Revista Latinoamericana de Microbiología **(2001) 43:27-35**
Asociación Latinoamericana de Microbiología

Identification of Anaerobic Nonsporeforming Gram-Positive Bacilli by Biochemical Tests and Gas-Liquid Chromatography

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ABSTRACT. There are many methods to identify anaerobic nonsporeforming bacilli: histological, bacteriological (biochemical test, microsystem API 20 A), serological, cell wall composition analysis, molecular methods and gas-liquid chromatography (GLC). A comparison between biochemical tests and gas-liquid chromatography was made in this study for the identification of this group of microorganisms. GLC conditions were established with the aid of reference strains. These conditions were then applied to ten strains which were previously identified by biochemical tests. Strains were grown in PYG broth and fermentation end products were analyzed, volatile and non volatile fatty acids. Their qualitative determination was made by comparing the retention time of known standards and the chromatographic pattern of reference strains. In addition, a semiquantitative analysis was made. The results of identification by biochemical tests were: five strains belonged to *Actinomyces* genus; three were *Propionibacterium acnes*; one *Propionibacterium granulosum* and one *P. propionicum*. By the GLC only seven strains were identified: four were *Actinomyces* and three *P. acnes.* Only six strains showed identification correlation by both biochemical tests and GLC. GLC is a presumptive identification method that can be used along with other complementary tests for a definitive identification at genus level.
Keywords: Anaerobes, Identification, gas-liquid chromatography

RESUMEN. Hay varios métodos para la identificación de los bacilos anaerobios Gram positivos no esporulados: histológicos, bacteriológicos (pruebas bioquímicas en tubo, microsistema API 20 A), serológicos, análisis de la composición de la pared celular, métodos moleculares y cromatografía gas-líquido (CGL). En este estudio se hizo una comparación entre las pruebas bioquímicas y la cromatografía de gases para la identificación de este grupo de microorganismos. Se establecieron las condiciones para la CGL usando cepas de referencia y se aplicaron para estudiar diez cepas problema, previamente identificadas por pruebas bioquímicas. Las cepas fueron cultivadas en caldo PYG y se analizaron los productos finales de fermentación de la glucosa, ácidos orgánicos volátiles y no volátiles. Se hizo su determinación cualitativa por comparación de sus tiempos de retención con los de patrones conocidos, el patrón cromatográfico de cepas de referencia y un análisis semicuantitativo. Los resultados de la identificación por pruebas bioquímicas fueron los siguientes: cinco cepas pertenecieron al género *Actinomyces*, tres fueron *Propionibacterium acnes*; una *Propionibacterium granulosum* y una *P. propionicum*. Por cromatografía de gases se identificaron solamente siete cepas: cuatro correspondieron a *Actinomyces* y tres a *P. acnes.* Sólo en la identificación de seis cepas hubo correlación entre las pruebas bioquímicas y la CGL. La CGL es un método de identificación presuntiva que puede usarse junto con otras pruebas complementarias para llegar a ser confirmativa a nivel de género.
Palabras claves: Anaerobios, Identificación, cromatografía gas-líquido

INTRODUCTION

Anaerobic nonsporeforming Gram-positive bacilli are found within section 15 of Bergey's Manual, where can be founded *Actinomyces, Arachnia, Propionibacterium, Bifidobacterium, Eubacterium* and *Rothia* genera included.[17] Until now, there have been several changes in the classification of this group, for example, *A. propionica* now is *Propionibacterium propionicum* within the genus *Actinomyces*; *A. israelii* serotype II was changed to *A. gerencseriae*; and *Bifidobacterium eriksonii* was changed to *B. dentium.*[9,15]

Actinomyces is involved in several pathological processes, one of them being actinomycosis which may be localized in the cervicofacial, thoracic, and abdominal regions. Sometimes it is associated with lachrymal canaliculitis, periodontal disease, caries, inflammatory pelvic disease, and pyogenic abscess of the liver. Also, *P. propionicum* may be isolated from subjects with actinomycosis and lachrymal canaliculitis. The presence of *Propionibacte-*

rium spp. has been reported in cases of uveitis, endophtalmitis, pulmonary abscesses; bone, joint, and central nervous system infections, endocarditis, and it is also associated with SAPHO (sinovitis, acne, pustulosis, hyperostosis, and osteomyelitis) syndrome.[4,5,6,15]

