Dehydrogenase Patterns in the Study of Bacteroidaceae

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Enzyme patterns were obtained by starch-gel electrophoresis of cell-free extracts from organisms in the family Bacteroidaceae. Esterases, phosphatases, and lactic and succinic dehydrogenases were detected but offered no possibility for classification purposes. Alpha-glycerophosphate dehydrogenase and 6-phosphogluconate dehydrogenase were not detected. Zymograms of malic, glutamic, and glucose-6-phosphate dehydrogenases separated Bacteroides species from Sphaerophorus and Fusobacterium species. Malic dehydrogenase and glucose-6-phosphate dehydrogenase were found in Bacteroides but not Sphaerophorus and Fusobacterium. These dehydrogenase zymograms placed three gram-negative, nonsporeforming anaerobic rod isolates with the Bacteroides species. A close correlation was found between the classification of Bacteroidaceae by zymogram analysis and a numerical taxonomy scheme previously published from this laboratory.

The use of protein and enzyme patterns as taxonomic tools has received increased emphasis during the last several years. Green, Goldberg, and Blenden (3) found that starch-gel electrophoresis of esterases and naphthlamidases would differentiate the pathogenic Leptospira from the saprophytes. Esterase zymograms have been found to be characteristic for some species of Mycobacterium (2). Protein patterns obtained by polyacrylamide gel electrophoresis were found to be useful for species characterization of Streptomyces (4).

The classification of the gram-negative, nonsporeforming, anaerobic rods has long been unsatisfactory. This investigation was undertaken with two objectives in mind: (i) to determine whether strains of certain species of Bacteroides, Sphaerophorus, and Fusobacterium could be identified or separated from one another by means of their enzyme patterns and (ii) to determine whether unidentified isolates belonging to the family Bacteroidaceae could be placed into a recognized genus by enzyme pattern similarity.

1 This is a portion of a dissertation submitted by Kenneth C. Keudell to the faculty of the University of Missouri Graduate School in partial fulfillment of the requirements for the Ph.D. degree in Microbiology, August 1969. This work was presented in part at the 69th Annual Meeting of the American Society for Microbiology, Miami Beach, Fla., 4–9 May, 1969.

MATERIALS AND METHODS

Cultures. A total of 39 strains of representative Bacteroidaceae genera were used (Table 1). They were obtained from six sources. A Sonnenwirth (Jewish Hospital of St. Louis, St. Louis, Mo.) supplied Eggetherella thetaiotamicron (245), S. freundii (B149), and Fusobacterium (B272). B. melaninogenicus (B536) was supplied by S. M. Finegold (Veterans Administration Hospital, Los Angeles, Calif.). The following strains were received from E. M. Barnes (Food Research Institute, Norwich, England): S. funduliformis (Boerens), S. necrophorus (Fievez, N252), and S. necrophorus (Fievez, N117). Strains B1, B2, and B5 were isolated from human feces by D. Henges (University of Missouri, Columbia, Mo.). The following strains were obtained from the stock culture collection at this laboratory: B. symbiosus ATCC 12829, S. varius ATCC 8501, F. polymorphum ATCC 10953, and group 4 poultry isolates EBF 60/50 and EBF 60/43. All of the other strains were obtained from W. E. C. Moore (Virginia Polytechnical Institute, Blacksburg, Va.). With one exception (B. symbiosus), all of the strains studied were from human or poultry sources.

All strains were grown in an anaerobic Reinforced Clostridial Medium (RCM) broth (Oxoid), except for B. melaninogenicus, which was grown in RCM broth supplemented with 1 ml of heme (50 mg of heme per 100 ml of distilled water) per liter and 0.1 ml of menadione (100 mg of menadione per 20 ml of 95% ethyl alcohol) per liter.

