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Salient Features of *Haemophilus vaginalis*

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A total of 78 strains of *Haemophilus vaginalis* were examined for 104 features. All strains fermented dextrin, maltose, and starch. Additionally, more than 90% of the strains fermented galactose, glucose, and ribose. Arbutin, cellobiose, melibiose, rhamnose, and salicin were not fermented by any of these strains. None of the strains acidified any of 14 alcohols or alkalinized any of 25 organic salts and amides. More than 90% of the strains hemolyzed human blood agar and hydrolyzed hippurate. No strain hemolyzed sheep blood agar. A recommendation is included for those minimal features that best differentiate *H. vaginalis* from other oxidase- and catalase-negative, gram-negative organisms.

Although numerous reports have been published during the last 20 years on the biochemical reactions of *Haemophilus vaginalis*, this organism still remains poorly characterized. Part of the problem in characterization can be ascribed to descriptions of true *Haemophilus* species mistaken for *H. vaginalis* (1). However, differences in methodology for determining biochemical features appear to be primarily responsible for these conflicting results. Additionally, although some studies have used a large number of biochemical tests to characterize this species, diagnostic features have been determined on relatively few strains (5). Thus, there is limited significance to these biochemical profiles.

Carbohydrate reactions are a striking example of variability caused by differences in methodology. Dextrose fermentation, for instance, was reported as being positive for 100% of 63 strains when buffered single substrates were used (7). However, when cystine Trypticase agar was used as the basal medium, only 2 of 16 strains were positive (14). Similar variability has been reported for fermentation of sucrose. One study listed four of five strains tested as sucrose positive (5), and another reported all of 67 isolates as sucrose negative (6). Variability of reactions has not been limited to carbohydrate fermentations. Hemolysis, nitrate reduction, and growth in the presence of tellurite have been reported with differing results (3, 5, 10, 13, 15).

In an attempt to better delineate the biochemical features of *H. vaginalis*, we report here 104 features of 78 strains. We also note those features that are most useful for identification of this species.

MATERIALS AND METHODS

Strains. *H. vaginalis* strains CV 208, CV 165, CV 594, and CV 3-7 were supplied by K.-H. Wong, Center for Disease Control, Atlanta, Ga.; strain 9760-2-73 was supplied by M. Bissett, California State Department of Health, Berkeley; strains BSC T 7 and BSC T 4 were obtained from B. S. Criswell, Baylor Medical College, Houston, Tex.; and strains V9660, V8094, V7644, V8821, V7854, and V9477 were supplied by L. V. Holdeman, Virginia Polytechnic Institute, Blacksburg. A total of 22 strains were obtained as clinical isolates from the Clinical Microbiology Laboratory, University of California, Los Angeles, and 43 strains were obtained as clinical isolates from the Student Health Center, California State University, Northridge. All strains were maintained on vaginalis agar (7). This medium is composed of Columbia agar base (Baltimore Biological Laboratory, Cockeysville, Md.) and 1% proteose peptone no. 3 (Difco Laboratories, Detroit, Mich.). Human blood (5%, vol/vol) preserved with citrate, phosphate, and dextrose is added to the autoclaved and cooled medium. The cultures were transferred every 48 h and were incubated at 35°C in an atmosphere containing 5% CO_2 and elevated humidity.

Fermentation media. Fermentation of carbohydrates was determined in a modified medium of Dunkelberg et al. (5). The composition of the modified medium was 2% proteose peptone no. 3, 0.002% phenol red, 0.5% agar, and 1% carbohydrate (added as a 10% filter sterilized solution to the autoclaved and cooled medium). The pH of the medium was adjusted to 7.3 before autoclaving, and 3 ml was dispensed into screw-cap test tubes (13 by 100 mm). The tubes were inoculated with a stab of 48-h growth from a vaginalis agar plate. The tubes were incubated in air at 35°C and examined daily for 5 days.

Acidification of alcohols. The acidification of alcohols was tested by the buffered substrate procedure of Greenwood et al. (7), except that the volume was increased to 0.5 ml to decrease effects of dehydration. The tubes were incubated at 35°C in a humidified incubator. The following alcohols were tested at a final concentration of 1%: 1,4-butanediol, 1,2,4-butanetriol, isobutanol, secondary butanol, tertiary butanol, ethanol, ethylene glycol, glycerol, methanol, tertiary pentanol, 1,3-propanediol, normal propanol, propylene

glycol, and 2-propanol.

