Prototrophic Thermophilic Bacillus: Isolation, Properties, and Kinetics of Growth

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A thermophilic bacillus (minimal growth temperature 41°C, optimal 55 to 58°C, and maximal 65°C) was isolated from a manure pile. It is very similar to Bacillus stearothermophilus, but it differs in its inability to hydrolyze starch. The thermophilic isolate is a prototroph which grows in a minimal medium consisting of glucose, ammonium salt, phosphate buffer, and inorganic salts. At all temperatures studied (low to high), the same minimal nutritional requirements prevailed. The Arrhenius constant for growth was found to be 15,000 and 13,500 cal/mole in the minimal and rich media, respectively.

Thermophilic bacteria have been found almost everywhere (1, 14), including deep layers in the ocean (6, 7) and arctic regions (8).

Nutritional requirements of many thermophilic strains have been investigated and frequently found to be complex (2, 4, 5, 9, 10). Successful cultivation at the high temperatures depended upon the composition of the medium, the degree of complexity usually being parallel to the increase of the growth temperature. This paper deals with the isolation and characterization of a thermophile, isolated from a manure pile, which is very similar to Bacillus stearothermophilus except for its inability to hydrolyze starch. Because the isolate grew abundantly in simple media, the effect of temperature on the nutritional requirements was also studied.

MATERIALS AND METHODS

Two types of media were used, a rich medium (RM) such as Trypticase Soy Broth (BBL) and a minimal medium (MM) of the following composition (in mg/liter): NaCl, KCl, and NH₄NO₃, 1,000 each; MgCl₂·6H₂O, 40; KNO₃, 200; glucose, 5,000; ethylenediaminetetraacetic acid, 80; Fe(III)SO₄·6H₂O, 25; MnSO₄·4H₂O, 2; CoSO₄·7H₂O, 0.2; ZnSO₄·7H₂O, 2; (NH₄)₂MoO₄·4H₂O, 1. [Microelements were prepared according to the method of Baker et al. (3).] A 100-ml amount of 1.0 M potassium K/K phosphate buffer (pH 7.0) was added, and the pH was adjusted to 7.0 with 10% KOH. Final volume was 1 liter. The same medium containing half the phosphate concentration was also used frequently.

Thermophilic bacillus was characterized according to the method described by Smith, Gordon, and Clark (18). All the tests were made at 55°C.

Effects of temperature and composition of the medium on growth were investigated by using 250-ml Erlenmeyer flasks which contained 50 ml of medium and which were fitted with side arms matched for turbidimetric measurements. Thus, repeated turbidity readings could be followed during growth without changing the volume of the culture and with small fluctuations in growth temperature. Incubation was carried out in a thermostatic water bath with reciprocal shaking (200 strokes/min).

The effect of temperature on the kinetics of growth was tested in both MM and RM at various temperatures, starting at 41 and going up to 65°C. The cells used as inoculum were grown logarithmically for at least 10 generations at the same temperature and medium to be employed. At time intervals, the turbidity of the cultures was read and recorded. From the data thus obtained, specific growth rates and generation times were calculated.

RESULTS

Isolation of the thermophile. Samples taken from manure piles (about 0.1 g per 10 ml of medium) were inoculated into the liquid RM and incubated at 55°C. After turbidity appeared, streaks were made on the RM agar and incubated at 55°C. Some of the colonies that grew out on the plates were picked and purified. Finally, a few colonies of a homogeneous morphology were selected. All of the colonies isolated by this procedure proved to be thermophilic and did not grow at or below 37°C for as long as 7 days. The main work reported below was done on one of these isolates.

Strain characteristics: cell morphology. The isolate is a slender gram-positive bacillus. It is actively motile, 4 to 6 μm long and 0.2 to 0.5 μm wide. Spores are subterminal, round to oval in shape, and rarely are formed. Predominantly unsolved sporangia were seen.
The bacillus grows well on nutrient agar or in nutrient broth and luxuriantly on Trypticase Soy Agar and Trypticase Soy Broth, but it does not grow under anaerobic conditions. On nutrient agar, the colonies usually are transparent and smooth, with entire margins; sometimes, opaque, rough, spreading colonies also appear. Their size may vary from small to 4 mm in diameter. In nutrient broth, growth begins with the formation of a thin pellicle, which afterwards spreads to give a turbid culture.

**Temperature of growth.** No growth was obtained after 7 days at 37 C. Optimal temperature was found to be between 55 and 58 C, maximal at 65 C.

**Biochemical tests.** When grown in nutrient broth containing 0.5% of carbohydrate, with phenol-red as indicator, acid without gas was produced from glucose, fructose, sorbose, raffinose, rhamnose, ribose, glycerol, mannitol, and sorbitol. Neither acid nor gas was formed from galactose, lactose, arabinose, and xylose. Urea was not attacked; catalase was positive. Indole was not formed, and acetoin formation was negative. Nitrates were not reduced to nitrites. The final pH after growth in glucose-broth was about 5.5. No growth was obtained in nutrient broth containing more than 3% NaCl.

**Nutritional requirements.** The thermophile grows luxuriantly in the commonly employed complex media such as RM broth and Brain Heart Infusion broth (Difco). Good growth (at 55 C) was also obtained in a simplified medium consisting of 0.5% yeast extract, 0.5% casein hydrolysate (vitamin-free), and minerals. Therefore, we tried to determine whether a chemically defined medium would also satisfy the growth requirements of the thermophile.

Growth requirements proved astonishingly simple, amino acids, vitamins, purines, and pyrimidines being not essential for growth (Table 1). Thus, the thermophile was found to be a prototroph which grows well over the entire range of temperatures tested in a mineral medium consisting of glucose, ammonia, phosphate buffer, and mineral salts; the exact composition of MM is given in Materials and Methods.