Enzyme preparation. Seed cultures were prepared by inoculating 10 ml of RCM broth with the specific organism and incubating for 24 to 48 hr at 37 C.
Table 1. Cultures used in this study

| Culture | Original source |
|---------|-----------------|
| Eggerthella thetaiotaomicron | 245 (Reinhold) |
| E. thetaiotaomicron | 249 (Reinhold) |
| Bacteroides thetaiotaomicron | 12 (Beersens) |
| Eggerthella convexus | 274 (Reinhold) |
| Bacteroides convexus | Au 21-27 (Mitsuoka) |
| B. convexus | E384 (Beersens) |
| B. convexus | E102 (Beersens) |
| B. fragilis | NTCC 9343 |
| Fusocillus | B209 (Finegold) |
| Bacteroides putredinis | (Suzuki) |
| B. symbiosus | ATCC 12829 |
| Bacteroides species | JH-4 (Sonnenwirth) |
| Bacteroides species | 05231-JH (Sonnenwirth) |
| Bacteroides species | 714172-MR (Sonnenwirth) |
| Bacteroides species | (Moore) |
| B. melaninogenicus | B536 (Finegold) |
| B. fundiformis | B540 (Finegold) |
| Sphaerophorus funduliformis | (Beersens) |
| Bacteroides necrophorus | NTCC 7155 |
| Sphaerophorus necrophorus | N252 (Fievez) |
| S. necrophorus | N117 (Fievez) |
| S. pseudonecrophorus | B130 (Beersens) |
| S. pseudonecrophorus | B176 (Beersens) |
| S. freundii | B149 (Beersens) |
| S. varius | ATCC 8501 |
| Group 1 poultry isolate | EBF 59/96p (Barnes) |
| Group 6 poultry isolate | EBD 1/4 (Barnes) |
| Fusobacterium | B272 (Finegold) |
| F. polymorphum | ATCC 10953 |
| Fusiformis fusiformis | F398 (Beersens) |
| F. fusiformis | F341 (Beersens) |
| Group 4 poultry isolate | EBF 61/61 (Barnes) |
| Group 4 poultry isolate | EBF 58/74 (Barnes) |
| Group 4 poultry isolate | EBF 61/42 (Barnes) |
| Group 4 poultry isolate | EBF 60/50 (Barnes) |
| Group 4 poultry isolate | EBF 60/43 (Barnes) |
| Isolate | B1 (Henties) |
| Isolate | B2 (Henties) |
| Isolate | B5 (Henties) |

* H. Beersens, M. M. Castel, and L. Fievez (Abstr., 8th Intern. Congr. Microbial., Montreal, 1962, p. 120) created the genus Eggerthella, which included some of the species placed in Bacteroides by other authors.

Samples (5 ml) of the culture were then inoculated into 1 liter of RCM broth and incubated for 18 to 40 hr at 37°C. Each culture was centrifuged at 12,000 x g at 4°C for 10 min to sediment the cells. The cells were washed once with 0.1 M phosphate buffer (pH 7.0), filtered through glass wool in a Buchner funnel, collected in an Erlenmeyer suction flask, washed again in the phosphate buffer, and resuspended in 40 ml of the buffer for cell rupture.

Cell-free extracts were prepared with a French pressure cell (American Instrument Co., Bethesda, Md.) and a hydraulic press (F. S. Carver, Inc., Summit, N. J.). The pressure was raised above 15,000 psi and slowly released so that the pressure was maintained above 10,000 psi while the cellular material was collected in a precooled flask in an ice bath. After two passages through the pressure cell, the cellular debris was removed by centrifugation at 17,300 x g at 4°C for 50 min. The supernatant fluid was dialyzed overnight (cellulose dialyzer tubing, Arthur H. Thomas Co., Philadelphia, Pa.) at 4°C against three changes of deionized water, lyophilized, and stored at -50°C until subjected to electrophoresis.

Protein determination and electrophoresis. The amount of protein in all preparations tested was determined by the method of Lowry et al. (6). A protein concentration of approximately 5 to 10% per sample was subjected to electrophoresis and subsequent enzyme detection.

Vertical starch-gel molds were prepared by the method of Smithies (8). The starch-gel was prepared by increasing the concentration of the hydrolyzed starch (Connaught Medical Research Laboratory, Toronto, Canada) to 1% above the recommended amount on the label. The starch was prepared in 0.03 M borate buffer (pH 8.6) and poured into a vertical starch-gel mold (Buchler Instruments, Inc.). If reagents were to be added to the gel before pouring, it was done after heating and before degassing.

