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Rapid Identification of Obligately Anaerobic Gram-Positive Cocci Using High-Performance Liquid Chromatography

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High-performance liquid chromatography was evaluated as a rapid means of identifying obligately anaerobic gram-positive cocci of medical interest. Isolates were inoculated into a defined chemical medium consisting primarily of amino acids and were incubated aerobically for 1 h at 35°C. After removal of organisms, the supernatant fluids were derivatized for 1 min at room temperature by the addition of *o*-phthalaldehyde. The total time required to run a chromatogram was approximately 50 min. Standardized peak heights for each medium component and any new peaks formed were calculated for each isolate and compared with those for uninoculated control medium. Multiple isolates of various species of anaerobic gram-positive cocci gave consistent patterns of medium utilization that could be used for identification purposes. This method can easily be adapted for laboratory use and has the potential for automated microbial identification.

In recent years, clinical microbiologists have shown increased interest in the role that anaerobic bacteria play in human disease. Gram-positive cocci have been reported to account for 20 to 40% of all anaerobes isolated in clinical laboratories (1, 7, 14, 18). While anaerobic gram-positive cocci have not received the same attention as other anaerobic genera because of their relative susceptibility to most antimicrobial agents, species identification is important for correlation of these organisms with certain diseases. Endocarditis, pericarditis, and mediastinitis caused by *Peptostreptococcus magnus* (*Peptococcus magnus*) have been reported, as have infections involving bones and joints, with or without the presence of foreign bodies (1, 3, 12).

Chromatographic methods to improve the identification of *Peptostreptococcus* (*Peptococcus*) species have involved gas-liquid chromatography of volatile and nonvolatile metabolic products and profiles of cellular fatty acids (4, 10, 17). In this report, we evaluated high-performance liquid chromatography (HPLC) for the rapid identification of obligately anaerobic gram-positive cocci by analyzing changes in the composition of a defined chemical medium following aerobic incubation for 1 h.

MATERIALS AND METHODS

***Peptostreptococcus* and *Staphylococcus saccharolyticus* (*Peptococcus saccharolyticus*) isolates.** Seventy-three gram-positive anaerobic coccal isolates were obtained from the American Type Culture Collection, Rockville, Md., North Carolina Baptist Hospital, Winston-Salem, N.C., Virginia Polytechnic Institute, Blacksburg, Va., State Laboratory of Public Health, Raleigh, N.C., Miles Laboratories, West Haven, Conn., and East Carolina University, Greenville, N.C. The following species were tested (number of isolates): *P. magnus* (12), including ATCC 29328; *S. saccharolyticus* (3), including ATCC 14953 (type strain); *P. asaccharolyticus* (20), including ATCC 29743; *P. prevotii* (8), including ATCC 9321 (type strain); *P. micros* (10), including ATCC 33270 (type strain); *P. anaerobius* (14), including ATCC 27337 (type strain); and "*Gaffkya anaerobia*" (6). Identification of all isolates was confirmed by using the RapID-ANA (Innovative Diagnostics Systems, Inc., Decatur, Ga.) and Minitek (BBL Microbiology Systems, Cockeysville, Md.) systems.

The cocci were grown anaerobically at 35°C on prereduced Columbia blood agar plates (Carr Scarborough, Atlanta, Ga.) in GasPak jars (BBL Microbiology Systems) for 40 to 48 h. These plates were used to inoculate the defined chemical medium and also for performing spot indole tests with *p*-dimethylaminocinnamaldehyde (11). To confirm the lack of indole production by some *P. prevotii* isolates, we tested these organisms by incubating them in tryptophan broth anaerobically for 72 h, extracting the broth with xylene, and adding Kovacs reagent.

Defined chemical medium and processing procedure. A defined chemical medium (Table 1) consisting of amino acids, an amino sugar, a dipeptide, and trace elements (Sigma Chemical Co., St. Louis, Mo.) was formulated. All the components were added to sterile 20 mM potassium phosphate buffer (pH 7.8 to 8.0) to a final volume of 100 ml and filtered through a 0.45-μm-pore disposable filter apparatus (Nalgene Labware Div., Nalge/Sybron Corp., Rochester, N.Y.). The filtered medium was dispensed in 0.5-ml quantities into sterile tubes and frozen at −70°C until needed. Each anaerobic coccal isolate was inoculated into the defined chemical medium with a sterile swab to correspond to the approximate turbidity of a McFarland no. 10 standard and was incubated aerobically for 1 h at 37°C. The defined medium was then centrifuged to remove bacterial cells, and the supernatant fluids were placed on ice to stop further enzymatic reactions. If specimens could not be tested on the same day, the supernatant fluids were stored frozen at −70°C.