It can be observed that the frequency of these infections has increased, so it is important to identify the ethiological agents accurately, specially when the treatment has not produced the expected results.[4,5,11]

As it is known, there are different methods to identify the above mentioned bacteria. Among the most used ones are the bacteriological (biochemical tests in tube, API20A microsystem), serological, and histological methods, although the analysis of the cell wall composition, molecular biology tests, and the analysis of fermentation products of glucose are useful.[7,10,18,19]

Identification by biochemical tests, particularly for this group of microorganisms, is not final, even though a big group of substrates were used.[7]

In serological identification the fluorescent antibody technique is employed. This technique is fast although presumptive, specially when monoclonal antibodies are not used.

It is worth to note that, when cell wall composition is used for the identification, it is necessary to analyze the sugars and aminoacids by paper or thin layer chromatography. It is important to remember that the cell wall must be free of contaminants.[16]

Other authors have used the gas-liquid chromatography method (GLC) to identify anaerobic microorganisms. This is done by taking advantage of their capacity to produce metabolites, such as organic acids and alcohols, produced by glucose fermentation.[2,3,10,14]

This methodology is sensitive, fast, and useful for the final identification to genus level, as well as of great value to determine the species, since less biochemical tests are used (compared to the traditional method). This cause savings of time and costs. Furthermore, it is of great value in the definitive identification of anaerobic non spore forming Gram-positive bacilli.[3,6,11,13] However, it is necessary to remember that, in order to identify them by this method, the following information must be available: Gram stain, presence or absence of spores, cell morphology, oxygen susceptibility, and production of catalase and indol.

After knowing the characteristics of the several methods, the this work establishes the use of GLC and biochemical tests methods for the identification of a group of anaerobic nonsporeforming Gram-positive bacilli, correlating the results obtained by both of them.

MATERIAL AND METHODS

Reference and isolated strains. *Actinomyces israelii* CDCW838 Ser2; *Actinomyces naeslundii* CDC X454; *Actinomyces odontolyticus* ATCC 17929*; Propionibacterium acnes* CDC 14369; *Propionibacterium acnes* ATCC 6919.

Test strains were isolated from tonsils with the following codes: 1015 ENCB; 1028 ENCB; 1033 ENCB; 1016 ENCB ; 1010 ENCB; 1031 ENCB; 1011 ENCB; 1012 ENCB; 1003 ENCB; 1003 (small colony*)* ENCB.

Recovery and identification of the strains. Lyophilized strains were rehydrated in Tioglycolate broth NIH (Bioxon No. 284-1). Strains were grown in tripticase soy agar (Bioxon No. 108-1) to observe catalase production, and GC agar (BBL No. 11275) enriched with isovitalex (BBL No. 11875) to determine macroscopic and microscopic morphology, and the influence of oxygen in its growth. The identification of the test strains was made by conventional biochemical tests and by GLC. In GLC the production of organic acids was detected. All solid media were incubated at 37°C in anaerobic conditions using the Gas-Pak system (BBL No. 70304).

The tests carried out were: carbohydrate fermentation (adonitol, arabinose, glycerol, rafinose, sucrose, and xylose) in tioglycolate medium without indicator (Bioxon No. 245-1), indol production, nitrate to nitrite reduction, and esculin and gelatin hydrolysis. The anaerobic conditions were obtained by using screw cap tubes (16 x 150 mm) sealed with mineral oil and incubated at 37°C during 3 to 7 days.

Organic acids production and extraction. Production of organic acid test was made in peptone-yeast extract-glucose (PYG) broth as follows: from a culture in medium GC, a collect was made in 1 ml of PYG until turbidity was equal to that of tube No 5 of McFarland nephelometer. With the collected material a culture in PYG was made and after growth, the broth was centrifuged. The extraction and methylation procedures were made according to Rizzo[14] with some modifications: 1g of NaCl, 0.1 ml of heptanoic acid (52.72 mg/ml), and 0.15 ml of 50% H_2SO_4 were added to 4 ml of supernatant and the extraction was made with ether in order to obtain the volatile organic acids. To extract the non volatile ones, 0.1 ml of benzoic acid (24.4 mg/ml) and 4 ml of methanol were added to 2 ml of supernatant the mixture was put on dry ice for 45 min and centrifuged. To the supernatant 0.8 ml of 50% H_2SO_4 was added and the mixture was refluxed at 80ºC for 30 min. Two ml of distilled water were added and the mixture was extracted with chloroform.