About 35 to 40 μlitters of each sample was added to the appropriate sample slot in the starch-gel. The electrode vessels contained 0.3 M borate buffer, pH 8.6. Electrophoresis was carried out at 4°C for 14 hr at a constant voltage of 7.5 v/cm. After electrophoresis, the gel was removed from the mold, sliced longitudinally, and placed with the cut surface up in trays for enzyme detection.

Enzyme identification. Esterases, phosphatases, and the following dehydrogenases were assayed in this study: malic (EC 1.1.1.37), glutamic (EC 1.4.1.2), succinic (EC 1.3.99.1), lactic (EC 1.1.1.27), glucose-6-phosphate (EC 1.1.1.49), 6-phosphogluconate (EC 1.1.1.43), and α-glycerophosphate (EC 1.1.1.8). The method for their detection in starch-gels has been described previously by Green, Goldberg, and Blenden (3).

An arbitrary scale from -2 to 15 was chosen to compare electrophoretic enzyme band mobilities of selected enzymes among the various tested organisms. Each consecutive number division is equivalent to a distance of 7 mm. An Rf value could not be determined since there was no known boundary.

RESULTS

Dehydrogenases. Alpha-glycerophosphate dehydrogenase and 6-phosphogluconate dehydrogenase were not detected in any of the cultures examined. Esterases, phosphatases, lactic dehydrogenase [nicotinamide adenine dinucleotide (NAD)-linked], and succinic dehydrogenase were
With only a few exceptions, malic dehydrogenase (MDH) was found in all of the Bacteroides species but not in Sphaerophorus and Fusobacterium species. Figures 1 to 4 are diagrammatic representations of thezymograms.

Glutamic dehydrogenase (GDH), NAD-
linked, was found in *Bacteroides*, *Sphaerophorus*, and *Fusobacterium* species (Fig. 5 to 8). Most of the *Bacteroides* species contained multiple molecular forms of the enzyme, but *Sphaerophorus* and *Fusobacterium* contained, at most, one band.

The presence of glucose-6-phosphate dehydrogenase (G6PDH) was detected in most species of *Bacteroides* but not in species of *Sphaerophorus* and *Fusobacterium* (Fig. 9 to 12). *S. pseudonecrophorus* was an exception.

**Zymogram analysis.** Zymograms of MDH, GDH, and G6PDH were analyzed by placing organisms with similar enzyme patterns in com-

![Diagram of glutamic dehydrogenase enzyme pattern.](image)

**Fig. 5.** Diagram of glutamic dehydrogenase enzyme pattern. (A) Isolate B5; (B) isolate B2; (C) isolate B1; (D) *Sphaerophorus* necrophorus N117; (E) *S. necrophorus* N-252; (F) *S. funduliformis* (Beerens); (G) *S. freundii* B149; (H) *Fusobacterium* B277; (I) *B. melaninogenicus* B536; (J) *Eggerthella* thetaiotaomicron 245.

![Diagram of glutamic dehydrogenase enzyme pattern.](image)

**Fig. 6.** Diagram of glutamic dehydrogenase enzyme pattern. (A) *Eggerthella* thetaiotaomicron 249; (B) *Bacteroides* thetaiotaomicron 12; (C) *E. convexus* 274; (D) *B. convexus* E384; (E) *B. convexus* E102; (F) *B. convexus* Au 21-27; (G) group 4 poultry isolate EBF 6%6; (H) *Fusocillus* B209; (I) *Bacteroides* species JH-4; (J) *B. fragilis* NTCC 9343.

![Diagram of glutamic dehydrogenase enzyme pattern.](image)

**Fig. 7.** Diagram of glutamic dehydrogenase enzyme pattern. (A) Group 4 poultry isolate EBF 6%4; (B) group 4 poultry isolate EBF 6%63; (C) *Fusiformis fusiformis* F341; (D) *F. fusiformis* F398; (E) group 6 poultry isolate EBD 4%; (F) *Bacteroides* funduliformis, B540; (G) group 4 poultry isolate EBF 6%60; (H) *Sphaerophorus* pseudonecrophorus B176; (I) *S. pseudonecrophorus* B130.

