An Approach to the Taxonomy of Gram-positive Anaerobic Cocci. 5. The Metabolism of Amino Acid

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Abstract:
This study showed that the metabolism of amino acids was useful in differentiating between the species of Gram-positive anaerobic cocci (GPAC). The best results were obtained from those whose taxonomic positions or biochemical reactions were in doubt. These include *Peptococcus (Pc.) asaccharolyticus, Pc. aerogenes*,, *Pc. magnus, Pc. anaerobius, Pc. variabilis, Pc. niger* and *Peptostreptococcus (Pst. micros.* For these species which are easily recognized by carbohydrate fermentation amino acid utilization was less discriminatory. It should be noted, however, that the most frequently isolated GPAC from clinical materials are those which have a high capacity to utilize amino acids.
This study has revealed that the carbohydrate fermenters are less commonly encountered in clinical specimens. This indicates that the species which are more active against amino-acids are more commonly pathogenic, and this phenomenon should therefore be exploited to identify and classify the GPAC.

Introduction:
The first report of micro-organisms arranged in packets was in 1842.[1] However, the first isolation of the GPAC was, in 1893, from a case of suppurative bartholinitis.[2] GPAC are a heterogeneous group of organisms defined by their morphological appearance and their inability to grow in the presence of oxygen. GPAC are part of the normal flora of the mouth, upper respiratory and gastrointensinal tracts, female genitourinary system and skin.[3-7]
GPAC are commonly present in human clinical specimens; data from four surveys of anaerobic infections are consistent that they account for about 25 to 30% of all anaerobic isolates. They are not involved in any single specific disease process; rather they may be present in a great variety of infections involving all areas of the human body. These infections may range in severity from mild skin abscesses, which disappear spontaneously after incision and drainage, to more serious and life-threatening infections such as brain abscess, bacteremia, necrotizing pneumonia, and septic abortion. Infection by GPAC usually involves invasion of devitalized tissue by organisms that are part of the normal flora of the affected tissue or of the surrounding area.[8-11]
GPAC have been poorly studied for several reasons; which include an inadequate classification, difficulties with laboratory identification, and the mixed nature of the infections from which they are usually isolated. GPAC have been fair game for amateur taxonomists. The classification has always been very unsatisfactory; but a few chemical tests can be helpful. Hare and co-workers put forward the original scheme to classify the GPAC into nine groups according to fermentation of carbohydrates (glucose, fructose, maltose and sucrose) and organic acids.
All strains of the nine groups are Gram-positive (except group V which is Gram negative) cocci arranging either in clusters or in chains, with the exception of group VI and VII showing no particular arrangement, under the microscope.
On the basis of fermentation of carbohydrates and organic acids group I is characterized by its ability to ferment glucose, fructose and maltose. Group III and VI ferment all carbohydrates tested, the former group differs from the latter in the fermentation of pyruvate. Group IV is readily separated from other groups, since fructose is the only sugar utilized. Both group II and IX do not ferment carbohydrates, but the former is different from the latter in the fermentation of pyruvate and citrate (group II produces gas from pyruvate and citrate). The remaining groups VII and VIII are separated from the others on the basis of the fermentation of glucose and fructose only, and the only difference between these two groups is the production of gas from carbohydrates (glucose and fructose) and pyruvate by group VIII. [12-15] (Table 1).

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Table 1: Fermentation characteristics of anaerobic Gram-positive cocci.

Group	Morphology	Fermentation of carbohydrate				Fermentation of organic acid		
		Glucose	Fructose	Maltose	Sucrose	Pyruvate	Citrate	Lactate
(Str. Putridus)	Gram-positive chains, cooci small	AG	AG	AG	-	G	-	-
II	Gram-positive, clusters	-	-	-	-	G	G	-
III	Gram-positive, clusters, large cocci	AG	AG	AG	AG	G	-	-
IV	Gram-positive, clusters	-	A	-	-	G+	-	-
V	Gram-positive, clusters	-	-	-	-	G	-	-
(Veillonellase?)								
VI	Gram-positive chains and clusters	A	A	A	A	-	-	-
VII	Gram-positive chains and clusters	A	A	-	-	-	-	-
VIII	Gram-positive clusters	AG	AG	-	-	-	-	-
IX	Gram-positive clusters	-	-	-	-	-	-	-

A = acid; G = Gas; + = moderate amount.