Gas-liquid chromatography analysis. The analysis of the organic acids was carried out in a gas chromatograph (Perkin Elmer Sigma 3B) equipped with a flame ionization detector (FID). The carrier gas was N_2 (20 ml/min). The analysis of organic acids was made by using 12% FFAP on Chromosoto W/AW 80-100 mesh packed in a glass column (180 cm; internal diameter, 3 mm). Aliquots of the ethereal extract (6 µl) and chloroformic extract (5 µl) were injected. For volatile organic acids (VOA) the oven temperature was 100°C for 2 min and was increased then by increments of 39°C/min to the final temperature of 215°C for 4 min; For non volatile organic acids (NVOA) the oven temperature was 60°C for 2 min and it was then raised 22°

C/min up to 215°C for 6 min. The inyection port temperature was 220°C for the VOA and 160°C for NVOA-methyl esters, and the detector temperature was 250°C.

Each time that chromatographic analysis of the acids produced by a strain was done, a chromatographic profile of a control tube was made. Control tubes consisted of PYG broth without inoculum.

For the identification of organic acids, a reference mixture containing 1 meq (in 100 ml of distilled water) of each of the following acids: acetic, propionic, butyric, isovaleric, and heptanoic acid for the reference solution of VOA; and lactic, succinic, and benzoic acid for the reference solution of NVOA. Before obtaining the chromatographic profile of these solutions, they were subjected to the same extraction procedure used for the culture supernatants of the strains.

Semiquantitative analysis of the organic acid production. There are diverse reports of the amounts of acids produced by the several strains employed in this study; so, here it was necessary to establish some semiquantitative criteria that allowed to define how much acid was being produced by a given strain. The way that production of the several acids was calculated, was to evaluate the relative height of each one of the acids produced by the strains with regard to the internal control (heptanoic or benzoic acid).

The production criteria based on the amount of acid produced expressed in relative height were: 0.0-0.01 (no production); 0.01-0.05 (traces); 0.05 - 0.20 (small production) and 0.20 or more production.

RESULTS

Bacteriological identification. Colonies of the reference strains of genus *Actinomyces* are smooth, convex, with complete regular edges, except for *A. israelii* which has dented edges. *P. acnes* shows smooth, pulvinar colonies with complete regular edges.

Colony morphology of the test strains, 1015, 1028, 1033, and 1016 was found to be like that of *A. naeslundii* and *A. odontolyticus*, while the test strains 1003, 1003 small colony, 1010, and 1031 showed a colony morphology similar to that of *P. acnes*. Test strains 1011, and 1012 showed different characteristics, their colonies were smooth, convex and with irregular edges.

The results of biochemical tests are shown in Table 1. Test strains 1015, 1028, and 1033 were identified only to genus level, while all other ones were identified to species level.

Analysis of the metabolites produced by GLC. In Fig. 1, the reference solutions of volatile, and non volatile organic acids are shown. Fig. 2 to 7 show the chromatographic profiles of the metabolites produced by one of the reference strains, and by two of the test strains, from a total of 15 strains tested. Each Fig. includes the chromatographic profile of the control (culture medium without inoculum).

Experimentally we found that PYG medium contains some contaminant substances in small amounts, which have similar retention times to those of some acids tested; thus, the sources of said substances were determined. It was found that yeast extract contains substances with retention times similar to those of acetic acid and succinic acid. On the other hand, peptone, yeast extract, and resazurine contain a substance with a retention time similar to that of lactic acid.

Semiquantitative analysis of metabolite production. Semiquantitative data of production of the diverse acids by the reference strains are shown in Table 2. As it can be noted, *A. naeslundii* produces a higher amount of lactic acid than *A. israelii* and *A. odontolyticus*. It also can be observed that *Actinomyces* does not produce propionic acid, but *Propionibacterium* does.

In Table 3 the semiquantitative data of the metabolites produced by the test strains are shown, as well as the identification of these strains based on such data. Test strains 1015, 1028, 1033, and 1016 have the same semiquantitative chromatographic profile than *Actinomyces* genus. Test strains 1003 small colony, 1010, and 1031 have a qualitative chromatographic profile that matches with that of *P. acnes*. Test strains 1011 and 1012 showed a high production of lactic and acetic acids, and traces of succinic acid. Finally, test strain 1003 produced high amounts of acetic, propionic, and succinic acid, but did not produce lactic acid. The results shown in Tables 2 and 3 were obtained in quadruplicate.

