SPORE-BEARING LACTIC ACID BACTERIA
ISOLATED FROM RHIZOSPHERE

II. TAXONOMIC STUDIES ON THE
CATALASE-NEGATIVE STRAINS

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Seven strains of catalase-negative, spore-bearing, lactic acid-producing bacteria were isolated from rhizosphere of various wild plants. They are mesophilic, facultatively anaerobic, gram-positive rods with peritrichous flagella and oval endospores at terminal to sub-terminal, and produce dl-lactic acid from glucose, fructose, sucrose, trehalose, and inulin in the homo-fermentative way, lowering the pH value of the medium to 3.8-3.2.

Among the hitherto known organisms, only Sporolactobacillus inulinus Kitahara et al. may be the closely related species to the new isolates.

From the rhizosphere of wild plants, catalase-positive and -negative strains of spore-bearing lactic acid bacteria were isolated. Descriptions of the former organisms were given in the preceding paper (1).

In this paper, catalase-negative strains, which can hardly be distinguished from Lactobacillus Beijerinck except for sporulation and flagellation, are described and a new genus is suggested in order to classify the catalase-negative spore-bearing facultative anaerobes, which can neither be listed as a member of Bacillus Cohn nor Clostridium Prazmowski.

MATERIALS AND METHODS

The same as described in the preceding paper (1).

RESULTS

Isolation of Bacteria

Seven cultures of the following strain numbers and origins were obtained:

1 The material presented in this paper is a part of the thesis submitted by Ooki Nakayama to the University of Tokyo, in partial fulfillment of the requirements for the degree of Doctor of Agricultural Science, and was in part presented at the Annual Meeting of the Kanto Division of the Agricultural Chemical Society of Japan, October 11, 1966.
M-3 Soil around the root of a wild vetch (*Vicia sepium* L.)
M-10 a horsetail (*Equisetum arvense* L.)
M-11 a plantain (*Plantago major* L.)
M-12 a styptic grass (*Hydrocotyle rotundifolia* Roxb.)
M-13 an emerald grass (*Omphalodes krameri* Franch)
M-16 a dandelion (*Taraxatum platycarpus* Dahlst)
M-17 a starwort (*Aster indicus* L.)

**Morphology**

Cells grown on the yeast extract-peptone-glucose broth are rod shaped with rounded ends, sometimes slightly bent, especially in M-3, M-13, and M-17. Usually not in chains. Dimensions are given in Table 1. Motile with

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vegetative cells</th>
<th>Spores</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Width (μm)</td>
<td>Length (μm)</td>
</tr>
<tr>
<td>M-3</td>
<td>0.4-0.7</td>
<td>3.0-5.0</td>
</tr>
<tr>
<td>M-10</td>
<td>0.5-0.6</td>
<td>1.5-5.0</td>
</tr>
<tr>
<td>M-11</td>
<td>0.7-0.8</td>
<td>2.5-3.5</td>
</tr>
<tr>
<td>M-12</td>
<td>0.7-0.8</td>
<td>1.2-4.5</td>
</tr>
<tr>
<td>M-13</td>
<td>0.4-0.6</td>
<td>1.5-4.0</td>
</tr>
<tr>
<td>M-16</td>
<td>0.4-0.7</td>
<td>2.0-3.5</td>
</tr>
<tr>
<td>M-17</td>
<td>0.4-0.7</td>
<td>1.5-4.0</td>
</tr>
</tbody>
</table>

**Fig. 1.** Spore-bearing lactic acid bacteria. M-16 cells grown on glucose broth for 18 hr at 30°. Stained by Toda's method.

**Fig. 2.** Spore-bearing lactic acid bacteria. M-16 cells grown on glucose broth for 36 hr at 30°. Stained by Toda's method.

**Fig. 3.** Spore-bearing lactic acid bacteria. M-3 cells and sporangia on synthetic medium with 10% glucose, incubated for 5 days at 30°. Stained by phenol-Fuchsin. Phase contrast.

**Fig. 4.** Spore-bearing lactic acid bacteria. M-16 cells and sporangia on yeast extract-peptone-starch agar, incubated for 4 days at 30°. Stained by phenol-Fuchsin. Phase contrast.

