An Approach to the Taxonomy of Gram-positive Anaerobic Cocci. 6. The Metabolism of Amino Acids

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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 these whose taxonomic position or biochemical reaction 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 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 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.

The conventional carbohydrate fermentation tests and other classical methods have proven unsuccessful in distinguishing between the various anaerobic cocci.[13-15] It was therefore necessary to examine other methods at 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 stand 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 of 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. The cophenetic correlation coefficients (r) were determined using the methods described by Sneath and Sokal. 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 theeronine (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 glutamc acid completely except two strains utilized more than 23.9% of that compound; Group B is 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. The cophenetic correlation coefficient for this dendogram was 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 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 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:

Group C:

Herein, Group C joined with group A, D, and F at a similarity level of 80.9% and separated from these groups at 87.7% S. The main character of Group C was the capacity to utilize glycine. This group includes the species of *Pc. magnus*, *Pc. anaerobius*, *Pc. variablilis*, *Pst. micros* and *Sarcina (S) veniriculi*. All organisms in Group C were able to utilize serine in addition to glycine. However, they vary in the utilization of threonine and arginine according to the species. The ability of *Pc. anaerobius* (variabilis) and *Pc. glycinophilus* to utilize glycine was reported by Douglas and Cardon et al. respectively and Dure and co-workers, which strengthen the present results.[28-35]

Rogosa considered *Pc. glycinophilus* as a synonym of *Pc. anaerobius*, since glycine was the only substrate utilized.[36] In contrast the present data showed that the species of *Pc. glycinophilus* differs from that of *Pc. magnus, Pc. anaerobius* and *Pc. variabilis* in the inability to utilize threonine. The cell wall of *Pc. glycinophilus* also differs from those organisms in containing serine and lower amounts of alanine and glycine. This is consistent with the findings of Durre et al that *Pc. glycinophilus* and *Pc. magnus* (anaerobius) are genotypically unrelated and are different in their nutritional requirements. The former grew optimally in the presence of 0.1% yeast extract, whereas *Pc. magnus* requires 1% yeast extract and Tween 80 in addition.[30] Therefore, the utilization of threonine is a sufficient characteristic to differentiate *Pc. glycinophilus*.

There is controversy as to whether *Pc. magnus, Pc. anaerobius* and *Pc. variabilis* are the same or different organisms. The name *Pc. anaerobius* was rejected by Holdman and co-workers since both species of GPAC; *Pc. anaerobius* and *Pst. anaerobius* share the same epithet anaerobius.[37] In contrast, Rogosa (1974) combined the species *Pc. variabilis* and *Pc. glycinophilus* under the name *Pc. anaerobius* whilst Skermand and co-workers considered the *Pc. variabilis* (ATCC 14956) as *Pc. magnus*.[36-38] It was found from the present study that the strains *Pc. anaerobius* (ATCC 14955); *Pc. variabilis* (ATCC 14956) and *Pc. magnus* (ATCC 29328) share a 98.5% S.; and they are unique in their utilization of amino acids and their cell wall composition. To overcome such obvious confusion therefore the epithet magnus is used for *Pc. variabilis* as proposed by Holdman and co-workers as well as for the strain anaerobius.[37]

The current classification of the species of *Pc. magnus* and *Pst. micros* is not clear, since those organisms are biochemically inert and

possess similar metabolic profiles, because their separation relied mainly on the cell size only.[37] The present results demonstrated that *Pst. micros* was distinguishable from *Pc. magnus* in its inability to utilize threonine and the ability to utilize arginine. Such separation is in accordance with the results of the cell wall analysis herein, since the main difference between the species *Pst. micros* and *Pc. magnus* is the occurrence of aspartic acid and ornithine in the former and lysine and serine in the latter. Such evidence will provide a much more reliable separation for those organisms.

Herein, the species *Pc. glycinophilus* was indistinguishable from *Pst. micros* on the basis of amino acid utilization although, the former species was isolated from mud. The present findings are in agreement with the recent study of Cato and co-workers.[39]

These workers have proposed that *Pc. glycinophilus* is a late subjective synonym of *Pst. micros*, because: 1. the soluble cellular protein of both strains is similar; according to the results of a poly-acrylamide electrophoretic analysis; 2. these species produced the same amounts of acid and alkaline phosphatase; 3. both cultures converted glycine to acetate, amonia and carbon dioxide in PY-1% glycine; 4. both strains possess a similar % G+C content (27% for *Pst. micros* and 28% for *Pc. glycinophilus*), and 5. the DNA of *Pc. glycinophilus* significantly hybridized with that of *Pst. micros* (84% homology). Thus, the distinction between these two organisms is blurred, but the present study showed that the cell walls of *Pc. glycinophilus* contained a higher amount of alanine and glycine and possessed lysine, whereas *Pst. micros* contained aspartic acid and ornithine. Therefore, such organisms need more clarification.

