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Phosphatase Activity of Anaerobic Organisms

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Anaerobic organisms were tested for phosphatase activity in different pH ranges. Several groups of organisms displayed characteristic patterns. *Bacteroides fragilis*, *B. melaninogenicus*, and *B. ruminicola* produced phosphatase with strongest activity at pH 8.6. *Fusobacterium mortiferum* was the only species of this genus to show strong hydrolysis. The enzyme was active in both acid and alkaline ranges. The activity of gram-positive organisms was variable, the most active groups being *Clostridium perfringens*, *Peptostreptococcus intermedius*, *P. micros*, and *Peptococcus constellatus*. The incorporation of phosphatase activity into the identification scheme of anaerobes seems feasible. There was a correlation of hydrolysis with several important pathogens.

Phosphatase activity has proved to be valuable in the identification of numerous bacteria (3). More specifically, phosphatase was applied to the characterization of *Staphylococcus aureus* (1) and in the differentiation of *Serratia* and *Enterobacter* (9). The pathogenicity of staphylococci has been correlated somewhat successfully with this biochemical property (7). More recently the phosphatase activity of *Candida* species and other yeasts was evaluated (8). There has been little information describing phosphatase production by anaerobic organisms. Bray and King (3) demonstrated phosphatase activity in certain *Clostridium* species; however, anaerobic nonsporeforming rods and cocci were not tested.

Anaerobic isolates obtained from clinical specimens during the past three years were employed in this investigation. Attempts were made to evaluate phosphatase production as a tool in identification and, possibly, as an indicator of virulence.

MATERIALS AND METHODS

Test organisms. With a few exceptions test organisms were obtained from recent clinical specimens and stock cultures of clinical isolates. Several strains were provided by the Anaerobic Reference Laboratory at the Center for Disease Control. Identification of the bacteria was made by performing appropriate biochemical tests and gas chromatography analysis as described by the Anaerobic Laboratory of the Virginia Polytechnic Institute and State University (6). The test organisms were cultured on brain heart infusion agar (BBL), supplemented with 5% defibrinated sheep blood, 0.5% yeast extract, and 0.5 μg of menadione per ml. Cultures were grown in GasPak jars by using the evacuation-replacement method (flushed three times) to provide an atmosphere of 85% N_2, 10% H_2, and 5% CO_2. Organisms incubated at 37 C for 48 h were tested for phosphatase production.

Phosphatase production. Phenolphthalein monophosphate (Sigma Chemical Co.) was the substrate chosen for the assays. Enzymatic hydrolysis led to the liberation of free phthalein which produced a light pink to a deep magenta color in alkaline solution.

The following buffers were prepared for the enzyme studies: 0.1 M acetate, 0.1 M citrate, and 0.1 M tris(hydroxymethyl)aminomethane (5). Phosphatase activity was determined by a modified procedure of Cannon and Hawn (4). The organisms were suspended in 1.0 ml of buffer to provide a turbidity equivalent to a no. 3 McFarland standard. Suitable suspensions were made using sterile cotton swabs; 0.2 ml of a 0.1% aqueous solution of phenolphthalein monophosphate was added to the suspension. The tubes were incubated in a 37 C waterbath for 30 min. Two or three drops of 0.4 N NaOH, depending on the buffer, were added to stop the reaction and provide a pH of approximately 11.0, which produced a stable color. The tubes were visually observed to determine the extent of phosphatase activity. Uninoculated tubes were included as controls.

RESULTS

Phosphatase activity of different anaerobic isolates was determined in 0.1 M acetate buffer, pH 5.2. Strong enzymatic activity was observed with several species of gram-negative rods, gram-positive cocci, and gram-positive sporulating rods (Tables 1 and 2).

The 27 strains of *Bacteroides fragilis*, representing all subspecies, gave moderately positive reactions. Three strains each of *B. ruminicola* and unidentified *Bacteroides* species also dis-

TABLE 1. *Phosphatase reaction of gram-negative anaerobic isolates cultured for 48 h and tested in a pH 5.2 acetate buffer*

Organism	No. tested	Phosphatase activity[a]
B. fragilis	27	+
B. melaninogenicus	7	−w
B. pneumosintes	3	−
B. corrodens	4	−
B. clostridiiformis	1	−
B. ruminicola	3	+
Bacteroides species	3	+
F. mortiferum	9	+
F. varium	3	−
F. nucleatum	2	−
F. gonidiaformans	4	−+
F. necrophorum	1	−
F. russii	1	w
F. symbiosum	4	−w
F. naviforme	1	w
V. parvula	2	−
V. adolescens	1	w

[a] +, Moderate to strong reaction; −, no reaction or trace only; w, weak reaction; −+, majority negative, occasional positive; −w, majority negative, occasional weak positive.