However, this conventional carbohydrate metabolism has proven unsuccessful in distinguishing between the various anaerobic cocci.[15] It was therefore necessary to examine other methods of characterization.

As long ago as 1936 Kluyver and Van Neil suggested that the species of the genus peptococcus were chemoheterotrophic and capable of fermenting protein decomposition products.[16]

Rogosa accepted few species (*Pc. asaccharolyticus*, *Pc. aerogenes* and *Pc. anaerobius*) which fermented some amino acids (glycine, serine, threonine, glutamate and histidine).[17] The production of isobutyric, butyric isovaleric and isocaproic acids by certain GPAC (*Pst. anaerobius*, and *M. elsdenii*) in peptone yeast extract with or without glucose indicates decomposition of nitrogen compounds, since these products are not from carbohydrate fermentation.[18]

Moreover, the related anaerobes, namely the genus Clostridium, demonstrate utilization of amino acids.[19]

It was therefore logical to attempt a detailed investigation of GPAC amino acid utilization pattern with a view to develop reliable diagnostic tests.

Materials and Methods:

Sources of GPAC strains:

Out of a collection of 116 anaerobic GPAC from a variety of clinical sources (at St. Lukes Hospital, Guilford; St. Thomas Hospital, London), fifteen strains were selected for the amino acid study to include representative from each of these species identified in an earlier study, together with a number of type culture collection reference strains.[18,20]

Cultures were checked for purity and had been subjected to a series of standard differential tests based on the recommendation of Holdeman and Co-workers in the Virginia Polytechnic Institute (VPI) manual.[18] Pure isolated colonies, from fresh isolates and reference strains, on pre-reduced blood agar were picked to 10 ml volumes of cooked meat media in screw capped bottles, and incubated for 18 hrs at 37°C under an anaerobic atmosphere, contained 85% N, 5% H and 10% Co_2, in the anaerobic cabinet. The bottles containing the cultures were firmly screwed and stored at 4°C in the dark.

Medium:

The medium used for the study of the utilization of amino acids by GPAC strains was modified after Elsden and Hilton (1979).[21] The medium contained (per 100 ml distilled water): Casamino (Difco, Cat. No. 0230.02-1) 39. Trypticase (BBL Cat. No. 11920), 0.1 g; Yeast extract (oxiod, code L21) 0.1 g; L-tryptophan 0.1 g; salt solution, 4 ml; resazurin solution, 0.4 ml; vit. k_1 and haemin solution, 1 ml; Tween 80, 0.02 ml; Cysteine hydrochloride, 0.05 g.

The salt solution was prepared by adding 0.2 g of each $CaCl_2$ and $MgSo_4$ mixed in 300 ml of distilled water until dissolved. Five hundred ml of distilled water was added. The remaining

salts; K_2HPo_4, 1.0 g; KH_2Po_4, 1.0 g; $NaHCo_3$, 10.0 g; and NaCl 2.1 g were added. Two hundred ml of distilled water was added and mixed and stored at 4°C.

Vitamin K_1 and haemin in final concentration of 0.5 – 1 µg/ml and 5 µg/ml respectively in the medium enhance the growth of anaerobic cocci. Hence these compounds were added to the medium. In order to avoid amino acid contamination which might derive from the finger prints, glassware and water, the method of Hamilton and Nagy (1972) was used as follows:

1. Micro-gloves were worn during handling of samples.
2. All glass ware used were cleaned with soap and water, soaked in 5% $NaHCO_3$, dried at 160°C then wrapped in aluminum foil and fried at 500°C for 6 hours.
3. Triple deionized water was used for the preparation of reagents.

Inoculation and incubation:

The maintained cultures in cooked meat medium were streaked onto freshly prepared Blood Brain Heart Infusion Agar (BBHIA), and incubated at 37°C for 24 hr. The casamino acid medium was directly and heavily inoculated from pure cultures (24 hr) of blood agar plates by scrapping the colonies from the surface of the agar using a sterile platinum loop.