OPA derivatization. *o*-Phthalaldehyde (OPA), which rapidly reacts with primary amines to form OPA derivatives (15), was used for derivatization of the defined medium. The OPA reagent consisted of 80 mg of OPA dissolved in 2.25 ml

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TABLE 1. Defined chemical medium

Component	Concn (μg/ml)
p-Aminophenyl-α-D-glucopyranoside	500
L-Seryl-L-leucine	200
L-Asparagine	200
L-Proline	200
L-Serine	200
L-Arginine hydrochloride	300
L-Alanine	200
L-Histidine	300
L-Phenylalanine	200
L-Leucine	100
L-Lysine	200
Trace factors and metals	
$CoCl_2$	0.1
$CaCl_2$	2.0
$ZnSO_4 \cdot 7H_2O$	1.6
$CuSO_4 \cdot 5H_2O$	0.9
$MgSO_4$	50.0
$FeCl_3$	5.0
$MnCl_2$	4.0
Pyridoxamine dihydrochloride	0.25
Pyridoxal hydrochloride	0.25
Nitrilotriacetic acid	40.0

of HPLC-grade methanol (Fisher Scientific Co., Pittsburgh, Pa)–50 μl of 2-mercaptoethanol–250 μl of a saturated boric acid solution (pH 9.2). This solution was prepared fresh daily and protected from light.

The derivatization procedure was performed under ambient conditions and consisted of the following components: 100 μl of defined medium supernatant fluid, 100 μl of a saturated boric acid solution (pH 9.2) containing 200 μg of valine per ml as an internal standard, 50 μl of the OPA reagent, and 250 μl of methanol. The components were mixed on a vortex mixer (Fisher Scientific Co.) and allowed to react for 1 min prior to injection into the HPLC column. Valine was chosen as the internal standard because no significant utilization or synthesis of this amino acid was detected with the isolates tested.

Chromatography. The HPLC pumping system consisted of a model, 420 System Controller-Programmer (Beckman Instruments, Inc., Berkeley, Calif.) connected to two Beckman model 110B pumps to perform gradient elution. Only HPLC-grade water and methanol were used throughout. Solvent A consisted of 30 mM sodium phosphate buffer (pH 6.5); solvent B was methanol. The mobile phase was pumped at a flow rate of 1.0 ml/min by using the following program (time in minutes, percent solvent B): 0, 25; 9, 25; 18, 35; 25, 45; 32, 55; 38, 65; 48, 70; 53, 70; and 63, 25. Before injection of a new sample, the column was allowed to equilibrate with 25% methanol for at least 15 min.

Samples were injected into a Rheodyne model 7010 sample injector (Rainin Instrument Co., Woburn, Mass.) equipped with a 5-μl sample loop. The column used was an Ultrasphere-ODS (4.6 mm by 15 cm; Beckman) connected to an Ultrasphere-ODS precolumn. Column effluents were monitored at 340 nm, the excitation wavelength for OPA derivatives, with a model 153 fixed-wavelength spectrophotometer (Beckman). The spectrophotometer was connected to a linear single-channel recorder (200 series; Linear Instrument Corp., Irvine, Calif.) with a chart speed of 20 cm/h.

Peak height quantitation. The standardized peak heights of the medium components for both the uninoculated control and the actual test runs were determined by dividing the peak height of each component by the peak height of the internal standard (valine). The standardized peak height of each bacterial test medium component was then divided by the standardized peak height of the corresponding uninoculated medium component to assess medium utilization. The resulting value is referred to as the medium utlization index (MUI). Both test medium and uninoculated medium were run on the same day so that any day-to-day variability in peak heights caused by system deterioration would be taken into account. Lack of utilization of a medium component would give a theoretical MUI of 1.00, while complete utilization would give an MUI of 0.00. An MUI of greater than 1.00 could occur if a product produced by the anaerobic cocci coeluted with one of the medium components. Some new peaks that were not present in the original medium were formed during incubation. Since MUIs, as defined, could not be calculated for these new peaks, MUIs were arbitrarily defined as standardized peak heights, i.e., the new peak height divided by the internal standard peak height.