Correlation between identification by biochemical tests and GLC. A comparison between identification by biochemical tests and by GLC is shown in Table 4. Four different correlations were found:

a) Six strains showed identification correlation in both methods

b) One strain which identification does not match in both methods.

c) Two strains in which uncertainty existed by GLC, making impossible to establish a correlation with biochemical tests results.

d) One strain identified by biochemical tests, but could not be identified by GLC.

DISCUSSION

It is important to note that identification by biochemical tests of some genera such as *Actinomyces, Bifidobacterium, Eubacterium* and *Propionibacterium* is not conclusive, because they share many metabolic characteristics among them. Furthermore, the time required for a final result is 7 days. However, the advantage is that identification of the microorganism can be done until species level.

On the other hand, GLC technique has a high sensitivity as it detects very low concentrations of metabolites. The assay is carried out in approximately 56 h, including 48 h of incubation in PYG medium. However, in some

Table 1. Identification of the test strains by biochemical tests

Identification	Fermentation											
	Catalase	Adonitole	Arabinose	Glycerol	Glucose	Sucrose	Rafinose	Xylose	Indole production	Nitrate reduction	Esculine hydrolysis	Gelatin hydrolysis
Actinomyces sp. 1015 ENCB	-	-	+	-	+	+	-	-	-	+	+	-
Actinomyces sp. 1028 ENCB	-	-	-	-	+-*	+	-	-	-	+	+	-
Actinomyces sp. 1033 ENCB	-	-	+	-	+	+	-	-	-	+	+	-
Propionibacterium propionicum 1016 ENCB	-	+-	+	-	+	+	-	-	-	+	+	-
Propionibacterium acnes 1003 small ENCB.**	+	+	-	+	+	-	-	-	+	+	-	+
Propionibacterium acnes 1010 ENCB	+	+	-	+	+	-	-	-	+	+	-	+
Propionibacterium acnes 1031 ENCB	+	+	-	+	+	-	-	-	+	+	-	+
Actinomyces israelii 1011 ENCB	-	-	-	-	+	+	+	+	-	-	+	-
Actinomyces israelii 1012 ENCB	-	-	-	-	+	+	+	+	-	-	+	-
Propionibacterium granulosum 1003 ENCB	+	+	+-	+	+	+	+	-	-	-	-	-

*Scarcely positive ** Small colony

Table 2. Semiquantitative data of production of the diverse acids by the reference strains.

	A	P	L	S
A. israelii CDC W838 ser 2	Tr	No	Little	Yes
A. naeslundii CDC X454	Tr	No	Yes	Yes
A. odontolyticus TCC 17929	Tr	No	Little	Yes*
P. acnes ATCC 6919	Tr	Yes	Tr	Little
P. acnes CDC 14369	Tr*	Yes	Tr	Little*

A, Acetic acid; P, Propionic acid; L, Lactic acid; S, Succinic acid; *, Irregular; Tr, Traces (0.01-0-.05); Little, Little production (0.05-0.20); Yes, There is production (0.20 or more); No, There is not production.

Table 3. Semiquantitative data of production of the diverse acids by the test strains and their identification.

	A	P	L	S
Actinomyces sp. 1015 ENCB	Tr	No	Little	Yes
Actinomyces sp. 1028 ENCB	Tr	No	Little	Yes
Actinomyces sp. 1033 ENCB	Tr	No	Little	Yes
Actinomyces sp. 1016 ENCB	Tr	No	Little	Yes
P. acnes 1003 small colony ENCB	No	Yes	Little*	Yes
P. acnes 1010 ENCB	No	Yes	Little	Little
P. acnes 1031 ENCB	Tr	Yes	Little	Yes
Actinomyces sp. 1011 ENCB	Yes	No	Yes	Tr
Actinomyces sp. 1012 ENCB	Yes	No	Yes	Tr
Strain 1003 ENCB not identified by GLC	Yes	Yes	No	Yes

A, Acetic acid; P, Propionic acid; L, Lactic acid; S, Succinic acid; *, Irregular; Tr, Traces (0.01-0-.05); Little, Little production (0.05-0.20); Yes, There is production (0.20 or more); No, There is not production.

