An Approach to the Taxonomy of Gram-positive Anaerobic Cocci. 2. Carbohydrate Fermentation

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Abstract:
An attempt was made to classify gram-positive anaerobic cocci (GPAC) on the basis of their production of fatty acids from the fermentation of mannose, galactose and rhamnose by the use of gas liquid chromatography (GLC). The only species that could be separated were *Peptostreptococcus* (*Pst.) anaerobius* and *Megasphaera (M.) elsdenii*. Unfortunately the other species were indistinguishable using information obtained from the GLC analysis.

Introduction:
The first report of micro-organisms arranged in packets was in 1842.[1] However, the first isolation of the Gram-positive anaerobic cocci (GPAC) was, in 1893, from a case of supportive 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, (Table 1).[12-15]

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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 fermentation test and other classical methods have proven unsuccessful in distinguishing between the various anaerobic cocci.[15]

The objective of this study was to investigate the end products of fermentation of some sugars other than glucose such as galactose, rhamnose and mannose by GPAC in order to assess their potential role in taxonomy of GPAC.

Materials and Methods:

The sources of cultures were a total of 116 strains of GPAC isolated from clinical materials in St. Luke's Hospital, Guilford and St. Thomas's Hospital, London. All specimens were routinely examined for anaerobic bacteria. The reference strains, 24, were obtained from the American Type Culture Collection (ATCC), Virginia Polytechnic Institute and State University (VPI) and the National Collection of Industrial Bacteria (NCIB), (Table 2).

The specimens were streaked onto freshly prepared blood agar plates using a swab or a sterile platinum loop. The remainder of the specimen was incubated in chopped meat medium. The inoculated blood agar plates were placed in anaerobic jars. An inoculated blood agar with a culture of *Clostridium (Cl.) tetani* was incubated in the anaerobic jar to act as a control for adequate anaerobiosis.[15] A modified sachet containing platinum catalyst was reactivated each time the jar was used by heating to 160° for 90 minutes.

The anaerobic jars were prepared by the use of the evacuation-replacement method.[16,17] In this method the anaerobic jar was filled with freshened catalyst. A tube of freshly prepared methylene blue indicator was placed in the loaded jar. The jar was sealed and evacuated to an internal pressure of about -440 mmHg by water pump. After three series of partial evacuation the anaerobic jar was filled with a gas mixture containing 90% hydrogen and 10% carbon dioxide and left for 25 minutes. Since the catalysis creates a reduced pressure within the jar due to decrease in the amount of gases, the valve to the mixed gas supply was reopened and the jar was filled with mixed gas and incubated. The colourless appearances of the methylene blue in the indicator capsule and the presence of swarming growth in the *Cl. tetani* plate, were an indication of good anaerobiosis in the jar.

After 6 days' incubation, the various types of colonies were examined and marked. On the basis of primary screening using the Gram stain, the colonies of GPAC were picked, inoculated into chopped meat media (C.M.) and incubated for 24 hours. The cultures of GPAC were transferred to the microbiological laboratory in Surrey University for further study.

All strains were plated in parallel on three Brain Heart Infusion Agar (BHIA); one of which was incubated aerobically, the second in candle jar, and the third in an anaerobic atmosphere.

All plates in different atmospheres were incubated at 37°C for 6 days. After this period

of incubation, the strains were accepted as GPAC if they had grown only anerobically. One pure colony was picked and used for subsequent inoculation of C.M. medium and for Gram stain. The inoculated C.M. medium was incubated in an anaerobic cabinet overnight, stoppered firmly, and the culture was preserved at 4°C until needed.

The identification of the isolates was based on the method described by Holdeman et al, in the Virginia Polytechnic Institute Laboratory Manual of Anaerobes.[18] Accordingly, GPAC were identified on the basis of morphology, biochemical examination and the detection of the end products from the fermentation in peptone yeast extract glucose or chopped meat glucose media by means of Gas Liquid Chromotography (GLC).

Carbohydrate fermentation tests included glucose, galatose, rhamnose and mannose. The basal medium was peptone yeast extract containing 0.5 g for both peptone and tripticase; 1.0 g yeast extract; and 0.4 ml salt solution per 100 ml of distilled water.[18] The basal medium was supplemented, per 100 ml. with 0.5 µg of vitamin K_1; 0.5 mg of haemin; 0.05 g of cysteine hydrochloride as reducing agent; 0.0001 g of resazurin as an indicator of anaerobiosis; and 0.02 ml of Tween-80. All sugars were added to the basal medium in a final concentration of 1%.

A total of 47 cultures of GPAC were tested for their fermentation of glucose, galactose, rhamnose and mannose in peptone yeast extract. The products of carbohydrate fermentation volatile and nonvolatile fatty acids were detected by gas liquid chromatography.

Results:

Forty-seven organisms were examined for the production of the volatile and nonvolatile fatty acids from the fermentation of different sugars; glucose, galactose, rhamnose and mannose in peptone yeast extract medium. As reflected in Table 2 and 3, the end-products produced from the tested carbohydrate in the basal medium gave almost similar results to those of glucose for the isolates of each species. The results of all acids were reproducible except the following compounds, propionic, pyruvic and succinic acids. Acetic acid was the only consistent volatile fatty acid in the culture of *Pc. magnus, Pc. saccharolyticus, Pst. productus, Pst. micros* and *Str. intermedius,* whereas acetic acid together with butyric acids were observed in the cultures of *G. anaerobia, Pc. asaccharolyticus* and *Pc. prevotii. Pst. anaerobius* and *M. elsdenii* were unique in the production of acetic, isobutryic, butyric, isovaleric and isocaproic acids. However, *M. elsdenii* differs from *Pst. anaerobius* in the production of valeric and caproic acids.