RESULTS

Each of the species of gram-positive anaerobic cocci studied produced patterns of medium utilization that could be used for identification purposes. A chromatogram of the individual medium components of the uninoculated control medium is shown in Fig. 1A. Note that proline did not form a derivative, since it is an amino acid lacking a primary amino group. To illustrate medium utilization and production of new peaks, Fig. 1B shows a chromatogram of the defined medium after inoculation with *P. anaerobius* while Fig. 1C shows a chromatogram of the defined medium after inoculation with "*G. anaerobia*."

Table 2 and Table 3 show MUI data for *S. saccharolyticus* and the *Peptostreptococcus* species tested. The species included were those most frequently isolated in our clinical laboratory. The data are based on the average of two chromatograms per isolate. In addition to the 10 defined medium peaks shown in Fig. 1A, at least 7 observable new peaks were formed and are labeled A through G (Fig. 1B and C and Table 3). Peaks A and B eluted before asparagine, peak C eluted after histidine, peaks D and E eluted after alanine, and peaks F and G eluted between leucine and lysine.

The cocci studied could be identified by medium utilization criteria and the results of a spot indole test, since chromatograms for the anaerobic cocci showed various utilization patterns. *S. saccharolyticus* utlilized serine and arginine and produced peak F. "*G. anaerobia*" had a similar pattern of medium utlization but also produced a larger peak C (Fig. 1C and Table 3). *P. asaccharolyticus*, which is indole positive, utilized histidine and seryl-leucine. *P. prevotii* was an interesting species in that, for the isolates studied, two patterns of medium utilization were found. The first group (A) produced chromatograms very similar to those of *P. asaccharolyticus* isolates, i.e., histidine and seryl-leucine utilization (Table 2). The other group of *P. prevotii* strains (B) strongly utilized serine, alanine, seryl-leucine, phenylalanine, and leucine. A large peak D was also produced (Table 3). The MUI for the type strain of *P. prevotii* are given separately in Tables 2 and 3 for comparison. It can be seen that it neither utilized histidine, like the *P. prevotii* group A isolates, nor utilized alanine, phenylalanine, or leucine, like the *P. prevotii* group B isolates.