Alkalinization of organic salts and amides. These tests were performed as outlined above for the acidification of alcohols except that the initial pH of the basal medium was adjusted to 6.5. The following organic salts and amides were tested at a final concentration of 1%: acetamide, allantoin, asparagine, *n*-butyramide, formamide, glutamine, malonamide, nicotinamide, propionamide, succinamide, valeramide, acetate, adipate, iso-butyrate, *n*-butyrate, citrate, formate, hexanoate, maleate, malonate, mucate, propionate, saccharate, tartrate, and valerate.

Additional buffered substrate tests. Tests for indole, urease, beta-galactosidase, lysine decarboxylase, ornithine decarboxylase, hippurate hydrolysis, acetoin (Voges-Proskauer test), 2-ketogluconate, and phenylpyruvic acid were made with Key tableted media (Key Scientific Products Co., Los Angeles, Calif.). Inocula for the tests were obtained by harvesting 24-h growth on vaginalis agar plates. The individual tests were performed according to the recommendations of the manufacturer.

Nitrate reduction. The medium of Dunkelberg et al. (5) was used. Since initial results disclosed that many strains failed to grow in this medium, 2 drops of sterile rabbit serum (Grand Island Biological Co., Grand Island, N.Y.) was added to each tube to enhance growth. After 5 days of incubation, nitrite was detected with nitrite reagent (12). Negative tests were confirmed with zinc dust.

Catalase and oxidase tests. Growth from a 48-h chocolate agar plate (Clinical Standards, Torrance, Calif.) was placed on a glass slide with a wooden applicator stick, overlayed with a drop of 3% H_2O_2, and then observed for evolution of bubbles. For the oxidase test, growth was smeared with a platinum loop on a filter paper strip saturated with a solution of 1% tetramethyl-*p*-phenylenediamine dihydrochloride in 0.2% ascorbic acid.

Hemolysis. The hemolytic activity of the cultures was tested on both human and sheep blood agar. Both media were prepared with vaginalis agar (7); each contained 5% (vol/vol) of blood. Isolated colonies were examined daily for evidence of hemolytic activity. The plates were incubated for a total of 4 days.

Lipase and lecithinase. Egg yolk agar (8) was inoculated and then incubated anaerobically in a GasPak jar (Baltimore Biological Laboratory). The plates were examined daily for 5 days for evidence of lipase and/or lecithinase activity.

Hydrolysis of gelatin. Hydrolysis of gelatin was determined by the film-strip method (12).

Hydrolysis of tributyrin. Medium for this was as follows: Columbia agar base, 17 g; proteose peptone no. 3, 4 g; dextrose, 2 g; and distilled water, 400 ml. Tributyrin (4 ml) was added to the autoclaved and cooled medium, the mixture was shaken, and 15 to 17 ml was poured per plate. Plates were inoculated and incubated at 35°C in an atmosphere containing 5% CO_2 and elevated humidity. Isolated colonies were examined daily for evidence of tributyrin hydrolysis. The plates were incubated for a total of 5 days.

Hydrolysis of Tween 80. Medium for this contained: Columbia agar base, 17 g; proteose peptone no. 3, 4 g; $CaCl_2 \cdot H_2O$, 0.04 g; dextrose, 2 g; Tween 80, 4 ml; and distilled water, 400 ml. Plates were poured, inoculated, incubated, and scored as outlined above.

Hydrolysis of casein. Medium for this contained the following: Columbia agar base, 17 g; dextrose, 4 g; proteose peptone no. 3, 4 g; skim milk (Difco), 30 g; and distilled water, 400 ml. Plates were poured, inoculated, incubated, and scored as outlined above.

Hydrolysis of starch. This starch agar medium contained the following: Columbia agar base, 17 g; dextrose, 4 g; proteose peptone no. 3, 4 g; corn starch, 4 g; and distilled water, 400 ml. Plates were poured, inoculated, incubated, and scored as outlined above.

Hydrolysis of esculin. Esculin agar medium contained 0.3% beef extract (Difco), 0.5% proteose peptone no. 3, 0.1% esculin (Difco), 0.05% ferric citrate, and 1.5% agar. The medium was melted, dispensed in 3-ml amounts into test tubes (13 by 100 mm), autoclaved, and slanted. These tubes were inoculated heavily and examined daily for 5 days for evidence of esculin hydrolysis. A group D streptococcus was used as a positive control for this medium.