Discussion:

An attempt was made to classify GPAC on the basis of their production of fatty acids from the fermentation of mannose, galactose and rhamnose by use of GLC. The only species that could be separated were *Pst. anaerobius* and *M. elsdenii*. Unfortunately, the other species were indistinguishable using the information obtained from GLC analysis.

It is quite interesting to note that although certain species of GPAC are asaccharolytic, they still produce fatty acids in the medium. The development of end-products highlighted certain problems associated with the classification of GPAC, providing evidence that these organisms are biochemically active. They probably use compounds other than carbohydrates such as amino acids and other nitrogenous compounds (such as purines), all of which are supplements in the medium. The fermentation of carbohydrates and amino acids by anaerobes yield fatty acids. For example the species of *Cl. tetanosporum, Pc. glycinophilus* and *Pc. variablilis* produced acetic acid from glycine.[19,20] The production of butyric acid from histidine and threomine by *Cl. propionicum, Pc. aerogenes, M. lactilyticus* and *Cl. tetanosporum* was reported by several workers.[16,21]

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Table 2: The production of volatile fatty acids from glucose, galactose, rhamnose and mannose by 47 species of AGPC[1]

Speicies	Tested strains	Glucose								Galactose								Rhamnose								Mannose							
		A	Pc	ib	B	iv	v	ic	c	A	Pc	ib	B	iv	v	ic	c	A	Pc	ib	B	iv	v	ic	c	A	Pc	ib	B	iv	v	ic	c
G. anaerobia	2	2	1	0	2	0	0	0	0	2	0	0	2	0	0	0	0	2	0	0	2	0	0	0	0	2	0	0	1	0	0	0	0
M. elsdenii	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
Pc. asaccharoltticus	4	4	2	0	4	0	0	0	0	4	1	0	4	0	0	0	0	4	1	0	3	0	0	0	0	4	1	0	4	0	0	0	0
Pc. magnus	8	8	6	1	0	0	0	0	0	8	0	0	0	0	0	0	0	3	3	0	0	0	0	0	0	8	3	0	0	0	0	0	0
Pc. prevotii	6	6	4	0	6	0	0	0	0	6	2	0	5	0	0	0	0	6	2	0	6	0	0	0	0	6	3	0	6	0	0	0	0
Pc. saccharolyticus	2	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0	2	0	0	0	0	0	0	0
Pst. anaerobius	10	10	7	9	9	10	0	9	0	10	10	9	10	8	1	8	0	10	7	9	9	10	1	9	0	10	5	9	9	7	0	10	0
Pst. micros	4	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0	4	0	0	0	0	0	0	0
Pst. productus	5	5	3	0	0	0	0	0	0	5	1	0	0	0	0	0	0	5	1	0	0	0	0	0	0	5	4	0	0	0	0	0	0
Str. Intermedius	5	5	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	5	0	0	0	0	3	0	0

Symbols: A = acitic acid; Pc = propionic acid; ib = isobutyric acid; B = butyric acid; iv = isovaleric acid; v = valeric acid; ic = isocaproic acid; c = caproic acid;
I = number of srtrains positive.

Table 3: The production of nonvolatile fatty acids from glucose, galactose, rhamnose and mannose by 47 species of AGPC[1]

Speicies	Tested strains	Glucose			Galactose			Rhamnose			Mannose		
		Py	La	S	Py	La	S	Py	La	S	Py	La	S
G. anaerobia	2	0/2	2/2	2/2	0/2	1/2	1/2	0/2	1/2	1/2	0/2	1/2	1/2
M. elsdenii	1	0/1	1/1	1/1	0/1	1/1	1/1	0/1	1/1	1/1	1/1	1/1	1/1
Pc. asaccharoltticus	4	0/4	3/4	3/4	0/4	2/4	3/4	0/4	4/4	4/4	0/4	4/4	4/4
Pc. magnus	8	0/8	8/8	7/8	0/8	7/8	4/8	0/8	8/8	8/8	0/8	5/8	8/5
Pc. prevotii	6	1/6	6/6	6/6	0/6	6/6	6/6	0/6	6/6	6/6	0/6	6/6	6/6
Pc. saccharolyticus	2	0/2	2/2	1/2	0/2	2/2	2/2	0/2	2/2	1/2	0/2	2/2	2/2
Pst. anaerobius	10	2/10	10/10	8/10	0/10	9/10	9/10	0/10	10/10	10/10	0/10	10/10	9/10
Pst. micros	4	0/4	4/4	4/4	0/4	4/4	4/4	0/4	0/4	4/4	0/4	4/4	4/4
Pst. productus	5	0/5	5/5	3/5	0/5	5/5	5/5	0/5	5/5	5/5	0/5	4/5	4/5
Str. Intermedius	5	0/5	5/5	4/5	0/5	5/5	4/5	0/5	5/5	5/5	0/5	5/5	2/5

Symbols: Py = pyruvic acid; La = lactic acid; S = succinic acid. 1 = number of strains positive/number of strains tested.

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