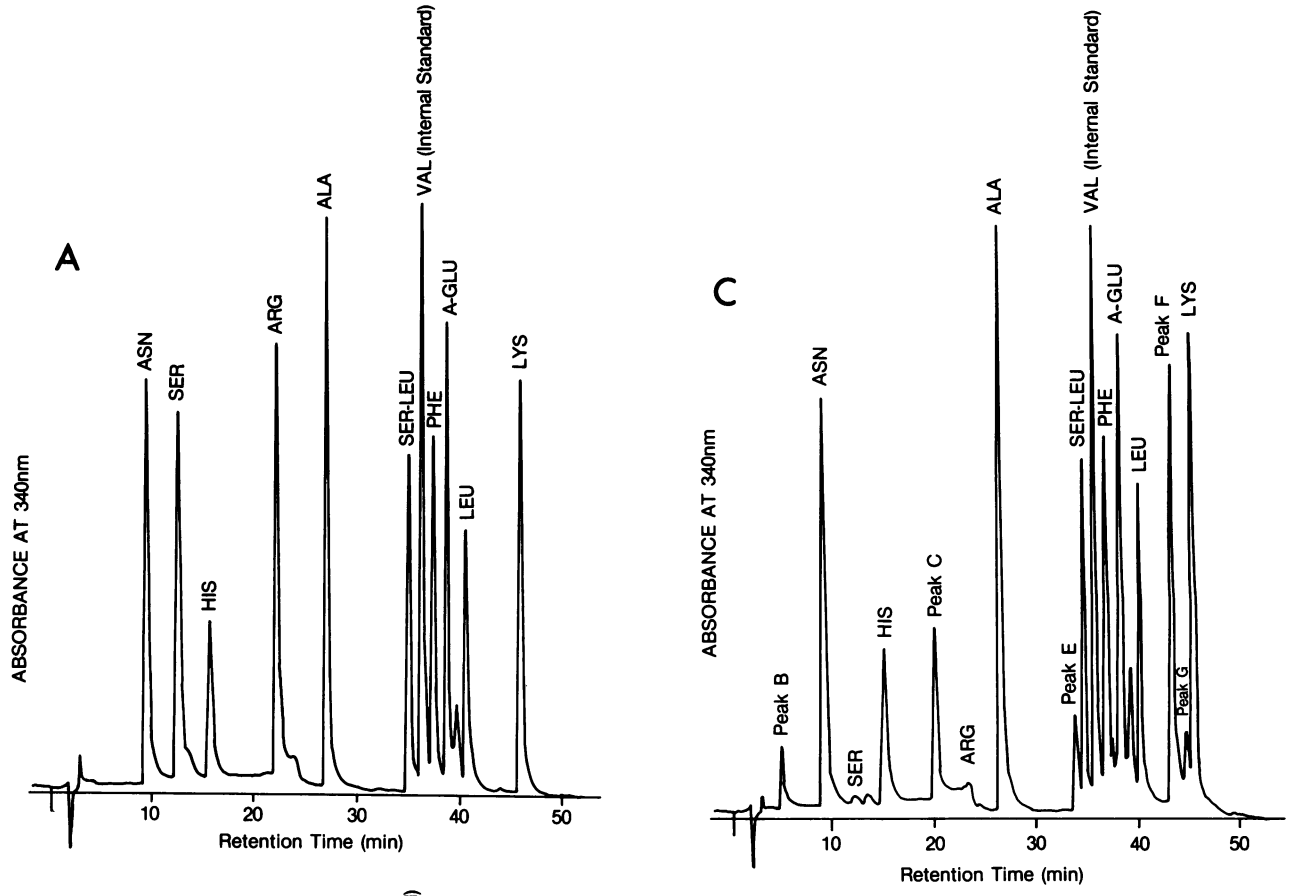

FIG. 1. (A) Chromatogram of derivatized uninoculated control medium. (B) Chromatogram of a derivatized *P. anaerobius* supernatant after 1 h of incubation. (C) Chromatogram of a derivatized "*G. anaerobia*" supernatant after 1 h of incubation. See Table 2, footnotes *a*, *b*, and *c*, for explanations of ARG, SER-LEU, and A-GLU.

The other peptostreptococci studied all showed consistent patterns of medium utilization. *P. magnus* showed very scant degradation of our medium. The only component utilized to any extent was the dipeptide seryl-leucine. *P. micros* did not utilize any component except seryl-leucine, which was utilized to a greater degree than by *P. magnus*. *P. anaerobius* utilized the medium components serine, alanine, seryl-leucine, phenylalanine, and leucine. Peak D was also produced. This pattern was similar to those of *P. prevotii* group B. However, *P. anaerobius* utilized the amino sugar to some extent, with the production of peak G. A dichotomous scheme to aid in identification is presented in Fig. 2.

DISCUSSION

The present study investigated the feasibility of using HPLC monitoring of a defined chemical medium to aid in the rapid identification of obligately anaerobic gram-positive cocci. The study included the major species considered to be pathogenic for humans, except for *P. productus*, which has been reported to be encountered less frequently (6). A 1-h incubation of the organisms with the medium seemed sufficient to produce characteristic chromatograms that could aid in species identification.

The defined chemical medium was formulated to consist mainly of amino acids. It was reasoned that this might