Growth on selected media. Vaginalis agar was prepared with either 0.01% potassium tellurite, 3% sodium chloride, or 1% bile. These plates were streaked and examined daily for evidence of growth. The plates were scored as positive if single colonies could develop within 5 days of incubation. With Rogosa agar, MacConkey agar, and Thayer-Martin agar, plates were inoculated and examined for evidence of growth. Positive reactions were scored as described above. For growth on acid and alkaline media, vaginalis agar was adjusted to either pH 4 or 8 and autoclaved. These plates were then streaked and examined for evidence of growth. Positive reactions were scored as described above.

Growth at 25, 30, and 42°C. Vaginalis agar was inoculated, and the plates were incubated at the appropriate temperature with 5% CO_2 and moisture. The plates were examined daily for 5 days for evidence of growth.

Growth in the presence of dyes, bacitracin, and ethyl hydrocuprein hydrochloride. A vaginalis agar plate was swabbed for confluent growth and then either dye tablets (Key Scientific Products Co.), bacitracin disks (Taxo A; Baltimore Biological Laboratory), or ethyl hydrocuprein hydrochloride disks (Taxo P; Baltimore Biological Laboratory) were placed on the surface of the agar. The plates were incubated for 24 h, and any zone of inhibition greater than 10 mm in diameter was recorded as positive.

RESULTS

Table 1 summarizes the results from the carbohydrate fermentation tests. All strains fermented dextrin, maltose, and starch. Additionally, more than 90% of the strains fermented dextrose, galactose, and ribose. Arbutin, cellobiose, melibiose, melizitose, raffinose, rhamnose, and salicin were not fermented by any of these strains. The other carbohydrates listed were fermented with variable results. Although most positive fermentation reactions were evident within 72 h, positive lactose reactions were not

TABLE 1. *Carbohydrate fermentation reactions of H. vaginalis*[a]

Carbohydrate	Test result	% Positive
L-Arabinose	−	9
D-Arabinose	−	5
Arbutin	−	0
Cellobiose	−	0
Dextrose	+	97
Dextrin	+	100
Fructose	+	81
Galactose	+	96
Inositol	−	0
Inulin	−	40
Lactose	−	14
Maltose	+	100
Mannose	+	82
Mannitol	−	0
Melizitose	−	0
Raffinose	−	0
Rhamnose	−	0
Ribose	+	99
Salicin	−	0
Sorbitol	−	0
Starch	+	100
Sucrose	−	10
Trehalose	−	4
Xylose	−	15

[a] Carbohydrate fermentation reaction data are based on 78 strains.

generally observed until day 5 of incubation. None of the strains acidified any of the 14 alcohols or alkalinized any of the 25 organic salts and amides.

Table 2 lists additional features of *H. vaginalis*. More than 85% of the strains hemolyzed human blood agar, hydrolyzed hippurate and starch, and grew at pH 8 and at 25, 30, and 42°C. Except for the tests for beta-galactosidase and lipase, which gave variable results, all other tests were uniformly negative.

The susceptibility of *H. vaginalis* to dyes and inhibitory agents is given in Table 3. The strains were uniformly susceptible to all of the dyes except pyronin. All strains were resistant to pyronin and also to ethyl hydrocuprein hydrochloride. Susceptibility to bacitracin was 96%.

DISCUSSION

The positive fermentation reactions reported here for dextrose, dextrin, fructose, galactose, ribose, and starch are in general agreement with those previously published (5, 6, 16), as are the negative reactions for polyhydric alcohols (6, 16). Lactose and trehalose were fermented by a few of our strains. This differs from the species description given in *Bergey's Manual of Determinative Bacteriology* (2) but agrees with data of Malone et al. (11).

Dunkelberg et al. (5) reported that *H. vaginalis* could grow in the presence of, but could not reduce, 0.01% potassium tellurite. Subsequently, Smith et al. (15) confirmed the inability of *H. vaginalis* to reduce tellurite, but found that

TABLE 2. *Features of H. vaginalis*[a]

Test or substrate	Test result	% Positive
Oxidase	−	0
Catalase	−	0
Indol	−	0
Urease	−	0
ONPG	+	53
Voges-Proskauer	−	0
Phenyl pyruvic acid	−	0
Lipase	+	43
Hydrolysis of:		
Hippurate	+	92
Tributyrin	−	0
Tween 80	−	0
Casein	−	0
Starch	+	100
Esculin	−	0
Gelatin	−	0
Growth at:		
pH 4	−	0
pH 8	+	95
25°C	+	85
30°C	+	100
42°C	+	100
$NO_3^- \rightarrow NO_2^-$	−	0
Hemolysis of:		
Human blood	+	96
Sheep blood	−	0
Decarboxylase		
Lysine	−	0
Ornithine	−	0
Gluconate	−	0
Lecithinase	−	0
Growth on selective media		
Tellurite (0.01%) agar	−	0
Sodium chloride (3%) agar	−	0
Bile (1%) agar	−	0
Rogosa agar	−	0
MacConkey agar	−	0
Thayer-Martin agar	−	0

[a] Biochemical reaction data are based on 78 strains.