![Diagram of glutamic dehydrogenase enzyme pattern.](image)

**Fig. 8.** Diagram of glutamic dehydrogenase enzyme pattern. (A) *Bacteroides* species 05231-JH; (B) *Bacteroides* species 714172-MR; (C) *Bacteroides* species (Moore); (D) *B. putredinis* (Suzuki); (E) *B. symbiosus* ATCC 12829; (F) *B. necrophorus* NTCC 7155; (G) group 4 poultry isolate EBF 6%43; (H) group 4 poultry isolate EBF 6%42; (I) *Sphaerophorus* varus ATCC 8501; (J) *Fusobacterium* polymorphum ATCC 10953.
family. The first phenon included species of *Sphaerophorus* and *Fusobacterium*, *B. melaninogenicus*, and three poultry isolates. The second phenon contained *Bacteroides* species. The third and fourth phena contained chicken, turkey, and

**DISCUSSION**

Barnes and Goldberg (1) identified four phena by numerical taxonomy of the *Bacteroidaceae* family groups. These groups were defined by the number of bands and the relative electrophoretic mobility of the bands as compared by an arbitrary scale.

![Diagram of glucose-6-phosphate dehydrogenase enzyme pattern](image-url)

**FIG. 9.** Diagram of glucose-6-phosphate dehydrogenase enzyme pattern. (A) *Bacteroides fragilis* NTCC 9343; (B) *Bacteroides* species JH-4; (C) Fusocillus 209; (D) group 4 poultry isolate EBF 6050; (E) *B. convexus* Au 21-27; (F) *B. convexus* E102; (G) *B. convexus* E384; (H) *Eggerthella convexus* 274; (I) *B. thetaiotaomicron* 12; (J) *E. thetaiotaomicron* 249.

**FIG. 10.** Diagram of glucose-6-phosphate dehydrogenase enzyme pattern. (A) Group 4 poultry isolate EBF 5574; (B) group 4 poultry isolate EBF 6163; (C) Fusiformis fusiformis F341; (D) *F. fusiformis*, F398; (E) group 6 poultry isolate EBD 142; (F) *Bacteroides* fundiformis B540; (G) group 1 poultry isolate EBF 5096; (H) *Sphaerophorus pseudonecrophorus* B176; (I) *S. pseudonecrophorus* B130.

![Diagram of glucose-6-phosphate dehydrogenase enzyme pattern](image-url)

**FIG. 11.** Diagram of glucose-6-phosphate dehydrogenase enzyme pattern. (A) *Fusobacterium polymorphum* ATCC 10953; (B) *Sphaerophorus varius* ATCC 8501; (C) group 4 poultry isolate EBF 5574; (D) group 4 poultry isolate EBF 6163; (E) *Bacteroides necrophorus* NTCC 7155; (F) *B. symbiosus* ATCC 12829; (G) *B. putredinis* (Suzuki); (H) *Bacteroides* species (Moore); (I) *Bacteroides* species 714172-MR; (J) *Bacteroides* species 05231-JH.

![Diagram of glucose-6-phosphate dehydrogenase enzyme pattern](image-url)

**FIG. 12.** Diagram of glucose-6-phosphate dehydrogenase enzyme pattern. (A) *Eggerthella thetaiotaomicron* 245; (B) *Bacteroides melaninogenicus* B536; (C) *Fusobacterium* B272; (D) *Sphaerophorus freundii* B149; (E) *S. fundiformis* (Beerens); (F) *S. necrophorus* N252; (G) *S. necrophorus* N117; (H) isolate B1; (I) isolate B2; (J) isolate B5.
duck isolates. These poultry isolates were a separate group and could not be related to the *Sphaerophorus-Fusobacterium* phenon or the *Bacteroides* phenon. It is interesting to relate the classification of the *Bacteroidaceae* emerging from numerical taxonomy with the data obtained from zymogram analysis.

**Bacteroides-Sphaerophorus-Fusobacterium.** Zymogram analysis separated the *Bacteroidaceae* into two major groups, the genus *Bacteroides* on one side and the genera *Sphaerophorus* and *Fusobacterium* on the other.

