5 × 10⁻ⁱ⁵ A, whereas 2.5 × 10⁻¹¹ A was used to detect and confirm trace metabolite presence.

Quantitation. Integration values compiled at the lower sensitivity were related to standard curves for conversion to micromoles per milliliter. Titrated aqueous solutions of SCFA were diluted in sterile human ascitic fluid, extracted, and chromatographed to obtain the standard values. No acids were detected in the ascitic fluid at 2.5 × 10⁻¹⁵ A prior to SCFA supplement. To ascertain the accuracy of the method, ascitic fluid was supplemented with known organic acid concentration at levels likely to be encountered in microbial culture. Mean recoveries for triplicate injections of all acids had an average relative error of 9% from the true concentrations. The relative standard deviation (precision) encountered was 10.2% based on replicate analyses of isovaleric acid. Acetic and propionic could be reliably detected to the 1-μmol/ml level, and all other acids to 0.1 μmol/ml.

A chromatogram of a mixed acid standard (Fig. 1) illustrates the clean and rapid resolution of the substances assayed. Two thousand chromatographic runs have been produced without substantial signs of column decomposition or alteration in quantitative capability.

RESULTS

Mean SCFA production by five strains of B. fragilis in five media after 72 h of anaerobic incubation is illustrated in Table 1. Acetic and succinic acids are the principal end products encountered, whereas propionic, isobutyric, butyric, and isovaleric acids are detected in much smaller quantities. (It is possible that the deflection listed as isovalerate may be an unresolved mixture of 2-methyl and 3-methyl butyric acids.) Valeric acid was not recovered. The dextrose-supplemented media (CMG, ASC-CMG and PYG) evidently serve as the best substrates to encourage SCFA production, notably that of acetic and succinic. Further, calculation of succinic-acetate ratios for all media indicated that succinate formation was substantially favored over that of acetate when glucose was present.

Evidence for the metabolism of lactic acid was observed in three media: ASC-CMG, PYG and PY. In the case of PY, all of the base-line lactate level (1.9 μmol/ml) had been consumed by 72 h. Concomitant with this decrease, a rise in propionic concentration from beneath detectable limits at zero time to 9 μmol/ml was recorded. Substantially elevated propionic acid concentrations were unique to PY.

Table 2 contains the mean SCFA levels in ASC-CMG at several intervals during 672 h of incubation. The characteristic primary end
Table 1. Concentrations of SCFA in five broth media after 72 h of anaerobic incubation

| Acids | Media   | ASC | CMG      | ASC-CMG | PYG | PY |
|-------|---------|-----|----------|---------|-----|----|
| A     | 8 ± 3.7 | 15 ± 2.6 | 11 ± 1.3 | 10 ± 1.3 | 8 ± 0.8 | 9 ± 1.8 |
| P     | <1      | 2 ± 0.6 | <1       | 2 ± 0.0  | 9 ± 1.8 |
| iB    | 0.1 ± 0.1 | 0.2 ± 0.03 | 0.2 ± 0.05 | 0.1 ± 0.02 | <0.1 |
| B     | 0       | < 0.1  | 0        | <0.1    | <0.1 |
| iV    | <0.1    | 0.8 ± 0.4 | 1.6 ± 0.42 | 0.1 ± 0.05 | 0.6 ± 0.12 |
| L     | 0.2 ± 0.26 | 2.2 ± 0.87 | -0.3 ± 0.24* | -0.6 ± 0.50 | -1.9 ± 0.00 |
| S     | 10.2 ± 4.77 | 46.0 ± 2.44 | 29.6 ± 4.77 | 24.4 ± 2.61 | 11.7 ± 3.70 |
| S/A   | 1.27*   | 3.07 | 2.69 | 2.44 | 1.46 |

* A, Acetic acid; P, propionic acid; iB, isobutyric acid; B, butyric acid; iV, isovaleric acid; L, lactic acid; S, succinic acid.

* Mean of five strains of B. fragilis expressed as micromoles per milliliter (values corrected for base-line acid).

* Standard deviation.

* Below base-line acid concentrations.

* Succinyl dimethyl ester/acetate.

Table 2. Concentrations of SCFA in ASC-CMG over 672 h of anaerobic incubation

| Acids | Hours |
|-------|-------|
|       | 0     | 24   | 48   | 72   | 168  | 336  | 672  |
| A     | 15 ± 0.9* | 25 ± 0.7 | 26 ± 1.3 | 26 ± 1.3 | 28 ± 1.7 | 31 ± 0.8 | 31 ± 3.5 |
| P     | 0     | <1   | 1    | <1   | 2 ± 1.1  | 3 ± 2.6 | 4 ± 2.9 |
| iB    | 0     | 0.1 ± 0.06 | 0.2 ± 0.1 | 0.2 ± 0.05 | 0.3 ± 0.07 | 0.3 ± 0.07 | 0.3 ± 0.07 |
| B     | 0     | <0.1 | 0.1 ± 0.06 | <0.1 | <0.1    | <0.1    | <0.1 |
| iV    | 0     | 1.1 ± 0.21 | 1.4 ± 0.38 | 1.6 ± 0.42 | 2.2 ± 0.59 | 2.8 ± 1.24 | 2.8 ± 1.22 |
| L     | 1.2 ± 0.08 | 1.2 ± 0.45 | 1.1 ± 0.33 | 0.9 ± 0.24 | 0.8 ± 0.53 | 0.8 ± 0.46 | 1.5 ± 0.92 |
| S     | 0.4 ± 0.05 | 27.5 ± 3.63 | 28.8 ± 2.64 | 30.0 ± 4.77 | 33.6 ± 2.28 | 35.1 ± 3.90 | 90.0 ± 11.36 |