The inoculated bottles were stoppered, mixed by inversion until even emulsions of bacteria were obtained and incubated at 37°C for 7 days in the anaerobic cabinet. All bottles were agitated manually each day. The control medium (which was uninoculated) was exposed to the same condition of incubation.

At the end of incubation, the viability and the purity of the cultures were checked by streaking on BBHIA plates and incubated for 3 days. The growing colonies were examined by Gram stain.

Preparation of the samples for amino acid analysis:

One ml of 4 N-perchloric acid was added to 10 ml of the incubated culture and centrifuged at 4000 rpm for 20 min. Perchloric acid precipitates proteins including bacterial cells. Excess perchloric acid was precipitated as the potassium salt by neutralizing the supernatant with 1 ml of 4 N-KOH, and then cooled immediately to 0°C in ice for 30 min and the precipitate of $KCLO_4$ was removed by centrifugation at 4000 rpm for 20 min.[21]

The clear deproteinized medium was kept at -20°C for the analysis of amino acids by thin-layer chromatography and amino acid analyzer.

Qualitative analysis of amino acids using thin-layer chromatography:

The developing solvents for the two dimensional thin layer chromatography (TLC) were as follows:

Solvent 1: Iso propanol; acetic acid; water [75: 10: 15 v/v].[23]

Solvent 2: Methanol; pyridine; 10 N-HCl; water [64: 8: 2: 14 v/v].[24]

Cellulose-coated aluminum sheets [20x20 cm; Merck Cat. No. 5552] were used in this study.[25]

For two dimensional thin layer chromatography, two stands on TLC tanks [30x22x13 cm] were used. To saturate the tank atmosphere with the solvents, two walls of each tank were lined internally with blotting paper, 100 ml of solvent was put into the tank for 24 hr before runs.

The TLC sheets were marked with seven original points 1.5 cm from the two sheet edges, and pencil lines were made 15.5 cm from and parallel to the sheet edges. The strips 4.5 cm wide were used for one-dimension chromatography for both the sample and the standard amino acids.

Two microliters of sample were spotted by Drummond micropipettes at the corner of the sheet (for two-dimensional run). Another two samples were spotted on the strip along with control standard solution of amino acids (for one-dimensional run). The spots on the sheet were left to dry at room temperature.

About 30 min. prior to the development of chromatograms in the first dimension, the old solvent (for saturation of tank atmosphere) was discarded and replaced by 100 ml of fresh solvent No. 1. After checking with a spirit level that each tank is horizontal, two duplicate plates were positioned carefully in the tank with their lower edges dipping into the solvent. All solvents were allowed to ascend until the solvent front was within 15.5 cm of the top. The chromatograms were then dried overnight at room temperature.

Before development of the plates in the second dimension, another chromatogram tank was presaturated with solvent No. 2. The old solvent was discarded and replaced by 100 ml of fresh solvent No. 2. The plates were then

developed at right angles to the first dimension until the solvent front was again 15.5 cm. above the starting position. The plates were removed and dried overnight at room temperature in a ventilated place.
The developed plates were transferred to a fume hood, supported in a vertical position and sprayed with 0.2% ninhydrin in acetone using a Shandon atomizer spray unit, which was held at a distance at about 30 cm from the plates. The spots were developed by heating in a convection oven for 15 min at 105°C.

Quantitative analysis of amino acids:
Quantitative analysis of amino acids was made with a Durram amino acids analyzer (Model D-500, Durram Instrument Corporation, 3950, Fabian Way, Palo Atlanto, California, USA) in Uppsala University, Sweeden.

The construction of the dendogram :
Dendograms were constructed using the method of unweighted-pair-group-arithmetic average (UPGMA) linkage by Sokal and Michener.[26] The cophenetic correlation coefficients (r) were determined using the methods described by Sneath and Sokal.[27] This coefficient measures the degree of distortion imposed on a hierarchical classification compared with the similarity (dissimilarity) matrix being clustered. All calculations were made using the CLUSTAN package version 2 and the BEST program on the prime 750 computer system of the University of Surrey.[24,27]

Results:
Casamino acids medium supported the growth of the strains of GPAC tested but those of *Pst. parvulus* (VPI 0546); *Str. morbillorum* (ATCC 27527) and *Pc. niger* (ATCC) showed scanty growth.