Table 4. Correlation of the results of identification by biochemical tests and gas-liquid chromatography (GLC).

Test strain	Biochemical tests	GLC
1015 ENCB	*Actinomyces* sp.	*Actinomyces* sp.
1028 ENCB	*Actinomyces* sp.	*Actinomyces* sp.
1033 ENCB	*Actinomyces* sp.	*Actinomyces* sp.
1016 ENCB	*Propionibacterium propionicum*	*Actinomyces* sp.
1003 Small ENCB	*Propionibacterium acnes*	*P. acnes*
1010 ENCB	*P. acnes*	*P. acnes*
1031 ENCB	*P. acnes*	*P. acnes*
1011 ENCB	*A. israelii* o *Bifidobacterium dentium*	*Actinomyces* sp.*
1012 ENCB	*A. israelii* o *B. dentium*	*Actinomyces* sp.*
1003 ENCB	*P. granulosum*	Not identified

*Qualitatively it does correspond to this genus, but semiquantitatively, does not.

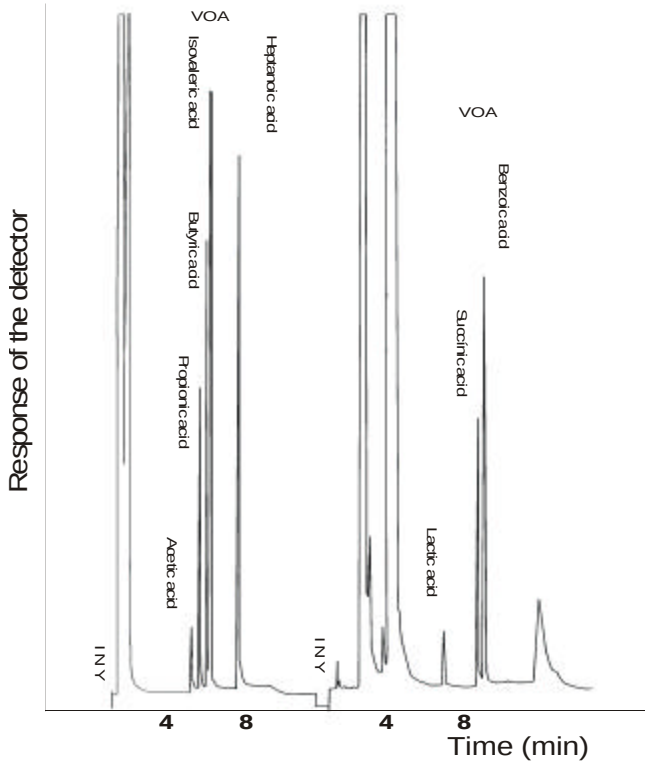

Fig. 1. Chromatographic profiles of reference solution of volatile organic acids (VOA) and no volatile organic acids (NVOA).

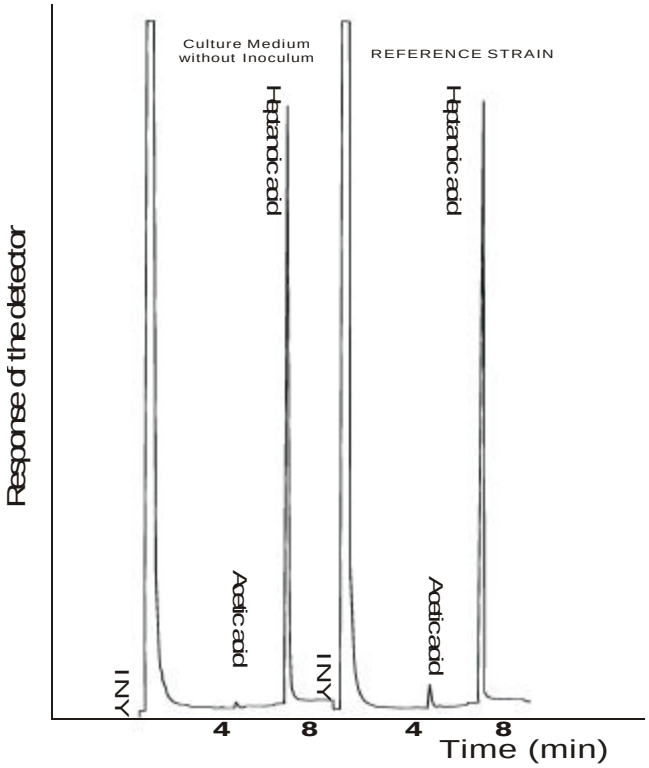

Fig. 2. Chromatographic profile of volatile organic acids produced by *A. Israelii* CDC W838 Ser 2 (Reference strain).

