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

PATRICIA CAUICH-SANCHEZ, FELIPE ALATRISTE-MONDRAZON, ELSA GARCIA-CANO,* CARLOS AQUINO-SANTIAGO

Departamento de Microbiología, Escuela Nacional de Ciencias Biológicas. Carpio y Plan de Ayala. Apartado Postal 4-862, México, D. F.

*Corresponding author E-mail pcauich@yahoo.com. Fax (5) 7296207

ABSTRACT. There are many methods to identify anaerobic nonsporforming 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

INTRODUCTION

Anaerobic nonsporforming 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. gerencierce; 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 canalculitis, periodontal disease, caries, inflammatory pelvic disease, and pyogenic abscess of the liver. Also, P. propionicum may be isolated from subjects with actinomycosis and lachrymal canalculitis. The presence of Propionibacte-
Identification of Anaerobic Bacilli by GLC

Test strains were isolated from tonsils with the following codes: 1015 ENCB; 1028 ENCB; 1032 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 Tri glycolate broth NIH
(Bioxon No. 284-1). Strains were grown in triplicate 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 micro-
scopic 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, glyc erol, rafinose, sucrose, and y-
lose) in tri glycolate medium without indicator (Bioxon
No. 245-1), indol production, nitrate to nitrite reduction,
and esculin and gelatin hydrolysis. The anaerobic con-
ditions 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 with
some modifications: 1g of NaCl, 0.1 ml of heptanoic
acid (52.72 mg/ml), and 0.15 ml of 50% H₂SO₄ 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 super-
natant the mixture was put on dry ice for 45 min and cen-
trifuged. To the supernatant 0.8 ml of 50% H₂SO₄ was
added and the mixture was refluxed at 80°C 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 N₂ (20 ml/min). The
analysis of organic acids was made by using 12% FFAP on
Chromos oto W/AW 80-100 mesh packed in a glass col-
umn (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°
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.

Colonies 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. included the chromatographic profile of the control (culture medium without inoculum).

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.

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

<table>
<thead>
<tr>
<th>Identification</th>
<th>Catalase</th>
<th>Adonitole</th>
<th>Arabinose</th>
<th>Glycerol</th>
<th>Glucose</th>
<th>Sucrose</th>
<th>Raffinose</th>
<th>Xylose</th>
<th>Indole production</th>
<th>Nitrate reduction</th>
<th>Esculine hydrolysis</th>
<th>Gelatin hydrolysis</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Actinomyces sp.</em> 1015 ENCB</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Actinomyces sp.</em> 1028 ENCB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+<em>-</em></td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Actinomyces sp.</em> 1033 ENCB</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Propionibacterium propionicum</em> 1016 ENCB</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em> 1003 small ENCB</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em> 1010 ENCB</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Propionibacterium acnes</em> 1031 ENCB</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td><em>Actinomyces israelii</em> 1011 ENCB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Actinomyces israelii</em> 1012 ENCB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td><em>Propionibacterium granulosum</em> 1003 ENCB</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

*Scarcely positive**   **Small colony
### Table 2. Semiquantitative data of production of the diverse acids by the reference strains.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>P</th>
<th>L</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. israelii CDC W838 ser 2</td>
<td>Tr</td>
<td>No</td>
<td>Little</td>
<td>Yes</td>
</tr>
<tr>
<td>A. naeslundii CDC X454</td>
<td>Tr</td>
<td>No</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>A. odontolyticus TCC 17929</td>
<td>Tr</td>
<td>No</td>
<td>Little</td>
<td>Yes*</td>
</tr>
<tr>
<td>P. acnes ATCC 6919</td>
<td>Tr</td>
<td>Yes</td>
<td>Tr</td>
<td>Little</td>
</tr>
<tr>
<td>P. acnes CDC 14369</td>
<td>Tr*</td>
<td>Yes</td>
<td>Tr</td>
<td>Little*</td>
</tr>
</tbody>
</table>

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.

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>P</th>
<th>L</th>
<th>S</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actinomyces sp. 1015 ENCB</td>
<td>Tr</td>
<td>No</td>
<td>Little</td>
<td>Yes</td>
</tr>
<tr>
<td>Actinomyces sp. 1028 ENCB</td>
<td>Tr</td>
<td>No</td>
<td>Little</td>
<td>Yes</td>
</tr>
<tr>
<td>Actinomyces sp. 1033 ENCB</td>
<td>Tr</td>
<td>No</td>
<td>Little</td>
<td>Yes</td>
</tr>
<tr>
<td>Actinomyces sp. 1016 ENCB</td>
<td>Tr</td>
<td>No</td>
<td>Little</td>
<td>Yes</td>
</tr>
<tr>
<td>P. acnes 1003 small colony ENCB</td>
<td>No</td>
<td>Yes</td>
<td>Little*</td>
<td>Yes</td>
</tr>
<tr>
<td>P. acnes 1010 ENCB</td>
<td>No</td>
<td>Yes</td>
<td>Little</td>
<td>Little</td>
</tr>
<tr>
<td>P. acnes 1031 ENCB</td>
<td>Tr</td>
<td>Yes</td>
<td>Little</td>
<td>Yes</td>
</tr>
<tr>
<td>Actinomyces sp. 1011 ENCB</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Tr</td>
</tr>
<tr>
<td>Actinomyces sp. 1012 ENCB</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>Tr</td>
</tr>
<tr>
<td>Strain 1003 ENCB not identified by GLC</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

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).

<table>
<thead>
<tr>
<th>Test strain</th>
<th>Biochemical tests</th>
<th>GLC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1015 ENCB</td>
<td>Actinomyces sp.</td>
<td>Actinomyces sp.</td>
</tr>
<tr>
<td>1028 ENCB</td>
<td>Actinomyces sp.</td>
<td>Actinomyces sp.</td>
</tr>
<tr>
<td>1033 ENCB</td>
<td>Actinomyces sp.</td>
<td>Actinomyces sp.</td>
</tr>
<tr>
<td>1016 ENCB</td>
<td>Propionibacterium propionicum</td>
<td>Actinomyces sp.</td>
</tr>
<tr>
<td>1003 Small ENCB</td>
<td>Propionibacterium acnes</td>
<td>P. acnes</td>
</tr>
<tr>
<td>1010 ENCB</td>
<td>P. acnes</td>
<td>P. acnes</td>
</tr>
<tr>
<td>1031 ENCB</td>
<td>P. acnes</td>
<td>P. acnes</td>
</tr>
<tr>
<td>1011 ENCB</td>
<td>A. israelii o Bifidobacterium dentium</td>
<td>Actinomyces sp.*</td>
</tr>
<tr>
<td>1012 ENCB</td>
<td>A. israelii o B. dentium</td>
<td>Actinomyces sp.*</td>
</tr>
<tr>
<td>1003 ENCB</td>
<td>P. granulosum</td>
<td>Not identified</td>
</tr>
</tbody>
</table>

*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.
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. In this study we observed that, in fact, peptone has a substance with the same retention time as lactic acid. Resazurine 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 indolet) 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 *Actino- myces* 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.\(^5,15\)

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.\(^2,8,15\)

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.

**REFERENCES**

58:151-155.