The quantitative analysis of both the uninoculated and inoculated media revealed that 11 out of 39 strains of anaerobic cocci utilized all the threonine available. A further four cultures utilized only 45.8% of that compound. It would be possible then to group the remaining organisms according to their ability to utilize threonine (P=0.01) into (1) weak utilizers (one strain); (2) non-utilizers (13 strains) and (3) those which produce threonine (10 strains).

In the case of serine, 84.2% (32 strains) of the organisms utilized all the available amounts of that amino acid; with the exception of two of the 32 strains which utilized more than 62.8% of serine. One replicate strain which degraded 19.6% of serine was considered a weak utilizer (P=0.01) and another strain was a non-serine utilizer, whereas 5 strains (16.3%) were serine producers. Thus serine produces a clear cut differentiation of these strains and therefore should be investigated further as a diagnostic test.

The GPAC tested were separated into three main categories on the basis of the utilization of glutamic acid; Group A composed of 12 strains (31.6%) which utilized glutamic acid completely except two strains utilized more than 23.9% of that compound; Group B was non-glutamic acid utilizer and contained 9 strains (21%), and Group C represented 18 cultures (47.4%) were considered as glutamic acid producing organisms.

None of the GPAC were able to utilize the following amino acids significantly; aspartic acid, valine, methionine, isoleucine and lysine. Therefore when the quantitative data of these amino acids were subjected to the statistics analysis the strains were subdivided into weak utilizers; non-utlizers and amino acid producers.

It was also of interest to regroup the GPAC according to their ability to utilize glycine. It was noted that of these cultures, 11 (29%) utilized glycine. Among these 9 utilized glycine within the range of 70-100% and only two strains utilized 67.5% of the amino acid. Two cultures degraded glycine weakly (22.1%) whereas 23 strains were unreactive. However, three strains (7.9%) were glycine producers.
Only six strains (13.2%) were able to decompose alanine, one utilized 89% of the given amino acid and five species utilized more than 44.9% of alanine. On the other hand 52.6% of the GPAC were entirely non-reactive, whilst two strains (5.2%) were weak utilizers and another 11 strains (8.2%) were alanine producers. Histidine was utilized by 12 (31.6%) species of GPAC. Histidine remained unattacked by 8 (21%) species, whereas 18 (47%) cultures produced histidine.
Twelve strains (31.6%) of the tested GPAC utilized tryptophan within the range of 76-100%. In contrast 18 strains (47.4%) did not attack tryptophan, whilst 8 strains (21%) added that compound to the medium.

Nineteen strains of the GPAC were able to utilize arginine completely. Seven strains also utilized significant amounts of arginine (40.6%).

Nine strains were non-arginine utilizers, whilst three strains were arginine producers.

It is interesting to note that leucine, tyrosine and phenylalanine were utilized by only three cultures, whereas proline was utilized by one strain in addition to the same three cultures. The rest of GPAC were either non-utilizers or producers for those amino acids.

Three ninhydrine-positive compounds, which were not detectable in the uninoculated medium, were produced by certain species of GPAC. These compounds were identified by the amino acid analyzer. The first was ornithine which was produced by 15 strains. The second was α aminobutyric acid which was produced by 10 strains. The third was γ-amino-butyric acid produced by only four strains.

The dendrogram produced by Unweighted-pair-group-arithmatic-average (UPGMA) linkage method for the raw data analysis revealed that a cophenetic correlation coefficient of 0.906. It was evident from the dendogram that at a 4.460 dissimilarity level, all strains grouped into two main phenons which could be subdivided to give seven groups at a dissimilarity level of 0.645, these groups were labeled A-G. Four strains did not cluster into any group. Ten strains were replicate and gave an overall dissimilarity of 0.184 of Euclidean distance.