**Fig. 5.** Spore-bearing lactic acid bacteria. M-17 sporangia on yeast extract-peptone-trehalose broth, incubated for 7 days at 30°. Stained by phenol-Fuchsin.
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peritrichous, sometimes polar-like flagella (Figs. 1 and 2). Gram-positive to variable. No capsules.

Spores are ellipsoidal, situated at terminal or subterminal. Sporangia are distinctly swollen (Table 1, Figs. 2-5).

**Cultural Features**

Fermentable glucids are necessary for growth, both in broth and solid cultures.

Glucose-agar plates: Small white colonies are formed; in the presence of calcium carbonate, each colony is surrounded by a distinct transparent halo.

Glucose-agar slants: Growth is scant to moderate, translucent to white, with butyrous consistency.
Spore-bearing Lactic acid Bacteria Isolated from Rhizosphere

Glucose-agar stab: Growth moderate, filiform.
Semi-solid media: Vigorous swarming along the stick line with neither aerophilic nor aerophobic tendencies.
Glucose broth: Uniform turbidity with silky waves followed by flocculent sediments are noted. Lactic acid is accumulated until pH value of the broth becomes 3.8-3.2.

Physiology

Indole, hydrogen sulfide, or gas production, nitrate reduction, catalase activity, and chromogenesis are not detected. The other characteristics are shown in Table 2.

Fermentation of Glucose. Fermentation products from glucose are listed in Table 3. It is apparent that the isolates are homo-fermentative without exception.

<table>
<thead>
<tr>
<th>Strains</th>
<th>RG (%)</th>
<th>CG (%)</th>
<th>TA (%)</th>
<th>VA (%)</th>
<th>NA (%)</th>
<th>VA/TA (%)</th>
<th>LA (%)</th>
<th>AA (%)</th>
<th>LA/C (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M- 3</td>
<td>0.95</td>
<td>8.35</td>
<td>0.901</td>
<td>0.012</td>
<td>0.889</td>
<td>1.3</td>
<td>8.00</td>
<td>0.07</td>
<td>95.8</td>
</tr>
<tr>
<td>10</td>
<td>4.06</td>
<td>5.24</td>
<td>0.580</td>
<td>0.008</td>
<td>0.572</td>
<td>1.4</td>
<td>5.15</td>
<td>0.04</td>
<td>98.2</td>
</tr>
<tr>
<td>11</td>
<td>4.69</td>
<td>4.61</td>
<td>0.497</td>
<td>0.013</td>
<td>0.484</td>
<td>2.6</td>
<td>4.36</td>
<td>0.08</td>
<td>94.5</td>
</tr>
<tr>
<td>12</td>
<td>3.21</td>
<td>3.09</td>
<td>0.333</td>
<td>0.003</td>
<td>0.330</td>
<td>0.3</td>
<td>2.97</td>
<td>0.02</td>
<td>96.1</td>
</tr>
<tr>
<td>13</td>
<td>0.00</td>
<td>9.30</td>
<td>1.031</td>
<td>0.009</td>
<td>1.022</td>
<td>0.9</td>
<td>9.19</td>
<td>0.05</td>
<td>98.8</td>
</tr>
<tr>
<td>16</td>
<td>1.02</td>
<td>8.28</td>
<td>0.924</td>
<td>0.003</td>
<td>0.921</td>
<td>0.3</td>
<td>8.29</td>
<td>0.02</td>
<td>100.1</td>
</tr>
<tr>
<td>17</td>
<td>3.91</td>
<td>5.39</td>
<td>0.572</td>
<td>0.009</td>
<td>0.563</td>
<td>1.5</td>
<td>5.07</td>
<td>0.05</td>
<td>94.0</td>
</tr>
<tr>
<td>Control</td>
<td>9.30</td>
<td>0.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Abbreviations: RG, residual glucose; CG, glucose consumed; TA, total acids; VA, volatile acid; NA, non-volatile acid calculated; LA, lactic acid; AA, acetic acid.

