CHARACTERIZATION OF AN OROTIC ACID FERMENTING BACTERIUM, ZYMUBACTERIUM OROTICUM, NOV. GEN., NOV. SPEC.

J. T. WACHSMAN AND H. A. BARKER

Department of Plant Biochemistry, University of California, Berkeley, California

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Orotic acid has been found to function as a growth factor for Neurospora mutants (Loring and Pierce, 1944) and has been implicated in the pyrimidine biosynthesis of Lactobacillus bulgaricus (Wright et al., 1951) and liver (Weed and Wilson, 1951). These findings have focused considerable attention on the metabolism of this uracil derivative. An orotic acid fermenting organism was isolated in this laboratory from bay mud by Dr. Arthur Kornberg by the use of standard enrichment culture techniques (Lieberman and Kornberg, 1953). The latter investigators (Lieberman and Kornberg, 1954) demonstrated the conversion of ureidosuccinic acid to orotic acid and therefore the reversibility of the first two reactions involved in orotic acid degradation.

The work reported in this paper was designed to characterize this organism for purposes of classification. It has resulted in the proposal of a new genus, Zymobacterium, to accommodate rod shaped, anaerobic members of the family Lactobacteriaceae that form ethanol and carbon dioxide as the major end products of glucose fermentation.

METHODS

The orotic acid fermenting organism was at all times grown in the presence of a pyrogallol-sodium carbonate seal at 30 to 37 C. An orotic acid basal medium consisting of orotic acid, 0.2 per cent neutralized with NaOH; yeast extract, 0.025 per cent; sodium thioglycolate, 0.05 per cent; phosphate buffer (0.05 M) pH 7.4; MgSO₄·7H₂O, 0.02 per cent; FeSO₄·2H₂O, 0.0005 per cent; MnSO₄·4H₂O, 0.0005 per cent; Na₂MoO₄·2H₂O, 0.0005 per cent; and agar, 0.1 per cent was found to support adequate growth. The addition of agar is essential for growth on this medium.

Tryptone-glucose-yeast extract (TGY) broth containing tryptone, 2.0 per cent; glucose, 0.50 per cent; yeast extract, 0.05 per cent; sodium thioglycolate, 0.05 per cent; phosphate buffer (0.05 M) pH 7.4; and the above salts supports vigorous growth of this bacterium in the absence of agar. This broth was used in the following tests: gelatin liquefaction (12 per cent gelatin), nitrate formation (0.1 per cent KNO₃), starch hydrolysis (0.1 per cent soluble starch), and liquefaction of egg albumin (cubes of coagulated egg albumin). The following media were used to check indole production: tryptone-yeast extract containing 0.1 per cent tryptophan, the above medium with 0.5 per cent glucose, and the above medium with 0.2 per cent orotic acid. Kovás reagent was used to test for indole production, sulfanilic acid, and α-naphthylamine for nitrite formation, and Lugol's iodine solution for starch hydrolysis (Conn, 1949).

Skim milk containing sodium thioglycolate, 0.05 per cent, and brom thymol blue, 0.002 per cent, adjusted to pH 7.0, was used to determine the ability of the organism to coagulate casein. The media used for testing the utilization of various carbon sources are described in table 1.

Orotic acid concentration was measured spectrophotometrically. A solution of orotic acid at pH 7.4, containing 1 μg per ml, has an optical density of 6.88 at 280 μν. Ammonia was determined by titration after distillation from sodium borate solution.

Carbon dioxide was determined manometrically by the method of Peters and van Slyke (1932). Glucose was determined by the method of Folin and Malmros (1929), and lactic acid by a modification (Umbreit et al., 1945) of the Barker and Summersorn procedure (1941). Steam distillation and titration were used for the determination of total volatile acids. Volatile acids were identified by paper chromatography with the ammonia-ethanol solvent of Kennedy and Barker (1951) and detected by the use of the brom thymol blue spray of Brown (1950). The Pirie (1946) method was used for quantitative formic acid analysis. When both acetate and

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formate were present in the volatile acid fraction, acetate was estimated by difference.