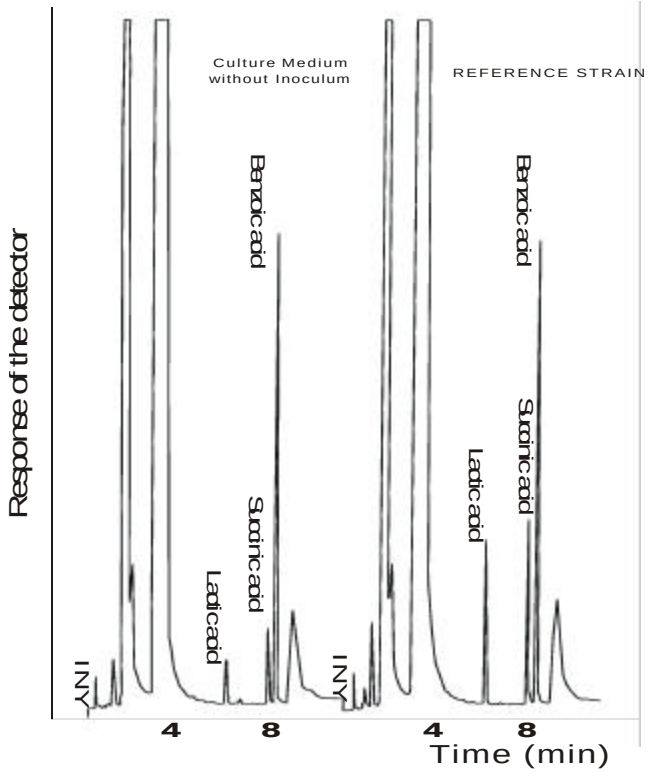

Fig. 3. Chromatographic profile of non volatile organic acids produced by *A. Israelii* CDC W838 Ser 2 (Reference strain).

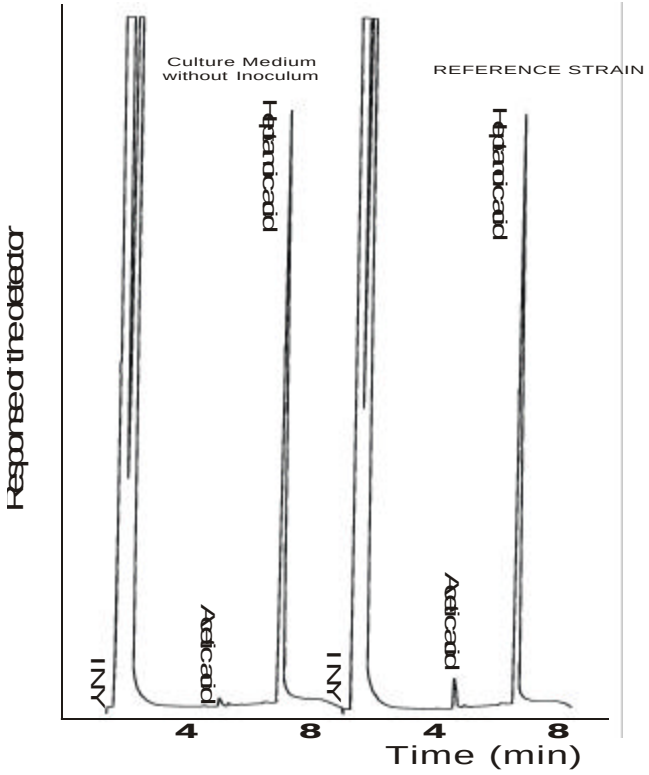

Fig. 4. Chromatographic profile of volatile organic acids produced by tested strain ENCB 1015.