TABLE 3. *Susceptibility of H. vaginalis to dyes and inhibitory agents*[a]

Dye or inhibitory agent	% Susceptible
Thionin	100
Basic fuchsin	100
Crystal violet	100
Azure A	100
Safranin	100
Pyronin	0
Bacitracin (A disk)	96
Ethyl hydrocuprein hydrochloride (P disk)	0

[a] Susceptibility data are based on 78 strains.

growth was inhibited by levels of tellurite lower than those previously reported. Our results confirm their findings, as none of the strains in our study grew in the presence of 0.01% potassium tellurite.

Lipase activity of *H. vaginalis* was first reported by Malone et al. (11). They stated that this feature, when present, was helpful in detecting *H. vaginalis* in mixed cultures. They noted, however, that lipase activity was not evident with all of their strains. Only 43% of our strains exhibited lipase activity. Beta-galactosidase activity also falls into this intermediate range of positive reactions. With only 53% of the strains positive, this feature is of limited diagnostic value. Additionally, there is no correlation between beta-galactosidase activity and any other variable characteristic (e.g., lipase). Hippurate, however, was hydrolyzed by more than 90% of our strains.

H. vaginalis has been described as beta-, alpha-, or nonhemolytic, depending on the species derivation of the blood examined (3, 9, 13). None of our strains showed any type of hemolytic activity on sheep blood agar. However, 96% produced a diffuse beta-hemolysis on human blood agar after 48 h of incubation. King (9) also reported that 55 of 59 strains of *H. vaginalis* showed this type of beta-hemolysis on rabbit blood agar. We have also noted that the majority of our strains are hemolytic on rabbit blood agar.

Table 4 lists the significant tests and results we feel are necessary for the presumptive identification of *H. vaginalis*. The hemolysis of human blood, but not sheep blood, is readily tested by initial isolation of organisms on a vaginalis agar, sheep blood agar bi-plate. Colonies showing diffuse beta-hemolysis on the vaginalis agar, but not on the sheep blood, are subcultured to a chocolate agar plate. Growth from this plate can be used for oxidase, catalase, and hippurate tests and for Gram staining. This minimum number of tests provides adequate differentiation of *H. vaginalis* from other oxidase- and catalase-negative, gram-negative organisms (e.g., *Haemophilus aphrophilus* or Center for Disease Control DF-1). Additionally, these tests also differentiate *H. vaginalis* from improperly gram-stained *Corynebacterium*, *Bifidobacterium*, and *Lactobacillus*.

TABLE 4. *Presumptive identification of H. vaginalis: recommended tests*

Test or substrate	Test result	% Positive
Oxidase	−	0
Catalase	−	0
Hemolysis of:		
Human blood	+	96
Sheep blood	−	0
Hippurate hydrolysis	+	92

It should be noted that starch fermentation has not been included as a screening feature. Because of the frequent isolation of starch-positive organisms with microscopic morphology resembling that of *H. vaginalis*, we feel that this feature is not sufficiently specific to warrant inclusion. Similarly, inhibition of *H. vaginalis* by hydrogen peroxide (4) is excluded since this test lacks specificity (14).

Because of the contradictory biochemical profiles of this organism in the literature, the impression has been that *H. vaginalis* is a species with heterogeneous characteristics. Our data, however, do not support this impression. Variable results were, indeed, obtained in tests for lipase, beta-galactosidase, and fermentation of six carbohydrates. However, nearly 100% of our 78 strains gave identical results with all other of the 104 features examined.

Since our observations reported here, as well as those of others, argue against placing this bacterium in any established genus, we believe a new genus should be created. Hence, we have recently submitted a manuscript to the *International Journal of Systematic Bacteriology* proposing creation of the new genus *Gardnerella* for inclusion of the organism currently designated as either *Haemophilus vaginalis* or *Corynebacterium vaginale*. This proposal is based on the results of a taxonomic study utilizing DNA hybridizations, biochemical analysis of cell wall constituents, and electron microscopy.

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