

## Production of Phenylacetic Acid by Anaerobes

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We tested 388 strains of anaerobes from 17 genera for the production of phenylacetic acid. The compound was found in only two genera (*Bacteroides* and *Clostridium*) but not within all the species of these genera.

Short-chain organic acids result from the various types of fermentation by anaerobes. These acids can be identified by gas-liquid chromatography (2). Although this technique is very useful in identifying specific groups or species, it can be of limited value within certain genera, since many species are known to produce the same acid pattern. Recently, several laboratories have identified one compound, phenylacetic acid (PAA), that can facilitate intragenus identification, particularly in the family *Bacteroidaceae*.

PAA can distinguish *Bacteroides vulgatus* (PAA negative) from all the other species of the *fragilis* group (7, 11) and *B. gingivalis* from nonoral isolates of a closely related species, *B. asaccharolyticus* (5, 8). The compound has also been found in *B. ruminicola* (1), *B. melaninogenicus* subsp. *macacae* (5), and *Clostridium botulinum* type G (9). More recently, PAA has been found in other species of *Clostridium* (M. Dezfulian, G. L. Lombard, R. J. Landry, and V. R. Dowell, Jr., Abstr. Annu. Meet. Am. Soc. Microbiol. 1981, C57, p. 272). PAA has been shown to be the main end product of phenylalanine metabolism with ruminal contents (6, 10).

The purpose of this work was to survey a wide range of anaerobes for the production of PAA, using two different techniques. This would permit us to determine whether the production of PAA can serve as a taxonomic character for easier intra- and intergenus identification.

The bacterial strains used, the number of each strain tested, and the sources from which they were received are listed in Tables 1 and 2. All of the isolates were grown and maintained in a modified Trypticase (BBL Microbiology Systems, Cockeysville, MD.)-yeast extract (TYE) medium originally described by Gibbons and MacDonald (3). The medium (pH 7.0) consisted of Trypticase (17 g/liter), yeast extract (3 g/liter), K<sub>2</sub>HPO<sub>4</sub> (2.5 g/liter), and NaCl (5 g/liter), supplemented with hemin (5 µg/ml) and vitamin K (10 µg/ml). In one experiment, glucose was added (0.2, 0.5, and 1% final concentration) to

the enriched TYE. All of the isolates were identified by procedures already described (4), including fatty acid determinations. All of the strains tested were grown at 37°C in an anaerobic chamber (Coy Laboratory Products, Ann Arbor, Mich.). The cultures were tested for PAA production after 48 h and in some cases after 5 days.

Two methods of analysis were tested. The first method was based on the methylation and extraction procedures described by Holdeman et al. (4). In this method, the samples were separated in a coiled glass column (1.8 m by 3 mm [inner diameter]) packed with 10% Resoflex LAC-1-R296 (Chromatographic Specialties, Brockville, Ontario, Canada) on acid-washed Chromosorb W (60 to 80 mesh; Johns-Manville Corp., Denver, Colo.). The temperatures were 140°C for the injection block and 125°C for the oven and detection block. Attenuation was 1,024, and analysis time was 11 min. PAA (Anachemia Chemicals Ltd., Montreal, Canada; concentration range tested, 10 to 500 µg/ml) standards were prepared in distilled water or added to an uninoculated medium and acidified, methylated, and extracted by the procedure described by Holdeman et al. (4) for nonvolatile fatty acids. The second method was described by Yamada and Carlsson (12). In this case, a one-ninth volume of freshly prepared 25% (wt/vol) *m*-phosphoric acid was added to the supernatant of the cultures, and a 5-µl sample was injected directly into a coiled column (1.8 m by 3 mm [inner diameter]) packed with Chromosorb 101 (100 to 120 mesh; Johns-Manville Corp.). The samples were run isothermally at 200°C, with the inlet at 200°C and the detector block at 240°C. Attenuation was 1,024. For this method, the standards were prepared in the same way as in the first method, except that they were not methylated or extracted. All samples for both methods were analyzed in a Hewlett-Packard model 5830A gas chromatograph equipped with a hydrogen flame ionization detector. Peaks