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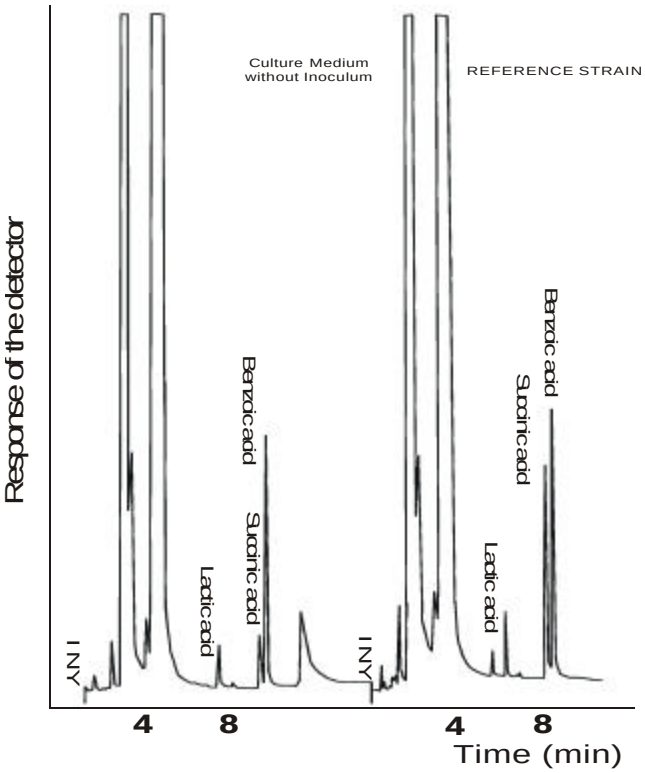

Fig. 5. Chromatographic profile of non volatile organic acids produced by tested strain, ENCB 1015.

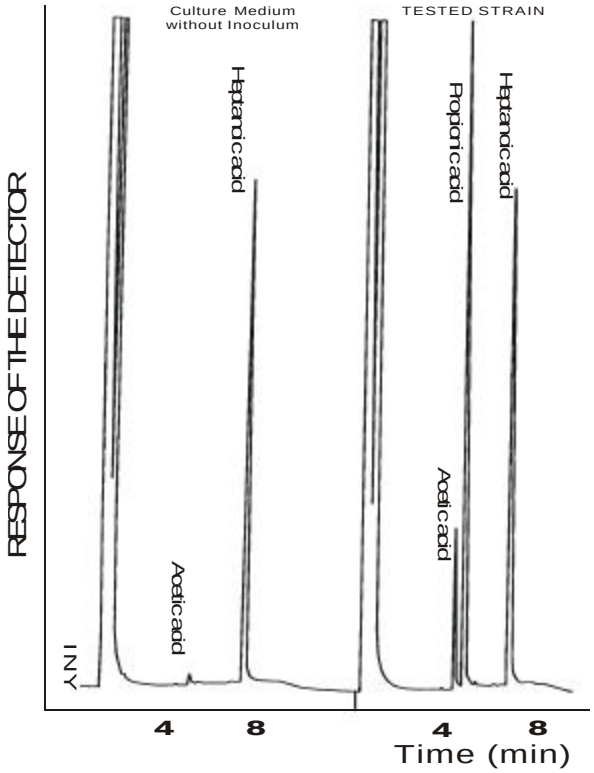

Fig. 6. Chromatographic profile of volatile organic acids produced by tested strain, ENCB, 1003.

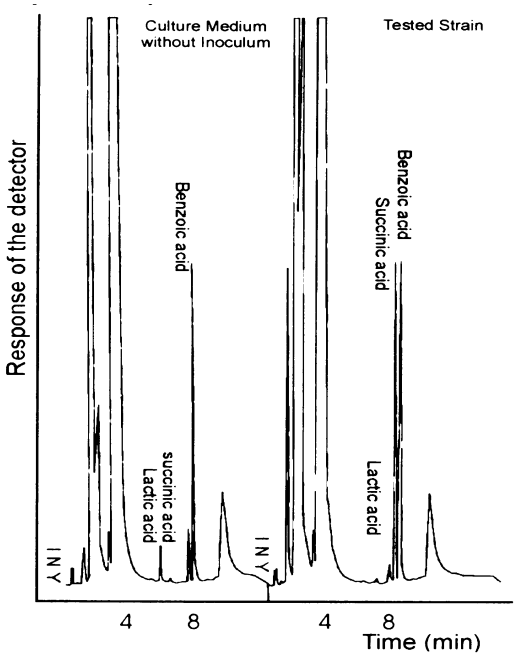

Fig. 7. Chromatographic profile of non volatile organic acids produced by tested strain, ENCB 1003

Fig. 7. Chromatographic profile of non volatile organic acids produced by tested strain, ENCB 1003

cases it must be considered as complementary to the traditional biochemical tests, if identification to species level is required.