TABLE 2. MUIs for *Peptostreptococcus* species and *S. saccharolyticus*

Organism (no. of strains)	Mean MUI ± SD of:									
	Asn	Ser	His	Arg[a]	Ala	Ser-Leu[b]	Phe	A-Glu[c]	Leu	Lys
P. micros (10)	0.95 ± 0.06	1.04 ± 0.08	0.99 ± 0.07	0.95 ± 0.05	1.03 ± 0.05	0.23 ± 0.15	0.99 ± 0.05	0.93 ± 0.09	1.72 ± 0.18	1.02 ± 0.07
P. magnus (12)	0.94 ± 0.08	0.93 ± 0.09	0.95 ± 0.07	0.90 ± 0.06	0.99 ± 0.04	0.67 ± 0.12	1.00 ± 0.06	0.96 ± 0.10	1.33 ± 0.15	1.01 ± 0.07
S. saccharolyticus (3)	0.90 ± 0.07	0.09 ± 0.02	0.90 ± 0.09	0.23 ± 0.20	0.94 ± 0.06	0.72 ± 0.04	0.99 ± 0.06	0.88 ± 0.09	1.21 ± 0.11	0.99 ± 0.05
P. anaerobius (14)	0.81 ± 0.09	0.08 ± 0.02	0.88 ± 0.06	0.96 ± 0.07	0.49 ± 0.24	0.04 ± 0.07	0.09 ± 0.06	0.67 ± 0.15	0.23 ± 0.15	1.00 ± 0.09
P. asaccharolyticus (20)	0.90 ± 0.08	0.72 ± 0.23	0.17 ± 0.20	0.93 ± 0.08	0.99 ± 0.06	0.48 ± 0.24	0.98 ± 0.05	0.82 ± 0.11	1.41 ± 0.24	0.97 ± 0.06
P. prevotii group A (4)	0.86 ± 0.06	0.61 ± 0.19	0.12 ± 0.11	0.91 ± 0.06	0.99 ± 0.09	0.57 ± 0.12	0.97 ± 0.06	0.74 ± 0.10	1.42 ± 0.12	1.00 ± 0.08
P. prevotii group B (3)	0.80 ± 0.08	0.31 ± 0.25	0.84 ± 0.11	0.94 ± 0.11	0.09 ± 0.05	0.47 ± 0.40	0.32 ± 0.34	0.94 ± 0.09	0.11 ± 0.03	0.96 ± 0.12
P. prevotii ATCC 9321 (type strain)	0.95	0.10	1.03	0.99	1.03	0.85	0.93	0.60	0.98	0.84
"*G. anaerobia*" (6)	0.95 ± 0.08	0.10 ± 0.10	0.92 ± 0.11	0.08 ± 0.04	0.94 ± 0.05	0.96 ± 0.08	0.99 ± 0.04	0.92 ± 0.17	1.08 ± 0.07	1.06 ± 0.10

[a] Arg, L-Arginine hydrochloride.
[b] Ser-Leu, L-Seryl-L-leucine.
[c] A-Glu, *p*-Aminophenyl-α-D-glucopyranoside.

provide additional taxonomic criteria that could be used for identification, since anaerobic gram-positive cocci are thought to use peptones or amino acids as their main nitrogen and energy sources (13). Leucine dissimilation to isovaleric and isocaproic acids has been reported for *P. anaerobius* (2), while serine and histidine utilization has been documented for *P. asaccharolyticus* (13).

As with our study of *Clostridium* species (5), preformed enzymes appear to be responsible for medium utilization, since all incubations were done aerobically. This conclusion is supported by the work of Schreckenburger and colleagues (9, 16), who described rapid aerobic biochemical tests for anaerobes that relied on the reaction of "preformed bacterial enzymes" with different substrates. Also, newer enzymatic tests, such as the RapID-ANA identification system, rely on aerobic incubation.

All species produced a consistent chromatogram, except for *P. prevotii*. The isolates of *P. prevotii* that were tested had patterns that strongly resembled those of other anaerobic cocci. Group B had patterns that were similar to that of *P. anaerobius*, except for amino sugar utilization. Group A had patterns very similar to that of *P. asaccharolyticus*, demonstrating strong histidine utilization. However, these isolates were spot indole negative. As confirmation, indole production was also tested with group A isolates by anaerobic incubation in tryptophan broth. All isolates were negative for indole production with this procedure. The patterns for both groups of *P. prevotii* isolates were unlike that of the type strain of *P. prevotii* (Tables 2 and 3).

The taxonomic characterization of the species *P. prevotii* is still unsettled. In the 8th edition of *Bergey's Manual of Determinative Bacteriology* (13), Rogosa states that *P. prevotii* should be considered "nomina confusa" and that the name should be rejected. He also states that the descriptions of *Micrococcus prevotii* (Foubert and Douglas 1948, 31) and *P. prevotii* (Douglas 1957, 477) are composite descriptions of two organisms, one being indistinguishable from *P. asaccharolyticus* (except for negative indole production) and the other being the organism "*G. anaerobia*." Our data for group A isolates support this assumption for similarities with *P. asaccharolyticus*.

The *P. prevotii* type strain (ATCC 9321) showed variable medium utilization reactions as compared with other *P. prevotii* isolates. Furthermore, it was identified as "*G. anaerobia*" by the RapID-ANA system. This same type strain also had biochemical reactions very different from those of the other strains of *P. prevotii* studied by Ezaki et al. (4). They found this organism to be urease, esterase (C-4), β-galactosidase, β-glucuronidase, and α-glucosidase positive, in contrast to all other strains of *P. prevotii* studied. The DNA-DNA homology between the type strain and the clinical strains of *P. prevotii* they tested ranged from 0 to 73%.