The dendogram produced on the basis of the analysis of two stale characters, strong utilization of amino acids coded as positive, very weak or no utilization was coded as negative gave a cophenetic correlation coefficient of 0.906. Seven groups were defined which contained the same strains with the exception of two strains of *Pc. prevotii* in group A. The strain 98 in the first dendogram was placed in group A and strain 13 was moved from A to group B. Replicate strains gave an overall similarity of 98.5% which indicates good reproducibility. The main characters of the groups and the single strains were summarized in Table 2.

Discussion:

The relationship between the strains suggested by cluster analysis based on dissimilarity or similarity values can profitably be compared with their position in the current classification,[28] although resemblance values have no correlation with taxonomic rank.[29] At present time the medically important GPAC constitute a problem in taxonomy. They show considerable variation in their morphology and they are generally inert when tested for carbohydrate fermentation.

This study showed that the metabolism of amino acids was useful in differentiating between the species of GPAC. The best results were obtained from those taxonomic position or biochemical reactions were in doubt. These include *Pc. asaccharolyticus*, *Pc. aerogenes*, *Pc. anaerobius, Pc. variabilis, Pc. niger* and *Pst. micros.* For those species which are easily recognized by carbohydrate fermentation amino acid utilization was less discriminatory.

It should be noted, however, that the most frequently isolated GPAC from clinical materials are those which have a high capacity to utilize amino acids. This study has shown this and that the carbohydrate fermenters are less commonly encountered in clinical specimens. This indicates that the species which are more active against amino acids are more commonly pathogenic and this phenomenon should therefore be exploited to identify and classify GPAC statistically, the amounts of amino acid utilized by GPAC were significant and reproducible.

Moreover, these results were in agreement with those obtained by other workers on the metabolism of individual amino acids by some strains of GPAC.

Table 2: Main differential characters of the 7 groups and four individual strains of anaerobic Cocci.

Characters	Phenons							Strains			
	A	B	C	D	E	F	G	74	27340	27824	112
Aspartic acid											
Threonine	+	V	V			+	+				
Serine	+	+	+	+		+	+	+	+		
Glumatic acid		+					V				
Proline							+			+	
Glycine			+				+				
Alanine							+	+	+	+	
Valine											
Methionine											
Isoleucine											
Leucine							+				
Tyrosine							+				
Phenylalanine							+				
Histidine		+		V			V	+	+		
Lysine							V				
Tryptophane		+					+	+			
Arginine	+	V	+	+							+
Ornithine			+	V							+
α-aminobutyric acid *		V					+				
γ-aminobutyric acid *							+			+	
No. of strains	2	8	9	9	3	1	3	1	1	1	1

A. *Gafkya anaerobia, Pc. prevotii.*
B. *Pc. asaccharolyticus, Pc. aerogenes, Pc. prevotii.*
C. *Pc. magnus, Pc. anaerobius, Pst. Micros, Pc. variabilis.*
D. *Str. intermedius, Str. constellatus, Pc. saccharolyticus, M. elsdenii.*
E. *Str. morbillorum, Pc. niger, Pst. parvulus.*
F. *V. Parvula.*
G. *Pst. anaerobius.*
Strain 74 – *Pst. productus.*
Strain ATCC 27340 – *Str. morbillorum.*
Strain 27824 – *Pst. productus.*
Strain 112 – *Str. intermedius.*
Test reaction:
+ between 89-100% of strains fermented the test compound.
- no utilization or production.
V between 22-78% of strains fermented the test compound.
* Ornithine, α- AbA, and GABA were not incorporated in the medium but produced by certain strains.