<table>
<thead>
<tr>
<th>Strains</th>
<th>H₂O (%)</th>
<th>ZnO (%)</th>
<th>Rotation</th>
<th>Lactic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>M- 3</td>
<td>18.12</td>
<td>27.25</td>
<td>dl</td>
<td>DL</td>
</tr>
<tr>
<td>10</td>
<td>17.95</td>
<td>27.41</td>
<td>dl</td>
<td>DL</td>
</tr>
<tr>
<td>11</td>
<td>18.07</td>
<td>27.07</td>
<td>dl</td>
<td>DL</td>
</tr>
<tr>
<td>12</td>
<td>18.03</td>
<td>27.31</td>
<td>dl</td>
<td>DL</td>
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<tr>
<td>13</td>
<td>18.10</td>
<td>27.29</td>
<td>dl</td>
<td>DL</td>
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<tr>
<td>16</td>
<td>18.04</td>
<td>27.26</td>
<td>dl</td>
<td>DL</td>
</tr>
<tr>
<td>17</td>
<td>18.02</td>
<td>27.28</td>
<td>dl</td>
<td>DL</td>
</tr>
</tbody>
</table>

Glucose-agar stab: Growth moderate, filiform.
Semi-solid media: Vigorous swarming along the stick line with neither aerophilic nor aerophobic tendencies.
Glucose broth: Uniform turbidity with silky waves followed by flocculent sediments are noted. Lactic acid is accumulated until pH value of the broth becomes 3.8-3.2.

Table 3. Fermentation products from glucose.

Table 4. Optical characteristics of lactic acid produced.
Isomeric Type of Lactic Acid. Contents of crystal water and zinc oxide, and optical rotation of zinc lactate are given in Table 4.

Aerobic Metabolism of Glucose. Metabolic products from glucose in shaken culture are given in Table 5. Neither increase in volatile acids nor decrease in lactic acid compared with the stand culture can be found except in the case of strain M-17.

Titratable Acidity and Cell Population. Acid production and viable cell counts of the isolates grown in the glucose broth containing 2% of sodium acetate are given in Table 6. It is noted that the strains M-11, -13, -16, and -17 produce more than three times as much acid as B. coagulans P-22,
which produces 0.48% of lactic acid in the same condition.

Salt Tolerance. Salt tolerance expressed in the titratable acidity of glucose broth containing various amounts of sodium chloride are shown in Table 7. Salt tolerance of these strains are comparable to the catalase-positive members described in the preceding paper (1).

Sporulation. Degree of sporulation on various media is presented in Table 8. It can be seen that the catalase-negative strains sporulate less actively than the catalase-positive members described in the preceding paper (1).

**DISCUSSION**

**Identification**

There is no doubt that these isolates should be classified into the family Bacillaceae Fischer, because of the rod-shaped cells, sporulation, and peritrichous flagellation. Sub-dividing system of the family Bacillaceae, however, is somewhat different with individual workers.

In the Bergey’s Manual (2) and KRASSILNIKOV’s Handbook (3), two genera, *Bacillus* and *Clostridium*, are found under the family Bacillaceae. In PREVOT’s Handbook (4), there is an additional genus *Plectridium*, which was incorporated into genus *Clostridium* in Bergey’s Manual and into genus *Bacillus* in KRASSILNIKOV’s Handbook.

Genus *Plectridium* Fischer, which was proposed for the strict anaerobes forming terminal spore of such a special shape as seen in tetanus rods, is not related to our isolates because our isolates are not strict anaerobes and their spores are sometimes situated at the sub-terminal.

Genus *Clostridium* Prazmowski, represented by the type species *Cl. butyricum* Prazmowski or *Cl. pasteurianum* Winogradsky, is characterized by the following points.

Morphology: Swollen sporangia and granulose reaction.
Cultural features: Strictly anaerobic or aero-tolerant.

Physiology: Gas and volatile acids or solvents under anaerobic conditions, or proteolytic.

In Bergey's Manual, granulose reaction is not emphasized that Clostridium tetani and related species were entered into this genus. On the other hand, cultural features were ignored by Krassilnikov, so that B. polymyxa and related organisms were counted as members of genus Clostridium in his Handbook.