In GLC other factors should be considered. There are reports that peptone and yeast extract of the culture medium (PYG) where metabolites are produced, contain acetic, lactic, and succinic acids.[1,2,3] In this study we observed that, in fact, peptone has a substance with the same retention time as lactic acid. Resarzurine and yeast extract also show it. In yeast extract small amounts of substances that have the same retention times that those of acetic and succinic acids show. This must be considered mainly when the results of acetic acid production are interpreted, as the recovery percentage is low (42.8%) (data not showed), and the production thereof by genera *Actinomyces* and *Propionibacterium* is only traces, as it was observed in the reference strains used. This may lead to a wrong interpretation of whether there is production or not of acetic acid. Due to this, it is necessary to investigate a way to reduce these impurities in the culture medium.

With regards to the identification by both of the methods, the following was found: in strain 1003, a mixed culture of two different strains was found. One of them was 1003 (small colony) strain, which was identified by biochemical tests as *P. acnes* (this will be discussed below), and the other one was identified, also by biochemical

tests, as *P. granulosum*. This last one showed a peculiar pattern by GLC because, in addition to production of propionic acid, it produced acetic and succinic acid (see Table 2) which does not correspond with the chromatographic profile of the reference strain *P. acnes* (Table 1). Besides, it seems to metabolize the substance with a retention time similar to that of the lactic acid present in the PYG medium. Due to these findings, it would have been necessary to determine the chromatographic profile of a reference strain of *P. granulosum* in order to compare it with that of this strain, although according to reports of Holdeman and col.[8], it does correspond to that identification.

On the other hand, Table 3 shows that the strains 1003 (small colony), 1010, and 31031 could be identified by GLC as well as by biochemical tests as *P.. acnes*. Note that for this species the information obtained by the chromatographic profile and the complementary tests (catalase, colony and microscopic morphology, influence of O_2 and indole) was enough to identify the test strain up to species level. According to the biochemical tests, the strains 1015, 1028, and 1033 belonged to genus *Actinomyces*, and it was not possible to identify the species because the tests chosen were not selective enough. For these strains, the information obtained by GLC allowed to identify them as *Actinomyces* as they had the same profile that the reference strains.

According to the results of the biochemical tests made in the strains 1011 and 1012, which were compared to those reported by Dowell and Hawkins[2] and Slack and Gerencser [16] , among others, both of the strains may either be *A. israelii* or *Bifidobacterium dentium*, so, more biochemical tests are required in order to differentiate between these two possibilities. By GLC, the strains 1011 and 1012 showed a profile, at qualitative level, similar to that of *Actinomyces*, but they are semiquantitatively different because in this study the reference strains of *Actinomyces* produced traces of acetic acid and a little lactic acid. However, the test strains produced a large amount of acetic and lactic acid. Due to this, there is a possibility that they belong to *Bifidobacterium* genus as in literature is reported that this genus produces mainly acetic and lactic acid.[6,15] This agrees with the results obtained. To confirm the identity of these strains by GLC, reference strains belonging to this genus must be available so that comparisons of the chromatographic profiles can be done.

The strain 1016 was identified as *P. propionicum* by biochemical tests, and as *Actinomyces* by GLC. This discrepancy can be explained if we consider that, at biochemical test level, there are only two differential tests, adonitol and esculin hydrolysis, and if the reaction is scarcely positive (see Table 1), it is easy to draw a wrong conclusion. In this case, the GLC confirms the genus, as according to the literature *P. propionicum* produces propionic acid, and *Actinomyces* does not.[2,8,12]

Although the number of strains is small, it can be observed that there is a correlation between both methods in 6 of the 10 strains studied. In addition, by the GLC technique and with the support of some biochemical tests (catalase production, colony and microscopic morphology, response to O_2, and indole), it was possible to identify 7 of the 10 test strains to genus level.

GLC together with the above-mentioned complementary biochemical tests made possible the confirmatory identification, at genus level, of anaerobic nonsporeforming gram positive bacilli, with the advantage that this can be done in less time 56 h. The traditional methodology used for the identification of this group of bacteria is troublesome, time consuming, and some times the results are difficult to interpret if the species of the microorganism is to be determined.

AKNOWLEDGMENTS

EGC was a fellowship of COFAA, IPN.

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