The biochemical identification of "*G. anaerobia*" also presents some difficulties. Rogosa (13) states that the "second discordant element of *P. prevotii* is indistinguishable from the organism with the rejected name '*Gaffkya anaerobia*.' " Also, a group of organisms previously referred to as "*G. anaerobia*" has been tentatively reassigned to the genus *Peptostreptococcus* as *P. tetradius* (4). Urease production has been cited as a criterion for identifying *P. tetradius* both by Ezaki et al. (4) and in the RapID-ANA identification manual. Urease production could easily be tested with our defined medium by the addition of urea and the observation of a large ammonia peak, as was done in a previous publication with *Clostridium sordellii* isolates (5). A medium containing urea was tested with the type strain of *P. prevotii*, which is known to produce urease. It produced a large ammonia peak (peak E) with an MUI of 0.70 (data not shown). Consequently, if urease production is a criterion for

TABLE 3. New peaks formed by *Peptostreptococcus* species and *S. saccharolyticus*

Organism (no. of strains)	Mean MUI ± SD of peak:						
	A	B	C	D	E	F	G
P. micros (10)	0.05 ± 0.02	0.08 ± 0 02	0.07 ± 0.03	0.00 ± 0.00	0.06 ± 0.02	0.10 ± 0.03	0.04 ± 0.01
P. magnus (12)	0.07 ± 0.03	0.09 ± 0.02	0.07 ± 0.02	0.00 ± 0.00	0.05 ± 0.02	0.12 ± 0.05	0.03 ± 0.01
S. saccharolyticus (3)	0.01 ± 0.01	0.07 ± 0.02	0.09 ± 0.02	0.00 ± 0.00	0.19 ± 0.05	0.71 ± 0.27	0.11 ± 0.03
P. anaerobius (14)	0.08 ± 0.03	0.08 ± 0.03	0.06 ± 0.02	0.53 ± 0.14	0.31 ± 0.07	0.03 ± 0.01	0.20 ± 0.11
P. asaccharolyticus (20)	0.04 ± 0.02	0.11 ± 0.06	0.05 ± 0.01	0.00 ± 0.00	0.20 ± 0.08	0.04 ± 0.02	0.08 ± 0.04
P. prevotii group A (4)	0.04 ± 0.01	0.07 ± 0.02	0.04 ± 0.01	0.00 ± 0.00	0.18 ± 0.07	0.03 ± 0.01	0.05 ± 0.01
P. prevotii group B (3)	0.04 ± 0.03	0.10 ± 0.06	0.08 ± 0.03	0.51 ± 0.37	0.24 ± 0.09	0.02 ± 0.01	0.05 ± 0.01
P. prevotii ATCC 9321 (type strain)	0.05	0.07	0.07	0.00	0.10	0.10	0.28
"*G. anaerobia*" (6)	0.00 ± 0.00	0.10 ± 0.04	0.24 ± 0.07	0.00 ± 0.00	0.17 ± 0.03	0.73 ± 0.10	0.15 ± 0.05

the identification of *P. tetradius*, perhaps our isolates of "*G. anaerobia*," which were urease negative, should be identified as *P. prevotii*. This would form a third pattern (group) for this species. Further studies are clearly warranted to place these organisms in their correct taxonomic niche.

Utilization of the defined medium by gram-positive anaerobic cocci produced new peaks (Table 3) with retention times similar to those of the new peaks formed by *Clostridium* species (5) utilizing a similar defined medium. At the present time, we do not know if these peaks are pure products or a mixture of products. Peak A was formed only in trace amounts. This peak is believed to be formed by deamination of asparagine, forming aspartic acid. Since none of the gram-positive anaerobic cocci studied utilized asparagine to any significant extent, a small peak A would be expected. Peak B appears to be background material from the original inoculation plate, since incubation of the organisms in 20 mM potassium phosphate buffer also produced this peak. Peak C is related to arginine utilization, since elimination of arginine from the medium caused it to decrease. Also, citrulline has the same retention time as peak C, and citrulline is known to be a product produced from arginine catabolism. Peak D has the same retention time as 5-aminopentanoic acid, a reduction product of proline. Peak E is believed to be related to ammonia production from the breakdown of amino acids, since the addition of ammonium hydroxide instead of bacterial supernatant to the derivative solution resulted in a peak with the same retention time. A greenish-yellow color produced after derivatization of *P. anaerobius* supernatants and the supernatant fluids of other