Ethanol was determined by microdiffusion (Widmark, 1922). Glycerol and 2,3-butanediol were estimated by the procedure of Voris et al. (1940) after removal of ionic substances by means of Dowex anion and cation exchange resins. Ethanol was identified by oxidation of the neutral volatile fraction with acid dichromate and characterization of the resulting acetic acid by the Duelaux method. No carbonyl compounds were present in the neutral volatile fraction derived from a glucose culture.

RESULTS

Morphology. The orotic acid fermenting organism forms long bead-like chains of gram positive rods when grown on an orotic acid basal medium or on tryptone-glucose-yeast extract broth (figure 1). Young cultures are characterized by short rods (1.2 to 2.0 µ in length by 0.35 to 0.60 µ in diameter) with tapering ends, whereas the cells of older cultures appear coccoid. The organism is nonmotile and grows in the form of a rather granular sediment in liquid media. Spore formation has never been observed. When grown on tryptone-glucose-yeast extract agar under strictly anaerobic conditions, small, round, convex colonies are formed.

Cultural characteristics. This bacterium fails to grow on the surface of a tryptone-glucose-yeast extract agar slant in the presence of air and appears quite exacting in its demand for anaerobiosis. Excellent growth was obtained in the presence of a sulfhydryl reducing agent when protected from oxygen by a pyrogallol-carbonate seal. Cells from a 72 hour culture on tryptone-glucose-yeast extract agar failed to produce visible bubbles of oxygen when mixed with 3 per cent hydrogen peroxide; the organism is therefore catalase negative.

Tests for liquefaction of gelatin or coagulated egg albumin, hydrolysis of starch, and production of nitrite or indole were negative. The test media, however, did support excellent growth. No growth could be detected microscopically in the milk medium after 14 days of incubation.

Data on the ability of various carbon sources to support growth are summarized in table 1.

Utilization of orotic acid. The organism was grown on the orotic acid basal medium (0.2 per cent orotic acid), and the residual orotic acid was determined by measuring the optical density at 280 nm. Orotic acid concentration fell from an initial value of 12.8 µM to 1.1 µM per ml after 7 days of incubation.
TABLE 2

Products of glucose fermentation

<table>
<thead>
<tr>
<th>COMPOUNDS</th>
<th>GLUCOSE</th>
<th>NO GLUCOSE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>m\mu per ml</td>
<td>m\mu per ml</td>
</tr>
<tr>
<td>Glucose, fermented</td>
<td>20.0</td>
<td>—</td>
</tr>
<tr>
<td>Products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactate</td>
<td>2.1</td>
<td>1.1</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>25.2</td>
<td>4.0</td>
</tr>
<tr>
<td>Ethanol</td>
<td>25.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Formate</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Acetate</td>
<td>11.5</td>
<td>4.6</td>
</tr>
</tbody>
</table>

Carbon recovery, per cent...73

A more rapid decomposition of orotic acid was obtained with a medium consisting of tryptone, 0.5 per cent; yeast extract, 0.05 per cent; sodium thioglycolate, 0.05 per cent; phosphate buffer (0.05 M) pH 7.4; salts; and orotic acid, 0.2 per cent. After inoculation the decrease in optical density at 280 m\mu was measured against inoculated samples of the same medium without orotic acid. All of the substrate (12.8 m\mu per ml) was decomposed by the third day of incubation. Ammonia formation in the presence of orotic acid was 23.0 m\mu per ml, whereas 2.9 m\mu of ammonia per ml were formed in the absence of orotic acid. Assuming that the difference is the result of the breakdown of orotic acid, 1.57 m\mu of ammonia are formed per m\mu of orotic acid used.

Fermentation of glucose. In a preliminary experiment glucose was found to be decomposed very slowly in a medium consisting of salts, yeast extract, 0.09 per cent; glucose, 1.0 per cent; and sodium thioglycolate, 0.05 per cent. The rate of decomposition could be increased by the use of more complex media. The medium finally selected (table 2) was a compromise between that with a high yeast extract-peptone concentration in which glucose is used rapidly, and that with a low concentration in which glucose is used slowly.