Our isolates are characterized as follows:

Morphology: Swollen sporangia but no granulose reaction.

Cultural features: Neither strictly anaerobic nor aerotolerant, but can grow on the surface of a glucose-agar under atmospheric pressure, as well as any members of genus Lactobacillus Beijerinck. Catalase negative.

Physiology: Neither gas, volatile acids, nor solvents, but only lactic acid under anaerobic conditions, not proteolytic.

It can be pointed out that only two features, i.e., swollen sporangia and not very aerophilic nature, are common between our isolates and the genus Clostridium. It is difficult to identify them with any species of the genus Clostridium according to either Bergey’s Manual, in which anaerobic nature was accepted as the generic key, or Krassilnikov’s key, in which granulose

Table 8. Degree of sporulation in relation to the culture media.

<table>
<thead>
<tr>
<th>Yeast extract (1%)-peptone (1%) broth</th>
<th>M-3</th>
<th>M-10</th>
<th>M-11</th>
<th>M-12</th>
<th>M-13</th>
<th>M-16</th>
<th>M-17</th>
</tr>
</thead>
<tbody>
<tr>
<td>without carbohydrates</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>with glucose (0.2%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; (1%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>&quot; (10%)</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>with dextrin (1%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>with soluble starch (1%)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>&quot; &quot; , CaCO₃</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td>++</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

Yeast extract (1%)-peptone (1%)-agar slant

without carbohydrates                 | -   | -    | -    | -    | -    | -    | -    |
| with glucose (0.2%)                   | -   | -    | -    | -    | -    | -    | -    |
| " (1%)                               | -   | -    | -    | -    | -    | -    | -    |
| with soluble starch, CaCO₃            | +   | +    | ++   | +    | +    | +    | +    |

Synthetic broth for lactic acid bacteria, with CaCO₃

with glucose (0.2%)                   | -   | -    | +    | ++   | -    | -    | +    |
| " (10%)                              | +   | -    | -    | -    | -    | -    | -    |

For abbreviations see footnote to Table 2.
reaction was emphasized.

Another genus, *Bacillus* Cohn, represented by the type species *B. subtilis* Cohn emend. Prazmowski, was characterized as aerobic, catalase-positive, and generally proteolytic members in Bergey’s Manual. Although Krassilnikov dealed *Bacillus* as a heterogeneous genus, in which any spore-forming rods other than *Clostridium* can be included, it may be considered as only a conventional way.

Our isolates, which are catalase-negative and not proteolytic, cannot be identified as any species of genus *Bacillus* according to Bergey’s Manual.

From the above-mentioned considerations, the authors prefer to set a new genus in order to classify our isolates. As to the known species, *Sporolactobacillus inulinus* Kitahara et Suzuki may be classified in this new genus together with the present organisms.

*Descriptions of Catalase-negative Strains.*

Strains: M-3, M-10, M-11, M-12, M-13, M-16, and M-17.

- Rods, 0.3 to 0.8 by 1.7 to 5.0 μ, some slightly bent, with ends rounded, usually not in chains. Motile with peritrichous flagella, which sometimes appear polar-like. Gram-positive to variable. Do not capsulate.
- Spores, 0.9 to 1.4 by 1.2 to 2.1 μ, ellipsoidal, terminal to sub-terminal.
- Sporangia definitely swollen.
- Gelatin stab: No change in general. Variation: Slow liquefaction (M-10).
- Glucose-agar colonies: White, pin-point. Chalk dissolved.
- Agar slants: Scant or no growth.
- Glucose-agar slants: Scant to moderate growth, translucent to white, butyrous.
- Glucose-agar stab: Growth moderate, filiform.
- Broth: Scant or no growth.
- Glucose broth: Turbid. Sediment flocculent. pH 3.8 to 3.2.
- NaCl broth: Growth in 2% NaCl. Scant or no growth in 5%.
- Milk: Slowly acidified and coagulated. Casein not hydrolyzed. Variation: No acid (M-3, M-10 and M-16).
- Potato: No growth.
- Indole not produced.
- Acid but no gas from glucose, fructose, maltose, sucrose, dextrin, inulin, and trehalose. Acid from galactose, mannose, lactose, glycerol, sorbitol, or mannitol by certain strains; no acid from α-methylglucoside.
- Starch not hydrolyzed.
- Anaerobic, facultative.
- Temperature relations: Optimum, about 30°. Maximum, between 40 and 45° or 30 and 40° (M-3 and M-11).
- Source: Isolated from rhizosphere of wild plants.
- Habitat: Perhaps distributed in soil.
- Specific characters: Facultatively anaerobic, catalase negative. No growth
on media without carbohydrates. pH of glucose, sucrose, or inulin broth becomes less than 4.0. Racemic lactic acid is produced from glucose in the homofermentative way. Motile, sporangia swollen.