TABLE 2. *Phosphatase reaction of gram-positive anaerobic isolates cultured for 48 h and tested in a pH 5.2 acetate buffer*

Organism	No. tested	Phosphatase activity[a]
P. magnus	10	−w
P. prevotii	14	−w
P. asaccharolyticus	13	−+
P. constellatus	1	+
P. micros	5	+
P. anaerobius	11	−w
P. intermedius	7	+
G. anaerobia	4	−w
C. perfringens	6	+
C. plagarum	1	+
C. putrificum	1	−
P. acnes	4	−
B. adolescentis	2	−
Bifidobacterium species	6	−
Lactobacillus species	2	±
E. rectale	1	−
E. lentum	1	−
E. cylindroides	2	−
Eubacterium species	8	−w

[a] +, Moderate to strong reaction; −, no reaction or trace only; w, weak reaction; −+, majority negative, occasional positive; −w, majority negative, occasional weak positive; ±, positive or negative.

played phosphatase activity. Several species which were negative included *B. pneumosintes*, *B. corrodens*, and *B. clostridiiformis*. *B. melaninogenicus* showed either weak or negative hydrolysis.

Within the *Fusobacterium* genus, *F. mortiferum* gave a uniformly strong phosphatase reaction. One strain of *F. gonidiaformans* was moderately positive, whereas the other three strains were negative. Either negative or slight activity was demonstrated with *F. varium*, *F. nucleatum*, *F. necrophorum*, *F. russii*, *F. symbiosum*, and *F. naviforme*.

The phosphatase production of the anaerobic cocci was variable. The gram-negative cocci, *Veillonella*, displayed either weak or no phosphatase activity. Gram-positive cocci showed species-specific reactivity. Strong phosphatase activity was demonstrated for *Peptostreptococcus intermedius* (all strains were microaerophilic), *P. micros*, and one strain of *Peptococcus constellatus* (microaerophilic). The other cocci evaluated, *P. anaerobius*, *P. magnus*, *P. prevotii*, *P. asaccharolyticus*, and *Gaffkya anaerobia*, displayed little activity, except for a single strain of *P. asaccharolyticus*.

Among the gram-positive rods, *Clostridium* species produced more active enzyme than nonsporulating species. All six *Clostridium perfringens* strains were strongly positive, as was the single strain of *C. plagarum*. The one strain of *C. putrificum* tested was negative. On the other hand, the only nonsporeforming bacillus demonstrating good phosphatase activity was one *Lactobacillus* isolate. Strains of *Propionibacterium acnes*, *Bifidobacterium adolescentis*, *Eubacterium rectale*, *E. lentum*, *E. cylindroides*, and unidentified *Eubacterium* species gave weak or negative phosphatase reactions.

Several additional buffers were employed to provide a wide pH range to test phosphatase. The activity of several anaerobic organisms displayed characteristic reactions which aid in their identification (Table 3).

Organisms which showed little or no phosphatase activity at pH 5.2 were usually nonreactive at other pH values. *B. melaninogenicus* was an exception, since its phosphatase activity became strong at high pH values. This property of increasing enzyme activity in the alkaline range was also observed with *B. fragilis* and *B. ruminicola*.

F. mortiferum was easily differentiated from other gram-negative anaerobic organisms by its equally intense phosphatase activity in acid and alkaline ranges. Other *Fusobacterium* species

tested gave negative or weak reactions.

The gram-positive isolates were generally less reactive for phosphatase. However, *P. intermedius* and *P. micros* were strong phosphatase producers in both acid and alkaline ranges and the *C. perfringens* reactions were intense at all pH values tested.