In other experiments, it was found that alanine, aspartate, or glutamate could serve as single carbon and nitrogen sources for the growth of the thermophile.

**Effect of temperature on growth.** To investigate the effect of temperatures between 41 and 65 C on growth, an inoculum from the stationary phase of the thermophile was used.

Growth obtained in the MM and RM broth at 43 C is shown in Fig. 1; that at 55 and 65 C is shown in Fig. 2. It can be seen that the temperature determined the rate of growth. In both media, the shortest lag was observed at 55 C and the longest at 43 C. At the respective temperatures, the lag was almost twice as long in the minimal medium. Although the growth rates differed at the various temperatures, similar turbidities were reached in all cultures regardless of the temperature employed.

**Effect of temperature on growth kinetics.** Little is known of the effect of temperature on growth rates of thermophilic bacilli (13, 16). From the experiments in MM and RM, generation times and specific growth rates (k) were calculated (Table 2). Over the temperature range tested, growth was about 1.5 times faster in RM. The

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**Table 1. Growth of the thermophile in various media at different temperatures**

<table>
<thead>
<tr>
<th>Medium</th>
<th>Factors supplemented</th>
<th>Turbidity (Klett units) after 6 hr at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>43 C</td>
</tr>
<tr>
<td>Trypticase Soy Broth</td>
<td>None</td>
<td>150</td>
</tr>
<tr>
<td>Minimal</td>
<td>None</td>
<td>80</td>
</tr>
<tr>
<td>Minimal</td>
<td>0.5% Casein hydrolysate a</td>
<td>140</td>
</tr>
<tr>
<td>Minimal</td>
<td>0.5% Yeast Extract (Difco) b</td>
<td>125</td>
</tr>
</tbody>
</table>

a Inoculum obtained from cells grown at the same temperature and medium to be used was transferred in amounts sufficient to give an initial turbidity of about 30 Klett units. A 420-nm filter was used for cultures grown in MM, and a 660-nm filter for those in RM medium; 100 Klett units corresponds to 145 and 135 µg/ml of bacteria (dry wt) at 420 and 660 nm, respectively.

b Vitamin Free Casamino Acids (Difco).
Table 2. Effect of temperature and medium composition on the kinetics of growth of the thermophile

<table>
<thead>
<tr>
<th>Growth temp</th>
<th>Generation time (min)*</th>
<th>Rich medium</th>
<th>Minimal medium</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>41</td>
<td>83 (0.50)</td>
<td>132(0.31)</td>
<td></td>
</tr>
<tr>
<td>43</td>
<td>60 (0.67)</td>
<td>114(0.35)</td>
<td></td>
</tr>
<tr>
<td>49</td>
<td>44.5 (0.94)</td>
<td>84(0.49)</td>
<td></td>
</tr>
<tr>
<td>53</td>
<td>32 (1.29)</td>
<td>46(0.91)</td>
<td></td>
</tr>
<tr>
<td>55</td>
<td>21 (1.96)</td>
<td>36(1.16)</td>
<td></td>
</tr>
<tr>
<td>58</td>
<td>27 (1.45)</td>
<td>42(0.97)</td>
<td></td>
</tr>
<tr>
<td>63</td>
<td>34 (1.20)</td>
<td>53(0.78)</td>
<td></td>
</tr>
<tr>
<td>65</td>
<td>60 (0.68)</td>
<td>102(0.40)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses express the specific growth rates.

The genus Bacillus shows a considerable strain variability. According to Smith et al. (18), "practically any bacterial character may vary"; this statement applies particularly to the strain isolated by us.

Table 3 provides a comparison between B. coagulans, B. stearothermophilus, and the thermophilic isolate resembling the second organism. Sporangia of the isolate are predominantly unswollen, and sporulation occurs rarely, thus showing two characteristic features of B. coagulans. On the other hand, the colony size and form, the ability to grow at 65°C and to hydrolyze gelatin, the degree of NaCl tolerance, and the inability to form acetoin are typical features of B. stearothermophilus. Similar difficulties were encountered by Marsh and Larsen (15) in classifying some of the strains isolated by them.

According to Ruth E. Gordon, who examined the organism isolated by us (personal communication), it resembles very much the strain described by Daron (11). Our preliminary experiments revealed that Daron's strain is similar to ours also with respect to its ability to grow in the MM.

Baker et al. (2, 4), who studied the nutritional requirements of several thermophilic strains, found that they frequently require methionine alone or in combination with other amino acids and vitamins. Addition of yeast extract, beef extract, and casein hydrolysate caused a further increase in growth of those strains. On the other hand, Campbell and Williams (9) found a decrease in the nutritional requirements of several thermophiles when the temperature was increased. Our results show that (i) our strain is a prototroph which grows in an MM consisting of glucose as sole carbon source and ammonia as the only utilizable nitrogen source and (ii) that the prototrophic nature of the organism did not change over the entire range of temperatures from...
41 to 65°C, suggesting that under conditions tested all of its biosynthetic activities are thermostable. Another, less likely, possibility is that alternative biochemical pathways operate at the different temperatures employed.

The Arrhenius-constant values of 15,000 and 13,000 cal/mole obtained for the thermophile grown in MM and RM, respectively, are essentially similar to those given by others for mesophiles and psychrophiles (12, 17); the values seem to be influenced by the composition of medium rather than by the temperature of growth or nature of the organism.

ACKNOWLEDGMENT

We express our thanks and appreciation to Ruth E. Gordon for her invaluable help in the identification of the thermophilic isolate as a starch negative strain of *B. stearothermophilus*.

LITERATURE CITED