**Relations to the Other Bacteria**

*Sporolactobacillus inulinus*, which was proposed as a sub-genus of *Lactobacillus* by Kitahara et al. (6), is only hitherto known bacteria to be thought as closely related species to our organisms.

The major differences between our isolates and *Sporolactobacillus inulinus* Kitahara et al. (6) are as follows: (1) The former produce dl-lactic acid in the main but the latter produces l-rotatory lactic acid. (2) The former lowers pH value of glucose broth to below 3.8, but the latter cannot lower the pH value less than 4.4.

The other related organism is *Bacillus racemilacticus* Nakayama et Yanoshi, which is distinguished from the present organisms in the catalase activity, aerobic metabolism, and the shape of sporangia.

If sporulation and flagellation were ignored, *Bacillus coagulans* (5) and *Sporolactobacillus inulinus* Kitahara et al. (6) would be similar to *Lactobacillus thermophilus* or almost similar to *L. leichmannii*, respectively. On the other hand, our isolates could be identified with an inulin-fermenting variant of *L. plantarum* if spores and motility were overlooked.

In the case of our organisms, it was so difficult to detect spores unless yeast extract-starch-agar was used as the sporulation medium that we had considered them as motile lactobacilli during the early days. It will be reasonable, therefore, to compare our organisms with hitherto known motile lactobacilli, e.g., motile *L. pentoaceticus* reported by Fred et al. (7) and Weinstein et al. (8), *L. delbrueckii* by Vankova (9), *L. casei* and its related organisms by Deibel et al. (10) and Langton et al. (11), *L. plantarum* and its related organisms by Cunningham et al. (12), Hays et al. (13), Harrison et al. (14), Mann et al. (15), Gemmell et al. (16), and Nonomura et al. (17), and short rods by Thornley et al. (18).

Among them, motile *L. pentoaceticus*, *L. delbrueckii*, *L. casei*, and Thornley’s organisms are easily distinguished from the vegetative stage of our isolates by the type of fermentation, optimum temperature and catalase, isomeric type of lactic acid produced, growth on media free from carbohydrates, and final pH of glucose broth.

Although minor differences can be pointed out, such as growth at 45° and excessive production of L(+-)-lactic acid in Harrison and Hansen’s and Mann and Oxford’s organisms, the motile strains of *L. plantarum* are the most related organisms to our isolates among the non-spore formers. For further considerations, isomeric type of lactic acid produced by our organisms before isomerization must be determined.

Any species of genus *Lactobacillus* are apparently more closely related to our organisms than any species of *Pediococcus*, *Streptococcus*, or *Leuconostoc*,
which are now classified into the family Lactobacillaceae together with *Lactobacillus*.

If physiological characteristics were more emphasized than morphology, our organisms should, therefore, be classified into the family Lactobacillaceae. However, there is no reason to exclude our organisms from the family Bacillaceae. If our organisms are excluded from Lactobacillaceae, it is quite reasonable, that a question would naturally arise as to whether *Streptococcus*, *Leuconostoc*, and *Pediococcus* as well may stay in the family Lactobacillaceae or not. This problem will be discussed elsewhere.

The authors thank Prof. Emeritus K. Sakaguchi, Profs. K. Arima, and H. Iizuka of the University of Tokyo, Prof. K. Kitahara of the Tokyo Agricultural University, and Prof. Y. Ohara of Yamanashi University, for their helpful suggestions and encouragements.

REFERENCES