An important observation obtained from MDH was that *Sphaerophorus* and *Fusobacterium* species do not contain this enzyme but that *Bacteroides* species generally do. All three strains of *B. thetaiotaomicron* (245, 249, and 12) lacked a band for MDH with a mobility between 6 and 8 on the scale, but all three strains did contain a band located between 4 and 6. All other species of *Bacteroides* contained a band located between 6 and 8 on the arbitrary scale. This might be useful for separating *B. thetaiotaomicron* from other *Bacteroides* species, but many more strains and species must be examined before it can be considered conclusive. However, these data support those of Reinhold (7). By investigating carbohydrate fermentations, she found that *B. thetaiotaomicron* strains were different from *B. convexus* strains. Joyner and Baldwin (5) reported finding malate dehydrogenase in *B. ruminicola* and *B. amylophilus*.

The genus *Bacteroides* fell into two major groups with respect to GDH patterns. Both these major groups contained two or more bands for GDH. Another group was identified (*B. putredinis* and *Bacteroides* species 05231-JH, EBF 61/42, and EBF 60/43) which contained only one band for GDH, which was common to all groups of *Bacteroides* tested. It appears that an organism may be tentatively identified as a *Bacteroides* by the GDH zymogram if one or two bands are detected, with one of the bands located between 5 and 7 on the electrophoretic mobility scale. The *Sphaerophorus-Fusobacterium* group was identified by one band for GDH which was always less than 5 on the mobility scale.

The *Bacteroides* group was generally characterized by two bands of G6PDH activity. The *Sphaerophorus-Fusobacterium* group was negative for G6PDH, with the exception of *S. pseudonecrophorophus* (B130 and B176). These two strains contained a band located between 6 and 8 on the arbitrary electrophoretic mobility scale.

The cultures labeled as *Bacteroides* JH-4, 05231-JH, and 714172-MR and the isolates B1, B2, and B5 consistently grouped with the *Bacteroides* by means of their MDH, GDH, and G6PDH zymograms. These results provide further evidence that they are indeed *Bacteroides*.

The studies by Barnes and Goldberg (1) indicated that *B. symbiosus* ATCC 12829 shows little resemblance to other *Bacteroides*. The zymogram of MDH placed *B. symbiosus* with the *Sphaerophorus-Fusobacterium* group and the zymogram of G6PDH placed it with *S. pseudonecrophorophus*. *B. symbiosus* was placed within the *Bacteroides* group only by the GDH zymogram. This evidence tends to support that obtained by numerical taxonomy.

Both numerical taxonomy and zymogram analysis placed *Sphaerophorus* and *Fusobacterium* in the same group. This study would support the proposal of the Bergey’s Manual Committee for Gram-negative Anaerobes that *Sphaerophorus* and *Fusobacterium* be considered as one genus with the name of *Fusobacterium*.

**B. melaninogenicus.** The taxonomic position of *B. melaninogenicus* is difficult to define. Barnes and Goldberg (1) indicated that this organism resembles *Sphaerophorus* and *Fusobacterium* species more than *Bacteroides*; therefore, *B. melaninogenicus* has been placed in phenon 1. In these studies, *B. melaninogenicus* B536 contained one band of MDH and as such could not be placed within the *Sphaerophorus-Fusobacterium* group. However, the zymogram of GDH and G6PDH grouped *B. melaninogenicus* with *Sphaerophorus-Fusobacterium*. The zymogram analysis of *B. melaninogenicus* was inconclusive and further work on more strains must be conducted.

**Poultry isolates.** The group 1 poultry isolate (EBF 59/96p) fell within phenon 1. It did not fall consistently into a single group by zymogram analysis.

The group 4 poultry isolates were placed into a separate phenon by Barnes and Goldberg (1). For each of the three dehydrogenase zymograms, these poultry isolates could be found within either the *Bacteroides* group or the *Sphaerophorus-Fusobacterium* group.

The group 6 poultry isolate (EBD 1/4) closely resembles a *Sphaerophorus* species by the numerical analysis of Barnes and Goldberg (1). The MDH and G6PDH zymograms tended to place EBD 1/4 into the *Bacteroides* group. However, the GDH zymogram placed EBD 1/4 with the *Sphaerophorus-Fusobacterium* group.

This study has demonstrated a close correlation between the taxonomic grouping of certain species of *Bacteroidaceae* by numerical taxonomy and the taxonomic grouping by zymogram analysis. The value of zymograms in identifying and classifying the *Bacteroidaceae* can be more firmly established as more strains and more species are examined by this technique.
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