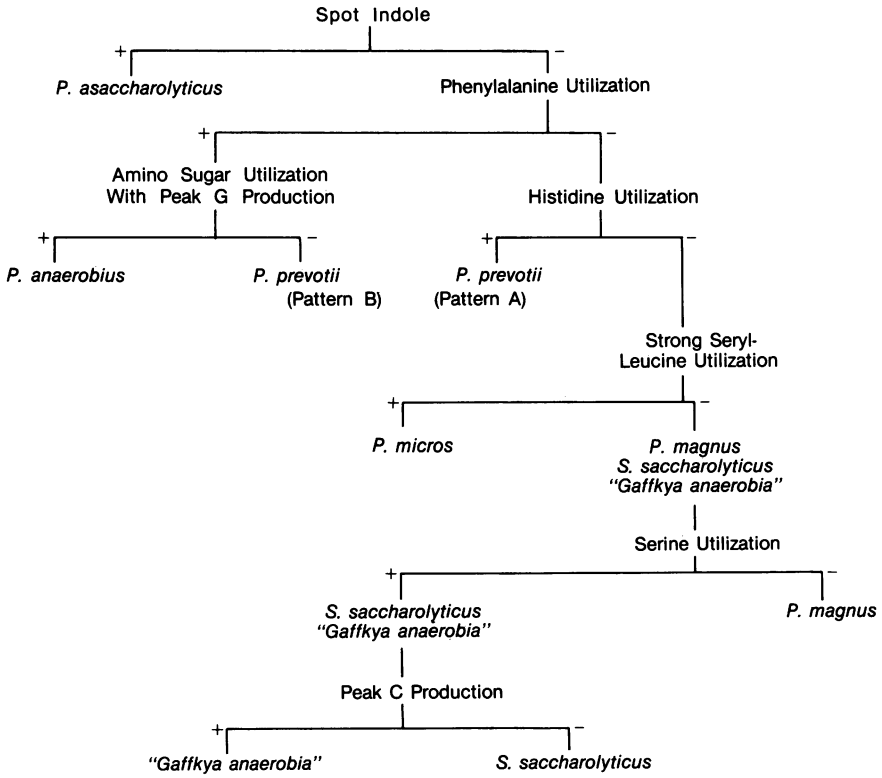

FIG. 2. Dichotomous scheme for identifying gram-positive anaerobic cocci with MUIs.

strong amino acid utilizers was also probably related to ammonia production. The MUI data (Tables 2 and 3) support this conclusion, since there was a positive correlation between amino acid utilization and peak E production. *P. magnus* and *P. micros* isolates, which were the most unreactive with our medium, also produced the smallest peak E. Peaks F and G were the most difficult to resolve with our system and appear to represent multiple products. Peak F production correlates with arginine breakdown. Ornithine, a breakdown product of arginine, has the same retention time as this peak. Peak G is believed to be related in part to amino sugar breakdown, since this peak was produced in the greatest quantity when *p*-aminophenyl-α-D-glucopyranoside was utilized most. This peak also had a high standard deviation, which is believed to be due to the variability in the utilization of *p*-aminophenyl-α-D-glucopyranoside by isolates of *P. anaerobius* (standard deviation, 0.15). This would be a weak point in the identification scheme presented in Fig. 2. Also contributing to the high standard deviation of peak G may be another component or components that appear to contribute to this peak, since peak shoulders were observed.

A recent taxonomic change based on DNA guanine-plus-cytosine content (4) has been proposed for the anaerobic gram-positive cocci. All former species of *Peptococcus*, except for *P. niger*, have been transferred to the genus *Peptostreptococcus*. However, in DNA-rRNA hybridization studies, Huss et al. (8) showed that the differences in melting points between homologous and heterologous hybrids, using *P. micros* and *P. anaerobius* with *P. magnus* and *P. asaccharolyticus*, were too high to justify their position in the same genus. This conclusion was also supported by comparative sequence analysis of the 16S rRNAs of *P. asaccharolyticus* ATCC 14963 (type strain) and *P. micros* ATCC 23195. The data indicated that the two species were not related at the genus level. Huss et al. (8), however, did not use the type strains of either *P. micros* or *P. anaerobius* in their rRNA-DNA studies.

A more extensive investigation of the species included in this study will be necessary to evaluate the usefulness of this system for identification purposes. However, as was shown with various clostridial species, this method provides a simple way to analyze amino acid breakdown by organisms that are mainly asaccharolytic. It also has the potential for automated microbial identification by computer handling of the data generated.

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