* A, Acetic acid; P, propionic acid; iB, isobutyric acid; B, butyric acid; iV, isovaleric acid; L, lactic acid; S, succinic acid.

* Mean of five strains of B. fragilis expressed as micromoles per milliliter ± standard deviation.

products of B. fragilis strains, acetate, and succinate are well developed within 24 h, and five other acids are detected in smaller quantities. The concentration of lactic acid was reduced below base-line levels at 72, 168, and 336 h, but increased to 1.5 μmol/ml at 672 h. Copious quantities of succinic acid (90 μmol/ml) were recorded at 672 h, whereas concentrations of the other six SCFA generally remained unchanged from 168-h levels. This progressive increase in succinic acid on extended incubation was also noted in ASC and CMG, but not in the PY or PYG media.

Figure 2 illustrates the relationship between glucose concentrations, total SCFA production, and viable cell counts in PYG. From an initial concentration of 658 mg%, total mean glucose catabolism over 168 h was 130 mg%. This represents 19.7% of the available monosaccharide. The maximum rates of glucose metabolism, SCFA production, and bacterial growth all occurred within the first 24 h of incubation.

The pH of the medium decreased from 6.9 to 4.9 within this period. Comparison of monosaccharide and SCFA levels observed at 24 and 168 h revealed that 75% (97 mg%/130 mg%) of the glucose consumption and 78% ([28.1 μmol/ml]/[38.6 μmol/ml]) of the SCFA generation oc-
curred within the first day of incubation. Acetic and succinic acids accounted for 93% ([28.1 μmol/ml] [30.2 μmol/ml]) of the total end product micromoles encountered in the initial 24 h. As viable cell recovery began to decrease, both the rate of glucose metabolism and SCFA evolution were diminished.

**DISCUSSION**

The observations described in this report confirm (4) that acetic and succinic acids are the primary SCFA end products of *B. fragilis* in a variety of media and are readily detected at 24 h. Similar chromatograms are produced with and without glucose. The presence of monosaccharide, however, appeared to induce not only a larger quantity of end products and at a faster rate, but also to favor increased succinic production relative to acetic. In vitro production of characteristic SCFA patterns in human ascitic fluid suggests that gas-liquid chromatographic analysis for end products in clinical pus specimens may give clues to the presence of anaerobes (3).

The source of SCFA in glucose-supplemented media may be both monosaccharide and amino acid. Acids detected in PY and ASC, where <5 mg% glucose was present, were probably derived from amino acids. Previous studies by Loesche and Gibbons (7) with *Fusobacterium nucleatum* indicated that glucose did not repress the metabolism of amino acids, since both substrates were simultaneously degraded.

The specific and complex metabolic origin of SCFA has been explored by many investigators. Rumen microorganisms apparently incorporate peptide carbon into microbial protein while degrading free amino acid supplement to metabolic end products (10). Acetic acid can be derived from glycine (11) and from an assortment of other amino acids (7). Methionine and threonine (7) as well as succinate or lactate (5, 6) can be sources of propionic acid. The observed disappearance of lactate in three media may be explained by its conversion to propionate. Butyric acid has been shown to derive from several amino acids (7), whereas isobutyrate may originate from valine (2) and isovalerate from leucine (2, 8). Catabolism of aspartate, serine, and threonine may generate lactic acid (7). Succinogenic bacteria have many pathways to pursue due to the wide distribution of succinyl coenzyme A in amino acid and carbohydrate metabolism. Additionally, 14CO₂ incorporation into succinate has been reported during carbohydrate fermentation by rumen *Bacteroides* (1).

In this study, substantial concentration increments for succinic acid were detected between 336 and 672 h in certain media. The increases in succinate over this time interval for ASC, CMG, and ASC-CMG media were, respectively, 48.8, 57.2, and 54.0 μmol/ml. The concentration of succinate in PYG and PY remained nearly identical to the relatively low 48-h levels. The accumulation of succinate in "spent" media may be due to a shift by the remaining metabolizing cells exclusively toward those pathways which include carboxylation of three-carbon fragments or exothermic hydrolysis of the thioester.

These cited metabolic observations point to the sources of end-product variation among media. Composition and competitive utilization (9) of amino acids in various available pools may direct the relative proportions of *B. fragilis* end products.

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