DISCUSSION

Anaerobic organisms vary in phosphatase production. Several groups displayed characteristic enzymatic activity over a pH range of 4.0 to 8.6. The amount of hydrolysis was related to several test conditions. The age of the culture has been shown to affect enzyme activity with aerobes, and maximum hydrolysis was obtained employing young cultures (2). To allow adequate growth of the more fastidious anaerobes, 48-h cultures were employed in this study. However, reproducible and consistent results were obtained on cultures of 5 days, whereafter a decline in phosphatase activity was observed.

Since no one buffer can be used over a broad pH range, different buffers were employed. Thus, enzyme activity was related to the presence of different ions in different pH ranges. The magnitude of hydrolysis was also dependent on the reaction time. Our results were based on a 30-min reaction time. These were found not to differ significantly from results after longer incubation.

Employing a 0.1 M acetate buffer, pH 5.2, for the determination of phosphatase proved indicative for all but one group of organisms. Strains of *B. melaninogenicus* displayed little activity at this pH; however, in the alkaline range strong reactions were observed.

The evidence of alkaline phosphatase production for certain members of *Bacteroides* provides a simple test for rapid identification. An increase in activity which was maximum at pH 8.6 was demonstrated in all strains of *B. fragilis* (all subspecies), *B. ruminicola*, and *B. melaninogenicus*. Several other *Bacteroides* species which were tested did not have detectable phosphatase.

The selection of a suitable pH for phosphatase determination varies for the particular test organism. At pH 5.2, *F. mortiferum* can be differentiated from *Bacteroides* and other *Fusobacterium* species by its characteristic intense reaction. On the other hand, negative reactions at this pH should be correlated with alkaline reactions when *B. melaninogenicus* is suspected.

When a gram-positive organism is indicated by Gram stain and colonial morphology, buffers of pH 5.2, 7.2, or 8.6 may be used with equal success for phosphatase evaluation. The enzyme was demonstrated to be specific for *P. intermedius* and *P. micros*. (A single strain of *P. constellatum* tested was positive, but not considered meaningful.) At pH 8.6 all seven strains of *P. intermedius* showed decreased activity,

TABLE 3. *Typical phosphatase reaction of selected anaerobic species cultured for 48 h and tested in different pH ranges*

Anaerobic isolates	Relative phosphatase activity				
	pH 4.0	pH 5.2	pH 6.0	pH 7.2	pH 8.6
B. fragilis	++	++	++	+++	++++
B. ruminicola	+	++	+	+++	++++
B. melaninogenicus	+	−	+	++	+++
B. corrodens	−	−	−	−	−
B. pneumosintes	−	−	−	−	−
F. mortiferum	++	++++	++++	++++	++++
F. varium	−	−	−	+	−
F. symbiosum	−	−	+	−	−
F. gonidiaformans	−	−	−	+	+
V. parvula	−	−	−	−	−
P. intermedius	−	++++	++	++++	++
P. micros	−	++++	+	++++	++++
P. anaerobius	−	−	−	−	−
P. magnus	−	−	−	−	−
P. prevotii	−	−	−	−	−
P. asaccharolyticus	−	−	−	−	−
C. perfringens	+++	++++	++++	++++	++++
B. adolescentis	−	−	−	−	−
E. cylindroides	−	−	−	−	−
P. acnes	−	−	−	−	−

whereas five strains of *P. micros* remained strongly active. Whether this difference is reliable in distinguishing between the two organisms remains to be seen.

Several conclusions can be drawn regarding the properties of phosphatase enzyme(s) of several anaerobes. The reactions of *F. mortiferum*, *P. intermedius*, *P. micros*, and *C. perfringens* indicated the presence of several enzymes possessing different specificities or a single highly active enzyme with a broad spectrum. In active *Bacteroides* species, only an alkaline phosphatase was detected.

In conclusion, phosphatase was variable for anaerobic organisms; however, specific activity was observed with several groups. The results obtained were highly reproducible. Thus, the incorporation of this property into the identification scheme of anaerobes is feasible. It can successfully differentiate the biochemically alike organisms, *F. mortiferum* and *F. varium*, as well as indicate particular gram-positive cocci. The association of phosphatase activity with many clinically significant pathogens, i.e., *B. fragilis*, *B. melaninogenicus*, and *C. perfringens*, may prove a useful correlate of pathogenicity.

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