TABLE 1. Gram-positive organisms tested for phenylacetic acid

| Strain                              | No. of strains | Source   |
|-------------------------------------|----------------|--|
| <i>Actinomyces viscosus</i>         | 48             | Own isolates                                   |
| <i>A. naeslundii</i>                | 51             | Own isolates                                   |
| <i>A. israelii</i>                  | 11             | Own isolates                                   |
| <i>A. odontolyticus</i>             | 18             | Own isolates                                   |
| <i>Arachnia</i> sp.                 | 10             | Own isolates                                   |
| <i>Bifidobacterium</i> sp.          | 15             | Own isolates                                   |
| <i>Clostridium sporogenes</i>       | 1              | Laval University <sup>a</sup>                  |
| <i>C. bifermentans</i>              | 2              | A. M. Bourgault, <sup>b</sup> Laval University |
| <i>C. septicum</i>                  | 1              | Laval University                               |
| <i>C. sordellii</i>                 | 2              | A. M. Bourgault, Laval University              |
| <i>C. difficile</i>                 | 2              | A. M. Bourgault                                |
| <i>C. putrificum</i>                | 1              | Laval University                               |
| <i>C. histolyticum</i>              | 1              | Laval University                               |
| <i>C. aminovalericum</i>            | 1              | A. M. Bourgault                                |
| <i>C. lentoputrescens</i>           | 2              | A. M. Bourgault                                |
| <i>C. perfringens</i>               | 4              | A. M. Bourgault                                |
| <i>C. paraperfringens</i>           | 1              | A. M. Bourgault                                |
| <i>C. limosum</i>                   | 1              | A. M. Bourgault                                |
| <i>C. innocuum</i>                  | 1              | A. M. Bourgault                                |
| <i>C. sporogenes</i>                | 2              | A. M. Bourgault                                |
| <i>C. novyi</i>                     | 1              | A. M. Bourgault                                |
| <i>Eubacterium</i> sp.              | 3              | Laval University                               |
| <i>Lactobacillus</i> sp.            | 9              | Laval University                               |
| <i>Peptococcus</i> sp.              | 6              | A. M. Bourgault                                |
| <i>P. magnus</i>                    | 8              | A. M. Bourgault                                |
| <i>P. prevotii</i>                  | 1              | A. M. Bourgault                                |
| <i>Peptostreptococcus productus</i> | 8              | Laval University                               |
| <i>Propionibacterium</i> sp.        | 7              | Laval University                               |

<sup>a</sup> Microbiology, culture collection.

<sup>b</sup> Infectiology, Laval University Hospital.

were recorded and integrated with a Hewlett-Packard GC terminal model 18850A (chart speed, 2.5 cm/min).

We were unable to detect PAA by the method described by Yamada and Carlsson (12). Higher oven temperature, increased injection volume, or longer analysis time did not help in detecting PAA. This might have been because this compound needs to be concentrated (by extraction) to be quantitatively recovered or that this type of column is not suitable for this compound.

Of the 17 genera of anaerobes tested, PAA was found in only two genera, *Bacteroides* spp. and *Clostridium* spp. (Table 3). The data obtained confirm and extend previous results on gram-negative anaerobic rods. *B. gingivalis* can be distinguished from *B. asaccharolyticus* and other human oral black-pigmented *Bacteroides* species. On the other hand, strains of asaccharolytic black-pigmented *Bacteroides* spp. isolated from the gingival sulcus of various animals were all found to be PAA positive. We also found that *B. ruminicola* produced PAA. However, our results indicate that we cannot distinguish between the two subspecies of *B. ruminicola* tested with this characteristic. It is important to note

that the medium used for this survey did not contain glucose. Allison (1) found that *B. ruminicola* produces increased quantities of PAA when the glucose content in the medium is increased. This last observation could explain the low values found for the PAA-positive strains other than *B. gingivalis* and asaccharolytic black-pigmented *Bacteroides* spp. isolated from animals. However, in one experiment, we added glucose to the medium and tested three species, *B. fragilis*, *C. difficile*, and *C. sordellii*, for PAA production. The results showed no increase in PAA production for the *Clostridium* strains in the glucose-supplemented medium. However, the *B. fragilis* strain tested showed highly reduced levels of PAA when grown in higher concentrations of glucose. This supports the observation of Van Assche (11), who found that some members of the *B. fragilis* group produced more PAA in a peptone-yeast extract medium without glucose.

Of the clostridia tested, seven species were found to produce PAA. It is interesting to note that most of the PAA-positive clostridia have a complex pattern of fermentation products, including acetic, formic or propionic, isobutyric,