Data on the fermentation products formed in the presence and absence of glucose are presented in table 2. Titration of the ether extractable nonvolatile acid fraction indicated that no fixed acid other than lactic acid is formed in appreciable amounts from glucose. Neither glycerol nor 2,3-butylen glycol could be detected in the neutral nonvolatile fraction by oxidation with periodate; residual glucose accounted for the observed periodate reduction. Only trace amounts of acetic or diacetyl were found by the procedure of Westerfeld (1945).

The relatively low carbon recovery (73 per cent) reported in table 2 is calculated on the assumption that the utilization of glucose does not inhibit the breakdown of amino acids and available substrates. If amino acid decomposition is suppressed completely, the calculated carbon recovery would be 88 per cent. It is possible that one or more minor fermentation products have been overlooked.

At least 59 per cent of the carbon of glucose can be accounted for as carbon dioxide and ethanol, and a maximum of 5 per cent as lactic acid. Since the material giving a positive test for lactic acid in the Barker and Summerson procedure (1941) was not characterized further, it cannot be stated conclusively that lactic acid is a product of the fermentation. Several other compounds are known to react like lactic acid under conditions of this colorimetric determination.

DISCUSSION

The morphology and gram positive staining of the orotic acid fermenting bacterium indicate that it belongs in either the family Lactobacteriaceae or Corynebacteriaceae, as defined by Breed et al. (1948). Although the distinction between these families is not clear-cut, we favor placing the organism in the Lactobacteriaceae because the description of this family is more definitive and because the rather uniform shape and staining properties of the organism agree more closely with the description of this family. Within the family, the organism obviously falls in the tribe Lactobacilleae because of its rod shape.

The main problem is the choice of a genus in which to place the orotic acid fermenting organism. In the Lactobacilleae there are four
genera, *Lactobacillus*, *Propionibacterium*, *Butyribacterium*, and *Microbacterium*. The latter genus is eliminated from consideration because it is defined to include aerobic, catalase positive bacteria, whereas our organism is an obligate anaerobe and does not produce catalase. The organism is excluded from the genera *Propionibacterium* and *Butyribacterium* by its inability to produce either propionic or butyric acids. Placement of the organism in the genus *Lactobacillus* also appears undesirable because it ferments sugars feebly and produces little if any lactic acid from glucose. The yield of ethanol, 1.3 moles per mole of glucose fermented, is considerably larger than that observed with the heterofermentative *Lactobacillus* and *Leuconostoc* species, which produce a maximum of one mole per mole of glucose (DeMoss et al., 1951).

Since the orotic acid fermenting organism cannot be placed properly in any of the genera of the Lactobacillaceae as described by Breed et al. (1948), we are faced with the choice of leaving the organism unnamed or of creating a new genus to accommodate it. We have decided on the latter alternative.

The name *Zymobacterium* is chosen for the new genus because it implies an ability to carry out an alcoholic fermentation and also harmonizes with the nomenclature of associated genera. The genus is defined as follows: Rods. Nonsporeforming. Gram positive. Anaerobic or microaerophilic. Ferment carbohydrates. The main products of glucose fermentation are ethanol and carbon dioxide. Catalase negative.

The type species is *Zymobacterium oroticum*, nov. sp., having the following characteristics: Rods, 0.35 to 0.60 by 1.2 to 2.0 μ, with tapering ends, usually in long intertwined chains. Nonmotile. Anaerobic. Glucose, fructose, galactose, sucrose, lactose, maltose, arabinose, mannitol, and orotic acid are fermented in a complex medium. Glutamate, lactate, and glycerol are not attacked. In addition to ethanol and carbon dioxide, small amounts of acetate, and possibly lactate and formate, are formed from glucose.

**SUMMARY**

An orotic acid fermenting anaerobic bacterium has been characterized and placed in the new genus *Zymobacterium* as the species *Z. oroticum*, nov. sp. The new genus, which is placed tentatively in the family Lactobacteriaceae, is defined so as to include gram positive, anaerobic, nonsporeforming rods that form predominantly ethanol and carbon dioxide from glucose.

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