The inability of the strain *S. ventriculi* to utilize arginine divided it from *Pc. micros*, and there are many other characters which support their separation. *S. ventreculi* is a carbohydrate fermenting coccus. The base composition of *S. ventreculi* is 30.6.[36] This value is higher than the range 27-28 obtained from the determination of the species of *Pst. micros*.[39]

It was observed that the fresh isolates (13, 98) which identified as *Pc. prevotii* according to Holdman et al (1977) were different from each other and from the reference strain (ATCC 14952) in their utilization and production of amino acids.[37] Both strains of *Pc. prevotii* (13, 98) were clustered in Group A, however, on the basis of the utilization of amino acids strain 98 was clustered in Group C, whereas strain 13 clustered with group B.

However, in both cases the reference strain was clustered in Group B. This evidence supports the contention of Rogosa that *Pc. prevotii* provides a link between *Pc. asaccharolyticus* and *Pc. magnus* (anaerobius). Rogosa considered *Pc. prevotii* as nomina confusa, since the characteristic which differentiated *Pc. prevotii* and *Pc. asaccharolyticus* was the indole production.[36] On the other hand the species of *Pc. prevotii* and *G. anaerobia* were combined under the name Micrococcus Prevotii.

However, from these results *G. anaerobia* differs from *Pc. prevotii* in its inability to break down glutamic acid and histidine. According to Holdman and co-workers strains ascribed to *M. prevotii* represented two different organisms, the saccharolytic strains were classified as *G. anaerobi* and non-saccharolytic cultures as *Pc. prevotii*.[37]

These findings are consistent with the present results. It would therefore appear that the species of *Pc. prevotii* and *G. anaerobia* are quite different species.

Group B:

Group B did not separate from Groups A, C, D and F until a 75% level of similarity. This group is characterized by the ability to utilize at least 5 amino acids by each strain. Group B contains the species of *Pc. asaccharolyticus, Pc. magnus, Pc. indolicus* and *Pc. prevotii.* These strains shared a similarity of 89.4% and were able to utilize serine, glutamic acid, histidine, and tryptophan but varied in the utilization of threonine and arginine according to the species. The present data showed that *Pc. indolicus* was indistinguishable from *Pc. aerogenes*, on the basis of the utilization of amino acids. Holdman et al noted that *Pc. indolicus* differed from *Pc. aerogenes* and *Pc. asaccharolyticus* in the ability to coagulate plasma.[37]

The present study showed that *Pc. asaccharolyticus* (ATCC 14963) strains were able to degrade threonine, serine, glutamic acid and histidine.

From these results the strains of *Pc. asaccharolyticus* (ATCC 14963 and strain No. 25) were separated from the other organisms in Group B at similarity of 89.4% and characterized by their ability to utilize threonine. *Pc. asaccharolyticus* (ATCC 29743 and the fresh isolates 25, 26) were split from the strains of *Pc. aerogenes*, *Pc. indolicus* and

Pc. prevotii at a similarity of 94% since they were unable to utilize arginine. This is in contrast with Rogosa (1974) who stated that the strains of *Pc. asaccharolyticus* and *Pc. aerogenes* were unique in their metabolism of amino acids. However, the method used for this study was not the same as that of Rogosa.[36] In this study all test amino acids were incorporated in the medium, while Rogosa examined each amino acid individually.[36]

The quantitative results obtained during the study of the cell wall of GPAC showed that the species of Group B varied markedly from each. Speculation indicates that those organisms which are combined in a sole species of *P. asaccharolyticus* could be separated easily on the basis of their ability to utilize a battery of amino acids rather than the detection of the intermediate products, from a mixture of carbohydrate and amino acids. These byproducts are unstable and not necessarily of comparable taxonomic value in the absence of information on the mechanisms of their formation.

In conclusion, this study showed that the metabolism of amino acids was useful in differentiating between the species of Gram-positive anaerobic cocci. 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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