TABLE 2. Gram-negative organisms tested for phenylacetic acid

| Strain   | No. of strains | Source  |
|--|----------------|---|
| <i>Bacteroides asaccharolyticus</i>                      | 1              | E. C. S. Chan <sup>a</sup>  |
| <i>B. gingivalis</i> (human)                             | 10             | Own isolates  |
| <i>Bacteroides</i> spp. (oral origin)                    |                |   |
| dog  | 14             | Own isolates  |
| cat  | 31             | Own isolates  |
| jaguar   | 14             | Own isolates  |
| <i>B. melaninogenicus</i> subsp. <i>melaninogenicus</i>  | 2              | Own isolates  |
| <i>B. melaninogenicus</i> subsp. <i>intermedius</i>      | 5              | Own isolates  |
| <i>B. fragilis</i>                                       | 7              | Own isolates  |
| <i>B. distasonis</i>                                     | 6              | Own isolates  |
| <i>B. thetaiotaomicron</i>                               | 2              | Own isolates  |
| <i>B. ovatus</i>   | 1              | Own isolates  |
| <i>B. vulgatus</i>                                       | 7              | Own isolates  |
| <i>B. ruminicola</i> subsp. <i>ruminicola</i>            | 1              | M. P. Bryant <sup>b</sup>   |
| <i>B. ruminicola</i> subsp. <i>brevis</i>                | 1              | M. P. Bryant  |
| <i>B. ureolyticus</i>                                    | 1              | S. M. Finegold <sup>c</sup>                                       |
| <i>Campylobacter</i> sp.                                 | 8              | Own isolates  |
| <i>Capnocytophaga ochracea</i>                           | 4              | J. G. Collee, <sup>d</sup> S. M. Finegold, M. Sebald <sup>e</sup> |
| <i>Fusobacterium nucleatum</i>                           | 37             | Own isolates  |
| <i>F. necrogenes</i>                                     | 1              | S. M. Finegold  |
| <i>F. necrophorum</i>                                    | 1              | S. M. Finegold  |
| <i>F. gonidiaformans</i>                                 | 1              | S. M. Finegold  |
| <i>F. naviforme</i>                                      | 1              | S. M. Finegold  |
| <i>F. mortiferum</i>                                     | 1              | S. M. Finegold  |
| <i>F. varium</i>   | 3              | S. M. Finegold  |
| <i>F. novum</i>  | 1              | Own isolates  |
| <i>Lachnospira multiparus</i>                            | 1              | M. P. Bryant  |
| <i>Leptotrichia buccalis</i>                             | 2              | J. G. Collee, own isolates  |
| <i>Selenomonas ruminantium</i> subsp. <i>ruminantium</i> | 1              | M. P. Bryant  |
| <i>S. ruminantium</i> subsp. <i>lactilytica</i>          | 1              | M. P. Bryant  |
| <i>S. sputigena</i>                                      | 2              | Own isolates  |
| <i>Succinimonas amyolytica</i>                           | 1              | M. P. Bryant  |
| <i>Succinivibrio dextrinosolvans</i>                     | 1              | M. P. Bryant  |

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butyric, isovaleric, valeric, and isocaproic acids.

The one result that is still unexplained is that oral black-pigmented *Bacteroides* strains produce much more PAA than do the other PAA-positive species. The quantities involved are so large that there is a possibility that the compound could be used as a diagnostic tool in oral infections involving these bacteria. We are currently investigating this possibility and are studying the metabolism of PAA in *B. gingivalis*.

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TABLE 3. Phenylacetic acid-positive species

| Strain  | Phenylacetic acid detected ( $\mu\text{g/ml}$ ) |                    |
|---|---|--------------------|
|   | Mean <sup>a</sup>                               | Range <sup>b</sup> |
| Gram negative                                 |   |                    |
| <i>Bacteroides gingivalis</i> (human)         | 5,000   | 3,550–6,610        |
| Oral black-pigmented <i>Bacteroides</i> spp.  |   |                    |
| dog   | 3,560   | 1,870–6,240        |
| cat   | 6,460   | 1,440–22,000       |
| jaguar  | 7,320   | 3,280–13,500       |
| <i>B. fragilis</i>                            | 203   | 20–787             |
| <i>B. distasonis</i>                          | 170   | 75–232             |
| <i>B. thetaiotaomicron</i>                    | 240   | 154–308            |
| <i>B. ovatus</i>                              | 257   | 210–309            |
| <i>B. ruminicola</i> subsp. <i>ruminicola</i> | 69  |                    |
| <i>B. ruminicola</i> subsp. <i>brevis</i>     | 46  |                    |
| Gram positive                                 |   |                    |
| <i>Clostridium aminovalericum</i>             | 286   |                    |
| <i>C. bifermentans</i>                        | 156   |                    |
| <i>C. difficile</i>                           | 221   | 199–243            |
| <i>C. lentoputrescens</i>                     | 413   | 128–698            |
| <i>C. paraperfringens</i>                     | 52  |                    |
| <i>C. perfringens</i> (2/4)                   | 23  | 21–24              |
| <i>C. sordellii</i>                           | 473   | 276–669            |

<sup>a</sup> Mean value for each species or group of organisms.

<sup>b</sup> Range of values for each species or group of organisms.

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