Group E:
The organisms in Group E are carbohydrate fermenters with the exception of *Pc. niger* which is apparently inert in the medium supplied. It was found from this study that not all members of Group E could be recovered from the medium, and the analysis of the incubated medium showed that none of the test amino acids were utilized.
However, there were increases of the amount of certain amino acids which probably indicate that the incubated bacteria were autolysed. Since *Pc. niger* was unable to metabolize carbohydrate and amino acids here, it will be easily recognized among GPAC.[18,30] The only character which distinguishes *Pc. niger* depends upon the production of caproic acid from peptone yeast extract glucose medium.[18,31]
On the other hand the analysis of the cell wall preparation of the species of *Pst. parvulus* and *Str. morbillorum* differ from that of *Pc. niger*

in containing lysine as a major amino acid, whereas the strains of *Str. morbillorum* differs markedly from the species of *Pst. parvulus* in the presence of serine and threonine in their cell walls. The species of *Pst. parvulus* was claimed to be a strain variant of *Pst. micros*.[17,32] The present study clearly separates those two species on the basis of amino acid utilization. The separation of *Pst. parvulus* from *Pst. micros* in group C is supported by the results of Romond and Co-workers that the % GPAC of *Pst. parvulus* (44.0) was higher than that of *Pst. micros* (28.3).[32,33]

This study also showed that *Pst. parvulus* species are distinct from *Pst. micros*, since the cell walls of the former contained lysine and the latter contained serine and ornithine. However, *Pst. parulus* is also distinguishable from the other organisms in Group E on the basis of fermentation of carbohydrates.[18]

Species of *Str. morbillorum* are also separatable from other members of Group E on the production of large amounts of lactic acid from carbohydrate. This separation is confirmed by other characters studied in this study by the cell wall analysis which showed that walls of *Str. morbillorum* possess a high amount of alanine.[19,32]

Other GPAC strains with affinity to Group E:

Strain 112 was identified according to Holdman et al., as *Str. intermedius*.[18] According to the present analysis strain 112 showed a great affinity with Group E, however, this strain differs from group E in its ability to utilize arginine.

Strain ATCC 27824 *Str. morbillorum* showed only 87.7% similarity with the other neotype strain *Str. morbillorum* (ATCC 27527). The present study showed that these two strains are quite different organisms. However, the reference strain (ATCC 27527) showed with Group G, the capacity to utilize proline and alanine to produce α-amino-butryic acid (GABA) although it differs from Group G. It should be pointed out that the molar ratio of alanine in the cell walls of *Str. morbillorum* strain (ATCC 27824) is higher than that of *Str. morbillorum* (ATCC 27523) by one mole. Whether these strains of streptococcus are nonspecific or whether they represent different species, remains to be determined.

Both strains of *Pst. productus* (74 and ATCC 27340) were characterized by their utilization of alanine and histidine, but they varied in the utilization of serine. Separation of *Pst. productus* from the other cocci supports the findings of Rogosa and Holdman and Co-workers on the basis of carbohydrate fermentation. [17,18]

In consistence with the study of the cell walls of GPAC, the composition of cell wall hydrolysate of *Pst. productus* contained a limited number of amino acids (glutamic acid, alanine, DAP, muramic acid glucosamine in molar ratios 1.4: 0.8: 0.6: 5) and the presence of DAP is a characteristic feature of *Pst. productus*.

In the present work group A, D and F are characterized by their ability to utilize a very limited number of amino acids (not more than two). Like Group E, all strains in these groups except Group F are carbohydrate fermenters.

Group A and F:

These groups joined a similarity level of 94.5, each being quite distinct from Group D at 88.6% of similarity. The results from this study indicate that the strains of *Goffkya (G.) anaerobia* in Group A and those of *Veillonella (V) parvula* in group F were able to utilize threonine and serine, but the strains of *G. anaerobia* differ from *V. parvula* in their ability to utilize arginine. On the other hand, the cell wall components of V. parvula differ from those of *G. anaerobia* in containing aspartic acid and ornithine. These findings are consistent with the proposal of Rogosa and Holdman et al.[17,18] Although Rogosa did not recognize *G. anaerbia* in the family *Peptococcaceae*, he classified *V. parvula* in the family *Veillonellaceae* on the basis of Gram reaction.[17] According to Holdman et al, *G. anaerobia* differs from *V. parvula* in its ability to retain Gram stain and ferment carbohydrate.[18]

It seems safe to assume therefore that arginine utilization is a useful character for the separation of the organisms within group A and F.

Group D:

Members of Group D differ from those in Group A and F in their inability to use threonine. Although species of *Str. constellatus* shared a similarity of 94.5% with those of Group D, they were distinguishable in their ability to degrade histidine. Whilst the species of *Pc. saccharolyticus*, *M. elsdenii* and *Str. intermedius* shared a 98.5%, S. since they utilize serine and arginine, they could not be separated from one another.

On balance, with respect to the molar ratios of the amino acids and amino sugar components

of the cell walls, members of group D together with the species of *Pc. glycinophilus* unlike other GPAC, contained high amounts of alanine. In general all species in this group except *Str. constellatus* strain (ATCC 27573) and *M. elsdenii* possessed an identical composition. Therefore, it seems likely that those organisms belong to a separate family. However, these organisms are easily identified on the basis of their utilization of carbohydrate, although their taxonomic position is still confusing. *Pc. saccharolyticus* is related to coagulase negative staphylococci and not to the genus peptococcus according to the cell wall analysis and the DNA-DNA homology studies.[17,18,23] It is now widely considered that the species of *Str. constellatus*, *Str. intermedius*, and *Str. marbillorum* belonging to the genus Streptococcus, since they produce lactic acid as a major product from glucose, although *Str. constellatus* has been designated under the genus Peptococcus.[17,18]
M. elsdenii beling to the family Veillonellaceae,[17] whilst Holdman and co-workers[18] recognized *M. elsdenii* as Gram-variable cocci. The broad taxonomic conclusion which can be drawn from the present study is that Group D are distinct from the species of Peptococcus and Peptostreptococcus. From the clinical point of view, the species of Group D are usually isolated from clinical materials together with peptococci and peptostreptococcus and form a considerable confusion in their separation particularly with those species of the genus Peptostreptococcus which are carbohydrate fermenters. It should be recommended therefore that all these cocci should be identified in one scheme.

Group G:
In the present study, Group G was readily separated from all anaerobic cocci at a level of 50.5% S. This group contains three strains of *Pst. anaerobius* and possesses certain characteristics that are unusual for species of anaerobic cocci. These included the capacity to metabolize proline and leucine and the aromatic amino acids; tyrosine and phenylalanine and with production of GABA, in addition to threonine, serine, alanine, glycine with production of α-aminobutyric acid. The activity of *Pst. anaerobius* against tyrosine as reported by Lambert and Moss,[34] and leucine by Brits and Wilkinson[35] strengthen the present study.

The production of isocaproic acid in PYG medium and the utilization of leucine by the species of *Pst. anaerobius* possibly indicates constitutive enzymes of catabolism of these amino acids, since the utilization of leucine to isocaproic acid by anaerobes was reported by Elsden and Hilton.[21] In the current classification of GPAC this evidence agrees with the view of Smith (1975),[36] who recognized the species of *Pst. anaerobius* and *Pst. putridus* as different organisms, although Rogosa (1974)[17] considered many names such as *Pst. putridus* and *Pst. foetidus* as synonyms of *Pst anaerobes*, wheras Holdman et al. recognized *Pst. anaerobius* only.[18] Unfortunately, the strain *Pst. putridus* was not available during the course of this study. The separation of *Pst. anaerobius* is based on few characters including the detection of the short chain fatty acids by gas liquid chromatography, which are questionable.[37] Therefore, in order to provide clear separation of the species *Pst. anaerobius* as well as other members of GPAC which are non-carbohydrate fermenters, the amino acid utilization pattern is of value.

In conclusion, this study demonstrated that the metabolism of amino acids was useful in differentiating between the species of GPAC. The best results were obtained from those whose taxonomic position or biochemical reactions were in doubt. These include *Pc. asaccharolyticus*, *Pc. aerogenes, Pc. magnus, Pc. anaerobius, Pc. variabilis, Pc. niger* and *Pst. micros*. For those species which are easily recognized by carbohydrate fermentation, amino acid utilization was less discriminatory. It should be noted, however, that the most frequently isolated GPAC from clinical materials are those which have a high capacity to utilize amino acids. This study has shown that the carbohydrate fermenters are less frequently encountered in clinical specimens. This indicates that the species which are more active against amino acids are more commonly pathogenic, and this phenomenon should therefore be exploited to identify and classify GPAC.

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